Proc. Nat. Acad. Sci. USA
Vol. 71, No. 3, pp. 657–661, March 1974

Mechanism of IgM Polymerization

(J chain/J stoichiometry)

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Communicated by Esmond E. Snell, October 19, 1973

ABSTRACT **The stoichiometry of J chain in pentamer IgM has been determined by measuring the radiolabeled thiols in the constituent chains after complete reduction and alkylation of the polymer. One mole of J was found to be disulfide bonded to 1 mol of pentamer. The linkage of J chain in IgM has been determined by correlating the J disulfides cleaved with the subunits released after limited reduction and alkylation of the polymer. The analyses showed that: (*a*) Significant amounts of monomers, as well as small quantities of dimers, trimers, and tetramers, were generated by the reducing conditions employed. (*b*) The number of J disulfide bonds broken did not correspond to the extent of depolymerization. (*c*) No J disulfides were cleaved in the J-containing dimer products of the limited reduction. These data demonstrated that the J chain is located as a disulfide clasp between two of the IgM monomer subunits. From the observed linkage, the assembly of IgM is postulated to proceed by a series of sequential disulfide exchanges beginning with the formation of the J-containing dimer.**

The polymerization of the immunoglobulins presents an intriguing problem because the process determines both the secretion of IgA and IgM polymers from cells and their specialized biological properties after secretion. An understanding of the mechanism of polymerization is, therefore, a necessary step for understanding the biological significance of the polymers. The first clue was obtained when a small polypeptide, the J chain, was found to be disulfide bonded to the polymerized IgA and IgM species, but absent from all monomeric species (1, 2). The linkage role of the J chain has been confirmed in a series of subsequent investigations. For example, biosynthetic studies showed that J and the IgA or IgM monomers are synthesized in the same cell and the polymer is assembled just prior to its secretion (3, 4). Recombination experiments demonstrated that J is not merely associated with the polymers, but is necessary for correct assembly (5). Stoichiometric measurements on IgA established that each polymer, independent of its size contains, and therefore requires, only 1 molecule of J chain (6).

On the basis of these data, two different mechanisms of polymerization have been postulated (7). In the "bracelet" model J provides an extended backbone to which each monomer subunit is disulfide bonded. In the "clasp" model J is disulfide bonded to only two adjacent monomers and induces direct S–S bonding between the other monomers. To distinguish these mechanisms, a method of stepwise reduction and differential radioalkylation was developed which permitted the cleavage of J chain disulfides to be correlated with the release of polymer subunits. The present paper describes this method and its application to solving the mechanism of IgM polymerization.

Abbreviations: DTE, dithioerythritol; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Purification of IgM. A human Waldenströms macroglobulin was purified by repeated euglobulin precipitation (8) followed by filtration through a Sepharose 6B column equilibrated with Tris-saline buffer (0.02 M Tris·HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, 0.02% Na azide). The main protein peak was concentrated by ultrafiltration (Diaflo UM-10 membrane). The final preparation contained only pentamers and a few percent larger aggregates as measured by velocity sedimentation. J chain isolated from this IgM preparation by preparative polyacrylamide gel electrophoresis (7) had an amino-acid composition in good agreement with published values (9).

Reduction of IgM and Differential Radioalkylation. For limited reduction, the purified IgM was precipitated in deionized H_2O and redissolved in 0.2 M Tris·HCl, pH 8.0, 2 mM EDTA, at a concentration of 10 mg/ml. One-milliliter aliquots of this solution were reduced for 80 min at room temperature with concentrations of dithioerythritol (DTE) ranging from 0.05 to 0.3 mM. All solutions were thoroughly flushed with N_2 and the reduction was performed in closed tubes under an atmosphere of N_2. The IgM was alkylated at 0° with neutralized [^{14}C]iodoacetic acid (30 μCi/μmol, Amersham and Searle, diluted with recrystallized iodoacetic acid to a specific activity of 9.38×10^6 cpm/μmol). A 3.2-fold molar excess over the DTE concentration was used in each case.

To remove any excess [^{14}C]iodoacetic acid before complete reduction and alkylation, 1-mg samples of the partially reduced IgM preparations were precipitated with 12.5% Cl_3CCOOH and the precipitates were washed 3 times with cold ethanol. The precipitates were dissolved in 9 M urea, 0.2 M Tris·HCl, pH 8.0, 2 mM EDTA to a concentration of 3.1 mg/ml, and reduced with 50 mM DTE at 45° for 2 hr. The free −SH groups were alkylated at 0° by adding a 3-fold molar excess of [^{3}H]iodoacetic acid (95 μCi/mol, Amersham and Searle, recrystallized with cold iodoacetic acid to a specific activity of 7.59×10^5 cpm/μmol). The alkylation was stopped after 30 min by precipitating the protein with 12.5% Cl_3CCOOH. The pellets were washed 3 times with cold ethanol and redissolved in 0.11 ml of 9 M urea, 0.1% NH_4OH.

Isolation of Dimers Formed by IgM Reduction. Dimers were separated from the other products of limited IgM reduction by electrophoresis on sodium dodecyl sulfate (SDS)–3.5%

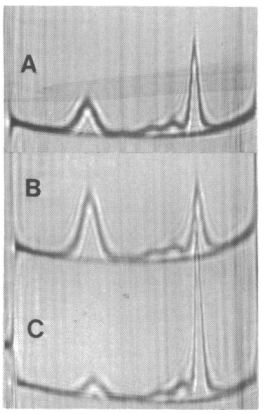

FIG. 1. Schlieren patterns of the products of limited IgM reduction. IgM reduced with (*A*) 0.15 mM DTE; (*B*) 0.2 mM DTE; and (*C*) 0.1 mM DTE. Exposures taken 40 min after reaching a speed of 59,780 rpm; T = 20°; 4.7 mg of protein per ml, Tris-saline buffer.

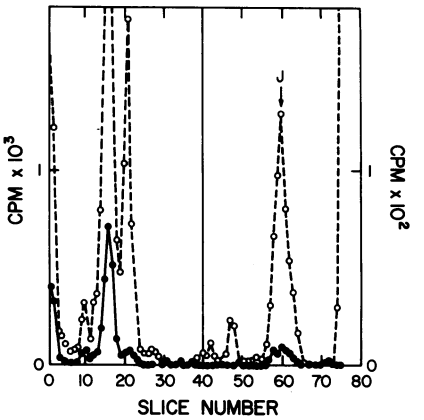

FIG. 2. Electrophoresis of IgM after limited reduction and ^{14}C alkylation followed by complete reduction and ^{3}H alkylation. Alkaline urea 4.15% polyacrylamide gels; ●——● = ^{14}C cpm; O- - -O = ^{3}H cpm.

polyacrylamide gels. After electrophoresis the gels were frozen and the dimer-containing slices removed. The pooled slices were ground in a tissue homogenizer with 4 volumes of 9 M urea, 0.2 M Tris·HCl, pH 8.0, 2 mM EDTA. After 2 hr at 4° the eluate was removed by centrifugation and the elution was repeated. The protein in the combined eluates was concentrated by ultrafiltration, dialyzed exhaustively against the elution buffer, and then completely reduced and alkylated with [^{3}H]iodoacetic acid as described above. The excess ^{3}H label was removed by dialysis against the elution buffer.

Analytical Methods. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to the method of Fairbanks *et al.* (10), and the gels were stained using the procedure of Weber and Osborn (11). Alkaline urea electrophoresis was carried out on 4.15% polyacrylamide gels according to the method of Reisfeld and Small (12). The gels were frozen, sliced, and prepared for radioactivity measurements as described previously (7). All values given were corrected for background and cross-contamination between the ^{14}C and ^{3}H counting. Sedimentation analysis was performed on a Beckman Spinco model E analytical ultracentrifuge equipped with schlieren optics. Double sector cells with quartz windows were used in a AnD rotor. Relative amounts of protein were determined from planimetric measurements of the areas under the peaks. No correction was applied for radial dilution.

RESULTS

In order to correlate the cleavage of J disulfide bonds with the release of polymer subunits, it was necessary to have (*a*) a method for selectively and progressively reducing intersubunit disulfides, (*b*) a means of assaying the reduction products, and (*c*) a quantitative method for determining the number of reacted half-cystines in J chain. The first requirement was satisfied by treating pentamer IgM with limiting amounts of dithioerythritol (DTE). Using these conditions, Beale and Feinstein (13) had shown that the intersubunit bonds in IgM were preferentially cleaved and the yield of monomer was proportional to the concentration of reducing agent.

The cleavage products were assayed by velocity sedimentation analysis. The monomer fraction could be readily separated and quantitated because of its size and relatively large yield, and the remaining components, dimers, trimers, tetramers, and residual pentamers, were sufficiently resolved to permit their measurement with reasonable accuracy.

The third requirement, the measurement of J disulfide bond cleavage, was more difficult to meet because the J chain represents a very minor fraction of pentamer IgM, 1.7% by weight, and is quantitatively released only by reduction and alkylation in denaturing solvents. A method of differential radioalkylation provided a solution to these problems. The −SH groups generated during the limited reduction of the pentamer were alkylated with [^{14}C]iodoacetic acid. The partially reduced preparations were then completely reduced in 9 M urea to permit quantitative isolation of the component chains, and the −SH groups generated in this step were alkylated with [^{3}H]iodoacetic acid. The chains were separated by electrophoresis on alkaline urea polyacrylamide gels and their radioactivity was determined. The total ^{14}C and ^{3}H

TABLE 1. *Cleavage of IgM by DTE*

DTE concentration (mM)	Yield of reduction products*				Residual pentamer*
	Monomer	Dimer	Trimer	Tetramer	
0.1	14.3	1.6	9.0	14.3	60.8
0.15	31.1	1.9	8.7	11.9	46.4
0.2	49.1	2.5	8.5	8.9	31.0

* % of total protein determined from schlieren patterns.

TABLE 2. *Stoichiometry of J chain in IgM*

Chain	Total cpm		Total nmoles recovered		
	^{3}H	^{14}C	[^{3}H]-CMCys*	[^{14}C]-CMCys*	Chain
μ (slices 1–18)	12,824	3,109	16.9	0.331	1.23
L (slices 19–30)	4,629	276	6.10	0.029	1.23
J (slices 55–64)	521	42	0.686	0.004	0.115

* CMCys = carboxymethylcysteine.

TABLE 3. *Number of reacted half-cystines per chain after limited reduction of IgM with DTE*

DTE concentration (mM)	Molar content of [^{14}C]CMCys			
	μ			
	Observed	Intersubunit	L	J
0.1	0.276	0.224	0.0240	0.0363
0.15	0.482	0.366	0.0527	0.209
0.2	0.733	0.539	0.0959	0.558

label in each chain provided a measure of its yield, while the ^{14}C label per chain provided a measure of the half-cystines alkylated after the limited reduction step. For these determinations values of 14, 5, and 6 were used for the half-cystine contents of μ, light, and J chains respectively.

The J chain was originally reported to contain seven half-cystine residues based on carboxymethylcysteine analysis after complete reduction and alkylation (7). This number was subsequently reduced to six when the analyses were recalculated using an experimentally determined extinction coefficient for carboxymethylcysteine and correcting for small amounts of contamination by methionine sulfoxide. A value of seven half-cystines would make an odd number of —SH groups available for intersubunit bonds and would, therefore, require the presence of a free —SH in intact IgM, either on a μ or the J chain. In contrast, a value of six half-cystines would allow all the —SH groups to be disulfide bonded. To distinguish these possibilities, intact IgM was alkylated with [^{14}C]iodoacetic acid in the presence of 7 M guanidine, and then completely reduced and alkylated with [^{3}H]iodoacetic acid. When the component chains were isolated by alkaline urea polyacrylamide gel electrophoresis, the [^{14}C]carboxymethylcysteine was distributed equally between the μ and light chains, 0.039 and 0.041 mol per mol respectively, indicating that disulfide bridges had not been formed between a small percentage of the μ and light chains. Since no ^{14}C label was found in the J chain, and no additional ^{14}C label in the μ chains, these data were taken as further evidence that 6 was the correct value for the half-cystine content of J.

The cleavage of IgM achieved with limited DTE reduction is illustrated by the schlieren patterns shown in Fig. 1 and the yields of products summarized in Table 1. As the DTE concentration was raised from 0.1 to 0.2 mM, the amount of monomer produced increased from 14.3 to 49.1% and the amount of pentamer cleaved from 39.2 to 69.0%. In contrast, the yields of the intermediate size components remained constant, averaging 2.0% for dimers, 8.8% for trimers, and 11.7% for tetramers. This distribution of reduction products confirmed the previous findings of Beale and Feinstein (13) and suggested that the breaking of the first intersubunit bond in a pentamer made the remaining intersubunit disulfides in that molecule more susceptible to reduction than those in intact pentamers. As a result, the intermediates were rapidly converted to monomer subunits.

The results of the differential radioalkylation analyses are illustrated in Fig. 2 and Table 2. The data were obtained from an IgM preparation which was initially reduced with 0.1 mM DTE and ^{14}C-alkylated and then completely reduced and ^{3}H-alkylated. It is evident from the gel pattern (Fig. 2) that a good separation of μ, light (L), and J chains was achieved by electrophoresis. Some of the μ chain remained as aggregate

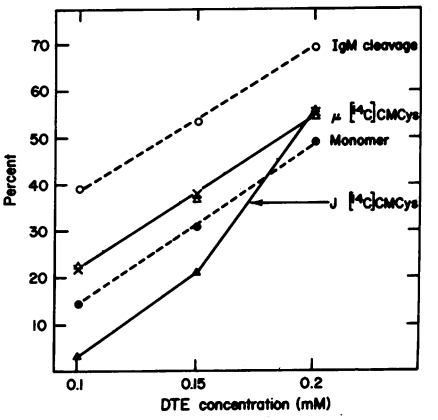

FIG. 3. Correlation of disulfide cleavage with depolymerization. ▲——▲ = % of J chains with one [^{14}C]CMCys per chain; △——△ = % of μ chains with one [^{14}C]CMCys per chain; O---O = % cleavage of IgM; ●---● = % release of monomers; ×——× = theoretical [^{14}C]CMCys-incorporation in μ chains based on the yields of IgM cleavage products.

which barely penetrated the gel, but there was no detectable loss from the top of the gel. When the yields were calculated (Table 2), the μmoles of μ chain recovered were identical to those of light chain as would be expected from their presence in equal numbers in the polymer IgM. The μmoles of J recovered were a factor of 10 less. Similar results were obtained in eight other experiments, giving an average yield for J chain of 0.96 ± 0.03 mol per mol of pentamer. These stoichiometric measurements established that only one J chain was present per pentamer and thus a maximum of six J half-cystines were available for intersubunit linkage.

The half-cystines alkylated after the limited reduction step were calculated from the ^{14}C content of the chains. The results are given in Table 3 for each concentration of DTE used. The values for the μ chains were found to reflect not only half-cystines involved in intersubunit bonds, but also a few involved in intrasubunit linkage. The evidence for μ-light reduction was the presence of [^{14}C]carboxymethylcysteine in the light chains. The amounts detected (Table 3) indicated that 2.4 to 9.6% of the μ-light bonds either had not been properly formed in the intact pentamer or were cleaved during the limited reduction step. The evidence for the cleavage of μ-μ intrasubunit bonds was obtained from analyses of the monomers formed by limited IgM reduction. Electrophoresis on SDS polyacrylamide gels showed that 10% of the monomers dissociated into μ-light pairs and thus had both μ-μ intrasubunit bonds cleaved. Assays of the ^{14}C content of the remaining intact monomers showed that 5% had one μ-μ intrasubunit bond cleaved. These values were consistent with results obtained in similar studies (14). The numbers of alkylated half-cystines contributed by the breakage of μ-light and μ-μ intrasubunit bonds were then subtracted from the total [^{14}C]-carboxymethylcysteine content of the μ chains to obtain the number of alkylated half-cystines resulting from the cleavage of intersubunit bonds (Table 3).

When the cleavage of μ and J chain disulfides was correlated with the release of IgM subunits, the relationships shown in Fig. 3 were obtained. In this figure, the cleavage data have been expressed as the percentage of chain containing one [^{14}C]carboxymethylcysteine per mol in order to facilitate comparison of the data with the results from sedimentation

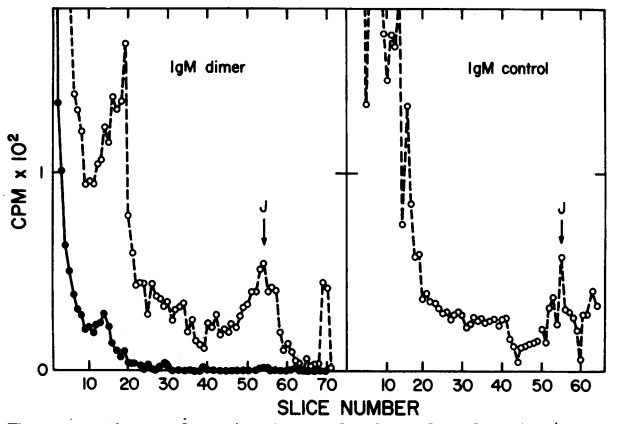

FIG. 4. Electrophoresis of completely reduced and alkylated IgM dimers and IgM control on alkaline urea 5% polyacrylamide gels. ●——● = ^{14}C cpm; O- - -O = ^{3}H cpm.

analysis. The correspondence between the cleavage of μ chain intersubunit disulfides and depolymerization was striking. The percentage of μ chain labeled increased proportionally to the DTE concentration and paralleled the generation of reduction products. More importantly, the absolute number of μ intersubunit bonds cleaved accounted for the observed amounts of reduction products. When the theoretical incorporation of carboxymethylcysteine into μ chain was calculated from the yields of reduction products and the assumption of two ^{14}C-labeled residues per monomer, dimer, trimer, and tetramer, the results agreed within 2% with the experimentally determined values (Fig. 3).

In contrast to the μ chain data, no correspondence was observed between the cleavage of J chain disulfides and depolymerization. After reduction with 0.1 mM DTE, 39.2% of the pentamer was cleaved, 14.3% to monomers. This cleavage was accompanied by the incorporation of very small amounts of ^{14}C into J chain, equivalent to 3.6% of the J containing one reacted half-cystine. Similarly, after the reduction with 0.15 mM DTE, the percentage of J chain labeled was significantly less than the percentage of monomer released. At 0.2 mM DTE, the large increase in the ^{14}C content of J was found to reflect the release of 2.5% free J containing six labeled half-cystines per mol (7, 9) These data were not compatible with a bracelet model for J linkage which would require the cleavage of at least one J-μ disulfide for every monomer subunit generated.

To pinpoint the location of J in the pentamer structure, studies were carried out on the dimers formed by reduction of IgM with 0.1 mM DTE. The dimers were separated from the other reduction products by electrophoresis on SDS polyacrylamide gels and then eluted with 9 M urea. When rerun on SDS gels, the isolated preparation migrated as a single band with the same mobility as the original fraction. Analyses of the ^{14}C radioactivity showed that the dimer was recovered in an 80% yield and that each μ chain contained 0.43 residues of [^{14}C]carboxymethylcysteine, in reasonable agreement with the theoretical value for the dimer of 0.5 residues per μ chain.

The J content of the dimers and the extent of cleavage of J disulfides were determined by differential radioalkylation. The electrophoretic pattern obtained is shown in Fig. 4 along with the pattern obtained for an IgM control carried through the same procedures used for the dimer isolation. Although these procedures impaired the resolution of the samples, a J chain peak comparable to that of the pentamer control was clearly discernible in the dimer gel. The measurements of radioactivity showed that J was present in 63% of the dimers and contained no detectable amounts of ^{14}C label. Thus, none of the J disulfides were broken during the limited reduction which released the dimers. These data demonstrated that J can be linked to only two monomer subunits and its remaining four half-cystines are linked in intrachain bonds.

DISCUSSION

From structural studies, mammalian IgM was known to contain five identical IgG-like subunits linked by heavy chain disulfides to form a circular polymer (15, 16, 17). On the basis of this structure, two different models have been postulated to explain the linkage of J chain and its role in IgM assembly (7). The first model proposed that J serves as a bracelet linking each monomer subunit and the polymer is closed by an in-

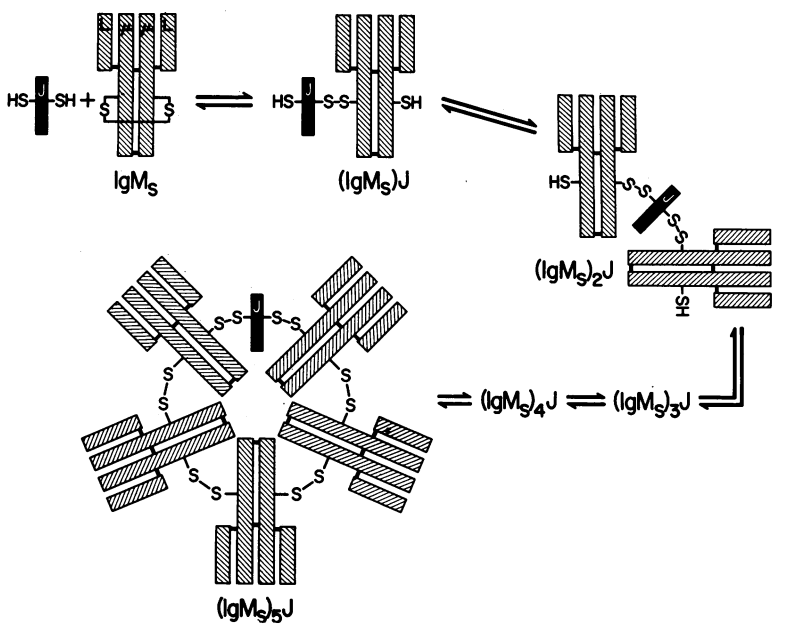

FIG. 5. The clasp model of J linkage in pentamer IgM and the postulated disulfide exchanges leading to its formation. IgM_s = monomer of pentameric IgM.

tra-J S-S bond. The alternate model proposed that J serves as a clasp between two monomers and the cyclic arrangement of the polymer is completed by direct S-S bonding between the other subunits.

The results presented in this paper provide strong support for the clasp model of J linkage (Fig. 5). Evidence was obtained first from the measurements of J chain stoichiometry. The finding of one J chain per pentamer limited the possible J-μ disulfides to six, the half-cystine content of J. Although this number allowed a bond to be formed between J and one μ chain in each monomer, the remaining half-cystines on the alternate μ chains and the extra half-cystine in J could not be arranged in intersubunit bonds which completely preserved the symmetry and closed structure of the IgM polymer.

More compelling evidence was obtained from the measurements of disulfide cleavage in J chain after limited reduction of IgM. The number of J bonds broken was found to be considerably less than the number of monomer subunits released, in direct contradiction to the predictions of a bracelet model. Moreover, none of the S-S bonds were found cleaved in the J chain isolated from the dimer product of IgM reduction. These results eliminated the possibility that J was covalently bonded to more than two of the IgM subunits. The only alternative to the dimer clasp model was the possibility that J was linked to a single monomer by insertion in an intrasubunit μ-μ bond. Although this linkage could not be rigorously excluded, it was highly unlikely since J-monomer complexes have not been detected in the monomer product of IgM reduction either by differential radioalkylation or by electrofocusing (18) techniques.

The clasp structure determined for IgM pentamer involves two kinds of disulfide bridges, μ-μ and J-μ. The finding that the μ-μ intersubunit bonds were more susceptible to reductive cleavage, particularly in the first step of depolymerization, suggested that the role of J chain is to initiate rather than to terminate the formation of the polymer. On this basis, the assembly of IgM can be explained by a series of sequential disulfide exchanges beginning with the formation of a J containing dimer (Fig. 5). The available evidence indicates that the potential intersubunit half-cystines are linked in an intrasubunit bond (19, 20). The intracellular J chain is assumed, therefore, to contain two free thiols which either never form an intra-J bond or are generated from an intra-J bond by an external oxidation-reduction system. The first intermediate would be formed by an exchange between a J chain thiol and a monomer μ-μ bond, yielding a J-μ disulfide and a free –SH on the opposing μ chain. In rapid succession a similar reaction would occur between a second J thiol and another monomer subunit to produce a J-linked dimer. The μ chain thiols would then induce additional exchanges with other monomers to form larger polymers.

If the assembly of IgM proceeds by disulfide exchange, the question arises as to how the polymerization is limited to the formation of pentamers. One possible explanation is that the closing of the polymer requires an oxidation step coupled with secretion in which a pentamer is the only acceptable substrate. A second explanation is that steric constraints on the intersubunit bridges within the polymer prevent the closing of any nonpentameric form either by oxidation or by exchange. The pentamer restriction does not hold for the IgM from all species. The catfish macroglobulin has been found to be tetrameric (21) and the *Xenopus*, hexameric (22). Since both these proteins contain J chain, the differences in polymer size presumably reflect changes in the biosynthetic pathway or in the structures of the respective μ chains.

To obtain an efficient yield of IgM, the disulfide-sulfhydryl equilibrium must be shifted toward the completion of the reaction. This shift can be accomplished by an excess of monomer reactant or by the rapid removal of the pentamer products. Studies on mouse myeloma lines synthesizing IgM indicate that both means are utilized by the cell. The intracellular immunoglobulin was found to consist of a large monomer pool and little or no polymers of any size, while the extracellular immunoglobulin was found to be predominantly completed pentamers (19). In contrast, no significant pool of free J chain has been detected within cells (3, 4), indicating that the supply of J is the rate determining step in polymer assembly. Thus, the synthesis of J chain is not only required for the initiation of polymerization, but it also provides a means for the cell to regulate the total output of polymeric immunoglobulin.

This investigation was supported by U.S. Public Health Service Research Grant R01 AI 07079 from the National Institutes of Health. The human Waldenströms macroglobulin was very generously provided by Dr. H. H. Fudenberg of the Medical School of the University of California, San Francisco.

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