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Non-covalent Association of IgM Subunits Produced by Reduction and Alkylation

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Summary. Purified IgM isolated from the serum of mice bearing the transplantable plasmacytoma MOPC 104E was reduced and alkylated and then analysed by sucrose density gradient centrifugation and by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. On partial reduction a mixture of IgM subunits was obtained in the absence of covalently linked 19S IgM. When examined under dissociating conditions this mixture was found to consist of disulphide-linked 7S subunits (IgM_s), small amounts of HL subunits and oligomeric IgM of a size intermediate between monomeric and pentameric IgM. In the absence of a dissociating agent and on sucrose density gradient, however, the mixture resolved into a 19S and a 7S peak. The 19S peak consisted primarily of oligomeric IgM and IgM_s with small amounts of HL subunits. Thus alkylated IgM_s and HL subunits of IgM can associate through non-covalent forces to form a molecule sedimenting at 19S, providing oligomeric forms are present. In the absence of oligomeric forms, IgM_s, HL subunits and heavy and light chains sediment at about 7S. The products of partial reduction which sediment at 7S and 19S could also be isolated by preparative polyacrylamide gel electrophoresis. When this was done J chain was absent in the former and present in the latter, raising the possibility that J chain does not disulphide bond to each of the five IgM_s subunits, constituting an IgM molecule. Thus, within cells secreting IgM, J chain would be expected to mediate the formation of an oligomeric form of IgM. Once the oligomeric structure has been assembled, then non-covalent forces between this and IgM_s subunits will cause the formation of a 19S structure, thereby facilitating the final assembly through disulphide bonds.

INTRODUCTION

Studies on the structure of mammalian IgM (review by Metzger, 1970) have shown the molecule to consist of five radially arranged, disulphide-linked 7S subunits (IgM_s). Each IgM_s subunit is composed of two heavy chains and two light chains, also linked together by disulphide bonds. In addition, the fully assembled 19S molecule contains a third polypeptide chain, the J chain, which is perhaps necessary for linking the IgM_s subunits together (Halpern and Koshland, 1970; Mestecky, Zikan and Butler, 1971; Parkhouse, 1972; Della Corte and Parkhouse, 1973).

The conversion of 19S IgM to a 7S subunit by reduction with sulphydryl reagents was

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first demonstrated in 1957 (Deutsch and Morton, 1957). Since then it has been well established that free heavy and light chains formed from IgM by reduction will associate through non-covalent interactions to give a 7S component composed of two heavy and two light chains (Metzger, 1970). However, there is no tendency to form higher molecular weight aggregates through non-covalent interactions, and this is also true of IgM_s molecules in which the interchain disulphide bonds are not reduced (Cooper, 1967; Suzuki and Deutsch, 1967; Morris and Inman, 1968; Beale and Feinstein, 1969; Chavin and Franklin, 1969; Parkhouse and Askonas, 1969; Miekka and Deutsch, 1970; Dolder, 1971). In order to generate polymer molecules from reduced samples of IgM, a general procedure has been to remove the reducing agent to allow reoxidation and reformation of disulphide bridges (Jacot-Guillarmod and Isliker, 1962; Harboe, 1967; Suzuki and Deutsch, 1969; Harboe, Solheim and Deverill, 1969; Parkhouse, Askonas and Dourmashkin, 1970).

Upon selective reduction of IgM with mercaptans, it is noteworthy that the only products found in significant amounts are either 19S or 7S when examined in non-dissociating solvents (Morris and Inman, 1968; Beale and Feinstein, 1969). Similarly, within cells secreting IgM, the disulphide-linked monomeric IgM_s subunits accumulate in the absence of 19S IgM or oligomeric forms intermediate between monomer and pentamer (Parkhouse and Askonas, 1969). The failure to find appreciable amounts of fully assembled IgM in cells actively secreting the pentamer suggests that the polymerization of IgM_s subunits occurs very shortly before or simultaneously with secretion. At this time J chain is incorporated into the molecule (Parkhouse, 1972), possibly through the involvement of a disulphide exchanging enzyme (Della Corte and Parkhouse, 1973).

The absence of non-covalent interactions between IgM_s subunits is curious in view of the rapidity and precision with which polymerization occurs in IgM-secreting cells. It was for this reason that the products of partial reduction of IgM were carefully analysed. The major finding to be presented is that 7S subunits of IgM can associate through non-covalent forces to give a molecule sedimenting at 19S, providing oligomeric forms of IgM, intermediate in size between monomer and pentamer, are present.

MATERIALS AND METHODS

Plasma cell tumours

These were supplied by Dr M. Potter and maintained in BALB/c mice by subcutaneous transfer of 1-mm^3 tumour pieces.

Preparation of myeloma proteins

Mice bearing the tumours MOPC 104E and Adj PC5 were used as a source of IgM and IgG2a respectively. The myeloma proteins were prepared from serum as previously described (Knopf, Parkhouse and Lennox, 1967; Parkhouse and Askonas, 1969). Purified radioactive myeloma proteins were isolated from culture supernatants of myeloma cell suspensions incubated *in vitro* with [^{3}H]leucine (Knopf *et al.*, 1967; Parkhouse and Askonas, 1969).

Reduction and alkylation of IgM

Purified IgM was reduced for 1 hr at room temperature with dithioerythritol, cooled to 0° and then alkylated by adding iodoacetamide (50 per cent molar excess over added -SH) in the presence of 0·2 M Tris–HCl, pH 8·0.

Preparative polyacrylamide gel electrophoresis

The sample was loaded on to a preparative polyacrylamide gel (4·0 per cent (w/v)) acrylamide, 16 mM Tris, 50 mM glycine, pH 8·7) and electrophoresis was conducted at 220 V and 200 mA. The preparative gel measured 90 × 50 mm (diameter × length) and the design of the apparatus has previously been described in detail (Brownstone, 1969).

Analytical polyacrylamide gel

This was done with either the sodium dodecyl sulphate system (Summers, Maizel and Darnell, 1965) or the alkaline urea system (Reisfeld and Small, 1966).

Protein

This was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

RESULTS

Samples of purified MOPC 104E IgM (3 mg/ml) were reduced with 0·5 mM and 2·0 mM dithioerythritol, alkylated with iodoacetamide and then analysed by sucrose density gradient centrifugation (Fig. 1). Radioactive secreted IgM and IgG were added to the samples to serve as internal 19S and 7S markers. At the lower concentration of reducing agent the major sedimenting species were localized in the 19S and 5–7S regions of the

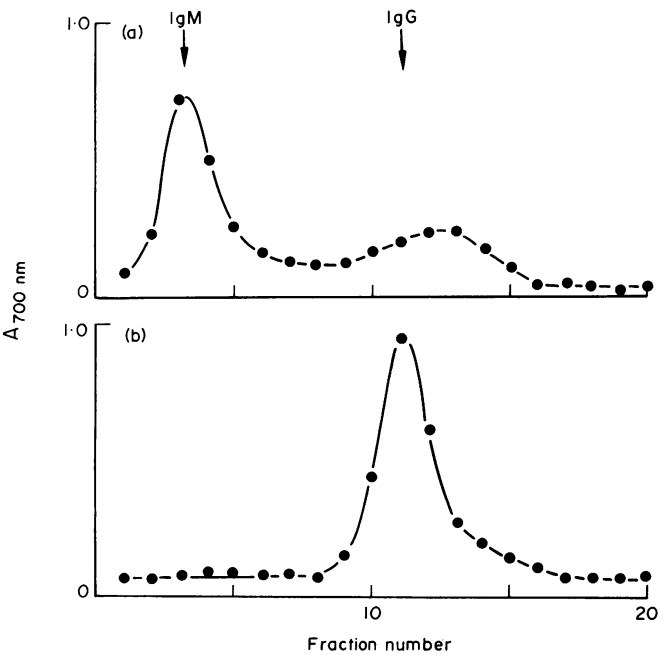

FIG. 1. Sucrose density gradient analysis of reduced and alkylated IgM. The IgM sample was reduced with dithioerythritol and then alkylated with iodoacetamide as described in the test and Materials and Methods section. Purified [^{3}H]leucine-labelled IgM and IgG2a were added as internal markers and the samples were centrifuged in a 10–30 per cent (w/v) sucrose gradient prepared in 130 mM NaCl–4 mM KCl–10 mM sodium phosphate, pH 7·4 (Spinco 41 rotor, 200,000 *g* for 18 hr). After fractionation the samples were assayed for protein by the Lowry procedure (Lowry *et al.*, 1951) and for radioactivity by scintillation counting. The position of the radioactive markers is indicated in the figure and the blue colour developed in the protein estimation was measured at 700 nm. (a) IgM reduced at 0·5 mM dithioerythritol and alkylated. (b) IgM reduced at 2·0 mM dithioerythritol and alkylated.

gradient, with little material in between (Fig. 1a). With 2 mM dithioerythritol all of the isolated IgM had been converted to a 7S form (Fig. 1b). The various fractions were then examined by polyacrylamide gel electrophoresis in the presence of a dissociating reagent, sodium dodecyl sulphate (Fig. 2). The purified IgM (Fig. 2a) was partially reduced by 0·5 mM dithioerythritol to a mixture of oligomers, IgM_s, HL subunits and a component subsequently identified as serum albumin (Fig. 2b). Under these conditions there was no

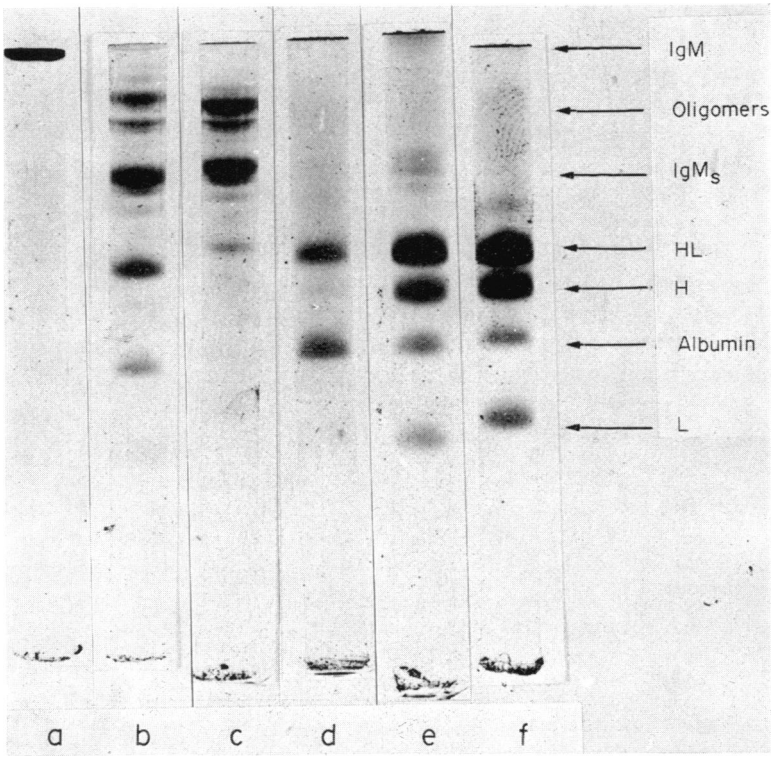

FIG. 2. Sucrose density gradient fractionation of reduced and alkylated IgM. Analysis by sodium dodecyl sulphate–polyacrylamide gel (4·25 per cent (w/v) acrylamide). Purified IgM (a) was reduced with 0·5 mM dithioerythritol, alkylated with iodoacetamide (b), and separated into 19S (c) and 5–7S (d) fractions by sucrose density gradient centrifugation (see Fig. 1a). On reduction with 2 mM dithioerythritol (e), the sample sedimented entirely in the 7S region of the sucrose gradient (f) (See Fig. 1b).

residue of 19S IgM, but it was this mixture of molecular species which yielded the 19S and 5–7S peaks on sucrose density gradient. When these two peaks from the sucrose gradient were subjected to electrophoretic analysis, the surprising result was that the 19S peak contained oligomeric material, IgM_s and traces of HL subunits (Fig. 2c), but no covalently bonded 19S materials. The slower sedimenting 5–7S peak contained HL and serum albumin (Fig. 2d). Upon treatment of IgM with 2 mM dithioerythritol, the molecule was reduced to HL subunits and heavy and light chains (Fig. 2e), which sedimented entirely at 7S (Fig. 2f) in a sucrose density gradient.

In order to confirm the finding that reduced and alkylated subunits of IgM could associate through non-covalent interactions, MOPC 104E IgM (2·5 mg/ml) was reduced with 0·5 mM dithioerythritol, alkylated with iodocetamide and then applied to a preparative

polyacrylamide gel (Fig. 3). Four pools (A,B,C,D) were made as indicated on the figure, and these were analysed by electrophoresis under dissociating conditions and by sucrose density gradient centrifugation. The first peak collected, pool A, was identified as albumin on the basis of its position on sodium dodecyl sulphate–polyacrylamide gels (Fig. 4c), and because it was precipitated by specific rabbit anti-(mouse serum albumin). Pool B consisted predominantly of HL subunits with some IgM_s (Fig. 4d), and sedimented at 4–6S (Fig. 5). The remaining material (Pools C and D), which did not contain 19S IgM, was

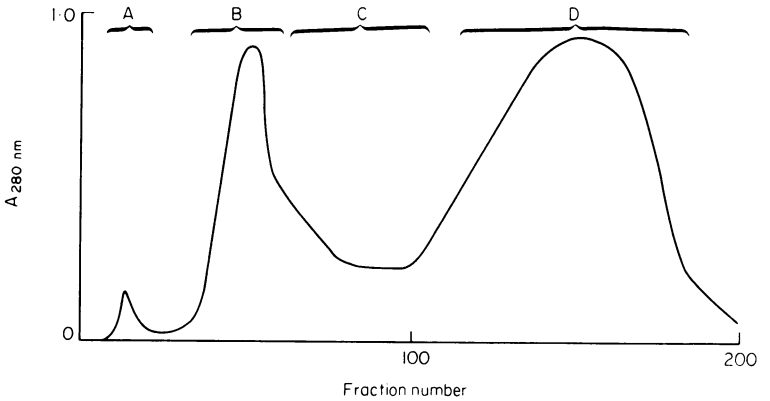

FIG. 3. Fractionation of reduced and alkylated IgM by preparative polyacrylamide gel electrophoresis. The IgM was reduced with 0·5 mM dithioerythritol, alkylated and applied to preparative polyacrylamide gel. Fractions were pooled as indicated in the figure. Details in the text and Materials and Methods section.

largely accounted for by oligomeric IgM and IgMs (Figs. 4e, 4f), but sedimented at 19S in the absence of dissociating reagent (Fig. 5). In order to check that there had been no formation of covalent bonds during the course of the sucrose density gradient sedimentation, samples were taken from the fractionated gradients and re-examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. It is clear that both Pool B and Pool D are unchanged (compare Fig. 6c and 6d with Fig. 4d and 4f) after sedimentation and that there is no evidence for the formation of covalently 19S IgM in Pool D. Upon reduction with 10 mM dithioerythritol in 9 M urea followed by electrophoresis in alkaline–urea gels, J chain was found in Pools C and D, but not in Pools A and B.

DISCUSSION

The most important point to emerge from these studies is the finding that alkylated subunits of IgM can associate through non-covalent interactions to form a molecule which sediments at 19S. Whilst such a structure may include IgM_s and HL subunits, it is essential that oligomeric forms are included. Such oligomeric forms may be dimers of IgM_s as judged from the position of the material on sodium dodecyl sulphate polyacrylamide gels and the results of electron microscopic analysis (Parkhouse *et al.*, 1970). However, the identification of the exact molecular nature of the oligomeric material awaits accurate determination. Its isolation will require dissociating conditions because it is clear from the results of sucrose density sedimentation and preparative polyacrylamide gel electrophoresis that non-covalent association occurs between subunits of IgM produced by reduction and alkylation.

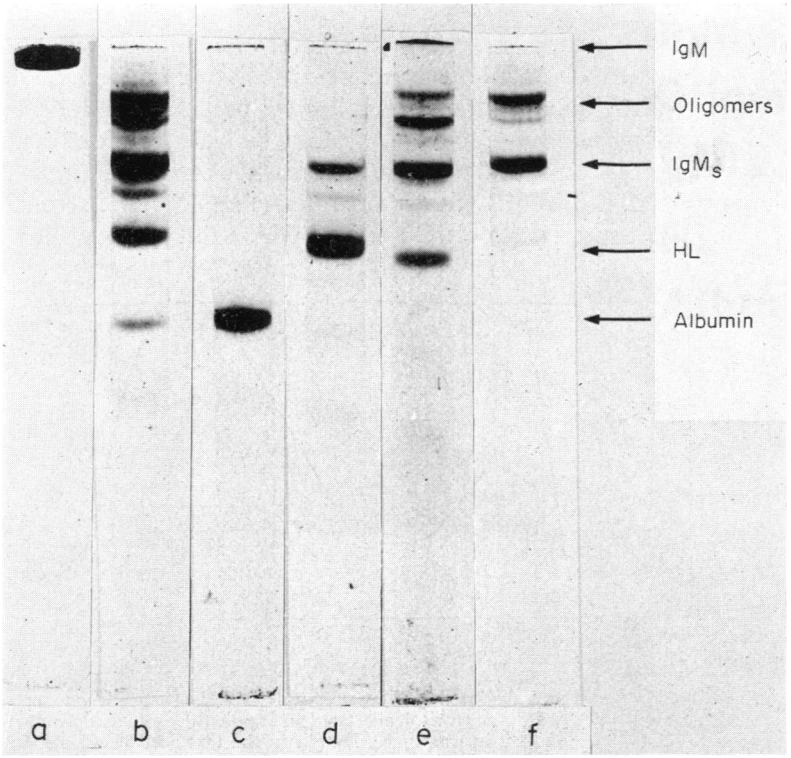

FIG. 4. Fractionation of reduced and alkylated IgM by preparative polyacrylamide gel electrophoresis. Analysis by sodium dodecyl sulphate–polyacrylamide gels (4·25 per cent (w/v) acrylamide). The IgM (a) was reduced with 0·5 mM dithioerythritol, (b) alkylated and applied to preparative polyacrylamide gel (See Fig. 3) to give Pools A (c), B (d), C (e) and D (f).

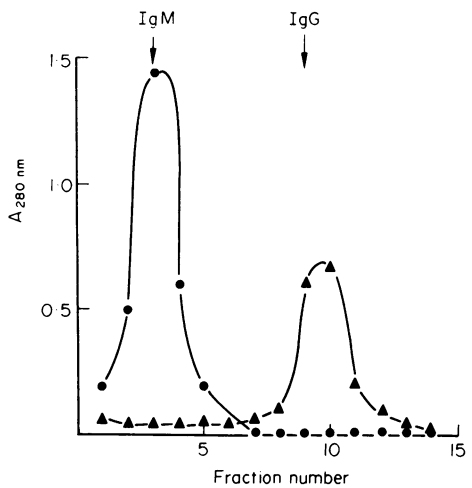

FIG. 5. Fractionation of reduced and alkylated IgM by preparative polyacrylamide gel electrophoresis (See Figs 3 and 4). Analysis by sucrose density gradient centrifugation of Pool B at 2 mg protein/ml (▲) and Pool D at 5 mg protein/ml (●). Purified [^{3}H]leucine-labelled IgM and IgG2a were added as internal markers and the samples were centrifuged in a 10–30 per cent (w/v) sucrose gradient prepared in 130 mM NaCl–4 mM KCl-10 mM sodium phosphate, pH 7·4 (Spinco SW39 rotor, 85,000 *g*, 16½ hr). After fractionation the samples were assayed for protein by spectroscopy and for radioactivity by scintillation counting. The position of the radioactive markers is indicated in the figure.

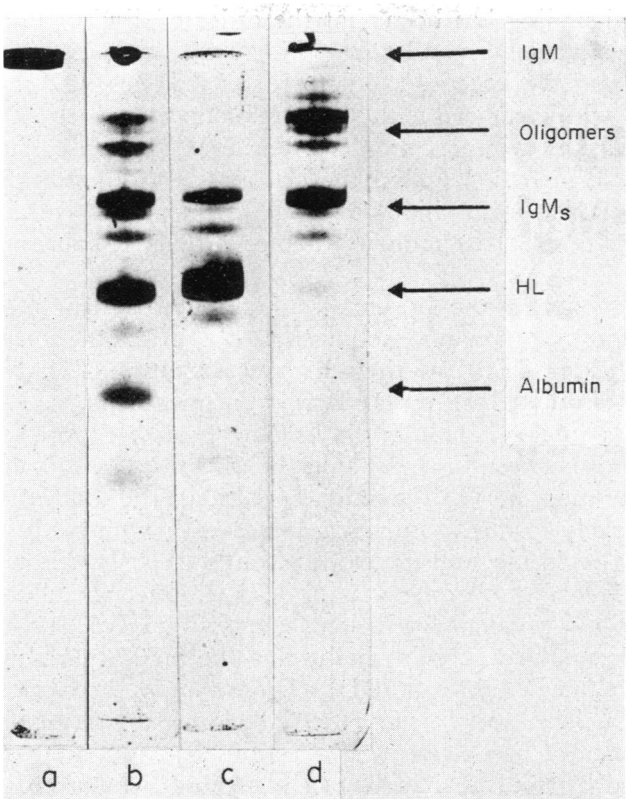

FIG. 6. Fragments of IgM prepared by reduction and alkylation. Analysis by sodium dodecyl sulphate–polyacrylamide gels (4·25 per cent (w/v) acrylamide). The IgM was reduced with 0·5 mM dithioerythritol, alkylated, fractionated by preparative polyacrylamide electrophoresis (See Figs 3 and 4) and samples of Pools B and D were loaded on to sucrose density gradients (See Fig. 5). (a) Purified IgM. (b) IgM reduced with 0·5 mM dithioerythritol and alkylated. (c) 4–6S peak of preparative polyacrylamide peak B recovered from sucrose density gradient. (d) 19S peak of preparative polyacrylamide peak D recovered from sucrose density gradient.

In previous studies with IgM, the products of partial reduction have been characterized in non-dissociating solvents as consisting predominantly of 19S and 7S species, with little material sedimenting in between (Morris and Inman, 1968; Beale and Feinstein, 1969). From the data presented above it seems possible that such 19S components consisted of a mixture of IgM fragments. One exception was a purified anti-Forssman IgM which yielded a spectrum of molecular species after partial reduction and when examined by sedimentation in a non-dissociating medium (Frank and Humphrey, 1969). In this case, however, the isolation procedure involved an extraction step with alkali, which could possibly have resulted in denaturation of the molecule with subsequent changes in its physical properties. In addition, the concentration of protein applied to the sucrose gradient was very low.

When reduction results in the cleavage of inter-IgM_s subunits bonds so that oligomeric forms are no longer present, then the material sediments entirely at 7S as a result of non-covalent interaction between HL subunits and between heavy chains and light chains. In the absence of oligomer, then, there is no tendency for the formation of larger aggregates.

The results may be interpreted with relevance for the biosynthetic assembly of the IgM

molecule. A general finding has been that the order of disulphide bond formation between immunoglobulin subunits and polypeptide chains reflects the reduction sensitivity of the molecule (Bevan, Parkhouse, Williamson and Askonas, 1972). Thus the first disulphide bond formed within the cell is the most resistant to reduction, whilst the last disulphide bond formed is the most sensitive. For IgM, therefore, the heavy-light chain disulphide bond is formed first within the cell and is the most resistant to reduction. Conversely, the inter-IgM_s disulphide bonds are the most sensitive to reduction and their formation constitutes the final assembly step in biosynthesis (Askonas and Parkhouse, 1971; Parkhouse, 1971).

Upon partial reduction of the IgM molecule and subsequent preparative polyacrylamide gel electrophoresis, two major fractions were obtained. One consisted predominantly of HL subunits with some IgM_s and the other was a mixture of oligomers, IgM_s and HL subunits. Since J chain was absent in the former but present in the latter, it appears that the disulphide bonds linking J chain in the IgM molecule are more resistant to reduction than disulphide bonds between IgM_s subunits. Bearing this in mind, and noting that IgM_s was liberated under these partial reduction conditions, the following may be deduced: that J chain is unlikely to form disulphide bonds with each of the five IgM_s subunits constituting an IgM molecule, and therefore within the cell J chain would be expected to mediate the formation of an oligomeric form of IgM. Once the oligomeric structure has been created, then non-covalent forces between this and IgM_s subunits will cause the formation of a 19S structure, thereby facilitating the final assembly through disulphide bonds. Formation of the J chain-containing oligomer must therefore be the rate limiting step in IgM biosynthesis. We may assume that IgA dimers are formed in a similar manner. However, the absence of non-covalent interactions between monomer and dimer IgA molecules (indicated by the fact that they sediment independently), does not favour the formation of larger polymers.

In a careful study of reduction intermediates of polymeric immunoglobulins, it has been convincingly demonstrated that the J chain links only two subunits together; remaining intersubunit disulphide bonds are between heavy chains (Chapuis and Koshland, 1974). The conclusions drawn above are in agreement with this model.

After completion of the work presented in this paper, a similar study was published by Tomasi (1973). He showed that subunits of human IgM produced by reduction and alkylation would associate through non-covalent interactions to give a molecule sedimenting at 18S, but he did not describe the molecular composition of the reduced IgM.

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