Complement-Mediated Hemolysis by Subunits of IgM Antibody

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ABSTRACT

IgMs* subunits with intact inter-μ chain disulfide bonds were isolated from specifically purified rabbit IgM anti-DNP antibody after mild reduction and alkylation, and separated from IgMs with broken inter-μ chain disulfide bonds by recycling gel filtration on Sepharose-4B. Quantitative measurements of the ability of these subunits to mediate hemolysis by complement show that both are hemolytically active. The hemolytic activity of the IgMs with intact inter-μ chain disulfide bonds is comparable to that of specifically purified IgG anti-DNP antibody, and is about 2,000 times less than that of intact IgM antibody. Loss of the inter-μ chain disulfide bonds leads to a further 2.5-fold reduction in activity.

The well-known loss of hemolytic activity which occurs when IgM antibodies are cleaved into IgMs subunits was studied quantitatively in specifically purified antihapten antibodies by Onoue, *et al.* (1965), and in anti-Forssman antibodies by Amiraian and Ferris (1968). In both studies, the results showed a total loss of activity within the limits of sensitivity of the methods employed. This seems odd in view of the structural similarity between IgMs and IgG antibodies, which are hemolytically active, albeit much less so that intact IgM antibodies.

Subsequant structural studies on the reductive cleavage of IgM (Beale and Feinstein, 1969; Mukkur and Inman, 1970; Egorov, *et al.*, 1971) have shown that the conditions used by earlier authors to produce IgMs break several disulfide bonds in addition to the inter-subunit bonds. Thus, it seems possible that the loss of hemolytic activity observed when IgM antibodies are reduced in this manner is due not to dissociation into IgMs but to additional structural changes in the IgMs. We have reinvestigated this problem with special attention to the integrity of the inter-μ chain disulfide bond(s) within IgMs, using specifically purified rabbit IgM anti-DNP antibodies.

MATERIALS AND METHODS

The production, purification, and characterization of rabbit IgM anti-DNP antibody have been described (Kuo, *et al.*, 1976). The same report also contains a description of the preparation of sheep erythrocytes with ε-DNP-aminocaproic acid and of the hemolytic assay employed in this study. The results of such assays are expressed in tems of AbH50 (Mayer, 1961): 1 AbH50 is difined as the amount of antibody required to lyse 1/2 of 10^9 cells under syandard conditions. Antibody concentrations were measured spectrophotometrically on the basis of the extinction coefficient $E_{278}^{1\,mg/ml}=1.30$

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* The nomenclature of immunoglobulins follows that recommended by the WHO Committee on Nemenclature of Human Immunoglobulins (*Immunochemistry* 7, 497, 1970). Other abbreviations: DNP-, 2,4-dinitrophenyl; DTT, dithiothreitol; SDS, sodium duodecyl sulfate; TRIS, tris-(hydroxymethyl)-aminomethane; TBS, 0.15 *M* NaCl buffered with 10 m*M* tris-HCl, pH 7.5.

given by McDonough and Inman (1970).

IgM antibody was reduced in 0.30 *M* TRIS-HCl, pH 8.0, with low concentrations of DTT as specified in the text, and alkylated at pH 7.0 with a 50% molar excess of Na-iodoacetamide. Hemolytic assays were performed after removal of the reducing and alkylating agents by dialysis for 24 h against 500 volumes of TBS, pH 7.5.

Reduced, alkylated IgM preparations were subjected to recycling gel filtration on a Separose-4B column (2.5×105 cm) in 0.5 *M* NaCl, buffered at pH 8.0 with 10 m*M* TRIS-HCl, to which 0.02% NaN_3 was added as a preservative. The column was operated at room temperature by upward flow at 14 ml/h. Effluents were analysed continuously at 280 nm in a UV absorbance detector (Chromatronix, Inc.) and collected in 5 ml fraction.

Products were characterized in physical-chemical terms by analytical ulyracentrifugation in a Spinco Model *E* ultracentrifuge equiped with absorption optics, monochromator, and photoelectric scanner, and by electrophoresis in polyacrylamide gel containing 0.1% SDS according to Weber and Osborn 1969), modified by omitting the stacking gels and reducing agents. To determine the relative concentrations of different components, the stained gels were examined in Gilford gel scanning system (Model 242) at 630 nm; areas under the peaks were measured by planimetry.

RESULTS

Effect of Low Concentrations of DTT—Reduction of rabbit IgM antibody at 1 mg/ml with DTT concentrations between 0.1 and 0.5 m*M*, followed by alkylation, produced mixtures of intact IgM, IgMs, oligomers and half molecules of IgMs, and some free polypeptide chains as previously observed for human IgM by Beale and Feinstein (1969). Our data showed very little dissociation at 0.1 m*M* DTT, suggesting that rabbit IgM may be somewhat more resistant to reductive cleavage than human IgM; however, this result may have been due in part to the presence of atmospheric oxygen in the solutions in this experiment. The extent of reduction and the loss of hemolytic activity are compared as a function of DTT concentration in Fig. 1, showing that the proportion of residual hemolytic activity decreases more slowly than the proportion of intact IgM molecules. This suggests that hemolytic activity is associated with at least some of the reduction products.

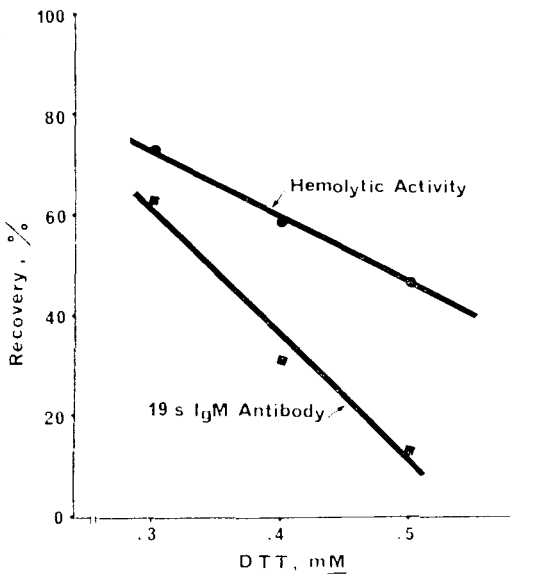

Fig. 1. Percentages of residual intact IgM (■) and hemolytic activity (•) remaining after reduction with varying concentrations of DTT, followed by alkylation with a 50% molar excess of iodacetamide.

Characterization of Cleavage Products—A sample of IgM antibody (0.42 mg/ml) was reduced under N_2 with 0.6 m*M* DTT, alkylated, and examined by gel filtration of Separose-4B. The chromatogram, shown in Fig. 2, indicates that most of the hemolytic activity of the mixture was associated with the first protein peak; slight but definite activity was also found in the second peak. The three peaks were pooled as indicated in Fig. 2 and concentrated by

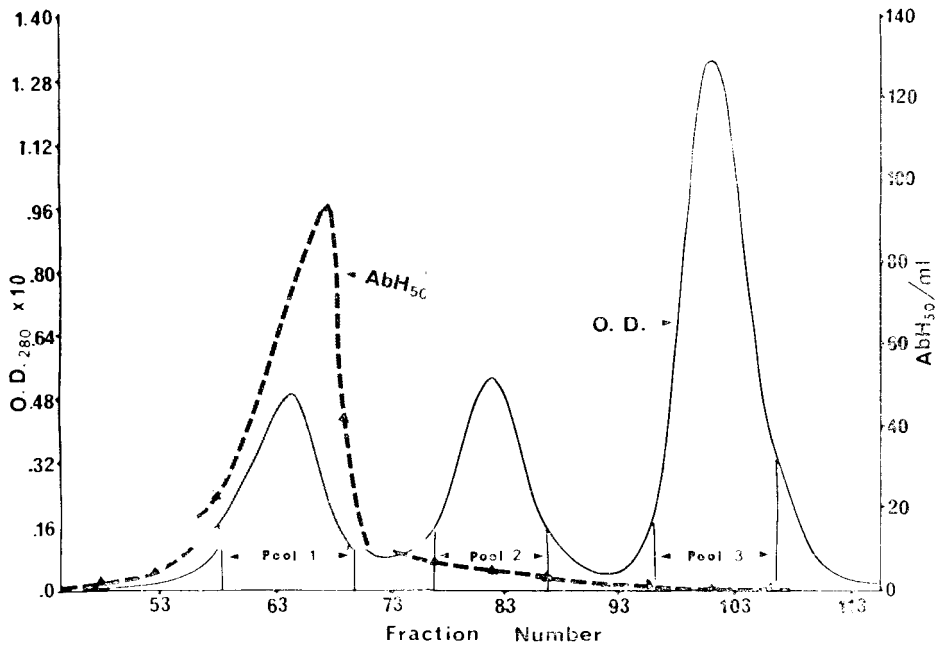

Fig. 2. Sepharose-4B chromatography of the products of reductive cleavage of IgM antibody with 0.6 m*M* DTT. *Solid line*: O.D.$_{280}$; ▲: hemolytic activity of fractions (5 ml).

ultrafiltration for ultracentrifugal analysis. Both pools 1 and 2 showed polydispersity, giving a broad boundary which sedimented at an average $S_{20,w}$ of 15.3 *S* for pool 1 and 4.4 *S* for pool 2; pool 3, which was devoid of hemolytic activity, contained no macromolecules absorbing at 280 *n*m.

As shown above, under the conditions of reduction, little intact IgM should have remained. The first peak observed by gel filtration should therefore contain primarily oligomers of IgMs; this expectation is confirmed by ultracentrifugal analysis. The coincidence between protein concentration and hemolytic activity in this peak suggests that the oligomers are active. When the hemolytic activity is expressed in terms of protein concentration, the protein in this peak is found to to be only half as active as intact IgM antibody; thus the smaller oligomers of IgMs are less effective at mediating hemolysis than the native pentamers.

The polydispersity and low average sedimentation coefficient of pool 2 suggest the presence of half molecules of IgMs, formed by reversible dissociation of IgMs molecules whose inter-μ chain disulfide bonds had been broken; such dissociation has been described at the protein concentration prevailing in our analysis by Egorov, *et al.* (1971). In addition, this pool might also contain intact IgMs and traces of trailing oligomers from the preceding peak. From this evidence, it is impossible to tell whether the hemolytic activity associated with this peak is due to intact IgMs, its half molecules, or contaminating oligomers. This question was resolved by recycling gel filtration.

Another sample of IgM antibody (1.2mg/ml) was reduced under N_2 with 0.6 m*M* DTT, alkylated, and applied to the Sepharose-4B column. The first half of the second peak was recycled 4 time to produce the chromatogram in Fig. 3, which shows two clearly though in-

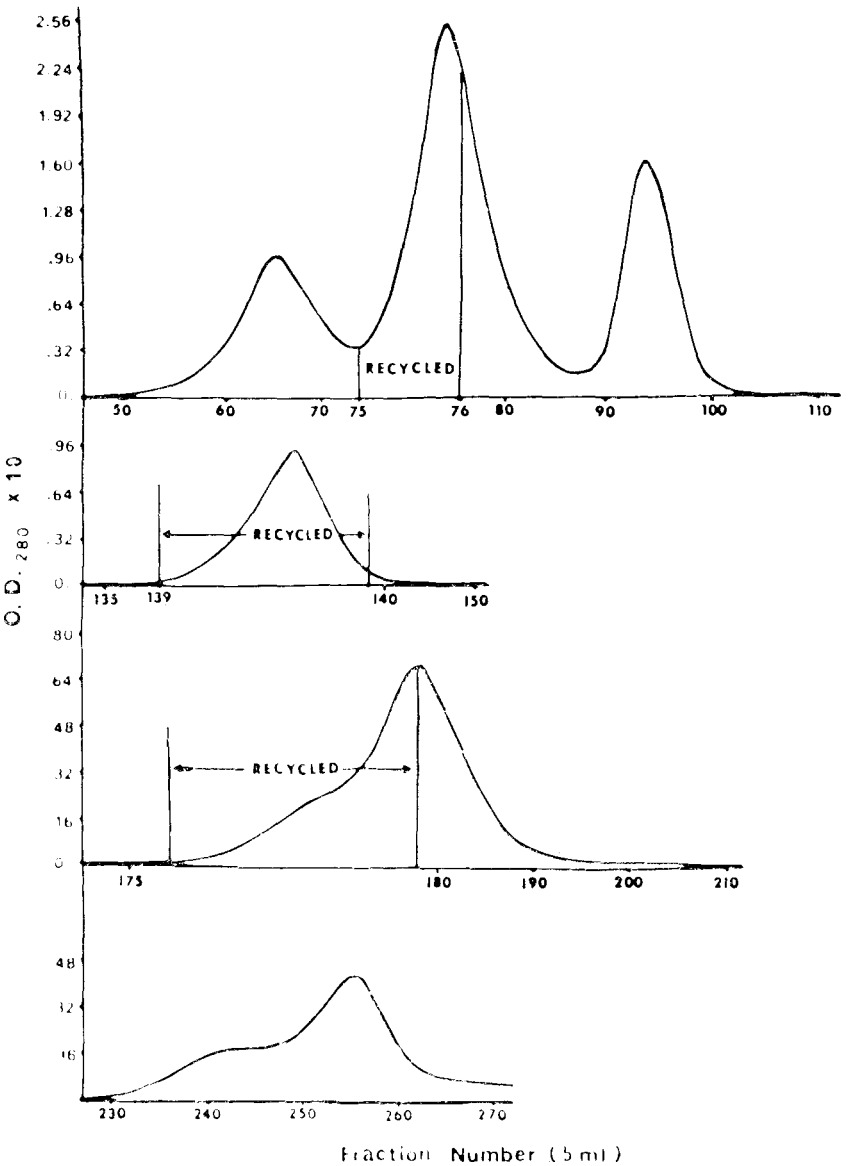

Fig. 3. Recycling gel filtration of IgMs on Sephaorse-4B.

completely resolved peaks in the 4th cycle, which were eluted. Individual fractions were dialysed against TBS in the cold, and their protein contents were determined from O. D. measurements at 230 nm on the basis of an extinction coefficient $E_{280}^{1\,mg/ml}=7.37$, measured on a sample of intact IgM antibody. Hemolytic assays were performed on every 3rd fraction, and the results were expressed as specific hemolytic activity, AbH50/mg of protein.

The results, superimposed on the elution pattern in Fig. 4, demonstrate the presence of 2 species of subunits, both of which are hemolytically active although to different degrees. We hypothesized that the first peak, with a hemolytic activity of about 5 AbH50/mg, represents IgMs with intact inter-μ chain disulfide bonds, whereas the the second peak, with a

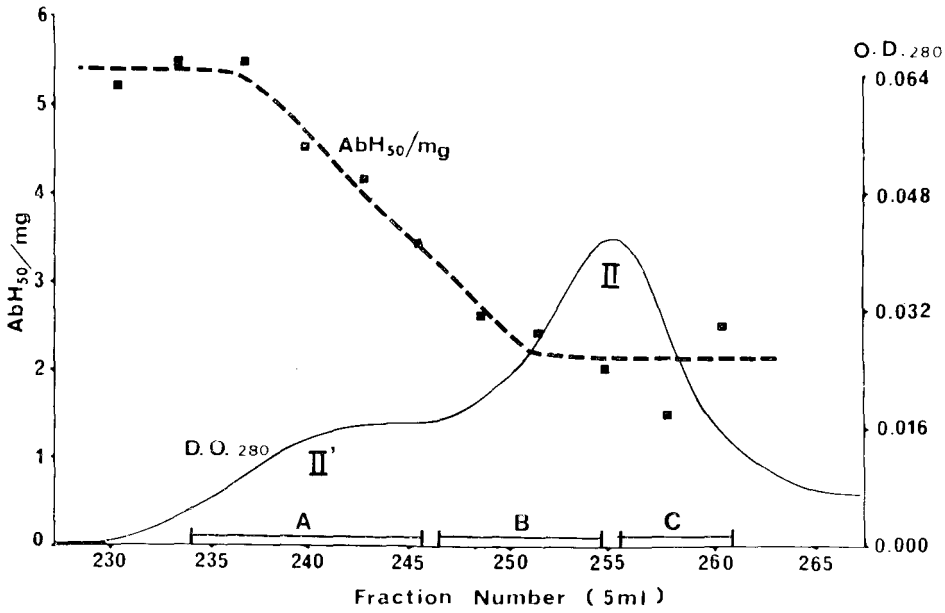

Fig. 4. Hemolytic activity of IgMs isolated by recycling gel filtration of Sepharose-4B. *Solid line*: O. D.$_{280}$; ■: specific hemolytic activity per mg of protein.

hemolytic activity of about 2 AbH50/mg, represents reversibly dissociating IgMs molecules with broken inter-μ chain disulfide bonds, which were shown by Solheim and Harboe (1972) to retarded during gel filtration.

To test this hypothesis, we pooled fractions from this run as shown in Fig. 4. Each pool was concentrated by ultrafiltration to 1 ml and subjected to SDS-polyarcrylamide gel electrophoresis in order to determine the number and molecular weight(s) of its constituent(s). The following molecular weight markers were used to establish the calibration curve: intact IgM antibody, specifically purified rabbit IgG anti-DNP antibody, crystallized bovine serum albumin showing monomers, dimers, and trimers; and β-lactoglobulin.

The stained gels are shown in Fig. 5. Pool A shows a single protein, migrating at a rate corresponding to a molecular weight of 180,000; pool C also shows a single protein except for a trace of slower material, but its mobility corresponds to a molecular weight of 88,000; pool B is a mixutre of the two. All 3 pools show some high molecular weight material which does not enter the gel. We take this to represent aggregates formed during concentration, as its concentration increases with increasing total protein concentration; since Fig. 4 shows decreasing hemolytic activity per mg protein in pools A, B, and C, it is unlikely that the aggregates account for this activity.

DISCUSSION

In contrast to the observations of earlier authors (Onoue, *et al.*, 1965; Amiraian and Ferris, 1968), we find that mild reduction and alkylation of rabbit IgM antibody does not lead to a total loss of hemolytic activity. The results in Fig. 1 demonstrate that the residual hemolytic activity in partially reduced IgM preparations cannot be attributed solely to residual intact IgM antibody. Upon fractionation of the mixture, we find that most of the remaining hemolytic activity is due to oligomers of IgMs; but monomeric IgMs retains a distinct

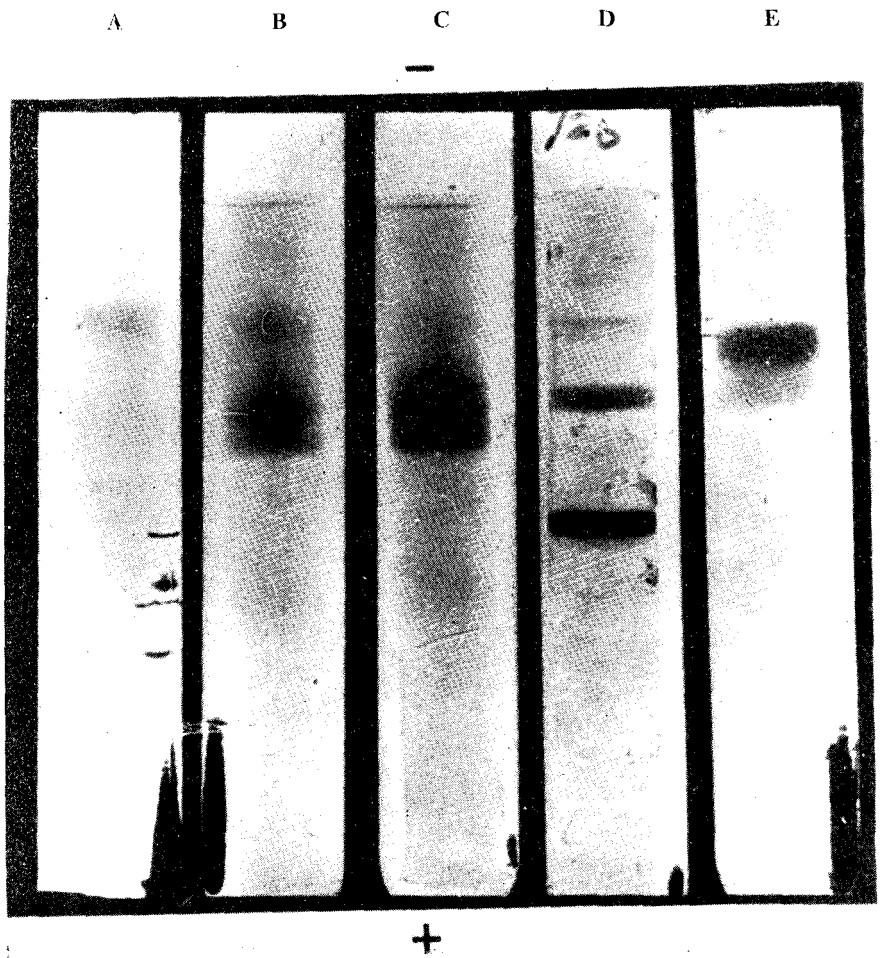

Fig. 5. SDS-polyacrylamide gel electrophoresis patterns of IgMs pooled as shown in Fig. 4 (Gels A, B, C). *Gel D*: Bovine Serum Albumin. *Gel E*: Rabbit IgG anti-DNP antibody. Traces of protein which did not enter gels A-C probably represent aggregated material produced during concentration.

hemolytic activity whose extent depends on the integrity of the inter-μ chain disulfide bond(s). The specific hemolytic activity of IgMs with one or both inter-μ chain intact is in the range observed for specifically purified IgG anti-DNP antibody (Hoffmann, 1974), although it amounts to only 0.06% of the 8380 AbH50/mg observed for intact IgM antibody (Kuo, *et al.* 1976). When the inter-μ chain disulfide bonds are briken, a further 2.5-fold drop in hemolytic activity is observed. The resulting activity of 2 AbH50/mg protein must be characteristic of the reversibly associating half IgMs molecules, since our preparation of this material did not show free H or L chains on SDS-polyacrylamide gel electrophoresis (Fig. 5).

The failure of previous authors to detect residual hemolytic activity in reduced, alkulated IgM antibody preparation may have been due to one or both of two causes: (1) assay sensitivity, and (2) conditions of reduction.

Both Onoue, *et al.* (1965) and Amiraian and Ferris (1968) used 0.1 *M* 2-mercaptoethanol for reduction: on the basis of the findings of

Mukkur and Inman (1970), one would expect a total of 3 disulfide bonds per IgMs molecule to be broken under these conditions. Mukkur and Inman (1970) found that such reduction led to the release of about half of the light chains in 6.7 *M* guanidine-HCl and concluded that one of the μ-L chain disulfide bonds is more labile to reduction than the other μ-L chain or the second intra-subunit μ-μ chain bond. Since we isolated IgMs molecules with both μ-μ chain disulfide bonds broken which did not liberate L chains in SDS, it seems to us that their observation may be due to disulfide interchange between a broken μ-μ and one of the μ-L chain bonds.

In any event, it appears that reduction with 0.1 *M* 2-mercaptoethanol would produce IgMs lacking either both inter-μ chain disulfide bonds or one μ-μ and one μ-L chain bond. Our results show that IgMs antibody with both inter-μ chain bonds broken shows a 4,000-fold reduction in hemolytic activity compared to intact IgM antibody. We have no data on IgMs molecules in which one inter-μ and μ-L chain bond are broken, but it seems safe to assume that such molecules would be no more active that IgMs with all inter-chain disulfide bonds intact, which we found to be 1600 times less active than intact IgM antibody. We were able to measure these low activities on isolated preparations of the subunits, a procedure not attempted by Onoue, *et al.* (1965) or Amiraian and Ferris (1968).

It is not immediately evident why IgMs with broden inter-μ chain disulfide bonds should lose so much of its hemolytic activity compared to IgMs with one or both inter-μ chain disulfide bonds intact. Perhaps a clue to this problem may be found in the observation of Egorov, *et al.* (1971) that intact IgMs shows an $s^{\circ}_{20,w}=7.3\,S$, compared to 6.7 *S* for the associated form of IgMs with broken inter-μ chain disulfide bonds. Assuming no change in molecular weight, their results suggest that intact IgMs is more compact than IgMs with broken inter-μ chain bonds. The loosened tertiary and quaternary structure implied by this result may account in part for the loss of hemolytic activity.

The great loss of hemolytic activity on conversion of IgM antibody into IgMs suggests a requirement for the formation of clusters of several IgMs molecules for the initiation of the complement sequence, as is the case for IgG (Borsos, *et al.*, 1968; Thompson and Hoffmann, 1974). The fact that IgMs molecules with intact inter-μ chain disulfide bonds have about as much hemolytic activity as IgG antibody supports the inference that the functional differences between IgM and IgG antibodies are due primarily to the fact that IgM is pentameric and not to any fundamental differences in premary structure. This inference was first suggested from the findings of Ishizaka, *et al.* (1968) that complexes of soluble antigen with IgM antibody were less efficient at fixing complement than those formed with IgG antibody, in contrast to the situation which prevails with particulate antigens.

If there is a requirement for the formation of clusters of IgMs molecules in order to initiate the complement sequence, an additional explanation for the lower activity of IgMs with broken inter-μ chain disulfide bonds may be provided by the fact that such molecules would exist largely as half IgMs molecules in the concentration range (0.02-0.08 mg/ml) employed in the hemolytic assay (c. f. Egorov, *et al.*, 1971); this would pose an additional barrier to the assembly of an active cluster of IgMs molecules.

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IgM 抗體小單元對補體作用產生之溶血

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摘 要

兎子對抗二硝基醛 (Dinitrophenol) 的免疫球蛋-M (Immunoglobulin M, IgM) 抗體是用專一元提煉法從抗血清分離出來。IgM 抗體含有五個相同的小單元 (Subunit) 經由雙硫鍵 (Disulfide bond) 環連起來。這種小單元稱的 IgMs，利用暖和的還原作用 (Reduction) 及烴化作用 (Alkylation) 使雙硫鍵體折斷而把 IgMs 彼此分散開來，然後這些散開的 IgMs 小單元再用重複凝膠濾過法 (Gel filtration) 把鏈間雙硫鍵 (Inter chain disulfide bond) 完整及折斷的 IgMs 再彼此分離出來。

定量測定結果顯示這兩種 IgMs 都具有與補體 (Complement) 作用產生之溶血能力 (Hemolytic Activity)。不過，這些鏈間雙硫鍵完整的 IgMs 之溶血能力只有原來 IgM 抗體之溶血能力之二千分之一，同時約相等於 IgG 抗體之溶血能力。鏈間雙硫鍵折斷之 IgMs 的溶血能力又比鏈間雙硫鍵完整的 IgMs 要小 2.5 倍。