Dissociation of Noncovalently Held Polymers of Human and Rabbit IgM[1] (34857)

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(Introduced by W. J. Payne)
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IgM is the largest of the immunoglobulins. It has a molecular weight of about 900,000, and appears to comprise five structurally similar subunits (1).

When human IgM molecules are treated with mercaptoethylamine (2) they dissociate into their subunits, IgM_s. Under the buffer conditions usually employed, reaggregation of the subunits to IgM size generally is not observed. After minimal digestion with papain, subunit-like proteolytic fragments called IgM_p may be formed (3). These fragments do not tend to reassociate either. When joined by intersubunit disulfide bonds, it thus appears that the subunits which comprise IgM have little tendency to associate with one another noncovalently to form a molecule as large as intact IgM.

The sedimentation coefficient of IgM ranges from 17–19 S. However, upon isolation and centrifugation of IgM from pathological human serum, one or more components which sediments faster than IgM usually is observed in schlieren patterns (1–3). Immunological analyses may indicate the presence only of IgM. Apparently then, even though the subunits themselves do not tend to aggregate noncovalently, some type of polymerization of intact IgM occurs either in the serum or as a consequence of the handling procedures.

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In the work reported here, the nature of the bonds involved in aggregation of human IgM has been reinvestigated. In addition, similar studies were done for the first time with purified rabbit IgM.

Materials and Methods. Plasma was obtained from patient Aug. who suffered from Waldenström's macroglobulinemia. IgM was prepared by the dropwise addition of the plasma to 10 times its volume of water. The precipitated protein was recovered by centrifugation at 13,200*g* for 10 min. The precipitate was taken up in 0.05 *M* tris (hydroxymethyl)aminomethane-0.50 *M* NaCl buffer, pH 8 (Tris–saline), or 0.32 *M* NaCl made 0.001 *M* in Na_3BO_3, pH 8 (0.32 SB), to a final volume about 75% that of the original plasma volume. After repeating this procedure, approximately 150 mg of the crude IgM were filtered through a column (2.5 × 125 cm) of either Bio-Gel P-200 equilibrated with 0.32 SB, or Sepharose 4B equilibrated with the Tris–saline buffer. The protein which constituted the leading two-thirds of the fraction represented by the peak of the eluted material from several columns was pooled, concentrated by Diaflo ultrafiltration through a UM-10 membrane, and then 150 mg were refiltered through a column (2.5 × 125 cm) of Bio-Gel P-300. The protein representing the eluted material constituting the leading edge of the single peak was pooled to obtain human IgM enriched in the faster sedimenting aggregates.

IgM was also prepared from the pooled sera of nonimmunized rabbits as previously described (4). Immunoglobulins and some other serum proteins were precipitated with a final concentration of 18% Na_2SO_4. The insoluble material was dissolved in a buffer

comprising 0.16 *M* NaCl–0.001 *M* Na_3BO_3, pH 8, and reprecipitated. The precipitate was taken up in a small volume of the buffered saline solution and dialyzed overnight against a solution of 0.0175 *M* $NaH_2PO_4 \cdot H_2O$ containing 0.1325 *M* NaCl at pH 6.9. After loading the material onto columns of DEAE–cellulose (Whatman DE 52), a mixture of proteins was eluted with the phosphate-buffered saline solution and discarded. An IgM-enriched fraction was recovered with 0.0175 *M* $NaH_2PO_4 \cdot H_2O$ solution made 0.1525 *M* in NaCl, pH 6.9. The fraction was purified further by rechromatography on DEAE–cellulose equilibrated with a solution of 0.15 *M* NaCl buffered with 0.05 *M* Tris, pH 8.6. Purified IgM was prepared by double filtration through Bio-Gel P-200 of the material from the DEAE–cellulose column using 0.32 SB as the dispersal phase.

Immunoelectrophoresis was done on glass plates (11.5 × 5.7 cm) covered with 1% agarose to give a gel depth of about 2 mm. The gel was buffered with 0.05 *M* barbital, pH 8.6. Rabbit IgM was electrophoresed at 160 V for 180 min using 0.05 *M* barbital, pH 8.6, as conducting buffer. The plates were developed with anti-whole rabbit serum antiserum (AWRS), an antiserum specific for the Fcγ region of IgG (γG) and an antiserum directed against rabbit immunoglobulins (IM).

The immunoelectrophoretic analysis of the human IgM was done on the glass plates layered with 0.85% ionagar buffered with 0.1 *M* barbital containing 0.4 *M* NaCl at pH 8.2. The unusually high salt content of the gel was required to prevent the euglobulin IgM from becoming insoluble in the gel. Electrophoresis was done at 160 V for 90 min with a conducting buffer of 0.1 *M* barbital adjusted to pH 8.2. The plates were developed with anti-human serum antiserum and anti-homologous IgM antiserum made specific for IgM by exhaustive absorption with human IgG (anti-Aug.).

Protein samples were centrifuged in a Spinco Model E analytical ultracentrifuge which was equipped with schlieren optics and an electronic speed control. All examinations were made at 20° and 60,000 rpm using the An-H rotor.

The concentration of proteins was determined in the Hitachi Perkin-Elmer Model 139 spectrophotometer. $E^{1\%}_{1\,cm,\,280\,m\mu} = 12$ (1) was used for calculation of concentrations of both human and rabbit IgM.

For studies involving the dissociation of polymers, samples of IgM were dialyzed in 4 *M* guanidine-HCl (Mann, Ultra-Pure) solutions, pH 7, for 12 hr at 4°. Prior to dialysis, the protein concentration was adjusted to 6.0 mg/ml.

Results. The purity of the IgM samples was determined immunoelectrophoretically. With respect to the human IgM, a single precipitin line raised with anti-IgM antiserum identified the protein as IgM, and a single line raised with anti-whole human serum antiserum indicated the absence of any other serum components (Fig. 1A). Similarly, only one line appeared when the electrophoresis plate of rabbit IgM was developed with either anti-whole rabbit serum or anti-rabbit immunoglobulins anti-sera (Fig. 1B). The absence of IgG was also demonstrated.

The schlieren patterns of the human IgM preparation are shown in Fig. 2A. The IgM was highly enriched in polymers. The material which sedimented at 15.4 S was IgM and that sedimenting at 20.5 S and 24.5 S represented various aggregated forms of IgM. When a portion of the IgM which was dialyzed against 4 *M* guanidine–HCl solution was examined by ultracentrifugation (Fig. 2B), most of the protein sedimented as a single component. It was assumed that the main component (Fig. 2B) represented IgM, and further that most of the aggregated protein had dissociated. Another preparation of human IgM was dialyzed against 6.7 *M* guanidine solution. At this concentration of dissociating agent, most of the aggregated material dissociated; but a very small portion still remained in the polymeric form.

Attempts were made to dissociate the polymers using the less stringent conditions of low ionic strength buffers adjusted to acid pH. The experiment initially was attempted in 0.1 *M* sodium acetate buffer, but the protein was extensively denatured and became

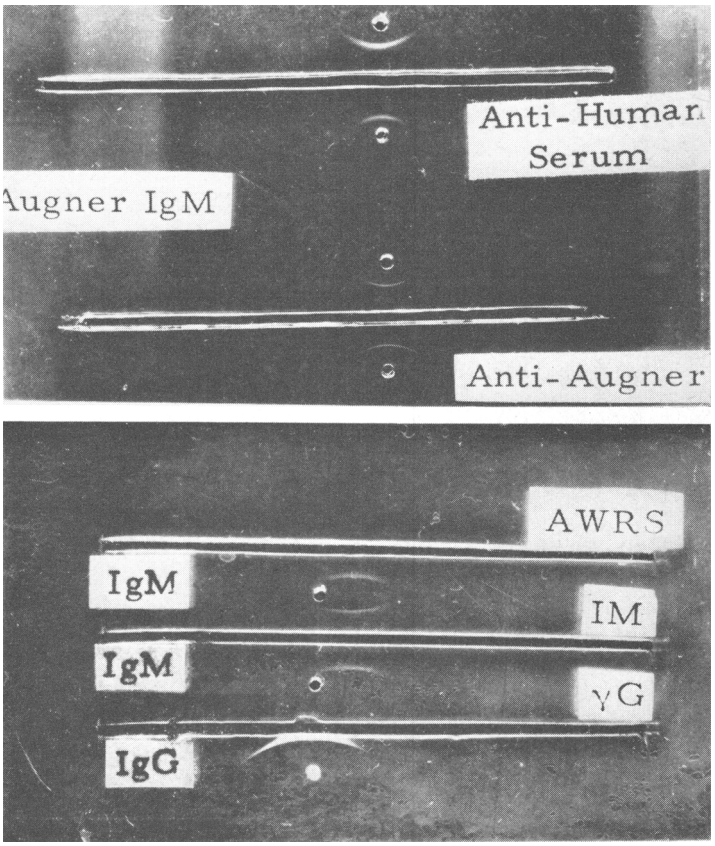

FIG. 1. Immunoelectrophoretic analyses of human and rabbit IgM preparations. Top. Human IgM (Aug.). The anode is at the left. Bottom. Rabbit IgM. The anode is at the right. Conditions and the identification of the antisera are given in *Materials and Methods*.

insoluble. Another sample rich in polymeric forms was dialyzed against 0.32 SB and the pH was lowered with 1 *M* HCl to values ranging from 1.9–4.0. When observed in the ultracentrifuge no change in the relative concentration of the components was noted.

The schlieren pattern shown in Fig. 3A is that of the rabbit IgM preparation. The major component with a sedimentation coefficient of 16.0 S was IgM. A minor component with a sedimentation coefficient of 26.9 S also was present, and was considered to be an aggregate of the IgM. After dialysis against 4 *M* guanidine–HCl solution, the IgM preparation again was examined in the analytical ultracentrifuge. The schlieren pattern is shown in Fig. 3B. The protein sedimented as a single component which apparently was nonaggregated IgM. Similar results were obtained when 6.7 *M* guanidine solution was the dispersal phase.

It was noted that both the human and the rabbit sample were able to repolymerize when the guanidine was gradually removed by dialysis against 0.32 SB (schlieren patterns not shown). There was, however, extensive loss of material due to insolubility.

Discussion Aggregates frequently are observed in preparations of human IgM (1–3). Kunkel (5) was not able to dissociate apparently similar IgM aggregates with urea, but he did observe reduction in their size after treatment with mercaptan reagents. He concluded that the faster-sedimenting components were polymers of the IgM that were covalently bound. In contrast, Suzuki and Deutsch (6) demonstrated that a certain degree of dissociation was brought about by

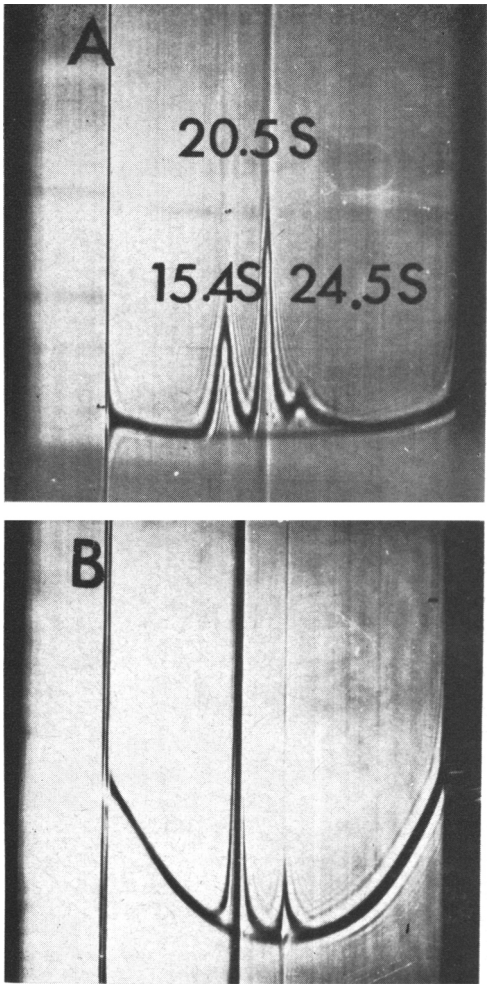

FIG. 2. Schlieren patterns of human IgM. A. IgM was centrifuged in 0.32 SB at 7 mg/ml. Picture was made 12 min after reaching 60,000 rpm. B. IgM was centrifuged at a concentration of 7 mg/ml in 4 *M* guanidine–HCl solution, pH 7. Photograph was made 80 min after reaching 68,000 rpm. Sedimentation is from left to right.

urea. They concluded that the polymers were noncovalently associated.

To our knowledge, no similar studies have been carried out with rabbit IgM. Since it was possible to prepare rabbit IgM enriched in the faster-sedimenting aggregates, we decided to determine if these aggregates, and those from human IgM preparations, were covalently- or noncovalently-linked polymers.

After dialysis against 4 *M* guanidine–HCl solutions it was readily demonstrated by analytical ultracentrifugation that the faster-sedimenting material in the preparation of human IgM had largely dissociated. Since IgM itself does not dissociate in 5 *M* guanidine solution (7), the material must have depolymerized to the more slowly sedimenting IgM. A small amount of aggregate failed to dissociate. Most likely, this could be attributed to use of an insufficient concentration of guanidine in the solution. It was also

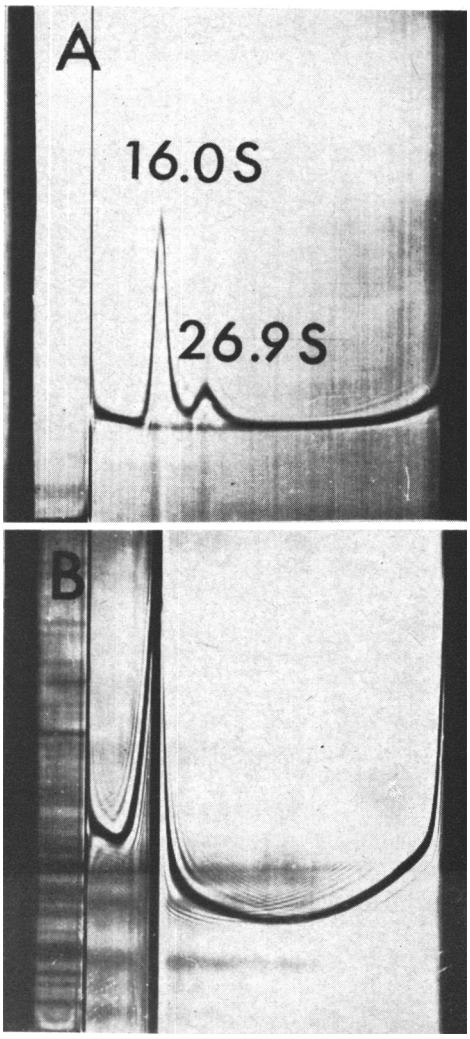

FIG. 3. Schlieren patterns of rabbit IgM. A. IgM was centrifuged in 0.32 SB at 6 mg/ml. Photograph was made 16 min after reaching 60,000 rpm. B. IgM was centrifuged in 4 *M* guanidine–HCl solution, pH 7. Photograph was made 48 min after reaching 60,000 rpm. Sedimentation is left to right.

possible that some of the aggregates were associated covalently. Due to the lack of a sufficient quantity of highly aggregated IgM, these possibilities were not investigated further.

When the rabbit IgM preparation was treated similarly, it was observed in the schlieren pattern that all the aggregated material had dissociated to a single component, ostensibly IgM. Thus, it appeared that aggregates of rabbit IgM comprise IgM molecules joined by noncovalent interactions.

Our results allowed us to support the conclusions of Suzuki and Deutsch (6) and Franklin (8) that the faster-sedimenting material frequently associated with human IgM preparations comprises IgM molecules held together mainly by noncovalent forces. A similar situation also obtained in the case of aggregates of rabbit IgM, except that all the bonds holding together the aggregated IgM in the preparations studied evidently were noncovalent.

Summary. IgM was prepared from normal rabbit serum and from the serum of a human patient (Aug.) suffering from Waldenström's macroglobulinemia. In each of the preparations, there were one or more components which sedimented in the analytical ultracentrifuge much faster than IgM. Such large components found in preparations of human IgM have been attributed in the past to both covalently and noncovalently associated polymers of IgM. Studies with the rabbit system have not previously been done. It was found that most of the faster-sedimenting materials in the preparations of human IgM, and all of that in preparations of rabbit IgM, dissociated to IgM size in 4 *M* or 6.7 *M* guanidine–HCl solutions, pH 7. It was concluded that all of the faster-sedimenting material accompanying the rabbit IgM, and most of that attending the human IgM, was an aggregation of the respective IgM held by noncovalent bonds.

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