OCCURRENCE AND PROPERTIES OF A MACROGLOBULIN DIMER IN SOME HYPERGLOBULINEMIC SERA*

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Isolated Waldenström macroglobulins or γM-globulins usually are of high electrophoretic homogeneity but contain three molecular weight components sedimenting at 18S to 19S; 25S to 29S, and 32S to 38S. They comprise from 65 to 85, 10 to 20, and 3 to 8%, respectively, of the isolated protein (1, 2). Small amounts of γG-globulins are also present as a contaminant in these systems. This latter material is not a 6S to 7S subunit of the γM-globulins and it may be readily removed by appropriate gel filtration.

A significant number of Waldenström macroglobulinemic sera are seen that possess a component sedimenting in the 21S to 22S range. The crystalline γM-globulin isolated by Kratochvil and Deutsch (3) is an example of this. When present, this component usually comprises only small amounts of the total γM-globulins. One such γM-globulin preparation, however, was found to be composed of over 40% of this 22S material and was studied in some detail along with two others that contained smaller amounts. The results of these studies indicated that this material was a dimer of the 19S components and that the complex was joined by noncovalent bonds. The 22S component is reduced to 7S subunits by sulfhydryl reagents as is the 19S material and also reaggregates in part when the sulfhydryl reagents are removed. Some of the physical-chemical and immunological properties of this γM-globulin system are delineated.

EXPERIMENTAL

Three macroglobulinemic sera containing the unusual 22S component were available. Their euglobulin fractions precipitating after dilution of these sera with 10 volumes of distilled water were removed and dissolved in 0.15 M NaCl and then reprecipitated by dilution. After 3 to 4 such precipitations, 1% solutions of the proteins in 0.15 M NaCl were lyophilized or maintained as solutions near 1°C. The small amount of contaminating γG-globulins in the preparation was removed by passage of the neutral solution of the protein in 0.15 M NaCl over a Sephadex G-200 column.

The reductive conversion of the γM-globulin to 8S material by 0.1 M solutions of 2-mercaptoethanol and of cysteine were carried out at pH 7.8 to 8.0 at room temperature (4).

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When stabile 7S subunits were desired the reduced protein was alkylated by adding a two-fold excess of iodoacetoamide at pH 8. Reaggregation of the nonalkylated 8S subunits of the γM-globulins was carried out by removing the sulfhydryl reagent by extensive dialysis at 2 to 4°C against 0.2 ionic strength, pH 7.4 potassium phosphate buffer or by passage of the protein solution over columns of Sephadex G-25 that had been equilibrated with this buffer.

A partial conversion of the reduced-alkylated 7S subunits to 3.5S material (5) was accomplished by dialysis overnight against pH 4, 0.02 ionic strength sodium formate buffer. The extent of conversion to 3.5S protein varied for different γM-globulins. The L and H chain material formed were separated by passage over columns of Sephadex G-100 at pH 4. Since the initial separation, particularly of residual 7S and H chain material, was not complete, the H and L chain fractions in 0.10 to 0.15 ionic strength potassium phosphate buffer of neutral pH were further purified by passage over columns of Sephadex G-100, G-150, or G-200.

Moving boundary electrophoresis, diffusion, and sedimentation velocity and equilibrium centrifugation experiments employed the well known Spinco instruments. The velocity sedimentation studies were carried out in 0.2 ionic strength buffers. Potassium phosphate was employed at pH 7.4, sodium formate at pH 4, sodium acetate at pH 5, Tris-HCl at pH 8 to 9 and $NaHCO_3$—Na_2CO_3 at pH 10 to 11. Sedimentation rates were extrapolated to zero protein concentration. Unless otherwise indicated, the ultracentrifuge diagrams presented represent experiments that were carried out in the pH 7.4, 0.2 ionic strength potassium phosphate buffer and at rotor speeds of 59,780 RPM.

Starch gel electrophoresis employed the method of Smithies (6).

Double diffusion precipitin reactions in agar gel were performed by the Ouchterlony technique (7) and microimmunoelectrophoresis experiments utilized the procedure of Scheidegger (8).

The γG-globulin fraction of rabbit antisera to type I and II Bence-Jones proteins and to various macroglobulins was separated by chromatography on columns of DEAE-cellulose and by ethanol fractionation. Rabbit and monkey antibody (9) to macroglobulins was absorbed with a mixture of normal γG-globulins and type I and II Bence-Jones proteins to provide an antibody with γM-globulin H chain specificity.

RESULTS

Sedimentation Properties.—Ultracentrifuge diagrams illustrating the sedimentation properties of three γM-globulins preparations (RJ, CB, and CO) containing variable amounts of the 22S component and one (AU) lacking this material and representative of the more usual γM-globulin system are shown in Fig. 1. The RJ preparation is seen to contain relatively large amounts of the 22S material. Since this component was present in the serum source[1] at a concentration near 1.5%, it was utilized almost exclusively in the studies to be described.

The concentration dependencies of the various RJ γM-globulin components are shown in Fig. 2 and the compositions of the four γM-globulin preparations whose sedimentation patterns are presented in Fig. 1 are given in Table I. The 22S component of the RJ preparation constitutes from 35 to 40% of the γM-globulins. No change in the ratio of this component to the other γM-globulins was occasioned by repeated precipitations at low ionic strengths.

[1] We wish to thank Dr. David D. Kliewer, Kahului, Hawaii, for generous amounts of this serum.

It was of interest to determine whether the noted 22S component was similar to the one of similar sedimentation constant seen in some rheumatoid arthritic sera (10). Efforts to dissociate it into 19S and 7S components as described for the rheumatoid material (10) were unsuccessful. It was noted, however, that

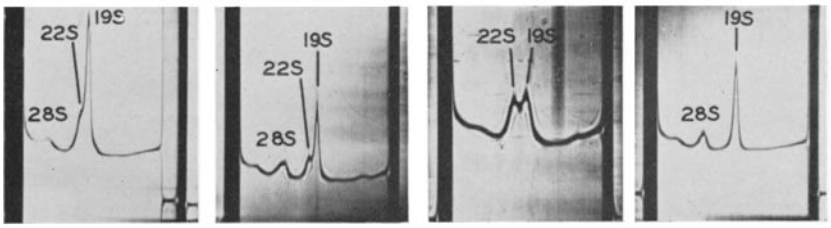


FIG. 1. Sedimentation diagrams of three γM-globulin preparations CO, CB, and RJ containing various amounts of the 22S component and one (AU) which lacks this material. The direction of sedimentation is to the left and the pictures shown were taken 32 min after reaching speed (59,780 RPM.).

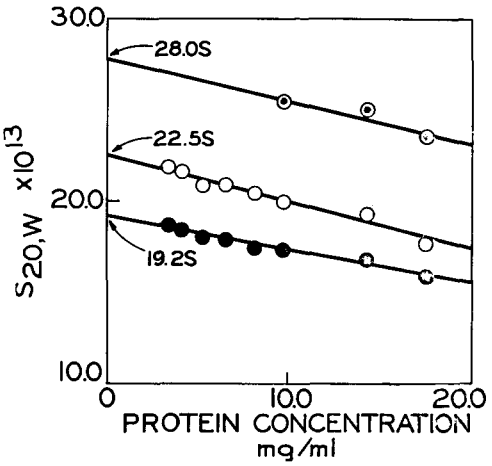


FIG. 2. The dependency of sedimentation rate on protein concentration for the various RJ γM-globulin components.

the 22S component of the macroglobulinemic sera could be reversibly converted to 19S material as a function of pH. Fig. 3 illustrates such effects. At pH 4 there is almost a complete disappearance of the 22S component with a corresponding increase in 19S material. The latter component constituted 38% of the γM-globulins at pH 7.4 and 84% at pH 4. The level of the 28S component showed no change over this pH range.

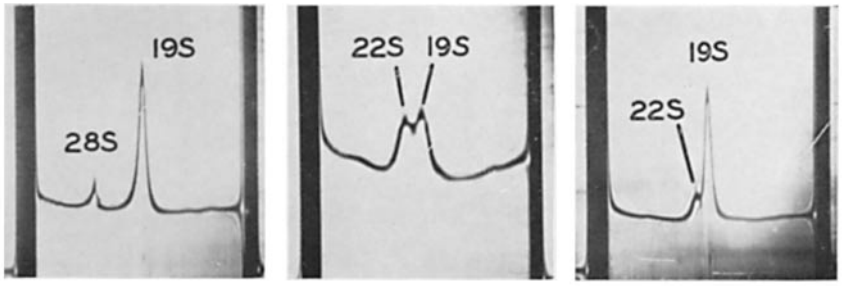
A partial conversion of 22S to 19S material occurs at pH 11. It is to be noted

from Fig. 3 that the 28S component is almost completely lost at this pH. When the acid or alkaline-treated RJ γM-globulins are brought back to pH 7.4, there is a restoration of the 22S and 28S components to their original level. A similar result was obtained for the 22S component of the CB γM-globulin preparation. The components of the usual γM-globulins show no change in the amounts of

TABLE I

The Composition of Four γM-Globulin Preparations

γM-globulins	Level of various components			
	19S	22S	28S	35S
	%	%	%	%
RJ	38.6	37.5	21.2	Trace
CO	72.9	18.4	8.1	Trace
CB	56.3	15.3	21.4	Trace
AU	67.4	0	23.5	9.1



pH 4.0 pH 7.4 pH 11.0

FIG. 3. The effect of pH on the sedimentation properties of RJ γM-globulin. The experiments were carried out on 1.2% solutions of protein at 20°C. The direction of sedimentation is to the left and all photographs were taken 24 min after reaching speed (59,780 RPM.).

the various components over the pH range 3 to 11. The acid stability of Waldenström γM-globulins has been previously noted (11).

The concentration of 22S material was also found to depend on temperature. Sedimentation studies were conducted on samples held for 12 to 15 hr at temperatures from 0° to 40°C. Some of the results obtained are shown in Fig. 4. It can be seen that at 40°C there is a decrease in the amount of the 22S component. At this temperature the 19S material comprises near 80% of the protein. The 28S and 32S components disappeared at 40°C but were reformed when the sample was brought to room temperature. This result indicates that

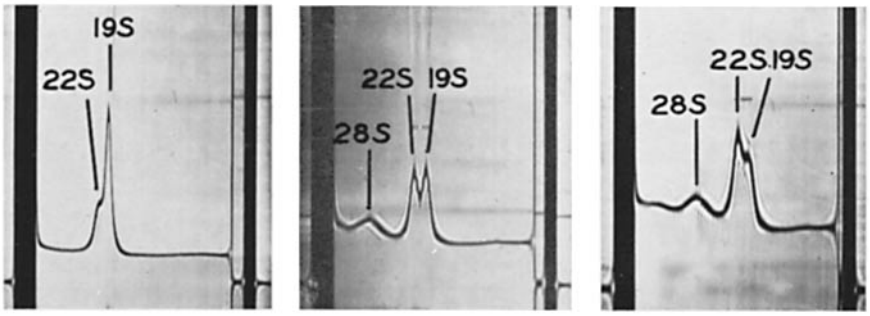


FIG. 4. The effect of temperature on the sedimentation properties of RJ γM-globulin. The experiments were carried out on 2% solutions of the protein at pH 7.4. The direction of sedimentation is to the left and the photographs were taken 24 min after reaching maximum speed (59,780 RPM).

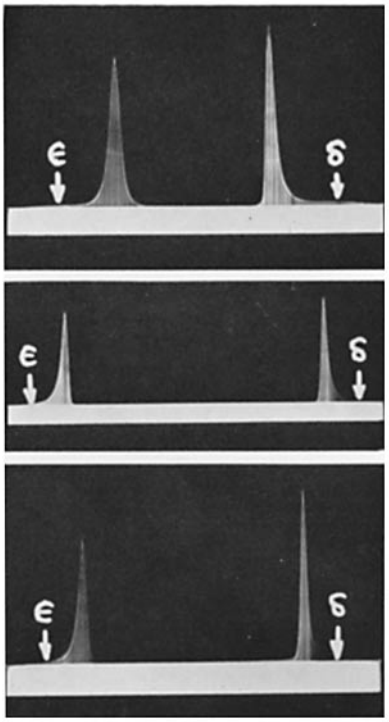


FIG. 5. Moving boundary electrophoretograms of the RJ γM-globulin preparation at 0.15 ionic strength (0.13 M KCl and 0.02 ionic strength buffers). Top, pH 3.9 sodium formate; middle, pH 8.0 Tris-HCl; and bottom, pH 10.0 Na_2CO_3-$NaHCO_3$. The times of electrophoresis were 300, 250, and 220 min at potential gradients of 2.0 v cm^{-1}.

the latter are also polymers of the 19S material. An increased amount of the 22S component is seen at 0°C.

Moving boundary electrophoretograms of the RJ γM-globulins in 0.15 ionic strength buffers of various pH are shown in Fig. 5. A single, symmetrical component is seen. The result for the pH 4 experiment indicates that no significant change in the acid-base properties occurs upon dissociation of the 22S into the 19S component and also suggests that the 19S components of the dimer are identical.

Attempts were made to determine if protein sulfhydryl groups were involved in the noted molecular transitions. The addition of iodoacetamide to a concentration of 0.02 M in the pH 4 and 10 buffer solutions did not affect the reformation of the 22S component when the pH was returned to neutrality

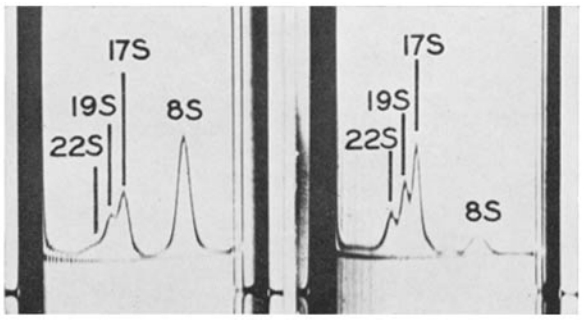


FIG. 6. The sedimentation diagrams of the reaggregated RJ γM-globulins produced from, left, L-cysteine; and right, 2-mercaptoethanol reduction products. The photographs shown were taken 24 min after reaching speed (59,780 RPM). The experiments were perforemed at 20°C in 0.15 ionic strength (0.02 μ, pH 7.4 potassium phosphate buffer and 0.13 μ KCl).

Increasing the ionic strength of the pH 7.4 potassium phosphate buffer to 0.75 by addition of KCl did not change the relative amounts of 19S and 22S components.

Dissociation-Reaggregation Studies.—The RJ γM-globulins are dissociated into 8S subunits on treatment with mercaptan and these aggregate upon removal of the mercaptan in a manner similarly noted for other γM-globulins (1, 3). The extent of reaggregation varies, however, depending on the type of mercaptan employed. Fig. 6 shows the ultracentrifuge patterns of reaggregated RJ γM-globulin in which L-cysteine and 2-mercaptoethanol had been used as reducing agents. A much larger amount of 8S protein, which will also be referred to as "reduced protein", remains in the cysteine treated system. It should also be noted that a component sedimenting near 17S and not present in the native material comprises the major portion of the reaggregated γM-globulins.

The 8S components of the reaggregated protein system can be readily separated from the macroglobulins by passage over Sephadex G-200 columns. In a

typical experiment, 40 ml of a 2% solution of RJ γM-globulins were treated with 0.1 M L-cysteine at pH 8.0 for 5 hr at room temperature. The reduced protein was then dialyzed exhaustively against pH 7.4, 0.15 ionic strength potassium phosphate buffer to remove the cysteine and to effect reaggregation of the sample. The dialyzed protein was then applied to a 4.5 × 65 cm column of Sephadex G-200 which was connected to another column of this size by capillary tubing. The elution pattern obtained is shown in Fig. 7 A. Ultracentrifugal analysis of the fractions isolated indicated that incomplete separation had been obtained. 3% solutions of the reaggregated fraction (300 mg) and of the 8S fraction (330 mg) whose separations are illustrated by Fig. 7 A, were further individually fractionated on 4.5 × 65 cm columns of Sephadex G-200 to provide the results shown in Figs. 7 B and 7 C respectively. The ultracentrifuge diagram of the main chromatographic component of Fig. 7 C is shown in Fig. 8 A and reveals a single boundary.

The reaggregated macroglobulins from the chromatogram shown in Fig. 7 B were brought to pH 4 and to pH 10. At these pH values they undergo breakdown into a series of lower molecular weight components instead of the conversion of 22S into 19S materials noted for the native protein. Furthermore, the temperature transitions noted for native γM-globulin components (see Fig. 4) no longer obtain. This indicates that the reaggregated γM-globulins do not possess all of the properties of their native counterparts.

When the isolated "reduced" proteins whose ultracentrifuge pattern is shown in Fig. 8 A are reduced with mercaptoethanol and then reaggregated by removal of the dissociating reagent, a series of reaggregation products are formed (see Fig. 8 C). All of these components, however, sediment below 19S.

Immunochemical Studies.—Ouchterlony reactions of the RJ proteins are presented in Fig. 9. It can be seen that the native system when reacted with antisera to a type I Bence-Jones protein gave two zones of precipitation whereas a type I γG-globulin gave one band showing a reaction of identity with one of the components of the γM-globulins. Absorption of the antibody preparation with the myeloma globulin removed the antibody directed against one of the macroglobulin components. A series of 10 type I myeloma γG-globulins gave a reaction of identity with the same precipitin band of the RJ γM-globulins.

The "reduced" and "reduced-alkylated" RJ γM-globulin gave a single zone of precipitation. However, it can be seen from Fig. 9 that the reaggregation products of the reduced protein (Fig. 8 C) and those of the native protein (Fig. 6) give very complex precipitin patterns, a minimum of three bands being evident.

The reactions of the RJ γM-globulins and various of its derivatives with rabbit antibody to the H chains of γM-globulins are shown in Fig. 9 C. A single zone of precipitation is given by each protein except in the case of the L chain proteins where no reaction occurred.

The complexities of the immunochemical reactions of the RJ proteins are

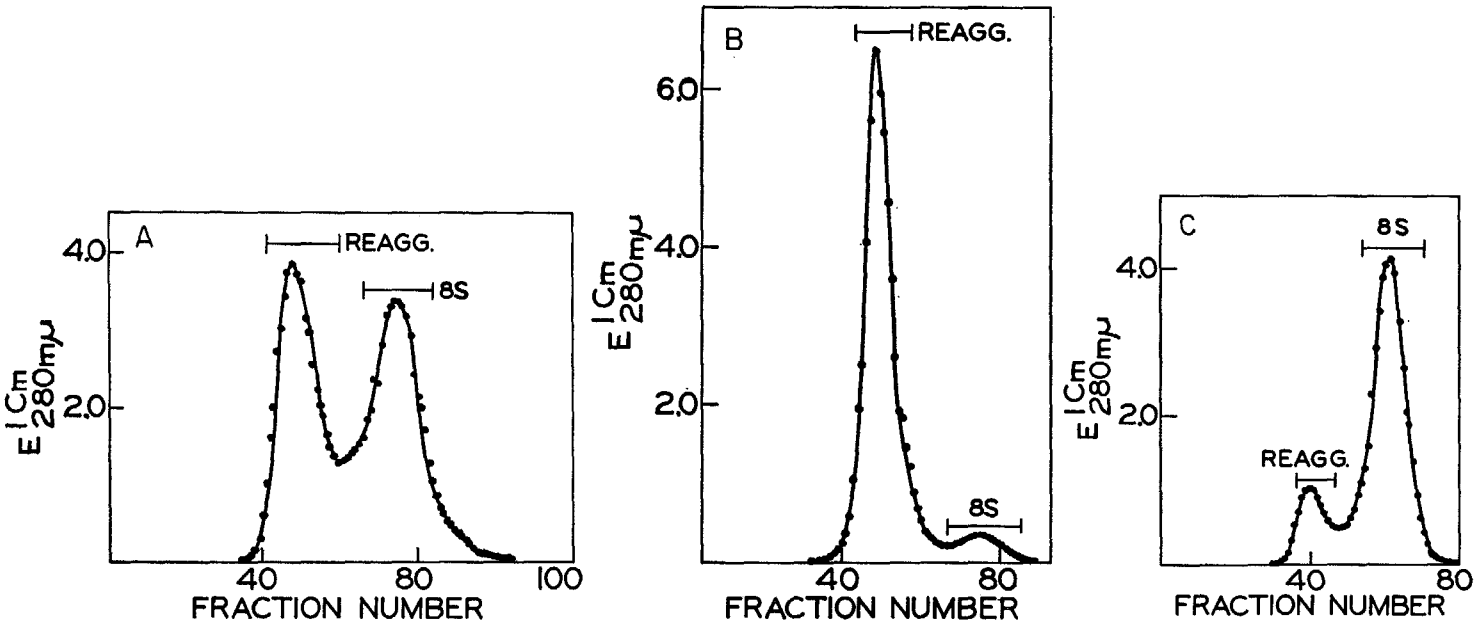


FIG. 7. Separation on Sephadex G-200 of the reaggregated L-cysteine produced subunits of RJ γM-globulin. A, complete system; B, rechromatography of the reaggregated (REAGG.) protein fraction of A; and C, rechromatography of the 8S protein of A.

further illustrated by immunoelectrophoretic studies, the results of which are presented in Fig. 10. A single zone of precipitation is given by the RJ γM-globulins in reactions with antibodies to native γM-globulins, to the H chains of γM-globulins and to Bence-Jones proteins (L chains) of serological type I. No precipitin reactions resulted with antisera to the Fc fragments of γG-globulins. The 6.7S reduced-alkylated proteins gave two zones of precipitation with the anti–γM-globulin and with the H chain antibody but not with L chain antibody.

The "reduced" (7.8S) and the "reduced-alkylated" (6.7S) subunits of the RJ γM-globulins showed marked differences in their reactions with antibody to

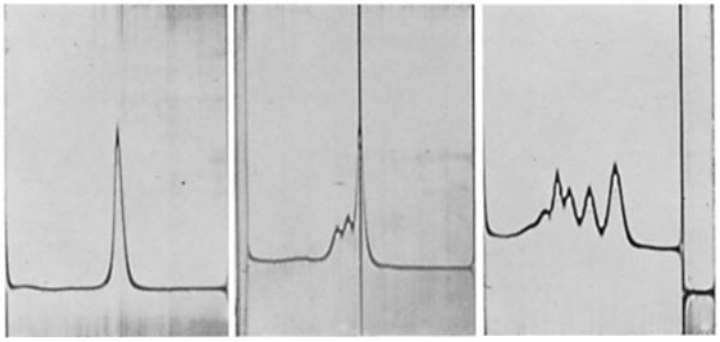


FIG. 8. A, Ultracentrifuge diagrams of the 8S; B, the reaggregated; and C, the reaggregation products of reduced 8S protein. The experiments were carried out at 20°C in 0.02 ionic strength pH 7.4 potassium phosphate buffer containing 0.13 M KCl. The photos shown were taken 32 min after reaching speed (59,780 RPM).

γM-globulins and to H chain protein. The results of Fig. 10 reveal that the "reduced proteins" (7.8S) gave a single zone of precipitation whereas two zones, one of which gave a reaction of partial identity, were noted for the "reduced-alkylated" (6.7S) protein. These latter proteins also showed a much longer zone of specific precipitation with H chain antibody than did the "reduced" (7.8S) proteins. Both proteins gave similar reactions with antibody to type I L chains. It appears that the 6.7S material may be formed by the splitting out of L chain material from the 7.8S molecule (12). This apparently results in the exposure of an antigenic grouping not available in the 7.8S protein. Although the result is not visible in the photo presented as Fig. 9 B, the 6.7S protein forms a slight spur with the 7.8S material when they are reacted with an antibody preparation to a type I Bence-Jones protein.

Further Ouchterlony experiments showed that each of the three γM-globulins shown in Fig. 1 which contained a 22S component gave two precipitin

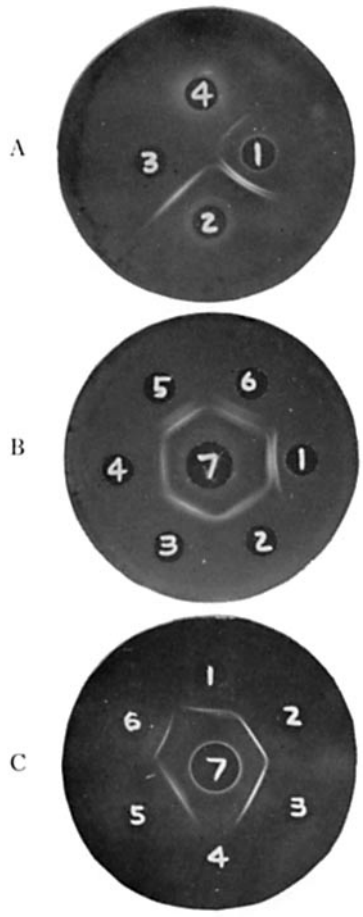


FIG. 9. Ouchterlony experiments with various RJ γM-globulin fractions.

Experiments	Contents of wells						
	Center (7)	1	2	3	4	5	6
A		γM	Ab_{BJ-I}	MM-I[4]	Absorbed Ab^{2}_{BJ-I}		
B	Ab^{1}_{BJ-I}	γM	MM-I[4]	6.71S	7.85S	Reagg.[5]	Reagg.[6]
C	Abγ M-H-chain[3]	γM	6.71S	7.85S	L chain	H chain	Reagg.[6]

[1] Ab_{BJ-I}, rabbit antibody to a type I Bence-Jones Protein.

[2] Absorbed Ab_{BJ-I}, rabbit antibody to a type I Bence-Jones Protein absorbed with the type I myeloma γG-globulin (MM-I).

[3] Abγ M-H chain, rabbit antibody to the H chain of γM-globulin.

[4] MM-I, a myeloma γG-globulin of serological type I.

[5] Reagg., reduced and reaggregated products of 7.85S protein.

[6] Reagg., reduced and reaggregated products of γM-globulins from which residual 7.85S protein had been removed.

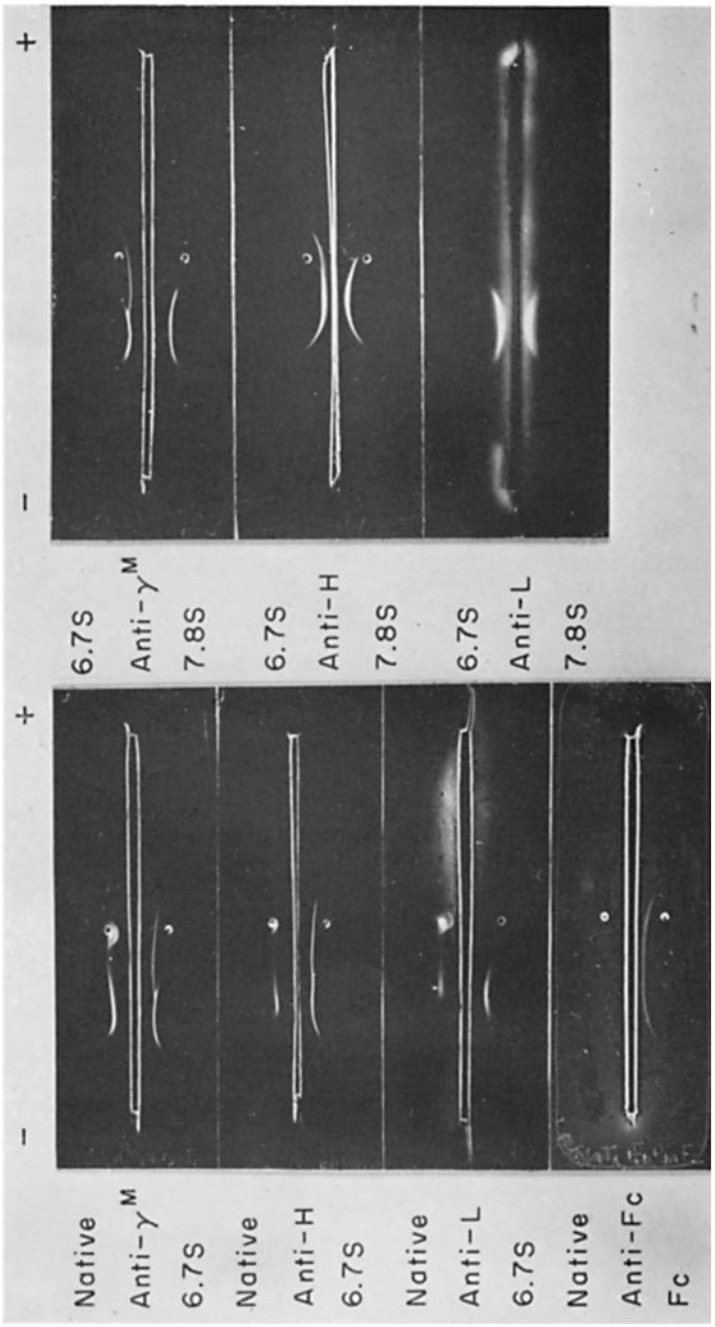


FIG. 10. Microimmunoelectrophoretic studies of the various RJ γM-globulin fractions.

bands with the antisera to the type I Bence-Jones protein employed whereas other γM-globulins gave only one band.

The dissociation of the 22S component by mild acid and alkali and by elevations of temperature suggested the possibility that this material was an antigen-antibody complex in which both components were 19S molecules. Various experiments were performed in attempting to prove this. If the 7S "reduced-alkylated" products of these γM-globulins retained either an antibody combining or an antigenic site present in the 19S component they might be able to form a complex with or modify the 22S component. The "reduced-alkylated" 7S subunits of the RJ γM-globulins as well as those of a series of other γM-globulin preparations not possessing the 22S component were added to the RJ γM-globulin system. The formation of an antigen-antibody complex in which one of the components was a 7S molecule and the other a 19S one would be expected to diminish the amount of 22S antigen-antibody complex. No change in the concentration of this component was noted in any case.

Additional experiments involved studies of mixtures of γM-globulin preparations lacking the 22S component with the RJ γM-globulins. If any of these had functioned as an antigen or as an antibody they might have increased the amount of 22S component. These experiments were uniformly negative.

Binary mixtures of 6 γM-globulins lacking the 22S material did not lead to the formation of such a component. Thus no evidence for an antigen-antibody reaction between different γM-globulins could be deduced from these experiments.

DISCUSSION

The 19S and 22S components studied in the present investigation behave like other γM-globulins in being readily converted by reducing agents into components sedimenting near 8S. These reduction products undergo extensive reaggregation when the mercaptan-dissociating reagent is removed and a large portion of the 22S material is reformed. However, it no longer behaves as 22S material in terms of its being able to reversibly dissociate into a 19S component.

It is not known whether 22S γM-globulin components are present in normal human serum nor is their biological significance known. This component is definitely not related to the 22S component seen in the sera of some rheumatoid arthritic patients. The patient RJ whose serum contained near 1.5% of the 22S material showed a disease process typical of Waldenström macroglobulinemia. The other macroglobulinemic patients whose sera showed much smaller amounts of this 22S material also could not be clinically distinguished from the usual macroglobulinemic syndromy.

The 22S γM-globulin component thus appears to be distinctly different from the usual γM-globulin seen in elevated amounts in macroglobulinemia. It is

difficult to relate these 22S components to those of the single acid-sensitive macroglobulinemic protein system studied by Rees (13).

The Ouchterlony result which showed the presence of two serological type I antigens in the RJ γM-globulins is difficult to explain. It appears to be related to the presence of a second type I antigen present in only one of the RJ γM-globulins. Antigenic components not present in native myeloma γG-globulins have been found in their pepsin digests (14, 15) and in Bence-Jones proteins (16). Other γM-globulins have been also previously shown to give complex immunochemical reactions with antibodies to Bence-Jones proteins and with antibodies to Fab fragments of γG-globulins (9). It is possible that the type immunochemical reactions experienced with the γM-globulins containing 22S components are indicative of availability of a L chain antigenic component which is buried in the γG-globulin molecule.

SUMMARY

A 22S human γM-globulin component has been subjected to various study. It appears to be a dimer of 19S proteins and can undergo reversible molecular transitions which are both pH and temperature dependent. The 22S material regenerated from its 8S subunits no longer undergo similar transformations. Efforts to demonstrate that the 22S material was an antigen-antibody complex of 19S molecules were unsuccessful. Complex precipitin reactions in gels are given by macroglobulins containing this component with antibody to L chains.

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