ANTIGENIC RELATIONSHIPS BETWEEN IMMUNE GLOBULINS AND CERTAIN RELATED PARAPROTEINS IN MAN*

By EDWARD C. FRANKLIN,‡ M.D., and DENIS R. STANWORTH,§ Ph.D.

(From the Department of Medicine and Rheumatic Diseases Study Group, New York University School of Medicine, and the Third Medical Division, Bellevue Hospital Center, New York)

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It is now generally accepted that the serum antibodies in man and several other species are associated with a group of structurally and functionally closely related proteins known collectively as the "immune globulins" (1, 2). In man these consist of the two major molecular size types of γ-globulins, *i.e.*, 7S and 19S γ-globulins, as well as the β_{2A}-globulin recently isolated by Heremans and coworkers (3). It now appears probable that the 19S γ-globulin is identical to the β_{2M}-globulin defined by immunoelectrophoresis (2). Certain proliferative states of the plasmocytic and lymphocytic cell systems are associated with the production of a group of closely related paraproteins. These include the γ- and β-myeloma proteins associated with multiple myeloma and a group of high molecular weight macroglobulins found in macroglobulinemia of Waldenström. In addition, the antigenically related Bence Jones proteins of smaller molecular size are frequently seen in the urine in both conditions (1, 2).

While each of these proteins possesses a certain degree of unique antigenic specificity, their serological cross-reactivity can be readily demonstrated (3–5). However, studies to elucidate the nature of this cross-reactivity have been hampered by the structural complexity of these proteins. The recent finding that human 7S γ-globulins can be reproducibly degraded to smaller units which differ from each other in antigenic properties by the method described by Porter (6) offers a possible approach to a clearer definition of the antigenic relationships between these proteins (7).

In the present report experiments are described comparing the antigenic properties of these proteins to the two major antigenically distinct fragments of 7S γ-globulin. The results indicate that all of these proteins cross-react with 7S γ-globulin and each other by virtue of certain determinant groups associated with those fragments of 7S γ-globulin that carry the antibody-combining

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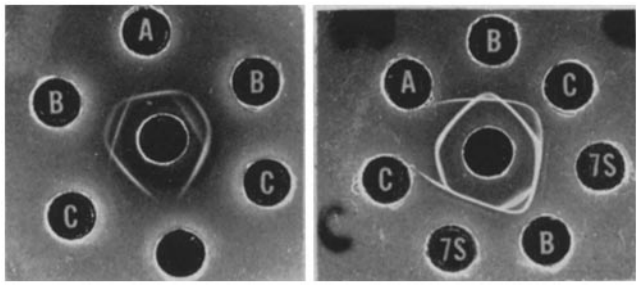
‡ Senior Investigator, Arthritis and Rheumatism Foundation.

§ Wellcome Travelling Research Fellow.

sites, while the individual antigenic specificity of 7S γ-globulin is associated primarily with the fragment richest in carbohydrate which is devoid of antibody-combining activity.

Materials and Methods

1. Antigens.—(*a*) *7S γ-globulin* was generally prepared by ultracentrifugation of a 5 per cent (*w/v*) solution of Cohn fraction II γ-globulin (Lederle C586, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York) in a No. 40 rotor of a Spinco model L ultracentrifuge as previously described (8). The supernatant fluid so obtained was free of 19S γ-globulin by ultracentrifugal analysis and gave only a single line with antisera to γ-globulin or whole serum by double diffusion in agar. In several experiments 7S γ-globulin was isolated from individual sera by batch chromatography using DEAE[1]-cellulose (9) or by pooling the slowest moving fractions obtained by starch zone electrophoresis (4).



(*a*) (*b*)

FIG. 1. Ouchterlony double diffusion plates comparing fragments A, B, C, of 7S γ-globulin with native 7S γ-globulin (7S) and each other. Antiserum to 7S γ-globulin is in the center well. (*a*) The outer line seen with fragment B is due to a small contaminant of fragment A. (*b*) A pure preparation of fragment B gives only a single line which penetrates the lines due to A and C.

(*b*) *19S γ-globulins:* Normal 19S γ-globulin was obtained from outdated plasma by precipitating the euglobulin fraction by dilution with ten volumes of distilled water followed by repeated ultracentrifugation. The corresponding paraproteins were obtained from the sera of five subjects with macroglobulinemia of Waldenström. The exact method of preparation and criteria of purity of each of these proteins has been described in detail previously (10). Final purification was frequently achieved by zone centrifugation in a saline density gradient formed from layers of 21, 14, and 7 per cent saline (11).

(*c*) *β_{2A}-myeloma proteins* were isolated by starch zone electrophoresis from the sera of six patients with multiple myeloma. In five the zone corresponding to the peak of the β-globulin was used while in the sixth, a more rapidly migrating fraction was employed because of the presence of contaminating γ-globulin in the peak tube. Myeloma proteins which resembled the normal protein closely were used rather than the normal protein because of lack of sufficient pure preparations of the latter.

(*d*) *Bence Jones proteins* (*BJP*) were isolated from the urines of three subjects with multiple myeloma by precipitation with 35 per cent saturated ammonium sulfate. Each protein gave

a single homogeneous peak on paper electrophoresis and ultracentrifugation and did not require further purification.

2. Fragments of 7S γ-Globulin.—The method of degrading 7S γ-globulin and isolating the fragments, as well as a description of their properties, has been given in detail (7) and will be summarized briefly for the sake of clarity.

Treatment of human 7S γ-globulin by the method of Porter results in the formation of fragments with molecular weights of 40,000 to 50,000 and $S^{o}_{20,w}$ of 3.5S. These fragments can be resolved into three major chromatographically distinct components called A, B, and C by chromatography on carboxymethyl-cellulose followed by a second separation on DEAE-cellulose. Fragment B which corresponds to the F component of Edelman (12) is richest in carbohydrate and devoid of antibody-combining activity, while fragments A and C contain the antibody-combining sites. Immunologic analyses by double diffusion in agar have failed to demonstrate any serologic differences between fragments A and C. These fragments give a reaction of complete identity, but lack some of the determinant groups of the native 7S γ-globulin (Fig. 1). Fragment B forms an additional line which appears to be antigenically distinct from that formed by fragments A and C. The outer line present in most of the preparations of fragment B used in these studies is probably due to small amounts of fragment A present as a contaminant since it gives a reaction of identity with the precipitin line of fragment A (Fig. 1 *a*). Recently, however, it has been possible to obtain fragment B free of contaminating proteins. Such preparations give only a single precipitin line (Fig. 1 *b*) which cross-reacts with 7S γ-globulin and shows a reaction of non-identity with fragments A and C.

3. Antisera.—Antisera were prepared in rabbits by the subcutaneous route using complete Freund's adjuvant.

(*a*) *Antisera to 7S γ-globulin* (*anti. 7S γ-glob.*) represent single bleedings from two animals injected repeatedly with 7S γ-globulin prepared from Cohn fraction II (see Antigens).

(*b*) *Antisera specific for 7S γ-globulin* (*anti. "7S"-abs.*) were prepared by absorption of antiserum to 7S γ-globulin with pure normal 19S γ-globulin. Such antisera no longer react with 19S γ-globulin, β_{2A}-myeloma globulin, or Bence Jones protein, and have been characterized in detail previously (8).

(*c*) *Antisera specific for 19S γ-globulin* (*anti. 19S abs.*) were prepared to three different pathologic macroglobulins, each of which cross-reacted strongly with the normal 19S γ-globulin fraction and were made specific for the 19S fraction by absorption with 7S γ-globulin (4). In one instance several additional faint lines were present in the absorbed antiserum which were readily removed by an additional absorption with small amounts of α_2-globulin.

(*d*) *Antisera to 3.5S fragments of 7S γ-globulin* (*anti. A, anti. B, anti. C*) represent single bleedings. They were prepared against the preparations shown in Fig. 1 *a*. Antisera to A and C gave only a single line against each of the fragments while those against B gave one line with fragments A and C and an additional more intense line with fragment B. These findings are consistent with the presence of a common antigenic component in all these fragments and an additional antigenically distinct one in fragment B.

(*e*) *Antisera specific for fragment B* (*anti. B abs.*): Because of the presence of small amounts of fragment A in the preparation of fragment B used for immunization, antisera specific for fragment B were prepared by absorption of this antiserum prepared to fragment B with fragments A or C. These antisera no longer reacted with fragments A and C and usually gave only a single line with fragment B.

4. Analytical Techniques. These are similar to those employed in previous studies and have been described in detail previously. They consist of (*a*) gel diffusion and precipitation in agar Ouchterlony (4), (*b*) starch zone electrophoresis and paper electrophoresis, and (*c*) ultracentrifugation in a Spinco model E ultracentrifuge (11).

RESULTS

Antigenic cross-reaction of the 7S with 19S γ-globulins, β_{2A}-globulins, and Bence Jones proteins has been demonstrated by a number of observers (4, 1, 5). Fig. 2 confirms this finding and demonstrates a reaction of partial identity between 7S γ-globulin and each of the β_{2A}-globulins and Bence Jones proteins used in this study. Similar cross-reactions have also been shown for each of the macroglobulins used and have been described in a previous publication (10). The spur on the 7S precipitin line indicates clearly that these proteins lack determinant groups present in the 7S γ-globulin used to produce the antiserum. In an effort to delineate more clearly the nature of the deter-

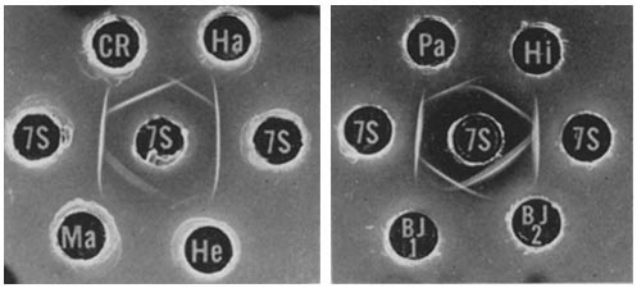
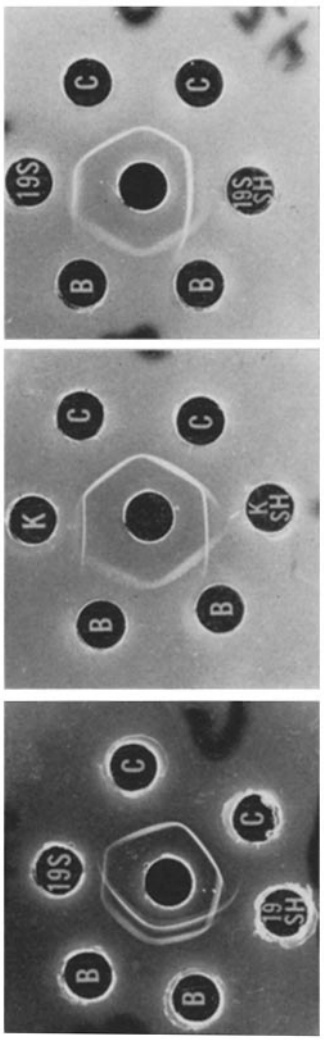


FIG. 2. Ouchterlony double diffusion plates comparing six β_{2A}-myeloma proteins (Cr, Ha, Ma, He, Pa, and Hi) and two Bence Jones proteins (BJ-1, BJ-2) with 7S γ-globulin (7S) using an antiserum to 7S γ-globulin in the center wells.

minant groups on the 7S γ-globulin responsible for the cross-reaction with each of these proteins, four distinct types of experiments were performed, all of which indicate that the cross-reacting groups are associated with fragments A and C while most of the antigenic specificity of the 7S γ-globulin is associated with fragment B.

1. Comparison of 19S γ-globulins, β_{2A}-Globulins, and Bence Jones Proteins with Fragments of 7S γ-Globulin Using an Antiserum to 7S γ-Globulin.—Fig. 3 compares the reaction of normal 19S γ-globulin, a pathological macroglobulin, and their 7S monomers with papain fragments B and C of 7S γ-globulin. Fragment C forms a reaction of partial identity with each of the paraproteins and gives a reaction of complete identity with the normal 19S γ-globulin and its monomer, indicating that the cross-reacting determinant groups on the 7S molecule are associated with this fragment and also fragment A which has similar antigenic properties. On the other hand the line unique to fragment B penetrates through the 19S lines without being deviated by them, thus indicating that it contains the antigenically unique part of the 7S molecule distinguishing it from the 19S fraction. The presence of some contaminating fragment A in



(a) (b) (c)

FIG. 3. Comparison of native normal 19S γ-globulin (19S) and its mercaptoethanol-dissociated monomer (19S SH) and a pathological macroglobulin (K) and its monomer (KSH) with fragments B and C of 7S γ-globulin. Antiserum to 7S γ-globulin in center wells. (*a*) Impure fragment B was used. The inner line due to fragment B penetrates the macroglobulin lines; the outer line represents fragment A. K contains trace amounts of 7S γ-globulin. (*b* and *c*) A pure preparation of fragment B was used which gives only a single line.

the preparation of fragment B used in Fig. 3 *a* accounts for the presence of the outer line. Fragment B shown in Figs. 3 *b* and 3 *c* is free of this material and gives only a single precipitin line with antiserum to 7S γ-globulin. Similar results were obtained with each of four other pathological macroglobulins. However, the degree of cross-reaction varied. The normal 19S γ-globulin showed a reaction of complete identity while several of the paraproteins showed significant antigenic deficiency compared to fragments A or C.

Fig. 4 demonstrates a similar relationship between the papain fragments of 7S γ-globulin and four representative β_{2A}-myeloma proteins and two representative Bence Jones proteins. In each case the line due to C and the related

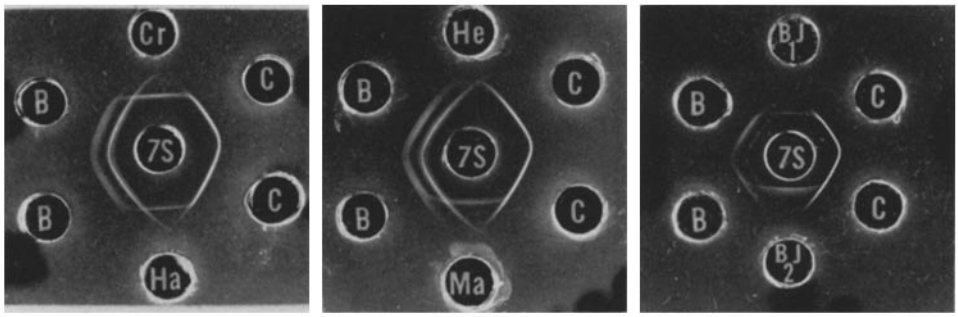


FIG. 4. Comparison of six β_{2A}-myeloma proteins and two Bence Jones proteins with fragments B and C of 7S γ-globulin. The inner line due to fragment B penetrates the lines due to the paraproteins while the outer one represents contaminating fragment A. Antiserum to 7S γ-globulin is in the center wells.

line in fragment B demonstrates a partial and in one case (He) complete cross-reaction with the other proteins, while the sharp line specific for fragment B crosses the line due to the β_{2A}-globulin or Bence Jones proteins without being affected by it, thus showing a reaction of non-identity. The distinct spur seen on fragment C indicates that here too the relationship is one of cross-reaction rather than complete identity. In every instance, when an antiserum to 7S γ-globulin was used, the spur was present on the precipitin line due to the fragment formed from it, thus indicating some serologic deficiency of the Bence Jones protein, β_{2A}- or 19S γ-globulin compared to it. However, the presence of additional antigenic groups unique for each of these proteins appears likely and has been demonstrated for the 19S γ-globulins and β_{2A}-globulin with specific antisera prepared to these proteins.

2. Reaction with Anti. B Abs. Specific for Fragment B and with Antisera to Fragments A or C.—Fig. 5 *a* shows the reaction of normal 19S γ-globulin with antisera to fragments A and C. Similar patterns obtained with each of the β_{2A}-globulins and Bence Jones proteins showed a reaction of partial identity

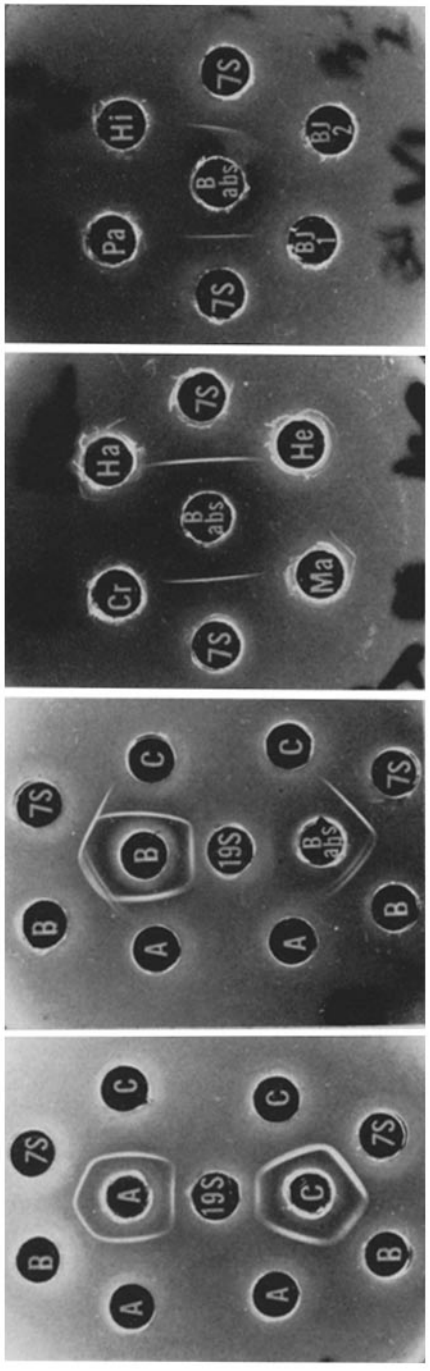


FIG. 5. (*a*) Comparison of normal 19S γ-globulin (19S), 7S γ-globulin (7S), fragments A, B, C of 7S γ-globulin using antiserum to fragment A (top) and to fragment C (bottom) in the center wells. (*b*) Same proteins compared to antiserum to fragment B after absorption with fragment A (B abs.) (bottom), and before absorption (top). (*c* and *d*) Reaction of six β_{2A}-myeloma proteins and two Bence Jones proteins and 7S γ-globulin with antiserum specific to fragment B (B abs.). None of these except 7S γ-globulin reacts with the antiserum specific for fragment B.

with 7S γ-globulin and fragments A or C. In each instance the spurs were significantly greater than those seen with the 19S γ-globulins. Fig. 5 *b* shows that unabsorbed antiserum to fragment B reacts well with 19S γ-globulin and each of the fragments. Following absorption with fragment A the antiserum no longer reacts with fragments A or C while it continues to react strongly with fragment B and native 7S γ-globulin. However, absorption has also removed all the antibodies reactive with normal 19S γ-globulin and each of the pathologic macroglobulins studied. Figs. 5 *c* and 5 *d* show that none of six β_{2A}-globulins and two Bence Jones proteins gave a precipitin line when tested with the same antiserum. Similar results were obtained with a third Bence Jones protein.

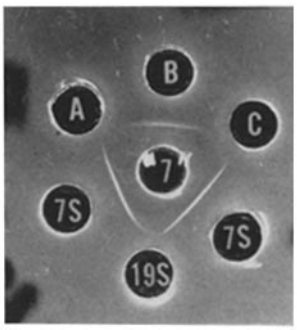


FIG. 6. Comparison of fragments, A, B, C, 7S γ-globulin, and 19S γ-globulin with antiserum to 7S γ-globulin absorbed with 19S γ-globulin. Following absorption there is no reaction with 19S γ-globulin or with fragments A and C.

3. Reaction with Antiserum to 7S γ-Globulin Absorbed with Fragments A or C.—The results obtained with this antiserum were similar to those obtained above with Anti. B abs. Only 7S γ-globulin and fragment B gave a single line which showed a reaction of identity, while none of the Bence Jones proteins, 19S γ-globulins, or β_{2A}-globulins reacted with the antiserum.

4. Reaction of Antiserum to 7S γ-Globulin Absorbed with Normal 19S γ-Globulin.—Fig. 6 illustrates that absorption of antiserum to 7S γ-globulin with pure normal 19S γ-globulin results in loss of reaction not only with the protein used for absorption, but also with fragments A and C. However, fragment B and 7S γ-globulin continue to react strongly. Similar experiments were not performed with the Bence Jones proteins or the β_{2A}-globulins since only antigenically incomplete paraproteins were available for study. However, the presence of distinct spurs on fragments A and C extending beyond the precipitin lines due to the paraproteins when tested with antiserum to 7S γ-globulin makes it appear unlikely that any of these proteins would have removed all of the antibodies to A and C. Studies to determine the behavior of normal β_{2A}-globulin will have to await the isolation of sufficient amounts of pure protein.

These experiments demonstrate that one type of structural relationship between these proteins is through certain common antigenic groups associated with fragments A and C of 7S γ-globulin. In order to determine whether some relationship also exists between the β_{2A}-myeloma proteins and Bence Jones proteins and that part of the 19S molecule which is antigenically distinct from the 7S γ-globulin, studies have been performed with three antisera to three different macroglobulins closely related to normal 19S γ-globulin. Each of these antisera was absorbed with 7S γ-globulin and no longer reacted with it or any of the fragments. When each of these antisera was allowed to react with the β_{2A}-myeloma proteins or the Bence Jones proteins no precipitin lines appeared. These findings suggest that there was no demonstrable antigenic relationship between these proteins and those groups on the macroglobulin responsible for its antigenic specificity, and that all cross-reacting antibodies in these antisera were removed by absorption with the 7S γ-globulin. Preliminary results with 3.5S units produced from 19S γ-globulins by papain digestion are consistent with this interpretation, but a definitive answer to this question will have to await the isolation of those fragments of the 19S molecule which carry the antigenic specificity and studies with antisera to the antigenically complete normal 19S γ-globulin.

DISCUSSION

The close resemblance in some of the physicochemical and functional properties of the several members of the family of immune globulins and related paraproteins has raised many questions concerning the structural relationship that might account for these similarities as well as the mode of synthesis of these proteins. Because of their large size and complex structure these proteins do not lend themselves readily to complete structural analyses such as those recently completed on smaller proteins such as insulin, ribonuclease, and the normal and abnormal hemoglobins. However, some insight into these problems can be gained by the somewhat cruder but relatively specific immunologic studies of these proteins and some of their structural subunits.

The results of the present studies demonstrate that each of these proteins is related to 7S γ-globulin by virtue of some common antigenic determinant groups associated with those fragments of 7S γ-globulin which are known to contain the antibody-combining site. Careful analyses of these reactions, generally obtained with the paraproteins and not the normal protein reveal, however, that in most instances there existed only a partial cross-reaction and not a reaction of identity. However, since the normal 19S γ-globulin appeared to give a reaction of identity with fragment C it is not possible to be certain that this would not also be true for the normal β_{2A}-globulin. While the techniques employed in this study do not permit accurate quantitation of the degree of cross-reaction of different proteins, it would appear that the 19S γ-globulins

cross-react with the fragments and native 7S γ-globulin more completely than do the β_{2A}-globulins or Bence Jones proteins employed in this study. The antigenic groups which render the 7S γ-globulin molecule distinct appear to be associated primarily with the carbohydrate-rich fragment B which is devoid of antibody-combining sites. The observation of a similar antigenic relationship between 7S and 19S γ-globulins in the rabbit through common determinant groups on fragments I and II of 7S RGG (13) suggests that a similar relationship between various immune globulins may also exist in other species of animals.

To date little is known about the location of the determinant groups of the 19S γ-globulin and β_{2A}-globulins which confer antigenic specificity to these proteins. However, preliminary experiments with the 3.5S fragments obtained by papain digestion of a pathological macroglobulin suggest that these groups are associated with a small carbohydrate-rich fraction of the molecule. (20).

7S γ-globulin differs from the 19S γ-globulin and the β_{2A}-globulin in at least three major biological properties. It has been shown to cross the placenta in man, rabbit, and certain other species (14, 15), to fix to the skin of the guinea pig thus permitting its detection by the P. C. A. technique (16), and to react specifically with rheumatoid factors, a group of proteins which may represent antibodies to altered 7S γ-globulin (11). Neither β_{2A}-globulin nor 19S γ-globulin crosses the placenta (18) and neither they nor the Bence Jones protein can fix to the skin (19) or react with rheumatoid factor (20). While the structural features which confer these properties to the 7S γ-globulin are not known, it seems likely that they may be due to some property associated with fragment B which corresponds to Porter's fragment III of RGG, since each of these properties of the intact molecule appears to be retained by this fragment when tested alone. Thus, Brambell, Hemmings, Oakley, and Porter (21) have shown that fragment III of RGG crosses the placenta more readily than fragments I and II which are retained more completely in the maternal circulation. Ovary (22) has demonstrated that fragment III of RGG also fixes to the skin while fragments I and II fail to do so when tested alone. In the case of human γ-globulin the site necessary for skin fixation appears to be destroyed during digestion since none of the fragments has the ability to fix to the skin (19). Thirdly, when tested with fragments from human or rabbit 7S γ-globulin, rheumatoid factor reacts only with fragments B or III and not with the fragments containing the antibody-combining sites (17). This observation would explain the failure of other types of γ-globulin or β_{2A}-globulins to react with rheumatoid factor.

Antibodies have been demonstrated in the 7S and 19S fractions of γ-globulin, and in the case of the 7S fraction of rabbits and man, the antibody-combining site has been demonstrated to be associated with those fragments which carry the determinant groups common to all the immune globulins. While no definite

antibody has been associated with the β_{2A}-globulin fraction, the common structural relationship to the other two suggests that it too may contain antibodies. However, an exact answer to this question may have to await the isolation of these proteins in large amounts and a high state of purity.

The antigenic relationships between the various immune globulins shown in this work and in previous publications (3–5) by several observers indicate the limitations attached to the practice of employing antisera for the quantitative estimation of protein components in serum or mixtures. The results suggest that such quantitative techniques are of greater practical value in studying pure proteins free of contaminating cross-reacting substances.

SUMMARY

The antigenic properties of normal 19S γ-globulin, pathologic macroglobulins, β_{2A}-myeloma proteins, and Bence Jones proteins have been compared with 7S γ-globulin and the small 3.5S units derived from it by gel diffusion precipitin techniques.

These studies demonstrate that the determinant groups on the 7S γ-globulin molecule responsible for the cross-reaction with each of the other proteins are associated with the two fragments of 7S γ-globulin which have the antibody-combining sites.

The antigenic specificity of the 7S γ-globulin which distinguishes it from each of these proteins is associated primarily with the fragment that is richest in hexose and can not combine with antigen. However when compared with certain of the paraproteins additional antigenic specificity was also found to reside in the fragments with antibody-combining activity.

The finding of similar antigenic relationships in rabbit γ-globulins suggests that some of the biological properties associated only with the 7S γ-globulins and not with the other immune globulins may reside in the fragment which also carries the antigenic specificity of the protein.

The technical assistance of Frances Prelli is gratefully acknowledged.

Addendum.—Since completion of this report the monograph "Les Globulines Sériques du Système Gamma" by J. Heremans (1960, Brussels Editions Arscia S. A.), which describes similar results has come to our attention. Recent gel-diffusion precipitin studies with β_{2A}-globulin isolated from normal human serum by Heremans' method II and with low molecular weight γ-globulins from normal human urine have demonstrated the following: (*a*) a reaction of almost complete identity between the normal β_{2A}-globulin and fragment C of 7S γ-globulin, whereas the urinary γ-globulin showed a partial cross-reaction with this fragment; (*b*) a reaction of non-identity between each of the two proteins and fragment B, neither the β_{2A}-globulin nor the urinary γ-globulin reacting with absorbed antiserum to this fragment.

[1] DEAE, diethylaminoethyl.

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