Horse Anti-SI Immunoglobulins. I. Properties of γM-Antibody*

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ABSTRACT: Three major classes of antibodies (γG-, γA-, and γM-globulins) were isolated from horse antipneumococcus, Type I, serum. Antibodies of the γM-globulin type represented the bulk (82%) of those eluted from precipitates made with Type I polysaccharide (SI). The γM-antibody was separated from the γG- and γA-antibodies by gel filtration on Sephadex G-200. The γG- and γA-antibodies were then separated by chromatography on DEAE-cellulose. The γM-antibody contained class-specific antigenic sites as well as sites common to γG- and γA-globulins. At least a portion of the common sites could be located on the light chains isolated from γG- and γM-globulins. Depolymerization of the γM-antibody to 6.3S subunits required incubation for 30 min at 37° in concentrations of mercaptoethanol ≥0.075 M. These subunits were incapable of precipitating with antigen. However, oxidative reaggregation led to the production of components having higher sedimentation coefficients and to a concomitant restoration of ability to precipitate with antigen. Binding of the antigen by the 6.3S subunits was qualitatively demonstrated using radioimmunoelectrophoresis with ^{125}I-labeled SI. The binding was quantitated using tube coprecipitation of labeled SI by subunits precipitated with anti-γM-globulin serum. The subunits coprecipitated 83–99% of the labeled SI coprecipitated by intact γM-antibody precipitated in turn with anti-γM-globulin serum.

The γM-globulins constitute one of the three principal classes (γM, γA, and γG) of immunoglobulins now recognized (Kunkel, 1960; Cohen and Porter, 1964). Heidelberger and Pedersen (1937) first demonstrated the association of antipneumococcal antibody with these high molecular weight globulins present in horse serum. Kabat and Pedersen (1938) proceeded to show the presence of γM-globulin antibodies to pneumococci in the serum of other species, such as the pig and cow. Values for $s^0_{20,w}$ = 19.8 S and $M_{s,D}$ = 990,000 were calculated for horse macroglobulin antibody, and similar values were obtained for the macroglobulin antibodies of other species (Kabat, 1939).

Subsequently, only a few studies of either isolated normal γM-globulin or purified macroglobulin antibody have been undertaken (Franklin and Kunkel, 1957; Franěk, 1962). Much of our present knowledge concerning this molecule has been derived from investigation of those γM-globulins produced in large quantities by humans suffering from Waldenström's macroglobulinemia (Waldenström, 1944; Kunkel, 1960). Various samples of Waldenström's macroglobulin, having $s^0_{20,w}$ = 18 S, were found to be reductively depolymerized to subunits having an $s^0_{20,w}$ = 6.3 S with 0.1 M mercaptoethanol (Deutsch and Morton, 1957, 1958). Antigenic analyses have indicated that both normal and pathologic human γM-globulins contain an immunogenically unique moiety as well as a portion which cross-reacts with a part of γG-globulin (Franklin and Kunkel, 1957; Korngold and van Leeuwen, 1957). At least part of the immunologic cross-reactivity between human γG and γM-globulins can be explained by the presence of certain polypeptides (light chains) as structural elements of both immunoglobulins (Fahey, 1963; Migita and Putnam, 1963).

Many of the above properties of human γM-globulins are presumed to be shared by macroglobulin antibodies of many specificities present in the serum of many species. For instance, one widely accepted characteristic of macroglobulin antibody of certain specificities is its apparent loss of activity upon reduction with 0.1 M mercaptoethanol. Reduction appears to destroy the ability of macroglobulin of the appropriate specificity to agglutinate or immobilize bacteria (Bellanti *et al.*, 1963; Nossal *et al.*, 1964), to agglutinate erythrocytes (Fudenberg and Kunkel, 1957; Chan and Deutsch, 1960; Bauer and Stavitsky, 1961), to sensitize erythrocytes for complement hemolysis (Stelos and Taliaferro, 1959), to neutralize phage (Uhr and Finkelstein, 1963; Bauer *et al.*, 1963), and to specifically precipitate antigen (Josephson *et al.*, 1962; Benedict *et al.*, 1963). In only one of these studies was an attempt made to isolate specific macroglobulin antibody and to correlate apparent loss of activity with reductive depolymerization (Fudenberg and Kunkel, 1957). Retention of the antibody combining sites after reduction was suggested

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by another of the above studies (Chan and Deutsch, 1960) in which reduced Rh saline agglutinins rendered the appropriate erythrocytes Coomb's positive. More recently, Jacot-Guillarmod and Isliker (1962, 1964) have shown that reduced human anti-A and anti-B γM-globulins can still inhibit hemagglutination by intact antibody and specifically render the appropriate erythrocytes susceptible to agglutination by an anti-globulin serum. Onoue *et al.* (1964b) have now demonstrated binding of ^{131}I-labeled antigen by precipitates formed of 6 S subunits of rabbit macroglobulin antibody and anti-γM-globulin using the technique of radio-immunoelectrophoresis (Hochwald *et al.*, 1961). Thus, it appears that at least some of the subunits of γM-antibody that are produced by reductive depolymerization retain intact antibody combining sites. It therefore seemed worthwhile to isolate purified specific macroglobulin antibodies to a defined, soluble antigen and to quantitate the antibody activity retained by its subunits. Accordingly, purified antipneumococcal polysaccharide, Type I, antibodies were resolved into γM-globulin (82%) and "6.4S immunoglobulins" (18%). Both classes of antibody apparently were specific for the same polysaccharide molecule. This polysaccharide was extrinsically labeled with ^{125}I by a novel method. The reductively produced subunits retained at least 83% to 100% of their antigen binding activity as measured by their coprecipitation of the labeled polysaccharide together with anti-γM-globulin. Moreover, the subunits could be partially reaggregated by oxidation to yield precipitating antibody.

Materials and Methods

Immunization of the Horse. Hyperimmune serum was obtained by immunizing a horse with saline suspensions of heat-killed *Diplococcus pneumoniae*, Type I, that had been initially passed through mice until they had become heavily encapsulated. The horse (500 kg) received a primary stimulation with three 30-mg portions of heat-killed pneumococci given intramuscularly (rump) on days 1, 20, and 29. Serum from trial bleedings had a low agglutinating titer and showed only a trace of precipitins after these injections. An injection of 120 mg of bacteria was made into the jugular vein on day 48 and thereafter on days 63, 97, 113, 118, 158, 165, 180, 219, 362, and 431.

The precipitating antibody level has remained at about 1.5 mg/ml over this period, and there has been only a slight increase in the level of agglutinins that are resistant to reduction by 0.1 M mercaptoethanol. At most, these resistant antibodies account for only 18% of the total specific antibody.

Soluble Antigens

Preparation of Type Specific Polysaccharide. D. pneumoniae, Type I, were grown for 24–36 hr in 4-l. batches of brain heart infusion broth (Difco) enriched to contain 1% sheep blood. The bacteria were then harvested by centrifugation and washed several times with saline to remove traces of the medium. The bacteria were then suspended in 1 l. of phosphate-buffered saline, pH 7.2, and deproteinized by several cycles of mixing with chloroform in a Waring blender. The clear aqueous layer containing the polysaccharide was concentrated ten- to twentyfold by pressure dialysis against distilled water. The polysaccharide was then precipitated by adding 1.5 volumes of 95% ethanol. After two reprecipitations, the polysaccharide was lyophilized from a distilled water solution. The material appeared relatively homogeneous by sedimentation velocity analysis and had a sedimentation coefficient of 3.0 S. Hereafter, the polysaccharide from Type I pneumococci will be referred to as SI.[1]

Isolation of Purified Antibodies. A quantitative precipitin curve was obtained for the serum from each bleeding, and these data were used to determine the amount of polysaccharide needed for precipitation of the maximum amount of antibody. The optimal amount of SI was then added to a large volume of the serum. The mixture was incubated at 37° for 1 hr and then at 2° overnight. The precipitate was collected by centrifugation and washed twice with cold saline. A quantity of acetate buffer, pH 3.5, $\mu = 0.05$, equal to one-half the original serum volume, was added, and the precipitate was suspended and allowed to stand at room temperature for 1 hr. The remaining precipitate was then removed by centrifugation. The supernatant now contained about 1 mg of protein for each milliliter of serum used. The extinction coefficient ($E^{1\%}_{280\ m\mu}$) of 15.0, found for horse γM-globulin (McDuffie and Kabat, 1956), was used to determine protein concentrations. The dissociated protein was about 82% γM-globulin while the balance consisted of immunoglobulins which sedimented with $s_{20,w} = 6.4$ S. This slower sedimenting fraction will be referred to as "6.4 S immunoglobulins."

Separation of γM-Antibody from the "6.4S Immunoglobulins." The technique of molecular sieving in columns of Sephadex G-200 (Pharmacia), equilibrated in 0.1 M phosphate buffer, pH 6.8, was used to separate the various immunoglobulins. The proteins eluted from the polysaccharide were equilibrated with the same phosphate buffer by dialysis, and 4-ml portions (10 mg) were applied to a column of Sephadex G-200 (2.5 cm × 60 cm). Fractions of approximately 4.7 ml were collected.

Fractionation of "6.4S Immunoglobulins." The pool of "6.4S immunoglobulins" from the above G-200 column was equilibrated with 0.01 M phosphate buffer, pH 7.5, and applied to a DEAE-cellulose column, 0.7 cm (i.d.) × 14 cm, equilibrated in the same buffer. A homogenous fraction of γG-antibody was not retained by the column and was collected after one column volume of buffer had been passed. Further protein fractions were obtained by stepwise elution with this buffer which was made 0.05, 0.1, 0.2, and 0.3 M in NaCl.

Preparation of Normal Horse γG-Globulin. Serum was collected from a nonimmunized horse and the globulins

[1] Abbreviation used in this work: SI, polysaccharide from Type I pneumococci.

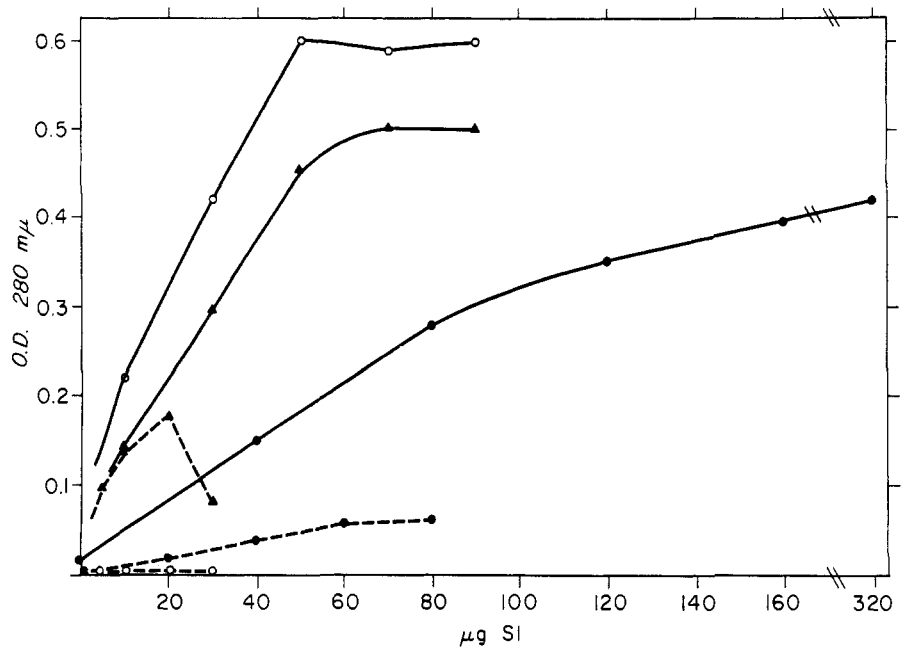

FIGURE 1: Quantitative precipitin curves obtained for sera taken at various times after initiation of immunization with Type I pneumococci. Sera used for the precipitin curves were taken on days 97 (○), 165 (▲), and 431 (●). Precipitin curves obtained following reduction of the sera with 0.1 M mercaptoethanol are indicated by the broken lines. The indicated amount of SI was added to 0.5 ml of untreated or reduced antiserum. The washed precipitates were dissolved in 1.0 ml of 0.1 N NaOH for optical density measurements at 280 mμ.

were precipitated by making the serum 37% saturated with ammonium sulfate. After standing in the cold overnight, the precipitated proteins were collected by centrifugation and washed several times with cold 37% saturated ammonium sulfate. The precipitate was then dissolved in 0.00525 M phosphate–borate buffer, pH 8.4 (Press *et al.*, 1960), then dialyzed overnight against the same buffer. The soluble globulins were applied to a triethylaminoethyl-cellulose (Bio-Rad Labs, Richmond, Calif.) column equilibrated in the same buffer. That protein fraction which was not retained by the column was concentrated by pressure dialysis and was only γG-globulin.

Reduction of Immunoglobulins

Depolymerization of γM-Globulin to Subunits. Whole serum was reduced by making it 0.085 M in mercaptoethanol (Morton and Deutsch, 1957) and incubating it at 37° for 30 min. To alkylate the free sulfhydryl groups, a 10% excess of monoiodoacetamide (Mann Research Labs, New York) was added following reduction, and excess reagents were removed by dialysis against 0.1 M phosphate buffer, pH 6.8. Reduction of γM-antibody was carried out by adding the desired amount of a stock solution of 0.85 M mercaptoethanol to the protein in a 0.1 M phosphate, pH 6.8, buffer and proceeding as for whole serum. Concentration of the stock solutions of reducing agent was determined by titration with 5,5-dithiobis(2-nitrobenzoic acid) (Aldrich Chemical Co., Milwaukee, Wis.), according to the method of Ellman (1959).

Reaggregation of γM-Subunits by Reoxidation. The γM-antibody (2.5 mg/ml) was reduced in 0.1 M phosphate buffer, pH 6.8, at a final concentration of 0.1 M thioglycolate (Sigma Chemical Co., St. Louis, Mo.) upon incubation for 60 min at 37°. Complete reduction to subunits was confirmed by alkylation of a small portion of the reaction mixture and subsequent sedimentation analysis. The balance of the reaction mixture was passed through a column of Dowex 1-X8-acetate equilibrated in 0.1 M sodium acetate. The subunits were then allowed to reassociate while being concentrated to 5–7 mg/ml by pressure dialysis against 0.14 M phosphate–borate buffer, pH 8.0.

Separation of Polypeptide Chains. Either γG- or γM-globulin was dialyzed against 0.55 M Tris buffer, pH 8.2. The solution was then made 0.75 M in mercaptoethanol and the globulin was reduced, alkylated, and fractionated according to the method reported by Fleishman *et al.* (1962). A column of Sephadex G-100, equilibrated in 1 M propionic acid, was used for fractionation.

Specific Precipitation and Coprecipitation of SI. Data for quantitative precipitin curves were obtained by adding increasing volumes of a 1 mg/ml solution of SI to 0.5 ml of the test serum or a saline solution containing 0.5 mg of purified γM-antibody. The mixtures were incubated at 37° for 1 hr and then at 2° overnight. The

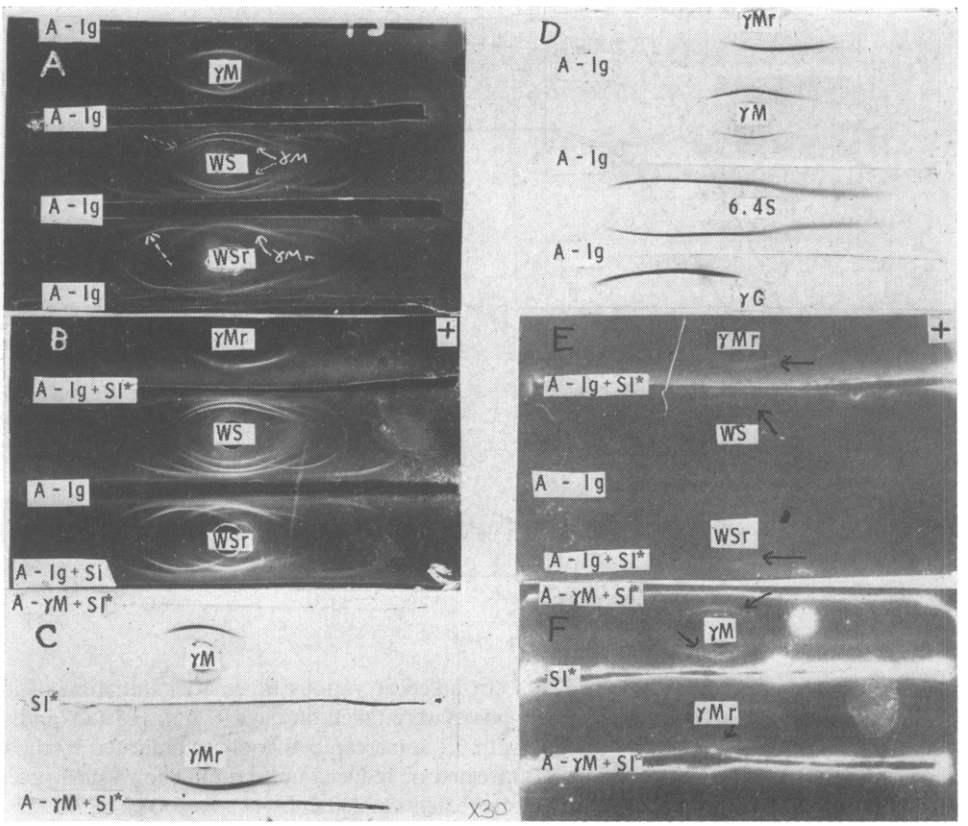

FIGURE 2: Immunoelectrophoretic and radioimmunoelectrophoretic analyses of anti-SI and immunoglobulins. The symbols used indicate: A-Ig, rabbit antiimmunoglobulins; A-γM, goat anti-γM-globulin; γM, γM-antibody; γMr, γM-antibody reduced with 0.085 M mercaptoethanol; WS, whole horse antiserum; WSr, horse antiserum reduced with 0.1 M mercaptoethanol; 6.4S, "6.4S immunoglobulins"; γG, γG-globulin from normal horse serum; SI*, Type I pneumococcal polysaccharide labeled with ^{125}I. Pictures E and F show radioautographs made of the plates shown in B and C, respectively.

precipitates were collected by centrifugation, washed twice with cold saline, and then dissolved in a convenient volume of 0.1 N sodium hydroxide. The protein present in the precipitate was determined by optical density measurements at 280 mμ.

For quantitative coprecipitation experiments, the SI polysaccharide was labeled with ^{125}I using triphenylphosphorus (Peninsular ChemResearch, Inc., Gainesville, Fla.) by a novel method (W. C. Hill and J. J. Cebra, in preparation).

The amount of ^{125}I-labeled polysaccharide required to give the maximum amount of precipitate with a given amount of intact γM-globulin and the amounts of rabbit antiserum (anti-horse immunoglobulins) required to maximally precipitate γM-antibody or normal γG-globulin (and "6.4S immunoglobulin") were determined separately in advance. For quantitation of the coprecipitated labeled polysaccharide, 0.5-mg amounts of intact γM-antibody, reduced and alkylated γM-antibody, normal γG-globulin, "6.4S immunoglobulin," or other horse globulin preparations, were mixed with the amount of labeled polysaccharide that was optimal with respect to the intact γM-antibody. Each globulin solution was set up in triplicate with SI. After incubation of the mixtures for 1 hr at 37° only the tubes containing intact γM-antibody or "6.4S immunoglobulin" showed precipitation. At this time the amount of rabbit antibody required to precipitate all of the horse immunoglobulin in a given tube was added to all tubes except one set which served as a control for amount of polysaccharide which could be directly precipitated by intact γM-antibody. After further incubation at 37° for 1 hr and at 4° overnight, the precipitates were centrifuged and washed several times with cold saline. Two precipitates derived from each immunoglobulin were dissolved in 1.0 ml of hyamine, placed in vials with 5.0 ml of scintillation fluid (Kinard, 1957), and counted in a liquid scintillation counter (Packard Instruments Co., Series 314A). The third precipitate from each set was dissolved in 0.1 N sodium hydroxide and the optical density at 280 mμ was measured.

Immunodiffusion, Immunoelectrophoresis, and Radioimmunoelectrophoresis

The various immunodiffusions and immunoelectrophoreses were carried out on glass slides (either 1 × 3 or 2 × 3 in.) covered with a 2-mm layer of 0.8% ion-ager

made up in 0.05 M Veronal buffer, pH 8.6. Electrophoresis was for 2.5 hr at 2 ma/cm (width).

For radioimmunoelectrophoresis, the same slides and method of electrophoresis were used. These slides were developed by filling the troughs with a 1 mg/ml solution of ^{125}I-SI plus the appropriate goat or rabbit anti-horse globulin. The developed gel was washed in saline, then in water, dried, and stained with amido black. Radioautography was carried out by placing the slide in contact with Kodak industrial X-ray film (Type KK) for 1 3 weeks. The principle of radioimmunoelectrophoresis has been previously described by Hochwald *et al.* (1961).

Zone Electrophoresis in Starch Gel. Electrophoresis of γM-antibody, before and after its reduction, was carried out in starch gel made up in 8 M urea and a formate buffer (pH 3.0) according to Edelman and Poulik (1961).

Preparation of Specific Reagent Sera

Goat Anti-horse γM-Globulin. A goat was injected twice with two 9.25-mg amounts of γM-antibody in adjuvant given 4 weeks apart. The bulk of the precipitating antibodies formed reacted with the class-specific portion of the γM-globulin molecule. A small amount of the antibody cross-reacted with the other immunoglobulins.

Rabbit Anti-horse Immunoglobulins. An antiimmunoglobulin serum with broad specificity was prepared by injection of the unfractionated immunoglobulins that were eluted from the SI polysaccharide. About 3 mg of the globulins was emulsified in complete Freund's adjuvant and injected into multiple subcutaneous sites (Bernier and Cebra, 1965). The antisera reacted with common antigenic sites as well as with the class specific sites of horse γM-, γG-, and γA-globulins.

Rabbit Anti-horse Light Chain. Light chain from either γG- or γM-globulin was emulsified with complete Freund's adjuvant and was injected at multiple subcutaneous sites as described above. The resulting antisera were specific for light chains.

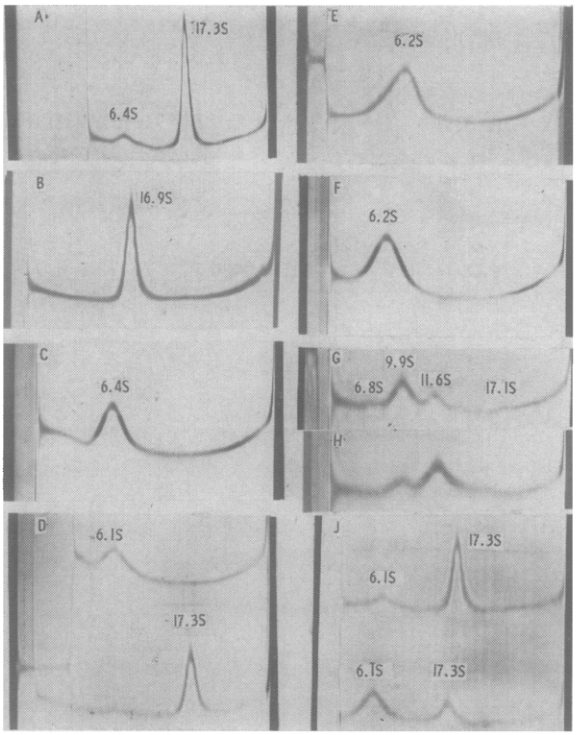

FIGURE 3: Sedimentation analyses of anti-SI antibodies and their products. Speed was 56,100 rpm unless otherwise indicated. Time after reaching rotor speed is indicated in parentheses. A, antibodies as eluted from the polysaccharide SI (20 min); B, γM-antibody freed of contaminating "6.4S immunoglobulins" (50,740 rpm, 24 min); C, "6.4S immunoglobulins" (40 min); D, γM-antibody, unreduced (lower) and reduced (upper), in 0.075 M mercaptoethanol (32 min); E and F, samples shown in A and B, respectively, after reduction in 0.1 M mercaptoethanol (60 min); G and H, reaggregation of γM-subunits obtained by reoxidation (42 min); J, γM-antibody reduced with 0.04 M (upper) and 0.06 M (lower) mercaptoethanol (32 min). The solvent was 0.1 M phosphate, pH 6.8, and the $s_{20,w}$ values are indicated.

Results

Reaction of Horse Antisera with SI. Some representative quantitative precipitin curves obtained for various bleedings of the immunized horse are shown in Figure 1. This figure also shows that the level of serum antibodies attained after the second intravenous injection remained nearly constant over a 68-day period despite four additional injections of antigen. Thereafter, the antibody titer declined only to about 70% of its maximum value in the 273 days following the last injection of antigen. During the immunization period there was only a modest increase in the level of antibodies that retained their ability to precipitate antigen following treatment with 0.085 M mercaptoethanol.

Figure 2, A and B, shows some typical immunoelectrophoretic patterns of the globulins in two of the whole sera, before and after incubation with 0.085 M mercaptoethanol. The solid arrows indicate the positions of the precipitin arcs given by γM-globulin and its reductively produced subunit. These components were identified in the patterns obtained for whole serum by their position (see accompanying precipitin arcs given by standard γM-globulin and its subunit on the same plates) and by their ability to bind radiolabeled antigen as described below. A more noticeable change in the electrophoretic patterns of whole serum upon its reduction is indicated in these same figures by the broken arrows. After reduction of whole serum an unidentified component, which does not seem to be an immunoglobulin, showed a marked decrease in anodic mobility and appeared to move toward the cathode.

Resolution of Purified Antibodies by Gel Filtration. Gel filtration of the mixed immunoglobulins eluted from the SI on columns of Sephadex G-200 separated these antibodies into a major, rapidly eluted com-

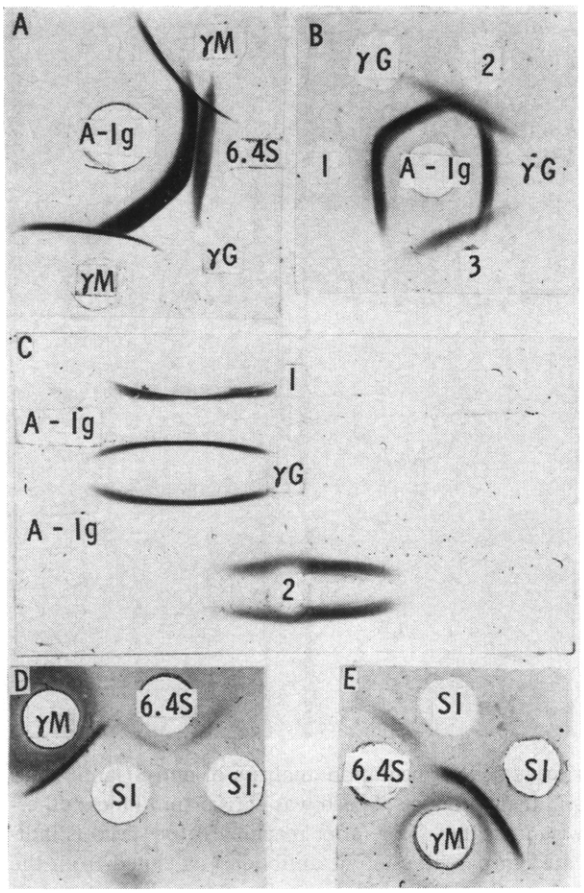

FIGURE 4: A comparison of the anti-SI immunoglobulins by immunodiffusion and immunoelectrophoretic procedures. The symbols used indicate: γM, γM-antibody; 6.4S, "6.4S immunoglobulins"; γG, γG-globulin; 1, 2, and 3, components of "6.4S immunoglobulins" eluted from DEAE-cellulose in 0.01 M phosphate buffer and by 0.2 M or 0.3 M NaCl made up in phosphate buffer, pH 7.5, respectively; SI, type specific polysaccharide from *D. pneumoniae*, Type I; AIg, rabbit anti-horse immunoglobulins.

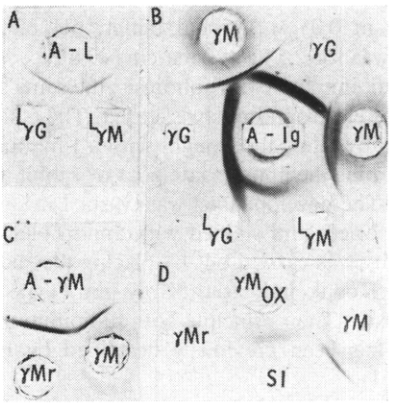

FIGURE 5: A comparison of γM-antibody, reduced γM-antibody, and light chains from γM-antibody by antigenic analysis and a demonstration of the ability of the reaggregated γM-subunits to precipitate with the antigen. Symbols used indicate: A-L, rabbit anti-light chains; $L_{\gamma G}$ and $L_{\gamma M}$, light chains isolated from γG-globulin and γM-antibody respectively; γM, γM-antibody; γG, γG-globulin; A-I_g, rabbit anti-immunoglobulins; A-γM, goat anti-γM-globulin; γMr, γM-antibody reduced with 0.1 M mercaptoethanol; γM_{ox}, γM-subunits reaggregated by reoxidation; SI, Type I pneumococcal polysaccharide.

ponent and a minor, more slowly eluted component. Antigenic and ultracentrifugal analyses indicated that the fraction eluted at the exclusion volume was homogeneous γM-globulin. The fractions eluted after the macroglobulin were also pooled and concentrated. These antibodies were contaminated by a very small amount of γM-globulin, which was regularly removed by a second gel filtration through the same column. Figure 3, A, B, and C, shows the schlieren patterns obtained upon sedimentation analyses of the purified antibodies before gel filtration and after their separation into γM-antibody and "6.4S immunoglobulins," respectively. The value of $s^0_{20,w}$ for γM-antibody calculated from values of $s_{20,w}$ obtained over the concentration range 0.5–5.0 mg/ml was 18.3 S.

Immunologic Characteristics of the Purified Antibodies. Antigenic analyses of the antibodies eluted from SI and fractionated on G-200 columns and of chromatographically isolated normal horse γG-globulin are shown in Figure 4, A and B. The anti-horse immunoglobulin sera used reacted with antigenic sites that are shared between γM- and γG-globulins. The immunodiffusion plate depicted in Figure 4A clearly showed the presence of antigenic sites specific for each of the classes of horse immunoglobulins (see reaction of partial identity with double spurring).

Comparison of the "6.4S immunoglobulin" fraction with γM-antibody and normal γG-globulin (Figure 4A) indicated that it contained two antigenically distinct components. One of these gave a reaction of identity with the γG-globulin while the other had sites that were antigenically distinct from both γG- and γM-globulin. To rule out the possibility that the antigenically unique sites on some of the more slowly sedimenting immunoglobulins were present on spontaneously produced subunits of γM-antibody, the gel diffusion shown in Figure 5C was prepared. This plate shows that the reductively produced subunits gave a reaction of identity with the intact γM-antibody and that no new antigenic sites were exposed upon reduction. Also, as can be seen in Figure 2D, the electrophoretic mobility toward the anode of the γM-subunits was only slightly greater than that of intact γM-globulins while the "6.4S immunoglobulins" were composed of two populations of molecules, one that migrated with the mobility of γG-globulins and a second that migrated faster toward the anode than the γM-globulins.

Fractionation of the "6.4S immunoglobulins" on

DEAE-cellulose clearly showed that this group was composed of two antigenically and electrophoretically distinct populations of molecules, both of which were distinct from γM-globulin. Immunoelectrophoresis (Figure 4C) of the components eluted with 0.01 and 0.2 M buffer clearly demonstrated the electrophoretic differences. The antigenic relationships of these two immunoglobulins to each other as well as to normal γG-globulin are shown in Figure 4B. The 0.1 M component was composed solely of γG-globulin while the 0.2 M component contained predominantly an immunoglobulin antigenically distinct from γG-globulin.

It seems probable that the immunoglobulin which had the fastest mobility toward the anode at pH 8.6 and which contained some antigenic sites distinct from those of γM- and γG-globulin was the well-known horse "T-globulin," which will be referred to as horse γA-globulin in accord with the newer nomenclature used by Rockey *et al.* (1964).

Light chains were prepared from both γG-globulin and γM-antibody by conventional methods. Gel diffusion studies using various antisera prepared against the individual light chains as well as antisera against the horse immunoglobulins all indicated that the light chains from the two classes of immunoglobulins were antigenically identical. This is indicated in Figure 5, A and B, where the cross-reactions of the light chains with γM- and γG-globulins are also shown.

The two purified antibody fractions, γM-antibody and the "6.4S immunoglobulins," were able to precipitate the SI preparation as shown in Figure 4, D and E. The "6.4S immunoglobulins" often produced a double precipitin band while the γM-antibody produced a heavier, single band. There was a noticeable inhibition of the formation of a dense precipitin band by the γM-antibody and SI as this band approached the precipitin band formed by the "6.4S immunoglobulins" and SI. It thus appeared that at least part of the γA- and γG-antibodies present in the "6.4S immunoglobulins" reacted with the same moiety precipitated by the γM-antibody.

Physicochemical Characteristics of the Purified Antibodies. Ultracentrifugal analyses of either the mixed antibodies eluted from SI or of purified γM-antibody after reduction with a final concentration of mercaptoethanol of 0.1 M showed a sedimenting boundary that had an $s_{20,w}$ = 6.2 S (Figure 3, E and F). Reduction of γM-antibody (2.5 mg/ml) with concentrations of mercaptoethanol of 0.04, 0.05, and 0.06 M gave increasing yields of the subunit but complete reduction to 6.1 S subunits required incubation to 0.075 M reducing agent for 30 min (Figure 3, D and J). The sedimentation patterns shown in Figure 3J are of γM-antibody incompletely reduced to subunits by concentrations of mercaptoethanol and 0.04 and 0.06 M, respectively. As can be seen, there were no components produced with sedimentation coefficient intermediate between 17.3 (intact molecule) and 6.1 S (subunit).

The intact γM-antibody and the reductively produced subunits were compared by starch gel electrophoresis at pH 3 in 8 M urea. Gel electrophoresis under

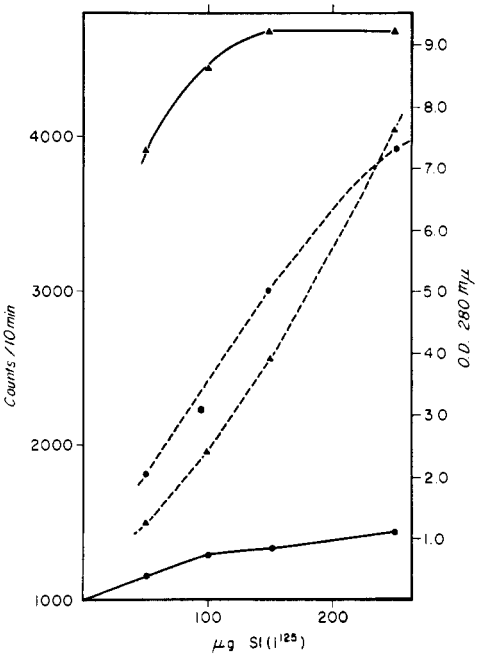

FIGURE 6: Precipitation of ^{125}I-labeled SI by intact γM-antibody alone and by intact γM-antibody plus anti-horse immunoglobulins. Increasing amounts of the ^{125}I-labeled SI were added to two sets of tubes containing intact γM-antibody. Then an amount of rabbit anti-horse immunoglobulins calculated to precipitate all of the γM-antibody was added to each tube of one set. The resulting precipitates were washed and dissolved in 0.1 N NaOH. The optical density at 280 mμ of the γM-antibody precipitated with SI directly is shown by (●—●), and that of the γM-antibody, SI, and anti-immunoglobulins by (▲—▲). Optical density values are those for the precipitates if dissolved in 1.0 ml of 0.1 N NaOH. The radioactivity precipitated by the intact antibody and by the anti-immunoglobulin plus antibody is indicated by (●- -●) and (▲- - -▲).

these conditions has been employed to resolve the heavy and light polypeptide chains of reduced γG-globulin (Edelman and Poulik, 1961). Under the above conditions the reduced subunits had a much higher mobility toward the cathode than the intact γM-antibody. A faintly staining second component was present after reductive depolymerization which moved rapidly toward the cathode. This second component was shown to be free light chain. A 7.5-mg amount of the subunit, prepared by reduction of γM-antibody with 0.1 M mercaptoethanol for 30 min and then alkylation with a 1.5 molar excess of iodoacetamide, was equilibrated with 0.5 M propionic acid 6 M in urea. This sample was applied to a column of Sephadex G-100 (2.5 cm × 45 cm) equilibrated on 0.5 M propionic acid 6 M in urea. The principal protein fraction emerged after the same volume required to elute intact γG-globulin. A second protein fraction, accounting for 7.5% of the total optical density units in the effluent, was eluted in the

TABLE I: Quantitative Coprecipitation of ^{125}I-Labeled SI by Anti-horse Immunoglobulins and γM-Antibodies, γM-Subunits, or Other Horse Immunoglobulins.

Horse Globulin Present[a]	Anti-horse Immunoglobulins Present	Expt 1 Counts/10 min[b]	Expt 1 $OD_{280m\mu}$[c]	Expt 2 Counts/10 min[b]
γM-Antibodies	−	5038	0.375	3057
		4685		2959
γM-Antibodies	+	5289	3.94	3619
		4929		3306
γM-Subunit	+	5098	4.58	2918
		5069		2842
γG-Globulin (normal)	+	ND[e]		1389
		ND		1393
γG-Globulin (immune)[d]	+	1843	3.76	ND
		2034		ND
6.4S Immunoglobulins	+	ND		4036
		ND		3551

[a] A 0.5-mg amount of γM-globulin or γM-subunits and a 0.8-mg amount of the other immunoglobulins was used to yield the same final amount of precipitate. [b] Net counts after correcting for background (457, expt 1; 449, expt 2). [c] Optical density expressed as that of a solution of the precipitate in 1.0 ml of 0.1 N NaOH. [d] γG-Globulin isolated from serum of the immunized horse by the same method used to isolate γG-globulin from the unimmunized horse. [e] Not done.

position of light chain. This second component was found to be identical with light chain prepared from either γG- or γM-globulin by conventional methods upon testing it with anti-light chain serum. Thus, the concentration of reducing agent required to depolymerize γM-antibody to subunits was also sufficient to release about 32% of its light chain from covalent bonding with heavy chain.

Retention of Activity by Reductively Produced Subunits. When reduced with 0.085 M mercaptoethanol, antibodies of the γM-globulin class lost their ability to precipitate with antigen (see Figures 2C and 5D). Electrophoresis of whole antisera, before and after reduction with 0.085 M mercaptoethanol, followed by development of the precipitin arcs of the various immunoglobulins with a mixture of anti-horse immunoglobulins and radiolabeled SI, resulted in distinct labeling of the precipitin arcs corresponding in position to those given by γM-antibody or its subunits, as shown by radioautography (Figure 2E). Likewise, both the isolated γM-antibody and the subunit derived from it bound labeled antigen as shown in the radioautographs depicted in Figure 2, E and F.

To quantitate the amount of radioactive antigen coprecipitated by the γM-subunit, the method of tube coprecipitation was employed. First it was shown that the addition of enough antiglobulin to precipitate all of the γM-antibody in the presence of different concentrations of antigen did not appreciably affect the amount of labeled polysaccharide found in the precipitates (Figure 6). In general, the amount of protein in the precipitates was at least 8.5 times greater in the presence of the antiglobulin than in its absence.

Table I, containing data from two separate experiments, reiterates the findings presented in Figure 6. In addition, it shows that the amount of labeled antigen coprecipitated by the γM-subunits, compared to that directly precipitated by the γM-antibody alone, amounted to 104 and 95.7% in experiments 1 and 2, respectively.

Since the presence of the rabbit anti-horse immunoglobulin might decrease the amount of specifically precipitated antigen and increase the amount of nonspecifically absorbed antigen, a more parallel comparison would be of the amount of labeled antigen coprecipitated by γM-subunits relative to that coprecipitated by the intact γM-antibody in the presence of the rabbit antiserum. On this basis of comparison, the γM-subunits coprecipitated 99.5% (experiment 1) and 82.2% (experiment 2) of the amount of labeled antigen coprecipitated by the intact antibody.

The counts precipitated by the two γG-globulin preparations were presumably due to nonspecific absorption. One of these γG-globulin preparations was prepared from normal serum while the other was prepared from whole immune serum but did not contain detectable anti-SI precipitins. Thus, the possibility exists that a fraction of the counts present in the large amounts of precipitate formed by the reaction of the rabbit antisera with either the γM-antibody or the γM-subunits was nonspecifically coprecipitated. However, the total amount of precipitate formed in both cases was almost the same, and therefore the amount of nonspecifically absorbed labeled antigen would presumably be about the same in both cases.

Finally, Table I also shows that the "6.4S immuno-

globulins" were capable of coprecipitating labeled antigen and that, comparing the counts coprecipitated by the normal γG-globulins, at least 63% of the labeled antigen present in the precipitate had been specifically coprecipitated. The "6.4S immunoglobulins" were slightly more effective at coprecipitating SI than either the intact γM-antibody or its subunits.

Reoxidation of the Subunits of γM-Antibody. Ultracentrifugal analysis of the γM-subunits formed by reduction is shown in Figure 3F. The subunit alone had a sedimentation constant of 6.2 S. After reoxidation of the subunits, sedimenting boundaries having $s_{20,w}$ = 6.8, 9.9, 11.6, and 17.1 S appeared, indicating that the subunits had reaggregated to various degrees (Figure 3, G and H). The reoxidized antibody was capable of forming precipitates with SI as can be seen in Figure 5D. Quantitative precipitin curves obtained for several preparations indicated that up to 42% of the reoxidized subunits could be precipitated by SI relative to the intact, untreated γM-antibody.

Discussion

Using a series of pathologic human macroglobulins, Deutsch and Morton (1957, 1958) first showed that γM-globulins were susceptible to reductive depolymerization with 0.1 M mercaptoethanol. A similar reduction of horse γM-antibody to subunits was found to occur on incubation for 30 min with concentrations of mercaptoethanol 0.075 M and higher. Incubation in mercaptoethanol at concentrations of 0.04 to 0.06 M resulted in only partial depolymerization. The concentration of reducing agent required to dissociate the γM-antibody is considerably higher than that required to halve the "5S product" of pepsin digestion of rabbit γG-globulin (0.008 M mercaptoethanol, pH 5, 1 hr, at 37°) (Mandy and Nisonoff, 1963). In fact, the concentration of reducing agent (0.075–0.1 M) required to depolymerize γM-globulin was very close to that (0.06 M) found by Fleishman *et al.* (1963) to be sufficient to reduce all of the interchain disulfide bonds in horse γG-globulin. Thus, it appears that reductive depolymerization of macroglobulin antibody requires cleavage of very resistant or very many disulfide bonds. Indeed, Jacot-Guillarmod and Isliker (1962) found that about 22 disulfide bonds were cleaved on depolymerization of human γM-globulin with 0.01 M borohydride. One might anticipate that some of the light chains of γM-globulin would be no longer covalently bound to the rest of the molecule after its depolymerization. Starch gel electrophoresis of reduced horse γM-antibody under conditions known to dissociate the chains of reduced γG-globulin (pH 3.0, 8 M urea) (Edelman and Poulik, 1961) did reveal the presence of a second, rapidly migrating component in the subunit mixture, which could have been free light chain. Free light chain was then isolated by dissociating it from the subunit in 0.5 M propionic acid 6 M in urea, and subsequent gel filtration.

Reduction of the horse γM-antibody to subunits resulted in loss of ability to precipitate antigen in solution or in agar gel. Through the years, the loss of a variety of activities indicative of antigen–antibody interaction upon reduction of whole serum or immune globulin has been taken to demonstrate the macroglobulin nature of the antibody under study (see introduction). However, a number of studies indicated that loss of precipitating or agglutinating ability upon reduction need not indicate inactivation of the antibody combining sites of γM-globulin (Chan and Deutsch, 1960; Schrohenloher *et al.* 1964; Onoue *et al.* 1964a,b). Using radioimmunoelectrophoresis we confirmed the retention of binding activity by the γM-subunits that were present in both reduced anti-SI sera or that were derived from purified, homogeneous γM-antibody by using ^{125}I-labeled SI and a goat anti-horse γM-globulin for coprecipitation. To quantitate the amount of binding activity retained by the γM-subunits, relative to their parent intact γM-antibody, quantitative tube coprecipitation was used. There was no significant difference between the amount of radiolabeled SI coprecipitated with anti-γM-globulin by the intact γM-antibody or the γM-subunit. Thus, our results indicate that reductive depolymerization of purified horse γM-antibody does not inactivate antibody combining sites.

Restoration of the ability to produce some of the secondary effects of γM-antibody due to its cross-linking properties, such as agglutination or precipitation, by oxidative recombination of its subunits has been reported by Jacot-Guillarmod and Isliker (1962). These workers succeeded in restoring the agglutination titer of reduced human anti-A or anti-B γM-isoantibodies by immediate dialysis of the reduction mixture against Veronal buffer, pH 7.4, according to the procedures used by Deutsch and Morton (1958) to oxidatively recombine subunits of Waldenström's macroglobulin. Likewise, Schrohenloher *et al.* (1964) have partially recombined the subunits of rheumatoid factor in the same manner, and the reoxidized preparations regained ability to precipitate with human γ-globulin. We have found that removal of thioglycolate from depolymerized horse γM-antibody on Dowex 1-acetate, followed by pressure dialysis against phosphate–borate buffer, pH 8.0, also leads to partial reassociation of subunits and restoration or precipitating ability. Thus, it seems that γM-subunits retain active antibody sites but that they are unable to cross-link particles or antigen. Even partial reassociation of the subunits restores significant precipitating ability. The subunits presumably cannot cross-link antigen either because they are univalent or because their sites are so oriented that they cannot combine with more than one high molecular weight antigen at a time.

Although the bulk of the antibodies (82%) precipitated from the horse anti-SI serum by the polysaccharide preparation were of the γM-class, γG- and γA-antibodies were also identified. The formation of antibodies of three classes (γG, γM, and γA) to a single antigen has been described for the human (Fahey and Goodman, 1964) and the rabbit (Onoue *et al.*, 1964a). Recently Rockey *et al.* (1964) have described the formation of a new 10S γ_1-antibody to the *p*-azophenyl-β-lactoside group along with γA-, γM, and three distinct

γG-antibodies by the horse. In our own anti-SI system, the actual specificities of the γA-, γG, and γM-antibodies have not been defined as narrowly. However, it appeared that at least a part of the γG- and γA-antibodies reacted with the same polysaccharide molecule that was precipitated by the γM-antibody. Since relatively large amounts of these separate classes of antibody are available, it should be possible to compare certain of their biologic properties, such as their opsonizing and complement-fixing activities.

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