The Interaction of Bovine Serum Albumin and Its Chicken Antibodies*†

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(Received for publication, January 29, 1962)

In 1937 Heidelberger and Pedersen (1) mixed assayed amounts of antigen (G) and antibody (A), and used ultracentrifugal analysis to determine the amount of free component, antigen or antibody, in the system. Somewhat later these experiments were extended by Pappenheimer, Lundgren, and Williams (2) with diphtheria toxin and its equine antibody to the point at which it could be demonstrated that the empirical composition of the dissolved complex in the region of antigen excess varies from AG to AG2, approaching the latter value in the region of high antigen excess. In short, the "valence" of the precipitating equine antibody is 2.

More recently, Singer, and Campbell (3) devised an alternative method for analyzing soluble complexes which depends upon the fact that at low pH, such as 2.4, the antigen-antibody bonds are broken. In this way it becomes possible to determine, ordinarily by either electrophoresis or sedimentation analysis, the amount of antigen from a total, measured at the low pH, which is bound to a given measured amount of antibody at pH 8.6. With the bovine serum albumin-rabbit anti-bovine serum albumin system and the ovalbumin-rabbit anti-ovalbumin system, it could be established with these methods that the valence of the precipitant is in each case 2.

The reports to which reference is made are representative of one approach to the problem. In other experiments, which have to do with hapten binding, the number of active sites on rabbit antibodies is again found to be two (4). Thus, even though the study of the combining power of antibodies formed in other species is now in its early stages, it was a matter of unusual interest to find a paper by Banovitz, Singer, and Wolfe (5) in which for the system BSA-chicken anti-BSA antibody, the mole ratio, r, of antigen to antibody in the complex, as determined largely by electrophoretic analysis, turned out to be relatively constant and not greater than approximately 0.9. No explanation is given of why r is relatively constant, but its low value is described as being the result of one of two situations: (a) either the antibody is a mixture of 1- and 2-valent species so that the degree of binding of BSA is less than in the rabbit system where all the antibody is bivalent, or (b) the antibody is essentially all bivalent but with relatively weak binding as compared to rabbit antibody. Other data which are set down are said not to be consistent with the presence of both 1- and 2-valent antibody, so the latter explanation is considered to be the more likely. Because of the two unexpected observations, relative constancy and very low value for r, it was decided to apply ultracentrifugal analysis for an independent reinvestigation. A possible key to an explanation was found in the recommendation of Goodman, Wolfe, and Norton (6) that an 8% salt medium was the optimal condition for precipitating the antibody from the serum, with the amount of precipitate formed depending in a dramatic manner upon the amount of salt present. Such behavior has not been observed when rabbit and horse sera are used in like manner. Then, during the progress of our research, we became aware of a report of Makino- don, Gengozian, and Canning (7) who demonstrated in the ultracentrifuge the existence of a normal serum macroglobulin which is occluded with the BSA-chicken anti-BSA precipitate, a protein which is not resolved on electrophoresis. This macroglobulin was reported to be a characteristic constituent of chicken sera, whether normal or immune; furthermore, it is occluded but not specifically bound in the BSA antigen-antibody complexes. Later on, after the completion of our experiments, it was noted that Orlans, Rose, and Marrack (8) question the existence of such a component in normal serum. They report that fowl antiserum to any one antigen contains two types of homologous antibodies, and assign molecular weights of approximately 800,000 and 180,000 to them. The latter, of normal size, showed but one combining site for antigen in antigen excess. We sought to conduct experiments under conditions that would permit the occurrence of an inert macroglobulin to be probed; if, at low pH, its amount to be quantitatively measured; and its presence in the antigen-antibody system taken into account. A description of such experiments, their results, and the conclusions we believe may be properly drawn from them form the substance of this report.

EXPERIMENTAL PROCEDURE

Preparation of Solutions of Soluble Complexes—Adult Arbor Acre White Rock chickens were treated by intravenous injection of 40 mg of crystalline BSA per kg of body weight. Eight days later, the birds, which had been deprived of food for 30 hours to reduce the lipid content of the serum, were bled by cardiac puncture. This procedure was found to give a high antibody titer.

* Supported by grants-in-aid from the United States Public Health Service (NIH-A3030(C6)) and the University of Wisconsin Research Committee, using funds provided by the Wisconsin Alumni Research Foundation. This work is taken from a thesis submitted by one of the authors (D. D. D.) to the Graduate School of the University of Wisconsin in partial fulfillment of the degree of Doctor of Philosophy.

† Presented before the American Association of Immunologists, Atlantic City, New Jersey, April 10 through 14, 1961.

The abbreviation used is: BSA, bovine serum albumin.

2 The BSA used throughout the experiments was obtained from Armour Pharmaceutical Company, Kankakee, Illinois, as Lot No. V 68802, and was used as received.
The blood was allowed to clot at room temperature, and the serum which rose to the top of the tubes was poured off and pooled. This serum was slightly hemolyzed and was designated Serum I. The remaining clot was allowed to stand in a refrigerator overnight and then was centrifuged at 5°. The remaining serum was pooled and designated Serum II. It showed more hemolysis than Serum I, although it was not markedly lyzed. Methyldemethate in the amount of 10,000 was added to both sera for preservation. The sera were stored in polyethylene bottles at 5°.

The equivalence point of Serum I was determined by a modified method of Heidelberger and Kendall (9). In duplicate tubes, 1.0 ml of 1% sodium chloride solution was added to 0.5 ml of serum, and 0.5 ml of BSA antigen dissolved in 1% sodium chloride was layered on top of this mixture. A series of 8 to 12 tubes was made up in this manner, the only difference being that the concentration of antigen was increased in each successive set of tubes. The final salt concentration was 1%. The reaction mixtures were incubated at 37° for 2 hours and then placed in the cold room overnight. The precipitates were centrifuged at 5° with a Servall angle head centrifuge. The supernatants were collected and analyzed for the presence of free antigen and antibody by the flocculation method. The precipitates were washed twice with 1% salt, and dissolved in 3.0 ml of 1 N sodium hydroxide. The concentration of this master solution was 1.865%. The mixture was incubated at 37° for 2 hours, and stored in the cold room overnight. After centrifugation, the resultant precipitate was washed twice with cold 1% salt, and suspended in a 0.2 ionic strength sodium chloride solution, pH 7.5. To this suspension were added 1.974 g of BSA dissolved in the 0.9% sodium chloride-phosphate buffer. The mixture was stirred for 2 hours at 37°, and at the end of this time nearly all of the precipitate had dissolved. The final volume of this solution was adjusted to 150 ml with the sodium chloride-phosphate buffer. The protein concentration of this master solution was 1.86%.

An aliquot of the master solution was dialyzed against a glycine-HCl buffer (pH 8.6; I/2 = 0.3) or the glycine-HCl buffer. Other solutions of the soluble complexes were performed in either a Veronal-NaCl buffer (pH 8.6; I/2 = 0.3) or the glycine-HCl buffer. Other solutions of the soluble complexes were prepared by addition of BSA solution to an aliquot of the master solution. All solutions at pH 8.6 are designated by unprimed letters, whereas the corresponding solutions at pH 2.4 are designated with primed letters.

For each solution, one aliquot was dialyzed against the Veronal-NaCl buffer, and a second against the glycine-HCl buffer. Equilibrium was reached in 2 days of constant stirring at 5° against multiple changes of buffer. The pH measurements for the protein solution were made with a Beckman pH meter. It was found that the pH of a solution originally at 7.5 fell to 2.5 within a half-day when dialyzed against one change of the glycine-HCl buffer, so that there was little doubt that the solution had reached equilibrium at the low pH in the time allotted for the attainment of equilibrium. For each solution, dilutions were made by adding a weighed amount of dialyzed buffer to a weighed sample of the complex solution. Each solution was studied in the ultracentrifuge at three or four different total protein concentrations, enabling correction for the Johnston-Ogston anomaly to be made.

**Preparation of Complexes from 8% NaCl Medium: Solutions E-F and E'-F'**—Except for slight modifications, the equivalence point of Serum II was determined in the same manner as that for Serum I. The final salt content of the precipitating medium was 8%. The BSA was dissolved in an 8% sodium chloride medium, and 2 volumes of 11.5% sodium chloride were added as diluent. The precipitate was washed twice with 8% NaCl, and dissolved in excess antigen, forming Solution E.

To an aliquot of this solution was added an equal volume of a saturated ammonium sulfate solution. The system was then half-saturated with respect to ammonium sulfate. The complexes were precipitated from solution upon standing at room temperature, and then centrifuged at 5°. Immediately after centrifugation, the precipitate was dissolved in the sodium chloride-phosphate buffer, to give Solution F. This procedure results in a preparation of complexes in lower antigen excess than would otherwise be possible. It is to be emphasized that the precipitate must be worked up immediately; if it is left standing for any length of time, great difficulty is encountered in trying to dissolve the precipitate.

**Velocity Sedimentation**—A Spinco model E ultracentrifuge was used in the sedimentation studies. The experiments were performed at 25.0° at a speed of 42,040 r.p.m. The temperature was controlled to within 0.05° and the speed to within 10 r.p.m. The optical system was equipped with a phase plate. A further aid in the analysis was the use of a double-sector cell, for this cell permits the simultaneous recording of the base-line and the pattern of the solution. This is very important for an analysis of this type where one component is present in much smaller amount than the others. The time between each exposure was measured with an electric timer, with zero time taken as the time that full speed was obtained. Most exposures were for 60 seconds. The relatively long exposure time was needed because the double-sector cell was used. The time, temperature, and angular velocity need be known only for the calculation of the sedimentation coefficients; they do not enter into the computation of the amounts of the constituents.

The area measurements were made on enlarged tracings and measured with a planimeter. The reproducibility of the apparent percentage of free antigen and apparent percentage of
total antigen represented a ±1% deviation. One criterion was used to ensure internal consistency in the measurement of the area under the resulting concentration gradient curves. The BSA boundary gradient curve was assumed to be Gaussian in form, and the leading half of the BSA experimental curve was drawn such that it would be symmetric about the maximum of the curve. Then the trailing side of the next component (soluble complex, pH 8.6; γ-globulin, pH 2.4) was drawn in such a way that it intersected the symmetric curve at a point where the area beneath this point just equaled the area above it. Then a perpendicular was dropped from the experimental curve through this point to the base line. A similar procedure was employed to determine the location of the minimal gradient between the macroglobulin and the middle component.

Once the apparent areas were measured, each was multiplied by the radial dilution factor \((x_H/x_0)^2\), where \(x_H\) is the position of the maximal point of the gradient curve, and \(x_0\) is the position of the meniscus. The values of the radial dilution correction for these experiments usually varied between 1.05 and 1.20. The apparent percentage of any constituent was calculated from the formula:

\[
\text{Apparent} \% \ A = \frac{\text{area of } A}{\text{total area}} \times 100
\]

To compute the true values we resorted to the procedure of plotting apparent percentage of constituent against total protein concentration and extrapolating to zero protein concentration. This intercept gives substantially the true percentage of the constituent, since the Johnston-Ogston anomaly vanishes in the limit of zero total protein concentration.

The slope of the curve of log \(x_H\) versus \(x_0^2\) provides a measure of the sedimentation coefficient. In practice, since the smallest constituent has a molecular weight of approximately 67,000, \(x_H\), the maximal point of the concentration gradient curve, and \(x_0\), the position of the square root of the second moment, do not differ significantly in value. The boundary gradient curves were nearly symmetrical. The calculated sedimentation coefficients are reported for the average concentration, \(\bar{c}\), between the first and last pictures within each experiment (11). The sedimentation coefficients were extrapolated to zero concentration to obtain the sedimentation coefficient extrapolated to zero concentration, measured at 25° in the buffer used.

A perceptive measure of the protein concentration dependence of the sedimentation coefficient is to plot \(s/s^0\) against \(\bar{c}\). The coefficient \(s\) is the sedimentation coefficient at the finite concentration, \(\bar{c}\), and \(s^0\) is the sedimentation coefficient extrapolated to zero protein concentration. Fig. 1 shows the results for all the components present in a typical set of solutions, A and A', and demonstrates the sensitivity of this plot.

**RESULTS**

*Sedimentation Analysis*—Solutions of soluble complexes in excess antigen, as well as immune and normal chicken serum, were examined in the ultracentrifuge. In general, the studies can be divided into three broad classes: those of (a) sera in media of neutral pH at the two different salt concentrations, (b) complexes in excess antigen at pH 8.6, and (c) solutions of former complexes which have been dissociated by dialysis to pH 2.4.

For the control experiments, sedimentation patterns at 25° for normal and immune sera were obtained. The normal (2-day-old) chicken serum was dialyzed against a 0.15 M NaCl solution in preparation for the study in the ultracentrifuge. Two macroglobulins, a faster one with \(s = 33\) S and a slower one with \(s = 25\) S, and present in nearly equal amounts were observed. In addition, the usual main globulin boundary and one representative of albumin made their appearance. The macroglobulins comprised 12% of the serum proteins, the albumin 67%, and the normal globulins some 21%, figures which are uncorrected for concentration dependence.

The immune chicken serum was also dialyzed against 0.15 M NaCl solution for a parallel experiment. Now three boundaries appeared, representative of a single macroglobulin with \(s = 25\) S, 10%; the normal globulins with \(s = 7\) S, 37%; and the albumins with \(s = 4.5\) S, 53%. The disappearance of the 33 S form of macroglobulin in favor of a 25 S form can take place in normal sera. Thus, if fresh normal serum is dialyzed first against a 1.5 M NaCl solution, and then redialyzed against a 0.15 M NaCl system and studied in the ultracentrifuge, it was found that but one macroglobulin boundary appears, but the over-all composition of the serum has changed but slightly; albumin makes up 66% of the protein content, the globulins 24%, and the macroglobulins 10%.

In all these situations there is a degree of uncertainty in the data given for both relative amounts of protein and sedimentation coefficients in that they have not been corrected for concentration dependencies; the point is that the relative amount of the macroglobulins was always substantially the same, some 10 to 12% of the total. Furthermore, it will appear that the amount of macroglobulin remains unchanged when solutions of the antigen-antibody complexes are investigated.

A typical schlieren diagram for a solution of complexes at pH 8.6 is shown in Fig. 2. Three components are readily distin-
Fig. 2. Typical sedimentation pattern for solutions of soluble complexes at pH 8.6. Sedimentation proceeds from left to right. Leading peak represents macroglobulin, middle peak represents complexes, and slowest moving peak represents free BSA.

Fig. 3. Typical sedimentation pattern for solutions at pH 2.4. Sedimentation proceeds from left to right. Leading peak represents macroglobulin, middle peak represents antibody globulin, and last peak represents the total BSA.

Fig. 4. Typical sedimentation pattern for solutions of complexes at pH 8.6, $\Gamma/2 = 0.3$. Exposure taken 4000 seconds after attaining full speed. The macroglobulin has reached the bottom of the cell. Exposures at a later time indicated complete resolution between the free BSA (trailing peak) and the complexes (leading peak).
Table I
Dependence of composition on total protein concentration for Solutions A and A’

<table>
<thead>
<tr>
<th>Solution</th>
<th>Total protein concentration (g/100 ml)</th>
<th>Antigen apparent %</th>
<th>Macroglobulin apparent %</th>
<th>Complex apparent %</th>
<th>γ-Globulin apparent %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.86</td>
<td>67.2</td>
<td>3.0</td>
<td>29.8</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.08</td>
<td>64.1</td>
<td>6.3</td>
<td>29.6</td>
<td></td>
</tr>
<tr>
<td>A’</td>
<td>0.71</td>
<td>62.6</td>
<td>7.9</td>
<td>29.5</td>
<td>14.1</td>
</tr>
<tr>
<td>A’</td>
<td>1.33</td>
<td>79.4</td>
<td>5.5</td>
<td>15.1</td>
<td></td>
</tr>
<tr>
<td>A’</td>
<td>0.79</td>
<td>76.6</td>
<td>7.7</td>
<td>15.7</td>
<td></td>
</tr>
</tbody>
</table>

Obtained for this system at 25°. At a later time, the faster moving complex piles up at the bottom of the cell, but the minimum in the boundary gradient curve between the free antigen peak and the complex peak very nearly reaches the base-line. Furthermore, the concentration of free BSA did not change during the course of the experiment, indicating that the rates of re-equilibration were shorter than the time necessary for separation. This constitutes a satisfactory proof that the schlieren diagrams may be used in the conventional manner to measure the concentration of each species.

Since relative concentrations are all that are required for our analysis, only the relative areas under the schlieren diagrams need to be measured. The measured area assigned to any component is the average value determined from three to eight exposures during an experiment. The macroglobulin, which is present in small amounts, travels so rapidly that many worthwhile pictures cannot be taken. Usually four to five measurements were made for it in every solution. The record of the analysis of Solutions A and A’ is suggestive of the mode of analysis for all the other solutions. This solution was the master solution from which Solutions B, C, and D were derived. The apparent percentages of the components, corrected for radial dilution, for these solutions are given in Table I.

Plots of apparent percentage of component versus total protein concentration are shown in Figs. 5, 6, and 7. The extrapolated values for Solution A are 59.8% BSA, and 10.9% macroglobulin. For Solution A’ they are 72.5% BSA, 11.0% macroglobulin, and 16.5% antibody γ-globulin. The percentage of complex can be obtained by difference. The amount of the antibody globulin, also calculated by difference, is 16.6%, a figure which agrees well with the extrapolated value. For the other solutions, the relative amount of antibody γ-globulin, Column 5, was always computed by difference. Again the percentage of antigen bound is the difference between the percentage of total antigen (from Solution A’), and the percentage of free antigen (from Solution A) or 72.5 - 59.8 = 12.7%. Since all the antibody present has reacted, the total percentage of antibody equals the percentage of antibody bound. This is 16.5%. The mole ratio of antigen to antibody, (Ag/Ab)exp, or r, is calculated by dividing the percentage of bound antigen by the percentage of bound antibody, and multiplying this ratio by the appropriate molecular weight ratio, 2.32. For Solutions A and A’, this ratio is (12.7:16.5) × 2.32 = 1.78.

Table II gives a summary of the experimental data. The

3 The molecular weight of the antigen was taken as 67,000; the corresponding datum for the γ globulin antibody was assumed to be 155,000.
TABLE II
Composition and mole ratio from sedimentation experiments and from Goldberg theory

<table>
<thead>
<tr>
<th>Solution</th>
<th>pH</th>
<th>Albumin</th>
<th>Complex</th>
<th>Globulin</th>
<th>Macroglobulin</th>
<th>Experimental mole ratio, ( r )</th>
<th>Theoretical mole ratio, ( r' )</th>
<th>G/A</th>
<th>Macroglobulin ( \times 100 ) total globulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8.6</td>
<td>59.8</td>
<td>20.3</td>
<td>16.5</td>
<td>10.0</td>
<td>1.78</td>
<td>1.84</td>
<td>10.2</td>
<td>38.5</td>
</tr>
<tr>
<td>A'</td>
<td>2.4</td>
<td>72.5</td>
<td>25.6</td>
<td>13.7</td>
<td>8.8</td>
<td>1.85</td>
<td>1.90</td>
<td>14.6</td>
<td>39.1</td>
</tr>
<tr>
<td>B</td>
<td>8.6</td>
<td>65.6</td>
<td>25.6</td>
<td>13.7</td>
<td>8.8</td>
<td>1.89</td>
<td>1.90</td>
<td>14.8</td>
<td>37.5</td>
</tr>
<tr>
<td>B'</td>
<td>2.4</td>
<td>77.5</td>
<td>22.7</td>
<td>12.5</td>
<td>7.5</td>
<td>1.95</td>
<td>1.94</td>
<td>24.8</td>
<td>41.3</td>
</tr>
<tr>
<td>C</td>
<td>8.6</td>
<td>69.8</td>
<td>27.8</td>
<td>12.5</td>
<td>7.5</td>
<td>1.96</td>
<td>1.97</td>
<td>24.8</td>
<td>38.0</td>
</tr>
<tr>
<td>C'</td>
<td>2.4</td>
<td>80.0</td>
<td>27.0</td>
<td>13.7</td>
<td>8.6</td>
<td>2.02</td>
<td>1.99</td>
<td>28.0</td>
<td>48.0</td>
</tr>
<tr>
<td>D</td>
<td>8.6</td>
<td>79.3</td>
<td>15.0</td>
<td>8.1</td>
<td>5.7</td>
<td>2.08</td>
<td>2.09</td>
<td>38.1</td>
<td>38.1</td>
</tr>
<tr>
<td>D'</td>
<td>2.4</td>
<td>86.2</td>
<td>24.0</td>
<td>8.6</td>
<td>5.7</td>
<td>2.08</td>
<td>2.09</td>
<td>38.1</td>
<td>38.1</td>
</tr>
<tr>
<td>E</td>
<td>8.6</td>
<td>67.4</td>
<td>24.0</td>
<td>13.7</td>
<td>13.3</td>
<td>2.14</td>
<td>2.16</td>
<td>41.8</td>
<td>41.8</td>
</tr>
<tr>
<td>E'</td>
<td>2.4</td>
<td>77.7</td>
<td>31.7</td>
<td>18.5</td>
<td>13.3</td>
<td>2.17</td>
<td>2.09</td>
<td>38.1</td>
<td>38.1</td>
</tr>
<tr>
<td>F</td>
<td>8.6</td>
<td>55.0</td>
<td>31.7</td>
<td>18.5</td>
<td>13.3</td>
<td>2.17</td>
<td>2.09</td>
<td>38.1</td>
<td>38.1</td>
</tr>
<tr>
<td>F'</td>
<td>2.4</td>
<td>68.8</td>
<td>31.7</td>
<td>18.5</td>
<td>13.3</td>
<td>2.18</td>
<td>2.16</td>
<td>41.8</td>
<td>41.8</td>
</tr>
</tbody>
</table>

![Fig. 8. Plot of antigen-antibody mole ratios versus G/A.](http://www.jbc.org/) Open circles are calculated from data for solutions prepared in 1% NaCl; solid circles calculated for solutions prepared in 8% NaCl. The upper solid curve was calculated from the Goldberg theory, assuming only bivalent antibody to be present. The lower curve was calculated for an equimolar mixture of univalent and bivalent antibody molecules. For definition of symbols on the ordinate, see "Sedimentation Analysis."

Dividing the percentage of total antigen by the percentage of total antibody, and multiplying this value by the ratio of the molecular weights, 2.32. It will be noted from Column 6 that the amount of macroglobulin in any given solution of complexes, A-A', B-B', etc., was not affected by the change in pH. At both pH values, however, the apparent percentage exhibited a very strong protein concentration dependence. Since the extrapolated values at both pH values were nearly the same, we averaged the extrapolated values for the computations. The ratio of macroglobulin to the total globulin is given in Column 10. It reflects the amount of macroglobulin that has been occluded in the precipitate. It is especially noteworthy that the amount does not seem to change for solutions prepared from the higher salt medium.

In Column 8 are tabulated the mole ratios predicted by the Goldberg (14, 15) theory which correspond to the several G/A ratios, assuming that all antibody present is bivalent, and that the antigen has six combining sites. Fig. 8 shows the agreement of the experimental data with the theoretical curve. The upper solid curve was calculated from the theory in which values of G/A between zero and 25 were chosen for the computation. It was assumed that all the antibody was bivalent. The lower solid curve was computed for the case of a system which would be composed of an equimolar mixture of univalent and bivalent antibody molecules.

Nature of Macroglobulin—It has only been in the last few years that much effort has been devoted to the study of macroglobulins. Most of the macroglobulins studied have been isolated from pathological sera. Normal human serum contains only 2 to 5% macroglobulin. A macroglobulin has been detected in normal rabbit serum, as well as in immune rabbit serum. Their electrophoretic mobilities lie between those of the β- and γ-globulins.

The immunological specificity of the pathological macroglobulins had been known before detailed studies on the "normal" macroglobulins were made. The pathological macroglobulins exhibit the usual specific immunological behavior, although they may be capable of cross-reacting with other macroglobulins. This indicates that these molecules have common antigenic properties. Antibodies in the antipneumococcal horse, pig, and monkey systems are found in the 20 S components. Thus, it may be possible that all the macroglobulins possess an antibody activity. To our knowledge, however, it has not been shown that BSA has ever produced antibodies in animal systems whose sedimentation coefficients are so large that they could be considered as macroglobulins. We are concerned, of course, with the possibility that the macroglobulin might have reacted chemically with the BSA in our system; that it may react with some other antigen is not relevant to our problem. It has been assumed that the macroglobulin was immunologically inert in the computation of the molecular ratio of bound antigen to bound antibody in the sedimentation experiments. The following observations strongly support this assumption.

When the solutions of soluble complexes, and solutions of complexes dissociated by dialysis to pH 2.4, were examined in the ultracentrifuge, the macroglobulin was present in both cases, but when similar solutions were examined by electrophoresis
only two maxima in the boundary gradient curves were observed. Neither of these two peaks could be caused by the presence of the macroglobulin alone. In electrophoresis at the several pH values the peak assigned to antibody globulin alone still included the macroglobulin of similar electrophoretic mobility. It could be either antibody or inert protein.

Sedimentation analysis of solutions of soluble complexes at pH 8.6 showed that a component with e - 25 S is present. It had the same sedimentation coefficient as the macroglobulin in either normal or immune serum. When solutions at pH 2.4 were studied in this way, the sedimentation coefficient of the macroglobulin fell from 25 S to 16 S. However, within experimental error, the amount of this heavier component did not change with pH. This decrease in the sedimentation coefficient could be attributed to an expansion of the molecule at pH 2.4. It could have been postulated that the macroglobulin dissociated when the pH was brought to 2.4, thus providing a smaller sedimentation coefficient. However, Müller-Eberhard and Kunkel (16) have reported that no such dissociation occurred even at pH as low as 1.9. These observations were the same for all solutions studied regardless of the amount of antigen in the system. Whereas the usual globulin fraction was increased from 20 to 37% of the total protein on immunization, the relative amount of the macroglobulin was unchanged.

Again, the observed amount of macroglobulin is the same at pH 8.6 and at pH 2.4. If the macroglobulin possessed antibody activity, the amount of the fact sedimenting component at pH 2.4 should be less than that at pH 8.6, because at the higher pH some antigen would be bound to it. The fact that no change in the amount of macroglobulin was detected implies that no antigen was bound to it; in other words, it is inert.

Another observation that augments the validity of our assumption is the observation of Makinodan, Gengozian, and Canning (7) that no macroglobulin antibody activity could be determined by the double serum agar diffusion technique. They performed over 100 analyses, and concluded that the macroglobulin was a nonantibody, i.e. inert, globulin. This technique, if properly used, is a very sensitive tool for the investigation of problems of this type. Such arguments have led us to conclude that the fastest sedimenting serum protein is an inert macroglobulin. The general physical behavior of this macroglobulin is similar to that observed for other macroglobulins.

**DISCUSSION AND CONCLUSIONS**

Basic to our arguments and calculations which lead eventually to the value of 2 for the combining power of chicken precipitating antibodies is the proposition that antigen-antibody bonds are completely dissociated in systems at pH 2.4. Indeed, there has been some question about this point, for recently Pressman and Yagi (17), in their studies of the effect of pH on the dissociation of protein antigen-antibody complexes, have concluded that the reaction is not complete at pH 2.4. This result is contrary to that of Singer and Campbell, and it is inconsistent with our own findings.

We turn to the analysis of the stock solution. By sedimentation analysis at this pH, it has been shown that the stock solution, Solution A', is composed of 72.5% BSA, 16.5% antibody globulin, and 11.0% macroglobulin. The protein concentration of this solution was found to be 1.865 g/100 ml, and the final volume was 150 ml. Therefore, there were 2.798 g of protein in the final solution. There was added 0.072 g of BSA to precipitate the antibodies from the serum. Since this antigen concentration is near the equivalence point, actually in very slight antigen excess, substantially all the antigen will be bound in the precipitating floccules. As previously mentioned, 1.974 g of BSA had been added to solubilize the precipitate, so 2.046 g of BSA were present in the final solution. By this conventional and independent method, it is seen that BSA comprises 73.1% of the total protein. The agreement is within experimental error. The value calculated from this second method might be expected to be somewhat high because we have assumed that all 72 mg of antigen has been bound in the precipitate. These data are indicative of complete resolution of antigen and antibody at pH 2.4.

Therefore, with an entirely conventional and well established method of analysis, the sedimentation velocity experiment, the mole ratio of BSA to its chicken antibody is shown to approach the limiting value of 2 as the relative amount of antigen in the system is increased. Furthermore, it is demonstrated that the observed mole ratios at the several G/A values agree well with the corresponding ratios which are calculated by using the Goldberg theory, assuming that all of the antibody present is bivalent, irrespective of whether the reacting substances are contained in 1% NaCl or 8% NaCl aqueous medium.

**SUMMARY**

Prior physical chemical studies of the soluble complexes of the bovine serum albumin-chicken anti-bovine serum albumin antibody system have led to conclusions which are either inconclusive (5) or inconsistent with the results which have been obtained for rabbit and horse antibody systems (8). It now appears that a macroglobulin, nonspecific in the aggregates, is occluded with the precipitates. On sedimentation analysis this macroglobulin is completely separated from the free antigen and complex. When allowance is made for its presence, and with the conventional methods of calculation, the mole ratio of antigen to antibody is here shown to approach the value 2 as the relative amount of antigen in the system is increased. Further, the observed mole ratios at intermediate finite values of G/A agree well with values computed from the Goldberg theory, assuming that all of the antibody is bivalent, irrespective of whether the reacting substances are contained in 1% NaCl or 8% NaCl aqueous medium.

**Acknowledgment**—We wish to express our thanks to Professor H. R. Wolfe and his staff for their generosity in connection with the provision of antisera and for their advice and interest in the research.

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