BIOPHYSICAL STUDIES OF BLOOD PLASMA PROTEINS
X. FRACTIONATION STUDIES OF NORMAL AND IMMUNE HORSE SERUM*

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A large portion of the antibody proteins of horse serum resides in a fraction possessing an electrophoretic mobility between the normal serum γ-globulins and the lipide-rich β-globulins (1–6). While this component has been designated β-globulin by Kekwick and Record (3) and has been shown to consist of two components (β₁ and β₂), it is analogous to the T component of van der Scheer and Wyckoff (4). We have described the corresponding antibody-rich protein fraction from normal human plasma as γ₁-globulin to distinguish it from the normal serum γ₂-globulin (7). We shall retain this terminology. The need for the separation of these two globulins in order that both their biological and physicochemical properties may be elucidated is evident.

Recently Smith and Gerlough (8) applied the low temperature ethanol fractionation procedures of Cohn et al. (9) developed for the fractionation of human plasma to the separation of the tetanus antitoxin from the plasma of hyperimmunized horses. They found the antitoxic activity to be associated with various fractions and concluded that the pepsin digestion methods of antibody recovery (10, 11) were more suitable for the concentration of such immune plasma systems than the ethanol type of fractionation. Other work on the ethanol fractionation of various animal sera (12–15) has indicated that the successful separation of any electrophoretically well defined protein entity from a given animal serum will require specific conditions and that the methods designed for human plasma cannot be applied to other animal plasmas in toto.

We have found it possible to develop conditions whereby the antibody content of the serum of hyperimmunized horses may be separated in high yield by the low temperature ethanol method in a single precipitation step. This fraction may, however, be divided into various electrophoretic components by subsequent refractionations. The methods of obtaining such

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fractions from normal and hyperimmunized horses and a description of
certain of their biological and physicochemical properties form the basis
of this report.

EXPERIMENTAL

Plasma or serum of normal and hyperimmunized horses was used as the
source material. The immune serum samples were usually aliquots of
relatively large pools of antiserum to either tetanus or diphtheria antitoxin.
In addition a pooled serum sample of two horses that had each been
immunized simultaneously with diphtheria, tetanus, and a heterologous gas
gangrene (vibriorn septique and Bacillus welchii) toxoid, and Hemophilus
pertussis and formalized pneumococcus type III vaccines was also studied.
While it was realized that type III pneumococcus vaccine is a poor antigen,
it was used because the type-specific polysaccharide is nitrogen-free and
allows for the ready determination, by quantitative precipitation, of the
antibody produced. Plasma samples were defibrinated by the addition of
sufficient calcium ion to permit clotting, followed by stirring to remove the
fibrin formed. These sera were then fractionated by means of the aqueous
ethanol precipitation techniques and as usual temperature, pH, protein
concentration, alcohol concentration, and ionic strength were carefully
controlled. The fractionation experiments were evaluated in terms of
electrophoretic composition and of protein and antibody yields resulting
from controlled variations of the several variables of fractionation. All
electrophoretic experiments at pH 8.6 were carried out in veronal buffer
of ionic strength 0.1 for 9000 seconds at a constant potential gradient of
6.0 to 6.5 volts per cm. The mobility experiments were performed in
solutions of ionic strength 0.1 in which sodium chloride made up 80 per cent
of the ionic strength, the remainder being the contribution of a univalent
buffer salt. Velocity sedimentation analyses were carried out with 0.7
per cent protein solutions in the oil turbine ultracentrifuge at 220,000 times
gravity, a schlieren optical method being used to record the position of the
moving boundaries as a function of time.

The antibody assays were obtained by the following tests. Preliminary
diphtheria antitoxin titers were obtained by Ramon flocculation and final
values by guinea pig intracutaneous (L+) tests. The antibodies to
Bacillus tetanus, vibriorn septique, and Bacillus welchii toxoids were as-
sayed by the standard mouse tests. The antibody to Hemophilus pertussis

1 All of the horse serum samples were supplied through the courtesy of Eli Lilly
and Company.
2 The sedimentation velocity experiments were performed by Mr. E. M. Hanson.
3 These assays were carried out in the laboratories of Eli Lilly and Company,
Indianapolis, Indiana.
was determined by agglutination procedures. Pneumococcus antibody assays were attempted by agglutination, by the capsular swelling or Quellung reaction, and by precipitin tests with the specific polysaccharide.

**Fractionation Results**

Preliminary experiments indicated that the electrophoretically heterogeneous \(\gamma\)-globulins and associated antibodies of horse serum could be removed almost quantitatively by precipitation with 25 per cent ethanol at pH 7.5 to 7.8. In addition to the \(\gamma\)-globulins these initial precipitates contained from 5 to 15 per cent of \(\beta\)-globulins. Subsequent experiments were carried out to remove these \(\beta\)-globulins and to provide a \(\gamma\)-globulin fraction made up of proteins having two electrophoretic constituents, the one described as \(\gamma_1\)-globulin and the other as \(\gamma_2\)-globulin. The fractionation conditions which evolved for this purpose are shown in the accompanying Diagram 1.

**Diagram 1**

1000 ml. serum, 3000 ml. H\(_2\)O; adjust to pH 7.7 (±0.1); add 50% EtOH to 25%; temperature -6° to -7°; centrifuge

Ppt. A; largely \(\gamma\)-globulins with some \(\beta\)-globulins

Suspend (dried protein or paste) in sufficient H\(_2\)O to give 1% solution; 0.05 m acetic acid added to pH 5.2 to 5.8; ethanol concentration 0 to 10%; centrifuge at 0° to -2°

Supernatant I

Ppt. A-A; largely \(\beta\)-globulins

Supernatant II; 0.5 m NaHCO\(_3\) added to pH 7.2 to 7.4; 50% ethanol added to 25%; centrifuge at -6° to -7°

Ppt. A-B \(\gamma\)-globulins

Supernatant III; discard

The electrophoretic diagrams of a typical series of fractions employing antidiphtheritic horse serum as starting material are shown in Fig. 1. From 80 to 100 per cent of the serum antibodies to diphtheria and tetanus toxins are usually found in Precipitate A. Considerable care must be taken to keep all precipitates as cold as possible during their removal and suspension prior to lyophilization to prevent marked destruction of antibody. Occasionally low yields (50 to 60 per cent) are experienced in the initial precipitate but, since the antibody is not found in the supernatant it appears that such marked losses are due to an improper handling of Precipitate
A, resulting in antibody destruction. In Table I are shown the results of various typical fractionations on diphtheria and tetanus antisera in terms of yields of protein and antibody. The antibody recovery data must

![Descending electrophoretic patterns of a hyperimmunized horse serum and fractions.](image)

**TABLE I**

Antibody and Protein Recoveries from Serum of Hyperimmunized Horses

<table>
<thead>
<tr>
<th>Antisera to</th>
<th>Units of antibody per 100 ml. plasma*</th>
<th>Weight of ppt. per 100 ml. serum</th>
<th>Units of antibody recovered per 100 ml. serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetanus toxoid</td>
<td>20,000</td>
<td>5.07</td>
<td>3.22</td>
</tr>
<tr>
<td>Diphtheria toxoid</td>
<td>45,000</td>
<td>5.14</td>
<td>3.35</td>
</tr>
<tr>
<td>&quot;</td>
<td>129,000</td>
<td>5.24</td>
<td>3.17</td>
</tr>
<tr>
<td>&quot;</td>
<td>65,000</td>
<td>5.55</td>
<td>3.36</td>
</tr>
<tr>
<td>&quot;</td>
<td>57,500</td>
<td>5.21</td>
<td>3.47</td>
</tr>
<tr>
<td>Tetanus toxoid</td>
<td>45,000</td>
<td>4.10</td>
<td></td>
</tr>
</tbody>
</table>

* Plasma diluted with anticoagulant.

be considered in relation to the shortcomings of the assay procedures. The data indicate that the major portion of the antibody is recovered in Precipitate A.

Refractionation of this precipitate to give Precipitate A-B may be ac-
compounded by excellent yields in some cases and by considerable losses of antibody in others. It appears again that losses of antibody at this point may be due to causes just mentioned. Approximately 10 gm. of protein for every 50 gm. of Precipitate A are lost in preparing Precipitates A-A and A-B under the conditions employed for their separation. It can be seen from Table I that the major portions of the antibody are recovered in Precipitate A or in its subfraction Precipitate A-B. Approximately 2 to 5 per cent of the initial serum antibody may be recovered by lyophilization.

**Diagram 2**

50 gm. Ppt. A, suspended in 4000 ml. H₂O; 0.05 M HAc added to pH 5.38; diluted to 5000 ml.; \( \mu = 0.0014 \); centrifuged at 0°

7.8 gm. (dry) Ppt. A-A

Supernatant IIA, volume 4910 ml.; 150 ml. 0.15 M NaCl and 8 ml. 0.5 M NaHCO₃ added to pH 5.83; 1267 ml. 50% EtOH added to 10% ethanol concentration; volume 5068 ml.; \( \mu = 0.0061 \); centrifuged at -2°

15.9 gm. (dry) Ppt. A-1B

Supernatant IIB, volume 4950 ml.; 1650 ml. 50% EtOH added to 20% ethanol concentration (pH 5.83); \( \mu = 0.0046 \); volume 6600 ml.; centrifuged at -6°

8.5 gm. (dry) Ppt. A-2B

Supernatant IIC, volume 6482; 100 ml. 0.5 M NaHCO₃ added to pH 7.4; 500 ml. 95% EtOH added to 25% ethanol concentration; volume 7082, \( \mu = 0.01 \); centrifuged at -7°

9.9 gm. (dry) Ppt. A-3B

Supernatant III, discarded

of Supernatant I. The yield of antibody into Precipitate A-A is relatively low. In a typical experiment, Precipitate A-A showed 100 units of tetanus antitoxin per gm. while Precipitate A-B gave 8000 units per gm. Subfractionations of Precipitate A-A give products with very low antibody content. They are composed largely of proteins moving with an electrophoretic mobility of \(-4.0 \times 10^{-5}\) sq. cm. per volt per second (pH 8.6). Such findings indicate that the antitetanus activity of horse serum proteins does not ordinarily extend into the electrophoretic region of the horse serum designated as \(\beta\)-globulin in Fig. 1.

The sera of the two horses immunized to a series of antigens were pooled...
and fractionated to yield the usual initial antibody-rich Precipitate A. These precipitates were subfractionated and the distribution of the antibodies in the various fractions was studied. The subfractionation conditions are shown in Diagram 2.

The predominant feature of this subfractionation was the separation of the usual Precipitate A-B into three fractions, a $\gamma_1$-globulin, a $\gamma_2$-globulin, and a mixture of these two globulins.

In Fig. 2 are shown the electrophoretic compositions of these subfractions. It is readily apparent that the component labeled $\gamma_1$-globulin is relatively heterogeneous electrically and the fraction as a whole has a considerably higher electrophoretic mobility than does the $\gamma_2$-fraction.

Antibody and protein yield data are shown in Table II. As was anticipated, no antibodies to pneumococcus type III organisms were found in Precipitate A as tested by agglutination, capsular swelling phenomena, or by precipitin reactions with pneumococcus type III polysaccharide. The horses evidently failed to produce antibodies to the formalized pneumococcus vaccine which was employed. Antibody production to vibron septique toxoid was relatively low (less than 200 units per gm. of Precipitate A) and the subfractions were not assayed further. The remaining antibodies were present in relatively low titer but were of sufficient magnitude
to make possible a study of their distribution into the various fractions. Practically no antibody was found in the supernatant to Precipitate A. The subfractions of Precipitate A showed varying titers of the several antibodies studied. The initial precipitate removed at pH 5.2 and low ionic strength (Precipitate A-A) contained very small amounts of antitoxin but contained as much, or more, agglutinin for *Hemophilus pertussis* on a weight basis as did the parent fraction (Precipitate A). Approximately 10 to 15 per cent of the diphtheria and tetanus antitoxins was found in Precipitate A-1B with the remainder appearing in the more soluble protein (pseudoglobulin in nature) making up Precipitates A-2B and A-3B. The diphtheria antitoxin showed a relatively higher titer in the γ2-globulin fraction (Precipitate A-3B) than was true for tetanus antitoxin. The antitoxin to *Bacillus welchii* was found to be rather well dis-

| Antibody Content of Subfractions of Antibody Fraction of Hyperimmune Horse Serum |
|-----------------------------|----------------|----------------|----------------|
| Weight of fraction | Fraction | Units antitoxin per gm. protein | Hemophilus pertussis agglutinin titer |
| gm. | | Diphtheria | Tetanus | Bacillus welchii |
| 50 | A* | 3000 | 1000 | 300 | 1-1280 |
| 7.8 | A-A | 300 | 100 | −25 | 1-2560 |
| 15.6 | A-1B (γ1) | 1000 | 500 | 300 | 1-160 |
| 8.4 | A-2B (γ1 + γ2) | 5000 | 4000 | 400 | 1-160 |
| 9.8 | A-3B (γ2) | 8000 | 1000 | 500 | 1-1280 |

* The supernatant to Precipitate A contained less than 2 per cent of the antibody activity of this fraction.

tributed in all of these fractions. The *Hemophilus pertussis* agglutinins showed a high concentration in the γ2-globulin fraction as compared to the γ1 and the γ1+γ2-globulin subfractions. Such a finding might have been predicted from the electrophoretic studies of van der Scheer et al. (5). The high titer of this antibody in the euglobulin type of precipitate (Precipitate A-A) is, however, quite surprising in view of the small amount of γ2-globulin in this fraction and is in contrast to the antitoxin distribution in the same fraction.

**Quantitative Diphtheria Antitoxin Assays**—A γ1-globulin, a γ2-globulin, and a mixture of them (analogous in electrophoretic composition to Precipitates A-1B, A-3B, and A-2B respectively of Fig. 2) were prepared from pooled antidiphtheritic horse serum that assayed approximately 800 units per ml. The γ1-globulin preparation, however, was separated from a γ1+γ2-globulin mixture (Precipitate A-2B), since this fraction has a relatively higher antidiphtheritic titer in contrast to Precipitate A-1B (see
No attempt was made to recover large amounts of antibody, attention being focused on the recovery of electrophoretically well defined fractions. The \( \gamma_1+\gamma_2 \)-globulin fraction contained approximately equal amounts of the two component proteins. The antibody contents of these preparations were determined by the quantitative methods as elaborated by Heidelberger and associates (16). These results, summarized in Table III, show a diphtheritic antitoxin distribution in the various fractions that was analogous to that found by in vivo assay in similar fractions of the polyvalent horse serum (see Table II). The shape of the quantitative precipitin curves for these fractions was essentially the same as that obtained by Kabat (17) in plotting the Pappenheimer and Robinson (18) data for the diphtheria toxin-antitoxin (horse) reaction. In agreement with results of Kekwick and Record (3) the \( \gamma_2 \)-globulins were found to flocculate more readily with toxin than the \( \gamma_1 \)-globulins.

### Table III

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units of diphtheria antitoxin per gm. protein</th>
<th>Per cent antitoxin of fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma_1 )-Globulins</td>
<td>9000</td>
<td>10.4</td>
</tr>
<tr>
<td>( \gamma_1+\gamma_2 )-Globulins</td>
<td>6750</td>
<td>7.8</td>
</tr>
<tr>
<td>( \gamma_2 )-Globulins</td>
<td>3070</td>
<td>3.6</td>
</tr>
</tbody>
</table>

**Fractionation of Serum during Course of Immunization**—Changes in the plasma proteins from a single horse were studied during the course of immunization to diphtheria toxoid. Serum samples were collected before and at various times during the immunization period. Unfortunately the animal employed did not develop antitoxin above 400 units per ml. and the experiment was discontinued at this point. The serum fractions obtained do, however, show the shift toward the development of large amounts of \( \gamma_1 \)-globulin, as was expected from the previous serum electrophoretic studies of van der Scheer et al. (5, 6) and Kekwick and Record (3). Electrophoretic patterns and yields of some of the serum samples fractionated are shown in Fig. 3. A marked increase in the yield of Precipitate A-B as immunization continued is readily apparent. The predominating feature is the gradual increase of the \( \gamma_1 \)-globulin. The effect is most readily observed as the electrophoretic diagrams for Precipitates A-3B are studied. As the \( \gamma_1 \)-globulin content of the serum increases it likewise becomes

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4 We wish to thank Mr. Melvin Cohn, Department of Microbiology, New York University, College of Medicine, for carrying out these determinations.
more difficult to prepare a $\gamma_2$-globulin fraction (Precipitate A-3B) which does not show the presence of considerable $\gamma_1$-globulin. As previously noted by Kekwick and Record (3), there was an increased production of the $\gamma_2$-globulin during the initial stage of immunization. Thus, by the 7th day of immunization this component showed a marked enhancement without any increase in the amount of $\gamma_1$-globulin, but as immunization progressed the level of the $\gamma_1$-globulin rose.

Fig. 3. Descending electrophoretic patterns and yield data of serum fractions from a single horse during immunization to diphtheria toxoid. The numbers under patterns are the yields of precipitate in gm. for 100 ml. of serum.

Fractionation of Normal Horse Sera—Serum samples which had been taken from nine normal horses were analyzed electrophoretically and fractionated individually in order to study the variation in composition and yield that one might expect when working with the plasma from single animals. The yield data of the fractions obtained from the sera examined are shown in Table IV. It is apparent that a great deal of individual variation may be expected among so called "normal" animals. Another feature of these and previous experiments was the increased difficulty in removing the $\beta$-globulins associated with Precipitate A into Precipitate A-A. This is in rather marked contrast with the results obtained when serum from hyperimmunized animals is used.

Electrophoretic Mobility Studies—The electrophoretic mobilities of $\gamma_2$-
and γ1-globulin preparations obtained from antidiphtheritic plasma were determined for a series of pH values. The γ1-globulin fraction represents that portion of Precipitate A from an antidiphtheritic serum which was soluble at pH 5.2, ethanol 10 per cent and ionic strength of 0.002, but which was insoluble at pH 6.1. This globulin preparation is somewhat analogous to Precipitate A-1B of Fig. 2, except that it was far more homogeneous electrically than the usual Precipitate A-1B. The γ2-globulin was a frac-

Table IV

<table>
<thead>
<tr>
<th>Material</th>
<th>Per cent electrophoretic composition</th>
<th>Protein, gm. per 100 ml. starting serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>γ1 18-31, γ1 9-17, β 11-24, α1 10-22, α2 7-13</td>
<td>5-8, 1-1.7, 1-2, 1-5, 1-0.5</td>
</tr>
<tr>
<td>Average</td>
<td>22 14, 17 14, 9 24, 0-3 1-4</td>
<td>7.6</td>
</tr>
<tr>
<td>Range</td>
<td>γ1 29-46, γ1 25-31, β 15-35, α1 4-11, α2 0-3</td>
<td>25-31, 15-23, 7-15, 2-4, 0-2</td>
</tr>
<tr>
<td>Average</td>
<td>40 28, 22 8, 0-3 1-4</td>
<td>4.1</td>
</tr>
<tr>
<td>&quot; A-A</td>
<td>Range 6-0, γ1 23-36, β 40-57, α1 1-22, α2 0-5</td>
<td>0.2-1.1</td>
</tr>
<tr>
<td>Average</td>
<td>8 28, 46 12, 3 1</td>
<td>0.7</td>
</tr>
<tr>
<td>&quot; A-B</td>
<td>Range 53-73, γ1 21-32, β 7-15, α1 0-3, α2 0-1</td>
<td>1.8-3.5</td>
</tr>
<tr>
<td>Average</td>
<td>60 25, 12 1, 0 1</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Fig. 4. Descending electrophoretic patterns of γ1- and γ2-globulins and mixtures of these two proteins.

The electrophoretic patterns of these fractions in pH 8.6 veronal buffer of ionic strength 0.1 are shown as γ1- and γ2-globulins in Fig. 4. It can be seen that the γ1-globulin has a slightly asymmetric pattern. The marked electrical inhomogeneity in this fraction has been already mentioned and, even though the γ2-globulin preparation appeared to be more homogeneous from its electrophoretic diagram, the heterogeneity constant (19) of various preparations gave values in the neighborhood of $1 \times 10^{-6}$ sq. cm. per volt per second. The

mobility versus pH values for the two protein fractions are plotted in Fig. 5. The average isoelectric points of the $\gamma_1$- and the $\gamma_2$-globulin components in buffer solutions of 0.1 ionic strength are 5.6 and 7.6 respectively. With the exception of the $\gamma_1$-globulin fraction at pH 8.6 these proteins gave single, apparently symmetrical peaks over the entire pH range studied.

Our investigations indicate that these globulin fractions are merely proteins of closely related isoelectric point and mobility. Dependent upon the conditions of separation there may be obtained arbitrary fractions which contain antibody and which have electrophoretic mobilities from $-1.0$ to $-3.5 \times 10^{-5}$ sq. cm. per volt per second in buffer of pH 8.6 and ionic strength 0.1. Hence the $\gamma_1$- and $\gamma_2$-globulin fractions employed for the determination of isoelectric point are merely two fractions, the molecules in each being more closely related electrically than are those of the parent fraction (Precipitate A-B, Fig. 1). In addition to the patterns of the relatively homogeneous fractions, Fig. 4 also shows the diagrams for two mixtures of $\gamma_1$- and $\gamma_2$-globulins. It is readily apparent that a series of protein fractions showing variations in the average net charge at a given pH may be separated from horse serum. This fact is substantiated by the electrophoretic diagrams and analytical data for the hyperimmune horse serum antibody fractions of Smith and Gerlough (8).

Sedimentation Studies—Various preparations of $\gamma_2$- and of $\gamma_1$-globulins and mixtures thereof were studied in the Svedberg high velocity oil turbine ultracentrifuge. Approximately 80 per cent of these globulin fractions con-
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Sisted of material having a sedimentation constant \( (s_{20,w}) \) of 6.8 or 7.2 Svedberg units, respectively. The remaining 20 per cent of the protein in each case sedimented with velocities between 8 and 15 Svedberg units and exhibited no well defined molecular components in this range. Some schlieren patterns of these proteins obtained during velocity sedimentation experiments are shown in Fig. 6. The main component of both of these fractions was molecularly monodisperse, showing no increase in the apparent diffusion constant as sedimentation progressed. A mixture of the \( \gamma_1 \) and \( \gamma_2 \)-

![Fig. 6. Sedimentation patterns after 72 minutes at 220,000 times gravity of (A) \( \gamma_2 \)-globulin fraction, (B) \( \gamma_1 \)-globulin fraction.](http://www.jbc.org/)

globulins exhibited the molecular mass behavior to be expected of a system of this kind.

DISCUSSION

The proteins of horse serum which are concerned with antibody activity can be readily separated from the serum by means of ethanol fractionation. In this respect the \( \gamma \)-globulins from hyperimmune sera are more easily separated free of \( \beta \)-globulin than are those from normal sera. The antibody fractions are relatively heterogeneous electrophoretically and may be separated into a series of fractions, the major components of which may show electrophoretic mobilities anywhere from \(-1.0\) to \(-3.5 \times 10^{-5}\) sq. cm. per volt per second in veronal buffer at pH 8.6 and ionic strength 0.1. Since these protein fractions show antibody activity, we have called them all \( \gamma \)-globulins in preference to using a series of unrelated terms such as \( \gamma, \beta_3, \) and T component (8), or \( \gamma, \beta_1, \) and \( \beta_3 \) (3), as has been done previously. The findings of Kekwick and Record are in agreement with the results of our work insofar as they may be compared.
The antibodies to the antigens we have studied appear to be distributed electrophoretically throughout this γ-globulin region. The γ₁- and γ₂-globulin fractions both contain antibody to a given antigen. The marked concentration of antibodies which make up only a small per cent of the total protein is not as readily achievable as it would be if the antibody were contained in a small and electrophoretically distinct component. The antibodies in question possess the same solubilities as do the γ₁- and γ₂-globulin serum components and, in order quantitatively to remove serum antibodies, these proteins must be likewise separated. Hence in preparing antibody-rich precipitates from horse serum the investigator is limited by the amount of antibody per unit of starting serum. Some slight difference in the solubilities of a given antibody is indicated by the results of the sub-fractionations of Precipitate A-B, as shown in Table II.

Fractionation of such protein systems must take into account isoelectric point and solubility distributions. For the antitoxins it would appear that, in agreement with previous findings, these antibodies are water-soluble and that furthermore they seem to be associated largely with the γ₁-globulins. Thus the γ₂-globulin fraction (Precipitate A-3B, Fig. 4) is much lower in antitoxin content than the water-soluble portion of the γ₁-globulin (see Table III). Thus Precipitate A-1B of Table II contains far more γ₁-globulin than does Precipitate A-2B but the antidiphtheritic potency of the latter fraction is far greater. This is apparently due to the γ₁-globulin of Precipitate A-2B being largely pseudoglobulin in nature as contrasted to the euglobulin characteristics of the globulin in Precipitate A-1B. As a further consequence of these fractionation conditions, the γ₂-globulins of Precipitate A-3B would tend to be more pseudoglobulin in nature than the analogous component in Precipitate A-2B. Such evidence is further indication that the antitoxic globulins of horse serum are more highly concentrated in the γ-globulins of lower isoelectric points and, as known before (20, 21), in the pseudoglobulin portions.

The bacterial antibodies as exemplified by the pertussis agglutinins show quite another behavior. Van der Scheer et al. (5) have indicated that bacterial antibodies appear to follow the γ₂-globulin component rather than the γ₁-component. The findings of Tiselius and Kabat (1), however, have indicated that horse pneumococcus antibody was a constituent corresponding to the γ₁-globulin (T component). Unfortunately, the two horses immunized in our work did not produce antibodies to the pneumococcus vaccine employed. It is difficult to reconcile the high content of pertussis agglutinin in both Precipitates A-A and A-3B (Table II). The former fraction is quite low in γ₁-globulins and very low in γ₂-globulins, while the Precipitate A-3B fraction is essentially all γ₂-globulin. The small amount of γ₁- and γ₂-globulin found by electrophoretic analysis in Precipitate A-A
and the low content of antitoxin are consistent. The euglobulin nature of Precipitate A-A is in agreement with the presence of the anticarbohydrate and certain antiprotein globulins of horse serum in the water-insoluble fractions (22–24). However, Precipitate A-3B is essentially water-soluble and likewise shows a large amount of pertussis agglutinin.

A further factor that must be considered is the degree of immunization that a particular animal has undergone. It must be realized, too, that both the chemical nature of the antigen and its route of administration are important factors in determining the characteristics of the antibody formed (24). The data of Fig. 3 clearly indicate a great deal of variation in the fractions obtained under analogous conditions from a single horse serum as the period of immunization progresses. Since the antibody fractions of the horse appear to be so complex, it would appear that a great deal of information would be gained by a careful and extended study of the physiology and rate of production of the serum proteins.

The horse γ-globulins separated in this work have sedimentation constants \( s_{20w} \) in the neighborhood of 7 Svedberg units. This figure is in agreement with previous data (2, 3, 25, 26) for horse globulin. No component having \( s_{20w} = 18 \) Svedberg units was observed to be present in our fractions. Small amounts of such heavy protein material have been found in the sera of apparently normal horses (25). The horse pneumococcus antibody (27, 28) is known to possess a sedimentation constant \( s_{20w} = 18 \) Svedberg units. The \( \gamma_2 \)-globulin fractions gave values which were consistently slightly less than \( s_{20w} = 7 \) Svedberg units, while the \( \gamma_1 \)-globulin fractions always gave a somewhat higher figure. Approximately 20 per cent of the protein sedimented at a rate corresponding to \( s_{20w} = 8 \) to 15 Svedberg units as a relatively polydisperse mixture. The \( \gamma_2 \)-globulins from other animal plasmas also have been found to separate with varying amounts of this faster sedimenting material (15, 29, 30). It is not known whether such protein results from the fractionation conditions used or whether the \( \gamma \)-globulins exist as such in nature.

While the so called \( \gamma_1 \)- and \( \gamma_2 \)-globulin fractions were definitely heterogeneous on electrophoresis at pH 8.6, the peaks showed no tendency to give more than one main component over a wide pH range. As separated, the \( \gamma_1 \)-globulin fractions were far more heterogeneous in nature than the \( \gamma_2 \)-globulin fractions. However, the relatively homogeneous \( \gamma_2 \)-globulin fraction obtained represents only a small portion of the serum proteins usually designated as \( \gamma_2 \)-globulin (Fig. 1).

The ability of the \( \beta \)-globulins to separate more readily from hyperimmune sera is probably related to increased concentration of the \( \gamma \)-globulins. Thus while relatively constant amounts of \( \beta \)-globulin are precipitated in all sera, they represent a smaller per cent of the total globulin precipitated
from the immune systems. An evaluation of the amount of β-globulin in the serum or in an antibody fraction is difficult for, as seen from Fig. 1, it does not resolve well from the area described in an electrophoretic diagram as being due to γ1-globulin. The very low content of antibody in such fractions as Precipitate A-A (Fig. 1) strongly suggests that antibody activity is not associated with β-globulin.

**SUMMARY**

An antibody-rich protein fraction of hyperimmunized horse plasma which is made up largely of γ2- and γ1-globulins may be readily separated from serum by ethanol fractionation. The antibodies appear to be distributed among molecules showing a wide variation in electrophoretic mobility. The largest amounts of antitoxin appear to be associated with the water-soluble portions of the γ1-globulin fractions which is in contrast to the pertussis agglutinin.

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