THE PURIFICATION OF THE ANTIBODIES IN TYPE I ANTI-
PNEUMOCOCCUS SERUM, AND THE CHEMICAL
NATURE OF THE TYPE-SPECIFIC
PRECIPITIN REACTION

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Some years ago it was shown by Avery (1) that the protective substances
of antipneumococcus horse serum are intimately associated with the globulins. By
means of fractionation with ammonium sulfate it was demonstrated that the anti-
odies are distributed among the eu- and pseudoglobulin constituents. The
immune bodies were likewise found to be partially precipitated when antiserum is
saturated with sodium chloride, or when the electrolytes, normally present in the
serum, are removed by dialysis.

Later methods for the isolation of pneumococcus antibodies were directed
toward the dissociations of the immune precipitate obtained by the absorption
of antiserum with pneumococci of the homologous type, or with aqueous extracts
of the bacterial cells. The first of these studies was made by Gay and Chickering
(2) who found that aqueous extracts of pneumococci, when added to homologous
antiserum, yielded a precipitate which could be partially dissociated by means of
dilute sodium carbonate. Such solutions contained not only type-specific agglu-
tinins and precipitins, but protective antibodies as well. Later work by Huntoon
(3) and his associates led to the belief that the immune substances were probably
non-protein in nature.

Further advances in the identification of the chemical nature of antibodies
have been made as a result of the extensive investigations of Felton (4). It has
been shown by him that when pneumococcus antiserum is diluted with distilled
water, the major portion of the antibodies separates as an insoluble precipitate.
Not only can the immune bodies be precipitated by this means, but they can be
effectively separated from the serum by dilution with small quantities of ethyl
alcohol (5). The conditions under which antibodies can best be separated from
antiserum have been thoroughly investigated (6) and the product which has been
isolated has been found to have properties which definitely characterize it as a
globulin more basic than ordinary serum globulin (7).

The further purification of the water-insoluble globulin bearing the immune
bodies of pneumococcus antiserum has also been attempted by Felton (8). It has
been shown that fractions of the protein, varying in their solubility in different
concentrations of sodium or ammonium sulfate, likewise vary in their capacity to protect mice against infection with standard doses of homologous virulent organisms. It has been shown by Felton (9), furthermore, that it is possible by the adjustment of the hydrogen ion concentration to separate from a solution of the original antibody globulin an insoluble and inert euglobulin fraction.

In summarizing the results of the more important contributions concerning the chemical nature of pneumococcus antibodies, it can now be stated with fair certainty that the latter are modified serum globulins, and that they can be separated from immune serum by a variety of methods. Although when so isolated, the antibodies are relatively pure, they are without doubt still accompanied by inert serum globulin. The present communication deals with an attempt to separate from the water-insoluble globulin of immune horse serum the biologically active protein free from accompanying inert globulins.

In his extensive studies on the proteins of normal horse serum, Soerensen (10) has found that the globulins apparently occur as labile compounds of eu- and pseudoglobulin. By means of solubility measurements Soerensen has shown that these compounds are readily dissociable, and that by repeated fractional precipitation and dialysis the constituents can be partially separated as protein fractions which become increasingly richer in eu- or pseudoglobulin, as the case may be. Although the normal horse serum globulins probably have never been obtained as pure chemical entities, yet it has been possible to dissociate these serum complexes into constituents in which one or the other of the two fractions predominates. From the results of studies on the purification and fractionation of serum globulins, both from normal and from immune horse serum, Reiner and Reiner (11) have reached similar conclusions.

The fact that the globulins of horse serum are so readily dissociable has been made use of in the present attempt to separate the immune globulin of Type I pneumococcus antiserum from accompanying inert globulins by fractionation with ammonium sulfate. The insoluble globulin fraction obtained by the dilution of antipneumococcus horse serum with distilled water, followed by the precipitation of small amounts of inert protein at pH 4.8 (8), has served as source material. By repeated fractional precipitation and dialysis this protein mixture has been separated into relatively inert euglobulin fractions, and
active pseudoglobulin fractions containing the major portion of the antibodies present in the starting material. A second method has also been devised for the separation of inert protein from the active immune globulin. It has been found that when pneumococcus antibody solutions, prepared according to the method of Felton (8), are treated with 0.2 M potassium acid phthalate buffer at the appropriate hydrogen ion concentration, a precipitate of inert protein is formed, leaving in solution the immunologically active globulin. The latter can be further fractionated by ammonium sulfate and dialysis to yield an end-product, which in some instances contains as much as 90 per cent of protein precipitable by the homologous type-specific polysaccharide.

When finally separated from the more inert protein, the purified immune globulin has been found considerably more potent in antibody content than the parent substance. In addition to its increased biological activity, the immune globulin possesses certain properties which distinguish it from the globulins of normal horse serum. The following experimental work is an account of the methods used in purifying the immune protein, together with a description of certain of its chemical properties. Included in this study is an investigation of certain of the chemical groupings which are believed to be involved in the union between antibody and the type-specific polysaccharide.

**EXPERIMENTAL**

Preparation of Source Material.—Type I antipneumococcus sera which have been in this laboratory since 1918 were used in the following experimental work. The method of separation of the immune globulin, which served as source material, is essentially the same as that described by Felton (8). Antibody solutions were thus obtained which varied in the amount of protein precipitable by the type-specific polysaccharide, from 25 to 42 per cent, depending upon the serum employed.

Concentration of Type I Pneumococcus Antibody by Ammonium Sulfate.—By repeated fractionation and dialysis it was found that the water-insoluble globulin, obtained by the dilution of antiserum with distilled water, could be separated into relatively inert euglobulin fractions, and pseudoglobulin fractions which, on dialysis, remained soluble in very dilute buffer solutions at pH 5.0. When the latter were adjusted to pH 7.6 the antibody proteins precipitated from solution, leaving small amounts of inert pseudoglobulin in the supernatant fluid. Throughout the experiment, the various protein fractions were dialyzed against 0.005 M acetate buffer at pH 5.0. The method of fractionation is summarized in the following outline.
Fractions P₁S, P₂S, and P₃S, containing the major portion of the antibodies, were now combined and the hydrogen ion concentration of the solution adjusted to 7.6 with 0.1 N NaOH. The antibody proteins (designated as fraction A) were thus precipitated, leaving in solution 0.077 gm. of water-soluble inert globulin. Fraction A, the antibody protein, was now dissolved in 242 cc. of 0.9 per cent salt solution and further fractionated as follows:
By this method of fractionation it is possible to separate the source material into relatively inert euglobulin fractions, and a pseudoglobulin fraction with marked biological activity as determined by quantitative precipitin titration (12). This immune protein, in addition to its immunological reactivity, has certain other properties which distinguish it sharply from the source material and from the globulins of normal horse serum.

When the above method of fractionation was followed, the actual quantities of the inert protein which separated from the active pseudoglobulin showed wide variation with different lots of immune serum. The immunological activity, however, of the pseudoglobulin isolated
from different lots of antisera was invariably the same provided the conditions of fractionation were carried out as described. Thus from an antiserum, which on dilution yielded a water-insoluble globulin containing only 25 per cent precipitable protein as determined by the quantitative precipitin titration, a pseudoglobulin fraction containing 65 per cent precipitable protein was finally obtained. On the other hand, the immune protein obtained from a more potent antiserum, having initially 42 per cent precipitable protein, yielded a purified antibody containing 67 per cent precipitable protein.

Concentration of Type I Pneumococcus Antibody by Precipitation of Inert Globulin with Potassium Acid Phthalate Buffer.—By means of the foregoing procedure it has been possible to enhance the activity of the antibody so that approximately two-thirds of the protein is precipitable by the type-specific polysaccharide. This method of concentration, however, yields an end-product in which serologically inert proteins still persist, and which cannot be separated by refractionation with ammonium sulfate.

It has been found, however, that when a concentrated solution of the source material is treated with an equal volume of 0.2 M potassium acid phthalate buffer at a definite acid pH, which must be precisely determined for any given lot of antibody solution, an inert protein fraction separates as a precipitate, leaving in solution the major portion of the active antibodies. When the latter are precipitated by half saturation with ammonium sulfate, and the precipitated protein dialyzed at pH 7.6 against 0.001 M phosphate buffer, the antibody protein separates in a high state of purity. Thus it is possible to secure a solution of immune globulin containing between 75 and 90 per cent specifically precipitable protein, depending upon the serum which served as the original source material.

In order to establish the optimum conditions for the precipitation of the inert protein fraction, it is necessary, with each new lot of source material, to determine the appropriate hydrogen ion concentration of the buffer to be employed according to the following method.

100 cc. of 0.2 M potassium acid phthalate solution were measured into each of four flasks. To each were added 5.30, 8.66, 12.00, and 15.95 cc. of 0.2 M HCl solution respectively. The pH of each solution was found to be approximately
3.8, 3.7, 3.6, and 3.4 respectively. Into each of four 50 cc. centrifuge tubes were now measured accurately 5 cc. of antibody solution (prepared by the method of Felton (8)) containing 50 mg. protein per cc. To each tube was then added, with stirring, 5 cc. of the phthalate buffer solutions at the different hydrogen ion concentrations. The tubes were allowed to stand at room temperature for 5 minutes, and the precipitates centrifuged off and discarded. The clear supernatant liquids were neutralized cautiously with 1.0 M NaOH. To each solution was added an equal volume of saturated ammonium sulfate solution, and the precipitates collected by centrifugation. The precipitates were dissolved as completely as possible in 15 cc. of water, and in each tube the pH adjusted to 7.6. (It was observed, at this point, that in several of the tubes some of the protein failed to dissolve. It was later found that this phenomenon occurred only in those tubes where the conditions for the maximum precipitation of inert protein were not optimal.) The solutions were clarified by centrifugation and to each supernatant liquid was added 1 cc. of 0.1 M phosphate buffer at pH 7.6. The solutions were dialyzed at 5°C. against 0.001 M phosphate buffer at pH 7.6 until free from sulfate ions. The active insoluble antibody protein which separated on dialysis, was recovered by centrifugation. The supernatant liquids, containing small quantities of inert protein, were discarded. The precipitates of immune protein were dissolved in 15 cc. of saline and the activity of the antibody was ascertained by means of the quantitative precipitin titration.

The method of fractionation can be diagrammatically represented as follows:

1. 5 cc. of antibody solution (50 mg. protein per cc.) + 5 cc. of 0.2 M phthalate buffer at appropriate pH
2. Precipitate of inert protein + small amount of antibody (Discard)
3. Solution of antibody
4. Adjust to pH 7.0 + equal volume of saturated (NH₄)₂SO₄
5. Precipitate
6. Dissolve in 15 cc. H₂O and adjust to pH 7.6
7. Insoluble precipitate (Discard)
8. Solution of antibody
9. Dialyze against 0.001 M phosphate buffer at pH 7.6
10. Precipitate of antibody
11. Solution of inert globulin (Discard)
Quantitative protein determinations of the various antibody fractions precipitated at different hydrogen ion concentrations, and the immunological activity of the purified antibody protein are given in Table I.

From the results given in Table I, it can be seen that when the pH of the phthalate buffer is varied in increments as small as 0.1 of a pH unit the activity and yield of the purified antibody shows wide variation. This can be accounted for only by the fact that when the pH of the buffer is not suitable, the separation of active and inert protein is incomplete. It is of utmost importance, therefore, to establish the optimum condition for the precipitation of inert protein for each antibody solution under investigation before attempting to purify the entire solution. When these conditions have been established, the

### Table I

<table>
<thead>
<tr>
<th>pH</th>
<th>Amount of antibody protein recovered per cent</th>
<th>Amount of protein precipitable by acetyl polysaccharide in recovered antibody protein fraction per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4</td>
<td>13</td>
<td>48</td>
</tr>
<tr>
<td>3.6</td>
<td>25</td>
<td>61</td>
</tr>
<tr>
<td>3.7</td>
<td>37</td>
<td>76</td>
</tr>
<tr>
<td>3.8</td>
<td>27</td>
<td>64</td>
</tr>
</tbody>
</table>

remaining antibody solution can be rapidly purified. Thus one obtains ultimately a solution of purified antibody containing approximately 75–80 per cent of the total antibody present in the starting material, and containing between 75 and 90 per cent type-specific precipitable protein, depending upon the serum employed. From the water-insoluble antibody obtained from three separate lots of antisera, containing originally 35, 30, and 43 per cent specifically precipitable protein, we obtained purified antibody solutions having 76, 85, and 90 per cent specifically precipitable protein respectively.

When antibody solutions are purified by the above method, it has been found that several factors other than hydrogen ion concentration influence the precipitin titre of the end-product. Thus it has been observed that if the initial concentration of water-insoluble globulin
is varied, or if the precipitation with buffer is carried out at 0° instead of at room temperature, the percentage of protein precipitable by the type-specific polysaccharide is considerably decreased. In conclusion it may be said that the separation of inert globulin cannot be accomplished merely by adjusting the hydrogen ion concentration of the antibody solution to the appropriate value with acid, nor can it be accomplished by substituting acetate buffer for the phthalate buffer. The phthalate buffer appears to have a selective precipitating action upon the inert globulin. Felton (13) has also pointed out that inert globulin can be selectively precipitated from antibody solutions by certain metallic salts such as aluminum and zinc chlorides to yield an end-product of which 80 to 90 per cent of the total protein is precipitable by the type-specific carbohydrate.

**Biological Activity of the Various Protein Fractions Obtained by the Concentration of Pneumococcus Antibody**

The usual method of ascertaining the potency of pneumococcus antibody solutions is to determine the minimum amount necessary to protect mice against a standard dose of virulent pneumococci of the homologous type. In attempting to quantitate small increments of increase in the biological activity of antibody solutions this particular method is, for obvious reasons, inadequate. However, the quantitative measurement of type-specific precipitins as devised by Heidelberger and Kendall (12), affords an admirable means of determining small differences in the biological activity of pneumococcus antibody solutions. The adoption of this method has made it possible to follow the purification of the immune proteins, and to determine the activity of the protein fractions discarded during the fractionation process.

Thus the percentage of protein precipitable by the type-specific carbohydrate (acetyl polysaccharide of Pneumococcus Type I (14)) was determined in the discarded fractions E1 and E2 (Method I) and compared with the activity of the purified immune protein. The method of determining the maximum precipitable protein was as follows:

The fraction under investigation was so diluted that each cc. contained exactly 2.0 mg. of protein. Four samples of 5 cc. were pipetted into each of four centrifuge tubes. The latter were cooled to 0°C. and to each were added from a cali-
brated pipette, 5 cc. of a solution of the type-specific polysaccharide at 0°C. The concentration of polysaccharide was so chosen that the first tube contained a total of 0.5 mg., the second 0.2 mg., the third 0.1 mg., and the fourth tube 0.05 mg., of type-specific carbohydrate. The tubes were kept at 0°C. for 48 hours. The immune precipitate was removed by centrifugation at 0°C. and washed twice with 3 cc. portions of ice cold saline. The clear supernatant liquids were discarded, and the precipitated immune protein dissolved in a few cc. of 0.1 m NaOH. The solutions were next transferred quantitatively to Kjeldahl flasks, and the analysis for protein nitrogen was carried out in the usual way (15).

Thus for each fraction of protein investigated, the total content and percentage of active protein precipitated by the type-specific carbohydrate were determined. The activity of the various protein fractions isolated during the purification of antibody by the ammonium sulfate method is shown in Chart 1.

From the results given in the chart it can be seen that 42 per cent of the protein in the original antibody solution was precipitable by the type-specific carbohydrate. When subjected to repeated fractionation by means of ammonium sulfate, the amount of precipitable nitrogen was increased to a maximum of 67 per cent. By the method of fractionation with ammonium sulfate, it has not been found possible to increase the amount of precipitable protein beyond the value of 67 per cent. Although it has not been indicated on Chart 1, it has been found that a solution of water-insoluble globulin obtained from an antiserum of low titre and containing originally not more than 25 per cent specifically precipitable protein, can be concentrated by the method so that the final activity of the immune protein thus derived equals that of the protein obtained from the concentration of more potent source material.

Although the quantity of active protein discarded in fractions E₁ and E₂ is in each instance approximately half that of the source material, the actual quantity of immune protein lost represents only a small percentage of the total. It is of interest to note that the ammonium sulfate method of fractionation outlined above has never yielded an end-product containing more than 67 per cent of type-specific precipitable protein. The reason for this probably lies in the fact that there remains in the concentrated antibody an inert globulin intimately associated with the active constituent and inseparable from it by the
method described. It can be seen from Chart 1 that when this antibody is further purified by precipitation with potassium acid phthalate buffer, a considerable quantity of inert globulin is eliminated, and the activity of the antibody is increased to a value of 88 per cent type-specific precipitable protein. The remaining 12 per cent of protein may possibly be represented by other antibodies present in the original serum. It must be remembered that in antipneumococcus serum there are a number of antibodies reactive with constituents of the cell other than the capsular polysaccharide, and that the relative quantities of these antibodies vary from serum to serum. The protein precipitable

by the type-specific polysaccharide does not therefore represent the total antibody protein present in the concentrated mixture. It can be clearly demonstrated experimentally that the latter still contains antibodies for the cellular proteins of the pneumococcus and for the somatic carbohydrate as well (16).

Properties of the Immune Protein Recovered from Antipneumococcus Serum Type I

Both Felton and Reiner and Reiner have observed that the isoelectric point of the water-insoluble globulin isolated by them from pneumococcus antiserum is distinctly more alkaline than that of normal horse serum globulin. This property becomes even more striking and more sharply defined when the inert protein present in the globulin studied by these investigators is separated by the methods outlined above. The unusual alkaline isoelectric point of the purified immune proteins is perhaps its most characteristic physical property, one which may possibly be explained by the relatively high ratio of amino to carboxyl groups present in the protein molecule. In the absence of high concentrations of electrolytes the immune protein is insoluble in distilled water at its isoelectric point. The protein may be brought into solution by the addition of very dilute alkali or acid, or by bubbling carbon dioxide into an aqueous suspension of the protein. The antibody can be quantitatively precipitated from such solutions by adjusting the hydrogen ion concentration again to 7.6. This point of minimum solubility may be tentatively regarded as the isoelectric point of the antibody protein, although the migration of particles in a cataphoretic field has as yet not been observed. Unlike the globulins of normal horse serum, the immune globulin showed only 14.85 per cent of nitrogen when analyses were performed on dried, ash-free, samples of the material. Quantitative analyses have shown that the antibody protein contains approximately 2.75 per cent of lipoid (17) and a prosthetic carbohydrate grouping as revealed by the qualitative Molisch test; the immune globulin, therefore, cannot be regarded as a simple protein.
Distribution of Basic Amino Acids in the Immune Protein of Anti-pneumococcus Serum Type I

In view of the fact that the immune protein derived in the manner described has an isoelectric point lying so far in the alkaline range, it might be anticipated that the protein would contain a higher percentage of basic amino acids than would the globulin of normal serum. In order, therefore, to ascertain whether the purified antipneumococcus protein shows any marked difference in the distribution of basic amino acids, an analysis was performed according to the Van Slyke method as modified by Cavett (18). The results of these analyses are given in Table II.

From the results given in Table II it can be seen that aside from a slightly higher lysine content there appears to be no essential difference in the distribution of the basic amino acids of the concentrated pneumococcus antibody globulin as compared with normal serum.

### TABLE II

**Distribution of Basic Amino Acids in the Globulins of Normal and Immune Horse Serum**

<table>
<thead>
<tr>
<th>Amino acid determined</th>
<th>Normal serum globulin (total)</th>
<th>Concentrated pneumococcus antibody</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cavett's method</td>
<td>Analysis 1</td>
<td>Analysis 2</td>
</tr>
<tr>
<td>Amide N</td>
<td>9.46</td>
<td>9.22</td>
<td>9.21</td>
</tr>
<tr>
<td>Humin N</td>
<td>2.26</td>
<td>2.28</td>
<td>2.08</td>
</tr>
<tr>
<td>Cystine N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) precipitate</td>
<td>0.31</td>
<td>0.53</td>
<td>0.38</td>
</tr>
<tr>
<td>(b) filtrate</td>
<td>0.78</td>
<td>0.71</td>
<td>0.88</td>
</tr>
<tr>
<td>(c) total</td>
<td>1.09</td>
<td>1.24</td>
<td>1.26</td>
</tr>
<tr>
<td>Arginine N</td>
<td>8.98</td>
<td>9.02</td>
<td>8.58</td>
</tr>
<tr>
<td>Histidine N</td>
<td>5.85</td>
<td>5.55</td>
<td>6.40</td>
</tr>
<tr>
<td>Lysine N</td>
<td>8.31</td>
<td>9.90</td>
<td>10.04</td>
</tr>
<tr>
<td>Filtrate N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) amino</td>
<td>59.18</td>
<td>59.60</td>
<td>59.16</td>
</tr>
<tr>
<td>(b) non-amino N</td>
<td>6.37</td>
<td>3.90</td>
<td>4.67</td>
</tr>
<tr>
<td>Total N</td>
<td>100.7</td>
<td>100.0</td>
<td>100.5</td>
</tr>
</tbody>
</table>
Nature of the Specific Groups Involved in the Union between Type-Specific Antibody and the Capsular Polysaccharide of Type I Pneumococcus

In view of the marked alkaline isoelectric point of the immune protein, it seemed not unlikely that the free amino groups of the antibody protein might play some rôle in the union of the antibody molecule with the specific polysaccharide of Type I Pneumococcus. To investigate this possibility, one of the hydrogen atoms of the primary amino groups of the immune protein was replaced by an acetyl radical under conditions in which the protein was not denatured.

Thus 0.5 gm. of immune globulin was dissolved in 100 cc. of 0.16 M NaCl solution and 30 gm. of sodium acetate were added. Ketene gas, generated by the pyrolysis of acetone (19), was bubbled through the solution at 0°C. for 60 minutes. During this period the pH of the solution did not go lower than 5.0. After the acetylation was completed, the protein was precipitated from solution by saturation with solid sodium acetate. The precipitated protein was separated by centrifugation, and dialyzed until free from electrolytes. The solution was then analyzed for total nitrogen content. Amino nitrogen determinations revealed the fact that no free amino groups could be detected. An acetyl determination (20) performed on a dried sample of the ash-free acetylated protein showed that the latter contained one acetyl group for every primary amino group present in the original protein molecule. The acetylated protein in varying concentration was tested for the presence of type-specific precipitins reactive with the acetyl polysaccharide.

From the results of the qualitative precipitin titrations given in Table III it is seen that, by replacing one hydrogen atom of the primary amino groups with an acetyl radical, the immune protein loses to a great extent its capacity to precipitate the type-specific polysaccharide. It can also be seen that when relatively high concentrations of specific polysaccharide are added to the acetylated antibody a precipitate is formed. Although the amino groups of the immune protein may be involved in the union of antibody and the carbohydrate, yet it is evident from Table III that certain other reactive groups must likewise play a part. It is believed, however, that the amino groups in the native antibody protein molecule are concerned in the union of antibody with specific carbohydrate. Support for this view is provided by the results of the following experiments in which
the serological reactivity of the antibody is completely lost when a solution of the immune protein is treated with formaldehyde (21).

The reaction between the free amino groups of protein and formaldehyde presumably takes place according to the following equation.

\[
- \text{NH}_2 + \text{HCHO} \rightarrow - \text{N} = \text{CH}_2 + \text{H}_2\text{O}
\]

Thus 150 mg. of antibody protein, dissolved in 1.5 cc. of 0.85 per cent NaCl solution were treated with 1.5 cc. of normal NaHCO₃ solution. The mixture was cooled to 0°C. 0.75 cc. of 37 per cent neutral formalin was added and the solution was allowed to stand for 30 minutes at 0°C. The solution was diluted with 15 cc. of ice water, and rapidly dialyzed against cold water, until the dialysate no longer showed the presence of free formaldehyde when tested with silver nitrate and ammonia solution. After the addition of the proper concentration of sodium chloride the clear solution of formalized antibody protein gave no precipitin test in the presence of the type-specific polysaccharide. The serological specificity of the antibody protein could be restored, however, by adjusting the pH of the formalized protein to 4.0 and allowing the mixture to stand at 0°C. for several days. In Table IV may be seen the comparative results of the precipitin titrations of original antibody, formalized antibody, and the "deformalized" antibody.

From the results of the qualitative precipitin reactions given in Table IV, it can be seen that when the antibody protein is treated with formaldehyde the serological specificity is lost. This loss is presumably due to the fact that the free amino groups of the immune protein molecule are converted to the grouping C –NH₃. It has been well established that this grouping is stable only when the hydrogen ion concentration of the solution of formalized protein is maintained above 7.0. When the pH of the solutions is lowered the derivative presumably reacts with water and restores the grouping originally present according to the reaction

\[
- \text{N} = \text{CH}_2 + \text{H}_2\text{O} \rightarrow - \text{NH}_2 + \text{HCHO}
\]

The reaction of concentrated formaldehyde upon native protein, however, is without doubt not as simple as presented above. It is not improbable that other changes take place within the sensitive protein molecule when in contact with high concentrations of the reagent. Although the nature of such changes is not understood, it is significant that when the formalized protein is subjected to the influence of hydrogen ions, the amino groupings are restored, and the serological
### TABLE III

**Precipitin Reactions of Acetylated and Native Pneumococcus Antibody**

<table>
<thead>
<tr>
<th>Solution of pneumococcus antibody</th>
<th>Concentration of protein per cc.</th>
<th>Final dilution of Type I pneumococcus acetyl polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Native</td>
<td>5.0</td>
<td>++</td>
</tr>
<tr>
<td>Acetylated</td>
<td>5.0</td>
<td>±±</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>++</td>
</tr>
</tbody>
</table>

### TABLE IV

**Precipitin Reactions of Native, Formalised, and Deformalized Type I Pneumococcus Antibody**

<table>
<thead>
<tr>
<th>Substance tested</th>
<th>Concentration of protein per cc.</th>
<th>Final dilution of Type I pneumococcus acetyl polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original antibody</td>
<td>mg.</td>
<td>1:2,000</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>++</td>
</tr>
<tr>
<td>Formalized antibody</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>Deformalized antibody</td>
<td>5.0</td>
<td>±±</td>
</tr>
</tbody>
</table>
specificity of the immune protein is partially regained. It is likewise significant that the "deformalized" protein does not regain its full specificity under these conditions. Whether this is due to the fact that the reaction

\[ \text{NH}_2 + \text{HCHO} \rightarrow \text{N} = \text{CH}_2 + \text{H}_2\text{O} \]

is not completely reversible, or whether other chemical changes have taken place within the immune protein molecule during its contact with concentrated formaldehyde cannot be answered with certainty.

**Immunological Changes Accompanying the Esterification of the Deacetylated Polysaccharide of Type I Pneumococcus**

Studies carried out in this laboratory on the chemical nature of the type-specific polysaccharide derived from encapsulated microorganisms have revealed that all the specific carbohydrates thus far investigated contain uronic acids as constituents of the polysaccharide molecule. The occurrence of glucuronic acid or its isomers in these bacterial products suggests the possibility that the highly polar carboxyl group of the uronic acid and its stereochemical relationship to other groups in the polysaccharide molecule might not only determine to a considerable extent the specificity of the carbohydrate, but that the carboxyl itself might actually enter into chemical combination with homologous antibody when the two substances are brought together either in vivo or in vitro. Consequently, if the carboxyl group of the Type I specific carbohydrate were to be covered with an ester group the derivative either should no longer react with homologous antibody, or the reaction should be greatly diminished. In order to test the validity of this hypothesis, the following experiments were performed.

0.95 gm. of dried deacetylated Type I polysaccharide was suspended in 10 cc. of anhydrous ether and treated with 30 cc. of an ethereal solution of diazo methane, prepared from 3 cc. of nitrosomethyl urethane. The mixture was shaken at room temperature for 24 hours, and the precipitate was then filtered and washed with anhydrous ether. 1.00 gm. of substance was recovered. Analysis of the end-product of the reaction showed that the material contained 11.2 per cent methoxyl and only 0.3 per cent primary amino nitrogen. The substance was found to be soluble in water, and such solutions were quite neutral, indicating the absence of
free carboxyl groups. This material, when tested with Type I antipneumococcus serum, failed to precipitate, as indicated in Table V.

From the results of the precipitin tests it is seen that when the carboxyl group of the specific polysaccharide is esterified by means of diazo methane, the resulting methyl ester of the polysaccharide fails to precipitate in homologous antipneumococcus serum within the range of dilutions tested. It appears, therefore, that the presence of the carboxyl group in the type-specific polysaccharide is essential for complete specific union and precipitation with homologous antibody.

<table>
<thead>
<tr>
<th>Substance tested</th>
<th>Final dilution of substance tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:2,000</td>
</tr>
<tr>
<td>Original* polysaccharide</td>
<td>++±</td>
</tr>
<tr>
<td>Esterified polysaccharide</td>
<td>±</td>
</tr>
<tr>
<td>Saponified polysaccharide</td>
<td>-†</td>
</tr>
</tbody>
</table>

* Deacetylated form of Type I specific polysaccharide.
† Not done.

When the esterified polysaccharide is treated with dilute alkali at 100°C., the methyl ester grouping is removed as may be seen from the following experiment.

488.5 mg. of ester were dissolved in 20 cc. of water and 1 drop of 1 per cent phenolphthalein solution was added. 10 cc. of 0.1 N NaOH were added and the mixture was heated on a water bath for 30 minutes. The solution was cooled, and the excess NaOH was neutralized with 1.9 cc. of 0.1 N HCl. The neutral solution of polysaccharide was dialyzed until free from chloride ion, and then poured into 10 volumes of acetone. The precipitated polysaccharide was removed by centrifugation and dried. This substance was acidic in character, and had a specific optical rotation of +277.5°. The material had an acid equivalent of 580 when titrated to pH 7.0. The substance contained 4.82 per cent of nitrogen, and only 0.39 per cent of primary amino nitrogen. A methoxyl determination

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1 We wish to thank Mr. Frank H. Babers for assisting with the micro analytical determinations.
revealed the fact that the saponified derivative now contained only 5.21 per cent of methoxyl. From the results of the analysis of the esterified and the saponified products, it can be concluded that the former substance contains a methyl grouping bound on the primary amino group of the parent substance, a methoxyl group attached probably to one of the hydroxyl groups, as well as a methyl ester group coupled to the carboxyl grouping of the uronic acid. When the methylated compound is warmed with dilute alkali, the ester grouping is removed, and the resulting derivative still contains a methyl group substituted on the primary amino group, as well as a methoxyl group still covering an hydroxyl group. The latter compound, however, unlike the serologically inert ester, reacts readily with antiserum as can be seen in Table V.

It is obvious, therefore, that the presence of a free carboxyl group is of primary importance in rendering the Type I polysaccharide serologically reactive, and that the substitution of the NH$_2$ and OH groups in the polysaccharide molecule has a less pronounced influence upon serological specificity.

DISCUSSION

The earlier hypotheses concerning the nature of immune bodies have gradually given way to evidence accumulated largely during the past decade which at present definitely points toward the fact that the circulating antibodies are in reality modified serum globulins. Because the gross qualitative chemical tests for the detection of protein in biologically active fluids are so greatly exceeded in delicacy by the more subtle immunological tests, it is our opinion that the chemical evidence fails to support the hypothesis concerning the non-protein nature of antibodies. For years an analogous situation has existed in the attempt to define the chemical nature of enzymes, yet it has been only recently that the nature of these substances has been defined with certainty. An approach quite different from the absorption methods hitherto employed in the concentration of enzymes has made possible the isolation of crystalline proteins possessing the biological activity of crude enzyme mixtures. These crystalline proteins show both constant physical properties and biological action on repeated crystallization (22). The success of Northrop and his associates in isolating from crude enzyme solutions a crystalline protein possessed with unique chemical and biological properties has served as a stimulus to us in attempting to isolate the immune protein of antipneumococcus
serum free from inert constituents. That this objective has at least in part been achieved can be seen from the results of the foregoing experiments.

The precise method of Heidelberger and Kendall which permits the determination of small differences in activity of protein fractions has been used in this study as a means for determining the potency of antibody solutions. Although the quantity of immune precipitate formed by a given weight of type-specific polysaccharide is the measure of only one of the active constituents of antibody solutions, yet this measurement serves as an index of the activity of antibody concentration as a whole. The solubility properties of the other antibody proteins parallel so closely those of the type-specific immune body, that the former are found intimately associated with the latter and relatively more concentrated, as the purification progresses.

Many samples of water-insoluble globulin obtained by the dilution of antipneumococcus serum have been subjected to the methods of fractionation outlined in the experimental part of this communication. Although the initial concentration of the type-specific antibody in different samples of source material has been found to vary from 25 to 42 per cent, yet invariably the end-product never attains a value of 100 per cent of type-specific precipitable protein. The reason for this may lie in the fact that the remaining proteins not precipitable by the type-specific polysaccharide represent the other antibodies, and possibly small amounts of inert globulin which cannot be separated by the methods of fractionation employed.

One of the most striking observations encountered during the course of the experimental work was the fact that the purified antibody protein was found to have essentially the same percentage of basic amino acids as has the globulin of normal horse serum. Although the proportion of these amino acids is in each instance approximately the same, the normal globulin is biologically inert, whereas the immune protein possesses both chemical and immunological properties which distinguish it sharply from the former. By simple chemical experiments we have attempted to show that the biological activity of pneumococcus antibody protein is to a great extent dependent upon the presence of free amino groups in the protein molecule. When these groups are covered with a chemical radical the activity of the protein
molecule is lost. It has been further shown that upon removal of the substituted group the antibody molecule again becomes specifically reactive. Such experiments, however, fail to account for the immunological specificity which the protein exhibits. A possible explanation of the origin and specificity of antibodies has been suggested independently by Mudd (23) in this country, and by Breinl and Haurowitz (24) in Germany. In the opinion of these investigators, the normal course of serum globulin synthesis is altered in the animal body when antigen reaches the site at which synthesis takes place. Under the influence of the antigen the serum globulin is altered in a way characteristic for the foreign stimulus. When the modified globulin eventually encounters the antigen either in the circulation or in vitro interaction of the two is possible.

From the results of studies on the experimental transformation of specific types of Pneumococcus (25) it seems not unlikely that the synthesizing function of a cell can be specifically oriented by a given stimulus. When R forms of pneumococci, irrespective of the type from which they were derived, are grown in the presence of a specific activator contained in a cell-free extract of the S organism, the former are induced to synthesize the capsular polysaccharide of the same type as that from which the bacterial extract was prepared. If the nature of the activator is changed, however, the same R organism can be made to synthesize a capsular polysaccharide quite different in chemical constitution and in biological specificity from that which it produced when stimulated by the original activator. Thus it is seen that bacterial cells under the same general environmental conditions can, by means of specific stimuli, be directed to synthesize polysaccharides which are chemically distinct and biologically specific.

Although it is probable that the union of antibody with type-specific polysaccharide involves the interaction of polar groups of opposite charge, yet this concept alone does not explain the specificity of the reaction. There is, however, certain evidence to support the view that the specificity of serological reactions is governed by the arrangement in space of the polar groups of the reactive substances. It has been shown in this laboratory (26) that antigens prepared from the azo phenol glycosides of glucose and galactose react only in their homologous antisera. In respect to the number and nature of polar
groups these two glycosides are identical. Since the active groups
are in each instance the same, the mechanism underlying the union
of each glycoside with its homologous antibody must likewise be the
same. The specificity of this reaction therefore can be accounted for
only by known differences in the spatial arrangement of the polar
groups of the fourth carbon atom of each glycoside. In their studies
on the serological differentiation of steric isomers Landsteiner and
van der Scheer have arrived at similar conclusions (27). Since the
spatial arrangement of identical polar groups suffices to determine
serological specificity, it therefore seems justifiable to assume in
the case of the type-specific antibody of Pneumococcus that the
spatial arrangement of the polar groups in the immune protein may
likewise determine its specific capacity to react with the polysaccharide
of the homologous type.

In view of these considerations it is believed that the general mecha-
nism underlying the union of antibody and carbohydrate involves the
interaction of polar groups of opposite charge. Similar views have
indeed been expressed by Heidelberger and Kendall (28), by Marrack
(29), and by Haurowitz and Breinl (30). In the case of the Pneumo-
coccus Type I carbohydrate it is believed, on the basis of the evidence
presented, that the carboxyl groups of the polysaccharide are the domi-
nant groups which interact to form the immune precipitate. Whether
the carboxyl groups actually combine with the amino groups of the
antibody protein and whether the formation of an insoluble precipitate
involves further chemical change in the protein molecule, such as a spe-
cific denaturization, cannot be answered with certainty at the present
time. It is believed, however, that the specificity of this reaction is
determined by the stereochemical relationship of the dominant polar
groups in the reacting molecules, whether they be antigen or antibody.
If the spatial pattern of the polar groups of both antigen and antibody
is of exactly the correct order, then union occurs. If, however, this
relationship is disturbed by artificial means, as has been experimentally
demonstrated by covering the dominant polar group of either poly-
saccharide or antibody with a chemical radical, the pattern is destroyed
and union between them is either greatly modified or fails to take
place. When the original constitution of the reacting substances is
restored, however, serological specificity is regained.
In conclusion the authors wish to express their grateful appreciation of the helpful suggestions and criticisms of Dr. John H. Northrop and Dr. Oswald T. Avery.

SUMMARY

1. Methods for the concentration of Type I pneumococcus antibody have been outlined.
2. The physical and chemical properties of the purified antibody have been described.
3. The chemical basis of serological specificity has been discussed.

BIBLIOGRAPHY

202  ANTIBODIES IN TYPE I ANTIPNEUMOCOCCUS SERUM