pH Dependent Hemolytic Systems. III. The Physical Properties of the Serum Factors Involved, with Some Observations on Their Occurrence in Various Disease States in Man

By Stanley Yachnin and Frank H. Gardner

In recent years it has become apparent that apart from the heterogeneity of antibody molecules in terms of their specificity in serologic reactions, there exists a distinction, based on molecular weight, between small and large antibody molecules. The smaller molecules usually comprise what is quantitatively the largest component of the antibody content of serum, and are gamma globulins of the 7s\textsuperscript{20} variety with molecular weights of approximately 160,000. The larger molecules of the 19s\textsuperscript{20} variety normally comprise about 2 per cent of the total serum proteins. They have a molecular weight of approximately 1,000,000, migrate electrophoretically as fast gamma or slow beta globulins, and are referred to as macroglobulins. These two groups of proteins may be separated by various technics such as zone electrophoresis, ultracentrifugation, gradient elution from ion exchange resin columns, or immunoprecipitation technics utilizing diffusion in agar gels or immuno-electrophoresis. A wide variety of antibodies or antibody-like substances has been found to be associated with the macroglobulin fractions of both normal sera and the sera of individuals in various disease states.\textsuperscript{1}

In the course of an investigation concerning the nature of the red cell defect in paroxysmal nocturnal hemoglobinuria (PNH), various normal human serum factors were encountered, which, under appropriate conditions, caused agglutination and hemolysis of normal human red cells treated with various agents capable of altering the red cell stroma.\textsuperscript{2} Despite the arbitrary nature of the red cell alterations produced, a great degree of specificity in the altered red cell-serum factor interaction was observed.\textsuperscript{3} One of these serum factors, the "T" agglutinin, has long been recognized\textsuperscript{4} and has been considered by Burnet as an example of so-called "natural antibody."\textsuperscript{5} However, very little is known about the actual nature of the serum factors involved in these systems. In order better to define these serum factors, and to clarify whether in fact their nature is consistent with properties generally attributed to antibody, the experiments described herein were undertaken. The absence of the "T" agglutinin\textsuperscript{6} and the agglutinin for periodate-treated red cells\textsuperscript{7} from newborn sera led us to postulate that if they were in fact antibody molecules, they

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were likely to be of the macroglobulin variety since macroglobulins do not cross the placental barrier. The results support this hypothesis.

**Methods and Materials**

The methods used in collection and preservation of human red cells and serum have been described previously, as have the methods used in altered red cell preparation, the serologic technics used in the analysis of altered red cell agglutination and acid hemolysis, and the technic used for preparation of antibody eluates. The following agents were employed in the preparation of altered red cells: trypsin, ficin, papain, bromelin, cholera vibrio filtrate (RDE) and sodium periodate.

The methods used in the preparation of serum protein fractions by zone electrophoresis on starch and by zone ultracentrifugation in a sucrose density gradient were similar to those described by Fudenberg and Kunkel. The electrophoretic serum fractions were eluted from the starch block segments by washing with phosphate buffered saline (pH 7.4) in a Hirsch fritted glass funnel aided by gentle suction. After the removal of five successive fractions [1 (top) -5 (bottom)] from the ultracentrifuge tubes by means of a tube slicer, each fraction was dialyzed for 36 hours against buffered saline at 4 C. Dialysis was necessary since it was found that high concentrations of sucrose interfered with agglutinin readings. Protein concentrations were determined by Hewitt’s method using the Folin reagent in an alkaline copper solution. In order to obtain linear curves for protein concentration, it was necessary to allow color development to proceed in the dark.

Analytic ultracentrifugations of several sera and serum fractions were done using a Spinco Model E ultracentrifuge. To test the efficacy of macroglobulin separation by the density gradient technic, fraction 5 from a patient with macroglobulinemia was subjected to analytic ultracentrifugation, and was found to consist entirely of proteins with sedimentation coefficients of 19S or greater.

The Ouchterlony technic of double diffusion in agar gel and immunoelectrophoresis were utilized as aids in identifying serum protein components. A modification of the former, similar to that described by Crowle, was used to determine the concentration of certain serum proteins in the fractions prepared by ultracentrifugation or zone electrophoresis. Serial twofold dilutions of serum fractions were tested for their ability to form precipitin lines with an appropriate antiserum. At the same time a standard solution of purified protein fraction, either human serum albumin or human gamma globulin, was tested in an identical manner with the same antiserum. By comparing endpoints for the standard protein solutions with those of the test fractions, an approximation of the absolute concentration could be obtained; usually it was only deemed necessary to find the relative concentration of a particular serum protein in various fractions of a single serum aliquot, in which case the use of a standard was not necessary. The human serum albumin and gamma globulin (7S) used as standards gave only one precipitin line both on agar gel double diffusion and immunoelectrophoresis; they were also used to identify the appropriate serum constituent in whole serum or fractions thereof by these two methods. A rabbit antiserum to whole human serum prepared in this laboratory as well as a rabbit antimacroglobulin serum§ were used. The former gave at least 14 precipitin lines with normal human serum by immunoelectrophoresis. The latter, after absorption with 7S gamma globulin, was specific for macroglobulin. After many observations, it was found

*These determinations were kindly performed by Dr. S. Allerton, Department of Biophysical Chemistry, Harvard Medical School, Boston, Mass.

1 Normal serum albumin (human), Massachusetts Public Health Laboratories, Boston. Lot # HAB52C.


3 Kindly supplied by Dr. Hugh Fudenberg, Rockefeller Institute for Medical Research, New York, N. Y.
that the macroglobulin precipitin line formed with the antiserum to whole human serum could be identified by its characteristic position and configuration.\textsuperscript{12}

In order to determine the effects of sulphhydryl-containing compounds on the capacity of serum to agglutinate altered red cells, the following technic was used. Glutathione and cysteine HCl were dissolved in normal saline - TRIS [Tris (hydroxymethyl) aminomethane maleate] buffer, pH 8.2 (9 parts saline, 1 part buffer V/V). The concentration of cysteine was 60 mEq./L., and that of glutathione was 50 mEq./L. Serial twofold dilutions of an appropriate plasma were then prepared in normal saline, saline-TRIS buffer, saline-TRIS buffer plus glutathione, and saline-TRIS buffer plus cysteine. After allowing these serum dilutions to stand at room temperature for 30 minutes, appropriate altered red cells were aliquoted into each tube, and agglutinin titers determined as described (supra vide).

**Results**

1. **Zone Electrophoresis**

The characterization of altered red cell agglutinins by zone electrophoresis revealed that the serum factors involved were usually found in the fast gamma or slow beta protein fraction (fig. 1). On one occasion the peak of agglutinin activity was found to coincide directly with the gamma globulin peak (fig. 1, top). A secondary diminutive agglutinin region was consistently found which coincided with the peak concentration of the albumin fraction. That this biphasic agglutination was not caused by contamination of the albumin fractions by gamma globulin, and conversely, that albumin was not present in the gamma or beta globulin regions of most intense agglutination was readily proved by analysis of the electrophoretic fractions with the Ouchterlony technic. (This procedure of analysis is, in reality, a lengthy two-step method of immunoelectrophoresis.) To exclude the possibility that a small fraction of globulin agglutinin, not demonstrable by immunoprecipitation methods, had migrated into the albumin region, and that its activity was enhanced by the long-recognized capacity of albumin to intensify weak agglutinin reactions, an attempt was made to see if purified human serum albumin would enhance agglutinin activity in whole serum or serum globulin portions obtained electrophoretically. No such enhancement of agglutinin titer could be demonstrated. No agglutinin activity was demonstrable in commercially prepared human serum albumin; on the other hand, agglutinin activity was present, albeit in low titer, in chemically fractionated gamma globulin preparations (F II). The studies performed with serum fractions separated by ultracentrifugation proved conclusively that albumin per se had no part in the agglutinin activity of serum (vide infra). The presence of agglutinin activity in the electrophoretic albumin fraction cannot be adequately explained at present, but other workers have noted that eluates from starch block electrophoresis may display nonspecific, artefactual agglutinin activity even for normal red cells.\textsuperscript{13}

Preparation of eluates from coated altered red cells and parallel electrophoresis of the whole serum and eluate revealed that the migration of the agglutinin activity coincided nicely (fig. 2). No diminution in serum electrophoretic protein peaks could be demonstrated subsequent to absorption of serum with altered red cells, in contradistinction to findings previously noted.
Fig. 1.—The agglutinin titer for various artificially altered red blood cells was measured in serum fractions prepared by starch block electrophoresis. Top: Agglutinin activity of serum Fi for RDE-treated red blood cells. Bottom: Agglutinin activity of serum Ma for bromelin-treated and papain-treated red blood cells. The anode (and albumin fraction) are to the right. Note the appearance of a second agglutinin peak in the albumin fraction (see text).

with high titer cold agglutinins associated with abnormal electrophoretic protein peaks. In addition, attempts to demonstrate specific protein fractions in the eluates by immunoprecipitation technics were unsuccessful, despite lyophilization and concentration. Presumably these observations are reflections of the small amount of protein, on a weight basis, which these agglutinins must represent.
Fig. 2.—Agglutinin activity of starch block electrophoretic fractions from serum Ma for ficin-treated red blood cells (top). An eluate prepared from ficin-treated red blood cells coated in serum Ma was subjected to electrophoresis (bottom). The fractions were then tested for agglutinin activity, using fresh ficin-treated red blood cells. Agglutinin activity in the eluate migrates in the same fashion as that in whole serum, in this case as a beta globulin. The protein peak coincides with the free hemoglobin resulting from the elution procedure.

2. Density Gradient Ultracentrifugation

Sample results of the ultracentrifugation studies are summarized in table 1. The peak agglutinin activity was invariably associated with the bottom fraction (fraction 5), and frequently this was the only fraction in which agglutinin activity was demonstrable. Considering the dilution factor of approximately 4 inherent in the method, recovery of agglutinin activity was almost complete. The peak distribution of 7S gamma globulin was usually in fractions 3 or 4; 7S globulin was usually, although not invariably, absent.
Table 1.—The Agglutinin Titers of Serum Fractions Obtained by Density Gradient Ultracentrifugation vs. Various Types of Altered Red Blood Cells

<table>
<thead>
<tr>
<th>Serum</th>
<th>Fraction tested</th>
<th>Agent used to alter red cells</th>
<th>Agglutinin titer</th>
<th>Protein concentration mg./cc.</th>
<th>Relative Concentration of 7S globulin</th>
<th>Macroglobulin precipitin line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fi</td>
<td>WS</td>
<td>RDE</td>
<td>512</td>
<td>0.34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mi</td>
<td>WS</td>
<td>periodate</td>
<td>512</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Me</td>
<td>WS</td>
<td>ficin</td>
<td>512</td>
<td>4.8</td>
<td>3+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Be</td>
<td>WS</td>
<td>bromelin</td>
<td>64</td>
<td>2.0</td>
<td>3+</td>
<td>0</td>
</tr>
</tbody>
</table>

(WS = whole serum.)

in fraction 5, and when present was found in very low concentration. Albumin was present in greatest concentration in fractions 2 or 3 and only rarely was it present to any appreciable extent in the bottom fraction, thus supporting the conclusion previously reached that albumin alone was not responsible for the puzzling agglutination found in the albumin fractions on zone electrophoresis. In almost every instance, a readily identifiable macroglobulin immunoprecipitin line was demonstrable in fraction 5 by the Ouchterlony technic. Even though the agglutinin activity for RDE-treated red cells in serum Fi (fig. 1) coincided with the peak of gamma globulins on zone electrophoresis, nevertheless on ultracentrifugation analysis 7S globulins were absent from the bottom fraction, to which agglutinin activity was limited. This coincides with the observations of others concerning the wide variability in electrophoretic mobility of other macroalbumin red cell agglutinins.8

Eluates prepared from coated altered red cells were subjected to identical density gradient ultracentrifugation technics (table 2). The only measurable protein present was invariably associated with the neatly separated hemoglobin band resulting from slight hemolysis during the elution procedure; this band was approximately equal in position to that of serum albumin, consistent with their similar molecular weights. Outside the hemoglobin region, no protein could be demonstrated. Despite this, however, agglutinin activity was...
Table 2.—Density Gradient Ultracentrifugation Fractions Were Prepared from Eluates of Altered Red Cells Coated in Normal Serum. These Fractions Were Then Tested for Agglutinin Activity vs. Fresh Homologous Altered Red Cells. The Only Protein Detectable Chemically Coincided with Free Hemoglobin Resulting from the Elution Procedure

<table>
<thead>
<tr>
<th>Altered red cell type from which eluate prepared</th>
<th>Fraction</th>
<th>Agglutinin titer</th>
<th>Protein concentration mg./cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDE WE</td>
<td>1</td>
<td>neg.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>neg.</td>
<td>.80</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>.84</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>ficin WE</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>32</td>
<td>0</td>
</tr>
</tbody>
</table>

(WE = whole eluate.)

present. The agglutinin activity tended to descend less readily than when whole serum was used; agglutinin activity was present in trace amounts as high as fraction 2, and was highest and usually equal, in fractions 4 and 5. Theoretically, the lower protein concentration in the eluates should allow more rapid descent of the protein molecules. At this point one can only speculate that the elution procedure may have resulted in artefactual degradation of the macroglobulin moieties involved, without affecting appreciably their ability to agglutinate altered red cells, a situation quite different from that which results when the macroglobulins are cleaved by sulphhydryl compound.

Efforts to isolate hemolysin activity by ultracentrifugation technics met with less success. Only in one experiment involving the ficin-treated red cell system was reconstitution of acid hemolysis in ficin-treated red cell absorbed serum achieved. Hemolysin activity in this experiment was limited to fraction 5 (table 3). Previous work has shown that hemolysin activity is much more labile than agglutinin activity. The failure to isolate consistently hemolytic activity by this technic may be another manifestation of this relative lability. However, anticomplementary activity of the fractions was not excluded.

3. The Effects of Sulphhydryl Compounds

There has been ample demonstration of the dissociation of macroglobulins by sulphhydryl compounds, and coincidentally, their loss of potency as antibodies. 7S globulins, such as those which constitute incomplete "warm" antibodies are not affected by these reagents. When serum was pretreated with cysteine or glutathione, its capacity to agglutinate altered red cells was markedly diminished (table 4).

4. Other Studies

a. Acquired hemolytic anemia: Serum from a patient with acquired hemolytic anemia associated with high titer cold agglutinins was subjected to
electrophoretic and ultracentrifugal analysis. The peak of agglutinin activity for normal red cells was found in the beta globulin fraction, and almost all the agglutinin activity sedimented to the bottom (fraction 5) when subjected to density gradient ultracentrifugation (fig. 3). These findings are in agreement with those of other workers.5

The sera of patients with other varieties of hemolytic anemia were assayed for their content of altered red cell antibody. These included sera from patients with acquired hemolytic anemia of the "warm" antibody variety (with a negative indirect Coombs' test), paroxysmal nocturnal hemoglobinuria, and hereditary spherocytosis. The titers obtained were within the range of those encountered in normal sera.

b. Dysproteinemias: Serum from a patient with lymphosarcoma and macroglobulinemia contained low titers of altered red cell agglutinins, despite the fact that over 40 per cent of this patient's serum proteins were macroglobulins. The latter were isolated in almost pure state by density gradient ultracentrifugation, and identified as 19S (75 per cent), 26.7S (20 per cent), and 34S (5 per cent) globulins (fig. 4). All of the agglutinin activity of this serum was recovered in the macroglobulin fraction (fraction 5).

Sera from several multiple myeloma patients with and without large myeloma protein spikes by electrophoretic analysis were found to contain normal titers of altered red cell agglutinins.

c. Rheumatoid arthritis: The rheumatoid factor has been identified as a macroglobulin.15 Accordingly, 20 sera from patients with rheumatoid arthritis and positive latex fixation tests were studied; the titer of agglutinins or hemolysins for altered red cells was well within the normal range.

d. Agammaglobulinemia: The sera from three patients with congenital agammaglobulinemia were studied. One patient* was found to have no altered

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*Kindly supplied by Dr. David Gitlin, Children's Hospital Medical Center, Boston, Mass.
Fig. 3.—Serum from a patient with high titer cold agglutinins was subjected to starch block electrophoresis. The peak of agglutinin activity coincided with the abnormally prominent beta globulin region. (top). Serum from the same patient was subjected to density gradient ultracentrifugation. (bottom). The greatest portion of agglutinin activity was found in the macroglobulin fraction (bottom of tube, fraction 5). The slight rise in protein concentration in fraction 5 is abnormal and indicates an increase in macroglobulin content in this patient's serum.

The second patient was a 15 year old boy who had been diagnosed as having agammaglobulinemia eight years previously. At that time his serum contained only 15 mg. per cent gamma globulin. Approximately six months prior to study he was noted to have developed anti-A and anti-B isoagglutinins, these having been previously absent. He was...
admitted to the Children's Hospital Medical Center, Boston, Massachusetts, in May 1960, when he was found to have nephritis, hepatosplenomegaly and pancytopenia. At this time his serum contained approximately 150 mg. per cent 7S gamma globulin* which was considered to be a residual of exogenously administered protein (the last gamma globulin having been administered five weeks prior to this determination).

The third patient was a 5 year old boy with newly diagnosed congenital agammaglobulinemia (7S gamma globulin 63 mg. per cent) who had no other significant clinical abnormalities.

The agglutinin titers of the sera from these three patients for various types of red cells are shown in table 5. In addition, serum from Patient 2 contained

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* Determined by an immunologic technic (see Methods).
Table 5.—Agglutinin Titer of Serum from Three Patients with Agammaglobulinemia for Various Red Cell Types

<table>
<thead>
<tr>
<th>Patient</th>
<th>Ficin*</th>
<th>Bromelin*</th>
<th>Type of Red Cells</th>
<th>Periodate*</th>
<th>Trypsin*</th>
<th>“A”</th>
<th>“B”</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>32</td>
<td>1</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>2048</td>
<td>2048</td>
</tr>
</tbody>
</table>

*Denotes red cells altered by pretreatment with these agents.
†All these patients were type “O”.

Other workers have amply demonstrated that many categories of antibody consist either entirely or in part of macroglobulins of the 19S category. Included in these are certain pneumococcal antibodies of the horse and rabbit, and in man such diverse antibodies as cold agglutinins, heterophile antibody, certain isoagglutinins, Rh saline agglutinating antibody, leukagglutinins, Wassermann antibody, certain LE serum factors, thyroglobulin and penicillin antibodies. Certain other substances, whose antibody nature is in doubt are also of this category, i.e., conglutinin, the rheumatoid factor, and properdin.1

The studies herein reported amply support the conclusion that the human serum factors active against various types of altered human red blood cells are in this category of serum protein, and, as such, are reasonably considered as an example of “natural antibody.”

The absence of “T” and periodate7 agglutinins in the newborn suggests that these serum factors are acquired. Two mechanisms may be considered:

1. These antibodies represent a heterogenetic response to the multitude of antigens derived from animal, plant and bacterial polysaccharides. Such antigens may be similar to those ascribed to the formation of isoagglutinins.17 Recent studies with germ-free chicks18 have revealed that the appearance of the anti-B heteroagglutinins can be delayed or suppressed until a bacterial flora is introduced. In fact, only one type of bacteria is necessary to evoke this serum factor.

2. Natural antibodies to altered red cell membranes may be initiated by a response of the reticulo-endothelial (RE) system to a specifically damaged erythrocyte mosaic pattern. It is apparent that the RE system can recognize slight modifications in the stromal membrane. Human red cells modified by
Trypsin or myxovirus (Newcastle disease virus and PR8 influenza virus) can stimulate specific antibodies in the rabbit. Certain differences in the immune response of the rabbit were noted to red cells altered by various strains of myxovirus.\(^\text{19}\) Hence, the RE system may respond to red cells damaged in vivo by enzymatic or chemical alterations from bacteria, chemicals and viruses. The persistent phagocytosis of senescent or nonviable red cells with such alterations during the multiple exposures coincident with human growth might account for the variety of serum agglutinins and hemolysins described in this and other reports.\(^\text{2,3}\)

These serum factors, so readily available in the agglutination or hemolysis of autologous red cells with stromal alterations, cannot be classified with any certainty as related to an antibody response favoring either the “template” or the “clonal selection” theory. Their presence does suggest a delicate mechanism for antibody response to all types of antigenic sources.

The presence of agglutinin activity for altered red cells in certain albumin fractions of serum isolated by zone electrophoresis on starch has not been adequately explained. The absence of agglutinin activity in albumin-rich ultracentrifugal fractions and in chemically fractionated human serum albumin, as well as the failure of albumin to enhance agglutinin titers in whole serum or other serum fractions indicates that it is not caused by albumin \textit{per se}. It may be caused by an artefact of the method employed, or by the presence of trace amounts of globulin not detected by the methods used. The methods of immunoelectrophoresis\(^\text{20}\) and of combined electrophoresis and ultracentrifugation\(^\text{21}\) have amply demonstrated that small amounts of 7S and 19S gamma globulin may migrate electrophoretically as rapidly as alpha-2 globulins. The possibility also remains that some agglutinin activity may be associated with gamma globulins of the 7S variety, since in certain experiments agglutinin activity was not limited to the bottom fraction of density gradient ultracentrifugation. However, the number of such sera was few, and the titers obtained, when present in the higher fractions, were accountable for only a small portion of the total antibody content of the particular serum under study. Sulfhydryl compounds greatly reduced, but did not entirely destroy the agglutinating capacity of serum; this observation is also consistent with the possibility that a small portion of antibody activity may be caused by 7S globulins, since these are known to be resistant to the disulfide splitting action of sulfhydryl compounds.\(^\text{2}\)

The many close similarities between the PNH hemolytic system and the hemolysis of artificially altered red blood cells in human serum\(^\text{2}\) are even further enhanced by the realization that in both instances macroglobulin factors are involved. In the PNH system these macroglobulin factors are called properdin, but the properdin system may be one which involves properdin as natural antibody plus the classic components of the complement system.\(^\text{22}\) As the number of recognized macroglobulin antibodies increases, it is interesting to speculate, as others have done for the 7S globulins, that macroglobulins present in normal human serum may consist entirely of antibodies of varying specificity.

Altered red cell antibodies are not increased by the pathologic stimulus to
abnormal protein formation in various dysproteinemias, particularly macro-
globulinemia. This, together with their presence in normal titer in the sera
of patients with high titers of rheumatoid factor (another macroglobulin
moiety) bespeaks a certain specificity in the stimulus to their formation. In
favor of their antibody nature is the fact that they were absent in one case
of agammaglobulinemia, where the formation of circulating antibody is notorious ly impaired. This latter finding is at variance with another report demonstrat ing the presence of a trypsin-altered red cell agglutinin in agamma-
globulinemic serum. However, these workers were dealing with a reversible
agglutinin, which, as has been previously noted, is a different system from
the non-reversible trypsin-altered red cell agglutinin we have encountered.

Of great theoretic interest was the finding of relatively good titers of iso-
agglutinins, altered red cell agglutinins and hemolysins in our second and
third cases of agammaglobulinemia. It is well recognized that although the
19S globulins are made up of six 7S subunits, these subunits are by no means
identical with normal 7S globulin, as evidenced by a particularly rich carbo-
hydrate content and specific immunologic properties. The fact that an in-
dividual is genetically incapable of producing one variety of antibody mole-
cule (7S) while being able to produce another (19S) is in keeping with the
one gene-one protein hypothesis as related to genetic disorders of protein
production, and lends some support to the clonal theory of antibody forma-
tion. Patient 2, with agammaglobulinemia, developed many features of a
“collagen-like” disease which has been amply described in α- and hypogamma-
globulinemic states concomitant with the appearance of these macroanti-
bodies. This observation raises the question of whether such antibody forma-
tion might not be deleterious to the individual’s well-being, appearing as it
does in association with the development of a clinical disorder that could be
construed as autoimmune.

**Summary**

Agglutinins for various artificially altered red blood cells belong to the
class of 19S macroglobulins, which migrate electrophoretically as fast gamma
or slow beta globulins. The agglutinin activity of serum for altered red cells is
readily destroyed by sulfhydryl compounds. Hemolysins for altered red cells
are not readily recoverable from serum fractions prepared by starch block
electrophoresis or density gradient ultracentrifugation, but, when present,
are found to have the same properties. This information lends credence to
the concept of these serum factors as examples of “natural antibody,” although
the stimulus to their formation is not understood.

The sera from patients with various types of hemolytic anemias and various
dysproteinemias including macroglobulinemia were found to contain normal
amounts of altered red cell agglutinins and hemolysins.

The sera from three patients with congenital agammaglobulinemia were
studied. Two of these sera contained agglutinins and hemolysins for altered
red blood cells, as well as isoagglutinins and isohemolysins. The significance
of this finding is discussed.

The “T” agglutinin and the agglutinin for periodate-treated red cells, both
of which are macroglobulins, have been shown by other workers to be absent from newborn sera. Their inability to cross the placenta can be explained by their large size.

**Summario in Interlingua**

Agglutininas pro variabrum artificialmente alteratum erythrocytos pertine a! classe del macroglobulinas 19S que migra electrophoreticamente con le rapiditate de globulinas gamma o de lente globulinas beta. Le activitatem agglutininicum de sero con respecto a alterate erythrocytos es presumente destruite per compositos sulfhydrylic. Hemolysinas pro alterate erythrocytos non es presumente recuperabile ab fractiones seral preparate per electrophorese a bloco de amylo o ultracentrifugation a gradiente de densitate, sed quando illos es presente, il es trovate que illos ha le mesme proprietates. Iste information servir a corroborar le validitate del concepto que iste factoris seral es exemplos de "anticorpore natural", ben que le stimulo que precipita lor formation non es ben comprendite.

Le seros ab patientes con varie typos de anemia hemolytic e varie dysproteinemias (inclus macroglobulinemia) revelava un contento normal de agglutininas e hemolysinas pro alterate erythrocytos.

Esseva studiate le seros de tres patientes con congenite agammaglobulinemia. Duo de iste seros contineva agglutininas e hemolysinas pro alterate erythrocytos, si ben como isohemolysinas e isoagglutininas. Le signification de iste constatationes es discutite.

Le agglutinina "T" e le agglutinina pro erythrocytos tractate con periodato (que ambes es macroglobulinas) es absent in le sero de neonatos, secundo le constatationes de altere autores. Lor incapacitate de transversar le placenta pote esser explicate per lor grandor.

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