QUANTITATIVE DETERMINATION OF HEMAGGLUTININS FOR NORMAL AND TRYPsinIZED HUMAN FET BLOOD CELLS

Dissertation

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Adviser
activity of trypsinized red blood cells.

Data from hemagglutination studies, while providing valuable adjuncts for diagnosis of some hemolytic diseases, are likewise inadequate. Wheeler (1950) states simply that it seems likely that enzymes actually modify the surface of the red cells so as to diminish their suspension stability and to rearrange, modify or uncover antigens which in normal cells either do not react with certain antibodies or which react in a different manner.

Weiner and Katz (1951) reported the presence in normal human serum of a substance which inhibited the action of trypsin on red cells and further stated that none of the known hemagglutininogen loci were affected by trypsin. These workers offered the following hypothesis.

"The experiments with enzyme inhibitors support the idea that the substance on the surface of the red cells on which the enzyme acts is a protein. In any event the substance appears to be firmly bound into the surface of the red cell since multiple washings with saline either before or after treatment with enzymes fail to remove it. The clumping which occurs when enzyme treated cells are mixed with antisera containing univalent antibodies, therefore, appears to be related to conglutination rather than agglutination."

Weiner and Katz (1951) also found that if normal red
cells were sensitized with incomplete Rh antibodies and subsequently exposed to non-sensitized trypsin treated cells containing Rh agglutinogen, agglutination occurred. If, however, the normal sensitized cells were mixed with trypsinized cells not possessing the Rh agglutinogen agglutination was not observed. As the authors point out, these results may be due to partial reversibility of sensitization of normal cells thereby providing antibody to react with trypsinized cells. Nevertheless, they have interpreted this observation as further evidence of a conglutination mechanism.

It becomes apparent from a study of the literature that the changes produced on red cells by the action of trypsin, in the concentrations used, involve a relatively minor physical modification of the cell structure. Moreover, it is extremely unlikely that any profound chemical alteration of the cell ultrastructure is produced. The problem is, therefore, relegated to the field of immunology with its techniques for the distinction of slight structural differences in chemical compounds, and since the classical methods of serology have yielded little information concerning the modified activity of trypsinized cells, it seems appropriate that the immunologic nature of these cells be investigated by the application of immunochemical techniques.

While there are numerous ways of describing the
Intensity of serologic reactions, only those based on the determinations of the amount of specific precipitate either by direct weighing or by chemical analysis yield absolute results and meet the requirements of quantitative analytical chemistry. Other techniques like the serum dilution titer, the optimal proportions method, turbidity measurements, determination of the volume of specific precipitate, the optimal flocculation method give results in relative terms rather than in absolute units and are much less precise.

Analyses of washed specific precipitates were first carried out by Wu (1927, 1928) who attempted to calculate their composition with respect to antigen and antibody. However, the quantitative precipitin technique as an analytical method for the estimation of antibody was first elaborated and perfected by Heidelberger and Kendall (1929, 1933, 1935) and has been widely adopted as a reliable and strictly quantitative method. It is based on the analysis of the washed specific precipitate for N by the micro Kjeldahl method after complete precipitation of antibody by addition of antigen in slight excess. Heidelberger and Kendall (1935) investigated the quantitative course of the precipitin reaction between egg albumin and rabbit anti-egg albumin, and found the composition of the precipitate varies continuously with changes in the proportions of the reactants. When the ratio $\text{AbN: EaN}$ was
plotted against the antigen N added, a straight line was obtained which follows the general equation $\text{AbN pptd.} = a - bx$ where $x$ is the amount of antigen N added. The constants $a$ and $b$ in this empirical equation can be evaluated readily; $a$ equals the intercept on the $y$ axis and $b$ equals the slope of the line. Multiplying both sides of the equation by $x$ gives the equation: $\text{AbN pptd.} = ax - bx^2$. Equations of the same general type have been found applicable to various precipitin systems which have been studies quantitatively.

Heidelberger and Kendall (1935) developed a theory of precipitation assuming that both antigen and antibody are multivalent and that the reactions follow the mass law. For convenience of calculation the antibody reaction is considered as a series of successive bimolecular reactions which take place before precipitation occurs. These authors state that "no evidence of dissociation could be found experimentally over a large part of the reaction range."

The lattice concept of specific forces operative in flocculation is embodied in the statement that "the final precipitate, then, would in each case consist of antibody molecules held together in three dimensions by antigen molecules". From their assumptions the authors derived, for the region of antibody excess, the equation $y = 2Rx - \frac{R^2x^2}{A}$ where $y$ is the antibody in the precipitate in mg., $x$ the antigen in mg., $A$ the total antibody and $R$ the ratio of antibody to antigen at the equivalence point. It can be
seen here that $a$ in the empirical equation is equal to $2R$ and $b$ is equal to $R^2/A$.

A large body of accurate quantitative data was obtained on the precipitation of polysaccharides and protein antigens (also the agglutination of bacteria), and in the majority of the examined instances the equation gave satisfactory agreement between observed and calculated values in the range considered; it accounts for the variable composition of the precipitates and the approximate independence, in the authors' experiments, of changes in volume.

It may be noted that in the experiments of Goodner (1939) with antipneumococcus sera, when the amount of antibody precipitated was plotted against the quantity of antigen, the graph, instead of being a smooth curve, was a figure of linear segments each of which seemed to be related to a certain grouping in the antigen. With a cross reacting precipitation system similar results were obtained by Heidelberger et al. (1942)

A priori it would seem that difficulties in the way of a rigorous physico-chemical treatment would arise from the presence of antibodies of different reactivity in the same immune serum and of groups unequal in specificity and binding capacity in antigen molecules. With regard to the unhomogeneity of immune sera Heidelberger remarks that "although the anticarbohydrate is known to be a mixture of
antibodies of different reactivities it may be treated mathematically as if its average behavior were that of a single substance."

In addition to other variations, "low-grade" antibody fractions have been demonstrated by Heidelberger and Kendall that are not capable of producing precipitation but are included in precipitates formed by more potent antibodies; and there exist antibodies that precipitate only at low temperature.

A theory put forward by Kendall (1942) through different reasoning and avoidance of some assumptions of the original theory of Heidelberger and Kendall - which the authors felt to be oversimplified - arrived, for bivalent antibodies, at the same equation as above. The following of his postulates should be stated. "Both antigen and antibody may be multivalent with respect to each other .... all reactive groups upon a given molecule may have the same specificity or they may be different." "The maximum number of molecules of antibody bound by one antigen molecule may be determined by the number of reacting groups upon the antigen, or it may be limited by steric factors." "The reactivity of an antigen or antibody group upon the surface of a precipitate is the same as the reactivity of the same group on the surface of a molecule in solution." The equilibria are established "between the free and combined antigen and antibody groups
in the system" and "between reactive groups upon the surface of precipitates and the molecules in solution." Kendall concludes that the equation is valid for homogenous antibodies.

Another formulation derived by Hershey (1941), based upon kinetic considerations, expresses the equilibrium in the precipitin reaction between multivalent antigens and antibodies in terms of the valence, the initial molar concentration of the reactants, and the dissociation constant of the "specific valence," regarded as the main characteristic of individual immune sera. The theory was found to be compatible with experimental results and presumptive evidence for the valence of antibodies.

Heidelberger and Kabat (1937) followed the course of bacterial agglutination as a function of the amounts of bacterial suspension and antibody for the reaction of the type specific capsular polysaccharide on type I pneumococci with homologous antiserum. They observed that the ratio \( \text{AbN:S} \) reached an upper limit with decreasing amounts of suspension. In the region in which a change of ratio was observed the general type equation derived for the precipitin reaction was found to hold. No rigorous mathematical treatment of complex systems involving a variety of antibodies with different specificities (e.g. antiserum prepared against foreign erythrocytes) has been
attempted.

There is only one report in the literature dealing with precise quantitative measurement of antibody to red blood cells. Heidelberger and Treffers (1942) studied the reaction between rabbit anti sheep cell sera and the stromata of sheep erythrocytes, and were unable to show any direct correlation between serum dilution titers and absolute amounts of antibody N. These workers reported that the sheep cell stromata were slightly soluble in saline controls, a fact which may cause antibody values obtained by nitrogen difference to be slightly too high.

Quantitative work using human erythrocytes has not been reported in the literature. This is probably due to the difficulty met in freeing human erythrocyte stromata of hemoglobin. Moreover, human erythrocyte stromata are markedly soluble in saline. The literature contains a variety of procedures for the preparation of stromata for purposes of chemical analysis (Ballantine 1944, Ponder 1951, Parpart 1942, Berstein et. al. 1937, Jorpes 1932) or isolation of antigenic components on the red cell (Belkin and Weiner 1944, Calvin et. al. 1946, Evans et. al. 1950) Howe (1951) reported that the maximum yield of whole crude stromata from a 1:10 hemolysate was obtained at pH 5.5 to 5.8 and ionic strength 0.01. Howe further demonstrated that not all of the non hemoglobin stroma protein was found
to be involved in virus and isoagglutinogen receptors; and the portion showing least receptor activity was minimally soluble in the neighborhood of pH 5.0 to 5.8, being completely soluble on either side of this relatively narrow pH range.

Although the methods mentioned above have been valuable for chemical analysis and crude separation of specific antigenic components, they do not provide satisfactory material for quantitative hemagglutinin determinations in antisera prepared against intact human red blood cells, and the experimental portion of this paper deals in part with an attempt to prepare such material.
MATERIALS AND METHODS

Preparation of Normal Red Blood Cells: Freshly drawn whole human blood, type 0, Rh, was sedimented in an angle centrifuge at 2000 rpm. Plasma and white cells were drawn off and the red cells were washed three times in 0.85 per cent phosphate buffered saline, pH 7.4.

Trypsinized Red Blood Cells: One ml of washed packed red blood cells, type 0, Rh, was added to 1.5 cc of a 0.1 per cent trypsin-saline solution. This suspension was incubated for thirty minutes at 37 C. with frequent agitation. After centrifugation the supernate was removed and the treated cells were washed three times with cold saline. Before being used for experimental work, these cells were tested to insure that agglutination took place in human serum known to contain anti-Rh blocking antibodies but no saline agglutinin. Treated cells were also checked for nonspecific agglutination in normal human serum.

Preparation of Rabbit Antisera: These antisera were prepared according to the method of Baxter (1951). Four rabbits received three intravenous injections of 1.0 ml of a four per cent suspension of washed, group 0, Rh, red blood cells at intervals of four days. Another group of four animals were immunized with three intravenous injections of a four per cent suspension of trypsinized, 0, Rh cells. The rabbits were bled one week after the last injection and the sera from each group were pooled and stored at -10C.
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Hemagglutination Titers: Doubling dilutions of rabbit antisera were made in phosphate buffered saline. Two drops of a two per cent cell suspension were added to 0.2 ml of each serum dilution. Tests made with normal red cells were allowed to incubate for thirty minutes at 37°C. Tubes were then centrifuged at 1500 rpm for 1 minute and read for agglutination. When trypsin treated cells were used, tubes were centrifuged and read immediately after mixing.

Absorption of Antisera: Sera from rabbits immunized with both types of red cells were absorbed with homologous and heterologous cells. Absorptions were carried out at various temperatures according to the following outline.

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<tr>
<th>Absorption</th>
<th>Cells:Serum</th>
<th>Time</th>
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<tr>
<td>2nd</td>
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Preparation of Stromata: A variety of procedures for the preparation of erythrocyte stromata have been proposed by other investigators. These procedures have been employed in this investigation and are, therefore, outlined below. Because these methods were not designed primarily for the preparation of human stromata which could be used for
quantitative hemagglutinin determinations, we have developed a modified procedure which will be presented in detail.

A. **Heidelberger and Treffers (1942):** One liter of human whole blood was sedimented in a large (International) type centrifuge. The plasma and white cells were removed and the red cells were washed three times with 0.85 per cent saline. The packed cells were laked in twenty volumes of distilled water and then passed through a Scharples centrifuge. The stromata thus obtained were washed twice with water and twice with 0.85 per cent saline at room temperature followed by two more washings at 4 C. They were then resuspended in saline containing 0.4 per cent formalin and were washed two times before being used in tests.

B. **Parpart (1942):** One volume of washed packed cells was added to one volume of water and hemolysis allowed to take place. Eighty volumes of water saturated with CO₂ and cooled to 10 C were then added and the mixture was kept cold from this time on. The "ghosts" clumped and settled spontaneously in several hours. They were then washed repeatedly (6 to 10 times) with cold CO₂ saturated water, resuspended in saline containing 0.4 per cent formalin and stored in the refrigerator.

C. **Ponder (1951):** Washed cells were made up to a volume concentration of 0.07 in 1 per cent saline and were
kept at 37 C for 6 to 8 hours. The cells were then packed at about 4000 rpm for 30 minutes. The saline was removed and the packed cells were added to 125 volumes of distilled water. The mixture was allowed to stand at 4 C for 24 hours after which time the "ghosts" appeared as loosely sedimented layer in the bottom of the vessel. The supernate was removed and the sediment was washed twice with water at 4 C, resuspended in 0.4 per cent formol-saline and stored in the refrigerator.

Because stromata prepared by the procedures outlined above proved unsatisfactory for quantitative hemagglutinin studies, stromata were prepared by methods which did not involve osmotic lysis.

D. Washed packed erythrocytes were added drop-wise to five volumes of liquid nitrogen. The nitrogen evaporated immediately leaving behind a solid mass of frozen cells which were thawed out at 37 C and diluted with 5 volumes of saline. The mixture was centrifuged and the sediment washed twice with saline and added to 5 volumes of liquid nitrogen. After thawing and dilution with saline the stromata were washed until hemoglobin no longer appeared in supernatant saline. Washed stromata were stored at 4 C in formol saline.

E. One hundred ml of washed packed red blood cells were diluted with 100 cc of 0.9 per cent saline and placed in a Waring Blender for one minute. The mixture was then
poured into centrifuge tubes and spun down and washed several times with saline. The sediment was finally suspended in formol saline and stored in the refrigerator.

The use of saline for washing stromata is attended with the loss of some nitrogenous compound, presumably protein. This loss is readily apparent in progressively smaller yields of stromata as the washing procedure is continued. This point will be discussed in a following section of the paper but is brought out here to indicate the necessity of developing a technic in which distilled water can be effectively utilized for the preparation of satisfactory antigenic material for quantitative work. Such a procedure is outlined in detail in Figure I.

Quantitative Studies: The general procedure for the quantitative hemagglutinin determinations carried out in this investigation has been adapted from Heidelberger and Treffers (1942), and consisted in adding accurately measured amounts of stromata suspensions to conveniently and accurately measured volumes of antisera. The mixtures were incubated at 37 C with frequent agitation two hours after which they were placed at 4 C for 18 to 24 hours. Tubes were then centrifuged at 4 C at 2400 rpm for 45 minutes and the precipitates were washed three times with 0.85 per cent saline in the cold. Supernates and washings were recentrifuged and any deposits left were combined with the main precipitates. Control tubes of serum alone
and stromata alone were also run. All tubes were set up in duplicate unless otherwise stated. Precipitates were analyzed for nitrogen by the Kjeldahl method as outlined by Kabat and Mayer (1948), and all results are expressed as milligrams of antibody N. Separate nitrogen determinations were carried out on the supernates and washings of stromata blanks to obtain an estimate of the magnitude of losses due to solubility or mechanical removal of material in washing. Antibody N was determined as the difference between total N precipitated in antisera and the stroma N deposited in blank tubes after three centrifugations and washings.

**Antisera for Quantitative Studies:** Immediately before use in quantitative tests all antisera were centrifuged at 2500 rpm for one hour at 4°C in an International refrigerated centrifuge. Any particles of lipid at the top of the tube were carefully aspirated and the clear serum was carefully decanted from any sediment. Control tubes of serum and saline were set up with each analysis and any blank value obtained was deducted from the total agglutinin N.

**Determination of Stromata Solubility:** Solubility of stromata preparations was determined in 0.85 per cent saline and in normal rabbit serum buffered at pH 5.0 to 7.4. One milliliter of stroma suspension was added to one milliliter portions of normal rabbit serum adjusted to
various hydrogen ion concentrations by addition of HCl and diluted with two milliliters of phosphate buffered saline. Stroma blanks were likewise set up in buffered saline alone. The tubes were incubated at 37 C for two hours followed by eighteen hours at 4 C. Tubes were then centrifuged and the sediments were washed and analyzed for N as previously described.

**Determination of Combining Proportions - AbN;Stroma N:**

In order to determine an optimum amount of antigen for successive quantitative absorption studies, amounts of stromata varying in N content from slightly less than 0.2 mg to 1.5 mg were added to one milliliter portions of rabbit antiserum diluted with two milliliters of phosphate buffered saline. Blanks were run on one milliliter of serum alone, three milliliters of saline alone and varying amounts of stromata in saline. Tubes were incubated at 37 C for two hours followed by eighteen hours at 4 C after which they were centrifuged in the cold. The precipitates were washed and analyzed for N as previously described.

**Determination of Total Antibody N:** Triplicate portions of one milliliter of stromata suspensions were added to one milliliter portions of antiserum diluted with two milliliters of phosphate buffered saline. Incubation and N analysis were carried out as before and the main supernates of each serum tube were saved and absorbed a second time with one milliliter portions of stromata set up with new
blanks. In some cases a third absorption was attempted.

Preliminary studies concerning the suitability of stroma preparations for use in human antisera have been carried out. This preliminary work has centered about the effect of nonspecific absorption of serum proteins to trypsin treated stromata.

One volume of a two per cent suspension of trypsin-treated red cells was added to two volumes of normal human serum. The mixture was incubated at 37 C for two hours. Tubes were centrifuged and the cells were washed three times with 0.85 per cent saline and suspended in a volume of saline to make up a two per cent suspension. Two drops of this suspension were added to 0.2 ml of doubling dilutions of rabbit anti-human serum and also to rabbit anti-human globulin.
1000 cc whole blood

(1) centrifuge - remove plasma and white cell layer

(2) wash red cells 3 times with 0.9 per cent saline

(3) add packed cells to 40 volumes of cold distilled water

(4) allow to stand 1 to 6 hours - adjust pH to 5.0 to 5.5 by addition of HCl or saturation with CO₂ - stromata settle spontaneously

(5) remove supernate first by suction then centrifugation

(6) resuspend stromata in 10 volumes of cold water - agitate vigorously (mechanical stirrer) for two hours at pH 4.0 to 4.5 - adjust pH to 5.5 and remove supernate

repeat (6) until hemoglobin is no longer detected in washings

(7) resuspend stromata in saline containing 0.4 per cent formalin and store in the refrigerator (4 to 8 C.)

(8) wash preparation immediately before use and suspend uniformly in formol saline by use of a mechanical stirrer

Figure I. Preparation of human red cell stromata for quantitative hemagglutination studies.
EXPERIMENTAL RESULTS

The pooled sera of rabbits immunized with normal human red blood cells and pooled sera from animals immunized with trypsin treated red blood cells were examined for hemagglutinins to both types of cells before and after homologous and heterologous absorption. Results of this study are presented in Table I. Unabsorbed sera showed little specificity for homologous cells, the titers for trypsinized cells being somewhat higher with either type of antiserum.

It was concluded that if an antigenic difference between normal and trypsinized red blood cells existed, the specific antibody was masked by antibody to both types of cells. In an attempt to demonstrate antibody specific for trypsinized cells anti Trbc and anti Nrbc were absorbed with the homologous and heterologous cells and the absorbed sera were titrated with each type of cell.

Extensive trials indicated that complete absorption of undiluted serum of either type was very difficult even when homologous antigen was used. It was finally determined that maximum absorption occurred at room temperature (22 to 25 C.) while at either 37 C. or 4 C. the process was considerably less effective. If the sera were diluted twenty times in saline before absorption, antibody removal was markedly enhanced. Results of these experiments are
shown in Table Ia.

Data on the absorption of anti Trbc serum also presented in Table I indicate that maximal removal of antibody was obtained at room temperature by five to seven absorptions with fresh cells. When homologous cells were used in this manner, practically all antibodies for both kinds of cells were removed. On the other hand similar treatment with normal cells reduced the titer for these cells to a trace in undiluted serum, while the titer for trypsinized cells dropped very little. These facts would indicate that a specific antibody for trypsin treated cells was present in this serum.

It can also be seen from Table I that antisera to normal red blood cells were not completely absorbed by five additions of either homologous or heterologous cells. Further additions of trypsinized cells in an attempt to separate antibodies only for normal cells resulted in a reduction of titers for both kinds of cells. However, a specific antibody for trypsinized cells was obtained from the anti Nrbc by first diluting the serum to 1:20 and absorbing with normal cells. The specificity of this fraction was similar to the anti T described above showing only trace agglutination of normal cells at 1:20 but still leaving a titer of 1:320 for trypsinized cells.

Although data obtained from serologic and absorption studies on these hemagglutinin systems have supplied strong
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evidence for the existence of antigenic differences between normal and trypsinized human erythrocytes little information has been obtained concerning the nature of these differences. Moreover, the differences obtained with absorption of diluted and undiluted antisera, and the patterns of successive homologous or heterologous absorption of either type of antisera suggest the possibility that the antigenic differences may be in part a reflection of the quantitative distribution of various antigens on both types of cells. Furthermore, the extent of true cross reaction between any antigens present exclusively on one cell and antigens common to both types of cells must be considered.

Hemagglutination titers obtained by the classical method of serum dilution are at best subject to an error of a factor of two. This means, in effect, that there is a possibility of removing seventy-five per cent of the total antibody in a pure system without showing any significant change in the serum dilution titer, and greater errors may be encountered in complex systems. Moreover, the classical methods do not always permit the detection of small differences in cells possessing similar antigenic mosaics. Consequently, it has become desirable to carry out quantitative determinations of antibody nitrogen to serve as a basis for comparison of the antigenic nature of normal and trypsinized red blood cells.
The chief limitation of the quantitative agglutination method is the dependence of the method upon differences in nitrogen. Each value for agglutinin nitrogen involves two sets of nitrogen analyses. Since the nitrogen content of the antigen used is usually comparatively high, large errors will result when the amount of antibody nitrogen removed by a given amount of antigen nitrogen is but a small fraction of the total nitrogen. With intact erythrocytes, the amount of antibody nitrogen removed is so small a fraction of the total erythrocyte nitrogen that the method is impractical. By preparing stromata suspensions and eliminating the immunologically inert hemoglobin nitrogen the method has been used with moderate precision.

The first quantitative determinations carried out in this investigation were based on the work of Heidelberger and Treffers (1942). These workers investigated a system involving sheep erythrocyte stromata and rabbit anti sheep erythrocyte serum. Human erythrocyte stromata were prepared by lysis in distilled water followed by recovery of cell membranes in a Sharples centrifuge (40,000 r.p.m.) and several washings with water and saline. These stromata were investigated for their combining capacity in homologous and heterologous antisera at pH 7.4 and results are presented in Tables II and III and Figures II and III.
It is readily seen from Figure II that the combining ratio in homologous antisera increases with smaller amounts of stroma suspension, approaching a maximum of .18 as an upper limit. If the ratio, antibody N : stromata N, is plotted against the milligrams of stromata N added, a straight line with a negative slope of .05 is obtained. From these data one can derive an equation similar to that found by Heidelberger and Kabat (1937):

\[
\frac{\text{mg. antibody } N}{X} = a - bX
\]  

(1)

where X is equal to the milligrams of stromata N added and substituting the experimentally determined constants:

\[
\text{mg. antibody } N = .18X - .05X^2
\]  

(2)

Values of antibody N calculated from this equation are compared with the experimentally determined values in Table IIa.

The combining ratio of normal stromata in anti Trbc sera obviously do not lend themselves to any simple mathematical analysis. It is interesting to note, however, that when milligrams of antibody N are plotted against milligrams of stromata N added, the points on the graph can be divided into two groups as shown in Figure III.

Although the data presented here indicate that a suitable concentration of stromata could be selected for consecutive absorptions, examination of supernates and saline washings of stromata controls showed the presence of significant quantities of N (as high as .1 mg in
controls containing the highest amount of antigen). Since there is no way of knowing if the same amount of N is lost in antiserum, the data become unreliable for precise studies. This does not, in effect, invalidate the analysis of the course of hemagglutination as a function of the amount of stromata suspension added. Such analysis is based on relative values and its validity depends largely on the precision of analysis rather than on extreme accuracy.

Attempts have been made to prepare stromata which would be more satisfactory for quantitative work. Liquid N, distilled water, and mechanical disintegration using a Waring Blender have been employed to effect initial lysis of normal red cells and cells previously treated with trypsin. With all methods the initial product consisted of a paste like substance containing considerable quantities of hemoglobin. Microscopic examination of the sediments obtained with water or liquid N showed them to be composed of intact membranes whereas the sediment from the Waring Blender consisted almost exclusively of cell fragments. Also, stromata prepared with distilled water or liquid N show little change in volume as compared to the volume of packed cells from which they were prepared while the sediment obtained by mechanical treatment is equal in packed volume to approximately ten per cent of the starting material.
Stromata prepared by the various methods were subjected to washing with .85 per cent saline and this procedure resulted in considerable fragmentation of membranes, some fragments becoming so small as to present the appearance of a colloidal sol. Centrifugation following the addition of saline resulted in the separation of the stroma material into two fractions. One of these fractions consisted of relatively large particles and in the case of stromata from trypsinized cells this fraction showed pronounced agglutination in human anti Rh serum containing blocking antibody only. The second fraction consisted of much smaller particles, some of which were apparently in solution. However, despite the fact that saline preparations of this fraction showed definite turbidity, repeated centrifugation produced no sediment, nor were such fractions from trypsinized cells flocculated by the anti Rh serum. These results are consistent with those of Howe (1951) who demonstrated that the fraction lost in saline is poor in virus and isoagglutinogen receptors.

Since it is desirable to use both stroma fractions in the analysis of sera from rabbits immunized with intact erythrocytes, the use of saline as a washing medium became prohibitive, and subsequent washing was carried out with distilled water. It appeared, therefore that there was no advantage to any special treatment
prior to exposure to distilled water and this latter medium was adopted as a standard material for purposes of initial lysis. Several attempts to purify the cell membranes by alternate dispersion and re-sedimentation in water demonstrated that unless the hemolysates were adjusted to pH 5.0 to 5.5, there was a loss of some protein fraction of the stroma. Consequently the stroma suspensions were repeatedly dispersed in water at pH 7.0 to 8.0 and precipitated at pH 5.0 to 5.5. This procedure led to the loss of approximately 40 percent of the original nitrogen and little hemoglobin could be detected in washings after the second reprecipitation despite the fact that the stromata were obviously heavily contaminated with hemoglobin. Since previous observations in this investigation had revealed that dispersion occurred on the acid side of pH 5.5 attempts were made to utilize a lower pH for washing. The pH of freshly hemolyzed cells was brought to 4.0 by the addition of HCl and the suspension was vigorously agitated for two hours after which time stromata were flocculated by bringing the pH to 5.5. After repeating this process five times the product obtained was pale brown in color and consisted of a mixture of intact membranes and finely divided fragments. It has been found more recently that saturation of hemolystes with CO₂ reduces the pH to 4.0 to 4.5 and that on standing the CO₂ concentration is
spontaneously reduced reaching an equilibrium at 4 C. at which time the pH remains at approximately 5.5. Thus saturation with CO₂ may serve a dual purpose; reduction of the pH to 4.0 for dispersion of stromata and removal of hemoglobin, and reajustment of the pH to 5.5 for reprecipitation. Details of this procedure are outlined in Figure I.

In order to determine the pH range in which stromata prepared by this latter method could be safely employed for quantitative studies, the solubilities of preparations were determined in .85 per cent saline and in normal rabbit serum buffered at various hydrogen ion concentrations from pH 5.0 to 7.4. Saline solubility was determined by direct analysis of supernates and washings. Serum solubility was determined as the difference in total N added and N recovered in washed sediments. The results of these solubilities are presented in Figure IV. Serum solubility is generally lower and this is very likely attributable to the presence of naturally occurring hemagglutinins in normal rabbit serum. It is interesting to note that these differences are greater as solubility increases. This may be taken as evidence that even slight amounts of antibody tend to bind the stromata, thus magnifying the effect of the normal hemagglutinins at higher solubilities. This is important in considering the use of stroma blanks
as bases for antigen N added. From Figure IV it is obvious that pH 5.5 is the optimum point for quantitative determinations for not only is the solubility at a minimum but the values obtained in saline and serum are practically identical. Consequently hemagglutination titers of both rabbit antisera were redetermined at this pH and these titers did not differ significantly from those obtained at pH 7.4.

Quantitative hemagglutinin determinations were carried out at pH 5.5 involving homologous and heterologous absorption of both antisera. Triplicate portions of 1 ml. of stromata suspensions were mixed with 1 ml. portions of serum diluted with 2 ml. of phosphate buffered saline. Incubation and N analysis were carried out as previously described and the main supernates of each serum tube were recovered and mixed with a second portion of stromata suspension. Results are shown in Table IV. Attempts to carry out further quantitative absorptions on the supernates were discontinued since the amounts of antibody picked up by a third portion of stromata were so small as to fall within the limits of experimental error.

The results shown in Table IV parallel, in many ways, those obtained in routine absorption studies. The results of triplicate analyses are included to indicate the relatively high degree of precision which can be
obtained with this method. Complete removal of antibody is extremely difficult especially in anti Nrbc sera. Practically all antibody in anti Trbc sera was removed with two homologous absorptions of approximately .5 mg of stroma N, and although less antibody was removed from this same sera with stromata from normal cells, there was apparently little antibody for this type of cell remaining in the final supernate.

Preliminary studies have been carried out to determine the suitability of stromata preparations for quantitative determination of incomplete hemagglutinins in human serum. Although the trypsinization procedure used in this investigation does not induce panagglutinability in erythrocytes, little is known concerning the effect of normal human serum on treated cells. The suggestion by Weiner (1951) that trypsin may remove from the red cell some component found also in the serum introduces the possibility that trypsinization may be, at least in part, reversible. If this is true then the measurement of antibody N would be complicated by the addition of a non-antibody protein to the treated cell stromata.

Furthermore, Rosenthal (1950) had reported the presence in normal serum of an agglutinin like component for trypsinized cells. He found that if treated cells were added to fresh serum (cells and serum from same
individual) and spun down immediately, there was marked agglutination. The cells became dispersed on standing and their agglutinability was gradually lost over a period of one hour. Agglutination of this type was never observed to take place spontaneously and if, after one hour, the cells and serum were separated, and retested with fresh materials, it was found that the cells had lost their agglutinability in all normal sera whereas the serum would readily agglutinate fresh cells.

The technic of Rosenthal was used to examine a number of normal human sera. Four of sixteen samples of sera tested reacted as indicated in Table V.

Trypsinized cells which had been exposed to the action of the normal sera in the above tests were examined for non specific uptake of serum protein by removing them from the sera after two hours incubation at room temperature, washing them three times with saline, and mixing them with serum from rabbits immunized with human serum. As indicated in Table VI, this procedure resulted in marked agglutination of the cells even when the rabbit antiserum was diluted 1:2000. If similarly treated cells were added to sera from rabbits immunized with human globulin there was either no agglutination or a trace agglutination. These results indicate that the trypsinized cell picks up some serum component not associated with gamma globulin
INTRODUCTION

The classical methods of serology, the serum dilution titer, the optimal proportions method, the optimal flocculation method, turbidity measurements, and the determination of the volume of specific precipitate, give results in relative terms rather than in absolute units and do not meet the requirements of quantitative analytical chemistry. Consequently, while these methods are quite useful where quantitative answers are not required, the results obtained with them do not permit the mathematical treatment essential for kinetic studies or for the precise determination of the extent of serological relationship between chemically or biologically related antigens. Moreover, immunologists have long been aware of the failure to correlate the results of serologic tests with the extent of the biological activity of the antibody in question. The development of quantitative methods based on the determination of the amount of specific precipitate either by direct weighing or by chemical analysis has removed considerably these limitations and it is now possible to measure small amounts of serologically active substances with a degree of accuracy as great as that of many standard chemical analyses.
and it appears that the quantitative as well as qualitative aspects of this reaction must be elaborated before attempts are made to use stromata of trypsin treated cells for quantitative determinations of hemagglutinins.
<table>
<thead>
<tr>
<th>Antisera</th>
<th>Absorbed with</th>
<th>Titers against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N cells</td>
<td>T cells</td>
</tr>
<tr>
<td>Antib</td>
<td>Unabsorbed</td>
<td>1:1000</td>
</tr>
<tr>
<td>Trbc</td>
<td>Trbc 5X</td>
<td>1:2</td>
</tr>
<tr>
<td></td>
<td>Nrbc 7X</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Unabsorbed</td>
<td>1:2000</td>
</tr>
<tr>
<td>Antib</td>
<td>Trbc 5X</td>
<td>1:1000</td>
</tr>
<tr>
<td>Nrbc</td>
<td>Nrbc 5X</td>
<td>1:32</td>
</tr>
</tbody>
</table>

Table I. Hemagglutination titers of rabbit anti-Trbc with both types of cells before and after homologous and heterologous absorption at room temperature.
<table>
<thead>
<tr>
<th>Temp.</th>
<th>Antisera</th>
<th>Absorbed with</th>
<th>Titers against N cells</th>
<th>Titers against T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-Trbc</td>
<td>Nrbc</td>
<td>1:4</td>
<td>1:256</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trbc</td>
<td>1:16</td>
<td>1:64</td>
</tr>
<tr>
<td>37 C</td>
<td>Anti-Nrbc</td>
<td>Nrbc</td>
<td>1:256</td>
<td>1:256</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trbc</td>
<td>1:512</td>
<td>1:256</td>
</tr>
<tr>
<td>4C</td>
<td>Anti-Trbc</td>
<td>Nrbc</td>
<td>1:512</td>
<td>1:1024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trbc</td>
<td>1:32</td>
<td>1:32</td>
</tr>
<tr>
<td></td>
<td>Anti-Nrbc</td>
<td>Nrbc</td>
<td>1:512</td>
<td>1:512</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trbc</td>
<td>1:64</td>
<td>1:256</td>
</tr>
<tr>
<td>Room T</td>
<td>Anti-Trbc</td>
<td>Nrbc</td>
<td>-</td>
<td>1:512</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trbc</td>
<td>1:2</td>
<td>1:8</td>
</tr>
<tr>
<td></td>
<td>Anti-Nrbc</td>
<td>Nrbc</td>
<td>1:32</td>
<td>1:64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trbc</td>
<td>1:1024</td>
<td>1:128</td>
</tr>
</tbody>
</table>

Table Ia. Hemagglutination titers of anti-Trbc sera and anti-Nrbc sera after homologous and heterologous absorption at 37 C, 4 C and room temperature.
<table>
<thead>
<tr>
<th>Ströma N added</th>
<th>Total N pptd.</th>
<th>Antibody N by difference</th>
<th>Ratio antibody N: stroma N</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td></td>
</tr>
<tr>
<td>.168</td>
<td>.197</td>
<td>.029</td>
<td>.173</td>
</tr>
<tr>
<td>.317</td>
<td>.360</td>
<td>.043</td>
<td>.136</td>
</tr>
<tr>
<td>.485</td>
<td>.561</td>
<td>.076</td>
<td>.157</td>
</tr>
<tr>
<td>.802</td>
<td>.915</td>
<td>.113</td>
<td>.141</td>
</tr>
<tr>
<td>.951</td>
<td>1.077</td>
<td>.126</td>
<td>.133</td>
</tr>
<tr>
<td>1.119</td>
<td>1.267</td>
<td>.146</td>
<td>.130</td>
</tr>
<tr>
<td>1.268</td>
<td>1.423</td>
<td>.155</td>
<td>.123</td>
</tr>
<tr>
<td>1.436</td>
<td>1.593</td>
<td>.157</td>
<td>.109</td>
</tr>
<tr>
<td>serum salt</td>
<td>.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table II. Quantitative determination of the combining ratios of antibody N and stromata N using anti-Nrbc and normal stromata. pH 7.4
Figure II
<table>
<thead>
<tr>
<th>Stroma N added</th>
<th>Total N pptd.</th>
<th>Antibody N by difference</th>
<th>Ab N calculated equation</th>
<th>Difference in AbN values</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>.168</td>
<td>.197</td>
<td>.029</td>
<td>.029</td>
<td>.000</td>
</tr>
<tr>
<td>.317</td>
<td>.360</td>
<td>.043</td>
<td>.052</td>
<td>.009</td>
</tr>
<tr>
<td>.485</td>
<td>.581</td>
<td>.076</td>
<td>.075</td>
<td>.001</td>
</tr>
<tr>
<td>.802</td>
<td>.915</td>
<td>.113</td>
<td>.112</td>
<td>.001</td>
</tr>
<tr>
<td>1.077</td>
<td>1.077</td>
<td>.126</td>
<td>.126</td>
<td>.000</td>
</tr>
<tr>
<td>1.119</td>
<td>1.267</td>
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<td>.006</td>
</tr>
<tr>
<td>1.368</td>
<td>1.423</td>
<td>.155</td>
<td>.148</td>
<td>.007</td>
</tr>
<tr>
<td>1.436</td>
<td>1.593</td>
<td>.157</td>
<td>.156</td>
<td>.001</td>
</tr>
<tr>
<td>serum saline</td>
<td>.001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table IIa. Comparison of antibody N values for normal stromata in anti-Nrbc sera found experimentally and calculated by use of equation (2).
Table III. Quantitative determination of the combining ratios of antibody N and stromata N using anti-Trbc and normal stromata. pH 7.4

<table>
<thead>
<tr>
<th>Stroma N added</th>
<th>Total N pptd.</th>
<th>Antibody N by difference</th>
<th>Ratio antibody N: stroma N</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>mg</td>
<td>mg</td>
<td></td>
</tr>
<tr>
<td>.241</td>
<td>.287</td>
<td>.046</td>
<td>.191</td>
</tr>
<tr>
<td>.482</td>
<td>.539</td>
<td>.057</td>
<td>.119</td>
</tr>
<tr>
<td>.723</td>
<td>.832</td>
<td>.109</td>
<td>.151</td>
</tr>
<tr>
<td>.964</td>
<td>1.097</td>
<td>.133</td>
<td>.138</td>
</tr>
<tr>
<td>1.205</td>
<td>1.416</td>
<td>.211</td>
<td>.175</td>
</tr>
<tr>
<td>1.446</td>
<td>1.675</td>
<td>.229</td>
<td>.158</td>
</tr>
<tr>
<td>serum saline</td>
<td>.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure IV
<table>
<thead>
<tr>
<th>Absorption</th>
<th>Stroma N</th>
<th>Total N pptd.</th>
<th>Antibody N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>1st</td>
<td>0.562</td>
<td>0.698</td>
<td>0.137</td>
</tr>
<tr>
<td></td>
<td>0.556</td>
<td>0.690</td>
<td>0.702</td>
</tr>
<tr>
<td>Treated cells vs Anti-Trbc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td>0.532</td>
<td>0.600</td>
<td>0.071</td>
</tr>
<tr>
<td></td>
<td>0.528</td>
<td>0.602</td>
<td>0.600</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>0.208</td>
</tr>
<tr>
<td></td>
<td>0.563</td>
<td>0.576</td>
<td>0.022</td>
</tr>
<tr>
<td>1st</td>
<td>0.555</td>
<td>0.588</td>
<td>0.580</td>
</tr>
<tr>
<td>Treated cells vs Anti-Nrbc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td>0.532</td>
<td>0.530</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>0.528</td>
<td>0.536</td>
<td>0.532</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>0.498</td>
<td>0.544</td>
<td>0.038</td>
</tr>
<tr>
<td>1st</td>
<td>0.504</td>
<td>0.538</td>
<td>0.538</td>
</tr>
<tr>
<td>Normal cells vs Anti-Nrbc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td>0.424</td>
<td></td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>0.430</td>
<td>0.476</td>
<td>0.476</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>0.087</td>
</tr>
</tbody>
</table>

* Sample lost in transfer to Kjeldahl apparatus.
The quantitative technique first elaborated and perfected by Heidelberger and Kendall (1929, 1933, 1936) has been widely employed in the investigation of precipitin systems involving relatively pure antigens e.g. ovalbumin, pneumococcus type specific polysaccharides, and artificial conjugates such as azo-proteins. More recently the method has been extended to systems involving cellular antigens, and the high degree of accuracy obtained in the measurement of agglutinins has served as a basis for the recognition of antigenic differences in bacteria not previously distinguishable by serologic technics (Kabat and Mayer, 1948). Such results emphasize the necessity of a more extensive application of quantitative technics especially to systems where small antigenic differences may be related to important biological activities of cells.

An excellent example of this latter situation arises from the fact that human red blood cells properly treated with trypsin exhibit striking serologic properties not possessed by untreated cells. The ability of such treated cells to agglutinate directly in saline dilutions of incomplete antibody is of practical significance in the diagnosis of acquired hemolytic anemia, and may bear some relationship to the etiology of this disorder. However, it is apparent from a study of the literature that the changes produced on red cells by the action of trypsin
<table>
<thead>
<tr>
<th>Absorption</th>
<th>Stroma N</th>
<th>Total N pptd.</th>
<th>Antibody N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>.498</td>
<td>.596</td>
<td>.100</td>
</tr>
<tr>
<td></td>
<td>.504</td>
<td>.594</td>
<td>.612</td>
</tr>
</tbody>
</table>

**Normal cells vs Anti-Trbc**

<table>
<thead>
<tr>
<th></th>
<th>2nd</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>.424</td>
<td>.506</td>
<td>.074</td>
</tr>
<tr>
<td></td>
<td>.430</td>
<td>.498</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>.502</td>
<td></td>
</tr>
</tbody>
</table>

**Total**

|          |          |               | .174       |

Table IV. Estimation of total antibody N removed by normal and trypsinized cells from anti-Trbc sera and anti-Nrbc sera. PH 5.5
<table>
<thead>
<tr>
<th>Time after mixing</th>
<th>Serum dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1</td>
</tr>
<tr>
<td>Minutes</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>++++</td>
</tr>
<tr>
<td>5</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>++</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>75</td>
<td>-</td>
</tr>
</tbody>
</table>

Table V. Agglutination of trypsin treated erythrocytes with normal serum dilutions recentrifuged at stated intervals
<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Antigen</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nrbc</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit anti-human globulin</td>
<td>Trbc</td>
<td>1:64</td>
</tr>
<tr>
<td></td>
<td>Trbc *sensitized with normal human serum</td>
<td>1:128</td>
</tr>
<tr>
<td></td>
<td>Nrbc</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit anti-human serum</td>
<td>Trbc</td>
<td>1:128</td>
</tr>
<tr>
<td></td>
<td>Trbc *sensitized with normal human serum</td>
<td>1:4096</td>
</tr>
</tbody>
</table>

Table VI. Effect of normal human serum on trypsin treated human red blood cells.
DISCUSSION

Prior to the elaboration of precise quantitative immunochemical technics there was little definitive knowledge of the mechanism of serologic reactions or of the quantitative relationship existing between the intensity of the serologic reactions as described by the classic technics and the extent of in vivo biological activity of antibody. Moreover, because the endpoint of the serologic reactions involved a subjective measurement, there was no possibility of standardizing technics or of setting up criteria for comparison of different immunologic systems. There was, in fact, some question as to precisely what one measured by many of the earlier methods of antibody titration. Accordingly it was impossible to carry out any thorough investigation of the peculiar immunological changes which are known to accompany certain minor physical and chemical modifications of naturally occurring antigens.

The development of analytical methods in serology has provided a technic for measuring antibody which meets the requirements of quantitative analytical chemistry, and overcomes, in large measure, the objections found in the classical methods. However, most quantitative work has been restricted to studies on simple systems involving relatively pure antigens e.g. ovalbumin - anti-ovalbumin, pneumococcal capsular polysaccharide -
anti-pneumococcal polysaccharide sera. The investigation of these simpler immunologic systems has clearly established the value of quantitative technics, and there remains now the task of extending the application of such technics to the more complex systems which are so often implicated in disease processes.

The use of quantitative methods in hemagglutination studies has been limited by the absence of a suitable antigen. The results of the present investigation indicate that a suitable antigenic preparation is now available, but before discussing the value of such preparation it would be well to examine the data obtained by serum dilution titer and absorption studies in order to demonstrate the value of the use of combined methods.

The results of hemagglutination titrations presented in Table I offer strong evidence for the existence of antigenic differences between normal and trypsin treated human red blood cells. That such differences may exist is not surprising in view of studies involving protein molecular or cellular antigens which have been modified by mild denaturation, formalization, or conjugation with prosthetic groups. Such modified molecules or cells often exhibit striking immunologic differences from the native materials. Moreover, enzymic treatment of a complex chemical structure such as is involved in the mammalian erythrocyte introduces the possibility not only for the
modification of antigens but also for the destruction of surface antigens with the consequent uncovering of deeper lying chemical structures capable of serologic reaction. The important issue here is the relationship between the modified antigenic structure of the trypsinized cell and its ability to agglutinate directly in saline dilutions of incomplete antibody. It is significant that in sera from rabbits immunized with normal red blood cells one can, by proper methods of dilution and absorption, obtain a serum fraction showing only trace agglutination for homologous cells but relatively strong reactivity for trypsinized cells. It may be inferred from these results that the \textit{in vivo} destruction of the foreign erythrocytes creates conditions whereby the antibody forming mechanism is stimulated by antigenic groupings corresponding to those which are made manifest by \textit{in vitro} trypsinization. In this connection it is appropriate to recall that practically all normal human sera contain agglutinins for homologous red cells properly treated with certain proteolytic enzymes.

Extensive absorption of the anti-Nrbc sera with heterologous cells produces a serum fraction showing only slight reactivity for trypsinized cells accompanied by mild reduction of the titer for normal cells. These data suggest that some antigen(s) present on the normal
erythrocyte are removed by trypsin. This idea is reinforced by failure to demonstrate in anti-Trbc any similar specificity for normal cells.

The overall results of absorption studies justify the following simple analysis of the immune response to normal and modified cells. There is, on first inspection, the possibility of three general types of antibody involved: (a) antibody specific for antigens common to both types of cells (b) antibody specific for antigens uncovered or modified by trypsinization (c) antibody specific for antigen(s) present on the normal cell but not on the treated cell. Obviously anti-Trbc would contain only types (a) and (b) whereas anti-Nrbc could conceivably contain all three types of antibody. Such analysis implies that antibody specific for antigens uncovered or modified by trypsin would react only with treated cells. This concept is opposed to the results obtained with undiluted anti-Nrbc sera after homologous absorption. Here the titers for the two types of cells are approximately equal, a factor which necessitates a modification of the concept of correspondence between in vitro enzymation and in vivo destruction of the normal human cell in rabbits. In the former process the enzymic treatment is allowed to proceed to some arbitrary point before the cell reaches the antibody forming apparatus whereas in the latter case the degradation of the cell is
very likely carried out in the immediate vicinity of
the site of antibody formation thus establishing the
possibility of the formation of antibodies with dual
specificities. Such dual specificity is found in the
case of immunization with proteins containing prosthetic
groups; antibodies are found whose specificity is
directed simultaneously to protein groupings and
haptene groups. In the present situation the dual
specificity would involve the antigenic groups on the
surface of the red cell and the modified or uncovered
groupings produced by trypsin. It may appear from this
discussion that as the process of degradation of cellu-
lar antigens continues, a large variety of specificities
would be established. Actually, however, the degradation
may involve only a few discrete steps. It is interesting
to note here that in vitro trypsinization leads first
to agglutinability by incomplete antibody and then to
agglutinability by so called "T" agglutinins. This would
support the idea of at least two well defined steps in
the process of cell digestion. The same steps can be
observed in cells treated by other procedures, e.g.
treatment with periodate ions, Vibrio cholerae filtrates,
or papain. Animals immunized with cells subjected to
various methods of treatment produce antibodies specific
for cells treated by the same process as the immunizing
antigen. Again this can be attributed to arbitrary
termination of the various procedures. Sera from animals immunized with normal untreated cells show practically identical reactivity for all types of treated cells, titers for all types being somewhat higher than for the homologous cells. This may be taken as evidence that in vivo modification takes place in discrete steps resulting in the production of antibodies with dual specificity, part of the reactive site being directed toward chemical groupings exposed by a variety of digestion procedures with each procedure accompanied by some other unique change. (Data on comparison of periodate treated cells and vibrio treated cells may be found in a report by Moskowitz and Treffers (1950). Studies on comparison of trypsin treated cells and periodate treated cells have been carried out by Dodd and Baxter (1952).

It becomes apparent at this point that the data obtained by serum dilution titers and routine absorption studies provide evidence for the existence of antigenic differences between treated and untreated cells but the nature of these differences is largely a matter of conjecture and the desirability of precise antibody measurements becomes obvious.

The results of quantitative studies presented here show clearly that limitations are imposed largely by the nature of the antigen which must be used. Erythrocyte
stromata give relatively low antibody M: antigen N ratios and are soluble throughout the pH range in which antibody measurements may be safely carried out. The presence of large quantities of serologically inert nitrogen containing compounds in red cell stromata is undoubtedly responsible for the low combining ratio. A certain percent of such compounds is represented by residual hemoglobin. Failure to remove hemoglobin completely from stromata is probably significant in considering the relationship between hemoglobin and the membrane ultra structure in the intact erythrocyte, but since little is known about this relationship no logical procedure is suggested for complete separation of stromata and hemoglobin. The separation of other non-reactive nitrogenous compounds is at present unfeasible since this would involve an extensive fractionation of the stromata. Data for trypsinized stromata in Table IV show a much higher ratio in homologous antisera than in heterologous antisera, while normal stromata show greater combining proportions in the heterologous or anti Trbc sera. Such data serve to emphasize the enormity of the problem of separation of serologically active and non-reactive stroma constituents; for reactivity is apparently a function of the type of antisera as well as the innate structure of various constituents. It should be noted that the use of a low pH in washing stromata has
or certain other proteolytic enzymes, in the concentrations used, involve a relatively minor physical modification of the cell structure, and it is extremely unlikely that any profound chemical alteration of the cell ultrastructure is produced. The problem is, therefore, relegated to the field of serology and since the classical methods of serology have yielded little definitive information concerning the modified activity of enzyme treated cells, it seems appropriate that the antigenic nature of these cells be investigated by the application of quantitative immunochemical technics.

Studies of this type have been delayed by the lack of a suitable antigenic preparation for quantitative determination of hemagglutinins for human erythrocytes. Such preparation would be valuable for a number of immunologic studies on erythrocytes and would aid directly in the elaboration of the nature of the red cell ultrastructure. In the course of the present investigation a procedure has been devised for the preparation of normal and trypsinized human red blood cell stromata which can be successfully used for precise measurements of hemagglutinin, and the experimental portion of this paper deals with the development of this technic and presents a comparison of data obtained with quantitative technics and routine serologic methods.
resulted in a product which is considerably less contaminated with hemoglobin than those by washing at a neutral or alkaline pH.

The phenomenon of stromata solubility offers the advantage of a crude fractionation of stromata but as pointed out previously it has been essential in this work to utilize all of the erythrocyte constituents at least in early studies. Consequently solubility studies were carried out as indicated in Figure III. The recovery of N in both saline and serum shows a definite minimum at pH 5.5, and in addition the solubility is practically identical in the two media at this pH. As solubility increases on moving from this pH, there is a marked difference in the values obtained in saline and serum, solubility being lower in the latter medium. This is apparently due to the presence of normal hemagglutinins which tend to bind the soluble or non-sedimentable fractions into the main sediment. Solubility in serum is fairly constant in the higher pH range indicating that a certain portion of the stromata suspension cannot be influenced by the normal hemagglutinins. In regard to these normal antibodies it should be pointed out that in the rabbit sera used in these solubility studies the total hemagglutinin content was apparently less than .005 mg N per cc as indicated by results at pH 5.5. Evidently even very small amounts of antibody are capable
of binding certain stromata constituents not otherwise brought into the sediment produced by centrifugation. This is significant since normal hemagglutinins are undoubtedly present in the rabbit antisera used throughout this investigation. The fact that a relatively large portion of the non-sedimentable fraction of stromata remain in suspension even in the presence of antibody offers some justification for the use of stromata blanks as a basis for antigen N added in the higher pH range.

The results of experiments set up to determine the combining proportions of stromata and antibody (Tables II and III and Figures II and III) serve also as a means of following the course of hemagglutination as a function of the amount of antigen added. With the homologous system, anti-Nrbc and normal stromata, an empirical equation has been derived and with the exception of the second value given in Table IIa there is very good agreement with experimental data. This single point of disagreement is not easily explained because of the complexity of the system. However it is apparent that the course of homologous hemagglutination is similar to that found by Heidelberger and Kabat (1937) for pneumococcus suspensions and antibody specific for the capsular polysaccharide. Furthermore, the estimations of total antibody in anti-Nrbc sera (Table IV) show
.067 mg of antibody N for homologous cells and only .025 mg for heterologous cells. The difference between these two values, .062 mg is evidently the amount of antibody N specific for normal cells. This means that over seventy per cent of the antibody picked up by normal cells in anti-Nrbc sera is specific for antigens susceptible to destruction or modification by trypsin, and that the antibody in anti-Nrbc sera specific for treated cells has a nitrogen value of less than .025 mg since part of this latter value represents antibody N specific for antigens common to both types of cells. It is significant that in the estimation of total antibody N for homologous cells in anti-Nrbc sera the value of antibody N for the second absorption is higher than that obtained in the first absorption. The value for the first absorption, .038 mg, would lie, on the curve in Figure II, in the region which does not conform well with the equation set forth.

If, now, we consider that the first portion of the course of hemagglutination in the normal cell homologous system involves the simultaneous occurrence of at least three reactions i.e. reactions due to the three types of antibody postulated earlier in the discussion, it appears that in the region of extreme antibody excess there is a preferential absorption of the antibody which is directed to partially degraded cells. This interference with
absorption of normal cell antibodies is absent in the region of higher antigen concentrations as judged by the character of the reaction curve in Figure II. This idea is supported by the observation that in order to prepare from anti-Nrbc sera a fraction specific for trypsinized cells, the antisera must first be diluted to permit absorption in the region of lower antibody concentration. In regard to the latter phenomenon it is important to consider that while the antibody specific for partially digested cells appears to be preferentially absorbed in high antibody concentration by normal cells, it does not agglutinate these cells when obtained in a partially purified fraction. This supports the contention that this antibody acts as an incomplete type for normal cells.

Although it is difficult to formulate any explanation for this preferential absorption phenomenon at high antibody concentration, it is appropriate to recall that the antibodies found in hemolytic anemia are generally capable of agglutinating only trypsinized cells despite the fact that they are absorbed by normal cells. It may be inferred that the antibody for treated cells in anti-Nrbc sera acts as an incomplete antibody for normal cells. Also considering that the action of enzymes on red cells involves the destruction of one antigen and the simultaneous appearance of a new
antigen, it is very likely that loci of such antigens are extremely close if not overlapping. In any case, if the \textit{in vivo} destruction does involve, as postulated, the appearance of an antibody with a dual specificity, then it is possible that the absorption of such antibody by normal cells would prevent the subsequent absorption of normal cell antibody on the adjacent or overlapping loci, whereas the absorption of normal cell antibodies which have a narrower specificity may not hinder the subsequent absorption of antibodies with dual specificity. In the region of higher antigen concentration there would no longer be any competition for binding sites and the removal of normal cell antibodies would be markedly enhanced. In view of the differences in the absolute amounts of the two types of antibody it is necessary to assume that the number of loci for normal cell antibodies is greater than that for antibody with dual specificity.

The course of heterologous absorption with normal stromata obviously does not conform to those established by other workers for simpler systems and the kinetics of the reaction are far too complex to facilitate any valuable discussion. There appears to be at least two distinct reactions involved but further studies will be necessary to gain an understanding of these. The estimations of total antibody in anti-Trbc sera show that the antibody specific for trypsinized cells is not absorbed by normal
cells. This agrees with the concept that this antibody differs from the antibody obtained from diluted anti-Nrbc after homologous absorption. The latter type antibody more closely resembles that found in hemolytic anemia, since it is capable of not only agglutinating treated cells but also of reacting with normal cells without effecting agglutination.

Estimation of total antibody by successive quantitative absorptions at pH 5.5 is attended with a moderately high degree of precision and removes the objections to the titrations carried out at or near neutrality. Data obtained by this method parallel results of routine absorption studies. Homologous or heterologous absorption of either type of antisera is extremely difficult to carry to completion. This is especially true of anti-Nrbc sera. There is convincing evidence here for the existence of antigenic differences between treated and untreated cells. There is, however, no correlation between the serum dilution titers and the absolute amounts of specific antibodies. A number of factors may be involved here including the possibility of the occurrence of various incomplete antibodies including types which act as agglutinating agents for one type of cells but not for the other type, differences in binding strengths of antibody molecules and effects of stearic hindrance due to proximity of different antigenic loci.

The precision obtained in quantitative studies with
rabbit anti human erythrocyte sera by the use of the above method has prompted an investigation of the applicability of such technics to studies of incomplete hemagglutinins in human sera. Results of preliminary studies have demonstrated the absorption of some serum component in normal serum by trypsinized cells. Apparently this component is not associated with the gamma globulin fraction and its presence on treated cells does not interfere with the agglutination of such cells in the presence of incomplete hemagglutinins. It does, however, cause the addition of nonspecific N to immune aggregates in human hemagglutinating systems involving trypsinized cells. Furthermore, since in some types of hemolytic anemia the hemagglutinins are known to be specific for certain antigens, the use of all stromata components would entail the presence of large amounts of nonreactive N. Consequently it would be necessary to remove a good portion of such nitrogen to prepare a suitable antigenic preparation for quantitative studies. In the case of anti Rh sera the removal of the stromata fraction which is soluble in saline at or above neutrality would represent a first step in the purification of a suitable antigen since in the course of the present investigation it has been shown that this stromata fraction, even from trypsin treated stromata, shows no observable reaction with anti Rh sera. Suitable
preparations for other hemagglutination systems remain to be established.

Despite the fact that quantitative studies have not yet been carried out in human sera, a number of facts from the foregoing discussion may be used to some advantage in explaining the peculiar serologic activity of trypslnized cells in the presence of incomplete hemagglutinins in various hematologic disorders. The literature on the subject of enzyme treated cells contains little discussion on this point. Wheeler (1950) pointed out that excessive trypsinization of cells led to spontaneous agglutination in saline alone and suggested the possibility that mild trypsinization of cells may reduce their suspension stability thus rendering them more susceptible to agglutination than untreated cells. However, trypsin treated cells do not always agglutinate to higher titers than normal cells when tested with sera containing saline agglutinins for normal cells. Pickels (1946) reported that trypsin modified cells showed no increased ability to detect the antibodies against the antigens Lewis, Lutheran and S, and Wheeler (1950) mentioned that certain anti-Rh sera which contained mainly saline agglutinins showed no increased reactivity for treated cells. These data indicate that the increased agglutinability of treated cells is not dependent upon a decreased suspension stability but is
related to the nature of certain types of antibody. In the present investigation it has been shown that there is no direct correlation between the absolute amounts of antibody absorbed by trypsinized stromata from antisera and the serum dilution hemagglutination titers of such sera. It is logical, therefore, to conclude that agglutination of trypsinized cells is not directly associated with the total amount of antibody globulin on them. Wheeler (1950) also reported that treated cells appeared to absorb and elute more antibody than normal cells in anti-Rh sera. These findings were not based on quantitative determinations and in view of the absence of any correlation between quantitative measurements and routine methods of antibody estimation such findings must be viewed with caution.

Weiner (1951) proposed that the agglutination of trypsin treated cells by anti-Rh blocking antibody was in fact conglutination and was associated with the presence of some normal serum component. Data presented here demonstrate that trypsinized cells do absorb some normal serum component which does not interfere with the agglutinability of cells. However, Weiner (1951) also reported that normal Rh positive cells sensitized with Rh blocking antibodies and washed with saline would form agglutinates in saline when mixed with trypsin modified Rh positive cells not previously sensitized.
His theory implies, therefore, that the normal serum component responsible for conglutination is bound by untreated cells in the process of sensitization despite the fact that they do not agglutinate. There is at present no evidence that normal untreated cells absorb any non antibody protein from human serum. (Complement is assumed to be absent from the sera used by Weiner). Moreover trypsinized cells will often agglutinate in human sera diluted several thousand fold. At these dilutions the concentration of all serum components is so low that it is unlikely that any of them could act as true conglutinins. In any case the theory proposed by Weiner does not even consider that the phenomenon is dependent upon the presence of a special type of antibody.

Burnet and Anderson (1947) and Moskowitz and Treffers (1950) suggested the possibility that agglutinins for treated cells may be involved in the pathogenesis of various diseases such as blackwater fever or in intravascular agglutination. The latter workers offered the following hypothesis. "It is possible that agglutinins for altered red cells are implicated in the pathogenesis of various diseases. However as there may be many different agglutinins acting on cells altered by different agents, these agglutinins would not be observed if the sera were tested with cells altered by a single
The fact that samples of human blood cells contaminated with bacteria may be non-specifically agglutinated by human sera of any type has been well known to immunologists for many years. The phenomenon was observed by Thomsen (1926) and shown to be due to a bacterial enzyme by Friedenrich (1928). The serum component responsible, generally known as "T" agglutinin, is present in varying titer in practically all human and many mammalian sera, and can be readily removed by absorption with treated cells. (Burnet and Anderson, 1947). Burnet, McCrae and Stone (1946) and Stone (1947) showed that similar changes are produced by viruses of the mumps-influenza group and that very active bacterial enzyme preparations can be obtained from cultures of Vibrio cholerae. Stone (1947) reported that except for some minor points of detail the action of the V. cholerae agent on the red cell surface closely parallels that of the viruses of the influenza group. Subsequently this bacterial agent has been referred to in the literature as the receptor destroying enzyme.

Burnet and Anderson (1947) demonstrated that human erythrocytes treated with the receptor destroying enzyme of V. cholerae, when used for the immunization of rabbits, provoke the appearance of agglutinins, in high titer, which are not removed by absorption with corresponding
agent. As an example, the tubercle bacillus may form an enzyme which alters some of the infected host's red cells and these altered red cells could then serve as antigens, having become "foreign" to the body. The antibodies produced against these altered cells may then act on other altered cells causing intravascular agglutination. The agglutinins produced in such cases need not necessarily be panagglutinins, since they may agglutinate only red cells altered by the specific agent.

While this hypothesis has a certain plausibility, it neglects the fact that in diseases where agglutinins for treated cells are found (acquired hemolytic anemia) such agglutinins are capable of sensitizing normal cells. Their specificity is, therefore, not as restricted as proposed by the authors. This fact supports the contention that certain types of hemagglutinins possess dual specificity. The hypothesis offers an explanation for the appearance of auto-antibodies but it does not propose any explanation for the ability of such antibodies to react differently with treated and untreated cells, i.e. to agglutinate treated cells but only sensitize untreated cells.

The discussion above emphasizes the rather vague concepts held in regard to the immunologic differences between trypsin treated and untreated human red blood cells.
The data presented in this paper appear to offer some aid in removing some of the confusion prevalent in this particular field of hemagglutination. The following tentative explanation is, therefore set forth.

The presence of anti-Rh antibodies in the sera of human individuals can be invariably associated with two contributing factors. The red cells of such individuals do not possess the Rh antigens to which they have become sensitized, and at some time, red cells possessing this antigen(s) have been introduced into their circulatory systems. Obviously this antigen is "foreign" to the individuals in question and, therefore, elicits the production of specific antibody. The antibody of this type which first appears is usually capable of agglutinating enzyme treated or untreated cells in saline. As immunization proceeds there is often a marked or even complete reduction of saline agglutinin and the appearance of so called incomplete antibody. It is possible that the initial immune response is directed toward the Rh haptenic group, but as immunization continues, the foreign cells would be sensitized before reaching the antibody forming cells. Now as the process of erythrocyte digestion commenced, some of the Rh molecules would be covered with globulin molecules which would have to be removed before the group could be instrumental in directing the synthesis of antibody. This could conceivably
allow time for partial digestion of the cell surface. Consequently the antibody forming mechanism would be stimulated by the Rh grouping acting as a haptene for subsurface structures, and the resulting antibody molecules would possess the dual specificity previously mentioned. As more antibody was formed more Rh groups would be thus sensitized before digestion occurred and saline agglutinin would be gradually replaced by antibody with dual specificity. Because the reactive groups of this antibody would be directed partially to subsurface constituents of the erythrocyte, there would conceivably be some difference in the manner in which it was bound to trypsin treated and untreated cells. The position which the antibody took upon untreated cells might be such as to preclude the possibility of simultaneous reaction with two cells thus preventing any lattice type of agglutination. It is significant that in the sera of rabbits immunized with untreated human red blood cells antibody specifically reactive with treated cells appears, and when this antibody is allowed to react with normal cells, it evidently comes to lie in some unique position on the cell surface since it interferes with the course of normal cell antibody absorption. Moreover, although this antibody agglutinates treated cells, it is capable of only sensitization of normal cells. Furthermore this rabbit antibody is produced by animals injected with untreated cells.
and since the immunization procedure involved several injections, it is possible that some of the antigenic groups on the cells of later injections were sensitized prior to phagocytosis and digestion. In short, this particular rabbit antibody is in many ways comparable to the incomplete type of hemagglutinin found in anti-Rh sera of humans. The serum fraction isolated from rabbit anti-Trbc agglutinates treated cells but apparently does not even sensitize normal cells. However, as pointed out previously, this antibody is elicited by a cell on which a proteolytic enzyme has been allowed to act for an arbitrary period of time, so that although this digestion process exposes the subsurface groups necessary for agglutination by anti-Rh incomplete antibody, it is unlikely that it is strictly comparable to in vivo digestion. (This point has been discussed at length earlier) Moreover, because the human cell is completely foreign to rabbits, antibody would be formed with a specificity for the subsurface groups alone and would thus be capable of reacting only with treated cells. Antibodies with dual specificity may be present in anti-Trbc sera but undoubtedly special absorption procedures would be necessary to obtain any fraction containing these alone.

In the case of acquired hemolytic anemia there is the appearance of auto-hemagglutinins, and little is known concerning the initiation of events in the immunologic
phase of this disease. The suggestion by some workers that the initial events involve the action on red cells of endogenous or exogenous enzymes fails to account for two pertinent facts. First, the antibodies found in the serum of patients have a specificity for untreated as well as enzyme treated cells; and second, since there is a constant turnover of red cells in the body, it seems logical to suppose that the stromata of senile cells are picked up and digested by the phagocytic cells. Hence it may be erroneous to speak of enzyme treated cells as being "foreign" to the body. It is worth noting in this connection that the pathogenesis of acquired hemolytic anemia may well be very intimately related to the mechanism for the removal of stromata of normal senile cells. There is at present little known about this normal mechanism. At any rate the initial event in the course of the disease very likely involves some modification of the normal red cell surface with the subsequent appearance of hemagglutinins directed toward the modified component. The nature of the antibody at the time of diagnosis appears to conform to those for which a dual specificity has been postulated and this is probably the case.

The hypothesis elaborated on the basis of results obtained in this investigation is proposed as a working hypothesis for which there appears to be a very definite need. The results of quantitative studies presented and
discussed here indicate that the analytical methods adopted and developed in the course of the work could be quite profitably exploited further.
The immunologic nature of the change produced by the action of trypsin on human red blood cells has been studied by the combined use of the classical methods of serology and quantitative immunochemical technics. Serum dilution titer and cross absorption studies have been carried out in the sera of rabbits immunized with normal human red blood cells and in the sera of rabbits immunized with cells previously treated with 0.1 per cent trypsin. Data obtained in these studies indicated the existence of antigenic differences between normal and trypsinized cells.

Erythrocyte stromata have been prepared by a variety of methods for use in quantitative determination of hemagglutinins for normal and trypsinized cells. The relative merit of the various methods has been discussed and a procedure for the preparation of a suitable antigen has been elaborated.

The course of agglutination as a function of the amount of normal stroma added has been followed in rabbit anti-Nrbc sera and in rabbit anti-Trbc sera. This work was carried out at pH 7.4 and the data obtained were considered unreliable due to the relatively high solubility of the stromata in saline controls.

The solubility of erythrocyte stromata has been
determined in saline and in normal rabbit serum at various hydrogen ion concentrations and has been shown to be minimal at pH 5.5.

Quantitative estimation of total antibody for normal and trypsin treated cells has been carried out in both homologous and heterologous rabbit antisera at pH 5.5, and a moderately high degree of precision has been realized.

Results obtained by quantitative methods parallel those obtained in routine absorption studies, and offer strong support for the original contention that there are antigenic differences between normal and trypsin treated cells.

It has been suggested that the incomplete antibodies found in certain hematologic disorders are capable of agglutinating treated cells by virtue of their ability to act as divalent molecules in reactions with such cells. An hypothesis has been offered to account for this peculiar behavior as well as for the appearance of incomplete antibodies in the sera of individuals with acquired hemolytic anemia.


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normal cells. Moreover, the sera from rabbits immunized with normal human erythrocytes showed only slight activity for either normal or enzymized cells after homologous absorption whereas heterologous absorption led to pronounced reduction of the titer for treated cells with only slight change in normal cell titer. Moskowitz and Treffers (1950) found that periodate ions rendered cells panagglutinable and further showed that the agglutinin for periodate treated cells is distinct from the agglutinin for red cells rendered panagglutinable by the filtrates of cultures of Vibrio comma.

During the work with Vibrio cholerae filtrates it was incidentally observed that red cells so treated became specifically agglutinable in saline dilutions of incomplete anti-Rh sera (Pickles 1946). Morton and Pickles (1947) noted that crude and crystalline trypsin solutions would also produce a similar effect when Rh positive red blood cells were subsequently exposed to the action of anti-Rh sera. Quilligan (1948) independently found that a variety of proteolytic enzymes were active in this manner including trypsin, pepsin, papain and erepsin. Wheeler, Luhby and Scholl (1950) have found that chymotrypsin, bromelin (fresh pineapple juice) and Russel viper venom (Stypven) also produce similar results. These latter workers standardized a method using trypsin treated cells as a routine laboratory method for the detection of incomplete


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Rh antibodies.

Chu and Coombs (1947) reported a similarity in the action of cholera filtrates and viruses of the Newcastle group on red cells. Either agent could remove virus receptors, produce panagglutinability and induce susceptibility to agglutination by incomplete antibodies to Rh antigens.

Wright, Dodd and Bouroncle (1949) found that red cells previously treated with trypsin could be used to demonstrate incomplete antibody in the sera of patients with acquired hemolytic anemia, and in a later publication (1951) these same workers demonstrated the presence of incomplete cold agglutinins in the sera of normal individuals by trypsin cell techniques.

Ponder (1951) carried out physical studies on red cells treated with crystalline trypsin and brought out the following points: (1) the cell volume is slightly increased (2) post hemolytic membranes are unusually rigid and volume occupying (3) osmotic and mechanical fragility are slightly increased and (4) the electrophoretic mobility of treated cells is reduced. Bouroncle (1951) found no significant change in the osmotic fragility, mechanical fragility or morphology of erythrocytes treated with 0.1 per cent trypsin for 30 minutes at 37 C. These results of physical studies are obviously inadequate to advance any explanation for the modified