STUDIES ON INDIRECT STAPHYLOCOCCAL HEMAGGLUTINATION WITH SPECIAL REFERENCE TO HUMAN SERA

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ABSTRACT

STUDIES ON INDIRECT STAPHYLOCOCCAL HEMAGGLUTINATION WITH SPECIAL REFERENCE TO HUMAN SERA

by Earl C. Renshaw, Jr.

The first aim of this investigation was to determine some of the factors involved in the indirect bacterial hemagglutination procedure using the soluble polysaccharide antigen of Staphylococcus aureus. The second aim was to measure the hemagglutination titer of normal human sera to determine what relationship, if any, existed with active staphylococcal infections.

The antigens were prepared from the International-Blair series of phage propagating strains of Staphylococcus aureus as well as clinical isolates. After autoclaving and centrifuging off the cells a semi-purified product was obtained by ethanol flocculation. Biochemical analysis of the dried powder showed approximately 8% protein, 2.2% nitrogen, and 34% polysaccharide. Thermal testing showed no similarity to the Rantz type of antigen. Between 0.5 and 1.0 mg per ml of antigen was required to produce maximum sensitization of 5% human erythrocytes

at a pH of 7.4. Antigen attachment which was rapid and directly related to temperature was maximum after 30 minutes at 37 C. Electrolytes were required for adsorption of antigen, but the presence of manganese ions was contra-indicated. The erythrocytes apparently possessed specific sites of attachment for the antigen because the incompletely covered surfaces still permitted agglutination by homologous blood group antibodies. A final erythrocyte concentration of 1.25% was found to produce optimal hemagglutination.

The anti-globulin and enzyme modifications were much more sensitive than the basic hemagglutination procedure.

The hemagglutination titer of staphylococcal antibodies was determined on normal sera as well as that from hospitalized patients with various illnesses and diseases. The titer of normal adult serum varied from 1:16 to 1:1024, with an average range of 1:128 to 1:256. After examining some 200 specimens no correlation was found between the hemagglutination titer and patients with various illnesses and diseases, including staphylococcal infections of various types.

Hemagglutination titers of 30 cord blood samples were consistently lower than the corresponding maternal serum.

To explain this phenomenon the existence of "incomplete" antibodies was proposed.

In conclusion, the testing of several hundred different samples of human serum by the indirect bacterial hemagglutination technique indicates a high degree of sensitivity for staphylococcal antibodies which is, unfortunately, not accompanied by adequate specificity. The cause for this last consideration remains undetermined.

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By

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TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

During the past fifteen years there has been concentrated interest on the so-called staphylococcal problem. This activity has been due mainly to widespread outbreaks of staphylococcal infections, the emergence of antibiotic-resistant strains, and new methods of study, such as phage typing. However, information on staphylococcal infections in man and animals still remains fragmentary, even though investigations have been going on since the early days of bacteriology.

Much has been done on the serological testing of staphylococcal infections, but as yet, no test having a clear and simple interpretation has been devised.

According to Witebsky (1958), the indirect or passive hemagglutination procedure is, at present, the most sensitive serological method for the detection and titration of specific antibodies. He has estimated that under optimal experimental conditions, hemagglutination is 100 to 1000 times more sensitive than the precipitation reaction.

The present investigation was undertaken to determine some of the factors involved in the indirect bacterial

hemagglutination reaction using an extract from Staphylococcus aureus as a modifying antigen. The hemagglutination titer of staphylococcal antibodies in normal human populations was measured to determine the relationship, if any, to active staphylococcal infections.

HISTORICAL

During the past twenty years the investigations have shown that red blood cells provide a valuable tool in microbiological studies. Hirst (1941), as well as McClelland and Hare (1941), independently described viral hemagglutination. Since then numerous other studies on viral hemagglutination and hemagglutination-inhibition have shown their importance, both for identification and titration of hemagglutinating viruses, and for the detection and titration of the corresponding antibodies.

Bacteria, also, have been found to cause the agglutination of erythrocytes. Eight known types of bacterial hemagglutination and hemolysis reactions have recently been extensively reviewed by Neter (1956). The first or the direct hemagglutination reaction involves only bacteria (bacterial antigen) and erythrocytes. The other seven require antibody in addition to the antigen and erythrocytes. One of the latter methods, the indirect or passive bacterial hemagglutination reaction, forms the basis for the present study.

Indirect or Passive Bacterial Hemagglutination

The Australian investigators, Keogh, North, and Warburton (1947) first demonstrated that when a soluble antigen obtained from Hemophilus influenzae was adsorbed to the surface of red blood cells, they acquired a new serologic specificity and were agglutinated upon the addition of the homologous bacterial antiserum. Since then, this indirect bacterial hemagglutination procedure has been studied by numerous investigators from many parts of the world. These tests have proved to be extremely sensitive for the detection and titration of antibodies against bacteria, fungi, protozoa, and various other substances such as penicillin-protein complexes and certain foreign proteins.

A large number of bacterial species have been found to contain antigens which are readily adsorbed by untreated erythrocytes, rendering thereby these erythrocytes specifically agglutinable by homologous bacterial antibodies (Neter, 1956).

The soluble antigens used for adsorption were either crude extracts of the organisms, or solutions of partially purified carbohydrates, or lipopolysaccharides. In almost

all instances studied, the modifying antigens were polysaccharides, or contained polysaccharides as the serologically active group (Oeding, 1960).

Untreated erythrocytes from most warm-blooded animals were easily modified by the polysaccharide antigens, while the erythrocytes of cold-blooded animals, such as the alligator, were not agglutinated unless the modified erythrocytes had been treated with proteolytic enzymes such as ficin, trypsin, or papain (Neter et al., 1959a).

Little is known about the mechanism of attachment of the polysaccharide antigen to the erythrocyte. It has been shown, however, that electrolytes are required for adsorption, as modification does not occur in 5% glucose or 5% sucrose, but will take place in isotonic NaCl, KCl, sodium citrate, or Ringer's solution (Neter, 1956; Veno, 1957). At present not much is known about the receptors on the surface of the erythrocyte to which the polysaccharide is attached. Specific receptors are believed to exist because the normal erythrocyte antigens are not blocked on modified erythrocytes, nor does the treatment with receptor-destroying enzyme, or periodate, interfere with the modification (Hayes, 1951; Wright and Feinberg, 1952; Neter et al., 1952). Several bacterial antigens, either

simultaneously or consecutively, can be used to modify the erythrocytes, since one antigen apparently does not block the adsorption of another (Neter, 1956).

In the presence of complement and specific antibody, modified erythrocytes may be lysed. Not all erythrocytes are equally susceptible to lysis, as human erythrocytes are not lysed when modified with E. coli antigen, while modified sheep erythrocytes are readily lysed (Neter, 1956).

When protein or non-polysaccharide antigens are used, the erythrocytes must be treated before the antigen can be adsorbed. This may be done by pretreatment with tannic acid (Boyden, 1951; Stavitsky, 1954) or by chemical linkage with bisdiazotized benzene (Stavitsky and Arquilla, 1955).

It is presumed that the amount and location of the antigen on the erythrocyte surface plays a significant role in the agglutination reaction with homologous antibodies. Thus, the indirect hemagglutination system offers a distinct advantage over those tests utilizing cells with naturally occurring antigens in unalterable amounts (Neter, and Gorzynski, 1959).

This indirect, conditional, or passive type of hemagglutination readily lends itself to the study of many bacteriological, serological, and immunological reactions.

Indirect Staphylococcal Hemagglutination

Keogh, North, and Warburton (1943) reported that staphylococci, along with other bacteria, contained a phenol-insoluble polysaccharide that was readily adsorbed by most erythrocytes, and this event was followed by agglutination with staphylococcal immune serum. Later, other workers (Rountree and Barbour, 1952) confirmed that cultural filtrates and extracts of Staphylococcus aureus had the ability to sensitize erythrocytes.

The erythrocyte sensitizing material from staphylococci has been extracted by many different methods, none of which is as yet completely satisfactory.

Heat is one of the most commonly used methods for extraction of the polysaccharide antigen. Simple heating at 100 C for one hour, or autoclaving at 120 C for up to two and a half hours has been used (Oeding, 1954). Autoclaving is a very effective means of disrupting cells and the crude extract obtained is very rich in polysaccharides. However, this method is rather drastic, and degeneration and partial hydrolysis of the polysaccharide most likely occurs.

Phenol has been used extensively for extraction of polysaccharides from bacteria. The active material may

be found in the phenol-insoluble, water-soluble material after extraction with 90% phenol (Keogh et al., 1948), or in the phenol-soluble fraction after extraction with 88% phenol (Rantz et al., 1952).

Hayes (1951) extracted staphylococci with glycine, but Oeding (1955) considers that this method has disadvantages, as the glycine could only be separated from the polysaccharides by dialysis, and some of the active polysaccharide, being dialyzable, will be lost.

Extraction, by hot formamide (Oeding, 1955) is considered an optimal method; it produces a rich polysaccharide with little protein or mucoid material.

Other extraction procedures have employed hot hydrochloric acid (Rantz et al., 1952) and proteolytic enzymes (Pakula and Walczak, 1956).

Much work remains to improve the purification of the active principle. Most of the investigators have used simple culture filtrates or very crude extracts of staphylococci in their investigations. The sensitizing substance is resistant to autoclaving, and subsequent heating to 180 C in an oil bath, but is destroyed by both dilute acids and alkalis at room temperature (Oeding, 1957). It is

also resistant to trypsin, formamide, phenol, ethanol, acetone, and chloroform (Oeding, 1960).

Rountree and Barbour (1952) found that the sensitizing activity was lost after storage for a few weeks at refrigerator temperatures. Other observations (Neter, 1956) with polysaccharide antigens have shown that the sensitizing activity may be lost during purification, and Oeding (1957) found that the activity of staphylococcal polysaccharide was lost both during purification and storage. Oeding (1957) believes that this indicates a labile grouping in the active polysaccharide which is necessary for its sensitizing activity, but not for its precipitating activity. He also found that the sensitizing activity was destroyed before the precipitating activity when treated with acid or alkali, and considers that the sensitizing substance is bound to the polysaccharide material, or is some substance intimately associated with it.

A sensitizing substance with heterophile properties was described by Rantz et al. (1956) and confirmed by Neter and Gorzynski (1959). This antigen was present in staphylococci and a number of unrelated species of gram-positive bacteria, but completely absent in gram-negative

bacteria. Rantz and associates (1956) named it a non-species specific (NSS) antigen. This antigen was found to be non-dialyzable, trypsin resistant, heat stable in acid solutions, but labile in neutral and alkaline solution.

A similar or identical antigen, common to both staphylococci and streptococci, was described by Pakula and Walczak (1955), as well as Znamuowski and associates (1959).

Basically, the indirect hemagglutination reaction takes place in two stages. In the first stage, the soluble polysaccharide antigen is attached to the surface of the erythrocyte without causing visible change. This is called sensitization or modification of the erythrocyte.

In the second stage, or antigen-antibody reaction, there is a specific reaction between the modified erythrocytes and the homologous bacterial antibody, causing the agglutination of the erythrocytes.

There are two important modifications of the basic indirect hemagglutination procedure. These are the anti-globulin (Coombs) hemagglutination test, and the enzyme hemagglutination test (Meter et al., 1959b, 1959c, 1960).

Staphylococcal Antibodies

Staphylococci are very complex antigenically, and produce many different toxins. Antibodies to the various

fractions have been found in normal human sera, such as the following examples: anti-hyaluronidase (Bergquist, 1951), anti-hemolysins (Vahlquist et al., 1950), anti-leukocidin (Towers and Gladstone, 1958), anti-capsular (Wiley, 1960) and anti-coagulase (Rammelkamp and Lebovitz, 1956).

Most investigations of staphylococcal antibodies in human sera have been concerned chiefly with antitoxic antibodies, while less has been done on the detection of bacterial antibodies. Because of the many different techniques involved, it is difficult to determine whether the different types of antibodies reported are identical, similar, or completely different in specificity.

Rountree and Barbour (1952) first detected antibodies in human sera to the soluble polysaccharide of staphylococci.

Rantz et al. (1956) found antibodies against the heterophile (NSS) substance in human sera, as did Neter and Gorzyski (1959), Nunnery et al. (1959), Znamirowski (1959), and Pakula and Walczak (1955). By hemagglutination, Neter and Gorzynski (1959), also demonstrated "incomplete" antibodies in high titer and "complete" antibodies in low titer to staphylococci in human gamma globulin and pooled sera.

Jensen (1958) found antibodies in all human sera examined, as well as in human colostrum, against a particular staphylococcal substance which he called "Antigen A." This substance was an extract of Cowan Type I strain, and is probably a polysaccharide fraction.

Wiley (1960) tested sera from normal blood donors for capsular antibodies and found that 80% had demonstrable titers.

Beiser et al. (1958) found that human gamma globulin gave multiple bands by agar precipitation against extracts of all strains of staphylococci examined, and believes that this indicates the presence of small amounts of different group antibodies in human sera.

Dudgeon and Bamforth (1925) observed that staphylococci were agglutinated by sera from patients with serum sickness, and Seeliger and Sulzbacker (1956) demonstrated a cross-reactivity to Listeria monocytogenes.

Therefore, normal antibodies reacting with staphylococci are not necessarily specific, nor are they always due to previous staphylococcal infections.

A criterion reported frequently by many workers was based on the ability of staphylococcal antibodies to pass the placental barrier. Since the human fetus cannot

synthesize gamma-globulin, all antibodies present in the cord sera must have been derived from the maternal sera by passive diffusion through the placenta. This passive transfer of antibodies depends mainly upon two factors, the structure and function of the placenta and the characteristics of the antibody.

The 7S gamma-globulins and a number of antibodies associated with this fraction are able to diffuse readily, and the antibody titer of cord sera is of the same order of magnitude as that of the maternal sera.

However, with the larger 19S gamma-globulin molecules, the placenta presents a greater barrier to diffusion, which results in a much lower concentration of 19S globulins in the cord sera than in corresponding maternal sera (Franklin and Kunkel, 1958).

Many antibodies, however, are present in newborn infants with titers that normally equal or exceed up to five times the titer of the maternal sera, so some factor other than simple passive diffusion appears to be operating (Brambell and Hemmings, 1960).

Rountree and Barbour (1952) demonstrated that the antibody to the erythrocyte-coating polysaccharide of Staphylococcus was not able to pass the placental barrier.

Florman et al. (1959) also using an erythrocyte-coating polysaccharide from Staphylococcus, were unable to detect antibodies in cord sera. However, the antibodies were present in about one-half of the sera of persons six months of age, and they believed that a positive hemagglutination test in infants under six months, or a rise in titer in persons of any age, indicated a recent staphylococcal infection.

Nunnery et al. (1959) also found similar results and believed that the hemagglutination titer appeared to be influenced by age.

In the case of two staphylococcal antibodies that easily pass the placental barrier, the anti-hemolysins (Vahlquist et al., 1950) and the Rantz antibody (Neter et al., 1959c, 1960), there was a decrease in titer for the first few months of life, followed by an increase as the infant grew older.

Rammelkamp and Lebovitz (1956) found that the level of the coagulase-reacting factor was low before two years of age, but increased thereafter.

Quie and Wannemaker (1960) found in human sera an inhibitor of staphylococcal satellite proteolysis (Mueller phenomenon). This inhibitor is believed to be an antibody and has the ability to readily pass the placenta.

MATERIALS AND METHODS

General Materials

Strains. The International-Blair (Blair and Carr, 1953) series of phage propagating strains of Staphylococcus aureus were used as the source of soluble polysaccharide antigen.

Buffered saline solution. Buffered saline solution was prepared by mixing equal volumes of 0.15M NaCl solution with a solution (0.15M) of KH_2PO_4 and Na_2HPO_4, mixed in the proper proportions to produce the desired pH.

Erythrocytes. Human erythrocytes (blood group O, Rh-negative) were collected in 5% EDTA (disodium dihydrogen ethylenediamine-tetraacetate dihydrate), washed several times with buffered saline solution (pH 7.4) to remove all traces of plasma, and resuspended to a 10% concentration. These washed erythrocytes were used within 24 hours.

Serum specimens. Blood specimens were collected from blood donors, healthy persons, and hospital patients with various infections and diseases. Cord blood was collected immediately after delivery and the corresponding maternal specimens within 24 hours. After clotting, the serum was removed and inactivated by heating at 56 C for 30 minutes, and stored at -20 C.

Methods

Indirect hemagglutination procedure. Briefly, the indirect hemagglutination procedure was carried out as follows:

To an optimum amount of antigen solution was added enough of the stock erythrocytes to produce a 5% concentration. After an incubation period of 30 to 60 minutes at 37 C, the modified erythrocytes were washed three times with large volumes of buffered saline solution (pH 7.4) and resuspended to a 2% concentration.

Serum was serially diluted two-fold with buffered saline solution (pH 7.4), using separate pipettes for each dilution. To 0.2 ml of each serum dilution was added an equal volume of the modified erythrocytes. After incubation at 37 C for 2 hours, the tubes were centrifuged at 1300 rpm for one minute and read for hemagglutination according to the following scheme:

4+ = one large clump.

3+ = several large clumps.

2+ = many small clumps.

1+ = fine granular clumping.

- = no clumping, with complete resuspension of erythrocytes.

Controls consisted of serum plus unmodified erythrocytes and saline plus modified erythrocytes.

Preparation of staphylococcal antigen. The crude antigens were prepared as follows: One liter of Brain Heart Infusion Broth (Difco)[1] in a three-liter round-bottom flask was inoculated with 100 ml of an 18 hour culture of Staphylococcus aureus. After an incubation period of 18 to 24 hours at 37 C with continuous shaking, the cells were removed by centrifugation at 10,000 rpm for 30 minutes. They were then washed three times with buffered saline solution (pH 7.6) and resuspended in 100 ml of the buffered saline solution.

The resuspended cells were then autoclaved at 120 C for 30 to 60 minutes. After centrifugation at 10,000 rpm for 30 minutes, the clear yellow supernatant fluid was saved as the crude antigen.

This crude extract was used either directly to modify the erythrocytes, or to produce the partially purified polysaccharide antigen.

Purification of antigen. This purification was done by first adjusting the extract to a pH of 2.0 with 1N HCl, and allowing precipitation to continue for 18 hours at 4 C.

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1. Difco Laboratories, Detroit 1, Michigan.

After centrifugation the precipitate was dissolved in neutral saline solution. Acetate buffer (pH 5.6) was added, and the polysaccharide was flocculated with 95% ethanol. For example, 100 ml of solution received 10 grams of sodium acetate and 1.0 ml of glacial acetic acid; then eight volumes of 95% ethyl alcohol were added and flocculation was allowed to proceed overnight at 4 C.

After centrifugation, the flocculate was dissolved in about 100 ml of distilled water and the proteins removed with chloroform and butyl alcohol. For this procedure about 40 ml of chloroform and 8 ml of normal butyl alcohol were added to the solution. The solution was subsequently cooled to 4 C, and emulsified intermittently with a Waring blender ten to twelve times during a two hour period. The mixture was then centrifuged at 10,000 rpm for 10 minutes, and the aqueous phase retreated with chloroform and butyl alcohol until an emulsion was no longer formed.

The polysaccharide was then flocculated an additional five times with alcohol and acetate and finally dried *in vacuo* over anhydrous $CaSO_4$ at room temperature for 48 hours.

The dried antigens were stored in tightly sealed vials at -20 C.

Biochemical Analysis of Antigen

Some of the biochemical constituents of the various antigens were determined. The partly purified antigens were dissolved in distilled water at a concentration of 1.0 mg per ml, while the crude antigen solutions were used directly.

Nitrogen. Total nitrogen was determined by the micro-Kjeldahl method as described in Kabat and Mayer (1961).

Protein. Total protein was determined by both the Biuret reaction (Kabat and Mayer, 1961), and the Folin-Ciocalteu (Lowry et al., 1951).

Carbohydrate. Total hexoses were measured by the anthrone method as described in Kabat and Mayer (1961). In this procedure, 10 ml of anthrone reagent (2 g anthrone in 1 liter concentrated sulfuric acid) was pipetted into a large (25 x 200 mm) test tube in a cold water bath. The sample (5 ml) was then carefully layered above the reagent. Blanks and standards of glucose were included. Each tube was vigorously shaken until thoroughly mixed, then heated at 90 C for 16 minutes, cooled and the blue color read at 625 mμ.

Effect of pH and heat upon antigen. In order to determine whether the antigens used here were similar to the

non-species specific antigen of Rantz and associates (1956), the effects of pH and heat were determined. The antigen was dissolved in normal saline solution at a concentration of 10 mg per ml. Into each of three tubes was pipetted 10 ml of the antigen solution and the pH of each was then adjusted to 2.2, 7.0 and 11.5 with 1N HCl or 1N NaOH. Each of these were then divided into half, one-half was allowed to stand at room temperature (24 C) while the other was placed in a boiling water bath. After 20 minutes, the tubes were cooled in running tap water, and the pH of each was adjusted to 7.2 with normal HCl or NaOH. The hemagglutination procedure was then carried out in the usual manner on each sample.

Factors Influencing Hemagglutination

The following general procedure was used unless otherwise stated. Washed erythrocytes were added to the antigen solution to produce a 5% suspension. After an incubation period of 30 to 60 minutes at 37 C, the erythrocytes were washed several times with normal saline solution and resuspended to a 2% concentration.

Pooled inactivated human sera were used to determine the hemagglutination titers in all comparison studies. After

incubation and subsequent centrifugation at 1300 rpm for one minute, the cells were shaken gently and read as previously described.

Sensitization of erythrocytes. In order to determine the concentration of antigen needed to produce a maximum hemagglutination titer, the following experiment was devised. The polysaccharide antigen was added to buffered saline solution (pH 7.4) at the following concentrations: 10, 5, 2.5, 0.5 and 0.1 mg per ml. Stock erythrocytes were then added to form a 2% suspension and after an incubation period of 60 minutes, the hemagglutination test was carried out as previously described.

pH. In determining the effect of pH on antigen adsorption, the antigen solution (3 mg per ml) was adjusted with buffered saline solution. The following pH values were used: 6.0, 6.5, 7.0, 7.5, and 8.0. The stock erythrocytes were then added, as usual, and modification and hemagglutination were done as previously described.

Incubation time. The effect of incubation time on antigen adsorption was determined by allowing the antigen solution (5 mg per ml) and a 5% concentration of erythrocytes to warm in a 37 C waterbath for at least 15 minutes.

The erythrocytes were then added to the antigen solution, mixed thoroughly and samples removed at 5, 10, 15, 30, and 60 minutes. These samples were immediately washed with large volumes of cold buffered saline solution (pH 7.4), and hemagglutination tests done on each.

Incubation temperature. In order to determine the effect of the incubating temperature on antigen adsorption, two sets of four tubes each were prepared. One set contained in each tube 5 ml of antigen solution, while the other contained 1.0 ml of a 10% erythrocyte suspension. One tube of each set was incubated at the following temperatures: 4, 27, 37, and 45 C. After 15 minutes, the antigen solutions were poured into their respective tubes of erythrocytes, mixed thoroughly, and incubated for 30 minutes. Each tube was then washed with large volumes of buffered saline solution at their respective temperatures, and hemagglutination tests done as previously described.

Electrolytes. The requirement of electrolytes for antigen adsorption was determined by the following experiment. As diluents, the following isotonic solutions were prepared from reagent grade chemicals in water, triple distilled in glass;

A. Sodium chloride solution (0.15 M).

B. A mixture of 10% glucose and 15% lactose (equivalent to 1.0M) diluted 1 to 3 with distilled water before using (0.33M).

C. Calcium chloride solution (0.1M).

D. Trisodium citrate solution (0.1M).

E. Magnesium chloride solution (0.12M).

F. Manganese chloride solution (0.1M).

Mixtures of electrolytes and the glucose-lactose solution were employed in various proportions so as to keep the solutions isotonic with respect to the erythrocytes. These solutions were used as diluents only during the first or antigen adsorption phase.

The antigen was dissolved in water and dialyzed against distilled water at 4 C for 24 hours to remove any electrolytes that may be present. The antigen solution was then made isotonic by adding the proper proportion of glucose-lactose solution (1.0M) and then tested to determine its inability to modify or sensitize the erythrocytes. The various diluted electrolyte solutions (made isotonic with glucose-lactose solution) were added to equal amounts of the prepared antigen solution.

The erythrocytes were washed several times with the diluted glucose-lactose solution to remove all traces of saline solution and added to the electrolyte-antigen solutions to a 2% concentration. After an incubation period of one hour at 37 C, the erythrocytes were washed three times with the glucose-lactose solution, once with saline solution and finally resuspended in buffered saline solutions (pH 7.4). Hemagglutination tests were then done on each.

Concentration of sensitized erythrocytes. To determine the final concentration of erythrocytes that will give the maximum hemagglutination titer, 25 ml of antigen solution (5 mg per ml) was prepared. To this was added enough washed erythrocytes to give a 5% concentration. After an incubation period of two hours, the erythrocytes were washed three times with buffered saline solution and resuspended to varying concentrations with a hemagglutination test being done on each. The final erythrocyte concentrations for each set were 10, 5, 2.5, 1.25 and 0.62%.

Comparison of Enzyme, Anti-globulin, and Basic Hemagglutination Tests

The enzyme bromelin was prepared from the commercial

Bromelase (Dade Reagents, Inc.)[1] according to the instructions provided. This Bromelase solution was used to treat the erythrocytes by three different methods. In the first method, 1.0 ml of the solution was added to 1.0 ml of antigen solution (5 mg per ml). Washed erythrocytes were then added to give a 5% concentration. After an incubation period of 30 minutes, the erythrocytes were washed and resuspended to a 2% concentration.

In the second method, the erythrocytes were treated with Bromelase before being sensitized by the antigen. This was done by adding 1.0 ml of the Bromelase solution to 1.0 ml of a 5% suspension of erythrocytes. After an incubation period of 15 minutes at 37 C, the erythrocytes were washed thoroughly with normal saline solution and modification was carried out in the usual manner.

With the third method, the Bromelase was used to treat the erythrocytes after they had been modified by the antigen.

The normal or basic hemagglutination test was done as previously described, and after being read, the 2 plus positives and lower tubes were converted into the antiglobulin (Coombs) modification.

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1. 1851 Delaware Parkway, Miami 35, Florida.

In this procedure, the erythrocytes were washed at least three times with normal saline solution to remove all traces of serum. Two drops of human Anti-globulin Reagent (Dade Reagents, Inc.)[1] were added to each tube, and after centrifugation the tubes were read for agglutination.

Antibody Titer Determinations

The hemagglutination titers of staphylococcal antibodies were determined by using the previously described methods. The samples tested included the following:

1. Pooled normal sera, from 10 to 25 individual serum samples per pool.
2. Sera from healthy individuals.
3. Sera from patients with various illnesses and diseases.
4. Sera from patients with active and chronic staphylococcal infections.
5. Paired maternal and cord blood specimens.
6. Sera from diabetic patients.
7. Twenty-five specimens of human cerebrospinal fluid.

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1. 1851 Delaware Parkway, Miami 35, Florida.

RESULTS AND DISCUSSION

Nature of the Antigen

Stability during storage. Autoclaving is a very effective method for disintegrating bacteria and has been used widely in the study of bacterial antigens. Even though this is a rather drastic method and degeneration and partial hydrolysis of the polysaccharides must occur, Oeding (1954) has shown that the staphylococcal polysaccharides were especially resistant to heat and well able to withstand autoclaving at 120 C for over 2 1/2 hours.

The crude antigens prepared here were autoclaved 30 to 60 minutes as this appeared to produce as rich a polysaccharide extract as autoclaving for longer periods. This extract also contained lesser amounts of protein and mucoid materials.

The sensitizing activity of the polysaccharide antigen is believed to be very labile and may be easily lost during purification and storage. Rountree and Barbour (1952) found that their antigen solution lost its sensitizing ability after 4 to 6 weeks storage in saline at refrigerator temperatures, while Oeding (1957) found that the activity may be lost both during purification and storage.

The crude antigen extracts prepared here appeared to be more stable. They have been stored as long as six months at 4 C with very little loss of sensitizing activity, provided the extracts remained sterile and precipitation did not occur. These extracts, however, were easily contaminated with bacteria and fungi, which resulted in immediate loss of all sensitizing activity.

The partially purified antigens were much more stable in the dried state, and have maintained their activity for over one year when stored in tightly sealed vials at -20 C. Loss of activity during purification was variable, some antigens lost most of their activity fairly easily, while others did not appear to be affected.

Biochemical analysis. One hundred ml of the crude antigen extract solutions contained 130 to 250 mg of protein, 58 to 110 mg of nitrogen, and 25 to 40 mg of polysaccharide.

The analysis on a dry weight basis of one of the partially purified antigens gave 8% protein, 2.2% nitrogen, and 34% polysaccharide.

Non-relation to Rantz antigen. The non-species specific antigen of Rantz and associates (1956) is a polysaccharide antigen found in culture filtrates of

staphylococci, and appears to be common to many, if not all, of the gram-positive bacteria, and is completely absent in gram-negative bacteria. Staphylococci, hemolytic streptococci of groups A, C, and G, Streptococcus viridans, pneumococcus, enterococcus, and Bacillus subtilis have all been found to be potent sources of this non-species specific antigen (Neter et al., 1960). This antigen is heat stable at acid pH, but is inactivated at a pH of 7.0 and above.

Table 1 indicates that the antigens used here are not that of Rantz and associates as boiling for 20 minutes at alkaline conditions did not cause inactivation.

The Rantz antigen is a surface antigen that is easily dissolved in the fluid medium of actively growing organisms. Careful washing of the organisms most likely removes the soluble surface antigens, and autoclaving at alkaline conditions will destroy any Rantz antigen present.

Factors Influencing Hemagglutination

Several important factors influencing the mechanisms of the hemagglutination reaction were determined.

Sensitization of erythrocytes. The optimum concentration of antigen needed to produce maximum sensitization

of erythrocytes as shown in table 2, is between 0.5 and 1.0 mg per ml. One hemagglutinating unit of antigen will be defined here as 1.0 mg of the dry antigen per ml. Two or more hemagglutinating units of antigen were used in all experiments.

pH. In determining the effect of pH on antigen adsorption, good adsorption occurred between a pH range of 7.0 to 7.5, as indicated by table 3. However, as there was not sufficient quantitative difference within the pH range used, pH was considered not to be critical, and a pH of 7.4 was used routinely as this is the approximate pH of human blood.

Incubation time. Table 4 indicates that the polysaccharide antigen of Staphylococcus was attached almost immediately to the erythrocytes. Within the first 5 minutes sufficient antigen was adsorbed to produce a good titer, and after 30 minutes hemagglutination was maximum in each tube.

Incubation temperature. A direct relationship between incubating temperature and antigen adsorption was observed as indicated in table 5. No antigen adsorption occurred at 4 C, and the titer increased as the temperature increased. At 45 C the titer was 2 to 4 fold higher than at 37 C, and

the reaction within each tube was stronger. However, the erythrocytes are more fragile at this temperature than at 37 C.

Electrolytes. As shown in table 6, antigen adsorption occurred in 4 of the 5 electrolyte solutions. Of the monovalent cations tested (sodium chloride and sodium citrate), adsorption was maximum at isotonic concentrations, while with divalent cations (calcium chloride and magnesium chloride), optimal adsorption did not occur up to 0.1 molar. It should also be noted that partial sensitization occurred at lower concentrations of the divalent cations than with the monovalent cations.

No adsorption was found to occur in manganese chloride. It was also noted that when manganese chloride was mixed with various concentrations of sodium chloride that antigen adsorption still did not take place. It appears that manganese ions for some reason inhibit the adsorption of antigen to the erythrocytes.

In this experiment, a 5% glucose solution was first used as the diluent, but difficulties were sometimes encountered. Hemolysis of erythrocytes frequently occurred when they were resuspended in saline after being incubated

in the glucose solution. Non-specific agglutination of human erythrocytes often occurred upon centrifugation, or when the erythrocyte suspension was too concentrated.

An isotonic glucose-lactose solution was substituted for the glucose solution as it is known that lactose will not pass the erythrocyte cell membrane while glucose will pass readily. The lactose counterbalances the effect of the glucose which causes the erythrocytes to absorb fluids and become fragile when resuspended in saline (Strumia et al., 1960). Antigen adsorption did not occur in this solution, and non-specific agglutination was minimal and easily dispersed.

Concentration of sensitized erythrocytes. A final erythrocyte concentration per tube of 1.25% or lower was found to give the best results as indicated in table 7. As the erythrocyte concentration increased, the titers decreased and reading became more difficult.

Comparison of Enzyme, Anti-globulin, and Basic Hemagglutination Test

Staphylococcal antibodies may be detected by several hemagglutination methods, of which the enzyme (bromelin or trade-name, Bromelase), and anti-globulin (Coombs) modifications are the most sensitive.

Both the Bromelase and Coombs modifications show the same degree of sensitivity, in which the reciprocal units were increased about 10 times over the regular hemagglutination procedure, as indicated in table 8.

The question now arises concerning the type of antibodies that these hemagglutination methods are detecting. According to Neter and associates (1959a, 1959b, 1959d, 1960), two hypotheses may explain this phenomenon.

One theory is that the enzyme and Coombs modification are detecting "incomplete" antibodies while the basic or regular procedure detects "complete" antibodies. It is well known that incomplete Rh antibodies are detected only by special methods, such as an enzyme or Coombs test, and this might be the case here. A definite answer to this question must await further studies on the antibody molecules themselves, as an actual difference in the molecules of "complete" and "incomplete" antibodies must be proved before this theory can be accepted.

The other theory is that the enzyme and Coombs modifications are merely much more sensitive than the regular hemagglutination procedure, and all three modifications are detecting an identical antibody.

Because enzymes are equally effective before, during, and after sensitization, it is unlikely that they are making it possible for the attachment of more antigen to the erythrocyte. This observation suggests that the enzyme may remove some substrate from the erythrocyte surface and allows more antigen molecules to react with the antibodies.

The most likely inference is that the antigen is present on the surface of the erythrocyte at various loci. Some antigen molecules are able to fix antibody at a location which allows it to react with the antigen on the second erythrocyte, whereas, other antigen molecules, because of spatial arrangement, react with antibody which cannot become attached to antigen on another erythrocyte. Agglutination then can only occur if another antibody (anti-globulin serum) is added, or the surface structure is changed by the use of proteolytic enzymes.

Neter and associates (1959a) studied the effects of proteolytic enzymes on the hemagglutination of lipopoly-saccharide modified erythrocytes of cold-blooded animal species (alligator, axolotl, and caiman). They demon-strated that these erythrocytes were not agglutinated unless treated with proteolytic enzymes, and concluded that this reaction was due to interference with the

hemagglutination by proteins or protein-like material on the surface of the erythrocyte.

In an experiment using erythrocytes of the frog (Rana pipiens) it was found that the polysaccharide antigens used here were able to attach to the erythrocyte, but produced a lower hemagglutination titer than when the enzyme, Bromelase, was used. Identical titers were obtained when the enzyme treatment occurred before, during, or after antigen modification.

These results may indicate that the interference of protein or protein-like material is not as great on frog erythrocytes, or may be due to the use of a different antigen-antibody system.

Neter and associates (1959a) also found that lipopolysaccharide antigens were attached to certain sites on the surface of the erythrocyte and did not cover the entire surface of the cell, as the lipopolysaccharide modified human erythrocytes of group A or B were still agglutinated by homologous blood group antibodies.

In this study, human erythrocytes of various antigenic structures were modified with the staphylococcal polysaccharide antigens and then tested with blood typing

antiserum. Commercial antiserum (Dade Reagents, Inc.)[1] of the following types were used: anti-A, anti-B, absorbed anti-A, anti-M, anti-N, anti-Kell, anti-Rh_o, anti-Rh_o^1, anti-Rh_o^{11}, anti-rh^1, anti-rh^{11}, anti-hr^1, anti-hr^{11}, and anti-Cellano.

In no instance was the erythrocyte antigen blocked by the modifying polysaccharide antigen, and this appears to indicate that the staphylococcal polysaccharide antigens have specific sites of attachment.

Staphylococcal Antibody Titers

Normal human sera. The staphylococcal antibody titer of human sera varies considerably when determined by the indirect bacterial hemagglutination procedure. Antibody titers ranging from 1:2 to 1:2048 were found routinely in some 500 specimens of human serum. Pooled sera from normal adults had a titer of about 1:128 to 1:256 with individual serum specimens ranging in titer from 1:16 to 1:1024. Titers were generally from 2 to 10-fold greater if the antiglobulin or enzyme modifications were used in addition to the basic procedure.

In general, there appeared to be slight relationship between the titer and age. Children under one year of

1. 1351 Delaware Parkway, Miami 35, Florida.

age had titers averaging about 1:32, while older children had titers closer to those of normal adults. Elderly persons also appeared to have slightly higher titers than young adults.

Abnormal human sera. No correlation was found between the hemagglutination titer and patients with various diseases and illnesses. Serum was collected from hospital patients and pooled according to the general type of illness. These pools were made from patients with acute and subacute bacterial infections, acute viral infections, rheumatic fever, diabetes, and various types of leukemia and other malignancies. The titer of these pools was always within one or two tube-dilutions of the normal, and this is probably within the experimental error of the procedure. Since diabetic patients and those with malignancies are often susceptible to chronic staphylococcal infections, it had been hoped that some differences in the titer might have been found by the use of this sensitive procedure.

No direct correlation could be detected between the hemagglutination titer and staphylococcal infections. In fact, the highest titer obtained (1:2048) was from a middle aged woman with no known previous history of

severe staphylococcal infection. In general, the titers of individual serum specimens from patients with various types of staphylococcal infections were within normal values.

Several patients with acute staphylococcal infections were followed during the period of hospitalization. Serum specimens were collected every day, if possible, and all specimens were tested at the same time. Antigens made from staphylococci isolated from these patients, as well as the stock antigens, were used. In no instance was there a variation in titer that did not fall within experimental error.

In one experiment, the antibody titer of the author was determined for over a two year period. Serum samples collected at various intervals were tested against antigens from all strains of the International-Blair set, as well as the six strains of the Seto-Wilson (Seto and Wilson, 1953) series of Staphylococcus aureus of bovine origin. All strains of the International-Blair series gave hemagglutination titers of 1:16 to 1:32, whereas, those of the Seto-Wilson series gave titers of 1:8 to 1:16. However, with an antigen produced from staphylococci (phage type 47/54) isolated from the nasopharynx of this person, the

hemagglutination titer was 1:123. During this two year period there was no significant change in antibody titer against any of the antigens used.

These results indicate that there is at least one common antigen present among all the staphylococci tested, including both the human and bovine strains. More antibodies appeared to be detected against the human strains than against the bovine strains, and with the "homologous" strain of Staphylococcus, a significantly higher titer was obtained.

These results differed, however, from patients with staphylococcal infections, where antigens from staphylococci isolated from the patient gave the same titer as that obtained by using the stock antigens.

Cord blood. Staphylococcal antibodies were present in all specimens of cord blood tested, as shown in table 9. These data indicated that antibodies to the soluble polysaccharide fraction of Staphylococcus were able to pass the placental barrier, but in a much lower concentration than that found in maternal serum. No direct relationship was found between the titer of the maternal serum and that of the offspring.

Further studies on cord sera using the enzyme or anti-globulin modifications have showed a comparatively higher titer than that found when using the same modifications on normal adult sera. In general, these modifications increased the titer of cord sera by 3 to 5 tube-dilutions as compared with 2 to 3 tube-dilutions with normal adult sera.

When using fresh, non-inactivated sera, it was found that hemolysis occurred within the first few tubes of the test. Cord sera gave a stronger hemolytic reaction than normal adult sera, producing a higher hemolytic titer and to a stronger degree. As it is known that there is less complement in cord sera (Aron et al., 1959), a stronger hemolytic reaction may indicate a difference in the characteristic of the antibody involved.

Cerebrospinal fluid. Also included in this study were several specimens of human cerebrospinal fluid. Twenty of these specimens, which were considered normal by laboratory analysis, were negative for staphylococcal antibodies when tested by the enzyme or antiglobulin modifications. However, with five abnormal specimens, staphylococcal antibodies were detected in low titer (1:2 to 1:4). These specimens were included to determine if the enzyme or

anti-globulin modifications were sensitive enough to detect staphylococcal antibodies in cerebrospinal fluid. Normal cerebrospinal fluid has a total protein content of 15 to 45 mg per 100 ml, while the abnormal specimens tested here had protein concentrations ranging from 100 to 450 mg per 100 ml.

From these data it may be concluded that the passage of staphylococcal antibodies into the cerebrospinal fluid readily occurred but detection was possible only in samples with elevated protein.

Placental transfer of antibodies. Concerning the placental transfer of antibodies the present results differed from that of other workers (Rountree and Barbour, 1952; Florman et al., 1959; Nunnery et al., 1959), who failed to find antibodies to the soluble polysaccharide fraction of staphylococci in the majority of the specimens of cord blood. The reasons for the inconsistent results among these workers can only be surmised.

It is known that antibodies to numerous other micro-organisms are able to pass the placenta, differences among them as well as variations in placental permeability may prevent their transfer from mother to child.

Since the fetus and newborn infant cannot synthesize gamma-globulin, all antibodies present in cord blood are

believed to be of maternal origin. At the present time the route of transmission in humans is not known clearly as in the case of some other mammals, nor are the physiological processes involved in the passage of such large molecules across the maternal-fetal barrier understood. It has been assumed that antibodies are transferred by the direct route across the placenta from the maternal blood in the lacunae to the fetal blood in the allantoic capillaries of the villi (Brambell and Hemmings, 1960).

Hitzig (1959) demonstrated that normal gamma-globulin with a molecular weight of about 156,000 could readily pass the placenta, while macroglobulins with a molecular weight of about one million could scarcely pass through, if at all. It is also known, that infants born to mothers with agammaglobulinemia have the same condition themselves for several weeks.

Therefore, the 7S gamma-globulins and the antibodies associated with this fraction diffuse readily across the placental barrier, and the antibody titer of the cord sera is of the same order of magnitude as that of the mother.

With the larger 19S molecules, however, it appears that the placenta presents a greater barrier to diffusion,

and this, together with the inability of the fetus to synthesize antibodies, results in a lower concentration of 19S gamma-globulin in the cord sera than in the corresponding maternal sera.

Several recent reviews (Aron et al., 1959; Brambell and Hemmings, 1960; Vahlquist, 1960) have extensively covered the placental transfer of antibodies in man. Many antibodies are present in the serum of cord blood of newborn full-term infants at titers that are normally about equal, or exceed slightly those of the maternal sera. These include antitoxins (diphtheria and tetanus), antihemolysins (staphylolysins and streptolysins), antiviral antibodies (measles, polio, encephalitis), some complement-fixing antibodies (influenza and toxoplasmosis), and anti-Rh conglutinins.

Certain antibodies appear to be transmitted less readily than others and always occur in much lower concentration in cord blood. These include anti-bacterial antibodies (H. influenzae, enteric pathogens), some complement-fixing antibodies such as syphilis, and isoagglutinins (A and B).

Antibodies which have not been found in cord blood include certain antibacterial (typhoid "H," E. coli "H"

and "O"), allergens and skin-sensitizing antibodies, and the C-reactive protein antibody.

The transmission of the blood-group isoagglutinins from mother to fetus is of particular interest. There is much evidence that incomplete or blocking blood-group antibodies are of the 7S character and are transmitted much more readily than the complete or saline antibodies, which are 19S in character.

Perhaps the staphylococcal antibodies detected here are of the complete variety, and are passing the placental barrier in a lesser concentration.

Another theory states that the adult sera contain both complete and incomplete antibodies, and during pregnancy the incomplete antibodies are able to pass the placental barrier much more readily than the complete.

The results obtained here appear to favor the latter theory as there was no relationship between the titers of the mother and child. If the antibodies were all of the same character, the cord blood titers should have paralleled the maternal titers but this was contrary to the observed facts. Supplementing the regular procedure with enzyme or antiglobulin modification resulted in

higher titers comparatively for the cord sample over the corresponding maternal ones; this observation may indicate presence of an incomplete antibody. The stronger hemolytic reaction with cord sera may also denote a difference in the character of the antibody reacting here.

However, a definite answer to this question must await further studies on the nature of the antibody molecules themselves.

It is well known that staphylococci are rich in antigenic substances, although there is no ready means of determining the exact number. Oeding (1960), in his review of staphylococcal antigens, lists 14 distinct antigens that have been studied to date. He listed a number of antigens, named by letters a to n, many of which were heat-stable and possibly carbohydrate in nature. At least one of the heat-stable, carbohydrate antigens (d) was shared by all strains.

Jensen (1961), using gel -precipitation methods, found that serum from healthy persons always contained a normally occurring antibody against the Staphylococcus "Antigen A," as well as other staphylococcal antigens. He also found a total of eight different antibodies in human sera and up to six different antigens in some strains of Staphylococcus.

In sera from patients with staphylococcal infections, he was able to demonstrate antibodies against some seven different staphylococcal antigens simultaneously, and also found that the patient's serum contained antibodies more frequently and often in greater amounts than did sera from healthy donors.

Erythrocytes may adsorb a variety of antigens, and in view of the complex antigenicity of Staphylococcus, the hemagglutination titer obtained is probably a result of multiple antigen-antibody systems, both specific and non-species specific.

The result of these experiments indicates that the indirect bacterial hemagglutination test is extremely sensitive but as used here, relatively non-specific for the detection of staphylococcal infections. Further purification and selective adsorption or neutralization of the antigens may clarify this problem and allow specific antibodies to be detected.

SUMMARY

This study was concerned with the factors related to the indirect bacterial hemagglutination procedure in serological diagnosis of staphylococcal infections. Since this is a relatively new and sensitive technique various factors involved in a staphylococcal system were determined. Using a soluble, heat stable, polysaccharide fraction from Staphylococcus aureus as the modifying antigen and pooled normal human sera as the antibody, it was found that 0.5 to 1.0 mg per ml of antigen was required to sensitize maximally 5% human erythrocytes at a pH of 7.4. The attachment was rapid and directly related to the incubation temperature, 30 minutes at 37 C producing a maximum reaction. Electrolytes were required for sensitization and an erythrocyte concentration of 1.25% was found to be optimum.

The anti-globulin and enzyme modifications of the basic hemagglutination procedure were found to increase the antibody titer tenfold.

Antibodies to the soluble polysaccharide fraction of Staphylococcus were found in all specimens of human sera examined.

The hemagglutination titer of normal human sera varied from 1:16 to 1:1024, with an average range of about 1:128 to 1:256.

No correlation was found between the hemagglutination titer and patients with various diseases and illnesses, including staphylococcal infections of various types. Variable results were obtained because many antigen-antibody systems appeared to be involved in this sensitive procedure.

At least one common antigen was present among all the strains of Staphylococcus used.

Antibodies were present in all specimens of cord blood, but at a much lower concentration than that of the corresponding maternal serum. These antibodies may possibly be of the "incomplete" variety.

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Table 1. Confirmation of the absence of Rantz antigen by thermal testing at different pH values for 20 min.

Serum Dilution Reciprocal Units	pH 2.2		pH 7.0		pH 11.5	
	24 C	100 C	24 C	100 C	24 C	100 C
2	4+	4+	4+	4+	4+	4+
4	4+	4+	4+	4+	4+	4+
8	4+	4+	4+	4+	4+	3+
16	3+	3+	3+	3+	3+	2+
32	2+	2+	2+	2+	2+	1+
64	2+	1+	1+	1+	1+	1+
128	1+	±	1+	1+	±	-
256	±	-	±	-	-	-
512	-	-	-	-	-	-

Table 2. Determination of optimal concentration of antigen for maximal sensitization of erythrocytes.

Serum Dilution Reciprocal Units	Mg per ml of Antigen					
	10	5	2.5	1.0	0.5	0.1
2	4+	4+	4+	4+	4+	4+
4	4+	4+	4+	4+	4+	4+
8	4+	4+	4+	4+	4+	3+
16	4+	4+	4+	4+	3+	2+
32	3+	3+	3+	3+	3+	1+
64	3+	3+	3+	3+	2+	-
128	2+	2+	2+	2+	1+	-
256	1+	1+	1+	1+	±	-
512	-	-	-	-	-	-

Table 3. Determination of optimal pH range for maximum sensitization of erythrocytes.

Serum Dilution Reciprocal Units	pH				
	6.0	6.5	7.0	7.5	8.0
2	3+	4+	4+	4+	4+
10	2+	3+	4+	4+	3+
20	2+	2+	3+	3+	3+
40	1+	1+	2+	2+	2+
80	±	1+	1+	1+	1+
160	-	±	1+	1+	±
320	-	-	-	-	-

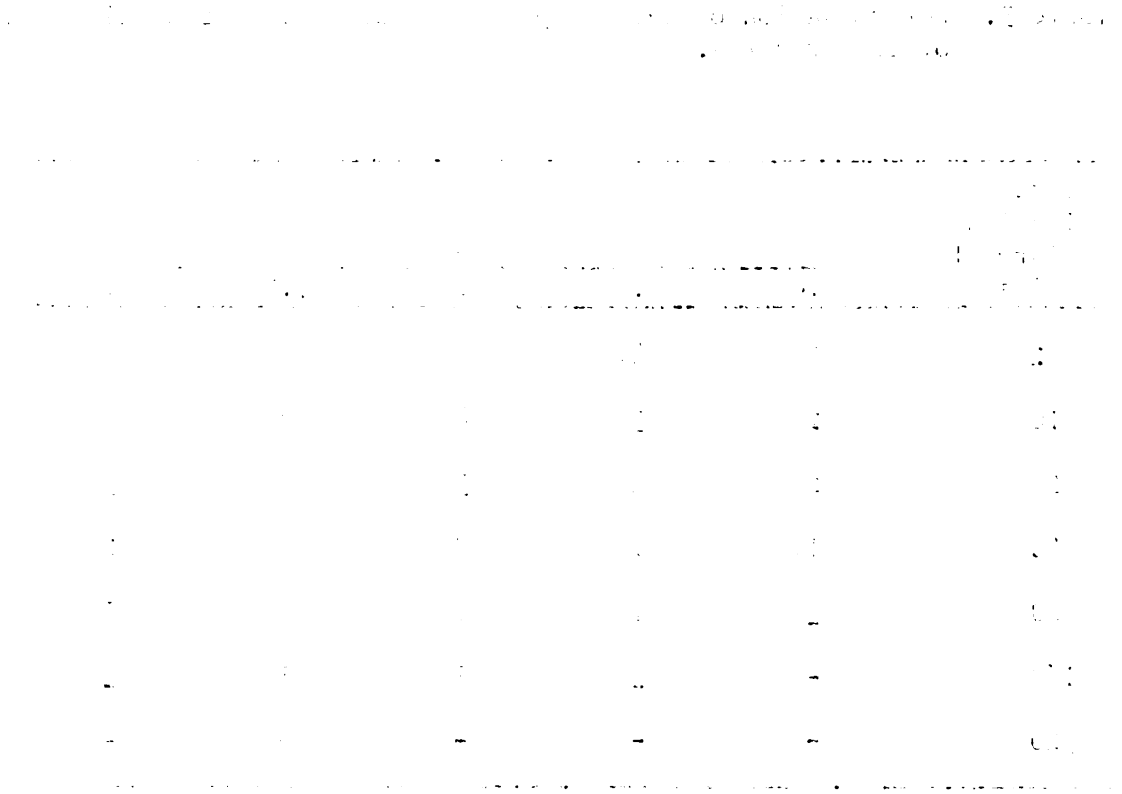

Table 4. Determination of optimal incubation time for maximum sensitization of erythrocytes.

Serum Dilution Reciprocal Units	Time in minutes				
	5	10	15	30	60
2	2+	3+	4+	4+	4+
4	2+	3+	3+	4+	4+
8	2+	2+	3+	4+	4+
16	2+	2+	2+	3+	4+
32	1+	2+	2+	3+	3+
64	1+	1+	1+	2+	2+
128	-	1+	1+	1+	2+
256	-	-	-	±	1+
512	-	-	-	-	-

Table 5. Determination of incubating temperature for maximum sensitization of erythrocytes.

Serum Dilution Reciprocal Units	Temperature			
	4 C	27 C	37 C	45 C
2	±	3+	4+	4+
4	-	2+	4+	4+
8	-	2+	3+	4+
16	-	1+	3+	3+
32	-	±	2+	3+
64	-	-	2+	2+
128	-	-	1+	2+
256	-	-	-	1+
512	-	-	-	±

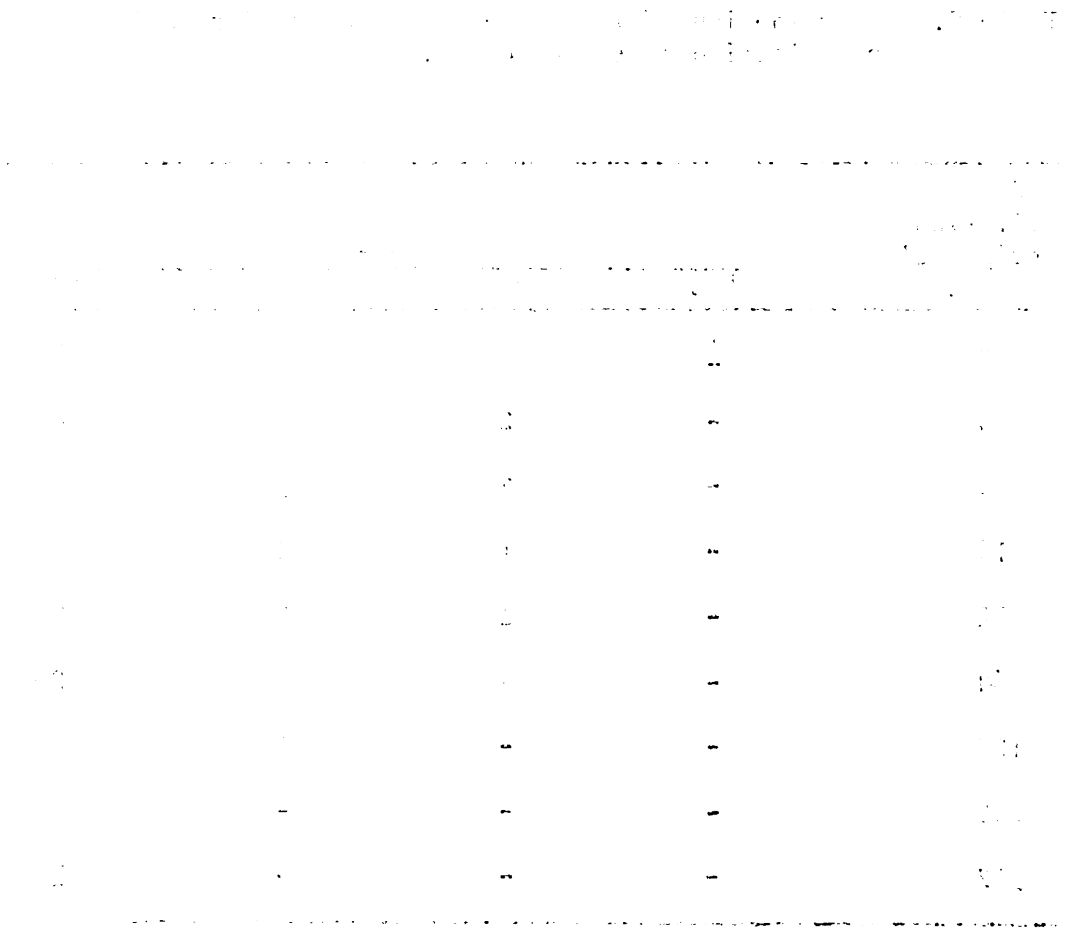

Table 6. Determination of electrolyte effect on sensitization of erythrocytes.

Sensitization of Erythrocytes	Electrolyte Concentration (Molar)				
	NaCl	$CaCl_2$	Sodium Citrate	$MgCl_2$	$MnCl_2$
Optimal	0.03 to 0.15	not optimal to 0.1	0.05 to 0.1	not optimal to 0.1	No Adsorption
Minimal	0.015	0.001	0.01	0.002	No Adsorption
Negative	0.008	0.0001	0.005	0.0002	No Adsorption

[illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 7. Determination of erythrocyte concentration for maximum hemagglutination.

Serum Dilution Reciprocal Units	Final Erythrocyte Concentration				
	10%	5%	2.5%	1.25%	0.62%
2	2+	2+	3+	4+	4+
4	1+	2+	3+	4+	4+
8	1+	1+	2+	3+	3+
16	-	1+	2+	3+	3+
32	-	±	1+	2+	2+
64	-	-	±	1+	1+
128	-	-	-	1+	±
256	-	-	-	±	-
512	-	-	-	-	-

Table 8. Comparison of the anti-globulin and enzyme modifications with the basic hemagglutination procedure.

Serum Dilution Reciprocal Units	Basic HA Procedure	Anti-Globulin Modification	Bromelase Modification		
			Before Sensitization	During Sensitization	After Sensitization
2	4+		4+	4+	4+
4	4+		4+	4+	4+
8	3+		4+	4+	4+
16	3+		4+	4+	4+
32	2+	4+	3+	3+	3+
64	1+	4+	3+	2+	2+
128	±	3+	2+	1+	1+
256	-	2+	2+	1+	±
512	-	1+	1+	-	-

Table 9. Range of staphylococcal hemagglutinins in maternal and cord sera.

Number of Samples	Serum Dilution (reciprocal units)									
	2	4	8	16	32	64	128	256	512	1024
Maternal Sera	0	0	0	1	2	4	9	8	4	2
Cord Sera	4	10	7	4	5	0	0	0	0	0

[redacted]

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