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CHAPTER I

INTRODUCTION

Direct Hemagglutination

It has been known for many years that red blood cells could be agglutinated by certain microbial agents. Kraus and Ludwig (1902) first described direct bacterial hemagglutination with Micrococcus and Vibrio species. Subsequently, a number of other bacteria has been described as possessing this activity. Fukuhara (1909) described this principle with Sarcina and Salmonella species, of Escherichia coli, Vibrio comma, and Corynebacterium diphtheriae. Guyot (1908) also described the hemagglutinative activity with Escherichia coli. The species of bacteria that hemagglutinate share no taxonomic relationships. Among the members of the family Enterobacteriaceae, for example, the Alkalescens-Dispar group are active (Gupta, 1950) and the other Shigella groups are inactive (Griffitts, 1948). Even within the same species, Kauffmann (1948) has shown that there are hemagglutinative and non-hemagglutinative strains.

The donor species of the red blood cells may play a part as to the hemagglutinative activity of certain bacterial species. Most strains of the Alkalescens-Dispar group are active with human cells,

while only a few strains agglutinate animal cells such as rabbit, sheep and guinea pig. The reason for these differences in activity of various bacteria toward erythrocytes from various animal species is not clear at the present time.

Many of the hemagglutinative antigens have been extensively studied. Fisher (1948 and 1950) studied the antigen of Hemophilus pertussis and found that it is heat labile and is inhibited by lipid components extracted from red blood cells. He was also able to show that the erythrocyte receptor for the H. pertussis hemagglutinin differs from the receptor for influenza virus (Fisher, 1949). Lowenthal and Lamanna (1951) were able to demonstrate that the hemagglutinative factor of Clostridium botulinum was distinct from its toxin. The hemagglutinative factor of Corynebacterium diphtheriae was found by Learned and Metcalf (1952) to be an antigenic lipid.

The receptor of the red blood cell which acts with the hemagglutinative principle of bacteria is not identical with the receptor of influenza virus because neither influenza virus nor the receptor destroying enzyme (described on page 4) interferes with direct bacterial hemagglutination. Since certain lipids inhibit bacterial hemagglutination, it is the general opinion that lipid components of the red blood cells may serve as receptors.

This hemagglutinative capacity is not restricted to bacteria nor are erythrocytes the only cells that can be agglutinated. Since the studies of Landsteiner and Raubitschek (1907) it has been known that the seeds of certain plants, such as Ricinus communis and a number of legumes, contain substances which agglutinate human red blood cells.

Boyd and Reguera (1949) suggested the use of seed extracts in the typing of human blood groups. Bachrach et al. (1957) studied extracts of plants of Israel and found 12 extracts that agglutinated red blood cells of all human blood groups and two that were active against groups A_1 and A_2. Agglutinative substances have been found in fungi by Bernheimer and Farkas (1953), and O'Conner (1945) showed the hemagglutination phenomenon with Rickettsia orientalis.

The direct principle of hemagglutination with viruses was described independently by Hirst (1941) and McClelland and Hare (1941). They observed that when fluid from an embryo infected with influenza virus was mixed with fowl red blood cells, gross clumping occurred. This discovery has had great practical importance for the identification and titration of viruses and as a method of concentration of viruses. It also serves as a model for studying virus-cell interaction. Hirst (1942_a) continued the work and devised a method to determine the hemagglutinative titer of infected fluid by the measurement of the number of red blood cells removed from the supernatant fluid. This method of titration does not have the widespread use to-day as does the sedimentation method of Salk (1944). However, the early work of Hirst (1942_b), established three facts concerning the hemagglutination phenomenon. They were: (1) hemagglutination is associated with the adsorption of virus to the cells, (2) the hemagglutinative titration gives a direct measure of the amount of virus present, and (3) there is an elution of the virus, when incubated at 37 C for several hours. The red blood cells from which the virus particles have eluted lose their power to readsorb virus, but the liberated virus retains its hemagglutinative power.

The essential reactions between influenza virus and the red blood cells are adsorption and elution with a modification of the red cell surface. Hirst (1942b) interpreted this sequence as an action of an enzyme incorporated at the viral surface. Separation of enzymatic activity from the viral particle has not been reported, but enzymes from filtrates of bacterial species have been obtained that have similar reactions toward the red cell surface. McCrea (1947) reported an enzyme in Clostridium welchii filtrates that was capable of rendering red cells insusceptible to hemagglutination by influenza viruses. Burnet and Stone (1947) demonstrated a more active enzyme preparation in Vibrio cholerae cultures. This enzyme has come to be known as the receptor destroying enzyme (RDE). In many respects it is identical with the enzyme of the influenza viruses.

Francis (1947) observed that all normal sera contained a substance capable of inhibiting hemagglutination by heated influenza virus B. This finding stimulated research on the characterization of the receptor of the red cell surface. When allantoic fluid infected with the Lee strain of influenza B virus is heated to 55 C for thirty minutes it does not lose its power of hemagglutination; it does lose its power of elution. The capacity of the virus to act as an enzyme has been lost (Briody, 1948). When heated virus was used to test the hemagglutinative inhibitory titers of normal and immune sera, very high titers were obtained with both types of sera. This inhibitor in normal serum, called the Francis inhibitor or alpha inhibitor, can be destroyed both by RDE and by active influenza virus (Anderson, 1948). This led to the discovery of a wide variety of physiological secretions and tissue extracts that

have a similar inhibitory activity (Burnet et al., 1947). All of these substances are of a complex nature and contain mucoid or mucoprotein material. Some of these mucoid materials have been found in human red blood cells (DeBurgh et al., 1948), mucus from human cervix (Burnet, 1948), human and pork lung (Rice and Stevens, 1957), meconium (Curtain et al., 1953), egg-white (Gottschalk and Lind, 1949), human urine (Tamm and Horsfall, 1950), and an extract from the submandibular salivary gland of the sheep (McCrea, 1951). Certain plant products such as apple pectin (Green and Woolley, 1947) will inhibit virus hemagglutination.

The inhibitor isolated from urine by Tamm and Horsfall in 1950 was found to be an unusual type of mucoprotein. It was soluble in distilled water, but became less soluble in saline as the salt concentration was increased. It behaved like the alpha inhibitor and was the first inhibitor preparation obtained in a chemically pure state (Tamm and Horsfall, 1952) (Perlman et al., 1952). The inhibitor in egg-white, on the other hand, could be partially purified by dilution in a large volume of distilled water where it was precipitated in the ovomucin fraction (Gottschalk and Lind, 1949). This ovomucin fraction was shown by Sharp et al. (1951) to contain three components, when subjected to electrophoresis, only two of which were biologically active.

In addition, there is, in normal human serum, another kind of inhibitor of hemagglutination designated "beta inhibitor" or "Chu inhibitor" (Chu, 1951). It can be readily demonstrated by using chicken red blood cells and unheated preparations of recently isolated influenza A virus which has not been adapted to mice. This inhibitor may

neutralize the infectivity of influenza, mumps, and Newcastle disease viruses. It is not inactivated by crystalline trypsin (Sampaio and Isaacs, 1953). In electrophoretic studies of serum, Tyrrell (1954) was able to separate specific antibodies, alpha inhibitor and beta inhibitor against influenza viruses. He demonstrated that the specific antibodies migrated with the gamma globulin, the beta inhibitor with the beta globulin and the alpha inhibitor with the alpha globulins.

Hirst (1949) and Fazekas de St. Groth (1949) observed that changes in the virus-red blood cell combination could be produced by treatment with very dilute periodate solutions. Fazekas de St. Groth treated red blood cells with metaperiodate and reported that, while adsorption of influenza virus occurred, spontaneous elution did not occur. Hirst treated the influenza virus with sodium periodate and determined also that it was adsorbed well on red cells but lacked the capacity for spontaneous elution. With larger amounts of periodate the cells become incapable of adsorbing virus. This evidence points strongly toward the likelihood that the specific configuration of certain carbohydrate units is responsible for adsorption of the virus and susceptibility to enzyme action. The action of the enzyme is not to break up the carbohydrate macromolecules but to split off functional groups. According to Gottschalk (1951), these functional groups are probably "N-substituted isohexosamine units" attached to a hexose unit of the main part of the molecule. The functional units may be chemically identical but differ in their accessibility to enzyme attack. These differences are probably responsible for the "receptor gradient" described by Burnet (1945) and the "inhibitor gradient" described by Stone (1949) and Hirst (1950).

When red blood cells are treated with the specific enzyme either in the form of active virus or RDE there is a characteristic sequence of changes. The cells show minor changes in their immunological reactivity (Burnet and Anderson, 1947), they lose their capacity to be agglutinated by viruses of the mumps-influenza-Newcastle disease group (Hirst, 1950), and they show diminished electrophoretic mobility (Hanig, 1948) (Ada and Stone, 1950).

While most of the direct viral hemagglutinative studies have been made with the influenza viruses, the range of hemagglutinative viruses is more extensive. All strains of influenza have been shown to agglutinate erythrocytes (Burnet and Bull, 1943). Newcastle disease virus (Burnet, 1942), pneumonia virus of mice (Mills and Dochez, 1944), and the viruses of mumps (Levens and Enders, 1945), fowl plague (Lush, 1943), vaccinia (Burnet, 1945) and ectromelia (Burnet and Boake, 1946) all agglutinate red blood cells. Under special conditions the Theiler virus strain GD VII (Lahelle and Horsfall, 1949), Japanese B encephalitis virus (Sabin and Buescher, 1950) and St. Louis, West Nile and Russian spring-summer encephalitis viruses (Sabin, 1951) will hemagglutinate.

Moolton and Clark (1951) reported the detection of herpes simplex virus in the circulating blood of patients exhibiting varying degrees of autohemagglutination and hemolytic anemia. They believed that their detection of virus in the red blood cell in vivo paralleled, in certain respects, viral hemagglutination in vitro. Their work has not been confirmed. Geller et al. (1953) state that hemagglutination with herpes simplex virus has not been demonstrated conclusively either in vivo or in vitro.

Cells other than erythrocytes may be agglutinated. Rosenthal (1943) found that Escherichia coli caused clumping of leukocytes, thrombocytes, spermatozoa, pollen and spores of molds.

Indirect Hemagglutination

Several types of indirect hemagglutination can be demonstrated as shown in Table I. They are (1) conditioned hemagglutination, (2) hemagglutination of erythrocytes treated with antigen-antibody mixtures, (3) red blood cell linked antigen hemagglutination, (4) the Thomsen-Friedenreich hemagglutination phenomenon, (5) hemagglutination by antibodies against chemically attached protein antigens, and (6) hemagglutination of tannic acid pretreated erythrocytes. Each of these types requires a modification or an alteration in the red blood cell. They also require the action of the specific antibody to produce agglutination in contrast to the direct hemagglutination in which case the specific antibody inhibits the agglutination.

Conditioned Hemagglutination

A large variety of bacterial species contain antigens that are readily adsorbed on untreated red blood cells, thereby rendering these modified erythrocytes specifically agglutinable by homologous antibodies. Almost without exception these erythrocyte modifying antigens contain polysaccharides as the active component. Little is known regarding the mechanism involved in the modification of the erythrocyte. The microbial antigen is adsorbed on specific receptors on the red blood cell surface thereby sensitizing the erythrocyte. The receptors for the bacterial antigens are, evidently, specific since the activities of A, B, or Rh

TABLE I

TYPES OF HEMAGGLUTINATION WITH MICROBIAL AGENTS

Types	Reaction Sequence								
Direct (bacterial or viral)	MA	+	RBC	=					HA
Indirect									
Conditioned	MA	+	RBC	=	modified RBC	+	Ab	=	HA
Treated with antigen-antibody mixtures	MA + Ab	+	RBC	=					HA
Red cell linked antigen	MA + complex*	+	RBC	=	modified RBC	+	Ab	=	HA
Thomsen-Friedenreich phenomenon	bacterial enzyme	+	RBC	=	altered RBC	+	RBC Ab	=	HA
Chemically attached antigens	MA + chemical complex	+	RBC	=	modified RBC	+	Ab	=	HA
Tannic acid pretreated cells	MA	+	tanned RBC	=	modified RBC	+	Ab	=	HA

* = Antibody against protein of RBC
MA = Microbial antigen
HA = Hemagglutination
RBC = Red Blood Cells
Ab = Antibody

antigens present on the red blood cells are not blocked when Escherichia coli is adsorbed on these erythrocytes (Neter et al.,1952). The treatment of red blood cells with RDE does not interfere with modification of the cell by bacterial polysaccharide (Hayes, 1951). Lipid structures probably serve as the receptors as it has been observed by Neter et al. (1955) that attachement of bacterial antigens to the cells can be inhibited by certain lipids such as lecithin, cholesterol and serum lipoproteins. It has been shown by Neter et al. (1952 and 1956) and Landy (1954) that several bacterial antigens can be adsorbed on the red blood cell without one antigen blocking the adsorption of another. This observation implies that there are receptors specific for each bacterial antigen. However, Spaun (1952) found interference between the O antigen and Vi antigen of Salmonella typhosa when these antigens were mixed. Spaun's observations have been confirmed by Landy and Ceppellini (1955). Neter and Zalewski (1953) demonstrated that electrolytes are necessary for the adsorption of these bacterial antigens on the red cells. They noticed that the modification did not occur in 5 per cent glucose solution but did occur in the presence of isotonic sodium chloride, potassium chloride or sodium citrate solutions. The modification took place in a few minutes to an hour at 37 C but only to a slight extent at 4 C. Neter et al. (1952) showed that erythrocytes from man, dog, rabbit, sheep, guinea-pig, rat and chicken were equally well modified by E. coli antigen. Boyden (1950) found that different amounts of the bacterial antigen were needed for modification of erythrocytes from different animal species.

With this modification, the red blood cells acquire a new serological specificity. Homologous bacterial antibodies cause specific

agglutination. The process is not limited to bacterial antigens nor to erythrocytes. Chang (1953) obtained from rickettsiae an erythrocyte-sensitizing substance (ESS) which modifies red blood cells and allows subsequent hemagglutination in the presence of homologous antibodies. A similar procedure has been demonstrated by Muniz (1950) with trypanosomes, by Kagan (1955) with schistosomes, by Vogel and Collins (1955) with Candida, by Norden (1949) with Histoplasma and by McEntegart (1952) with Trichomonas.

Boyden (1953) observed that the bacterial antigen could be adsorbed on leukocytes, and Adler (1952) succeeded in attaching bacterial antigens to heterologous bacteria. Bacterial antigens can also be adsorbed onto particles other than cells, such as collodion particles and ion exchange resins (Evans and Haines, 1954). Nelson (1953) described a reaction between bacteria and erythrocytes known as the immune-adherence phenomenon. He observed the attachment of spirochetes and Diplococcus pneumoniae to red blood cells if homologous antibodies and complement were present.

Hemagglutination of Erythrocytes Treated with Antigen-Antibody Mixtures

Boyden and Anderson (1955) made the interesting observation that mixtures of tuberculo-protein and certain rabbit antisera against the tubercle bacillus agglutinate normal sheep erythrocytes. They also found that stronger agglutination was obtained when the globulin fraction of antiserum was used than when whole serum was used. The suggested explanation is the removal of a normal serum inhibitor in the albumin

fraction. When complement was added to the system, hemolysis, not hemagglutination occurred. This reaction differs from that of the conditioned hemagglutination test in that the antigen is a protein instead of a polysaccharide. The reaction becomes dissociated upon repeated washings. The sensitization of the erythrocytes with the antigen-antibody mixture and subsequent hemagglutination takes place readily at both 37 C and 4 C. Middlebrook (1952) made a similar observation and assumed that the reaction was due to the red blood cell sensitizing polysaccharide. If this were the case, then it would differ from the reaction observed by Boyden.

Red Cell Linked Antigen Hemagglutination Test

A red blood cell linked antigen hemagglutination test was devised by Coombs et al. ($1945_{a,b}$) as a means of detecting weak and "incomplete" Rh agglutinins. These workers coupled nonbacterial protein antigens to incomplete Rh antibodies (Coombs et al., 1952), to ox red blood cell antibodies (Coombs et al., 1953), and to Forssman antibodies (Coombs and Fiset, 1954) by chemical means. By themselves such erythrocyte antibodies do not cause hemagglutination but they serve as carriers for the protein. Erythrocytes treated with such an antigen-antibody complex then become agglutinable in the presence of homologous antibodies to the artificially attached antigen. The application of the antiglobulin technique of Coombs has been described by Wilson and Merrifield (1951) with Brucella agglutinins. By this method they showed a significant titer in 17 cases which were negative by conventional methods. The Coomb's technique has also been applied by Morgan and

Schutze (1946) with Shigella shiga and Salmonella typhi and by Stewart and McKeever (1950) with "O" antigen suspensions of Salmonella typhi.

The Thomsen-Friedenreich Hemagglutination Phenomenon

Thomsen (1927) observed that occasionally a blood specimen with bacterial contamination would be changed so that the erythrocytes became panagglutinable. An extensive investigation of this phenomenon which is known as the Thomsen-Friedenreich hemagglutination phenomenon was carried out by Friedenreich (1930). Friedenreich demonstrated that the filterable fraction of bacterial cultures contained an active substance capable of transforming the latent receptors of the red blood cells to an active antigen, the "T antigen." Studies of the activity and the physical properties of the bacterial filtrate indicated that the substance was an enzyme or enzymes. T agglutinins are present in normal human adult sera but absent in the sera of newborns (Friedenreich, 1928). The changes in the red cells are very similar to those produced with influenza virus. Likewise, they are similar to the changes brought about in the red blood cells with the receptor destroying enzyme of Vibrio cholera. Both the virus and the RDE cause changes in addition to panagglutinability. Chu (1948) showed that many bacterial species cause panagglutinability of erythrocytes without some of the other changes associated with RDE. Study of the activity of bacterial enzymes which cause panagglutination has revealed important information on the chemical identity of the receptor. Other well known enzymes such as trypsin (Morton and Pickles, 1947), and ficin (Weiner and Katz, 1951) can effect these changes.

The biological significance of this pehnomenon lies in the fact

that contamination of a blood specimen with panagglutinative bacteria leads to erroneous results in blood grouping. Very little is known concerning the activity of this phenomenon *in vivo*, however, Wright and Stein (1951) were able to demonstrate a decrease in titer of T agglutinins in experimental anthrax infection in rabbits.

Hemagglutination by Antibodies against Chemically Attached Protein Antigens

A method was devised by Pressman *et al.* (1942) to attach protein antigens to erythrocytes by chemical linkage. The method employs bis-diazotized benzidine as the protein conjugating material. Stavitsky and Arquilla (1955) applied this method to the study of diphtheria toxin. Cole and Farrell (1955) coupled tuberculin purified protein derivative (PPD) to formalinized erythrocytes by this method and obtained agglutination of these cells when exposed to PPD antibody. Their method had advantages over some of the other methods, in that the formalinized erythrocyte preparation was stable. Cole *et al.* (1955) applied their technique to the sera of hospital tuberculous patients and noted significant titers in 92 per cent of the specimens. This hemagglutinative method warrants more extensive trial with other microbial agents both as a means of measuring bacterial protein and as a diagnostic criterion.

Hemagglutination of Tannic Acid Pretreated Erythrocytes

Boyden (1951) used a number of substances to treat the red blood cells in an effort to render them capable of adsorbing protein antigens. Certain inulin preprations were found to be effective, but tannic acid proved superior to all others. The technique involves pretreatment of

the red blood cells with very dilute solutions of tannic acid. The pretreated cells are then exposed to the protein antigen and thus acquire a new serologic specificity as evidenced by specific agglutination in homologous anti-protein serum. This method has been shown by Stavitsky (1954_a) to be very sensitive. He found that amounts as small as 0.001 microgram of diphtheria toxoid could be measured accurately. The homologous antibodies can be titrated by this hemagglutinative test and the antigens can be titrated by hemagglutination-inhibition. The tannic acid pretreated erythrocytes are capable of adsorbing two antigens simultaneously as shown by Stavitsky (1954_b) with diphtheria and tetanus toxoids. Polysaccharides may also be adsorbed onto the tannic acid treated cells but there is a difference in time and temperature. Chen and Meyer (1954) used a temperature of 37 C for one hour for polysaccharide adsorption and room temperature for 15 minutes for the protein adsorption. Not all protein antigens can be adsorbed on the tannic acid cells. Working with various chemical fractions of the tubercle bacilli, Boyden and Sorkin (1955) observed that some fractions were more active than others. They also observed that different antibodies could be detected by the tannic acid hemagglutination test depending on which chemical fraction was used to sensitize the tanned cells.

The tannic acid hemagglutination test has been used to study many antigen-antibody reactions. Boyden and Sorkin (1955) have studied the tuberculo-proteins extensively by this method. Stavitsky (1954_b) has used the method for assays of antitoxins against Corynebacterium and Clostridium. Boyden (1951) investigated the protein of Streptococcus pyogenes, and the proteins and toxins of the plague bacillus were

studied, with this method, by Chen and Meyer (1954), McCrumb et al. (1955), Landy and Trapani (1954) and Warren et al. (1955). Kagan (1955) employed this method with schistosome antigens, and Kagan and Bargai (1956) used the method in the study of trichinosis. Feinberg et al. (1956) applied this tannic acid method to the detection of antibodies to certain pollen extract antigens in the sera of treated and untreated hayfever patients. The tannic acid technique has been utilized to test for rheumatoid arthritis by Heller et al. (1954). They sensitized the tannic acid treated sheep erythrocytes with human plasma fraction II (gamma globulin) and added it to serial dilutions of the test serum. This was designated the FII test and should not be confused with the test in which untreated sheep cells were sensitized with non-agglutinating concentrations of rabbit serum containing sheep erythrocyte agglutinins (SEA).

The development of a new serological technique for the detection and study of herpes simplex virus and anti-herpes simplex agglutinins, and an evaluation of the critical factors involved in this technique are presented in this dissertation. Experiments which provide evidence for the specificity of the reaction will be described.

CHAPTER II

MATERIALS AND METHODS

Viral Antigen

The HF strain of herpes simplex virus, obtained from the Communicable Disease Center, Montgomery, Alabama, was used as seed inoculum. It had been propagated 60 times on the chorio-allantoic membranes (CAM) and 9 times in the yolk sacs (YS) of developing chick embryos, 3 times in the brains of white Swiss mice, and twice on the corneas of rabbits. Seed virus was inoculated into the YS of the chick embryo and the infected allantoic fluid was harvested upon death of the embryo. The virus infected allantoic fluid was partially purified and concentrated by differential centriguation at 450 X G for 15 minutes followed by centrifugation at 24,500 X G for two hours. This cycle was repeated three times in order to remove as much of the host protein as possible. After each high speed centrifugation pH 7.2 buffered saline was added to the sediment which was then incubated at 4 C overnight to allow resuspension of the virus. After the last centriguation the virus was resuspended in buffered saline to make a ten-fold concentration. The concentration and purification protocol is illustrated in Figure 1.

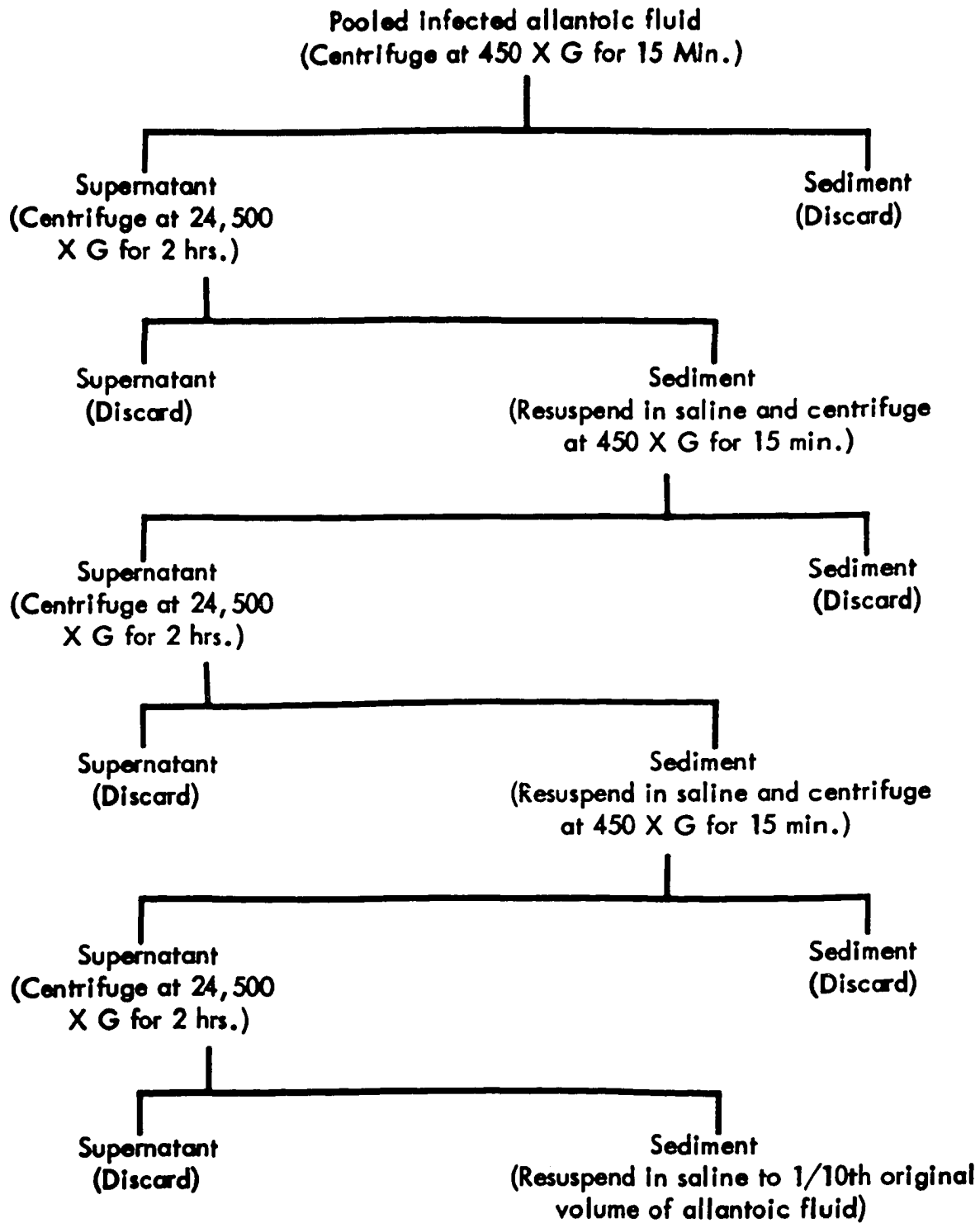

Fig. 1 - Protocol for concentration and purification of viral antigen by differential centrifugation.

Antisera

Pre-immunization sera were obtained from rabbits for use as controls. Herpes simplex virus antisera were produced in rabbits in response to a series of subcutaneous injections over a period of six weeks. A buffered saline suspension of herpes simplex infected mouse brain which had been inactivated with formalin (1 part per thousand) was injected during the first part of the immunization schedule and a buffered saline suspension of active virus infected mouse brain was injected during the last part. The immunization schedule is shown in Table II. Blood samples taken from the rabbits four weeks after the first injection were tested for hemagglutinative titer. Immunization was continued for two weeks more. Large quantities of post-immunization sera were obtained from the rabbits by cardiac puncture one week after cessation of inoculations. All sera were inactivated by heating at 56 C for 30 minutes and were adsorbed with tannic acid treated sheep erythrocytes to remove non-specific agglutinins.

Buffered Saline

Buffered saline was prepared by mixing equal volumes of 0.85 per cent saline and phosphate buffer solution. The phosphate buffer solution was obtained by mixing the proper amounts of 0.15 M Na_2HPO_4 and 0.15 M KH_2PO_4 to give the desired pH. Unless otherwise stated pH 7.2 buffered saline was used.

Erythrocytes

Whole sheep blood was collected at a local abattoir and stored

TABLE II

IMMUNIZATION SCHEDULE FOR PRODUCTION OF HERPES SIMPLEX VIRUS ANTISERA IN RABBITS

Time of Inoculation*	Amount of Inoculum	Type of Inoculum
1st week		
Mon.	0.5 ml.	Formalinized virus
Wed.	0.5 ml.	" "
Fri.	0.5 ml.	" "
2nd week		
Mon.	0.5 ml.	" "
Wed.	0.5 ml.	" "
Fri.	0.5 ml.	" "
3rd week		
Mon.	0.25 ml.	Active virus
Wed.	0.25 ml.	" "
Fri.	0.25 ml.	" "
4th week		
Mon.	0.25 ml.	" "
Wed.	0.25 ml.	" "
Fri.	0.5 ml.	" "
Rabbit sera tested for antibody content.		
5th week		
Mon.	0.5 ml.	Formalinized virus
Wed.	0.5 ml.	" "
Fri.	0.5 ml.	" "
6th week		
Mon.	0.5 ml.	Active virus
Wed.	0.5 ml.	" "
Fri.	1.0 ml.	" "
Sat.	2.0 ml.	" "
7th week		
Rabbits were bled for antisera.		

*Subcutaneous injections

in an equal volume of sterile modified Alsever's solution (Bukantz et al., 1946). Sterile pooled citrated chicken blood, rabbit blood and horse blood were obtained from Chappel Laboratories, West Chester, Pennsylvania. Human red blood cells (group "O") suspended in citric acid-dextrose solution (Strumia et al., 1947) were obtained from a local blood bank. The erythrocytes were washed three times in buffered saline, packed by centrifuging at 450 X G for 15 minutes and resuspended to the 2.5 per cent erythrocytes concentration used throughout this study.

Serum Diluent

A 1:100 dilution of pre-immunization rabbit serum in buffered saline was used for diluting the antiserum in the test. In one experiment normal chicken and sheep sera were also used to dilute the antiserum. All sera had been inactivated previously and adsorbed with tannic acid treated sheep erythrocytes. The use of the serum diluent prevents non-specific agglutination.

Tannic Acid

A 1:100 dilution of Mallinckrodt reagent grade tannic acid in 0.85 per cent saline was prepared and kept at 5 C as a stock solution. Dilutions for the various experiments were made as needed from this stock solution.

Tannic Acid Treated Cells

One volume of the suspension of 2.5 per cent sheep erythrocytes was mixed with an equal volume of freshly prepared 1:20,000 dilution of tannic acid. This mixture was incubated in a 37 C water bath for 10

minutes, centrifuged, washed once with one volume of buffered saline, and the resulting sediment was resuspended in one volume of the same saline.

Sensitization of Tannic Acid Treated Cells

Four volumes of pH 6.4 buffered saline, one volume of concentrated and purified virus, and one volume of tannic acid treated sheep erythrocytes were mixed in that order. The mixture was incubated for 15 minutes at room temperature, centrifuged, washed with two volumes of serum diluent and resuspended in one volume of the serum diluent. Three aliquots of tannic acid treated erythrocytes were sensitized with three dilutions of virus in buffered saline 1:10, 1:20, and 1:50, respectively. All of the tannic acid treated-virus sensitized erythrocytes were stored at 5 C and were used within 48 hours.

Adsorption of Immune Serum with Viral Preparation

Tannic acid treated and virus sensitized sheep erythrocytes were mixed in equal amounts with herpes simplex immune serum and allowed to incubate overnight at 25 C. The serum was removed, mixed with fresh tannic acid treated virus sensitized sheep erythrocytes, and again allowed to incubate overnight at 25 C. The virus which was adsorbed on the tannic acid treated erythrocytes had been inactivated prior to use. The serum was removed from the cells by low speed centrifugation followed by centrifugation at 24,500 X G for two hours to remove any excess virus. The serum was then sterilized by passing it through a Swinny filter. This serum will be referred to as tannic acid virus cell adsorbed serum (TVCS).

Preparation of Control Serum for Adsorption Studies

The above procedure was repeated with another aliquot of the same herpes simplex immune serum, except that the tannic acid treated sheep erythrocytes were not sensitized with the virus. This serum will be refered to as tannic acid cell adsorbed serum (TCS).

Hemagglutinative Titrations of Antisera

Serial dilutions of pre- and post-immunization sera were made in the serum diluent in 0.5 ml volumes. To each tube of the diluted sera 0.05 ml of the tannic acid treated-virus sensitized sheep erythrocytes were added. Results of titration were recorded according to Salk (1944) and Stavitsky (1954_a) after an incubation period of three hours at room temperature or after 16 hours at 5 C. A positive pattern of agglutination consisted of an uniform thin layer of red blood cells with irregular edges which covered the bottom of the test tube. The end point was the highest dilution of serum which gave this positive pattern. The negative pattern consisted of a round "button" of sedimented red blood cells with regular edges. Examples of positive and negative patterns are shown in Figure 2. The titration protocol is shown in Table III.

The controls, as shown in Table IV, include: (1) serum diluent plus tannic acid treated-virus sensitized cells, (2) serum diluent plus tannic acid treated cells sensitized by normal allantoic fluid concentrated in a similar manner as the virus infected allantoic fluid, (3) serum diluent plus tannic acid treated cells, (4) antiserum plus tannic acid treated cells, (5) antiserum plus untreated cells and virus, and

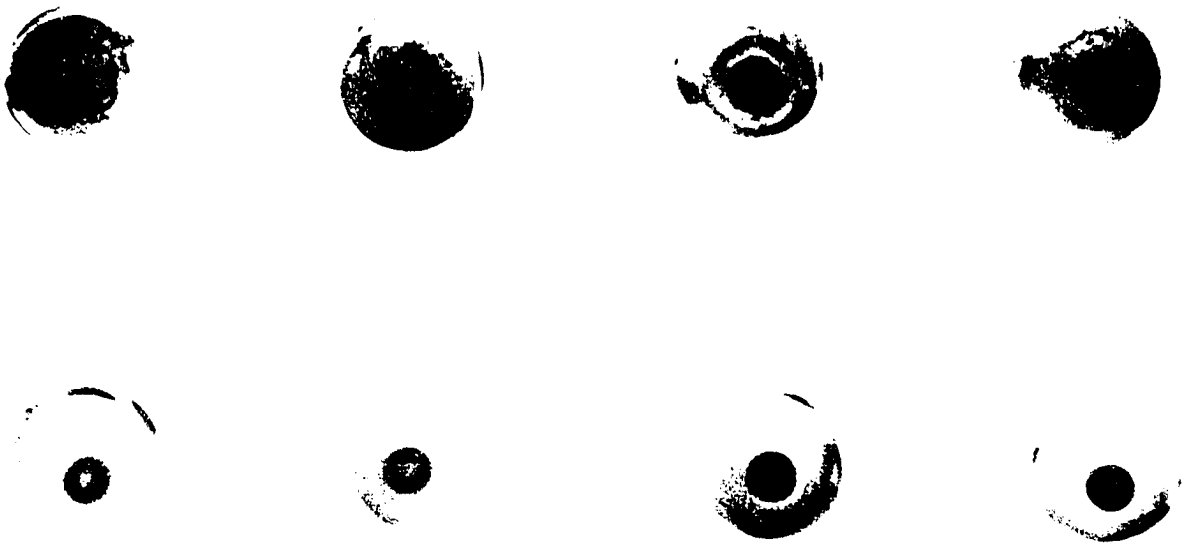

Fig. 2. Positive and negative sedimentation patterns. Top row shows positive patterns and bottom row shows negative patterns.

TABLE III

PROTOCOL FOR THE HEMAGGLUTINATION TITRATION OF ANTISERUM

Tube Number		1	2	3	4	5	6	7	8	9	10
1st	Diluted serum	0.5*	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6
Row	TAV 1:10	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
2nd	Diluted serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Row	TAV 1:20	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
3rd	Diluted serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Row	TAV 1:50	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
4th	Diluted serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Row	TAN	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

*Amounts shown are in milliliters.
TAV - Tannic acid treated-virus sensitized erythrocytes.
TAN - Tannic acid treated erythrocytes with normal concentrated allantoic fluid.

TABLE IV

PROTOCOL FOR THE CONTROLS USED WITH THE HEMAGGLUTINATION TITRATION OF ANTISERUM

Tube Number	1	2	3	4	5	6	7	8
Serum diluent	0.5*	0.5	0.5	0.5	0.5			
TAV 1:10	0.05							
TAV 1:20		0.05						
TAV 1:50			0.05					
TAN				0.05				
TAC					0.05	0.05		
Diluted Antisera						0.5	0.5	0.5
Untreated cells plus virus							0.05	
2.5% Suspension Sheep red cells								0.05

*Amounts shown are in milliliters.
TAV - Tannic acid treated-virus sensitized erythrocytes.
TAC - Tannic acid treated erythrocytes.
TAN - Tannic acid treated erythrocytes with normal concentrated allantoic fluid.

(6) antiserum plus 2.5 per cent cell suspension.

Virus Titrations in Presence of Saline, TVCS and TCS

Ten fold dilutions of 10^{-2} through 10^{-7} of the HF strain of herpes simplex virus were made in serum diluent. Each dilution of this virus was mixed with an equal volume of undiluted serum, TVCS or TCS. The mixtures of virus and serum were incubated in an ice-water bath at 8° C for one hour. After incubation, 0.05 ml of each mixture was inoculated onto the CAM of 12 days old developing chick embryos by the method described by Beveridge and Burnet (1946). These were incubated for 72 hours at 36 C, the CAM were harvested and infectivity of the membranes was determined. Herpetic plaques on the membrane denotes a positive reaction and a negative reaction was the lack of lesions on the membrane.

The controls included: (1) saline plus TVCS, (2) saline plus TCS, and (3) each dilution of virus plus saline. The protocol for the preparation of these mixtures is shown in Table V.

TABLE V

PROTOCOL OF MIXTURES FOR VIRUS INFECTIVITY TITRATIONS WITH TVCS, TCS AND SALINE

Tube No.	Virus Dilutions								
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	TVCS	TCS	Saline
1	0.5*						0.5		
2		0.5					0.5		
3			0.5				0.5		
4				0.5			0.5		
5					0.5		0.5		
6						0.5	0.5		
7	0.5							0.5	
8		0.5						0.5	
9			0.5					0.5	
10				0.5				0.5	
11					0.5			0.5	
12						0.5		0.5	
13	0.5								0.5
14		0.5							0.5
15			0.5						0.5
16				0.5					0.5
17					0.5				0.5
18						0.5			0.5
19							0.5		0.5
20								0.5	0.5

*Amounts shown are in milliliters.
TVCS - Serum adsorbed with tannic acid treated-virus sensitized erythrocytes.
TCS - Serum adsorbed with tannic acid treated erythrocytes.

CHAPTER III

RESULTS

Titration of Antisera

The results of typical hemagglutinative titrations obtained for the sera of 18 rabbits are shown in Table VI. The titers are expressed as the reciprocal of the serum dilutions. The titers ranged from 0 to 128. Titrations of each serum have been made several times with the same and different preparations of antigen and these titers are typical. Rabbit Number 18 in Table VI was immunized by the same procedure as the other 17 except that uninfected mouse brain, which was prepared in the same manner as the viral inoculum, was used instead of virus infected mouse brain. The control tests, performed according to the protocol in Table IV, were negative for hemagglutination.

The effect of the concentration of virus on hemagglutinative titers is shown in Table VII. When tannic acid treated sheep erythrocytes were sensitized by exposure to three different dilutions of the virus antigen, the hemagglutinative titers obtained varied directly with the concentration of the antigen.

The rabbits were bled during the immunization schedule at the end of four weeks to test the hemagglutinative titers. Table VIII shows the titers of sera before, during, and at the completion of, the immunization

TABLE VI

RESULTS OF TITRATION OF ANTISERA WITH HERPES SIMPLEX HEMAGGLUTINATION TEST

Rabbit Sera	Hemagglutination Titers of Sera*	
	Pre-immunization	Post-immunization
No. 1	0	32
No. 2	0	8
No. 3	0	16
No. 4	0	16
No. 5	0	16
No. 6	0	32
No. 7	0	16
No. 8	0	8
No. 9	0	8
No.10	0	4
No.11	0	32
No.12	0	16
No.13	0	64
No.14	0	32
No.15	0	32
No.16	0	32
No.17	0	128
No.18**	0	0

*Expressed as the reciprocal of serum dilution.

**Rabbit No. 18 was immunized with uninfected mouse brain instead of virus infected mouse brain.

of ten rabbits. It was not possible to demonstrate hemagglutinative antibodies in the sera of three of the rabbits at the end of four weeks. One week after the cessation of inoculations, however, antibodies were demonstrable in the sera of all ten rabbits.

Adsorption Studies as Evidence for Specificity

Hemagglutinative Titrations with TVCS, TCS and Saline

The hemagglutinative procedure was performed with the two sera, TVCS and TCS. Two-fold dilutions of the sera were made in serum diluent. Typical negative hemagglutinative reactions were obtained with titrations of TVCS, the serum from which all demonstrable specific antibodies had been removed by adsorption with herpes simplex virus - tannic acid treated erythrocytes. Positive hemagglutinative reactions were obtained with titrations of TCS, thus, demonstrating that the specific antibodies had not been removed from the serum adsorbed with tannic acid treated erythrocytes without virus. Table IX shows typical results obtained in hemagglutinative titrations with the two sera. It also shows results of the test using non-adsorbed serum as a positive control and using non-immune serum and saline as negative controls.

Virus Infectivity Titrations with Saline, TVCS and TCS

Typical results of repeated virus titrations with saline, TVCS and TCS are shown in Table X. The presence of herpetic lesions on the CAM was recorded as positive and the absence of herpetic lesions as negative. In the table, the numerator represents the number of positive

TABLE VII

EFFECT OF CONCENTRATION OF VIRUS ON HEMAGGLUTINATIVE TITERS OF ANTISERA

Post-immunization Sera	Hemagglutinative Titers with Varying Concentrations of Virus*		
	1:10 dilution	1:20 dilution	1:50 dilution
Rabbit No. 16	32	16	0
Rabbit No. 17	128	16	8

*Expressed as the reciprocal of serum dilution.

TABLE VIII

HEMAGGLUTINATIVE TITERS WITH SERA BEFORE, DURING AND AT COMPLETION OF IMMUNIZATION

Rabbit Number	Titers of Sera*		
	Before inoculations	After 4 weeks	After 7 weeks
1	0	4	32
2	0	4	8
3	0	16	16
4	0	0	16
5	0	4	16
6	0	32	32
7	0	8	16
8	0	0	8
9	0	4	8
10	0	0	4

*Expressed as the reciprocal of serum dilution.

TABLE IX

HEMAGGLUTINATIVE TITERS WITH TVCS, TCS AND SALINE

Sera	Hemagglutinative Titers*
TVCS	0
TCS	32
Saline	0
Unadsorbed immune	32
Unadsorbed non-immune	0

*Expressed as the reciprocal of the serum dilution.
TVCS - Serum adsorbed with tannic acid treated-virus sensitized erythrocytes.
TCS - Serum adsorbed with tannic acid treated erythrocytes.

TABLE X

VIRUS INFECTIVITY TITRATIONS WITH TVCS, TCS AND SALINE

Virus Dilution	TVCS	TCS	Saline
10^{-2}	4/4*	5/5	4/4
10^{-3}	6/6	3/5	5/5
10^{-4}	4/4	1/6	3/3
10^{-5}	5/5	1/3	5/5
10^{-6}	3/3	0/6	2/6
10^{-7}	0/4	0/5	0/6
Saline	0/2	0/5	—

*The numerator represents the number of positive membranes; the denominator the total number of embryos inoculated.

TVCS - Serum adsorbed with tannic acid treated-virus sensitized erythrocytes.

TCS - Serum adsorbed with tannic acid treated erythrocytes.

membranes; the denominator the total number of embryos inoculated with that particular mixture of virus and serum. It was demonstrated that the specific neutralizing antibodies were removed by adsorption with tannic acid treated-virus sensitized erythrocytes. In the immune sera which had not been adsorbed by the virus, the neutralizing antibodies remained. Results of titrations with saline-virus mixtures indicate the infectivity of the virus and can be compared with results of titrations of serum in which the neutralizing antibodies have been removed to show similarity between saline and adsorbed immune serum. It can be seen that there is 100 per cent infectivity with both TVCS-virus mixtures and saline-virus mixtures with the dilution of virus used as small as 10^{-5}.

Factors Influencing the Herpes Simplex Hemagglutination Test

In order to determine the optimum conditions necessary for the proper performance of this test, a number of factors involved in the preparation of reagents and the mechanics of the test were investigated. Two pools of sera were used. The first contained the pre-immunization sera of ten rabbits and will be referred to as the non-immune serum. The second pool contained the post-immunization sera of ten rabbits and will be referred to as the immune serum. These two pools of sera were used throughout these investigations in order to give a more uniform evaluation by the use of identical serum specimens. The concentration of virus used throughout these studies was 1:10 unless otherwise stated. The preparation of all materials and the procedures followed were as previously described except the modifications as will be pointed out in

each experiment that follows.

Effect of Size of Test Tube Used in the Test

The herpes simplex hemagglutination test was performed in triplicate, in which a different size test tube was used in each of the tests. The sizes of the test tubes were 10 mm X 75 mm, 12 mm X 75 mm and 13 mm X 100 mm. No differences were observed in titers of the sera obtained in the varicus sizes of test tubes and no differences were observed in the ease with which the sedimentation pattern could be read in the bottom of the tube. Because the 13 mm X 100 mm size test tube is standard equipment in most serological laboratories, it was chosen as the one to be used throughout this study.

Effect of Time of Incubation on the Test

The time which elapsed between the exposure of a serum to the tannic acid treated-virus sensitized erythrocytes and the reading of the sedimentation pattern was investigated for optimum results. Three different incubation times were employed: four hours, overnight and for three days. All tests were incubated at room temperature. The patterns obtained in four hours and overnight were comparable and resulted in identical titers. The test incubated for three days could not be read because the erythrocytes partially lysed and the sedimentation pattern was distorted.

Effect of Temperature of Incubation on Test

Four hemagglutination tests were performed which were alike except for the final incubation temperature. These tests were incubated at 4 C, 25 C, 37 C, and 56 C respectively for four hours at

which time no differences in titers were noted. After continued incubation overnight, the two tests incubated at 4 C and 25 C gave titers identical with those read at four hours, but lysis of the red blood cells incubated at 37 C and 56 C interferred with satisfactory readings of the test.

Effect of Concentration of Tannic Acid on Test

From tannic acid stock solution (1:200 dilution tannic acid in 0.85 per cent saline, pH 7.2) dilutions of 1:1000, 1:5000, 1:10,000, 1:20,000 and 1:50,000 were prepared. Sheep erythrocytes were treated with each of these dilutions of tannic acid before they were sensitized with the virus and used, subsequently, as the antigen in the hemagglutination test. Titers obtained with the various concentrations of tannic acid are presented in Table XI. It can be seen that the dilutions of 1:20,000 and 1:50,000 gave comparable results and that the use of higher concentrations of tannic acid resulted in a decrease or even elimination of hemagglutinative titer.

Effect on Test of pH of Saline in Virus Sensitization of Tannic Acid Treated Erythrocytes

Each of six aliquots of saline was buffered to a different pH, and to each aliquot virus and tannic acid treated erythrocytes were added. These preparations were then used as the antigens in the herpes simplex hemagglutination tests. The pH used for each aliquot and the results obtained are shown in Table XII. No hemagglutination occurred in test containing neutral or alkaline saline. The preparations with buffered saline of pH 5.2 and pH 5.8 gave titers of 4 and 8 respectively,

TABLE XI

THE EFFECT OF CONCENTRATION OF TANNIC ACID ON TEST

Concentration of Tannic Acid	Hemagglutinative Titers*	
	Immune Serum	Non-immune Serum
1:1000	0	0
1:5000	4	0
1:10,000	8	0
1:20,000	32	0
1:50,000	32	0

*Expressed as the reciprocal of the serum dilution.

TABLE XII

EFFECT ON TEST OF pH OF SALINE IN VIRUS SENSITIZATION OF TANNIC ACID TREATED ERYTHROCYTES

pH of Saline used in Virus Antigen Preparation	Hemagglutinative Titers*	
	Immune Serum	Non-immune Serum
pH 5.2	4	0
pH 5.8	8	0
pH 6.4	32	0
pH 7.0	0	0
pH 7.6	0	0
pH 8.2	0	0

*Expressed as the reciprocal of the serum diltuion.

whereas a titer of 32 was obtained with pH 6.4 buffered saline.

Effect on Test of Time of Exposure of Virus to Tannic Acid Treated Erythrocytes

To determine the optimum time for the sensitization of tannic acid treated erythrocytes with virus, buffered saline (pH 6.4), the virus, and tannic acid treated erythrocytes were mixed and the mixture was divided into seven equal portions. Each portion was allowed to incubate at room temperature for different periods of time which ranged from one minute to 24 hours. At the end of the designated time, each preparation was washed with the serum diluent, resuspended in serum diluent and then was used as the antigen in the herpes simplex hemagglutination test. The various incubation times during which the tannic acid treated erythrocytes were exposed to the virus and the influence of these times on hemagglutination titer are shown in Table XIII. Apparently a minimum exposure time of ten minutes was needed for adsorption of the virus on the erythrocytes; 30 and 60 minutes were equally effective. At six hours the non-immune serum gave a titer of 4, while the immune serum did not give as high a titer as those preparations incubated for shorter periods of time. In 24 hours the erythrocytes were partially hemolyzed and a satisfactory sedimentation pattern could not be seen.

Effect of Erythrocytes of Different Species on Test

Herpes simplex hemagglutination was performed with erythrocytes of five different species to determine if one kind was superior to another in the test. The erythrocytes of rabbit, sheep, chicken, horse

TABLE XIII

THE EFFECT ON TEST OF TIME OF EXPOSURE OF VIRUS TO TANNIC ACID TREATED ERYTHROCYTES

Time of Exposure	Hemagglutinative Titers*	
	Immune Serum	Non-immune Serum
1 minute	0	0
5 minutes	0	0
10 minutes	32	0
30 minutes	32	0
60 minutes	32	0
6 hours	16	4
24 hours	Not readable	Not readable

*Expressed as the reciprocal of the serum dilution.

and human (blood group "O") were treated individually with tannic acid and each was sensitized by the virus. When these preparations were used as the antigen in the herpes simplex hemagglutination test, the results shown in Table XIV were obtained. Sheep cells gave the most clear-cut results. The horse and human cells gave results comparable to the sheep cells with the immune serum but the sedimentation pattern with the non-immune serum was doubtful as it did not present the picture of either a clear-cut positive or negative reaction. The rabbit and chicken cells were hemolyzed when treated with tannic acid.

Effect on Test of Type of Serum Used for Serum Diluent

Normal rabbit serum, normal chicken serum, and normal sheep serum were tested for use as the diluent in the test. These sera were adsorbed with tannic acid treated sheep erythrocytes to remove any non-specific agglutinins. A 1:100 dilution of the serum of each animal species was used to dilute the antiserum in each titration. The titers obtained for the antisera thus diluted were reasonably comparable as shown in Table XV. However, when the diluent alone was mixed with the tannic acid treated-virus sensitized erythrocytes, absence of agglutination was obtained only with the rabbit serum. Both the chicken and sheep sera showed some agglutination in these control tubes. Because of the results obtained with the control tubes, rabbit serum was employed as the serum diluent in subsequent tests. An added advantage in using rabbit sera was that the pre-immunization sera from the rabbits could be used as the normal rabbit sera in the diluent.

TABLE XIV

EFFECT OF ERYTHROCYTES OF DIFFERENT SPECIES ON TEST

Erythrocytes Source	Hemagglutinative Titers*	
	Immune Serum	Non-immune Serum
Sheep	32	0
Horse	32	±
Human	32	±
Rabbit	Cells hemolyzed when treated with tannic acid.	
Chicken	Cells hemolyzed when treated with tannic acid.	

*Expressed as the reciprocal of the serum dilution.

±Reading doubtful. A typical positive or negative pattern was not obtained.

TABLE XV

EFFECT ON TEST OF TYPE OF SERUM USED FOR SERUM DILUENT

Serum Diluent	Viral Concentration	Hemagglutinative Titers*	
		Immune Serum	Non-immune Serum
NRS	1:10	32	0
NRS	1:20	16	0
NRS	1:50	0	0
NSS	1:10	64	4
NSS	1:20	32	4
NSS	1:50	0	0
NCS	1:10	64	4
NCS	1:20	32	0
NCS	1:50	16	0

*Expressed as reciprocal of the serum dilution.
NRS - Normal rabbit serum
NSS - Normal sheep serum
NCS - Normal chicken serum

Effect of Age of the Viral Antigen on the Test

Three preparations of viral antigen, prepared in a similar manner, were tested to see the effect of aging of the viral antigen on the test. The antigens were concentrated and partially purified by differential centrifugation and then maintained at 4 C until used in the tests. The three antigens were prepared in April, June and October of the same year. They were used in the herpes simplex hemagglutination test in December of the same year with a number of previously titrated immune and non-immune sera. Identical titers were demonstrated by all three antigen preparations. This experiment proved not only that the antigen remained stable when kept at 4 C, but also that different preparations of the viral antigen gave reproducible results.

Effect of Preservatives on the Viral Antigen

Viral antigen prepared as described above was separated into two aliquots. To one aliquot was added merthiolate to give a final concentration of 0.1 per cent. To the second aliquot formalin was added to give a final concentration of 0.3 per cent. These preparations were refrigerated at 4 C for approximately 25 months. They were then used as the antigen in the herpes simplex hemagglutination test with antisera which had been previously titrated. There were no differences in titers of the antisera when tested with freshly prepared or preserved virus. Nor was there any deviation in titers of the antisera between those titrations performed with formalinized virus or merthiolated virus.

Effect on Test of pH of Buffered Saline Used to Resuspend the Concentrated Virus

The last high speed centrifugation in the concentration and purification of the virus (fig. 1) gave a "button" of sedimented protein material which was resuspended in buffered saline to one-tenth the original volume of allantoic fluid. An experiment was devised to determine the effect of the pH of the buffered saline used to resuspend the virus. Six aliquots of virus infected allantoic fluid were concentrated and purified by differential centrifugation. The six aliquots were resuspended in saline buffered at pH values of 5.2, 5.8, 6.4, 7.0, 7.6 and 8.2, respectively and then used to sensitize tannic acid treated erythrocytes. The sensitized cells were then used in the herpes simplex hemagglutination test with antisera of known titer. The results obtained are shown in Table XVI. Those preparations of viral antigen in acid buffered saline did not produce clear-cut positive and negative sedimentation patterns, whereas those in pH 7.0 or above were well defined. Those preparations of viral antigen in acid pH gave false positive reaction patterns with the non-immune serum and correspondingly increased titers with the immune serum. Saline buffered to pH 7.2 was chosen as the suspending fluid for all viral preparations.

Effect of Methods of Preparation and Storage of Viral Antigen on Test

In this experiment several methods of preparation and storage

TABLE XVI

EFFECT ON TEST OF pH OF BUFFERED SALINE USED TO RESUSPEND THE CONCENTRATED VIRUS

pH of Buffered Saline	Hemagglutinative Titers*	
	Immune Serum	Non-immune Serum
pH 5.2	256	16
pH 5.8	256	16
pH 6.4	128	8
pH 7.0	32	0
pH 7.6	32	0
pH 8.2	32	0

*Expressed as reciprocal of the serum dilution.

of the viral antigen were used to establish not only the optimum methods but also the possible range of satisfactory methods. The methods investigated included (1) unconcentrated infected allantoic fluid, harvested and frozen at minus 20 C, (2) unconcentrated infected allantoic fluid, harvested and stored at 4 C, (3) concentrated infected allantoic fluid, diluted 1:10 and stored at minus 20 C, (4) concentrated infected allantoic fluid, diluted 1:10 and stored at 4 C, (5) concentrated infected allantoic fluid, diluted 1:100 and stored at 4 C, and (6) concentrated infected allantoic fluid, diluted 1:1000 and stored at 4 C. Each of these six viral preparations was obtained from the same pool of infected allantoic fluid and was maintained at the storage temperature until it was used to sensitize the tannic acid treated erythrocytes. The results of tests with immune and non-immune sera are shown in Table XVII. The titers obtained with the preparations of virus, unconcentrated at minus 20 C, concentrated diluted 1:10 at minus 20 C, and concentrated diluted 1:10 at 4 C, were identical. Use of the 1:100 and 1:1000 dilutions of the concentrated viral antigen stored at 4 C and of the unconcentrated antigen at 4 C yielded titers of 16, 8 and 4, respectively. The difference in titer between storage of unconcentrated preparations at minus 20 C and 4 C is 8-fold. No difference in titers was noted between concentrated preparations stored at minus 20 C and 4 C. However, differences in titers were noted among concentrated preparations which were diluted 1:10, 1:100 and 1:1000 and stored at 4 C. These titers were 32, 16 and 8 respectively.

TABLE XVII

EFFECT OF METHODS OF PREPARATION AND STORAGE OF VIRAL ANTIGEN ON TEST

Methods	Hemagglutinative Titers*	
	Immune Serum	Non-immune Serum
Unconcentrated Minus 20° C.	32	0
Unconcentrated 4° C.	4	0
Concentrated, diluted 1:10, Minus 20° C.	32	0
Concentrated, diluted 1:10, 4° C.	32	0
Concentrated, diluted 1:100, 4° C.	16	0
Concentrated, diluted 1:1000, 4° C.	8	0

*Expressed as reciprocal of the serum dilution.

CHAPTER IV

DISCUSSION

The development and evaluation of a new hemagglutinative method which is specific for herpes simplex virus has been presented. Application of the usual viral hemagglutinative technique as described by Hirst (1941) and McClelland and Hare (1941) to herpes simplex was found by most workers to be inactive. A contradictory finding was reported by Moolten and Clark (1951) who were able to isolate herpes simplex in the circulating blood. They believed that this detection of the virus in the circulating blood cell was the equivalent of the hemagglutination reaction in the test tube. Their work has not been confirmed and Geller et al. (1953) showed that herpes simplex virus will not agglutinate erythrocytes by the direct method. The method described here is an indirect type of hemagglutination involving the pretreatment of the erythrocytes with tannic acid. The tannic acid treated erythrocytes are capable of adsorbing the viral protein and will agglutinate when exposed to the homologus immune serum. This technique may prove to be useful in the diagnostic laboratory and most certainly can be adapted to other viruses which do not have the characteristic of direct hemagglutination.

The adsorption of all sera with tannic acid treated erythrocytes

was found to be essential in order to remove the non-specific agglutinins. This was done with the pre- and post-immunization sera and with the sera used as the diluents.

The amount of virus used in the sensitization of the tannic acid treated erythrocytes had a direct effect on the hemagglutinative titer. In Table VII it can be seen that there was a decrease in titer as smaller amounts of virus were used. This fact indicated that a specific antigen-antibody reaction had taken place.

As additional evidence for antigen-antibody specificity, studies were done with two sera, TVCS and TCS, which were prepared by adsorption techniques. The herpes simplex hemagglutination test and a virus infectivity technique involving viral neutralization were used to investigate whether a specific antigen-antibody reaction was involved. The negative results obtained in the hemagglutination tests with immune sera which had been adsorbed with viral antigen demonstrated that the specific antibodies were removed from those sera. The effect of adsorption of the sera on the infectivity of the virus also demonstrated that the specific neutralizing antibodies had been removed from the immune sera. The virus adsorbed serum (TVCS) did not neutralize the various dilutions of virus as shown in Table X. The 100 per cent infectivity of the CAM which were inoculated with the virus and serum (TVCS) mixture containing virus diluted from 10^{-2} through 10^{-6} showed that no demonstrable neutralizing antibodies were present. As a comparison, the immune serum adsorbed with tannic acid treated erythrocytes without virus (TCS) neutralized the virus. The results of these adsorption studies on the hemagglutinative reaction and virus infectivity add evidence to support the fact

that a specific antigen-antibody reaction is involved in this hemagglutination test with herpes simplex virus.

Since the antibodies which react in the hemagglutinative and neutralization procedures are both removed by the same adsorption technique, it appears that the neutralizing and hemagglutinating antibodies in the immune sera may be identical.

When performed under controlled conditions, the herpes simplex hemagglutination test has a high degree of specificity and sensitivity. Various factors influencing the test were investigated to determine the optimum conditions and the acceptable range under which it could be performed. While the test is restricted by certain factors such as an optimum hydrogen ion concentration and the concentration of tannic acid used to treat the erythrocytes, it can be operated efficiently under variable conditions, including age of viral antigen and temperature of incubation of test, which allow some diversity of performance and scope of application.

The size of the test tube used in the test is one of the variables that can be adjusted to the convenience of the person performing the test without affecting the results. Stavitsky (1954_a) investigated two sizes of test tubes, 10 X 100 mm and 13 X 100 mm, in his hemagglutinative technique with bacterial toxins and he also found that tube size did not influence the titers.

The temperature at which the test was incubated was another variable in that no one temperature gave consistently better results than the others. However, when the time of incubation was also taken into account, it was found that the higher incubation temperatures for

prolonged times were unsatisfactory. The combination of increased time and higher temperatures caused the erythrocytes to lyse with consequent destruction of the sedimentation pattern. The acceptable combination of time and temperature proved to be (1) all temperatures tested for four hours, or (2) incubation at 4 C and 25 C for four hours or overnight. Incubation for longer than overnight is not recommended because, regardless of the temperature, the older red cells tend to lyse and distort the sedimentation pattern. Incubation at room temperature (25 C) for four hours or overnight proved to be most convenient and required the use of no temperature controlling equipment, such as refrigerator or water bath. This combination of time and temperature was followed by Stavitsky (1954_a), but Boyden (1951) incubated his test at 4 C overnight.

The concentration of the tannic acid used to treat the erythrocytes must be weak enough to neither hemolyse nor agglutinate the cells during treatment. Boyden (1951) found that erythrocytes treated with 1:20,000 dilution of tannic acid and tuberculin PPD gave most satisfactory results in the presence of anti-H37Rv (strain of Mycobacterium tuberculosis) serum. A tannic acid dilution of 1:20,000 also gave good results in the herpes simplex hemagglutination test as shown in Table XI.

The pH of the saline used to sensitize the tannic acid treated erythrocytes with the virus must be acid for proper adsorption. A pH of 6.4 was found to be the best. This is consistent with the findings of Boyden (1951) and Stavitsky (1954_a). The time of exposure necessary for the adsorption of virus on tannic acid treated red cells had a

relatively wide range but required a minimum of 10 minutes. The optimum time was between 10 minutes and one hour. In less than ten minutes the adsorption does not take place and the free virus is, apparently, removed during the washing process following the adsorption step. If the sensitization period is prolonged for more than one hour, hemolysis and distortion of the sedimentation pattern occur. This rapid adsorption of the viral antigen on the erythrocytes differs from the conditioned type of hemagglutination where the antigen is a polysaccharide. From one to several hours are required for the adsorption of the polysaccharide antigen on untreated erythrocytes, while a protein antigen can be adsorbed on tannic acid treated erythrocytes in 10 minutes.

While sheep red blood cells gave excellent results in the herpes simplex hemagglutination test, it was also possible to employ erythrocytes from other animal species. In Table XIV it can be seen that horse erythrocytes and human (blood group "O") erythrocytes may be used but that the chicken and rabbit erythrocytes hemolyzed when treated with the tannic acid. The application of human erythrocytes has advantages in many laboratories because they are more easily obtained than those of various animal species. Sheep erythrocytes were used throughout this study. The reproducibility of the test will vary to a slight degree with different samples of red blood cells from the same animal species. Therefore, serum specimens that need comparative evaluations, such as acute and convalescent samples, should be examined by the same aliquot of red blood cells under identical conditions. It is always advisable in this test, as well as in any serological test, to include control sera, both positive and negative.

As a diluent in this test normal rabbit serum proved to be superior to sheep serum or chicken serum and, therefore, all serum samples tested in this study were diluted with it. All sera to be used as the diluent were adsorbed with tannic acid treated erythrocytes to remove non-specific agglutinins and then diluted 1:100 in saline. Boyden (1951) showed that when the serum diluent was used at a dilution of 1:100 the erythrocytes were protected. The use of the serum diluent prevents hemolysis and non-specific agglutination.

The viral antigens were prepared and stored by a variety of methods with acceptable results. The age of the viral antigen did not influence the titers obtained if it was stored at 4 C. It could also be preserved with formalin or merthiolate and then stored at 4 C until used with results comparable to fresh preparations. There were no differences in titers obtained when the antigen was stored at 4 C or when it was frozen at minus 20 C. Concentrated and purified viral antigens were superior to unconcentrated infected allantoic fluid. However, in one lot of viral antigen, as shown in Table XVII, unconconcentrated allantoic fluid frozen at minus 20 C gave results comparable with the concentrated and purified virus. This finding was probably due to the extremely high concentration of virus in the allantoic fluid. For reproducible results the concentrated and purified preparations were more dependable. The pH of the buffered saline used to resuspend the virus at the completion of the concentration and purification procedure did have some effect on the titers. In Table XVI, it can be seen that the virus resuspended in buffered saline pH 7.0 or above was superior to that in acid buffered saline. The virus resuspended in acid

buffered saline gave some false positive patterns in the non-immune serum. Saline buffered at pH 7.2 was chosen to be used throughout these studies.

This hemagglutination test is sensitive, specific and easy to perform. It has advantages of ease of preparation of reagents, and it requires small amounts of reagents. While there are limitations in the preparation of reagents and performance of the test, there are also some variables that will allow diversity in performance. The application of this technique has many possibilities. It had previously been explored with bacterial proteins and some general proteins, such as ovalbumin, horse serum globulin and albumin, and chicken serum globulin by Boyden (1951). He established that the technique was applicable to antigenic proteins in general. This technique may prove to be of diagnostic value with certain viruses that now require expensive neutralization and protection tests in order to establish a diagnosis. It has possibilities of application in the analysis of tissue culture fluids. In fact, virtually all antigenic proteins which are not hemolytic may be applicable to these methods.

CHAPTER V

SUMMARY

1. Herpes simplex virus, which by the usual technique of hemagglutination is inactive, has been shown to produce hemagglutination in the presence of specific immune sera.

2. When herpes simplex virus was adsorbed on sheep erythrocytes which were pretreated with tannic acid, the sensitized erythrocytes were agglutinated in the presence of specific immune serum.

3. The amount of virus used in the sensitization process had a direct effect upon the hemagglutinative titers of the immune serum.

4. The preparation of reagents and the procedures for the herpes simplex hemagglutination test have been described.

5. The antibody in herpes simplex immune sera can be removed by adsorption with tannic acid treated-virus sensitized erythrocytes. The removal of this antibody as demonstrated by both hemagglutinative and infectivity techniques indicates that the herpes simplex neutralizing and hemagglutinating antibodies are probably identical and that a specific antigen-antibody reaction is involved in the herpes simplex hemagglutination test.

6. Factors affecting the herpes simplex hemagglutination test were examined to show the range and optimum requirements necessary for

the proper performance of the test. Factors investigated were: (1) the size of the test tube employed in the test, (2) the time and temperature of incubation of the test, (3) the concentration of tannic acid used to treat the erythrocytes, (4) the pH of the saline applied in the sensitization of the cells with virus, (5) the time required for this sensitization, (6) the utilization of erythrocytes from different species, (7) the type of serum used for the serum diluent, and (8) various methods of preparation and preservation of the viral antigen.

7. The herpes simplex hemagglutination test, when controlled by limiting factors, is a sensitive, specific and reproducible new serological test.

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