ADAPTATION OF THE TANNIC ACID HEMAGGLUTINATION TEST FOR USE IN THE STUDY OF HEMOPHILUS PERTUSSIS ANTIGENS AND ANTIBODY

Thesis for the Degree of Ph. D.

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Bernice Carter Graham

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ADAPTATION OF THE TANNIC ACID HEMAGGLUTINATION TEST FOR USE IN THE STUDY OF *HEMOPHILUS PERTUSSIS* ANTIGENS AND ANTIBODY

By

Bernice Carter Graham

AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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The tannic acid hemagglutination test developed by Boyden (1951) is based upon the ability of washed red blood cells, modified by treatment with dilute tannic acid solution, to adsorb protein antigens as well as polysaccharide. These "sensitized" cells are then agglutinated when mixed with dilutions of homologous antisera. The test thus becomes useful in determining antibody titer of serum and in identifying antigens by checking with known antisera.

This study was carried out to determine whether the tannic acid hemagglutination test could be used in studying H. pertussis antigens and antibody as it has been used in the studies of antigens of M. tuberculosis (Boyden and Sorkin, 1955) and capsular antigens of P. pestis (Chen and Meyer, 1954; Landy and Trapani, 1954).

Three strains of H. pertussis were grown in liquid culture medium with constant shaking. At the end of the incubation period the liquid cultures were subjected to sonic oscillation treatment to break up the bacterial cells. A portion of this material was then centrifuged immediately to remove insoluble debris.

To gain some information concerning the antigenicity of these liquid H. pertussis cultures, mouse protection tests were carried out on these cultures before sonic treatment, after sonic treatment and on the supernatant fluids of the centrifuged, sonic treated cultures. Two of the three strains of H. pertussis tested showed a moderate amount of protection for mice whereas the third strain showed only a slight amount.

Sonic oscillation treatment did not decrease the protective effect. Supernatant fluids from sonic treated cultures contained slightly less protective antigen.

The sonic treated cultures and supernatant fluids from these cultures were found to contain hemagglutinins for sheep cells modified by treatment with tannic acid. These hemagglutinins appeared to increase with incubation up to 30 hours and then gradually decrease to a low titer after 96 hours of incubation. Cultures of strains carried through six or less transfers from the lyophilized state produced a much higher hemagglutinin titer than those of the same strains carried through 26 transfers on artificial media.

Protein precipitated by the addition of ammonium sulphate solution to the supernatant fluids of sonic treated H. pertussis cultures or to the supernatant fluids of sonic treated organisms suspended in salt solution could be used to "sensitize" tannic acid cells. These sensitized cells were then susceptible to agglutination by rabbit anti-H. pertussis or mouse anti-H. pertussis sera.

Mice inoculated with the supernatant fluid from a liquid culture of H. pertussis in two doses equivalent to 5 billion organisms per dose developed a hemagglutination titer of 1:600. Mice inoculated with the same supernatant fluid but in amounts equivalent to 0.06, 0.3 and 1.5 billion organisms per dose did not develop demonstrable hemagglutination titers in the same period of time.

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INTRODUCTION

Bordet and Gengou in 1906 recognized the close relationship between the organism, Hemophilus pertussis and the human disease, pertussis, or whooping cough. Investigations since that time have definitely established this organism as the cause. Development of knowledge concerning growth and antigenic characteristics of this bacillus has been stimulated by interest in the preparation of vaccines and other prophylactic agents. Most of these vaccines have been prepared from whole organisms assumed to contain the complete range of antigens present in the actively growing H. pertussis cells. In recent years, however, the tendency toward use of purified extracts and fractions of these organisms has been increasing. Preparation of both of these types of immunizing agents requires a knowledge of antigenic composition and methods of testing antigen and antibody. Such tests as complement fixation, precipitin, bacterial agglutination, hemagglutination, animal protection tests and clinical field studies have been applied to these various vaccine preparations in efforts to determine both their antigenic make-up and their suitability as human immunizing agents.

As interest in the chemical separation of antigenic fractions of H. pertussis grows, the need for new means of assaying types and quantities of antigenically active material increases also. It is with this thought in mind that the present study was undertaken. Keogh, North and Warburton (1947) developed the hemagglutination tests in which the action of H. pertussis, or extracts of H. pertussis, on washed, normal red blood cells is observed. These tests were also utilized by Ungar (1949), Fisher (1950) and Masry (1950). Normal red blood cells are believed to be capable

of adsorbing polysaccharide fractions but very little protein-containing material. Boyden (1951) noted that when red cells are given a preliminary treatment with dilute tannic acid they are rendered capable of adsorbing protein as well as polysaccharide. Cells which have adsorbed these antigenic materials then become susceptible to reaction with homologous antibody. Such reactions are made visible due to the fact that agglutination of the involved red cells usually occurs. Thus the use of tannic acid treated red cells, acting as an indicator, gives a clew to antigen-antibody reactions which might otherwise be undetected.

This type of antigen-antibody test has been used by Landy and Trapani (1954) for examination of purified capsular protein of Pasteurella pestis. They found the tannic acid hemagglutination test to be 20 to 50 times as sensitive as the complement fixation test and equally specific. Chen and Meyer (1954) also found that this test is highly specific for P. pestis protein and state that the protein fraction responsible for positive hemagglutination tests is also protective for guinea pigs. Boyden and Sorkin (1955) studied heated and unheated fractions of M. tuberculosis cells by utilizing this test. They found that cross hemagglutination-inhibition tests were also helpful in determining antigenic relationships of the different fractions.

Since the Boyden tannic acid hemagglutination test has been used successfully for the examination of fractions of other organisms, it seemed feasible to try to adapt it for use in the study of extracts and chemically purified fractions of H. pertussis. If such a method of testing is possible, it can be used to help identify unknown antigenic fractions or to check antibody content of H. pertussis immune sera.

HISTORICAL BACKGROUND

Interest in the study of the antigenic composition of Hemophilus pertussis developed rapidly after the establishment of this organism as the cause of whooping cough. Bordet and Gengou (1909) reported the discovery of a toxic reaction in animals produced by injection of either the whole organisms or extracts of these organisms. They concluded that this toxin was a form of endotoxin. They also noted that injection of this substance did not protect animals against subsequent infections of virulent organisms nor would the sera of these toxin-injected animals protect against or neutralize endotoxin given to other animals.

Shortly after the above report, Bordet and Sleeswyk (1910) noted that many H. pertussis cultures contained agglutinogens inasmuch as they were agglutinated by sera prepared in rabbits by inoculation of whole organisms. They observed a difference between freshly isolated strains and those which had become adapted to growth on ordinary nutrient agar without blood. The former were all agglutinated by homologous serum but not agglutinated by serums prepared by injection of strains grown on plain agar. The latter strains were not agglutinated by serum produced by injection of freshly isolated organisms but were, of course, agglutinated by their homologous antiserum.

Since that time many workers in this field have confirmed and extended these general findings in two of the principle antigenic components of Hemophilus pertussis - toxin and agglutinogen.

Most workers agree that all freshly isolated strains of H. pertussis appear to be homologous in type. Immunologic variations appear, however, as these cultures are transferred a number of times on laboratory media. Krumwiede, Mishulow and Oldenbusch (1923), using agglutination and agglutinin-absorption tests, found that a series of H. pertussis cultures fell into two distinct groups. Those freshly isolated from cases of whooping cough were included in one group whereas most of those maintained on culture media over a period of time were included in the second group. Leslie and Gardner (1931) made a study of these changes and reported the existence of four "phases" of H. pertussis, all of which were antigenically distinct. Practically all recently isolated strains were in phase I, with an occasional one falling into phase II. These strains were all toxic for guinea-pigs. So-called "laboratory" strains, those which had been transferred many times and which would grow easily on ordinary media, were either in phase III or phase IV. These strains were non-toxic for guinea-pigs.

Shibley and Hoelscher (1934) examined 98 strains of H. pertussis, 59 of which were freshly isolated and the remainder stock laboratory cultures. They observed the same differences between these two groups of strains as those described above and designated the smooth, encapsulated, recently isolated strains as S forms and those adapted to non-enriched laboratory media as R forms.

Straight and cross agglutination experiments of Toomey et al. (1935) confirmed the findings, in general, of Leslie and Gardner. Serum produced by freshly isolated strain had an agglutinin titer of 1:10,240 against its homologous strain but a negligible titer against old strains acclimated to

media without blood. Serum produced by old strains had relatively lower agglutinin titers (1:2,560) against homologous strains, and a titer of only 1:160 against heterologous, freshly isolated strains.

Lawson (1939), after an extensive study of the different types of H. pertussis, gave detailed descriptions of the morphological and cultural characteristics of smooth, intermediate and rough strains. Carrying this differentiation further by mouse protection tests, he found that vaccines prepared from smooth organisms gave much better protection to mice challenged intranasally with virulent cultures than vaccines made from rough variants.

The study of H. pertussis toxin was continued by Toomey and McClelland (1933) who checked the antigenicity of pertussis toxin by growing cultures of H. pertussis on veal brain infusion broth for 14 days, filtering the broth to remove the organisms and injecting 0.1 ml of the filtrate into 680 individuals. Local reactions were obtained which persisted for 24 hours. It is questionable whether these reactions were due to toxin present in the filtrate or to ingredients in the medium.

In 1937 Evans and Maitland carried out the most extensive study on pertussis toxin to that date. They prepared their toxic material by different methods, using organisms washed from Bordet-Gengou agar plates as the starting material. They found that the toxicity of living bacilli and of those killed by maintaining in a vacuum or by freezing and thawing was of the same order. Grinding of the bacterial suspension increased the toxicity fourfold. They are also responsible for noting the lability of the toxin and that it is completely destroyed by heating to 56 C for 30 minutes. They also found that formalin in a final concentration of

0.3 per cent destroys toxin in 20 hours. Toxin inoculated into rabbits was only slightly antigenic as tested by agglutination, complement fixation and precipitin tests. Toxin produced death in guinea pigs when injected intravenously and necrotic lesions in rabbits when injected intradermally. This type of skin necrosis appeared in rabbits immunized with H. pertussis as well as in non-immunized rabbits.

These authors were able to separate toxin from other antigens by mixing bacterial extracts with immune rabbit serum and removing the precipitate which formed. The supernatant fluid retained all of the toxic activity whereas the precipitate was non-toxic but antigenic.

Wood (1940) prepared her toxic substance by growing the organisms in serum broth, centrifuging off the bacilli and sterilizing the supernatant fluid by Seitz filtration. Tests on the latter agreed well with those carried out by Evans and Maitland. She found that it was impossible to produce precipitins or neutralizing antibodies in rabbits by intravenous, subcutaneous or intraperitoneal injections of toxic filtrates. She also found that a rough strain of H. pertussis after the twenty-first subculture on veal infusion agar was non-toxic for mice.

Flosdorf and Kimball (1940) separated toxin from agglutinogenic compounds by Seitz filtering a sonic extract of organisms and adjusting the pH to 4.5. Toxin was quantitatively precipitated at this pH and appeared undiminished in that portion of the precipitate which was soluble at pH 7.0.

Stean and Grant (1940) prepared pertussis endotoxin by freezing and thawing bacterial suspensions many times and extracting the dried residue with water. Purification was carried out by precipitating with two per cent

acetic acid at pH 4.0. This purified endotoxin contained very little agglutinogen as evidenced by the fact that injection into rabbits produced serum having an agglutinin titer of less than 1:100. Chemical analysis indicated a nitrogen content of 0.047 mg/ml and reducing sugar content of 3.4 per cent.

Flosdorf, Bendi and Dozois (1941), continuing their studies on the antigenicity of the H. pertussis toxin, are the first to mention that these organisms contain a heat stable as well as a heat labile toxin. They also claimed that weak antitoxin can be produced in rabbits after a long series of immunization.

Roberts and Ospeck (1944) developed a pertussis toxin-antitoxin neutralization test. They standardized toxin by determining the Minimal Lethal Dose for mice and with this known toxin standardized the antitoxin by mixing various proportions and testing by intradermal injection of the mixtures into rabbits. They felt that such a test would be useful in checking the antitoxin response in humans to disease or to vaccinations.

Smolens and Flavell (1947) produced a highly potent H. pertussis toxin by subjecting the organisms to sonic disintegration. Toxin prepared in this manner had a potency of at least 1000 Lethal Doses - 50 per cent (LD_{50}) per ml compared with a potency of only 50 LD_{50} per ml of toxin prepared by freezing and thawing. They found that toxin produced by sonic disintegration of the cells was antigenic and could be used to produce potent toxin-neutralizing rabbit antisera.

In 1949 Verwey and Thiele published the results of extensive studies on the antigenicity of H. pertussis toxic extracts. They prepared these

extracts by sonic disintegration of organisms grown in liquid medium. A portion of this extract was then mixed with immune serum to precipitate out the agglutinogen. Comparison of the whole sonic extract with the absorbed extract showed that the former was capable of protecting 50 per cent of mice against intracerebral infection whereas the latter absorbed extract, even when administered in larger doses, was completely incapable of protecting mice. When the unabsorbed extract was injected into rabbits both agglutinin and antitoxin were produced; when, however, the agglutinogen-absorbed sonic extract was injected, a very slight amount of agglutinin and no antitoxin were produced. This result was probably due to the small amount of agglutinogen still present in the absorbed sonic extract.

These authors believe that the presence of agglutinin mixed with the toxin is necessary for the production of any antitoxin. This theory might explain some of the contradictory results reported previously in which some workers state that toxin shows no evidence of antigenicity whereas others claim that toxin can act as an antigen on prolonged series of injections and cause antitoxic response.

Robbins and Pillemer (1950b) undertook the purification of pertussis toxin by harvesting the organisms in 0.05 M $CaCl_2$ at pH 6.5 and precipitating the toxin in the supernatant fluid with 15 per cent methanol at pH 4.4. The most active fraction prepared by this method contained 5260 LD_{50} per mg nitrogen and represented a 17.5-fold purification over the water extract.

Considering the complement-fixing antigens of H. pertussis it should be mentioned that Bordet and Gengou, in their original report in 1906,

described the appearance of complement-fixing antibodies in the serum of children with pertussis. Miller in 1934 carried out an extensive study of pertussis vaccines by testing the complement-fixing antibodies produced in rabbits receiving series of injections of these antigens. He found that vaccine composed of living organisms produced the same type of response as a formolized vaccine. The addition of colloidal aluminum hydroxide delayed and decreased this response. An extract prepared from ground, dried organisms with physiological salt solution produced a good response; so also did an extract prepared by treatment of frozen bacilli with 9 per cent sodium chloride solution and incubation for 24 hours at 37 C. A portion of this extract was also incubated for 24 hours at 56 C and produced complement-fixing antibodies of the same order. An extract prepared by alternately freezing and thawing a water suspension of dried bacilli through two cycles resulted also in a good response. Additional interesting information was gained when the preparation of this extract was repeated using stock strains of H. pertussis which had been isolated three months previously and kept at 37 C continuously since then with transfers every three days. Very poor response was produced by this extract. A saline extract which was passed through a Berkefeld sterilizing filter also proved to be ineffective in producing complement-fixing antibodies.

Production of agglutinin and examination of agglutinin-absorbing properties of vaccines and bacterial extracts have been the objects of many studies of antigenic components of H. pertussis.

Cruickshank and Freeman (1937) obtained an actively antigenic fraction by tryptic digestion of H. pertussis cells followed by filtration through a candle and precipitation with 68 per cent alcohol. This fraction was known to contain carbohydrate and probably enzymatically hydrolyzed protein. It immunized mice against experimental infection and probably contained "agglutinogen" as isolated by later workers.

Flosdorf, Kimball and Chambers (1939) examined sonic extracts of virulent, phase I H. pertussis cells for content of agglutinin absorbing substances by mixing them with high titer immune sera and removing the precipitate by centrifugation. It was found that these extracts absorbed agglutinins to completion.

A study of the pertussis agglutination titer of human sera was carried out by Miller and Silverberg (1939) who checked the sera of 101 children with negative whooping cough histories. Only ten of these were positive with a low titer of 1:20. Sera from 161 of 164 children vaccinated with phase I H. pertussis organisms were positive with a titer of 1:160 or above.

Flosdorf and Kimball (1940), starting with a sonic-disintegrated, Seitz-filtered extract of phase I organisms were able to separate several fractions by altering the pH, treating the supernatant fluid with saturated ammonium sulphate solution and then precipitating with saturated picric acid solution. All these fractions produced high agglutinin titers in rabbits.

Evans (1942) checked both the antitoxin and agglutinin production in rabbits by inoculation of a variety of antigens including:

1) bacterial extract prepared by freezing, thawing, extracting with salt solution and finally detoxifying by formalin; 2) the above extract detoxified by heating to 56 C for 20 minutes; 3) bacterial vaccines detoxified by formalin, and 4) bacterial vaccines detoxified by heating to 56 C for 30 minutes. The extract which was detoxified by formalin was the only one which produced antitoxin titers in rabbits and which protected rabbits from necrotizing effects of intradermal injections of toxin. Both extracts and bacterial vaccines produced agglutinins in high titer. The author expressed the opinion that vaccines for prophylactic use should produce antitoxic antibodies as well as antibacterial antibodies.

Smolens and Mudd (1943) prepared an extract by hydrochloric acid extraction of sonic disintegrated H. pertussis organisms at 56 C followed by precipitation of the active fraction with saturated ammonium sulphate solution and dialysis. Six intravenous doses of this extract into rabbits produced an agglutinin titer of over 1:2000. No toxin was present as tested by inoculation into rabbits.

A different type of extract was prepared by Hink and Johnson (1947) who treated acetone-dried and powdered organisms with 2.5 M urea. The supernatant fluid from this mixture, after dialysis, contained agglutinin-absorbing properties. It was toxic when injected intravenously into rabbits but non-toxic for mice. This extract proved to be very antigenic as it caused a sharp increase in agglutinin titer in rabbits and humans. They believed it also caused an active immunity since sera from injected rabbits or humans gave protection to mice against lethal doses of H. pertussis.

Smolens et al. (1947) believed that the agglutinogens present in the H. pertussis cells were an important factor concerned with protection against whooping cough. They tested for these antigens by mixing a bacillary suspension with a known amount of antiserum, centrifuging and checking the supernatant fluid for agglutinins still present. In this manner, they could determine the mg of bacteria required for 90 per cent absorption of agglutinins and could therefore compare various strains. There seemed to be only a general correlation between agglutinin titers and the absorbing potency of strains of H. pertussis. Some which were agglutinated to a high titer required 10 mg of bacteria for 90 per cent absorption of agglutinins, whereas others with equally high agglutination titers required only one or two mg for 90 per cent absorption. Rabbits which were injected with organisms of high absorbing potency produced serum with agglutinin titers of 1:12,000 whereas those which were inoculated with organisms of low absorbing potency produced a serum with a titer of only 1:800.

Polysaccharide, capsular and mouse protective antigens as well as hemagglutinins of H. pertussis have been studied individually and in conjunction with the toxin, complement-fixing antigens and agglutinogens of the organism.

Polysaccharide fractions from H. pertussis were examined for antigenic properties by Eldering (1942). These fractions were essentially protein-free as determined by negative biuret, Millon, xanthoproteic and Hopkins-Cole tests. She found that inoculations of these fractions into mice did not appreciably protect them against challenge doses of

H. pertussis organisms given intraperitoneally in mucin 14 days after vaccination.

Evans and Adams (1952) concentrated capsular material of H. pertussis organisms by washing it off the cells, freeze-drying and redissolving in small volumes of water. Using this as an antigen for rabbits they produced antisera which contained an average agglutinin titer of less than 1:320 and an average complement-fixing titer of less than 1:16. Antisera produced by inoculation of whole bacteria had an agglutinin titer of 1:15,000 and a complement-fixing titer of 1:128. Antisera from rabbits inoculated with the concentrated washings did not passively protect mice against intranasal instillation of organisms, whereas antisera from rabbits inoculated with whole organisms protected approximately 80 per cent of the mice. These authors therefore conclude that the antigens of H. pertussis which produce agglutinating, complement-fixing and protective antibodies are somatic rather than capsular.

Robbins and Pillemer (1950a) carried out a study on the antigenic composition of two strains of H. pertussis, 18323 and 29. They found these two strains differed markedly in mouse protective factors, strain 18323 having a Protective Dose - 50 per cent (PD_{50}) of 0.25 billion organisms and 29, a PD_{50} of 12.0 billion organisms. Strain 18323 contained agglutinin-absorbing antigens for both 18323 and 29 antisera whereas strain 29 contained specific agglutinin-absorbing antigen only and none for strain 18323. They could separate the protective antigens by subjecting a suspension of 18323 organisms to treatment with sodium acetate and sonic disintegration. A portion of the suspension subjected

to acid treatment at pH 2.0 and sonic disintegration contained agglutinin-absorbing antigen but no protective antigen. Mouse protective antigens could also be precipitated from a sonic extract by 40 per cent methanol at pH 4.5.

Pillemer, Blum and Lepow (1954) carried out an investigation of the factors influencing the liberation of soluble protective antigen from H. pertussis. They were able to achieve high yields by sonic disintegration of the cells and mixture of the supernatant fluid with red blood cell stromata. By carefully controlling the conditions of temperature, pH and ionic strength of the mixture the protective antigen was selectively adsorbed onto the stromata. Mice vaccinated with dilutions of this stromata were protected against intracerebral inoculations of the challenge culture of H. pertussis. The authors state on experimental evidence that their soluble protective antigen is not associated with pertussis hemagglutinin, toxin or agglutinogen. Tests for all three of these antigens carried out with the soluble protective substance were negative. They believe that soluble protective antigen could act as a prophylactic against whooping cough.

The demonstration of hemagglutinins in the hemophilus group of organisms was announced by Keogh, North and Warburton (1947). They showed that erythrocytes of mouse, fowl, man and other animals were agglutinated by saline suspensions of H. pertussis, H. parapertussis and Br. bronchisepticus and by supernatant fluids of broth cultures. These hemagglutinins were neutralized by human and animal immune sera. Keogh and North (1948) published results of further experiments on

hemagglutinins of H. pertussis. They grew the organisms in broth, separated them by centrifugation and tested both the supernatant fluid and suspensions of organisms for hemagglutinins by mixing twofold dilutions with washed chicken or human red cells. High hemagglutinin titers were found to correlate with high virulence in intranasal injection into mice. Vaccines prepared from the cultures showing high hemagglutinin titers, as well as the supernatant fluid containing hemagglutinin, had good immunizing properties. Human or animal sera from immunized individuals which contained anti-hemagglutinins protected mice when given intraperitoneally against intranasal challenge with virulent organisms. It was noted that high anti-hemagglutinin content did not necessarily coincide with high agglutinin content. Sera having low anti-hemagglutinin titer but high agglutinin titer did not protect mice when given intraperitoneally before challenge but did protect mice when instilled intranasally with the infecting dose.

Ungar (1949) was able to duplicate some of these results on hemagglutinin by using H. pertussis organisms washed off Bordet-Gengou agar medium. Making serial dilutions from a starting suspension of six billion organisms per ml he noted agglutination of human red cells through a dilution of 1:16. In his experimental procedures, hemagglutination did not occur at 37 C but did occur when the tubes were incubated for two hours at 46 C. He noted that the 24 and 48 hour growths demonstrated hemagglutinin but cells collected after 72 hours caused only very slight hemagglutination. All of 32 virulent strains of H. pertussis showed hemagglutinating activity. None of eight avirulent strains gave this

reaction. Ungar heated bacterial suspension to 56 C for 30 minutes with only a slight drop in hemagglutinating power. In a later report (Ungar and James, 1949) he stated that heating the suspension for three hours at 68 C caused complete loss of the property of agglutinating red cells.

Fisher (1950) continued observations on the hemagglutinating complex of H. pertussis culture supernatant fluids. He obtained anti-hemagglutinin by inoculating rabbits with their own red cells to which the hemagglutinin had been adsorbed. He found that heating the supernatant fluid to 56 C for 16 minutes destroyed its hemagglutinating power but not its ability to cause formation of anti-hemagglutinin in rabbits. Supernatant fluid heated to 56 C for 30 minutes still protected mice when injected intraperitoneally against challenge ten days later by intranasal instillation. Supernatant fluid heated as above was still adsorbed onto the surface of erythrocytes as evidenced by the fact that cells were agglutinated by H. pertussis immune rabbit serum in a dilution of 1:800.

Pertussis hemagglutinin, adsorbed on aluminum phosphate as a stabilizing agent, was used as a prophylactic antigen in a group of young children by Fisher et al. (1951). They noted the development of serum hemagglutinating titers of 1:12 to 1:32.

Masry (1950) undertook the extraction and purification of H. pertussis hemagglutinin. He confirmed the results of Fisher and Ungar, finding hemagglutinin in bacterial cells in the early stages of growth with a gradual decrease as the culture aged. On the other hand, he found that the supernatant fluid contained little or no hemagglutinin in the early stages but showed rapidly increasing titers up to the third

or fourth day, reaching a maximum on the eighth day. He extracted hemagglutinin from 24 hour growth of organisms by two methods:

1. Organisms were suspended in 2 M sodium chloride, removed by centrifugation and the supernatant fluid checked for hemagglutinin content. This averaged a titer of 1:128. Immune antisera absorbed with this extract showed a decrease in bacterial agglutination titer from 1:25,000 to 1:3,200. Antiserum prepared by injection of this extract had a bacterial agglutination titer of 1:4,000. Apparently this extract contained some agglutinogen as well as hemagglutinin.
2. Bacterial growth from solid medium was suspended in M sodium acetate solution and removed by centrifugation. The supernatant fluid had a hemagglutinin titer of 1:256. This hemagglutinin was precipitated with 40 per cent methanol in acid pH, which is the method used by Pillemer, Burrell and Ross (1947) for precipitation of protective antigen. This methanol extraction brought about considerable purification of the hemagglutinin. Before methanol extraction, extracts with a titer of 1:128 contained an average of 0.7 mg nitrogen/ml and after precipitation, extracts showing the same titer contained only 0.02 to 0.03 mg/ml.

The sodium chloride, sodium acetate and methanol extracts were all toxic by rabbit intradermal tests and on intravenous injection into mice. The hemagglutinating activity of all three of these extracts was destroyed by heating to 60 C for a few minutes. Activity also disappeared on filtration through a Seitz filter. Rabbits immunized with hemagglutinins in the form of either extract or bacterial suspension produced anti-hemagglutinin as determined by hemagglutinin-inhibition tests.

Red cells which had adsorbed to capacity the hemagglutinin from methanol precipitated extract were used to immunize mice by intraperitoneal injection. Practically no protection was afforded the mice when challenged with virulent organisms intranasally.

Anti-hemagglutinin was prepared in rabbits by injecting red cells to which hemagglutinin had been adsorbed. This serum had a titer of 1:1200. A mixture of this serum with 100 LD_{50} doses of virulent H. pertussis organisms inoculated intranasally into mice demonstrated that it had only a very slight protective activity. The same serum injected intraperitoneally into mice which were then challenged by intracerebral inoculation gave no protection against infection.

Difficulties and confusion in carrying out animal protection tests have been encountered by most workers in the study of H. pertussis antigens. This is due mainly to the fact that most laboratory animals are fairly resistant to infection with this organism and consistent production of symptoms and/or death has been difficult to achieve. Mice have been most frequently used for testing of protective antigen. Before the development of the intracerebral mouse test by Kendrick et al. (1947) vaccinated mice were challenged by intraperitoneal injection of large doses of living organisms supplemented with mucin to increase infectivity. Responses of mice to such challenges were often irregular and rarely could 100 per cent fatality in control mice be produced. A second method was that of Burnet and Timmins (1937) in which virulence and protection by vaccination was checked by intranasal instillation of living cultures. This method is still being used in some laboratories

at the present time. The mouse protection test of Kendrick using the intracerebral route of challenge has proven to give the most reliable results. A good correlation appears to exist between the substance responsible for protecting mice against intracerebral injection with H. pertussis and the antigen responsible for increasing the immunity of children to the disease.

Evans and Perkins (1953) investigated the agglutinin production in mice after vaccination with H. pertussis. They found that intraperitoneal inoculation produced a much higher agglutinin response than subcutaneous injection. One inoculation produced an agglutinin titer averaging only 1:160, whereas a second inoculation given approximately 14 days after the first resulted in an agglutinin titer averaging 1:3,200. A direct relationship existed between the dose and the level of agglutinin response. Based on a limited number of tests, they also showed that the dose of vaccine, in terms of equivalent numbers of organisms, necessary to immunize 50 per cent of the mice is indirectly proportional to the agglutinin titer attained. In other words, a whole bacterial vaccine requiring a dose of 0.43 billion organisms to immunize half the mice vaccinated produced an antiserum with an agglutinin titer of 1:3,000; a vaccine requiring a dose of 11.0 billion organisms produced an antiserum with an agglutinin titer of only 1:180.

These same authors (Evans and Perkins, 1954) were interested in examining the type of immunity developed in mice after H. pertussis vaccination. They challenged groups of mice by intracerebral inoculation at various time intervals after vaccination. With a dose of

2.5 billion H. pertussis organisms immunity developed very rapidly, 31 per cent of the mice being immune after five hours, 52 per cent after one day, 70 per cent after three days and practically 100 per cent after ten days. Sera taken from mice three days after vaccination did not offer any protection when given to other mice two hours before intracerebral challenge. Sera taken 1, 3 and 14 days after one inoculation showed very low agglutinating and complement-fixing titers. When a second equal inoculation was given 14 days after the first, sera taken 12 days after the second injection had agglutinin titers of 1:500 to 1:3,200 and complement fixation titers of 1:32.

The authors concluded that their results suggest that the immunity formed in mice as the result of a single injection of pertussis vaccine is of a type not associated with specific antibody production.

This conclusion was partially confirmed by further work carried out by Evans and Perkins (1955a). In these investigations they inoculated intraperitoneally a smaller dose of vaccine than that used in the previous experiments and extended the observation period to 40 days. They noted that at ten days, 95 per cent of the mice were protected, whereas at 14 days only 80 per cent survived. The percentage gradually increased again until at 30 and 40 days 95 per cent of the mice were protected. From these later results they suggested that the immune response might be made up of two curves, one due to an early, more transient type of immunity and a second due to the slower development of a true antibody type of immunity. Verification of this suggestion is aided by the fact that agglutinin production of mice inoculated with

whole bacterial vaccine was 8 times higher at 30 days than at 14 days and 16 times higher at 40 days.

In a separate report (Evans and Perkins, 1955b) these authors stated results of a comparison study of agglutinin production of mice inoculated with whole bacterial vaccine and with the soluble protective antigen of Pillemer. Injection of regular bacterial vaccine yielded an agglutinin titer of 1:5,000 whereas three samples of soluble antigen vaccine yielded an average titer of 1:34. These results confirmed Pillemer's own statement that soluble pertussis protective antigen contained very little agglutinogen.

MATERIALS AND METHODS

Hemophilus pertussis Strains

H. pertussis strains 10536, 18334 and 18323 were obtained as lyophilized cultures from the Division of Laboratories, Michigan Department of Health. Strains 10536 and 18334 are standard strains used in pertussis vaccine production and were chosen for this study as they have been used in other studies at the Laboratories dealing with nutritional requirements. Strain 18323 is virulent for mice on intracerebral inoculation and is used as the challenge culture in the standard mouse protection test.

H. pertussis vaccine 401 was obtained as a sample of bulk vaccine produced by the Laboratories and consisted of equal amounts of the following strains: 22490, 18297, 21763 and 18925. It was prepared by growing these organisms on Bordet-Gengou (B.G.) agar medium containing 16 per cent sheep blood, washing off the growth with physiological salt solution, centrifuging and resuspending the organisms in salt solution to a known concentration.

Media

The experimental cultures used in this study were produced in liquid medium. The seed culture medium was prepared according to the formula of Cohen and Wheeler (1946) and consisted of: Bacto Casamino acids, 10.0 g; NaCl, 2.5 g; KH_2PO_4, 0.5 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; soluble starch, 1.5 g;

$CaCl_2.2H_2O$, 13.0 mg; $FeSO_4.7H_2O$, 12.5 mg; $CuSO_4.5H_2O$, 7.5 mg; cysteine hydrochloride, 30.0 mg; yeast dialysate, 50.0 mg; distilled water, 950.0 ml. The pH of this medium was adjusted to 7.2 before autoclaving.

The medium used for the production of large numbers of organisms was a slightly modified Cohen-Wheeler medium developed by Dr. Birger Olson in his studies at the Laboratories on nutritional requirements of H. pertussis. This medium contains twice the amounts of Casamino acids and yeast dialysate and four times the amount of soluble starch of those in regular Cohen-Wheeler broth.

Both of these media were dispensed in 50 ml quantities into 500 ml wide-mouth Erlenmeyer flasks and autoclaved for ten minutes at 121 C.

Solutions

Physiological salt solution buffered at pH 7.2 was prepared by mixing 23.9 ml of 0.066 M KH_2PO_4, 76.0 ml of 0.066 M Na_2HPO_4 and 100 ml of 0.85 per cent NaCl solution. This solution is the one referred to whenever the term "buffered salt solution" is used.

Salt solution buffered at pH 6.4 was prepared by mixing 67.7 ml 0.066 M KH_2PO_4, 32.2 ml 0.066 M Na_2HPO_4 and 100 ml of 0.85 per cent NaCl solution.

Alsever's solution was prepared by dissolving 2.05 g dextrose, 0.80 g sodium citrate, 0.42 g sodium chloride in 100 ml distilled water and adjusting to pH 6.1 with 10 per cent citric acid.

The tannic acid stock solution was prepared by dissolving 1 g of tannic acid (Merck reagent grade) in 100 ml buffered salt solution. This

solution was kept for several weeks in the refrigerator. A 1:40,000 dilution of tannic acid in buffered salt solution was prepared just prior to use.

Antisera

Rabbit anti-H. pertussis serum was obtained from the Laboratories. It had a bacterial agglutination titer of 1:8,000. This serum was absorbed with an equal quantity of normal, washed sheep red blood cells.

Mouse anti-H. pertussis serum was prepared by intraperitoneal inoculation of 32 mice with vaccine 401 diluted to contain 5 billion organisms per dose. These mice received second and third inoculations of 5 billion organisms each at 14 day intervals. Twelve days after the final inoculation the mice were bled and the blood pooled. The serum was removed and absorbed twice with normal, washed sheep red blood cells. The serum was kept frozen until used.

Dilutions of antisera for checking agglutination of tannic acid treated and sensitized red cells were always prepared in buffered physiological salt solution containing one per cent normal rabbit serum as a stabilizing agent.

Mouse Tests

The mouse protection tests were carried out in general according to the method of Kendrick et al. (1947). Each H. pertussis culture, or extract from sonic treatment of cultures, was tested at three levels,

equivalent to 1.5 billion, 0.3 billion and 0.06 billion organisms per ml, inoculated intraperitoneally in one injection. Dilution was made in physiological salt solution. Thirteen mice weighing between 10 and 14 gm were used for each level. A reference vaccine prepared by the Laboratories and known to be comparable to the National Institutes of Health reference vaccine was injected into equal numbers of mice at the same three levels with each protection test carried out. Fourteen days after intraperitoneal injection, all mice were given an intracerebral injection of 0.03 ml containing approximately 100,000 virulent H. pertussis organisms of strain 18323.

The LD_{50} of this challenge culture was determined by intracerebral inoculation of 80, 400 and 2,000 organisms in three groups of ten mice each. A control group of ten mice also received an intracerebral inoculation of the challenge dose of 100,000 organisms. The mice were observed for a 14 day period after challenge and deaths recorded daily. The PD_{50} of the experimental cultures and reference vaccine were determined by the method of Reed and Muench (1938).

Preparation of Experimental H. pertussis Cultures

Lyophilized cultures were transferred to the surface of Bordet-Gengou agar plates containing 16 per cent sheep blood and incubated 3 to 4 days at 35 C. A second transfer was made to Bordet-Gengou agar slants and incubation continued for 48 hours. A final transfer to B.G. agar medium was made and incubated 24 hours at 35 C. After being checked microscopically for purity, the growth from each slant was washed off

with 1 ml of Cohen-Wheeler medium and transferred to flasks containing 50 ml of the seed culture medium. These flasks were incubated at 35 C for 24 hours on a Gump-type shaker which imparts a continuous rotary motion to the flasks. At the end of this incubation period the flasks were removed from the shaker and the contents were checked microscopically for contamination. The amount of growth in the seed flasks averaged 50 to 60 billion organisms per ml. One ml of seed culture was transferred to each of the production medium flasks. These flasks were incubated at 35 C for 30 hours, unless otherwise specified, with continuous shaking. After removal from the incubator, the contents of each flask were checked for purity and density of growth. Density determinations were made in a photelometer by comparison with the National Institutes of Health standard for Hemophilus pertussis and expressed in billions of organisms per ml. Flasks containing pure cultures of the same strain of organisms were pooled and the pH determined by potentiometer. The pH of the pool was adjusted to 7.0 - 7.1 by the addition of 0.2 N H Cl. The density of the pool was then re-checked by photelometer determination. If these cultures were not used immediately, they were kept frozen at -10 C.

Sonic Disintegration and Centrifugation

Sixty ml of the liquid cultures of H. pertussis prepared as described above were treated by sonic oscillation for 30 minutes in a Raytheon 10 KC sonic oscillator. At the end of this disintegration period, 50 ml of the culture was immediately centrifuged in a refrigerated centrifuge at 1 C

for 10 minutes at 6,000 r.p.m. Forty ml of the slightly opalescent, slightly yellow supernatant fluid was removed and immediately frozen. The compact, gray sediment was resuspended in the remaining 10 ml of supernatant fluid and also frozen. This sediment when examined microscopically on stained slide preparations consisted of gram-negative staining debris giving the impression of broken up bacterial cells. Very few whole bacilli were seen.

Preparation of Sheep Red Blood Cells and Treatment with Tannic Acid

The method used for the preparation of tannic acid treated red blood cells is essentially that of Boyden (1951) as modified by Stavitsky (1954a).

Fresh, defibrinated sheep blood was added to sterile Alsever's solution in the ratio of 1:1.2. The blood was used up to three weeks of age. Immediately before use, the blood cells were washed three or four times with physiological salt solution buffered at pH 7.2 and finally suspended in salt solution in two per cent concentration. One volume of this suspension was mixed with an equal volume of freshly prepared tannic acid solution, 1:40,000. This mixture was placed in a 37 C water bath and the flask rotated frequently during a ten minute period. The treated cells were then removed by centrifugation, washed once with one volume of buffered salt solution and resuspended to the original concentration, two per cent. These tannic acid treated sheep erythrocytes were usually used on the day of preparation but could be used the following day providing no hemolysis occurred after standing overnight in the refrigerator.

The suspension of tannic acid treated blood cells is much less stable than that of untreated washed cells and will auto-agglutinate in physiological salt solution. The addition of one per cent normal rabbit serum to the saline solution will stabilize the suspension and prevent this agglutination. The normal rabbit serum used for this purpose was absorbed twice with equal quantities of washed, packed sheep cells, inactivated by maintaining at 56 C for 30 minutes and Seitz filtered. Each preparation of tannic acid treated cells was checked by adding 0.05 ml to several tubes containing 0.5 ml buffered salt solution and to several tubes containing 0.5 ml salt solution with one per cent normal rabbit serum. The tubes were well shaken and allowed to stand undisturbed for several hours at room temperature. The patterns formed by settling of the cells to the bottom of the tubes were then checked. The cells suspended in saline solution only formed an even mat completely covering the rounded bottom of the tubes, indicating agglutination. The cells suspended in one per cent normal rabbit serum settled to a small, round, red "button" in the lowest portion of the bottom of the tube, indicating non-agglutination. A tannic acid cell suspension which did not give these typical patterns was discarded and tests carried out with this suspension were repeated with freshly prepared tannic acid cells.

Hemagglutination Tests Using Tannic Acid Treated Red Blood Cells

Tests for hemagglutination activity of *H. pertussis* extracts on red blood cells modified by treatment with dilute tannic acid were

carried out as follows: Twofold dilutions of the extract in physiological saline containing one per cent normal rabbit serum were made from 1:2 through 1:256. Five-tenths of a ml of each dilution was placed in clear 12 x 75 mm tubes and 0.05 ml of freshly prepared tannic acid red cell suspension added. Duplicate sets of tubes were always prepared. Control tubes containing diluent and cells only, and medium, diluted through the same range as the extracts, and cells were always included with each test. The tubes were shaken approximately 100 times to ensure thorough mixing of the contents. They were then left at room temperature for 1½ to 2 hours and overnight in the refrigerator. Final reading was made the following morning by observation of the type of pattern formed by the sedimented cells. An even, smooth mat completely covering the rounded bottom of the tube was considered agglutination equivalent to ++++. Occasionally very strong agglutination resulted in the edges of this mat being folded in an irregular fashion. Usually a tube showing this type of sedimented, agglutinated cells would also show good macroscopic agglutination of cells when the contents of the tube were shaken gently.

A round, evenly dispersed mat in the bottom of the tube surrounded by a regular red ring was considered agglutination equivalent to ++. A wider red ring with some irregular granulation in the center was considered agglutination of + or ± only. A round, red button of collected cells or a round, red doughnut-type ring was considered to show no agglutination. In determining titers of hemagglutinins, any reaction less than ++ was considered negative.

Sensitization of Tannic Acid Sheep Red Cells

One volume of the antigen dilution to be used as a sensitizing agent was mixed with four volumes of physiological salt solution buffered at pH 6.4 and one volume of two per cent tannic acid treated cells. A control tube containing one volume of salt solution, pH 7.2, in place of the antigen was always included with each sensitization preparation. The tubes were allowed to stand at room temperature with frequent mixing. Cells were then removed by centrifugation at 2,000 r.p.m. for four minutes and washed once with twice the original volume of salt solution containing one per cent rabbit serum. They were then resuspended in one volume of salt solution containing one per cent rabbit serum. These cells were checked for auto-agglutination by mixing 0.05 ml with 0.5 ml salt solution containing one per cent normal rabbit serum and checking the pattern formed on sedimentation. Antigens which caused agglutination of the tannic acid modified red cells when used for sensitization in this manner were unsuitable for use in the antigen-antiserum test (described below). This condition could frequently be corrected by diluting the antigen. These sensitized cells can be kept for two to three days at refrigerator temperature and will demonstrate the same agglutinative activity if no hemolysis appears.

Hemagglutination Tests Using Sensitized Red Cells and Antisera

Hemagglutination tests using sensitized cells prepared as described above were carried out as follows: Dilutions of antisera, which have been

absorbed with normal sheep cells, were made in buffered salt solution containing one per cent normal rabbit serum. Five-tenths of an ml of each dilution was transferred to clean 12 x 75 mm tubes. Where the quantity of serum permitted, each set of dilution was always carried out in quadruplicate, otherwise in duplicate. Sensitized tannic acid cells were then added in 0.05 ml quantities to each tube. Control cells, which were tannic acid cells mixed with salt solution only, were always added to several tubes containing the lowest dilution of antiserum. The tubes were then shaken vigorously approximately 100 times back and forth to ensure complete mixing and then allowed to stand at room temperature for $1\frac{1}{2}$ to 2 hours before being placed in the refrigerator overnight. Final reading was made the following morning by observation of the types of pattern formed by the sedimented cells as described previously.

RESULTS

Mouse Protection Tests

Mouse tests were carried out primarily to check the mouse protective antigens present in H. pertussis organisms of strains 10536, 18334 and 18323 when these cultures were produced during a 30 hour incubation period on a rotary-type shaker. Inasmuch as the supernatant fluids obtained after sonic disintegration of these cultures were used as antigens in the tannic acid hemagglutination tests, it was felt that any information concerning the antigenic composition of these fluids would be helpful. It was also of interest to determine the effect of sonic treatment and of centrifugation on the amounts of mouse protective antigen which these fluids contained.

It was found that these liquid cultures when tested shortly after preparation were toxic for mice on intraperitoneal inoculation. Toxicity was markedly increased by sonic disintegration of the organisms and was evident to an equal extent in the supernatant fluid. Heating to 56 C for 30 minutes completely eliminated this toxicity. Apparently the agent encountered was the thermolabile endotoxin of H. pertussis. Subsequent to the discovery of this difficulty, all liquid cultures and supernatant fluids used in these mouse tests were pre-heated to 56 C.

From the results of these tests, the PD_{50}, expressed in billions of organisms, of these cultures were determined.

Table 1 presents the compiled results of these mouse protection tests.

TABLE 1

Mouse Protection Tests on H. pertussis Cultures

H. pertussis strain	Type of Inoculum	PD_{50} Reference Vaccine / PD_{50} Test Culture
10536	Whole organisms in liquid culture medium	1.08
10536	Sonic disintegrated organisms in liquid culture medium	Toxic
10536	Supernatant fluid from sonic disintegrated organisms	1.00
18334	Whole organisms in liquid culture medium	1.00
18334	Sonic disintegrated organisms in liquid culture medium	1.00
18334	Supernatant fluid from sonic disintegrated organisms	0.85
18323	Whole organisms in liquid culture medium	0.39
18323	Sonic disintegrated organisms in liquid culture medium	<0.39
18323	Supernatant fluid from sonic disintegrated organisms	<0.39
Vaccine 401	Whole organisms in salt solution	1.27
401	Sonic disintegrated organisms from above vaccine	1.19

Results indicated in Table 1 show that H. pertussis culture of strain 10536 containing undisrupted organisms demonstrated a protective level comparable to the reference vaccine. The supernatant fluid obtained by centrifugation after sonic treatment of this same culture demonstrated a level of protection only slightly less.

The culture of strain 18334 containing whole organisms and a sample of this same culture after sonic disintegration of the cells both contained protective antigen equivalent to the reference vaccine. The supernatant fluid obtained by centrifugation of the sonic treated culture contained slightly less antigen than originally present.

The culture of strain 18323 proved to contain only a small amount of mouse protective antigen in comparison with the reference vaccine. After sonic treatment of this culture the amount of antigen dropped below the limit of measurement in the test performed.

Vaccine 401, produced by growth of organisms on Bordet-Gengou agar medium is included for purposes of comparison with liquid cultures. It will be noted that the sample containing whole organisms contains slightly more protective antigen than the reference vaccine. Sonic treatment of this same vaccine resulted in only a slight decrease of this antigen.

Hemagglutination of Tannic Acid Treated Red Blood Cells

Supernatant fluid from H. pertussis liquid cultures and extracts from sonic disintegrated cultures were tested for their ability to agglutinate normal, washed human, chicken, mouse and sheep red blood

cells. With the exception of an occasional agglutination of sheep cells by 1:2 and/or 1:4 dilutions of an extract no agglutination occurred with any of the culture supernatant fluids or extracts used throughout the dilution range, 1:2 to 1:256. These results are at variance with those obtained by Keogh and North (1948) and Masry (1950). In view of these negative results, attempts to demonstrate hemagglutinin by use of unmodified red blood cells were abandoned.

In the course of carrying out studies to determine the feasibility of adapting the Boyden tannic acid hemagglutination technique to the study of H. pertussis antigen-antibody reactions, it was noted that sheep red cells, modified by action of dilute tannic acid were strongly agglutinated by sonic extracts of H. pertussis liquid cultures. The titers of the hemagglutinin in these sonic treated cultures and supernatant fluids were determined by making serial dilutions and mixing them with tannic acid modified cells as described in the Materials and Methods section.

The results of these determinations are presented in Table 2.

TABLE 2

Hemagglutinin Titers of H. pertussis Extracts for Sheep Red Cells Modified by Tannic Acid

H. pertussis strain	Type of Extract	Density as Billion org./ml or Equivalent	Hemagglutinin Titer*
10536	Sonic disintegrated organisms in liquid culture	92.0	128
10536	Supernatant fluid from sonic disintegrated organisms in liquid culture	92.0	256
18334	Sonic disintegrated organisms in liquid culture	93.5	8
18334	Supernatant fluid from sonic disintegrated organisms in liquid culture	93.5	256
18323	Sonic disintegrated organisms in liquid culture	126.0	32
18323	Supernatant fluid from sonic disintegrated organisms in liquid culture	126.0	32
18323	Organisms removed from liquid culture by centrifuging, washed and resuspended in salt solution and sonic disintegrated	126.0	0
401	Sonic disintegrated organisms suspended in salt solution	20.0	0
---	Liquid medium used for seed culture	--	0
---	Modified liquid medium used for culture	--	0

* Hemagglutinin titer expressed in reciprocals of dilution

All of the liquid medium cultures used in the above hemagglutinin tests were incubated at 35 C for 30 hours with continuous rotary shaking as previously described. It was felt that more interesting information might be obtained by checking the density, pH and hemagglutinin titers of liquid medium cultures which were incubated at 35 C for various periods of time. The cultures were handled in the same manner as described above, except that several flasks were removed from the shaker and examined at the intervals stated in Table 3. The contents of the flasks were pooled, the pH adjusted to 7.0 - 7.1 and 60 ml samples were subjected to sonic disintegration and centrifugation. The hemagglutinin tests were carried out on the supernatant fluids.

The organisms of H. pertussis strains used in the previous tests were assumed to be in phase I. Microscopically they were small, coccoid, gram-negative bacilli showing capsular material and very few pleomorphic forms. They formed small dewdrop-like colonies on Bordet-Gengou agar containing 16 per cent sheep blood, showing hemolysis after two to four days of growth. They would not grow on five per cent sheep blood, veal infusion agar, nor on unenriched veal infusion agar. They were agglutinated by H. pertussis immune rabbit serum in a dilution of 1:4,000. They were utilized for production of cells in liquid medium within six transfers from lyophile.

Included in this experiment also were hemagglutinin tests on liquid cultures of H. pertussis strains 10536 and 18334 which had been carried through 26 transfers on artificial media. The purpose of this trial was to observe whether changes in hemagglutinin production paralleled changes

in other characteristics of the organism occurring after a series of transfers. These cells had been carried through five transfers on Bordet-Gengou agar containing 16 per cent sheep blood, four transfers on veal infusion agar containing ten per cent sheep blood, four transfers on five per cent sheep blood veal infusion agar, and 11 transfers on two per cent sheep blood veal infusion agar at four day intervals. These cultures, which were adapted to two per cent blood agar, would also grow on plain veal infusion agar and on Bordet-Gengou agar without blood. The growth from these unenriched media was scanty, very sticky, and was made up of gram-negative, pleomorphic cells, many of them greatly elongated. The growth from two per cent sheep blood agar was composed of typically short, coccoid-like bacilli with surrounding capsular material.

Cells from a 24 hour growth on two per cent blood agar (twenty-fourth subculture) were washed off the slant into a flask of 50 ml seed culture medium and the final production cultures were prepared exactly as described previously.

The results of these hemagglutination tests are presented in Table 3.

TABLE 3

Hemagglutinin Titers of H. pertussis Extracts of Cultures Incubated for Different Periods of Time

H. pertussis strain	Hours of Incubation	Density bil. org./ml	pH of Pool	Hemagglutinin Titer*
10536 (6th subculture)	10	<10	7.40	8
	24	65	8.30	128
	30	94	8.50	512
	48	93	8.84	256
	72	73	8.94	32
	96	69	8.55	16
	120	71	8.75	8
10536 (26th subculture)	10	<10	7.39	0
	24	88	7.30	32
	30	97	8.62	64
	48	clumps**	8.97	16
	72	clumps	9.00	16
	96	clumps	8.90	16
	120	clumps	8.72	0
18334 (6th subculture)	10	<10	--	8
	24	95	8.50	64
	30	136	8.80	256
	48	121	9.05	32
	72	110	8.93	--
	96	113	8.91	8
18334 (26th subculture)	10	<10	7.35	4
	24	88	8.30	32
	30	115	8.58	64
	48	100	8.98	32
	72	84	9.00	16
	96	85	8.96	0
	120	83	8.80	0

* Hemagglutinin titer expressed in reciprocals of dilution.

** Clumps formed during the growth of the culture which were difficult to break up in salt solution, so it was felt that density determination was inaccurate.

Hemagglutination of Tannic Acid Modified Red Blood Cells Sensitized with H. pertussis Extracts

The Boyden tannic acid hemagglutination test is usually carried out by allowing the antigen to be adsorbed onto the surface of the modified red blood cells, forming "sensitized" cells, and then mixing these cells in small quantities with increasing dilutions of the antiserum. Agglutination of the cells as evidenced by the pattern formed on the bottom of the tubes after standing indicates an antigen-antibody reaction which disturbs the stability of the cells causing them to agglutinate. Proper controls must always be included with each test to demonstrate that no agglutination is caused by the antigen alone or by the antiserum alone.

Difficulty was encountered in obtaining an antigen from the H. pertussis extracts which would be adsorbed onto the cells and which would not cause their agglutination without the addition of antiserum. All of the supernatant fluids from the sonic extracts caused a very strong agglutination of the modified red cells even when diluted 1:400, or 1:1,000 in some cases. When they were diluted beyond the concentration causing agglutination, no reaction was observed upon the addition of antiserum. Either the antigen responsible for reaction with antiserum and resulting agglutination of the cells was diluted out beyond the effective range, or the reaction was inhibited by the adsorption of other antigens onto the red cells. It, therefore, seemed reasonable to attempt various methods of concentrating and purifying the antigens adsorbed onto modified red cells. The following procedures were tried:

I. Dialysis

Dialysis of the supernatant fluids for 7 days against distilled water at 4 C did not affect their ability to agglutinate modified red cells in a dilution of 1:400.

II. Heat

Five ml samples of the supernatant fluid from sonic treated liquid culture of strain 10536 were heated for 10 minutes at the following temperatures: 60 C, 70 C, 80 C and 100 C. The fluids were then centrifuged, the supernatant fluids separated and the sediments resuspended in equal volumes of buffered salt solution. These fluids were then tested for their ability to agglutinate modified red cells. Results of these tests are presented in Table 4.

TABLE 4

Effect of Heating Extract of H. pertussis Strain 10536 on Ability to Agglutinate Modified Red Cells

Temperature C	Supernatant Dilution used for Sensitizing Red Cells		Sediment Dilution used for Sensitizing Red Cells
	1:10	1:100	1:10
60	++++	++	-
70	++++	++	-
80	++++	++	-
100	++++	++	-

The red cells treated with 1:10 dilutions of the resuspended sediments were not agglutinated, as indicated in Table 4. These cells were, therefore, tested for sensitization by mixing with 1:100 and 1:1,000 dilutions of immune rabbit serum and 1:100 immune mouse serum as well as with normal rabbit and mouse sera as controls. No agglutination occurred in any of the tubes.

III. Precipitation with Ethyl Alcohol

Portions of the supernatant fluid, pH 7.7, from sonic treated liquid culture of strain 10536 were mixed with 95 per cent ethyl alcohol to give the following final concentrations of alcohol: 30, 40, 50 and 60 per cent. The precipitates were removed by centrifugation, washed once in alcohol solution of the corresponding concentration and redissolved in buffered salt solution to the original volume. These alcoholic extracts were tested for agglutination of tannic acid modified red cells with the following results:

TABLE 5

Agglutination of Modified Red Cells by Alcoholic Fractions of Extract of H. pertussis Strain 10536

Fractions Obtained by Treating with Ethyl Alcohol in Following Concentrations	Dilution of Fractions used for Sensitizing Red Cells	
	1:10	1:100
30 per cent	+++	+
40 per cent	++	++
50 per cent	++	-
60 per cent	+	-

The red cells not agglutinated by 1:100 dilution of 50 per cent and 60 per cent alcohol precipitated fractions were tested for sensitization by adding to 1:100 and 1:1,000 dilutions of immune rabbit serum and 1:100 dilution of immune mouse serum. No agglutination occurred.

This alcohol fractionation experiment was repeated using a second sample of the same supernatant fluid which had been adjusted to pH 6.4. Results were essentially the same.

IV. Precipitation with Trichloracetic Acid

A sample of supernatant fluid from sonic treated liquid culture of strain 10536 was mixed with an equal amount of 10 per cent trichloracetic acid. The precipitate which formed was removed by centrifugation and redissolved in buffered salt solution. This extract in dilutions of 1:10 and 1:20 caused agglutination of tannic acid modified red cells. It was then dialyzed for 5 days at 4 C against distilled water. The dialysed extract in a dilution of 1:2 did not cause agglutination of the cells. When these sensitized cells were tested with immune rabbit serum, a strong agglutination occurred with the 1:100 dilution of serum, no agglutination with the 1:1,000 dilution and no agglutination with 1:100 dilution of mouse serum.

V. Precipitation with Ammonium Sulphate

A sample of supernatant fluid of sonic treated liquid culture of H. pertussis strain 10536 was mixed with an equal volume of

saturated solution of ammonium sulphate. The precipitate was separated by centrifugation and redissolved in buffered salt solution. Treatment with ammonium sulphate solution was repeated and the final precipitate was redissolved in salt solution to the original volume. This ammonium sulphate precipitated fraction actively agglutinated modified red cells in a dilution of 1:200. Dialysis for five days at 4 C did not affect its ability to agglutinate the cells. A dilution of 1:300 did not cause agglutination of modified red cells but apparently did sensitize them as agglutination occurred upon the addition of immune rabbit serum in a dilution of 1:100. No agglutination occurred with a 1:1,000 dilution of immune rabbit serum, 1:100 dilution of immune mouse serum, or with any of the normal sera controls.

Several samples of the supernatant fluids from other sonic treated liquid cultures of H. pertussis gave comparable results when treated with ammonium sulphate in the same manner.

Since this method seemed promising for the production of an antigen which would sensitize modified red cells to the action of antiserum, this procedure was tried using supernatant fluids from sonic treated saline suspensions of H. pertussis cells rather than from sonic treated liquid cultures. For this purpose vaccine 401, containing 20 billion organisms per ml was used. The supernatant fluid after sonic disintegration of these cells was treated with saturated solution of ammonium sulphate as described above. This fraction diluted 1:2 did not cause agglutination of tannic acid modified red cells. Tests for sensitization of the cells were carried out with immune rabbit and mouse sera with the following results:

TABLE 6

Agglutination of Modified Red Cells Sensitized with H. pertussis Extract by Rabbit and Mouse Antisera

Serum	Serum Dilution											
	1:10	1:50	1:100	1:200	1:400	1:600	1:800	1:1000	1:2000	1:4000	1:6000	1:8000
Immune Rabbit	++++	++++	++++					++++	++	+	+	+
Immune Mouse	++++	++++	++++	++++	++++	+++	++	±				
Normal Rabbit	-	-	-									
Normal Mouse	-	-	-									

These results indicate that some antigen of H. pertussis cells precipitated by 50 per cent ammonium sulphate was adsorbed by tannic acid treated cells and that a reaction occurred between this antigen and antibodies present in the sera of immunized mice and an immunized rabbit.

As stated previously, the mice were immunized by three inoculations of H. pertussis vaccine 401. It was felt that information on the ability of liquid cultures of H. pertussis and supernatant fluids of sonic treated liquid cultures to produce similar antibodies in mice would be of interest. Consequently, groups of mice were given intraperitoneal injections of various quantities of liquid culture of H. pertussis strain 10536 and

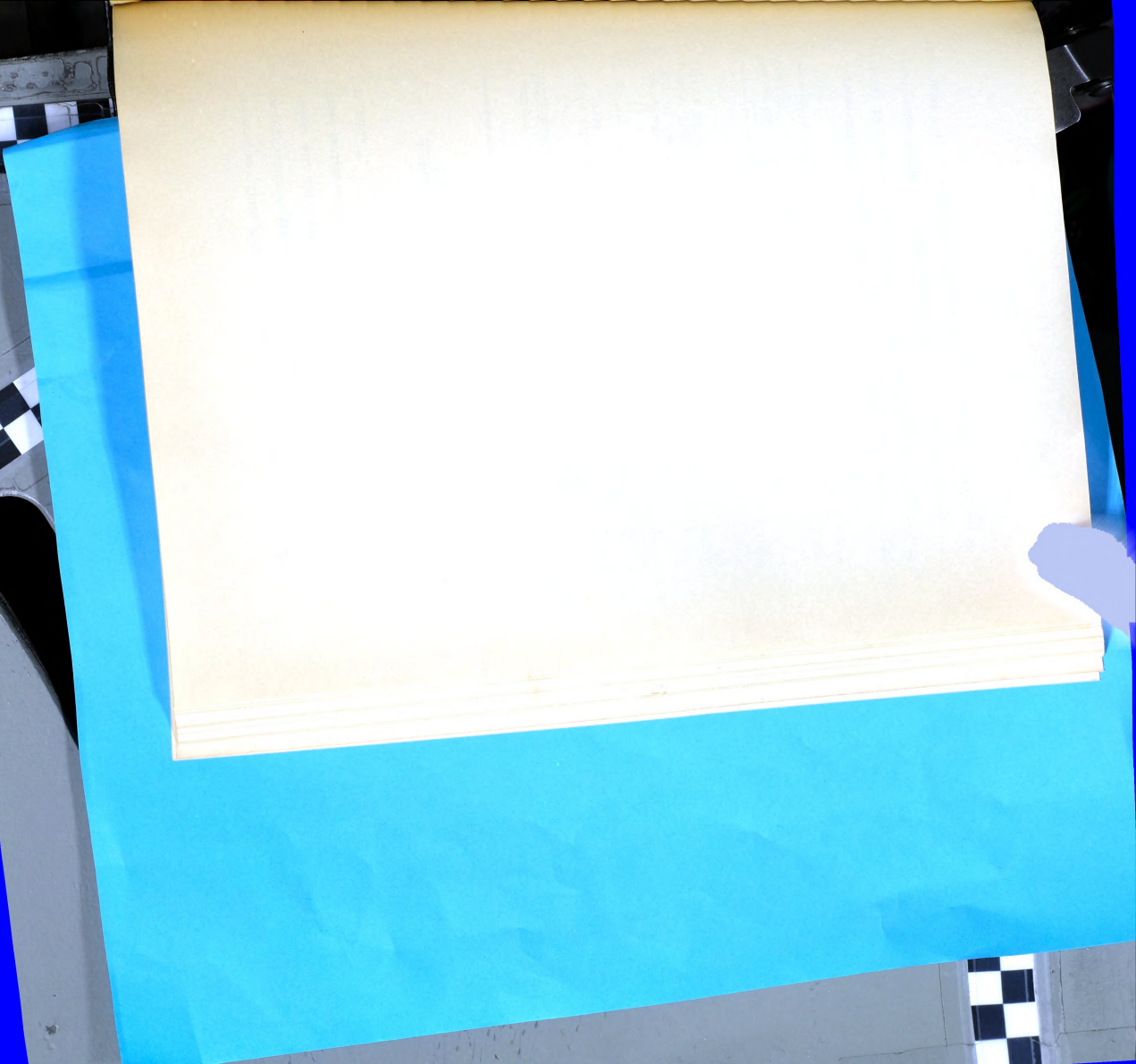
supernatant fluid from the same sonic treated liquid culture. The hemagglutination titers of the serum were determined by checking with tannic acid cells sensitized with 50 per cent ammonium sulphate precipitated antigen of vaccine 401 as in the previous test. Data are presented in Table 7.

TABLE 7

Hemagglutination Titers of Sera from Mice Inoculated with Liquid Cultures and Supernatant Fluid from Liquid Cultures of H. pertussis

Antigen Inoculated	Single Dose Billion org./ml	Number of Doses	Interval between doses Days	Interval between last dose and bleeding Days	Hemagglutination Titer
Liquid culture 10536	5.00	2	13	10	1:50
	5.00	3	1	10	0
Supernatant fluid of sonic treated liquid culture 10536	0.06	2	15	10	0
	0.30	2	15	10	0
	1.50	2	15	10	0
	5.00	2	13	10	1:600
Vaccine 401 (control)	5.00	3	14	14	1:800

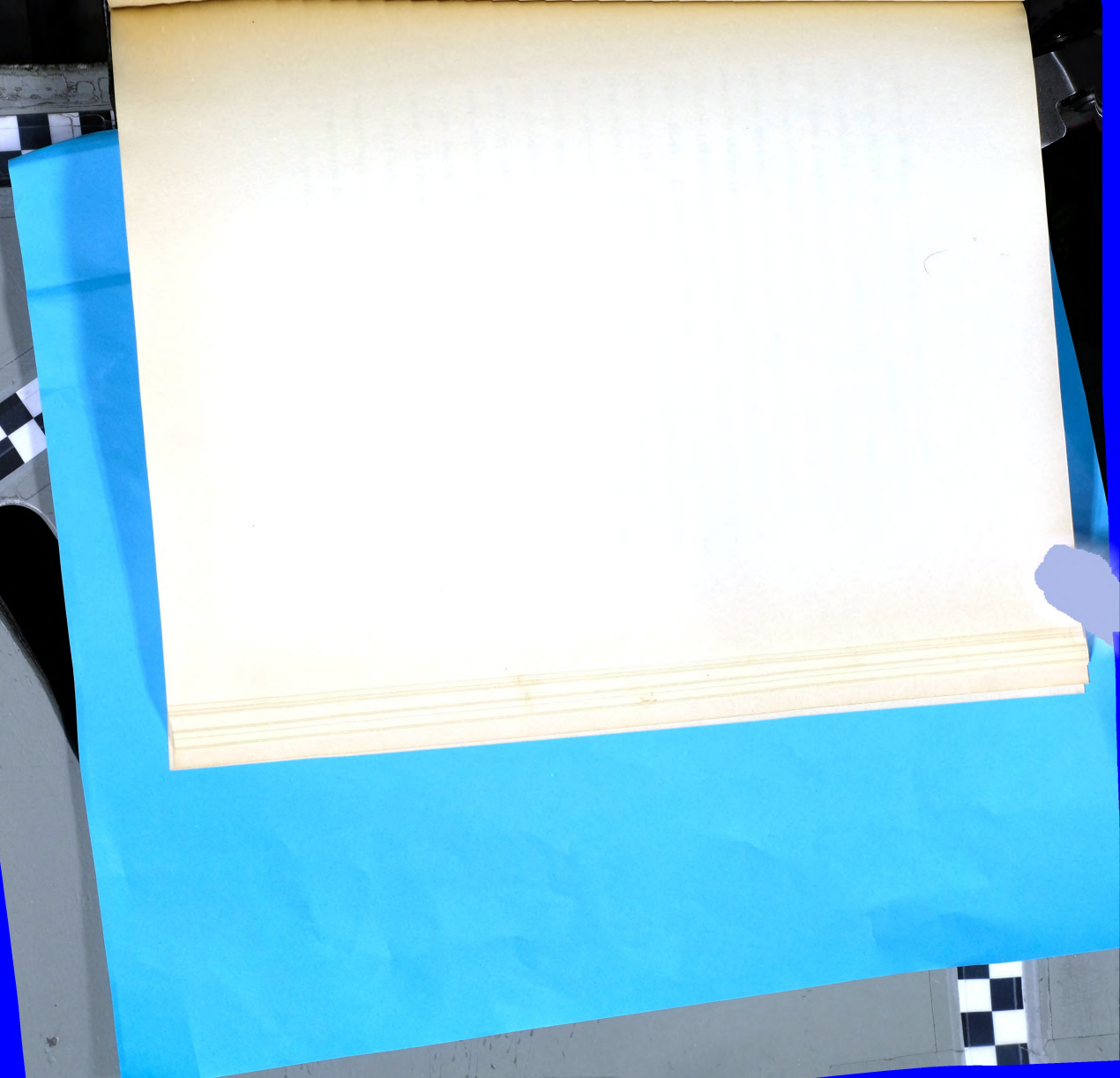
These results indicate that mice given two doses of 5 billion H. pertussis organisms 13 days apart and then bled 10 days following the second injection produced serum having an hemagglutination titer of 1:50. Mice which were given three doses of 5 billion organisms each of this same culture but on



three successive days and then bled 10 days following the last injection produced no antibodies demonstrable by the hemagglutination test employed. Mice which were given two doses of the supernatant fluid from the same culture which had been sonic treated and diluted to contain the equivalent of 5 billion organisms per dose and then bled 10 days after the second injection produced serum with an hemagglutination titer of 1:600. Mice which were inoculated with two doses of supernatant fluid diluted to contain 1.5 billion organisms per dose, or less, did not produce demonstrable hemagglutination antibodies.

DISCUSSION

The data presented in these results were collected mainly by examination of cultures of H. pertussis organisms grown in liquid medium with continuous rotary shaking. In this manner large yields of cells could be obtained in a short incubation time, usually thirty hours. Tests were carried out on the cultures of organisms suspended in the medium in which they were grown. This method was chosen so that any substance emitted into the medium during growth or any metabolic breakdown product would not be lost. In order to increase the concentration of these soluble metabolic products, as well as to procure other soluble contents within the bacterial cell, some method of completely breaking up the cell was necessary. The sonic oscillation method has been successfully used by Flosdorf, Kimball and Chambers (1939), Smolens and Flavell (1947), and Pillemer, Blum and Lepow (1954). In this study sonic oscillation was carried out on the complete liquid cultures which contained unused medium ingredients, breakdown products of bacterial action on ingredients, enzymes, substances excreted by the organism and bacterial cells. Insoluble materials, such as cell wall fragments, were removed immediately after sonic treatment by centrifugation. Tests using supernatant fluids from sonic treated cultures, therefore, were actually carried out on the soluble substances, or at least colloidally dispersed substances, present in the medium and in the cell contents.



Antigens responsible for protection of mice, as determined by the intracerebral mouse protection test, appeared to be present in two of the three liquid cultures tested. Treatment of these liquid cultures with sonic oscillation for 30 minutes did not appreciably decrease the amounts of these antigens. This finding is in agreement with that of Pillemer et al. (1954). The supernatant fluids from these sonic treated cultures still contained the antigens protective for mice but in decreased amounts. Apparently some of this antigenic material remained attached to the cell debris and was removed with the sediment.

Further examination of these sonic treated liquid cultures and supernatant fluids from sonic treated liquid cultures of H. pertussis revealed the fact that some substance is produced during growth which causes the agglutination of tannic acid modified sheep red blood cells. This substance is present in cultures containing sonic disintegrated cells and is present to a higher titer in the supernatant fluids from centrifuged sonic treated cultures. The substance does not appear to be present in the fluid obtained by the sonic disintegration of H. pertussis cells which have been washed free of medium and resuspended in salt solution.

This material which causes the agglutination of tannic acid modified sheep cells increases as the culture is incubated up to 30 hours and decreases gradually as the culture is further incubated. The decrease is not correlated with changes in pH as the pH gradually increases during growth from an initial of 7.2 to approximately 8.8 to 9.0 in 48 hours and remains relatively constant or falls off very slightly as incubation proceeds.

Increasing numbers of transfers on artificial media and adaptation of the cultures to growth on non-enriched media appeared to affect the ability of H. pertussis organisms to form the substance in liquid cultures which causes the agglutination of tannic acid modified red cells. H. pertussis strain 10536 after only six transfers on artificial medium produced a supernatant fluid with an hemagglutinin titer of 512, whereas the same strain carried through 26 transfers produced a supernatant fluid with an hemagglutinin titer of only 64. Essentially the same results were found with H. pertussis strain 18334.

It is impossible to determine the significance of this hemagglutinin activity of supernatant fluids of sonic treated cultures of H. pertussis based on this preliminary examination. These results cannot be compared with those on hemagglutinins of H. pertussis reported by Keogh, North and Warburton (1947), Fisher (1950) or Masry (1950) inasmuch as all of these workers used normal, unmodified red cells.

It would be interesting to compare the hemagglutinin activity for tannic acid treated cells of a number of H. pertussis strains with their mouse protective antigen content.

Several theories exist as to the effect of tannic acid on washed red blood cells. It has been suggested (Boyden 1951) that tannic acid alters the surface properties of the red cells creating an hydrophobic state in place of the usual hydrophilic state. Freund (1931) believed that tannic acid, like homologous antibody, brought about a change in the surface potential of the cells which resulted in their agglutination in the presence of certain electrolytes. Whatever the mechanism of the action

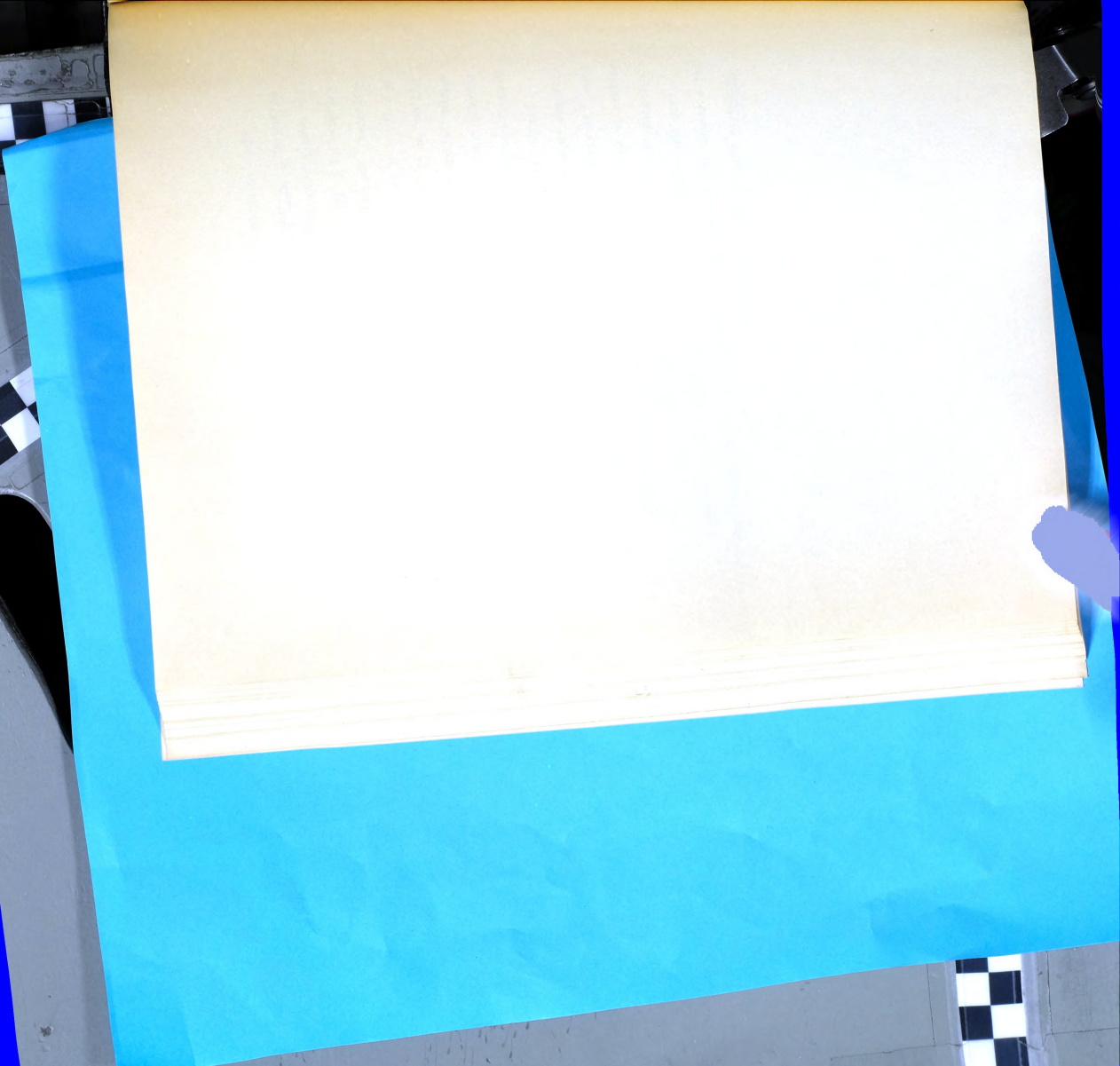
of tannic acid may be, it is certain that the altered surface of the red cells will adsorb protein molecules much more readily than untreated cells (Boyden 1951, Stavitsky 1954a). Since many bacterial antigens of interest are protein substances, it is this property of tannic acid treated cells which makes them useful as an indicator mechanism for demonstration of antigen-antibody reactions. It soon became evident that this method also had certain disadvantages. The sensitive adsorbing surface of the treated red cells would also take up a variety of protein species if a mixture happened to be present. Boyden (1951) and Stavitsky (1954a) stress the fact that high titers of antisera are obtained only when pure antigens are used for "sensitization" of the cells. This test is also subject to difficulty due to "inhibitors" - substances present in some biological fluids which prevent or interfere with the adsorption of antigen or with the antigen-antibody reaction.

In this study only preliminary attempts were made to concentrate the protein material in the supernatant fluids of the sonic treated H. pertussis cultures. The protein precipitants, trichloracetic acid and ammonium sulphate, yielded protein extracts which would slightly sensitize the modified red cells but only sufficiently to give a reaction with a low dilution (1:100) of immune rabbit serum. Protein precipitated extract from supernatant fluid of sonic treated cells suspended in salt solution was much more satisfactory. This extract sensitized cells to give a reaction with immune rabbit serum in a dilution of 1:2,000 and immune mouse serum in a dilution of 1:800. Apparently other materials present in the medium of the liquid cultures acted as interfering or inhibiting agents.

Since an antigen capable of sensitizing modified red cells to the action of H. pertussis antibody was now available, it was used to test the hemagglutination titer of sera from mice inoculated with liquid cultures of H. pertussis and supernatant fluid from these sonic treated cultures. Results of this test indicated that the supernatant fluid of a culture of strain 10536 when inoculated into mice in sufficient quantity did act as an antigen and that antibodies were produced which would cause agglutination of sensitized tannic acid red cells. Liquid cultures containing whole organisms also produced antibodies demonstrable by this hemagglutination procedure but in lower titer than the supernatant fluid.

Further work on the purification of the antigen from H. pertussis cultures capable of sensitizing tannic acid modified red cells should be done. No attempt has been made to determine the chemical nature of this antigen or to correlate it with those responsible for mouse protection, complement fixation, toxin or agglutinin-absorption.

From the results of these experiments, however, it is concluded that a tannic acid hemagglutination procedure can be utilized to demonstrate H. pertussis antigen-antibody reactions.



SUMMARY

Cultures of three strains of Hemophilus pertussis produced in liquid medium with continuous shaking were tested for their ability to protect mice against H. pertussis infection, and for their hemagglutinin activity for tannic acid treated red cells. A culture of one of these strains was tested for its ability to produce in mice antibodies demonstrable by a tannic acid hemagglutination test.

Two of the three strains tested by the intracerebral mouse test were protective for mice. Sonic oscillation treatment for 30 minutes to break up the bacterial cells did not affect their protective potency. Tests on the supernatant fluids of these sonic treated cultures showed a decrease in their protective antigens.

Sonic treated liquid cultures and supernatant fluids from these cultures of all three strains agglutinated washed sheep red blood cells which had been modified by treatment with tannic acid. Hemagglutinin titers ranged from 1:8 to 1:256.

Protein fractions from supernatant fluids of H. pertussis cultures were capable of sensitizing tannic acid modified red cells, which were then agglutinated by H. pertussis immune rabbit serum.

Liquid cultures and supernatant fluid from liquid cultures of one strain of H. pertussis inoculated into mice caused the production of antibodies which agglutinated sensitized tannic acid red cells.

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