THE EFFECT OF VARIOUS ENZYMES ON THE ANTIGENIC ACTIVITY OF HEATED AND UNHEATED BORDETELLA SUSPENSIONS

Thesis For The Degree Of Ph.D.

MICHIGAN STATE UNIVERSITY

Aina Valdmanis

1960

C 2

This is to certify that the

thesis entitled

The EFFECT of VARIOUS ENZYMES
on the ANTIGENIC ACTIVITY
of HEATED AND UNHEATED
BORDETELLA SUSPENSIONS

presented by

AINA VALDMANIS

has been accepted towards fulfillment
of the requirements for

Ph D degree in Microbiology
and Public Health

Walter N. Mack
Major professor

Date June 29, 1960

O-169

THE EFFECT OF VARIOUS ENZYMES ON THE ANTIGENIC ACTIVITY OF HEATED AND UNHEATED BORDETELLA SUSPENSIONS

By

AINA VALDMANIS

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1960

ABSTRACT

Very little is known about the chemical nature of Bordetella pertussis antigens, either the agglutinogen or the protection-inducing antigen. This investigation was undertaken to study the effect of various enzymes on B. pertussis antigens, both unheated and heated, in order to obtain information concerning their chemical composition. A comparative study was made of heat-stable antigens of Bordetella and certain other species for which the antigens have been at least partially defined. The effect of urea on Bordetella and Escherichia coli antigens was also determined.

Enzymes with various specificities were selected: pepsin, trypsin, chymotrypsin, beta amylase, acid phosphatase, cellulase, hemicellulase, lipase, and lysozyme.

Unheated B. pertussis suspensions were resistant to the action of the enzymes employed. Some denaturation, either by low pH or heating at 100°C, made the organisms susceptible to the action of proteolytic enzymes.

From the study of heated antigens it was noted that certain Bordetella antigenic factors were more heat-stable than others.

Agglutinability, agglutinin production in rabbits, and agglutinin-absorbing properties varied independently in their heat stability.

Heating cells to 100°C did not destroy certain Bordetella antigens, but separated them off the bacterial cell. The presence of these antigens in the separated supernatant fluids of heated bacterial suspensions was demonstrated by agglutinin absorption procedures.

The antigenic activity of the heated bacterial cells was reduced after separation from the supernatant fluid.

Serological reactions of Bordetella, Brucella abortus, Salmonella paratyphi B, and Escherichia coli 02 and 09 indicated that a method of antigenic analysis based on heat stability, which is successful with one bacterial species, cannot be expected to apply to a different species.

The effect of chymotrypsin on heated (100°C) cells was the loss of their agglutinability and agglutinin-producing property in rabbits, while little change was observed with other similarly treated bacterial species. These observations strongly suggested that Bordetella agglutinogens are proteins and chemically different from the O antigens of E. coli. Agglutinogen and protective antigen of B. pertussis were destroyed by 8 M urea solution. This finding was

further evidence that these antigens are proteins. The O antigens of E. coli 02 or 09, considered to be polysaccharide-lipid complexes, were resistant to the urea treatment.

ACKNOWLEDGMENTS

The author is indebted to Dr. Walter N. Mack, under whose supervision this study was made; to Dr. Grace Eldering for guidance and advice; to Dr. Pearl L. Kendrick for helpful suggestions; and to the Division of Laboratories of the Michigan Department of Health for providing space and facilities for the experimental work.

For the suggestion for the use of enzymes in the study of antigens, acknowledgment is made to Dr. William Verwey.

TABLE OF CONTENTS

LIST OF TABLES

TABLE Page

INTRODUCTION

Very little is known of the chemical nature of Bordetella pertussis antigens. Much has been learned from previous studies about the activity of such antigens as agglutinogen, hemagglutinin, protective antigen, toxins, and the histamine sensitizing factor. A schema has been described which explains the serological cross reactions within the genus Bordetella.

The most studied antigens are the agglutinogen and the protective antigen. Many attempts to separate the individual antigens have not been entirely successful. If the chemical nature of the protective antigen were known, it might be possible to work out the preparation of a better immunizing agent against whooping cough. In this study an attempt was made to learn something about the antigenic structure, using various enzymes as tools. Enzymes of different specificities were selected: proteolytic enzymes such as pepsin, trypsin, and chymotrypsin; enzymes with activity on polysaccharides and lipids; and others including lysozyme, acid phosphatase, and hemicellulase. If a specific antigen would be destroyed by a particular enzyme, some clue could be obtained as to its chemical nature.

The degree of heat stability of an antigen has been used in antigenic studies of other bacterial species. Such studies were applied to Bordetella in conjunction with enzyme studies and in comparison with other species. For example, Escherichia coli, Salmonella species, and Brucella abortus were included, since there is already considerable information concerning the chemical nature of their antigens.

REVIEW OF LITERATURE

The genus Bordetella includes three species, Bordetella pertussis, Bordetella parapertussis, and Bordetella bronchiseptica. The type species B. pertussis was isolated from a child with whooping cough and primarily described by Bordet and Gengou (1906). B. bronchiseptica was first isolated from the respiratory tract of dogs and was believed for a time to be the cause of canine distemper (Ferry, 1912). It is also commonly found in rabbits and has been recovered from the trachea and lungs of many other animals, including guinea pigs, ferrets, and monkeys. There are a few reported instances in which this organism was isolated from man and associated with clinical symptoms resembling those of whooping cough. The relationship between B. pertussis and B. bronchiseptica was first observed by Ferry (1918). Eldering and Kendrick (1938) described an organism which they isolated from a patient with whooping cough and which was serologically related to B. pertussis. They referred to the organism as the parapertussis bacillus. With the establishment of the genus Bordetella (Lopez, 1952), this organism became known as Bordetella parapertussis.

The cross protection afforded mice by these species was studies by Kendrick et al. (1953). Cross protection was demonstrated between B. pertussis and B. bronchiseptica, while mice immunized with B. parapertussis showed little protection against challenge with B. pertussis. B. parapertussis–vaccinated mice challenged with B. bronchiseptica showed good protection. The results of challenge with B. parapertussis were not included, because of the failure of this organism to kill mice when injected intracerebrally. Even so, these data suggested that B. pertussis and B. parapertussis are related more closely to B. bronchiseptica than they are to each other.

An antigen common to all species is the endotoxin described by Evans (1939). According to him, the endotoxin is indistinguishable among the three species but is distinct from the bacterial antigens. The same author (1942) demonstrated that, while extracts obtained by disrupting the bacilli and detoxifying with formaldehyde produce antitoxin and antibacterial antibodies in rabbits, suspensions of whole bacilli treated with formaldehyde or suspensions of heated bacilli as well as heated extracts produced only antibacterial antibodies.

Flosdorf and McGuinness (1942) described two toxins which were present in all species. One is thermolabile, being destroyed at 56°C within one-half hour, while the other is thermostable and is

only partially destroyed within one hour at 100°C. The toxins are not antigenic in man, although a mild degree of antigenicity by production of circulating antitoxin can be demonstrated in rabbits.

Lacey (1953) noted that the ionic composition of the medium and the temperature of incubation are important factors in the development of the antigens of B. pertussis, B. parapertussis, and B. bronchiseptica. No single environment allowed the simultaneous development of all potential antigens which were detectable by agglutination. According to Lacey (1953), at least two modes (from different environments) were necessary for the complete identification of any strain. The distribution of antigens in the two modes is represented as X antigens composed of several factors in the X mode and O and H antigens in the C mode. The O and H antigens have only one factor. The cross agglutination between species is usually through X antigens.

Another approach to the antigenic structure was based on heat stability. Kauffmann (1947) studied Escherichia coli and, based upon heat stability, separated the surface K antigens from the somatic O antigens. The O inagglutinability of E. coli was related to the presence of a K or capsular antigen. The K antigens were divided into separate L, B, and A antigens based on their heat stability. Smith and Bryant (1927) had described the capsular antigen,

found in E. coli strains of bovine origin, which corresponded to the A antigen later described by Kauffmann (1947). Kauffmann was the first to describe the thermolabile L antigens, which are mostly envelope or surface antigens. According to Kauffmann, the term "K" is used as a symbol for either envelope or capsular antigen. Antigens L, B, and A are substances which differ from each other in several respects. The only antigen which has been analyzed chemically is the A antigen, which proved to be a type specific capsular polysaccharide. Antigens L and B are heat-labile, and their antigenicity is destroyed at 100°C for 2-1/2 hours. Antigen A requires heating at 120°C for 2 hours to be destroyed. Wilson and Miles (1957) point out that all workers do not agree with Kauffmann's classification, and that all of these antigens may be regarded as heat-labile, somatic antigens with varying degrees of lability, depending on the test conditions. It should be noted that one strain can possess only one of these antigens—never a combination of two or more.

Following Kauffmann's method, Andersen (1953) demonstrated that B. pertussis, B. parapertussis, and B. bronchiseptica possess heat-labile K and heat-stable O antigens. Performing studies on suspensions of living organisms and cultures heated at 100°C for 2 hours, Andersen concluded that 100°C was insufficient to destroy the

surface K antigens. Cultures heated at 120°C for 1 hour showed complete O identity of B. pertussis, B. parapertussis, and B. bronchiseptica. Unheated suspensions of Bordetella did not stimulate O agglutinins when injected into animals. This observation indicated an unusual behavior of the heat-stable antigen of Bordetella. Animals injected repeatedly with suspensions of cells heated at 120°C for 1 hour produced sera with low titers. Cross reactions have been observed with various other Gram-negative bacilli considered to be unrelated. It was possible that the high temperature denatured the Bordetella antigen and made it quite nonspecific, resulting in cross reactions with similar preparations of other genera. Particularly, the denaturation of serum proteins by heat was described by Uwazuvi and quoted by Landsteiner (1936). Rabbits injected with homologous serum heated at 120°C for 1 hour produced antisera which precipitated with similarly treated serum proteins of various other species, as well as rabbit.

In considering heat-stable antigens, the type described by Boivin and Mesrobeanu (1933) should be included. These are protein-polysaccharide-lipid complexes, and they are found in Salmonella, Shigella, Neisseria, Brucella, and other groups.

Andersen's (1953) proposed antigenic schema for Bordetella was confirmed and extended by Eldering et al. (1957), postulating

fourteen K factors. For the purpose of this report an antigenic factor is defined as an agglutinogen which can be demonstrated as a specific entity by agglutinin absorption procedures. These factors explained the serological relationships among the species. Factors 1 through 6 were found in cultures of B. pertussis; Factor 7 was common in all Bordetella species. Factor 12 was specific for B. bronchiseptica, and Factor 14 for B. parapertussis. Factors 8, 9, and 10 were found in B. parapertussis and different strains of B. bronchiseptica. Factor 11 was found in some B. bronchiseptica cultures. Factor 13 was found in certain B. pertussis and B. bronchiseptica cultures. The most extensively studied member of the Bordetella group has been B. pertussis. Leslie and Gardner (1931) tested thirty-two strains by agglutination, agglutinin absorption, and agglutinin-producing procedures and showed that they all fell into one or another of four well-marked agglutinating groups called Phases I, II, III, and IV. Phases I and II were stated to be smooth, while Phases III and IV were dissociated or rough. Phase I organisms were cocco-bacilli, while the rough organisms were long filamentous forms.

Flosdorf (1941) studied the four serological phases of B. pertussis and demonstrated that Phase I agglutinogen was completely lost in Phases III and IV. Evidence suggested that Phase I organisms

were not merely Phase III or IV with a Phase I coating on the surface, but were antigenically different. Kasuga et al. (1953) compared B. pertussis antigens with antigens of E. coli described by Kauffmann. Based on the morphology of the colonies, capsular staining, and agglutinability, he decided that smooth cultures belonged to Phase I, intermediate phase, and Phase III, and rough cultures were designated R_1, R_2, and R_3. The antigenic structure consisted of the capsular heat-labile L and heat-stable S antigens and somatic, heat-stable O antigen. Phase I organisms had a well-developed capsule and the L, S, and O antigens. Suspensions of living organisms of this phase were agglutinated with K antiserum, but not with the O antiserum. The so-called O inagglutinability (Kauffmann, 1947) was eliminated by heating the suspensions at 100°C for 2 hours. The intermediate phase had less of the capsular material and less L and S antigens. Phase III had no capsule, and therefore no L or S antigen but only the O antigen, and it could be used to produce O antiserum. Strains with rough colonies lacked capsules and L and S antigens, and the O antigen varied. The study of heat stability of the antigens indicated that L antigen lost its agglutinability at 70°C for 30 minutes and its antigenicity at 100°C for 1 hour. The S antigen was destroyed by

heating at 120°C for 1 hour. The O antigen was damaged to some extent by heating at 120°C for 2 hours.

Someya and Kaneko (1955) found that the classification of the K antigen into L and S antigens by agglutination reactions was inadequate. From agglutination tests and agglutinin absorptions they concluded that K antigen may change its serological properties according to heating or other treatment. This conclusion was in agreement with the theory that antigens may all be classified as heat-labile, depending on the test conditions.

The whole cell of B. pertussis consists of many antigens, some of which may not be necessary for prophylaxis. If the protective antigen could be isolated, a better immunizing agent could probably be produced. Attempts have been made to identify the agglutinogen with the protective property of the vaccine. Evans and Perkins (1953) used four vaccines in a comparison of agglutinin production and protection-inducing properties for mice. There was a rough parallelism between agglutinin production and protective property. Whether or not agglutinogen is related to protective property is still an unanswered question.

Pillemer (1954) reported that the protective antigen could be extracted from B. pertussis cells by sonic disintegration and under special conditions could be adsorbed on human red cell stromata.

This antigen did not lose the protective property at 56°C for one hour, but was readily inactivated at 100°C. The protective antigen represented only a minute portion of the organism, less than 1 percent of the total nitrogenous material. From these experiments it appeared that the protective antigen was associated with the bacterial cell and was not related to the hemagglutinin nor agglutinogen. The ability of the protective antigen to produce agglutinins was studied by Evans and Perkins (1955). These authors demonstrated that, in comparison with the whole vaccine, the soluble antigen of Pillemer contained a small quantity of agglutinogen and produced a poor agglutinin response in mice. In this laboratory (Michigan Department of Health) it was demonstrated that Pillemer's antigen stimulated agglutinin production in rabbits. It seems difficult to compare tests carried out in two different animal species, where the number of injections, the dose, and the route by which the animals were injected varied. Field studies of Pillemer's antigen by the Whooping Cough Immunization Committee in England (1959) indicated good protection in children; however, the soluble antigen was more toxic than vaccine containing the whole cells. Agglutinin production in mice and in children was poor as compared with the vaccine.

Maitland and Guerault (1958), using saline solution extracts of B. pertussis, protected mice against intracerebral challenge. Animals

injected with these extracts produced agglutinins and were sensitized to histamine. This would indicate that these antigens are located on the bacterial surface. The chemical findings are consistent with the presence of a protein-lipid-carbohydrate complex and indicate a complex antigenic structure. Such a concept may explain the cross-agglutination results with unrelated species, when the antigens are similarly treated with heat at 120°C for 1 hour.

MATERIALS AND METHODS

Media

Bordet-Gengou (BG) medium containing 16 percent sheep blood was used for reconstituting lyophilized cultures of and for maintaining other cultures used as antigens. Bordetella cultures used in experiments with enzymes or in the study of the effect of various temperatures were grown in a semisynthetic medium prepared according to the method of Cohen and Wheeler (1946). This fluid medium was dispensed in 16 oz. French squares in amounts of 200 ml. and autoclaved at 120°C for 15 minutes. The Cohen-Wheeler medium was also used for growing cultures of Salmonella paratyphi B and Brucella abortus. For cultivation of Escherichia coli, veal infusion agar or beef extract agar was used.

Cultures

Bordetella pertussis 10 536, Bordetella pertussis—rough (Farrell), Bordetella parapertussis 17 903, and Bordetella bronchiseptica 88 were obtained from the Michigan Department of Health Laboratory in the lyophilized state. The cultures were suspended in small

amounts of 1 percent Casamino acid (Difco), plated on BG medium and incubated at 37°C for 3 days. Typical-appearing colonies were transferred to slants and subsequently to the BG medium layered in 16 oz. French squares. After incubation at 37°C for 48 hours the cells were washed off with saline solution, centrifuged, and resuspended in saline solution with merthiolate in 1:5000 dilution as a preservative. These suspensions were used as antigens in agglutination tests only.

For growing cultures of Bordetella in fluid medium, a seeding suspension was prepared by inoculating small amounts of Cohen-Wheeler medium with typical colonies from the BG plates of lyophilized cultures. After incubation at 37°C for 48 hours the purity of this culture was tested by Gram stain, and 5 ml. amounts were transferred to 200 ml. of Cohen-Wheeler medium. Cultures were incubated at 37°C for 48 to 60 hours, except for B. bronchiseptica cultures which, due to their more rapid growth, were incubated for only 24 to 36 hours.

After incubation the cultures were tested for purity by making Gram-stained preparations. The pure suspensions were pooled and centrifuged, and the sedimented cells were resuspended in saline solution with merthiolate in 1:5000 dilution. The density of the suspensions was standardized with a Bausch and Lomb "Spectronic 20"

colorimeter, already calibrated, using filter 530 Mu. These suspensions were kept in the cold room. The Salmonella paratyphi B culture was isolated in the diagnostic laboratory of the Michigan Department of Health in Grand Rapids, and was stored on veal infusion agar. One hundred milliliters of Cohen-Wheeler medium was inoculated with a loopful of the culture and incubated at 37°C for 3 days. Several French squares with 200 ml. of the Cohen-Wheeler medium were inoculated with this culture and incubated at 37°C. After 3 days' incubation the cultures were tested for purity by Gram stain. The centrifuged organisms were suspended in saline solution and the density was determined by the same procedure as in Bordetella. Brucella abortus strain number 19 was obtained from Dr. I. F. Huddleson of Michigan State University. A small amount of this culture, grown on a veal infusion agar slant, was used as inoculum for Cohen-Wheeler medium. A second transfer in this medium was made for growing the cells for use in this study. Brucella cultures were incubated at 37°C for 6 to 7 days. All cultures were grown under similar conditions to eliminate the variations resulting from different cultural conditions. Escherichia coli strains 02, K1 (L) and 09, K26 (A) were obtained from Dr. W. W. Ferguson of the Michigan Department of Health. These cultures were maintained on veal agar slants. For antigen preparation, blood plates were inoculated with each of

the strains and incubated at 37°C for 15 to 18 hours. A single, smooth colony was transferred to a veal infusion agar slant and incubated at 37°C for 15 hours. The growth was washed off with saline solution and 1 ml. amounts were used for seeding the beef extract agar slanted in 16 oz. French squares. After 15 to 18 hours' incubation, the cells were harvested in saline solution and centrifuged, and the sedimented cells were resuspended in saline solution. The suspensions either with or without merthiolate were kept in the cold room.

Enzymes

The enzymes selected according to their specificity were beta amylase, cellulase, hemicellulase, acid phosphatase, lipase, lysozyme, chymotrypsin (salt-free), pepsin (2x crystallized), and trypsin (2x crystallized and salt-free). These were purchased from Nutritional Biochemicals Corporation, Cleveland 28, Ohio. The optimal conditions for the activity of the various enzymes were followed as given by the respective workers: beta amylase, Myrback and Neumuller (1950); cellulase and hemicellulase, Pigman (1950); acid phosphatase, Bodansky (1937); lipase, Comfort and Osterberg (1934); lysozyme, Fishman (1951); chymotrypsin, pepsin, and trypsin, Smith (1951).

Whether or not a particular proteolytic enzyme is capable of hydrolyzing the peptide bonds of a protein or peptide substrate depends upon the specific chemical structure of the bond. According to Neurath (1957), pepsin splits peptide bonds where the NH groups are contributed by tyrosine or phenylalanine. Trypsin acts on peptide bonds in which the CO- groups are contributed by lysine or argenine. Chymotrypsin for its specificity requires the CO- groups of phenylalanine, tryptophane, or tyrosine.

Antisera

Antisera were prepared either in rabbits or mice. Rabbits were injected with vaccines, receiving six or more intravenous injections, the doses graded according to the weight of the animal. Injections were given a week apart, and trial bleedings were made periodically. The blood was collected by bleeding the rabbits from the heart. All sera were inactivated at 56°C for 30 minutes and preserved with merthiolate in 1:5000 dilution. The factor sera for Bordetella were prepared by multiple absorptions with appropriate cultures.

Agglutinin Production in Mice

Groups of mice were injected twice intraperitoneally with 0.5 ml. containing 5 billion organisms. The injections were 7 days apart. Fourteen days after the last injection the mice were anaesthetized and bled from the subclavian artery. The sera after separation from the cells were inactivated at 56°C for 30 minutes and preserved with merthiolate.

Agglutination Tests

Agglutination tests were performed with serial serum dilutions, starting with 1:10. Each tube contained 0.1 ml. of the diluted serum plus 0.1 ml. of antigen containing 20 billion cells per milliliter. The tubes were shaken for 3 minutes and incubated at 37°C for 1 hour. Before the tests were read, 0.5 ml. saline solution was added to each tube and the degree of agglutination was determined by viewing the tube with a hand lens against light with a dark background. The degree of agglutination was read as ±, +, 2+, 3+, and 4+. The end point was expressed as the highest dilution giving a 2+ reading. The 1 hour incubation was used in the K antigen-antibody system. The procedure of agglutination tests using O antigen-antibodies was the same, except the tests were incubated at 37°C

for 4 hours. Agglutination tests done with the factor sera followed the same method, but after 3 minutes' shaking the tubes were kept in the cold room overnight.

Agglutinin Absorption Tests

Serum in 1:10 dilution was mixed with a measured amount of packed bacterial cells. The mixture was incubated at 37°C for 5 hours with occasional shaking and held at 4°C for 24 to 48 hours. The absorbed serum was separated from the cells by centrifugation and tested for agglutinins. Absorption was repeated whenever indicated. Agglutinin absorption procedures were also performed by using bacterial suspensions. Serum in 1:10 dilution was mixed with an equal amount of the suspension and incubated at 37°C for 5 hours and held at 4°C for 24 to 48 hours. This method was useful in cases in which the agglutinogens were soluble in saline solution. The supernatant fluid from the heated vaccine was added to the antiserum in the proportion of 10:1.

Mouse Protection Tests

The protective properties of the vaccines were tested by the mouse protection test, using the procedure outlined by the National Institutes of Health (1948). The test was originally described by

Kendrick et al. (1947). Groups of mice were given graded doses of the material in one intraperitoneal injection contained in 0.5 ml. The usual doses were 0.06, 0.5, and 1.5 billion organisms. After 14 days the animals were challenged intracerebrally with a living B. pertussis 18-323 suspension containing 100,000 organisms per 0.03 ml. The animals were observed daily and recorded as paralyzed, dead, or surviving. The observation period ended 14 days after the mice were challenged, and all paralyzed mice were killed and counted as dead. The results were calculated by the Litchfield-Fertig method (1941), and the protection of the vaccine was expressed as the ED/50 (the dose protecting 50 percent of the mice) in billions of organisms.

RESULTS

Each experiment testing the effect of an enzyme on B. pertussis was repeated at least once, and many tests were repeated several times. The concentration of the individual enzyme, the optimal conditions, and the density of the vaccine were kept the same. While it would have been interesting to expand the study by varying the amount of enzymes, this was not possible.

The effect of the proteolytic enzymes on B. pertussis was determined by quantitative or qualitative Biuret tests on supernatant fluids. The Molisch test was used for carbohydrates in these fluids. Antigenic changes of the treated bacterial cells were determined by tests for agglutinability, agglutinin production, agglutinin absorption, and mouse protection. To demonstrate that any changes in antigenicity of the enzyme-treated cells were due to the enzyme action, control samples without enzyme were included in the tests and subjected to every step of the procedure.

The experiments are presented in the order in which they were conducted, starting with the pepsin and continuing through the trypsin, chymotrypsin, beta amylase, hemicellulase, cellulase, lipase, acid phosphatase, and lysozyme.

The Effect of Pepsin

The effect of pepsin was tested on B. pertussis 10536, suspended in saline solution adjusted to pH 2.2 with 1 N HCl. A 10 ml. sample with a density of 400 bil./ml. was mixed with 4 mg. pepsin and the mixture was incubated at 37°C for 2 hours. Another 10 ml. sample with the same density was used as a control. After incubation both samples were adjusted to pH 7.0 with 0.1 N NaOH and centrifuged at 4500 rpm. The bacterial cells were washed twice and resuspended in saline solution to the original volume. A sample of the pepsin-treated bacterial cells was heated at 120°C for 1 hour. Pepsin activity was tested on the 120°C B. pertussis 10536 vaccine and also on the B. pertussis pepsin-treated cells heated at 120°C.

Tests for agglutinability

The untreated and treated vaccines were tested for agglutinability using antisera produced with unheated vaccine, vaccine heated at 120°C for 1 hour, and pepsin-treated cells subsequently heated at 120°C for 1 hour. The results are given in Table 1.

The agglutinability of the pepsin-treated cells was destroyed or inhibited by the enzyme action. The control cells showed autoagglutination, an effect of the low pH. It was interesting to observe that the pepsin-treated cells were weakly agglutinated by the serum

TABLE 1

AGGLUTINATION RESULTS WITH PEPSIN-TREATED B. PERTUSSIS ANTIGENS[a]

Antigens; B. pertussis 10536 Suspensions	Titers with Antiserum Against B. pertussis 10536		
	Unheated Vaccine	120°C Vaccine	Pepsin-Treated 120°C Vaccine
Untreated	4000	–	–
Pepsin-treated	–	40	–
Control	Unsatisfactory—Clumped		
Pepsin-treated; heated at 120°C	–	320	1280
Pepsin-treated; 120°C cells plus additional pepsin treatment	–	40 (weak)	80 (weak)
120°C vaccine	–	320	640
120°C vaccine, pepsin-treated	–	–	–

[a]The dashes indicate no agglutination with 1:10 serum dilution. Titers are given as reciprocals of the highest dilution in which 2+ agglutination was observed.

made with the 120°C vaccine. When the pepsin-treated cells were heated at 120°C for 1 hour, the agglutinability was increased, giving the same reaction as the 120°C vaccine. These cells used in agglutination tests with serum produced with pepsin-treated cells heated at 120°C were agglutinated to a titer of 1:1280. The pepsin-treated

unheated cells did not agglutinate with this serum. By testing the effect of the second treatment with pepsin on the bacterial cells, previously treated with pepsin and heated at 120°C, the agglutinability was noticeably reduced, from 1:1280 to 1:80. Also, the 120°C vaccine, which was agglutinated by the serum made with homologous antigen as well as with the serum made with pepsin-treated cells heated at 120°C, lost its agglutinability after pepsin treatment. This result suggested that the denatured protein still played a part in the antigenicity of the heated material.

Chemical tests

The supernatant fluids from the enzyme-treated vaccines, the control suspensions, the uninoculated Cohen-Wheeler medium, the supernatant fluid from the vaccine, and the pepsin solution were tested for protein and carbohydrate by the Biuret and the Molisch tests, respectively. The results are shown in Table 2.

The results of the quantitative Biuret tests indicated that the supernatant fluid from the pepsin-treated sample contained three times the amount of the protein present in the control. In addition, the supernatant fluid from the 120°C vaccine treated with pepsin showed an increase of protein compared with the enzyme solution.

TABLE 2

RESULTS OF MOLISCH AND BIURET TESTS

Materials	Molisch Test	Biuret Tests	
		Qualitative	Quantitative
Supernatant fluid from the unheated vaccine (400 bil./ml.)	+	-	
Supernatant fluid from the pepsin-treated vaccine (400 bil./ml.)	-	+	100 mg./100 ml.
Supernatant fluid from the control (400 bil./ml.) . . .	-	-	20 mg./100 ml.
Cohen-Wheeler medium	+	-	
Supernatant fluid from 120°C vaccine (30 bil./ml.)	+	-	
Supernatant fluid from pepsin-treated 120°C vaccine (50 bil./ml.) . . .	-	+	60 mg./100 ml.
Supernatant fluid from 120°C vaccine used as control	-	-	negative
Pepsin solution, 0.04 percent	-	-	40 mg./100 ml.

In both cases the presence of protein in the supernatant fluid correlated with the loss of agglutinability.

Agglutinin production in mice

Groups of mice were injected with pepsin-treated cells, control cells, and the untreated vaccine. Also, supernatant fluids from the pepsin-treated sample, the control sample, and the vaccine were used in agglutinin production experiments. The mouse sera were tested for agglutinins for the untreated vaccine, and the results are given in Table 3.

The pepsin-treated cells separated from supernatant fluid stimulated agglutinins in 1:20 titer only, indicating almost complete loss of agglutinogen. The cells from the suspension which was used as a control, and subjected to pH changes and incubation in the same manner as the enzyme-treated sample, also showed poor agglutinin-producing property. This serum had a titer of 1:80, compared with 1:20 resulting from the pepsin-treated material. The serum from mice injected with the untreated vaccine had a titer of 1:640.

The supernatant fluid from the untreated vaccine stimulated agglutinins in a titer of 1:320. It was noted that the fluid was toxic and caused necrotic lesions at the site of injection. Supernatant fluids from the pepsin-treated sample and the control sample produced sera with equally high titers—1:320—although the amount of protein in the supernatant fluid from the pepsin-treated sample was greater

TABLE 3

AGGLUTININ PRODUCTION IN MICE

Antigens Used for Antiserum Production	Total Mouse Dose	Titer with Untreated 10536 Vaccine
Untreated vaccine	10 billions	640
Supernatant fluid from vaccine[a] . .		320
Pepsin-treated cells	10 billions	200
Supernatant fluid from pepsin-treated sample[a]		640
Control cells	10 billions	80
Supernatant fluid from control sample[a]		320

[a]Total mouse dose was equivalent to 1 ml. of supernatant fluid from a suspension containing 400 bil./ml.

than in the control. These results suggested that a very small amount of protein is necessary to produce agglutinins in mice.

Of even more significance, these results indicated that the agglutinogen was not destroyed by low pH or by pepsin, but was split off the bacterial cell and was present in the supernatant fluid.

Mouse protection tests

Tests for protective properties were carried out in mice using three graded doses of vaccine, and fourteen mice per dose. The

results are shown in Table 4. The results given in the table represent one experiment in which 250 mice were used. In two other tests similar results were obtained.

The protective property for mice was somewhat reduced but certainly not completely destroyed by pepsin. Interpretation was difficult because the low-pH treatment alone, without the enzyme, reduced the mouse protective property.

The loss of the protective property of the pepsin-treated cells was greater than that observed for the control vaccine. This difference was not greater than the accepted variability of the mouse protection test. These ratios were obtained consistently in repeated tests. The supernatant fluid from the pepsin-treated sample had less protective property than the control, as indicated by the ED/50 end points expressed as dilutions.

TABLE 4

THE RESULTS OF MOUSE PROTECTION TESTS WITH PEPSIN-TREATED AND CONTROL SAMPLES

Materials	ED/50
Untreated vaccine	0.42 billion
Pepsin-treated cells	1.11 billion
Control cells	0.78 billion
Supernatant fluid from the pepsin-treated sample	1:1.3 dilution
Supernatant fluid from control sample	1:5.6 dilution

Agglutinin absorption tests

Agglutinin absorption tests were performed according to the procedure outlined in the section on methods, using antiserum for unheated B. pertussis vaccine and absorbing with untreated cells, pepsin-treated cells, and control cells, respectively. One absorption with untreated cells removed agglutinins, while two absorptions with pepsin-treated or control cells were required for complete removal of agglutinins. This result indicated that twice the quantity of either pepsin-treated or control cells were necessary to absorb agglutinins from the same serum, as compared with the untreated vaccine. Absorptions of serum made with pepsin-treated 120°C cells with either 120°C vaccine or with the pepsin-treated 120°C antigen resulted in complete removal of agglutinins.

Discussion of results with pepsin

The effect of pepsin on B. pertussis was observed as a loss of agglutinability (Table 1). This result was in accord with Flosdorf and Kimball (1940), who suggested that the agglutinogen is a protein. This conclusion does not exclude the possibility of some other reactant groups associated with the protein molecule. If some of the peptide bonds were broken by the action of pepsin, the arrangement may have been changed; this could affect the agglutinability. Eldering

(1941), working with B. pertussis and related organisms, obtained a fraction which gave a positive Molisch test and negative tests for protein. This fraction precipitated in 1:100,000 dilution with the B. bronchiseptica antiserum, suggesting the presence of a hapten. These observations would indicate that, if the agglutinogen in B. pertussis is not all protein, at least its agglutinability depends on protein.

The results in Table 3 indicated that the agglutinin-producing property of the cells was lost after the pepsin treatment. The control cells also were poor in producing agglutinins. Haurowitz (1955) states that acid can denature protein by converting the COO- into an uncharged COOH and breaking the salt bridges.

The results with the supernatant fluids are of particular interest (Table 3), indicating a separation of agglutinogen from the bacterial cell. According to the results of the Biuret tests, the pepsin split off three times as much protein as did the acid treatment alone. Agglutinin production experiments in mice using supernatant fluids from pepsin and from control samples resulted in sera of the same titer. According to Landsteiner and Chase (1933), there is no clear evidence that partially hydrolyzed protein can retain its power to stimulate antibody production. The extent of the hydrolysis

in these supernatant fluids could not be determined with the available facilities.

From the results of the tests in mice it was observed that the protective property was reduced by pepsin activity. Part of this effect was the result of the lowered pH. In either case the reduction in the protective antigen was not great. The supernatant fluids from pepsin-treated and control samples had very slight protective activity for mice. These results were in contrast to those obtained in the agglutinin-producing experiments. Here the active component was in the supernatant fluid, while the cells were almost devoid of agglutinogen.

The pepsin-treated cells, after heating at 120°C and treating again with pepsin, lost their agglutinability, suggesting that some of the protein was not affected by pepsin until it was denatured by heat (Table 1).

The agglutinin-absorbing property of both the pepsin-treated and control cells was reduced, possibly because of changes in reactant groups, resulting in a monovalent antigen.

Since enzyme hydrolysis is frequently incomplete (Haurowitz, 1955), it would be surprising to have an "all or none" effect.

The Effect of Trypsin

Five milliliters of unheated B. pertussis 10536 vaccine containing 400 bil./ml. and adjusted to pH 8.0 was mixed with 10 mg. of trypsin, and the mixture was incubated at 37°C for 2 hours. A similar sample was incubated without the enzyme, as a control. After centrifugation the sedimented cells were washed twice with saline solution and resuspended to the original volume. The trypsin-treated cells did not show any loss in agglutinability when tested with antiserum made with the unheated vaccine. In addition, the supernatant fluid from the trypsin-treated sample gave a weakly positive Biuret reaction, as did the enzyme solution itself. The supernatant fluid from the control sample was negative. Because of these observations no further tests were performed, as it was evident that trypsin was not active on the vaccine.

Since trypsin does not act on native proteins, some denaturation of the protein was attempted by heating the bacterial suspension prior to the trypsin treatment. A sample of B. pertussis 10536 cells at a density of 250 bil./ml. was incubated at 56°C for one hour. It is known that this temperature does not destroy the protective property (Pillemer, 1954). After incubation, 4 mg. of trypsin were added to one 5 ml. sample and the other was used as a control. Both

samples were incubated at 37°C for 2 hours. The cells were centrifuged, washed twice with saline solution, and used in agglutination and mouse protection tests. The supernatant fluid from the trypsin-treated sample gave a weakly positive Biuret reaction, while the control and enzyme solutions were Biuret negative.

Tests for agglutinability

The results of the tests for agglutinability are given in Table 5. Agglutinability of the trypsin-treated cells was reduced about half as compared to the untreated vaccine or the control suspension. Doubling dilutions of serum were used, and therefore the observed difference in titer was only one tube in the test. There was a slight suggestion that trypsin may be active on denatured cell proteins.

TABLE 5

AGGLUTINATION WITH TRYPSIN-TREATED VACCINE

Antigens, *B. pertussis* 10536	Antiserum Produced with *B. pertussis* Unheated Vaccine
Untreated	4000
56°C, trypsinized	2000
56°C, without trypsin	4000

However, heating at higher temperatures would destroy the mouse protective property (Pillemer, 1954).

Mouse protective test

The results of the mouse protection tests are given in Table 6. The protective property for mice was not affected by the trypsin treatment.

Agglutinin production in mice

Mice were injected with the suspension of B. pertussis incubated at 56°C for 1 hour and trypsin-treated. The serum was tested for agglutinins using several antigens: the untreated cells, cells incubated at 56°C, and the incubated and trypsin-treated cells. All of these antigens were agglutinated by the serum to the same titer, 1:640. There was no loss of agglutinogen due to the trypsin treatment.

TABLE 6

THE RESULTS OF MOUSE PROTECTION TESTS OF TRYPSIN-TREATED VACCINE

Antigens	ED/50 (in billions)
B. pertussis vaccine heated at 56°C for 1 hour	.35
B. pertussis vaccine heated at 56°C for 1 hour and trypsin-treated	.21

Effect of trypsin on boiled vaccine

To test the action of trypsin upon further denatured protein, a 10 ml. sample of B. pertussis 10536 vaccine at a density of 200 bil./ml. was heated at 100°C for 1 hour. After centrifugation the cells were resuspended in saline solution at pH 8.0 and divided into two 5 ml. samples. One was mixed with 2 mg. trypsin and the other was used as a control. After incubation at 37°C for 2 hours, the cells were washed and resuspended in saline solution to the original volume, and the cells and the supernatant fluids were subjected to various tests. Also a 5 ml. sample of control cells from experiments with pepsin suspended in saline solution at pH 8.0 at a density of 200 bil./ml. was mixed with 2 mg. of trypsin and incubated at 37°C for 2 hours.

Trypsin activity on these cells indicated that the low pH which was used in experiments with pepsin had denatured the cell protein. Another sample of the control cells was incubated without the enzyme. After incubation the supernatant fluids were tested for protein and the washed cells were used in agglutination tests.

The results of quantitative Biuret tests are tabulated in Table 7. These results indicated that supernatant fluid from cells incubated at 56°C and trypsin-treated had a small amount of protein split off compared with the amount of enzyme protein added. However,

TABLE 7

RESULTS OF QUANTITATIVE BIURET TESTS

Materials	Quantitative Biuret Test
Supernatant fluid from 56°C vaccine, trypsin-treated	90 mg./100 ml.
Trypsin solution, 0.08 percent	80 mg./100 ml.
Supernatant fluid from trypsin-treated 100°C sample	100 mg./100 ml.
Supernatant fluid from trypsin-treated control sample	90 mg./100 ml.
Trypsin solution, 0.04 percent	40 mg./100 ml.

in the supernatant fluids from the 100°C vaccine and the control cells from the pepsin experiments the amounts of protein were much larger, indicating the trypsin effect increased with the denaturation of the cellular protein.

Tests for agglutinability

The results of agglutination tests are given in Table 8. B. pertussis 10536 cells heated at 100°C for 1 hour were agglutinated by the antiserum made with unheated vaccine, although the agglutinability was reduced. After the heated cells were treated with trypsin, the agglutinability was lost. The heat apparently changed the surface of the cell, and it was possible that the peptide linkages

TABLE 8

AGGLUTINATION RESULTS WITH TRYPSIN-TREATED CELLS

B. pertussis Antigens	Antiserum Produced with B. pertussis, Untreated
Unheated vaccine	4000
100°C vaccine	500
100°C vaccine, trypsin-treated	no agglutination
"Pepsin control" trypsin-treated	no agglutination

specific for trypsin action became available. It also was noticed that trypsin action on the control cells from the pepsin experiments which were autoagglutinable (Table 1) changed them into a smooth suspension. The quantitative Biuret test showed that the trypsin had split off some of the protein. It may be that the clumping of the cells without serum resulted from the alteration of the protein by acid.

Agglutinin-producing property

Attempts were made to produce agglutinins in mice, using 100°C vaccine treated with trypsin, and untreated. The results are tabulated in Table 9. The 100°C antigen produced agglutinins in mice for both heated and unheated antigens. The trypsin-treated

TABLE 9

RESULTS OF AGGLUTINATION TESTS WITH MOUSE SERA

B. pertussis Antigens	Antiserum for 100°C Antigen	Antiserum for Trypsin-Treated 100°C
Unheated	320	no agglutination
100°C	320	no agglutination
100°C, trypsin-treated	no agglutination	no agglutination

vaccine did not stimulate agglutinins, either for itself or the other vaccines tested.

Discussion of results with trypsin

Trypsin was inactive on the native cell protein. Heated bacterial cells lost their agglutinability and agglutinin-producing property after trypsin treatment (Tables 8 and 9). According to Hauro-witz (1950), the denaturation consists of an alteration of the specific internal structure of a protein molecule; the closely folded peptide chains unfold, making the specific peptide bonds accessible to the enzyme. Trypsin was active on the control cells from the pepsin experiments, changing the autoagglutinated suspensions into smooth ones and splitting off some protein. The results showing that trypsin

treatment increased protein in the supernatant fluid, suggested that the specific peptide linkages of arginine or lysine were present in B. pertussis protein.

The Effect of Chymotrypsin

A 15 ml. sample of B. pertussis 10536 vaccine at a density of 150 bil./ml. was centrifuged, the cells resuspended in saline solution to the original volume, and mixed with 10 mg. of chymotrypsin. A similar sample was prepared as a control. A sample of B. pertussis cells heated at 56°C for 1 hour at a density of 150 bil./ml. was also mixed with 10 mg. chymotrypsin. All these samples were incubated at 32°C for 4 hours. After incubation the cells were separated, washed twice with saline solution, and used in additional tests.

Tests for agglutinability

The results of agglutination tests are given in Table 10. The agglutinability of the chymotrypsin-treated cells was reduced, compared with the control. From the agglutination tests with the factor sera, it was apparent that all factors were affected to the same extent, the agglutinability being somewhat reduced. Heating the cells

TABLE 10

AGGLUTINATION TESTS WITH CHYMOTRYPSIN-TREATED CELLS USING B. PERTUSSIS UNABSORBED SERUM AND SPECIFIC FACTOR SERA

Antigens	Antiserum for B. pertussis				
	Unabsorbed	Absorbed Factor Sera			
		3	3–6	2–4	2–5
Untreated 10536 vaccine	4000	1500	3000	3000	2000
Chymotrypsin-treated cells ...	1500	200	100	500	200
Cells heated at 56°C and chymotrypsinized	2000		not done		
Control cells	4000	1500	2500	3000	2000

at 56°C did not increase the chymotrypsin activity, as shown by the agglutination test.

Agglutinin production in mice

Groups of mice were injected with 3 billion organisms in two injections, using the chymotrypsin-treated cells and the original vaccine. The results of agglutination tests with mouse sera are given in Table 11.

Serum produced with the chymotrypsin-treated cells did not agglutinate the untreated vaccine, but did agglutinate the homologous antigen. Serum produced with untreated vaccine agglutined the

TABLE 11

TESTS FOR AGGLUTININ PRODUCTION IN MICE WITH CHYMOTRYPSIN-TREATED ANTIGENS

B. pertussis Antigens	Antiserum Produced with B. pertussis	
	Vaccine	Chymotrypsin-Treated Cells
Untreated	640	no agglutination
Chymotrypsin-treated	640	160

untreated and chymotrypsin-treated cells. These results could be expected if chymotrypsin affected at least part of the outer layer of the cell surface.

Mouse protection tests

The results of the mouse protection tests are shown in Table 12. The fivefold difference in end points indicated some loss of protective property. However, from one experiment it was difficult to make a conclusion on a quantitative basis.

Discussion of the results with chymotrypsin

Chymotrypsin effect on B. pertussis indicated that the agglutinability and the mouse protective property were slightly reduced.

TABLE 12

RESULTS OF MOUSE PROTECTION TESTS WITH CHYMOTRYPSINIZED ANTIGENS

Antigens	ED/50 (in billions)
Untreated vaccine	.10
Chymotrypsin-treated vaccine	.52

From the agglutinin production tests (Table 11) it was concluded that chymotrypsin affected the surface antigen. No agglutinins were produced for the untreated vaccine, while the chymotrypsin-treated cells were agglutinated. If the chymotrypsin activity were related to the peptide bonds, it would indicate that tyrosine, phenylalanine, or tryptophane was present in the agglutinogen.

The Effect of Beta Amylase

B. pertussis vaccine, 200 bil./ml., was centrifuged, washed with saline solution, and resuspended in citrate buffer at pH 5.0. To one 5 ml. sample, 10 mg. of amylase was added and the mixture was incubated at 37°C for 2 hours. Another 5 ml. sample was incubated without the enzyme, as a control. Both samples were

centrifuged, and the sedimented cells were washed twice with saline solution. The resuspended bacteria and the supernatant fluids from both samples, as well as the enzyme solution, were tested according to plan.

Tests for agglutinability

The results of agglutination tests are given in Table 13. The amylase-treated and control vaccines were agglutinated by the 1:2000 serum dilution, compared with 1:4000 for the untreated vaccine. The slight loss of agglutinability probably could not be attributed to the action of the enzyme.

Chemical tests

The supernatant fluid from the enzyme-treated sample gave a positive Molisch test, while the control and the enzyme solutions

TABLE 13

AGGLUTINATION TESTS WITH AMYLASE-TREATED ANTIGENS

B. pertussis Antigen	Antiserum Produced with *B. pertussis* Unheated Vaccine
Untreated	4000
Treated with beta amylase	2000
Control	2000

were negative. Amylase is specific for starch digestion. The carbohydrate in the supernatant fluid from the enzyme-treated sample might have come from the small amount of starch in the organisms.

Mouse protection tests

The results of mouse protection tests are given in Table 14. The ED/50 values for the untreated and amylase-treated vaccines were not considered significant when groups of forty-two mice per antigen were used (National Institutes of Health, 1948). It may be concluded, therefore, that the amylase treatment did not affect the protective property of the vaccine. Mice injected with the supernatant fluid from the enzyme-treated sample as well as that from the control sample showed very little protection.

TABLE 14

RESULTS OF MOUSE PROTECTION TESTS OF AMYLASE-TREATED ANTIGEN

Antigens	ED/50
Untreated vaccine	0.62 billion
Amylase-treated vaccine	0.40 billion
Supernatant fluid from the amylase-treated sample	1:1.7 dilution
Supernatant fluid from the control sample	1:1.5 dilution

Agglutinin-producing property

As the agglutinability of the amylase-treated cells was not affected, it was not necessary to test the agglutinin-producing property.

Effect of amylase on 120°C vaccine

B. pertussis 10536 vaccine heated at 120°C for 1 hour was centrifuged and the cells resuspended in 5 ml. citrate buffer, at pH 5.0 with a density of 50 bil./ml. One sample was mixed with 5 mg. amylase and the other one without the enzyme; both were incubated at 37°C for 2 hours.

The cells, after washing with saline solution, were used in additional tests.

Tests for agglutinability

The results of agglutination tests are given in Table 15. The agglutinability of the amylase-treated cells and also the control suspension showed no change compared to the 120°C vaccine without amylase.

Agglutinin-producing property

Two groups of mice were given two doses of 120°C vaccine, and vaccine heated and amylase-treated, respectively. Neither the

TABLE 15

AGGLUTINATION TESTS OF THE 120°C VACCINES

B. pertussis Antigens	Antiserum Produced with B. pertussis 120°C Vaccine
120°C vaccine	320
120°C vaccine, amylase-treated	320
120°C vaccine, control	320

120°C vaccine nor the amylase-treated 120°C cells stimulated agglutinin production in mice.

The Effect of Hemicellulase

Five milliliters of B. pertussis vaccine was centrifuged and the cells were resuspended in 4 ml. of acetate buffer at pH 4.8. A 5 ml. suspension of the pepsin-treated cells and the control cells used in the pepsin experiment were centrifuged and the cells were resuspended in 4 ml. of acetate buffer at pH 4.8. To each of these samples 1 ml. of a hemicellulase solution containing 10 mg. was added, and the samples were incubated at 45°C for 5 hours. The density of the samples before incubation was approximately 50 bil./ml.

Control suspensions without the enzyme were included. After incubation the cells were washed and resuspended in saline solution to the original volume.

Observations on density and morphology

To observe any changes due to the action of the hemicellulase, density determinations and Gram stains were made. Table 16 presents the results of these experiments.

The only noticeable effect of the hemicellulase on the cells was on the sample previously treated with pepsin. The density of this sample was reduced by two-thirds as compared to the control.

TABLE 16

DENSITY AND CELL MORPHOLOGY OF HEMICELLULASE-TREATED VACCINE

Materials	Density (bil./ml.)	Gram Stain
Hemicellulase-treated cells	49	no change
Control cells	49	no change
Pepsin-hemicellulase-treated cells	17	damaged cells
Pepsin-treated-hemicellulase "control" cells	48	no change
Pepsin "control"-hemicellulase-treated cells	40	no change
Pepsin "control"-hemicellulase "control" cells	47	no change

The bacteria were broken up as was shown in a Gram-stained preparation. The other samples had lost some of the density, perhaps because of the numerous washings and centrifugations. It seemed that pepsin had to remove at least a part of the protein from the cell surface to allow the hemicellulase activity to take place.

Tests for agglutinability

Results of agglutination tests are given in Table 17. Hemicellulase did not affect the agglutinability of the vaccine. Cells which were previously treated with pepsin and then with hemicellulase were not agglutinable, but the agglutinability was lost after the pepsin treatment. The treatment of control cells from the pepsin experiments with hemicellulase resulted in smooth suspension, but the cells were not agglutinated.

Agglutinin production in mice

As the agglutinability of the hemicellulase-treated vaccine was not changed, the agglutinin-producing property in mice was not tested.

Mouse protection tests

Tests for mouse protective property were made on the control cells, pepsin-hemicellulase–treated cells, and the hemicellulase-

TABLE 17

AGGLUTINATION TESTS WITH HEMICELLULASE-TREATED ANTIGENS

Antigens	Antiserum Produced with B. pertussis 10536
Untreated vaccine	4000
Hemicellulase-treated cells	4000
Pepsin-hemicellulase-treated cells	no agglutination
Pepsin control-hemicellulase-treated cells	no agglutination

treated control cells from the pepsin experiments. The dilutions used in the mouse protection tests were based on the original density before the hemicellulase treatment, except for the pepsin-hemicellulase-treated cells which were based on 17 bil./ml. The results of mouse protection tests are given in Table 18.

When the mouse protection test of the pepsin-hemicellulase-treated or pepsin "control"-hemicellulase-treated cells was done with immunizing doses based on the original density, no protection was observed. However, if the immunizing doses of the pepsin-hemicellulase-treated cells were based on the final density, the protective property was little affected, compared with the control antigen. It seemed that a small number of the bacterial cells could

TABLE 18

RESULTS OF MOUSE PROTECTION TESTS WITH PEPSIN-HEMICELLULASE–TREATED ANTIGENS

Antigens	ED/50 (in billions)
Control cells	0.87
Pepsin-hemicellulase–treated cells (dilutions based on 17 bil./ml.)	1.1
Pepsin-hemicellulase–treated cells (dilutions based on 50 bil./ml.)	no protection
Pepsin "control"-hemicellulase–treated cells	no protection

resist the enzymatic action and also retained the property to protect the mice.

Discussion of the results with a combination of two enzymes

Hemicellulase was active only after the B. pertussis was acted upon by pepsin. The activity was noticed as reduced density of the suspension, indicating that the cells were ruptured. As the hemicellulase was a specific enzyme for hemicellulose, it would suggest that hemicellulose was present in the cell wall and that protein had to be removed before the hemicellulase could act on this more deeply located substance. Not all cells were affected by hemicellulase,

as indicated by the results (Table 16). Approximately 14 percent of the total density was recovered after the enzyme treatment. The mouse protective property of these cells was little affected (Table 18). It could be that these cells were resistant to pepsin treatment and because of that were not affected by hemicellulase.

The Effect of Cellulase

Several samples of B. pertussis suspensions including unheated vaccine, pepsin-treated vaccine, vaccine used as pepsin control, trypsin-treated vaccine, and 100°C vaccine were washed and resuspended in sodium acetate buffer at pH 4.5. All of these samples were prepared in duplicate, in 5 ml. volumes. One sample of each was mixed with 10 mg. of cellulase and the other was used as a control. After incubation at 45°C for 5 hours the cells were centrifuged, washed, and resuspended in saline solution to the original volume.

Effect of cellulase on density of suspensions

Densities were determined before and after the cellulase treatment; the results are tabulated in Table 19. The loss of density in any of these samples was not remarkable, except in the pepsin-treated sample, which after the cellulase treatment had lost

TABLE 19

EFFECT OF CELLULASE TREATMENT

Vaccines	Density (in bil./ml.)	
	Before	After
Cellulase-treated 10536 vaccine	40.2	38.9
Pepsin-cellulase-treated vaccine	38.9	25.0
Pepsin control-hemicellulase-treated vaccine	38.9	36.3
Cellulase-treated 100°C vaccine	40.2	31.0
Cellulase control vaccine	40.2	38.9
Pepsin-treated cellulase control vaccine	38.9	37.6
Pepsin control-cellulase control vaccine	38.9	37.6
Cellulase control 100°C vaccine	40.2	35.0

one-fourth of its density as compared to the control. This observation suggested that cellulase action on B. pertussis cells was not similar to the effect of hemicellulase.

Tests for agglutinability

Cellulase-treated vaccines and vaccines used as controls were tested for agglutinability, and the results are shown in Table 20. Cellulase-treated cells without any previous treatment did not show any loss in agglutinability. However, when the cells were previously treated at low pH or heated the agglutinability was lost. To test

TABLE 20

RESULTS OF AGGLUTINATION TESTS WITH CELLULASE-TREATED ANTIGENS

Antigens	Antiserum Produced with B. pertussis 10536
Untreated vaccine	4000
Cellulase-treated cells	4000
Pepsin-cellulase-treated cells	no agglutination
Pepsin-treated-cellulase control cells	no agglutination
Pepsin control-cellulase-treated cells	no agglutination
Pepsin control-cellulase control cells	unsatisfactory, clumped
100°C vaccine	500
100°C cellulase-treated cells	no agglutination

whether the agglutinogen was destroyed, groups of mice were injected for agglutinin production following the outlined procedure.

Agglutinin production in mice

The results of the agglutination tests are given in Table 21. Serum produced against vaccine treated first with pepsin and then with cellulase did not show agglutinins for either homologous antigen or the untreated vaccine. It was observed previously that pepsin destroyed the agglutinogen (Table 1). Serum made with pepsin control-cellulase-treated cells did not agglutinate the homologous antigen, but agglutinated the untreated vaccine to a titer of 1:80.

TABLE 21

RESULTS OF THE AGGLUTINATION TESTS WITH MOUSE SERA PRODUCED WITH CELLULASE-TREATED ANTIGENS

Sera Produced with	Homologous Antigens	10536 Vaccine
Pepsin-cellulase-treated cells	–	–
Pepsin control-cellulase-treated cells	–	80
Pepsin control-cellulase control cells	unsatisfactory	160
100°C vaccine	–	20
Cellulase-treated 100°C vaccine ...	–	–
100°C vaccine-cellulase control ...	–	–

A serum made with pepsin control-cellulase control cells agglutinated the vaccine to a titer of 1:160, which was not significantly different from the titer of serum made with pepsin control-cellulase-treated cells. These results indicated that cellulase did not destroy the antigenicity but affected the agglutinability only. The heated suspensions at 100°C for 2-1/2 hours did not stimulate agglutinin production in mice.

The Effect of Lipase

B. pertussis vaccine at a density of 200 bil./ml. was centrifuged and the sedimented cells were resuspended in 3 ml. distilled

water to which 1 ml. phosphate buffer at pH 7.0 was added. One sample was mixed with 10 mg. lipase and the other was used as a control. Both samples were incubated at 37°C for 24 hours. After centrifugation the sedimented cells were washed twice with saline solution and used in agglutination tests. The lipase-treated cells and the control cells did not indicate any loss of agglutinability as compared to the original vaccine; all of these antigens were agglutinated to the same titer (1:4000) by the serum made with untreated vaccine.

Based on these observations, no additional tests were performed with lipase-treated cells.

The Effect of Lysozyme

Two 5 ml. samples of B. pertussis 10536 vaccine at a density of 100 bil./ml. were washed and resuspended in 5 ml. of sodium acetate buffer with NaCl at pH 5.3. To one sample 10 mg. of lysozyme was added, and this mixture and the sample without the enzyme were incubated at 37°C for 2 hours (Fishman, 1951). Two 5 ml. samples of the same vaccine were prepared in the same buffer but at pH 3.8. One sample was mixed with 10 mg. of lysozyme and the other used as a control. Both were incubated at 45°C for 2 hours (Salton, 1957). After incubation the samples were centrifuged,

washed once with saline solution, and resuspended to the original volume, after which the density was determined. Any effect of lysozyme would result in lysis of bacterial cells and reduced density. This was not observed in these two treated samples. Another approach to the lysis of pertussis cells by lysozyme was by treating them first with acetone. A sample of B. pertussis vaccine at a density of 100 bil./ml. was centrifuged and the cells were mixed with two volumes of acetone and kept at room temperature for 1 hour. After separating the cells from the acetone by centrifugation, portions of these cells were suspended in two 5 ml. samples of phosphate buffer solution at pH 7.0. The density was adjusted to 100 bil./ml. One sample was mixed with 2.5 mg. of lysozyme and incubated at 37°C for 1 hour (Warren et al., 1955). The control suspension was incubated in the same manner. Two other 5 ml. samples of the acetone-treated cells were suspended in sodium acetate buffer at pH 3.8 and one was mixed with 2.5 mg. of lysozyme. This and the control sample were incubated at 45°C for 1 hour.

Another sample of acetone-treated cells suspended in distilled water was mixed with 2.5 mg. of lysozyme and incubated at 37°C for 1 hour. All of these samples were tested for changes in density after the cells were centrifuged, washed with saline solution, and resuspended to their original volumes, respectively. There was no

change in the density of any of these samples, indicating that lysozyme was not effective on pertussis cells under these test conditions.

Brown (1959) also did not obtain any lysis of certain strains of B. pertussis when treated with lysozyme.

Brumfitt, Wardlaw, and Park (1958) proposed that lysozyme hydrolyzes β 1-4 links between N-acetylmuramic acid and N-acetylglucosamine, and that the cell walls contain repeating units of these sugars.

It could be assumed from the results of the experiments with B. pertussis that the cell wall of this organism does not contain these sugars in this specific arrangement.

The Effect of Acid Phosphatase

The sedimented cells of B. pertussis 10536 vaccine were suspended in 6 ml. of saline solution at pH 4.6 with a density of 250 bil./ml. To a 3 ml. sample of this suspension, 5 mg. of acid phosphatase was added; the other 3 ml. sample was used as a control. To test the enzyme activity, a known substrate was mixed with 5 mg. of the enzyme. All of these samples were incubated at 37°C for 1 hour and 15 minutes. The amount of phosphorus was calculated from the supernatant fluids using the equation:

$$\frac{\text{density of unknown}}{\text{density of standard}} = \frac{X}{0.01 \text{ mg. phosphorus}}$$

The density of these samples was determined in a Leitz colorimeter by the method of Bodansky. The supernatant fluid from the sample treated with the enzyme contained 0.0152 mg. of phosphorus, while the control sample without the enzyme had 0.0092 mg. It is recognized that the samples were small and the difference slight, but one could not expect a large amount to be present in the quantity of sample used. These observations would agree with Smolens and Mudd (1943), who determined small amounts of phosphorus present in the acid extract from B. pertussis.

The acid phosphatase-treated and the control cells were used in agglutination tests, and no changes in agglutinability were observed with either sample.

The Effect of Heat

The heat stability of an antigen may vary depending on the temperature, time period, and density of the bacterial suspension. Kauffmann, in his studies of E. coli, used 100°C for 2-1/2 hours, and bacterial suspensions so treated were called O antigens. To observe the effect of heat on Bordetella, suspensions of cells were heated at 100°C for 2-1/2 hours. The density of the bacterial

suspensions and the amounts were kept constant. It is recognized that the same temperature for the same time period may have a different effect when the density of cells is lowered.

Ten milliliters of each of the suspensions—B. pertussis 10536, B. pertussis–rough (Farrell), B. parapertussis 17903, and B. bronchiseptica 88—at a density of 100 bil./ml. were heated at 100°C for 2-1/2 hours. After cooling, a sample containing 20 bil./ml. was prepared from each of the heated suspensions and used in agglutination tests.

Tests for agglutinability

The results of agglutination tests are given in Table 22. Heating at 100°C reduced the agglutinability of the antigens with sera produced with unheated vaccines. The cross agglutination between B. pertussis 10536 and B. parapertussis or B. bronchiseptica was lost after the heating. Cross agglutination still occurred between B. parapertussis and B. bronchiseptica. These observations suggested a study of the different antigenic factors and their heat stability. The rough B. pertussis culture was not agglutinated by its homologous serum nor by any other serum used in this study.

It will be recalled that the antigenic factors ascribed to the genus Bordetella are as follows:

TABLE 22

AGGLUTINATION TESTS WITH UNHEATED AND HEATED ANTIGENS

Antigens	Antiserum Produced with Unheated Antigens			
	B. pertussis	B. parapertussis	B. bronchiseptica	B. pertussis rough
B. pertussis				
unheated	4000	1000	500	–
100°C	1000	–	–	–
B. parapertussis				
unheated	1000	5000	2000	–
100°C	–	500	200	–
B. bronchiseptica				
unheated	1000	1500	4000	–
100°C	–	200	500	–
B. pertussis–rough				
unheated vaccine . .	–	–	–	–
100°C vaccine	–	–	–	–

B. pertussis 10536 – 1, 2, 3, 5, 7, 13

B. parapertussis 17903 – 7, 8, 9, 10, 14

B. bronchiseptica 88 – 7, 8, 9, 12, 13

From this schema it is possible to see the factors present in the three species which may be the basis for cross reactions. The results of agglutination tests with factor sera are given in Table 23.

TABLE 23

AGGLUTINATION TESTS WITH FACTOR SERA

Antigens	Titers with Bordetella Factor Sera					
	3	2–5	8	9	12	14
B. pertussis 10536						
unheated	500	1500	–	–	–	–
100°C	–	500	–	–	–	–
B. parapertussis 17903						
unheated	–	–	1500	100	–	200
100°C	–	–	200	40–80	–	–
B. bronchiseptica 88						
unheated	–	–	500	500	1000	–
100°C	–	–	500	100	–	–

These agglutination results indicated that some of the factors were more heat-stable than others. Factor 3 was completely lost in the heated suspensions, while either Factor 2 or Factor 5 or both lost only a part of the agglutinability. Factors 8 and 9 were at least partially heat-resistant, as indicated by the agglutination tests, explaining the cross reaction between the heated suspension of B. parapertussis and B. bronchiseptica antiserum, and also heated B. bronchiseptica and B. parapertussis antiserum. The common Factor 7 seemed to be heat-labile.

Agglutinin production in rabbits

The agglutinin-producing property of these 100°C suspensions was studied in rabbits. Two rabbits were used for each antigen and were selected only if they had no agglutinins for any of the antigens previous to the injections. Each rabbit received seven intravenous injections of 0.5 ml. suspensions, containing 30 bil./ml. given at weekly intervals. The sera from each pair of rabbits were pooled, inactivated at 56°C for 30 minutes, and preserved with 1:5000 merthiolate. The results of agglutination tests are given in Table 24.

The serum produced against B. pertussis 100°C vaccine had a titer of 1:1280 for the unheated antigen and 1:640 for the homologous one. Neither B. parapertussis 17903 nor B. bronchiseptica 88 was agglutinated by this serum. Antiserum produced with B. parapertussis 17903 100°C vaccine agglutinated both B. parapertussis and B. bronchiseptica unheated and heated antigens. No reaction was observed with B. pertussis 10536. Antiserum produced with B. bronchiseptica 88 100°C antigen agglutinated B. bronchiseptica 88 and B. parapertussis 17903 suspensions but not B. pertussis 10536. These observations also indicate the presence of heat-stable factors.

Agglutinin production in guinea pigs with the heated antigens was not successful.

TABLE 24

AGGLUTINATION TESTS WITH SERA MADE WITH ANTIGENS HEATED AT 100°C

Antigens	Antiserum Produced with 100°C Antigens		
	10536	17903	88
B. pertussis 10536			
unheated	1280	–	–
100°C	640	–	–
B. parapertussis 17903			
unheated	–	640	320
100°C	–	320	160
B. bronchiseptica 88			
unheated	–(20)	320	1280
100°C	–	160	640

Agglutinin absorption

The agglutinin-absorbing property of the 100°C vaccine was tested with sera against both unheated and 100°C antigens. The procedure was followed as outlined in the section on methods. The results of agglutinin absorption tests are given in Table 25.

All of the sera were absorbed twice, using the same amount of sedimented cells each time. Serum against unheated vaccine absorbed with 100°C antigen lost agglutinins for heated cells, but still

TABLE 25

AGGLUTININ ABSORPTION TESTS WITH B. PERTUSSIS

Antigens	B. pertussis Antisera			
	Unheated Antigen		100°C Antigen	
	Unabsorbed	Absorbed with 100°C Antigen	Unabsorbed	Absorbed with 100°C Antigen
B. pertussis				
unheated . .	4000	320	1280	–
100°C	1000	–	640	–
B. parapertussis				
unheated . .	1000	320	–	–
100°C	–	–	–	–
B. bronchiseptica				
unheated . .	1000	80	–	–
100°C	–	–	–	–

retained agglutinins for the unheated suspensions of all three species although in much lower titers. Agglutinins produced with B. pertussis 10536 100°C vaccine were absorbed with the homologous antigen. Here again, the antigen or antigens common to the three species appeared to be heat-labile.

Similar agglutinin absorption tests were performed with B. parapertussis 17903 and B. bronchiseptica 88 antisera. The results of these tests are shown in Tables 26 and 27. B. parapertussis 17903 100°C antigen absorbed all agglutinins from the serum made with heated suspension. All agglutinins against the heated antigens were removed from the serum made with unheated vaccine. Some agglutinins against the unheated suspension were still present,

TABLE 26

AGGLUTININ ABSORPTION TESTS WITH B. PARAPERTUSSIS SERA

Antigens	B. parapertussis Antisera			
	Unheated Antigen		100°C Antigen	
	Unabsorbed	Absorbed with 100°C Antigen	Unabsorbed	Absorbed with 100°C Antigen
B. parapertussis				
unheated . .	5000	320	640	–
100°C	500	–	320	–
B. bronchiseptica				
unheated . .	1500	80	320	–
100°C	200	–	160	–
B. pertussis				
unheated . .	1000	80	–	–
100°C	–	–	–	–

TABLE 27

AGGLUTININ ABSORPTION TESTS WITH
B. BRONCHISEPTICA SERA

Antigens	B. bronchiseptica Antisera			
	Unheated Antigen		100°C Antigen	
	Unabsorbed	Absorbed with 100°C Antigen	Unabsorbed	Absorbed with 100°C Antigen
B. bronchiseptica				
unheated . .	4000	80	1280	–
100°C	500	–	640	–
B. parapertussis				
unheated . .	2000	320	320	–
100°C	200	–	160	–
B. pertussis				
unheated . .	500	80	–	–
100°C	–	–	–	–

indicating that some of the agglutinin-absorbing property was lost during heating. The unabsorbed serum produced with the 100°C antigen agglutinated both heated and unheated B. parapertussis and B. bronchiseptica antigens. B. bronchiseptica 100°C antigen absorbed all agglutinins from the homologous serum. Serum made with the unheated vaccine had some agglutinins present after the absorption

with the 100°C vaccine, which reacted with the unheated suspensions. The presence of a heat-stable antigen common to B. parapertussis and B. bronchiseptica was confirmed by the results with the B. bronchiseptica antisera.

Again, these results indicated that Factor 7, common to the three species, was heat-labile.

Agglutinin absorption tests using whole bacterial suspensions

On the assumption that part of the antigenic material was in the suspension fluid, agglutinin absorption tests were performed using the whole suspension of the 100°C vaccine for absorption, rather than only the packed cells. In this case equal volumes of serum dilution and bacterial suspension were mixed. The results are shown in Table 28.

The 100°C bacterial suspensions were more effective in agglutinin absorptions than the sedimented cells from which the supernatant fluids had been removed. Agglutinins in low titer only were left in sera made with unheated vaccine against the unheated suspensions. This observation indicated that supernatant fluid of the heated suspensions possessed some absorbing property and was in agreement with Maitland's theory that agglutinogens are soluble in saline solution. To investigate this matter further, the 100°C

TABLE 28

RESULTS OF AGGLUTININ ABSORPTION TESTS USING THE WHOLE SUSPENSION

Antigens	Antisera against Unheated Antigen Absorbed with Homologous 100°C Suspensions		
	B. pertussis	B. parapertussis	B. bronchiseptica
B. pertussis			
unheated	40	40	40
100°C	—	—	—
B. parapertussis			
unheated	320	640	320
100°C	—	—	—
B. bronchiseptica			
unheated	40	40	80
100°C	—	—	—

suspensions were centrifuged and only the supernatant fluids were used for absorbing the antisera; the results are given in Table 29.

Two successive absorptions were carried out using the supernatant fluid as the absorbing agent. Final serum dilution after the second absorption was 1:40. Results of the agglutination tests in Table 29 indicates that supernatant fluids were effective in removing agglutinins against the unheated vaccines of B. pertussis

TABLE 29

AGGLUTININ ABSORPTION TESTS USING SUPERNATANT FLUID

Antigens	Antisera for Unheated Antigens Absorbed with Supernatant Fluid from Homologous 100°C Antigen					
	B. pertussis		B. parapertussis		B. bronchiseptica	
	Before	After	Before	After	Before	After
B. pertussis						
unheated . . .	4000	80	1000	–	500	–
100°C	1000	–	–	–	–	–
B. parapertussis						
unheated . . .	1000	160	5000	320	2000	320
100°C	–	–	500	–	200	–
B. bronchiseptica						
unheated . . .	1000	–	1500	80	4000	80
100°C	–	–	200	–	500	–

and B. bronchiseptica, and that agglutinins in low titer only were left for B. parapertussis. These findings suggest that the common factor or factors vary quantitatively. Supernatant fluid from heated B. parapertussis 17903 removed all agglutinins for B. pertussis. The absorbed B. bronchiseptica serum did not react with unheated or heated B. pertussis antigens.

Agglutination tests with strains containing different factors

To obtain more information on the heat-labile and heat-stable factors of B. pertussis, agglutination tests were performed with B. pertussis 5373 containing Factors 1, 3, 7, and 13, and with B. pertussis 5374 with Factors 1, 2, 5, 7, and 13. All of the previous experiments had been carried out with a single culture of B. pertussis 10536. This culture has been assigned Factors 1, 2, 3, 5, 7, and 13. The results of agglutination tests with strains containing different factors are given in Table 30.

TABLE 30

AGGLUTINATION TESTS WITH HEAT-LABILE AND HEAT-STABLE FACTORS OF B. PERTUSSIS

B. pertussis Antigens	Antisera for B. pertussis 10536		
	Unheated Antigen	100°C Antigen	Unheated, Absorbed with 10536 at 100°C
10536 unheated	4000	1280	320
10536 at 100°C	1000	640	–
5373 unheated	2560	–	320
5373 at 100°C	–	–	–
5374 unheated	1280	640	320
5374 at 100°C	640	320	–

The results indicated that Factors 3 and 7 are heat-labile; strain 5373 after heating at 100°C was not agglutinated by serum made with unheated 10536. The agglutinability of B. pertussis 5374 containing Factors 2 and 5 was not affected by heating at 100°C. The unheated as well as the heated antigens were agglutinated by serum produced with 10536 heated antigen. It would require a more extended study to confirm the heat stability of these factors.

The Effect of Heat at 100°C for 2-1/2 Hours on Suspensions of Salmonella paratyphi B, Brucella abortus 19, and Escherichia coli 02

Cultures of Salmonella paratyphi B, Brucella abortus 19, and Escherichia coli 02K1, all unrelated to Bordetella, were selected for a comparative study of their antigenic changes at the 100°C temperature. S. paratyphi B and E. coli 02 heated at 100°C for 2-1/2 hours are accepted as O antigens. Chemically these O antigens consist of carbohydrate-protein and lipid complexes (Kauffmann). Brucella abortus 19 possesses two antigens, A and M. Attempts at separation of the A and M antigens have been unsuccessful.

The soluble antigenic materials from Brucella species consist of phospholipid-polysaccharide and protein complexes (Dubos, 1955).

Suspensions of S. paratyphi B, Br. abortus 19, and E. coli 02 in amounts of 10 ml. with a density of 100 bil./ml. were heated at 100°C for 2-1/2 hours. These heated suspensions were used for agglutinin production in rabbits and for agglutination tests. The results of agglutination tests with related and unrelated organisms are shown in Table 31.

TABLE 31

RESULTS OF AGGLUTINATION TESTS WITH THE RELATED AND UNRELATED ORGANISMS

Antigens	Br. abortus Diagnostic Serum	Sera Made with 100°C Antigens		
		Br. abortus 19	E. coli 02	S. paratyphi B
Br. abortus 19				
unheated	2000	800	–	–
100°C	1500	1200	–	–
E. coli 02				
unheated	–	–	–	–
100°C	–	–	4000	–
S. paratyphi B				
unheated	–	–	–	1600
100°C	–	–	–	1600

Agglutinin production in rabbits

Sera against S. paratyphi B and Br. abortus 19 were prepared by injecting rabbits weekly with 0.5 ml. suspensions containing 30 bil./ml. Each rabbit received seven injections. It should be noted that rabbits were bled and sera was tested for agglutinins prior to injection, and only rabbits with no agglutinins or titers of not more than 1:8 for the antigens applied in this study were used.

E. coli 02 antiserum was prepared following the outline by Edwards (1955). The agglutinability of Br. abortus 19 suspension heated at 100°C was tested by using the diagnostic serum supplied by the Division of Laboratories of the Michigan Department of Health.

The agglutinability of S. paratyphi B 100°C antigen did not vary from the unheated one. The E. coli 02 unheated antigen was not agglutinated by the E. coli 100°C serum. This was explained by the presence of the L antigen covering the cell surface. Heating at 100°C destroyed the L antigen and made the cells O agglutinable. Br. abortus 19 possessed an antigen which was heat-stable, and the agglutinability of the unheated and heated suspensions was about the same. The titer of the serum was slightly higher with the homologous antigen. Agglutination tests using the sera produced with Bordetella unheated or 100°C suspensions and S. paratyphi B, Br. abortus 19, and E. coli 02 antigens gave negative results. The

S. paratyphi B, Br. abortus 19, and E. coli 02 antisera did not agglutinate unheated or heated Bordetella suspensions. It could be assumed that there were no antigenic relationships among these species.

Agglutinin absorption tests

Agglutinin-absorbing properties of 100°C suspensions of S. paratyphi B, Br. abortus 19, and E. coli 02 were tested by absorbing each of the antisera with its homologous antigen following the procedure outlined for Bordetella.

Discussion of the results with heated antigens (100°C)

According to Kauffmann (1947), S. paratyphi B somatic O antigens are protein-polysaccharide-lipid complexes. The agglutinogen of Br. abortus 19 is heat-stable and chemically also a protein-lipid polysaccharide complex. E. coli somatic antigens are the same complex, but according to Neter et al. (1956) a purified O antigen is a polysaccharide-lipid complex. The heat stability of proteins varies within a wide limit (Northrop et al., 1948). Frobisher (1944) stated that O antigens are heat-stable proteins in contrast to the labile flagellar antigens. However, it is possible that proteins denatured by heat are changed in their antigenicity or specificity. According to Neurath (1944), denaturation is any nonproteolytic modification of

the unique structure of a native protein, giving rise to definite change in chemical, physical, and biological properties.

B. pertussis 10536, B. parapertussis 17903, and B. bronchiseptica 88 antigens heated at 100°C were agglutinated by the antisera produced with their homologous unheated vaccines. The cross reactions between B. pertussis and B. parapertussis, and B. pertussis and B. bronchiseptica were lost during heating, but were still present between B. parapertussis and B. bronchiseptica.

In all cases the titers were lower than with the unheated antigens. It was interesting to note that a rough culture of B. pertussis, heated or unheated, was not agglutinated by serum from rabbits injected with the unheated rough culture. The antigenicity of this strain appeared to be lost.

Results of the agglutination tests suggested that some of the factors present were more heat-stable than others. The observation that only Factors 2 and 5 of B. pertussis and Factors 8 and 9 of B. parapertussis and B. bronchiseptica were heat-stable explained certain loss of cross reactivity after heating.

Antisera produced with 100°C antigens agglutinated the unheated antigens to a higher titer than the homologous antigens. This finding indicated that the agglutinin-producing property of some factors was more heat-stable than the agglutinability. The absorption

tests indicated that agglutinin-absorbing property was quite heat resistant. Agglutinin absorptions done with whole suspensions were more effective than with the sedimented cells. This observation suggested that part of the agglutinin-absorbing property was present in the supernatant fluid. Agglutinin absorption tests using the supernatant fluids proved this. The results with the 100°C antigens of S. paratyphi B and E. coli 02 were as expected.

The Effect of Heat at 120°C on the Antigens of Bordetella, S. paratyphi B, Br. abortus 19, E. coli 02, and E. coli 09

According to Andersen, the O antigens of Bordetella are stable to heat at 120°C for 1 hour, and are the same in all three species. Suspensions of B. pertussis 10536, B. parapertussis 17903, and B. bronchiseptica 88 at densities of 30 bil./ml. were heated at this temperature.

E. coli 09 possesses the capsular A antigen which is destroyed by heating at 120°C for 2 hours. Suspensions of S. paratyphi B, Br. abortus 19, and E. coli 02 at approximate density of 30 bil./ml. were also heated at 120°C for 1 hour to determine their antigenic changes at this temperature. All of these suspensions were tested for agglutinability and agglutinin-producing properties.

The antisera prepared with Bordetella 120°C suspensions were obtained from the Michigan Department of Health Laboratory. The agglutinin-producing properties of S. paratyphi B and Br. abortus 19 were tested by following the procedure outlined for Bordetella. The E. coli 09 O antiserum was produced according to the method of Edwards and Ewing (1955).

Tests for agglutinability

The results of agglutination tests are given in Table 32. The titers of all the sera made with the 120°C suspensions were relatively low. The highest titer observed was 1:640, obtained with E. coli 09 antiserum and the homologous antigen. All Bordetella 120°C antigens were agglutinated by the 120°C antisera, not only by the three Bordetella sera, but also by those produced with S. paratyphi B and E. coli 09. The Br. abortus 19 serum had titers of 1:40 and 1:20 for B. pertussis and B. bronchiseptica, respectively, and did not agglutinate B. parapertussis. The S. paratyphi B and Br. abortus 19 unheated and 100°C antigens as well as the 120°C antigens were agglutinated by the homologous serum. None of the other unheated and 100°C antigens were agglutinated by any of the antisera produced with 120°C antigens.

TABLE 32

RESULTS OF AGGLUTINATION TESTS USING 120°C SERA AND ANTIGENS OF RELATED AND UNRELATED SPECIES

Antigens	Antisera Prepared with Suspensions Heated at 120°C					
	10536	17903	88	Br. abortus	S. paratyphi	E. coli 09
B. pertussis 10536						
unheated	–	–	–	–	–	–
100°C	–	–	–	–	–	–
120°C	320	320	320	40	160	160
B. parapertussis 17903						
unheated	–	–	–	–	–	–
100°C	–	–	–	–	–	–
120°C	320	320	80	–	80	80
B. bronchiseptica 88						
unheated	–	–	–	–	–	–
100°C	–	–	–	–	–	–
120°C	160	80	160	20	40	40
Br. abortus 19						
unheated	–	–	–	80	–	–
100°C	–	–	–	80	–	–
120°C	–	–	–	80	–	–
S. paratyphi B						
unheated	–	–	–	–	80	–
100°C	–	–	–	–	160	–
120°C	–	–	–	–	80	–
E. coli 09						
100°C	–	–	–	–	–	–
120°C	–	–	–	–	–	640
E. coli 02						
100°C	–	–	–	–	–	20
120°C	–	–	–	20	40	160

Agglutinin absorption tests

Cross absorption tests were performed by using E. coli 09 serum and absorbing with E. coli 09, E. coli 02, and B. pertussis 10536 120°C antigens; the results are tabulated in Table 33. Before the absorption the E. coli 09 120°C cells were agglutinated to a titer of 1:640. After the serum was absorbed with E. coli 02 or B. pertussis 10536 the titer was somewhat lower. The absorption with the homologous antigen removed all agglutinins.

The observation that Bordetella suspensions heated at 100°C were still inagglutinable by antiserum prepared with 120°C antigens led to experiments in reheating these suspensions at 120°C for 1 hour.

TABLE 33

RESULTS OF CROSS ABSORPTION TESTS

Antigens	Antiserum Prepared with E. coli 09, 120°C, Absorbed with:		
	E. coli 09, 120°C	E. coli 02, 120°C	B. pertussis 10536, 120°C
E. coli 09, 120°C	–	320	320
E. coli 02, 120°C	–	–	–
B. pertussis 10536, 120°C	–	–	–

The results are shown in Table 34. E. coli 02 and Br. abortus 19, previously heated at 100°C for 2-1/2 hours, were reheated at 120°C for 1 hour and tested for agglutinability; the results are given in Table 35. The results given in Table 34 indicated that the sera produced with unheated antigen were lacking in antibodies which would react with antigens heated at 120°C.

TABLE 34

AGGLUTINATION TESTS WITH REHEATED BORDETELLA ANTIGENS

Antigens	Antiserum			
	Heated 10536, 120°C	Unheated		
		10536	17903	88
B. pertussis 10536				
100°C	–	1000	–	–
100°C–120°C	320	–	–	–
120°C	320	–	–	–
B. parapertussis 17903				
100°C	–	–	500	200
100°C–120°C	320	–	–	–
120°C	320	–	–	–
B. bronchiseptica 88				
100°C	–	–	200	500
100°C–120°C	160	–	–	–
120°C	160	–	–	–

TABLE 35

AGGLUTINATION TESTS WITH REHEATED E. COLI AND BR. ABORTUS 19 ANTIGENS

Antigens	Antiserum	
	E. coli 02 O	Br. abortus
E. coli 02		
100°C	2560	
100°C–120°C	1280	
Br. abortus 19		
100°C		1280
100°C–120°C		1280

Antigens heated at 100°C were agglutinated by antiserum against unheated vaccine but not by antiserum against the 120°C antigen. These two antigens did not show any changes in agglutinability after reheating the 120°C, and it was evident that they were heat-stable.

The results of agglutination tests with E. coli 09 are given in Table 36; these results indicated that serum produced with E. coli 09 120°C antigen did not agglutinate the unheated or 100°C suspensions, which were inagglutinable because of the capsular A antigen. Serum for the unheated E. coli 09 antigen contained both O and A

TABLE 36

AGGLUTINATION TESTS WITH E. COLI 09

Antigens	Sera Made with E. coli 09	
	Unheated Antigen	120°C Antigen
E. coli 09		
unheated	500	–
100°C for 2-1/2 hours	1000	–
120°C for 2 hours	1000	1000

antibodies and agglutinated the unheated, 100°C, and 120°C antigens.

Discussion of the results with 120°C antigens

From the comparative studies of the effect of heat at 120°C on Bordetella, S. paratyphi B, Br. abortus 19, E. coli 02, and E. coli 09, certain differences among these organisms became apparent. Br. abortus 120°C antigen, while still having agglutinability, did not stimulate agglutinin production. S. paratyphi B 120°C antigen had little agglutinability and produced a serum with poor agglutinin titer.

E. coli 09 120°C antigen was effective in stimulating agglutinin production in rabbits. B. pertussis, B. parapertussis, and B.

bronchiseptica 120°C antigens were agglutinated not only by their group antisera, but also to a low titer by antiserum produced with S. paratyphi B 120°C antigen and E. coli 09 120°C antigens. However, the S. paratyphi B and E. coli 09 120°C antigens were not agglutinated by the Bordetella antisera. These results suggested that S. paratyphi B and E. coli 09 antisera were specific while the Bordetella 120°C antigens and antisera were nonspecific.

E. coli 09 antiserum absorbed with E. coli 02 or B. pertussis 10536 120°C antigens retained the specific antigens for E. coli 09 (Table 33).

The results of the reheated Bordetella antigens gave evidence that 120°C definitely altered the agglutinability. Antigens heated at 100°C were agglutinated by the sera produced with unheated vaccine, while the 120°C antigens were not. After the 100°C suspensions were reheated at 120°C they lost agglutinability with antisera against the unheated vaccines and became agglutinable by the sera produced with 120°C antigens. Reheating at 120°C of B. abortus 19 and E. coli 02 did not change the agglutinability.

The results of the agglutination tests with E. coli 09 (Table 36) demonstrated the O inagglutinability of the unheated or 100°C antigens. The antiserum produced with unheated antigen contained capsular as well as O agglutinins. If the results of agglutination

test of E. coli 09 unheated and heated antigens were compared with the agglutination tests of Bordetella with antigens prepared in the same manner, it was seen that there was no similarity.

The Effect of Chymotrypsin on Heated Bacterial Suspensions

Because the optimal conditions for the activity of chymotrypsin include a pH near 7.0, this enzyme was selected for experiments with the heated bacterial suspensions. Five milliliters of the 100°C suspensions of the three species of Bordetella, S. paratyphi B, Br. abortus 19, and E. coli 02 were treated with chymotrypsin as described above. The supernatant fluids were used for quantitative tests for protein, and the results are shown in Table 37.

The protein in the supernatant fluid from the vaccine must represent soluble material washed off the cells (by shaking the bacterial suspensions). The amount of the protein in the supernatant fluid increased noticeably after the bacterial suspension was heated for 2-1/2 hours. After the treatment with chymotrypsin, more protein was present in the supernatant fluid and the amounts did not vary much among the species tested. Total nitrogen determinations were made in the Michigan Department of Health Laboratory, Lansing, and the results are shown in Table 38.

TABLE 37

QUANTITATIVE BIURET TESTS

Supernatant Fluids	Protein (mg./100 ml.)
B. pertussis 10536	
unheated vaccine (containing 100 bil./ml.)	15
100°C suspension (containing 100 bil./ml.)	60
100°C, chymotrypsin-treated (containing 100 bil./ml.)	110
100°C control sample (containing 100 bil./ml.)	5–8
B. parapertussis 17903	
100°C, chymotrypsin-treated (containing 100 bil./ml.)	120
B. bronchiseptica 88	
100°C, chymotrypsin-treated (containing 100 bil./ml.)	130
B. pertussis, rough	
100°C, chymotrypsin-treated (containing 100 bil./ml.)	130
S. paratyphi B	
100°C, chymotrypsin-treated (containing 100 bil./ml.)	100
Br. abortus 19	
100°C, chymotrypsin-treated (containing 100 bil./ml.)	90
Chymotrypsin solution (0.04 percent)	40

TABLE 38

NITROGEN DETERMINATION OF VARIOUS MATERIALS

Materials	mg.N/ml.	Calculated mg. protein/100 ml.
B. pertussis		
10536 unheated vaccine (100 bil./ml.)	0.46	287.5
10536 sedimented cells (100 bil./ml.)	0.34	212.5
supernatant fluid	0.022	13.75
B. pertussis 10536		
100°C suspensions (100 bil./ml.)	0.48	300.0
sedimented cells	0.29	181.25
supernatant fluid	0.22	137.5
chymotrypsin-treated cells	0.12	75.0
supernatant fluid from chymotrypsin-treated sample	0.25	156.25
Chymotrypsin solution (0.04 percent)	0.052	32.5

Comparing the results in Tables 37 and 38, the amount of protein in the supernatant fluid of the vaccine is practically the same. The results of the quantitative Biuret test and total nitrogen (Micro Kjeldahl) applied to the enzyme solution correlated. However, the supernatant fluid from the heated suspension had twice the amount of protein calculated from total nitrogen as compared to the amount determined by Biuret test. The supernatant fluid from

the enzyme-treated sample had about 50 mg. more protein based on the nitrogen determination. It must be assumed that such variations were within the limitations of the tests on such small samples. The results of the nitrogen determinations indicated that some protein was still present in the chymotrypsin-treated cells.

Tests for agglutinability

The results of the agglutination tests are given in Table 39. The agglutinability was lost after the cells were treated with chymotrypsin, while the control cells manipulated in the same manner

TABLE 39

RESULTS OF THE AGGLUTINATION TESTS WITH CHYMOTRYPSIN-TREATED CELLS

Antigens	Antiserum Produced with Unheated Vaccine		
	10536	17903	88
B. pertussis 10536			
100°C, chymotrypsin-treated	–	–	–
100°C, control vaccine	500	–	–
B. parapertussis 17903			
100°C, chymotrypsin-treated	–	–	–
100°C, control vaccine	–	500	200
B. bronchiseptica 88			
100°C, chymotrypsin-treated	–	–	–
100°C, control vaccine	–	200	500

but without the enzyme were still agglutinated by antisera for unheated vaccines.

To compare the effect of chymotrypsin on Bordetella with the effect on other species, experiments were carried out as described above, using suspensions of S. paratyphi B, Br. abortus 19, E. coli 02, and E. coli 09. Results with 100°C antigens are tabulated in Table 40. S. paratyphi B and E. coli 02 100°C vaccines treated with chymotrypsin lost some of their agglutinability compared with

TABLE 40

AGGLUTINATION TESTS WITH CHYMOTRYPSIN-TREATED ANTIGENS OTHER THAN BORDETELLA

Antigens	Antiserum for Culture	Titer
S. paratyphi B		
100°C, chymotrypsin-treated	S. paratyphi B	400
100°C, control cells	O	1600
Br. abortus 19		
100°C, chymotrypsin-treated	Br. abortus	1500
100°C, control cells		1500
E. coli 02		
100°C, chymotrypsin-treated	E. coli 02	500
100°C, control cells	O	2560
E. coli 09		
100°C, chymotrypsin-treated	E. coli 09	1000
100°C, control cells	unheated	1000

the controls. However, the Br. abortus 19 and E. coli 09 100°C antigens showed no evidence of loss in agglutinability related to chymotrypsin treatment.

Small amounts of the chymotrypsin-treated cells of Bordetella, E. coli 02, and Br. abortus 19 were reheated at 120°C for 1 hour and used in agglutination tests; the results are given in Table 41. Previously it was determined (Table 34) that Bordetella organisms reheated at 120°C after heating at 100°C became agglutinable by the serum produced with 120°C B. pertussis. E. coli and Br. abortus 19 could be reheated without losing agglutinability.

TABLE 41

AGGLUTINATION TESTS WITH ANTIGENS REHEATED AFTER CHYMOTRYPSIN TREATMENT

Antigens	Antiserum	Titer
B. pertussis 10536, chymotrypsin-treated + 120°C	B. pertussis 120°C	20
B. parapertussis 17903, chymotrypsinized + 120°C		—
B. bronchiseptica 88, chymotrypsinized + 120°C		—
Br. abortus 19, chymotrypsinized + 120°C	Br. abortus unheated	1500
E. coli 02, chymotrypsinized + 120°C	E. coli 02 O	640

Bordetella vaccines lost their O agglutinability when they were treated with chymotrypsin prior to reheating at 120°C. Br. abortus 19 did not show any loss of agglutinability, and E. coli 02 showed little change due to similar treatment.

Agglutinin production in rabbits with the chymotrypsin-treated antigens

The results of agglutinin production experiments with the various chymotrypsin-treated antigens are given in Table 42. The

TABLE 42

AGGLUTININ PRODUCTION WITH CHYMOTRYPSIN-TREATED MATERIALS

Antigens	B. pertussis Antisera	
	Chymotrypsinized Sample	Supernatant Fluid from Chymotrypsinized Sample
B. pertussis 10536		
unheated	80	640
100°C	80	160
100°C, chymotrypsinized	–	–
120°C	40	20
B. parapertussis 17903		
unheated	–	20
100°C	–	–
120°C	–	–
B. bronchiseptica 88		
unheated	–	–
100°C	–	–
120°C	–	–

B. pertussis sedimented cells treated with chymotrypsin stimulated agglutinins in very low titer compared with the supernatant fluid from the same sample. The agglutinogen apparently was split off rather than destroyed by the enzyme treatment. Neither of these sera agglutinated B. parapertussis or B. bronchiseptica. B. pertussis 10536 heated at 120°C was agglutinated to a low titer.

The results of agglutinin production experiments with the supernatant fluid from the 100°C 10536 vaccine are given in Table 43.

TABLE 43

AGGLUTININS PRODUCED WITH THE SUPERNATANT FLUID FROM THE 100°C 10536 VACCINE

Antigens	Antiserum Produced with Supernatant Fluid from 100°C 10536 Vaccine
B. pertussis 10536	
unheated	1280–2560
100°C	640
120°C	40
B. parapertussis 17903	
unheated	–
100°C	–
120°C	–
B. bronchiseptica 88	
unheated	40
100°C	–
120°C	–

Neither B. parapertussis 17903 nor B. bronchiseptica 88, heated or unheated, was agglutinated by the serum produced with the supernatant fluid from the B. pertussis 100°C suspension. The supernatant fluid from the chymotrypsin-treated sample (Table 42) produced serum with a lower titer than that produced with the supernatant fluid from the vaccine. The latter had less protein as determined by Biuret or nitrogen tests. The results of agglutinin production with control antigens are given in Table 44.

TABLE 44

AGGLUTININS PRODUCED WITH CONTROL ANTIGENS FROM THE CHYMOTRYPSIN EXPERIMENTS

Antigens	Antisera Produced with Control Antigens	
	Sedimented Bacteria	Supernatant Fluid
B. pertussis 10536		
unheated	1280	2560
100°C	640	640
120°C	40	–
B. parapertussis 17903		
unheated	–	40
100°C	–	–
120°C	–	–
B. bronchiseptica 88		
unheated	20	20
100°C	–	20
120°C	–	–

Since the vaccine used as control in the chymotrypsin experiment produced agglutinins in rabbits, it could only be concluded that the chymotrypsin-treated cells had lost their antigenicity because of the action of the enzyme.

Materials in Table 38 showed the difference in amount of protein. Some of these were used as antigens to relate the titer of antiserum with the given amount of antigen, and the results are shown in Table 45. These results showed that a very small amount

TABLE 45

TITERS OF ANTISERA PRODUCED WITH DIFFERENT AMOUNTS OF PROTEIN

B. pertussis Antigens Used for Antisera Production	Protein		Antigen B. pertussis 10536 Vaccine
	mg./ml.	Total Amount Used (mg.)	
Unheated	0.9	3.15	4000
100°C antigen	1.0	3.5	1280
120°C supernatant fluid	1.3	4.5	1280
100°C chymotrypsinized cells	0.25	0.87	80
100°C control cells	0.6	2.1	1280
100°C supernatant fluid from chymotrypsinized sample	1.25	4.55	640
100°C control supernatant fluid	0.08	0.28	2560

of protein was needed to stimulate agglutinins in rabbits. It was shown that 100°C vaccine was less antigenic than unheated vaccine containing the same amount of protein.

The agglutinin-producing property of the chymotrypsin-treated cells of other species was also determined, and the results are summarized in Table 46.

TABLE 46

AGGLUTININ-PRODUCING PROPERTY OF CHYMOTRYPSINIZED ANTIGENS

Antigens	Antiserum	Titer
S. paratyphi B		
unheated	S. paratyphi B	640
100°C O	chymotrypsinized O	640
100°C chymotrypsinized		80
Br. abortus 19		
unheated	Br. abortus 19	640
100°C	100°C chymotrypsinized O	640
100°C chymotrypsinized		640
E. coli 02		
unheated	E. coli 02	—
100°C	chymotrypsinized O	2560
100°C chymotrypsinized		640
E. coli 09		
unheated	E. coli 09	—
100°C	chymotrypsinized	—
120°C chymotrypsinized		80
120°C (O)		160

S. paratyphi B chymotrypsinized O antigen still produced serum which agglutinated the unheated and the O antigens. The homologous antigen was poorly agglutinated by this serum, indicating that chymotrypsin damaged the agglutinability of the cells but affected very slightly the agglutinin-producing property. Chymotrypsin-treated O antigen of E. coli 02 produced antiserum with a good titer. Br. abortus 19 cells treated with chymotrypsin produced serum which agglutinated equally well the unheated antigen, the 100°C antigen, and the antigen heated and chymotrypsinized. E. coli 09 chymotrypsin-treated cells produced agglutinins of low titer for the 120°C antigen, and for the same antigen treated with chymotrypsin.

Agglutinin absorption tests

The agglutinin-absorbing property of the chymotrypsinized B. pertussis 10536 cells was tested by using serum produced with B. pertussis 10536 100°C antigen and absorbing with the sedimented cells after enzyme treatment. It was shown that all agglutinins were removed, indicating that the enzyme had not destroyed the agglutinin-absorbing property of the antigen.

Similar experiments were carried out with E. coli 02, S. paratyphi B, and Br. abortus 19, with identical results; chymotrypsin did not destroy the ability to absorb agglutinins.

Absorption tests were conducted with E. coli 09 O serum and serum made with unheated antigens; the results are tabulated in Table 47. As shown in this table, E. coli 09 chymotrypsinized O antigens retained their agglutinin-absorbing property.

Discussion of the results with heated chymotrypsin-treated antigens

The denaturation of the bacterial proteins apparently made the organisms more susceptible to the action of enzymes. From the results of quantitative Biuret tests (Table 37) it was noticed that supernatant fluid from the 100°C B. pertussis suspension contained

TABLE 47

AGGLUTININ ABSORPTION TESTS OF E. COLI 09 SERA

Antigens	E. coli 09 O Serum		E. coli 09 Antiserum	
	Unabsorbed	Absorbed with Chymotrypsinized O Antigen	Unabsorbed	Absorbed with Chymotrypsinized O Antigen
E. coli 09				
unheated	–	–	500	320
100°C	–	–	1000	640
120°C O	1000	–	1000	–
120°C O, chymotrypsinized	100	–	100	–

four times the amount of protein present in the supernatant fluid from the vaccine. This could be explained if the heat denaturation had resulted in splitting the large molecules into subunits (Haurowitz, 1950) which would be detached from the cell. All supernatant fluids from the chymotrypsin-treated vaccines had increased amounts of protein. The sedimented cells from the 100°C antigen contained as much nitrogen as the supernatant fluid. After chymotrypsin treatment, the cells still contained some nitrogenous material. These chymotrypsin-treated cells had little ability to stimulate agglutinins, compared with the control cells.

From the results in Table 40, it was seen that S. paratyphi B and E. coli 02 chymotrypsin-treated antigens partially lost their agglutinability, while Br. abortus 19 and E. coli 09 (100°C) did not show any changes. These observations suggested that protein was an important antigenic constituent of Bordetella species, while the situation is different with S. paratyphi B and Br. abortus. It is well known that antigens of S. paratyphi B, E. coli, and Br. abortus contain polysaccharides and lipids in addition to the proteins. E. coli 09 capsular A antigen is a carbohydrate and was not affected by chymotrypsin. Previously reheated Bordetella antigens became O agglutinable but the chymotrypsin-treated cells after reheating at 120°C were not agglutinated by the serum produced with 120°C

antigen. Little change in agglutinability was observed with the chymotrypsin-treated and reheated E. coli 02 or Br. abortus 19 antigens (Table 41). The results showed that Bordetella O antigens were affected by the chymotrypsin treatment in comparison with E. coli 02 and Br. abortus 19 (Table 41).

The results with antisera production against the supernatant fluids of the 100°C vaccine, the chymotrypsin-treated and control samples, the chymotrypsin-treated cells and the control cells indicated that chymotrypsin-treated cells stimulated very little agglutinin production as compared with the other antigens. Chymotrypsin separated most of the agglutinogen from the cells without destroying its antigenicity. If the total amounts of protein used in individual antiserum production were compared (Table 45), it was found that not necessarily the highest amount of injected protein produced a serum with the highest agglutinin titer. It was noticed (Table 46) that S. paratyphi B chymotrypsinized O antigen stimulated agglutinins, while its agglutinability was reduced. The E. coli 02 chymotrypsinized O antigen was agglutinated to a lower titer with homologous antiserum than the untreated O antigen (Table 46).

With Br. abortus 19 no difference was shown among the differently treated antigens. E. coli 09 chymotrypsinized O antigen was poor in agglutinin production, possibly indicating that protein

was an important component in its antigenic structure. The agglutinin-absorbing property of the chymotrypsinized cells was not destroyed. B. pertussis 10536 chymotrypsinized cells removed all agglutinins against the 100°C antigen, while leaving some against the unheated one. This was observed with the 100°C antigen also. E. coli 09 chymotrypsinized O cells absorbed agglutinins against the O antigen, but not those against the capsular antigens. Br. abortus 19 chymotrypsinized 100°C antigen absorbed all agglutinins from the serum produced with 100°C or with unheated antigen.

The Effect of Chymotrypsin on the Agglutinability of Bordetella Suspensions Heated at 120°C for One Hour

Samples of B. pertussis 10536, B. pertussis–rough, B. parapertussis 17903, and B. bronchiseptica 88 antigens each with a density of 25 bil./ml. were heated at 120°C for 1 hour. Five-milliliter samples of these suspensions were centrifuged and the sedimented cells were resuspended in 2.5 ml. saline solution, mixed with 1 mg. of chymotrypsin and incubated at 32°C for 4 hours. A similar sample was incubated without enzyme. After incubation the cells were separated from the supernatant fluids, washed twice, and resuspended in saline solution for use in agglutination tests. The results of quantitative Biuret tests are given in Table 48.

TABLE 48

QUANTITATIVE BIURET TESTS OF THE SUPERNATANT FLUIDS FROM 120°C *BORDETELLA* SUSPENSIONS

Supernatant Fluids from:	Protein (mg./100 ml.)
Chymotrypsinized 120°C *B. pertussis* 10536	100
Chymotrypsinized 120°C *B. pertussis*–rough	90
Chymotrypsinized 120°C *B. parapertussis* 17903	80
Chymotrypsinized 120°C *B. bronchiseptica* 88	90
Control, 120°C *B. pertussis* 10536	(negative)
Chymotrypsin in solution (0.04 percent)	50

Chymotrypsin separated some of the protein from these heated cells. The amounts in the supernatant fluids were small, but there was a difference between the amount accounted for and the total protein.

Effect of heat at 120°C on density of suspension

It was noted that the densities of the bacterial suspensions appeared to be reduced after heating at 120°C. Photelometer readings were made, and the results are tabulated in Table 49. A noticeable loss of density was demonstrated after the suspensions had been heated at 120°C for 1 hour. Gram-stained preparations of these bacterial suspensions revealed many badly damaged cells,

TABLE 49

DENSITIES OF THE BORDETELLA SUSPENSIONS BEFORE AND AFTER HEATING AT 120°C

Bacterial Suspensions	Density of Suspensions	
	Unheated (bil./ml.)	Heated at 120°C (bil./ml.)
B. pertussis 10536 suspension	25	12.5
B. pertussis–rough	23.5	12.9
B. parapertussis 17903	23.4	12.4
B. bronchiseptica 88	30.0	12.5

although some bacilli appeared to be unchanged. The stained preparations indicated some clumping of the bacilli.

Tests for agglutinability

The washed cells, after the chymotrypsin treatment, were used in agglutination tests; the results are given in Table 50. The chymotrypsin effect on the 120°C Bordetella vaccines was indicated by the presence of protein in the supernatant fluids and the loss of agglutinability of the cells. These results were in contrast to those obtained with E. coli 09 heated at 120°C for two hours and treated

TABLE 50

AGGLUTINATION TESTS WITH 120°C BACTERIAL SUSPENSIONS TREATED WITH CHYMOTRYPSIN

Antigens	Antiserum Produced with B. pertussis 10536 120°C Antigen
B. pertussis 10536	
120°C, chymotrypsinized	–
120°C control	320
B. parapertussis 17903	
120°C, chymotrypsinized	–
120°C control	320
B. bronchiseptica 88	
120°C, chymotrypsinized	–
120°C control	160

with chymotrypsin. The agglutinability of this organism was reduced, but not destroyed.

Agglutinin absorption tests

Attempts were made to absorb agglutinins from the serum prepared with 120°C B. pertussis 10536 antigen using as the absorbing reagent the supernatant fluid from a vaccine with a density of 400 bil./ml. Part of this supernatant fluid was heated at 120°C for 1 hour and used in similar tests. The results of agglutinin absorption

tests are given in Table 51. A rabbit was immunized with supernatant fluid heated at 120°C. The rabbit received ten injections with a total of 1.0 mg. of protein as determined by the nitrogen content.

There was a definite difference in the titer of serum absorbed with unheated supernatant fluid and that absorbed with supernatant fluid heated at 120°C. This finding indicated that the supernatant fluid heated at 120°C had the property of removing the antibodies produced against the whole 120°C vaccine.

TABLE 51

AGGLUTININ ABSORPTION TESTS WITH SUPERNATANT FLUIDS

Antigens	Antiserum Produced with 120°C *B. pertussis*		
	Unabsorbed	Absorbed with Supernatant Fluids	
		Unheated	Heated at 120°C
B. pertussis 10536, 120°C antigen	320	320	40±
B. pertussis–rough, 120°C antigen	640	320	±

The heated supernatant fluid was used for agglutinin production experiments. The results of tests with antiserum produced with 120°C heated supernatant fluid from B. pertussis vaccine are given in Table 52. The heated B. pertussis supernatant fluid (120°C) stimulated agglutinins for the 120°C antigens of all three Bordetella species. Titers (1:80) were observed with B. pertussis vaccine, unheated and heated at 100°C.

TABLE 52

AGGLUTININ PRODUCTION WITH SUPERNATANT FLUID HEATED AT 120°C

Antigens	Antiserum Produced with 120°C Heated Supernatant Fluid from B. pertussis Vaccine
B. pertussis 10536	
unheated	80
100°C	80
120°C	640
B. parapertussis 17903	
unheated	–
100°C	–
120°C	320
B. bronchiseptica 88	
unheated	–
100°C	–
120°C	320

Discussion of the results with supernatant fluids

Supernatant fluid from B. pertussis vaccine, heated at 120°C, stimulated agglutinin production in rabbits and removed agglutinins from antiserum produced with 120°C B. pertussis antigen. These observations suggested that part of the antigenic material was present in the supernatant fluid, but did not act as an O antigen prior to heating.

The Effect of 8 Molar Urea Solution

Since urea in a concentrated solution is known to have a denaturing effect on proteins, experiments were performed to study its effect on Bordetella.

Five-milliliter samples of 200 bil./ml. suspensions of each of the three species—B. pertussis 10536, B. parapertussis 17903, and B. bronchiseptica 88—were centrifuged and the sedimented cells were resuspended in 5 ml. of 8 M urea solution, and shaken for 1 minute by hand. In each instance the suspension became very mucoid, with the apparent optical density noticeably reduced. In order to separate the organisms by centrifugation, it was necessary first to make a 1:2 dilution in saline solution. The sedimented cells were washed twice with saline solution and used in agglutination tests.

A suspension of a rough culture of B. pertussis treated similarly with urea did not lose its density, and the bacteria could be separated by centrifugation without dilution of the suspension.

Tests for agglutinability

The results of agglutination tests are given in Table 53. The treatment with urea destroyed the agglutinability of all three Bordetella species.

A suspension of B. pertussis 10536 was treated with urea, as above, but was not separated by centrifugation. This was used for

TABLE 53

AGGLUTINATION TESTS WITH UREA-TREATED MATERIALS

Antigens	Antiserum Produced with Unheated Vaccines		
	10536	17903	88
B. pertussis 10536			
untreated antigen	4000	1000	1000
urea-treated	40	–	–
B. parapertussis 17903			
untreated	1000	5000	1500
urea-treated	–	80	–
B. bronchiseptica 88			
untreated	500	2000	4000
urea-treated	–	20	80

mouse protection tests with dilutions for the injection of the mice based on the density of the untreated suspension. The results are given in Table 54.

The urea-treated material did not protect the mice against intracerebral challenge. The corresponding untreated vaccine had good protective property. It can be concluded that urea destroyed the protective property as well as the agglutinability.

To determine whether the agglutinin-stimulating property had also been affected, groups of mice were injected with the urea-treated B. pertussis suspension used in the mouse protection test. As before, the density was based on the original untreated suspension; that is, 200 bil./ml. Another group of mice was injected with the washed cells from the urea-treated vaccine. The density of this vaccine was 7 bil./ml.

TABLE 54

MOUSE PROTECTION TESTS

Antigens	ED/50 (in billions)
B. pertussis 10536 vaccine	.31
B. pertussis 10536 urea-treated suspension . . .	no protection (survivors = 1) (total = 39)

The resulting antisera in both experiments had titers of only 1:40 for untreated B. pertussis vaccine. The antisera from mice injected with untreated B. pertussis vaccine had titers of 1:640 for the homologous antigen. It is evident that the agglutinin-producing property was also destroyed by urea solution since neither the whole suspension nor the cells were antigenic.

To test whether the urea had also destroyed the heat-stable antigens, urea-treated B. pertussis, B. pertussis–rough, B. parapertussis, and B. bronchiseptica vaccines were heated at 120°C for 1 hour. These suspensions were used in agglutination tests with serum prepared with 120°C B. pertussis 10536 suspension.

The results of agglutination tests with urea-treated cells, heated at 120°C, are given in Table 55. The agglutinability of all three species of Bordetella was almost completely destroyed by the urea treatment.

For comparison, similar experiments were done with S. paratyphi B, E. coli 02, and E. coli 09; the results of agglutination tests are shown in Table 56. None of the suspensions of these organisms became mucoid as the Bordetella suspensions did, and the cells were easily separated without dilution. Also, no changes were observed in density. The separated cells were washed with saline solution and portions of S. paratyphi B and E. coli 02 were heated at 100°C

TABLE 55

AGGLUTINATION TESTS WITH 120°C
UREA-TREATED CELLS

Antigens	Antiserum Produced with B. pertussis 10536 120°C
B. pertussis 10536	
heated at 120°C	320
treated with urea, 120°C	40
B. pertussis–rough	
heated at 120°C	640
treated with urea, 120°C	–
B. parapertussis 17903	
heated at 120°C	320
treated with urea, 120°C	40
B. bronchiseptica 88	
heated at 120°C	160
treated with urea, 120°C	–

for 2-1/2 hours. A sample of E. coli 09 urea-treated cells was heated at 120°C for 2 hours. The O agglutinability of S. paratyphi B was greatly reduced by the urea treatment. No change was observed with E. coli 02 or E. coli 09.

TABLE 56

AGGLUTINATION TESTS OF UREA-TREATED ANTIGENS

Antigens	Antiserum for Culture	Titer
S. paratyphi B		
urea-treated	S. paratyphi B O	80
urea-treated, 100°C		80
E. coli 02		
urea-treated, 100°C	E. coli 02 O	1280
E. coli 09		
urea-treated, 120°C	E. coli 09 O	1000

Discussion of the experiments with urea

Urea solution at high concentration is used to denature proteins. A common effect of urea is to induce protein molecules to split (Gortner, 1953). The purpose of using 8 M urea in this study was to observe antigenic changes which would suggest a particular antigen to be a protein. With all three species of Bordetella, the urea treatment resulted in a very viscous mass as a result of molecular dissociation (Putnam, 1953). The agglutinability of the urea-treated cells was lost. In addition, the urea-treated material did not protect mice against the challenge. Agglutinins were not produced in mice injected with urea-treated vaccine material or the

separated cells from the treated vaccine. Urea-treated cells of B. pertussis–rough, B. parapertussis, and B. bronchiseptica heated at 120°C were not agglutinated by the serum made with B. pertussis antigen.

Urea-treated S. paratyphi B, heated at 100°C or unheated, retained little agglutinability, while E. coli 02 treated with urea and heated at 100°C did not show much change in agglutinability. Urea had no effect on the E. coli 09 antigen (Table 56). It was noticed that none of the other suspensions gave the viscous appearance. It was demonstrated that similar treatments of different organisms did not result in similar antigenic changes.

DISCUSSION

It is recognized that enzymes are important tools in the study of protein structure. Proteolytic enzymes, because of their wide range of specificity, can furnish many different hydrolysates from the same protein (Desnuelle, 1953). Dubos (1955) states that many types of bacteria are susceptible to the action of various enzymes and believes that enzymes used singly or in combinations may play an important part in the study of biological problems including cellular structure.

In the present work the effect of enzymatic activity on the antigenicity of the bacterial cell was studied in order to obtain some clue as to the chemical nature of various Bordetella antigens. The results of the experiments with proteolytic enzymes on B. pertussis indicated that agglutinogen and protective antigen were resistant to these enzymes. Some effect was noted after the application of pepsin, but this was shown to be due largely to the low pH rather than to the specific action of the enzyme.

While the results were not clear-cut, there was an indication of a stepwise action on the antigenicity of B. pertussis cells, treated

first with pepsin and then with hemicellulase. The evidence of activity of the hemicellulase after pepsin treatment of B. pertussis suggested that protein had been partially removed making hemicellulose, which is located more deeply in the cell, available for hemicellulase activity. The presence of hemicellulose or related substances may be the explanation for the toughness of the cell wall of B. pertussis. It was known that Bordetella species were resistant to lysis by freezing and thawing, and were extremely difficult to disrupt by grinding procedures. Hemicellulose, according to Gortner (1950), comprises an ill-defined group of polysaccharides, which accompany cellulose in the cell walls of plants. The same author also states that cellulose is present in bacterial polysaccharides. To explore this hypothesis further would require much more work, with the main goal the determination of the chemical structure.

The fact that proteolytic enzymes did not show activity on the B. pertussis antigens did not prove that the antigens were not proteins, but merely indicated that the antigens in native state were resistant to the enzyme activity. This observation led to the use of some method of denaturation prior to the enzyme application. Heating the antigen at 56°C for 1 hour did not affect the protective property of B. pertussis, but neither did it make the organisms susceptible to the action of trypsin or chymotrypsin. It seemed

that enzymes may not be so useful in the study of antigenicity as in the study of chemical structure, because the optimal requirements for the enzyme activity were frequently not favorable to antigen stability.

Kauffmann's work with E. coli or Salmonella classified the O antigens on the basis of heat stability at 100°C for 2-1/2 hours. Similar treatment of Bordetella indicated some loss of antigenicity but not complete destruction. This indication of some more heat-stable factors of Bordetella should lead to further study. It may well be that agglutinability, agglutinin-producing, and agglutinin-absorbing properties vary independently in their heat stability.

There was a suggestion by Maitland that the important antigen of B. pertussis was a polysaccharide-protein-lipid complex and that this applies to other Bordetella species. A polysaccharide-lipid complex would resist heating at 100°C, as was demonstrated in studies with E. coli, where the purified O antigen was a polysaccharide-lipid complex (Neter et al., 1956). The chymotrypsin treatment did destroy the agglutinability and agglutinin-producing property of Bordetella, but did not affect the agglutinability or agglutinin-producing property of E. coli 02. These observations suggested that protein was important in the antigenicity of Bordetella and that if polysaccharide were present in the Bordetella complex it was without antigenicity by itself. The possibility that all protein was

removed and the hapten-polysaccharide was made inactive, was ruled out because the chymotrypsin-treated B. pertussis 10536 cells still had nitrogenous material present. With the E. coli 09, which possessed a polysaccharide capsular antigen, chymotrypsin did not reduce the agglutinability with specific A antiserum. S. paratyphi B O antigenicity was partially affected by chymotrypsin treatment resulting in an antigen with reduced agglutinability although it still stimulated O agglutinins in high titer. It was known that the O antigen was a protein and therefore might be affected by chymotrypsin. Br. abortus 19 antigen was resistant to heat at 100°C and also was not affected by chymotrypsin. These observations suggested that polysaccharide might be responsible for the agglutinability and also for agglutinin production. According to Huddleson et al. (1941), the lipid fraction from Br. abortus was biologically inactive. These comparative results indicated that classification based on heat stability must be studied for each species of bacteria, and conclusions based on the study of one species did not necessarily apply to another species. Particularly the classification of Bordetella O antigen based on heating at 120°C for 1 hour seemed questionable due to the cross agglutination with other unrelated genera. It was demonstrated that the antigenicity of both S. paratyphi B and Br. abortus 19 was destroyed by heating at 120°C for 1 hour.

Urea treatment was selected because of its known effect in denaturing protein. In B. pertussis 10536, the agglutinogen and protective antigens were destroyed, strongly suggesting that both were protein in nature. By applying this urea treatment to B. parapertussis and B. bronchiseptica, the agglutinability was completely destroyed. The agglutinability of S. paratyphi B by its specific antiserum was reduced by urea treatment while no effect was noticed on E. coli 02 or 09.

SUMMARY

The activity of proteolytic enzymes on B. pertussis antigens was studied. The antigenicity of unheated B. pertussis suspensions, as measured by agglutinability, agglutinin production, agglutinin absorption, and mouse protection tests, was unaffected by pepsin, trypsin, or chymotrypsin.

Treatment with lipase, acid phosphatase, beta amylase, cellulase, hemicellulase, and lysozyme did not show any effect on the agglutinability of B. pertussis, and therefore, the effect of these enzymes was not tested further.

Heating at 100°C for 2-1/2 hours did not completely destroy the antigenicity of B. pertussis, B. parapertussis, or B. bronchiseptica. However, there was some indication that agglutinability, agglutinin-producing, and agglutinin-absorbing properties varied independently in their heat stability. It was shown by serological tests that heating separated part of the antigen from the bacterial cell. Chemical tests showed that nitrogenous material was removed from the cells and could be detected in the supernatant fluid.

Results with antisera against specific antigenic factors of Bordetella indicated that certain factors were more heat-stable

than others. For example, Factors 1, 3, 7, 12, and 14 were inactivated by heat at 100°C, while Factors 2, 5, and 8 were heat-stable at this temperature.

The action of chymotrypsin on Bordetella cells heated at 100°C released the agglutinogen from the cells, which were then inagglutinable and unable to stimulate the production of agglutinins. However, the agglutinogen was not destroyed since antigenicity of the supernatant fluids could be demonstrated.

In contrast to the effect on B. pertussis, chymotrypsin had little effect on the antigenicity of heated suspensions of Br. abortus, S. paratyphi B, and E. coli 02; these results indicated a difference in chemical nature of the specific antigens of these organisms.

Bordetella antigens heated at 120°C (120°C antigens), classified as O antigens, showed cross reactions with other unrelated bacterial species.

Studies of the effect of heat showed that the antigenicity of S. paratyphi B and Br. abortus 19 was destroyed at 120°C. E. coli 02 and E. coli 09 O antigens were resistant to this temperature.

Bordetella 120°C antigens were susceptible to chymotrypsin action, while O antigens of other genera were resistant. These findings suggested that the antigenic material was protein in nature in Bordetella.

Urea solution destroyed the agglutinogen and the protective antigen of B. pertussis, indicating again that these two antigens were proteins.

B. parapertussis and B. bronchiseptica, treated with 8 M urea solution, lost their agglutinability with antiserum made with unheated antigens, as well as with antiserum made with 120°C antigen.

Urea solution in similar concentration did not affect the O antigens of E. coli 02 or E. coli 09.

The basic differences demonstrated to exist between the classic O antigens of E. coli, and the 120°C antigens of Bordetella raised serious questions as to the validity of designating the latter as O antigens.

REFERENCES

Andersen, E. K. 1953. Serological studies on H. pertussis, H. parapertussis and H. bronchiseptica. Acta Pathologica et Microbiologica Scandinavica, 33, 202–24.

Bodansky, A. 1937. Determination of serum inorganic phosphate and of serum phosphatase. Am. J. Clin. Path., 1, 51–59.

Boivin, A., and Mesrobeanu, L. 1933. Technique la Preparation des Polysaccharides Microbiene specifiques. Compt. Rend. Soc. de Biol., 113, 490–92.

Bordet, J., and Gengou, O. 1906. Le Microbe de la coqueluche. Ann. Inst. Pasteur, 20, 731–41.

Brown, A. M. 1959. Growth idiosyncrasies of some Bordetella pertussis strains and the intracerebral virulence of these strains in mice. J. Gen. Microbiol., 20, 532–39.

Brumfitt, W., Wardlaw, C., and Park, J. T. 1958. Development of lysozyme-resistance in Micrococcus lysodeikticus and its association with an increased O-acetyl content of the cell wall. Nature, 181, 1783–1784.

Cohen, S. M., and Wheeler, M. W. 1946. Pertussis vaccine prepared with Phase I culture grown in fluid medium. Am. J. Public Health, 36, 371–76.

Comfort, M. W., and Osterberg, A. E. 1934. Lipase and esterase in blood serum; their diagnostic value in pancreatic disease. J. Lab. and Clin. Med., 20, 271–78.

Desnuelle, P. 1953. The general chemistry of Amino Acids and Peptides. In the Proteins, pp. 88–180. Edited by H. Neurath and K. Bailey. Academic Press Inc., New York.

Dubos, R. 1955. The Bacterial Cell. Harvard University Press, Cambridge, Massachusetts.

Edwards, P. R., and Ewing, W. H. 1955. Identification of Enterobacteriaceae. Burgess Publ. Co., Minnesota.

Eldering, G. 1941. A study of the antigenic properties of H. pertussis and related organisms. Am. J. Hyg., 34, 1–7.

Eldering, G., Hornbeck, C., and Baker, J. 1957. Serological study of B. pertussis and related organisms. J. Bacteriol., 14, 133–136.

Eldering, G., and Kendrick, P. L. 1938. A group of cultures resembling both Bacillus pertussis and Bacillus bronchisepticus, but identical with neither. J. Bacteriol., 36, 561–572.

Evans, D. G. 1939. The toxin of B. parapertussis and the relationship of this organism to H. pertussis and Br. bronchiseptica. J. Pathol. Bacteriol., 49, 563–70.

Evans, D. G. 1942. Antigenic properties of Haemophilus pertussis in relation to active immunization. The Lancet, i, 529.

Evans, D. G., and Perkins, F. T. 1953. An agglutinin production test in the study of pertussis vaccine. J. Pathol. Bacteriol., 66, 479–88.

Evans, D. G., and Perkins, F. T. 1955. Tests for agglutinin production by pertussis protective antigen. J. Pathol. Bacteriol., 69, 329–31.

Ferry, N. S. 1912. Bacillus bronchisepticus; the cause of distemper in dogs and similar disease in other animals. Vet. J., 68, 376.

Ferry, N. S. 1918. Studies relative to the apparent close relationship between Bact. pertussis and B. bronchisepticus. J. Bacteriol., 3, 193–208.

Fishman, N. H. 1951. Enzymatic Hydrolysis of Muco-polysaccharides. In the Enzymes, pp. 769–92. Edited by F. B. Sumner and K. Myrback. Academic Press, Inc., New York.

Flosdorf, E. W. 1941. Studies with H. pertussis: Agglutinogenic relationship of the phases. J. Bacteriol., 41, 457–71.

Flosdorf, E. W., and Kimball, A. C. 1940. Separation of the Phase I agglutinogen of H. pertussis from toxic components. J. Immunol., 39, 475–94.

Flosdorf, E. W., and McGuinness, A. C. 1942. Studies with Haemophilus pertussis: The antigenic structure of H. pertussis and its clinical significance. Am. J. Diseases of Children, 64, 43–50.

Frobisher, M. 1944. Fundamentals of Bacteriology. 3d ed. W. B. Saunders Co., Philadelphia.

Gortner, R. A. 1953. Outlines of Biochemistry. 3d ed. Edited by R. A. Gortner, Jr., and W. A. Gortner. John Wiley and Sons, Inc., New York.

Haurowitz, F. 1950. Chemistry and Biology of Proteins. 1st ed. Academic Press, Inc., New York.

Haurowitz, F. 1955. Biochemistry and Introductory Textbook. John Wiley and Sons, Inc., New York.

Huddleson, I. F., Andrey, W. B., Pennel, R. B., Stahl, W. H., Hamann, E. E., and Munger, M. 1941. Studies in Brucellosis. Michigan State College, Agricultural Experiment Station, Tech. Bull., 177, 29–34.

Kasuga, T., Nakase, Y., Ukishima, K., and Takatsu, K. 1953. Studies on Haemophilus pertussis. Kitasato Arch. of Exp. Med., 26, 121–42.

Kauffmann, F. 1947. The serology of the coli group. J. Immunol., 57, 71–100.

Kendrick, P. L., Eldering, G., Dixon, H. K., and Misner, J. 1947. Mouse protection tests in the study of pertussis vaccine. Am. J. Public Health, 37, 803–10.

Kendrick, P. L., Eldering, G., Nadolski, E. B., and Baker, J. 1953. Antigenic relationship of Haemophilus pertussis, the parapertussis bacillus and Brucella bronchiseptica as shown by cross protection tests in mice. J. Bacteriol., 66, 166–69.

Lacey, B. W. 1953. On the serology and taxonomy of Haemophilus pertussis, para-pertussis and bronchisepticus. Alti Vi congresso Internazionale Di Microbiologia, 2, 333–34.

Landsteiner, K. 1936. The specificity of serological reactions. Charles C. Thomas Publ., Springfield, Illinois.

Landsteiner, K., and Chase, M. W. 1933. Observations on serological reactions with albumose preparations. Proc. Soc. Explt. Biol. Med., 30, 1413.

Leslie, P. H., and Gardner, A. A. 1931. The phases of Haemophilus pertussis. Am. J. Hyg., 31, 423–34.

Litchfield, J. T., and Fertig, J. H. 1941. On a graphical solution of the dosage effect curve. Bull. Johns Hopkins Hosp., 69, 276–86.

Lopez, M. M. 1952. El Genera Bordetella, Microbiologia Espanola, 5, 1–5.

Maitland, H. B., and Guerault, A. 1958. Surface antigens of Haemophilus pertussis. Nature, 181, 122–23.

Myrback, K., and Neumuller, G. 1950. Amylases and the Hydrolysis of Starch and Glycogen. In the Enzymes, pp. 653–724. Edited by J. B. Sumner and K. Myrback. Academic Press, Inc., New York.

National Institute of Health. 1948. Minimum requirements for pertussis vaccine. Bethesda, Maryland.

Neter, E., Westphal, O., Luderitz, O., and Gorzynski, E. 1956. The bacterial hemagglutination test for the demonstration of antibodies to Enterobacteriaceae. Ann. N.Y. Acad. Sc., 66, 141–55.

Neurath, H. 1957. Some considerations of the multiple specificity of proteolytic enzymes. Ann. N.Y. Acad. Sc., 68, 11–23.

Neurath, H., Greenstein, J. P., Putnam, F. W., and Erickson, J. O. 1944. The chemistry of protein denaturation. Chem. Revs., 34, 157.

Northrop, F. H., Junitz, M., and Herriott, R. M. 1948. Crystalline Enzymes. Columbia Univ. Press, New York.

Pigman, W. 1951. Cellulases, Hemicellulases and Related Enzymes. In the Enzymes, pp. 725–44. Edited by J. B. Sumner and K. Myrback. Academic Press, Inc., New York.

Pillemer, L. 1954. Protective antigen of Haemophilus pertussis. The Lancet, 1, 1257–60.

Putnam, F. W. 1953. Protein Denaturation. In the Proteins, pp. 808–91. Edited by H. Neurath and K. Bailey. Academic Press, Inc., New York.

Salton, M. R. 1957. The properties of lysozyme and its action on microorganisms. Bact. Review, 21, 82–99.

Smith, E. L. 1951. Proteolytic Enzymes. In the Enzymes, pp. 793–872. Edited by J. B. Sumner and K. Myrback. Academic Press, Inc., New York.

Smith, T., and Bryant, G. 1927. Studies on pathogenic B. coli from bovine source. J. Exp. Med., 46, 133–40.

Smolens, J., and Mudd, S. 1943. Agglutinogen of B. pertussis, Phase I for skin-testing. J. Immunol., 47, 155–63.

Someya, S., and Kaneko, Y. 1955. Studies on the "K" antigens of Haemophilus pertussis. Japanese J. Med. Sc. and Biol., 8, 99–108.

Warren, G. H., Gray, J., and Bartell, P. 1955. The lysis of Pseudomonas aeroginosa by lysozyme. J. Bacteriol., 70, 614–19.

The Whooping Cough Immunization Committee of the Medical Research Council. 1959. Vaccination against whooping cough. Brit. Med. J., 1, 994–1000.

Wilson, G. S., and Miles, A. 1957. Topley and Wilson's Principles of Bacteriology and Immunity. 4th ed., pp. 763. The Williams and Wilkins Co., Baltimore, Maryland.