STUDIES ON THE GENUS BORDETELLA.

by

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C O N T E N T S.

EXPERIMENTAL AND RESULTS.

INTRODUCTION.

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The genus Bordetella as defined by Lopez (1952) consists of the three organisms Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. These species were formerly classified in the genus Haemophilus, but their inclusion in a new genus was suggested on the grounds of cultural and antigenic differences from other Haemophilus species.

Bord. pertussis was first isolated by Bordet and Gengou (1906) from children suffering from the disease known as whooping cough. Eldering and Kendrick (1937) isolated a second organism, Bord. parapertussis, which was also a cause of whooping cough, although the infection was generally of a mild type. The third member of the genus, Bord. bronchiseptica, has frequently been found in respiratory-tract infections of rabbits, guinea pigs, cats and dogs and has also been isolated from a few cases of whooping cough (Brown, 1926; Medical Research Council, 1951). The isolation of these species has never been easy, even using complex media rendered selective by the addition of penicillin, and few diagnostic laboratories obtain high percentage isolations of Bordetella strains from cases of whooping cough. Problems are also encountered in the laboratory culture of Bord. pertussis. This organism is difficult to grow

and maintain in a virulent state, but the other two species are more easily grown and can be cultured on most, simple laboratory media. Cultural problems are also reflected in vaccine production.

Whooping cough in young children causes high morbidity and since the discovery of the causal organisms, attempts have been made to develop suitable prophylactic agents. Whole cell vaccines are commonly used but possess many disadvantages, the most serious being the occasional occurrence of encephalopathy following vaccination. Much research has been carried out to obtain a satisfactory prophylactic material without such disadvantages.

The present work is an attempt to prepare a medium giving optimal growth of virulent Bord. pertussis, and, using this medium, to obtain a better knowledge of the chemical and immunological properties of Bord. pertussis antigens. An attempt is also made to clarify some of the antigenic relationships within the genus, using modern techniques of serological analysis such as gel diffusion and immunoelectrophoresis.

A historical introduction to the work is presented, to show the difficulties which earlier workers have encountered with this genus and to give an outline of our present knowledge.

GROWTH OF BORDETELLA SPECIES.

1) Virulent Bordetella pertussis.

a) Growth on solid media.

Media for the growth of Bord. pertussis have been developed for two main purposes, laboratory diagnosis and vaccine production. The species was first isolated on a medium containing 50% (v/v) rabbit or human blood, glycerol extract of potato and agar (Bordet and Gengou, 1906). The Bordet-Gengou media currently used for diagnostic purposes contain 15 - 50% sheep or horse blood, peptone, glycerol and potato extract solidified with agar. Modifications have been the use of lysed blood and Potato starch, while greater selectivity has been achieved by the addition of penicillin. Some workers showed that glycerol concentrations greater than 1% (v/v) were inhibitory and that the omission of glycerol from the medium did not cause a significant decrease in growth (Dawson, Farnworth, McLeod and Nicholson, 1951). These workers suggested that potato extract provided optimal concentrations of amino acids and peptides required for the growth of the organism and that the starch and protein were of minor importance. In view of the peptides and amino acids present in blood, this suggestion seems rather doubtful. While addition of aspartate or glutamate to Bordet-Gengou medium was beneficial, absence of potato extract, or use of serum instead of whole

blood gave poor growth of the organism. Moreover, some commercial peptones were found to have an inhibitory effect. Lacey (1954) suggested another medium for isolating Bord. pertussis and the other causal organisms of whooping cough. It contained a partially defined base of potato starch, salts, organic acids, glutamic and other amino acids. Blood was added to this base together with penicillin and 4:4 diamidinophenylamine dihydrochloride (M and B 938). The penicillin and the amine rendered the medium selective for the Bordetella group.

The relatively slow growth on Bordet-Gengou medium and absence of growth on simpler media led to the belief that Bord. pertussis had very complex nutritional requirements. It appeared that these were only satisfied in media containing large amounts of blood. It was also found that in the few cases where growth did occur on simpler media, the organism differed in some respects from that initially isolated. Although media containing blood are suitable for the laboratory diagnosis of Bordetella infections, they are too complex for use in growth studies or in work on the bacterial antigens. Bordet-Gengou medium is also very expensive due to its high content of blood.

With the development of simplified liquid media by Hornibrook (1939) attempts were made to introduce similar media solidified with agar or silica gel (Verwey, Thiele, Sage and Schuchardt, 1949; Mazloum and Rowley, 1955). While a liquid medium containing casein hydrolysate, starch and nicotinic acid supported growth, a similar medium solidified with agar failed to do so. The conclusions drawn from many experiments of this nature are that solidifying agents contain toxic components whose effects can be neutralised by the addition of whole blood or blood components. Charcoal has been added to media used for the growth of Neisseria meningitidis and caused greatly improved growth (Glass and Kennet, 1939). This prompted its use in studies of Bord. pertussis. Addition of charcoal to a solid medium based on the semi-defined medium of Cohen and Wheeler (1946) allowed satisfactory growth of the organism with a retention of antigenic characteristics even after 15 - 20 subcultures (Powell, Culbertson and Ensminger, 1951). Other adsorbents have also been tried but only a preparation known as "attaclay" resulted in as good growth as that obtained on charcoal-containing media (Ensminger, Culbertson and Powell, 1953.) Mazloum and Rowley (1955) observed that a similar medium prepared

with different charcoal required an inoculum of 10^6 cells to initiate growth. Growth was improved by the addition of horse blood or by a cell-free extract obtained from Staphylococcus aureus strains; this could be replaced by the enzyme catalase. Cytoplasmic material from certain catalase-producing bacteria had a less marked effect. The growth-promoting effect of catalase was destroyed by heating at 100° for 15 min. and could be replaced by haematin and protoporphyrin IX (Brumfitt, 1959). No satisfactory explanation has yet been given for the fact that catalase aids growth on solid media but has no such effect in liquid media. It can only be postulated that the difference in growth requirements in solid and liquid media is due to particular requirements for physicochemical conditions (e.g. oxidation-reduction potential).

It was claimed that Bord. pertussis would grow from large inocula on charcoal-casein hydrolysate agar media, but irregular results were obtained from small inocula (Rowatt, 1957b). In all these media, the charcoal acts by adsorbing factors which inhibited growth of Bord. pertussis and which were present in the agar or were formed during heat sterilisation of the medium. The differences in the results obtained by

different workers suggest that the type of charcoal used may have a marked effect on the growth obtained. Krasnopeutseva (1958) emphasised that high quality charcoal was necessary, but pointed out that different methods of preparation of the casein hydrolysate resulted in varying amounts of growth on charcoal-casein hydrolysate agar media. Billaudelle (personal communication) has claimed that different casein hydrolysates may affect growth in both solid and liquid media, and that laboratory-prepared acid hydrolysates were superior to the commercial products. This is probably because the hydrolysates prepared in the laboratory are incorporated directly into the medium and are not submitted to any drying procedures as are the commercial products. The drying processes must inevitably cause some degradation of the materials present, particularily the sulphur-containing amino acids cystine and cysteine. Sulphur and sulphides formed from these amino acids may be present in commercial products and may cause inhibition of growth. Brumfitt (1959) used "defatted" agar to solidify a casein hydrolysate medium, but was still unable to obtain satisfactory growth. He does not record how the "defatted" agar was prepared nor the amount or nature of the material removed from it. The result suggests that the inhibition might have

Table 1. Liquid culture medium of Hornibrook (1939).

Casein hydrolysate	7.0
NaCl	5.0
KCl	0.2
$CaCl_2$	0.2
$MgCl_2,6H_2O$	0.1
Na_2CO_3	0.5
Phosphate	0.25
Starch	1.0
Nicotinic acid	0.01
Sulphur source	0.01

The composition is expressed in grams per litre.

been due to degradation products from sterilisation rather than to substances present in the commercial agar.

b) Growth in liquid media.

The first liquid medium giving satisfactory growth of virulent Bord. pertussis, was that described by Hornibrook (1939). The contents of this medium are shown in table 1. Many liquid media have been described since, with variations in the salts and growth factors (Wilson, 1945; Cohen and Wheeler, 1946; Verwey et al., 1949; Ungar et al., 1950). Thus Verwey et al. (1949) found that calcium chloride was unnecessary and an increased phosphate content was provided to increase the buffering capacity. The medium which is in current use for vaccine production in several laboratories is that of Cohen and Wheeler (1946), which contains yeast dialysate as a source of growth factors. Using this medium Fisher (1948b) was able to obtain growth from an inoculum of 12 viable cells/ml. He found that neither the rate of multiplication nor the final yield was influenced by the size of the inoculum. This result is in contrast to those obtained by several other workers. Verwey et al. (1949) observed that inoculum size, incubation time, surface: volume ratio and agitation all had a very marked effect on the growth rate and on the final yield

of cultures. Billaudelle, Heden and Malmgren (1959) also found that inoculum size affected the final yield. The results varied with the size of the culture vessel and with the conditions of aeration employed. An inoculum of 16 x 10^7 organisms/ml. yielded fewer organisms in 1.5 l. cultures than did larger inocula. The final yield from such cultures was 5 x 10^9 bacteria/ml., but in 6 l. cultures the same inoculum yielded 36 x 10^9 cells/ml. No satisfactory explanation has been offered for this result, but it is likely that differences may be due to the varying extent of aeration of their contents.

The requirement for starch in casein hydrolysate media has been the subject of several studies. Hornibrook (1939) emphasised that starch was necessary but stated that it could be replaced by α or β dextran. The starch could also be replaced by amylose or by charcoal (James, 1949). A decrease in the starch content of media was observed during the growth of virulent strains although no decrease was noticed during the growth of avirulent strains. James also claimed that suspensions of virulent Bord. pertussis caused breakdown of starch but this has never been confirmed by other workers. As the organism shows no fermentative ability for simple carbohydrates, it seems unlikely that it would

break down a polysaccharide, the structural unit of which, the glucose molecule, is not utilised. As the starch can be replaced by metabolically inert materials (e.g. charcoal), the role of starch is probably that of adsorbing toxic materials present in the medium, as was suggested by Pollock (1947).

Growth of virulent Bord. pertussis has been reported in casein hydrolysate medium containing the anion exchange resin, Amberlite IR4B, instead of starch. (Kuwajima, Matsui and Asano, 1955). The resin was included in the medium at a concentration of 0.1% (w/v) and the optimum resin concentration varied for different strains. Resin concentrations greater than 1% (w/v) were usually inhibitory. Most anion exchange resins tested were able to support growth in the medium. The exceptions were those resins which were unable to withstand the heating involved in sterilisation of the medium (Kuwajima, Matsui and Kishigani, 1957). The growth-supporting effect was lost when the ion exchange capacity was destroyed by treatment with hydrogen peroxide. In an experiment with various forms of the resin Dowex 1, growth-supporting ability was limited to the more highly cross-linked types. Cation exchange resins were without effect. It is unfortunate

that no attempt was made to elute from the resins and identify any toxic materials which may have been adsorbed. It is possible that the resins act by removing either toxic fatty acids or sulphides from the medium.

Completely synthetic media were studied by several workers (Jebb and Tomlinson, 1955, 1957; Fukumi Sayama, Tomujawa and Uchida, 1953a), but in these media growth was extremely slow. The media are expensive to prepare and although they are useful in the study of growth requirements, they would be of little value for the growth of large quantities of bacteria.

Growth requirements.

Virulent Bord. pertussis grew in a sythetic medium containing 16 amino acids, growth factors, salts and starch (Proom, 1955). Not all the amino acids used were essential for growth, but an organic "sulphur source" was necessary and cystine was used in this case. Cystine could be replaced by cysteine or glutathione, but not by methionine, taurine or several other sulphur-containing compounds tested (Jebb and Tomlinson, 1957). The main amino acid metabolites were glutamic acid, serine, alanine, proline and leucine and in simple mixtures of amino acids, the glutamic acid could be replaced by succinate or

α-Ketoglutarate (Proom, 1955). Moreover, the addition of glutamic acid to casein hydrolysate media was shown by Rowatt (1957a) to result in increased yields of bacteria. Nicotinic acid was the only known essential growth factor required (Hornibrook, 1940), although addition of guanine, hypoxanthine, xanthine, biotin and haemin, either singly or together, stimulated growth (Jebb and Tomlinson, 1957).

Very little information is available on the growth requirements of Bord. pertussis and our knowledge of its metabolic activities is negligible. Cultivation of the virulent organism in good yield, under completely reproducible con conditions, is still difficult to achieve. It is certainly fastidious in growth and is greatly affected by toxic factors in the medium. As a result, we tend to know more about factors which inhibit growth than those which are essential.

2) Growth of avirulent Bord. pertussis.

These strains grew on meat digest agar or on casein hydrolysate media without starch, but often slowly and irregularly (Rowatt, 1957b). Avirulent strains grew in some batches of casein hydrolysate media which were unsuitable for virulent Bord. pertussis. This was probably due to the greater resistance of avirulent strains to inhibition by

colloidal sulphides, the most likely inhibitors present in batches of the casein hydrolysate which might have been overheated.

3) Growth of Bord. parapertussis.

This species grew in media containing a mixture of seven amino acids and required nicotinic acid as a growth factor; glutamic acid was essential for growth (Proom, 1955). The growth rate was intermediate between the rapidly growing Bord. bronchiseptica and the slower growing Bord. pertussis, in casein hydrolysate media. Rowatt (1955) showed that Bord. parapertussis had no requirement for starch. During growth, a brown water-soluble pigment was formed. This only occurred in the later stages of growth unless tyrosine was present in the medium in large quantities (Ensminger, 1953). The brown pigment was also formed by washed suspensions of the bacteria when incubated with tyrosine and glutamic acid and greater quantities were produeed at pH 7.4 than at lower pH values (Rowatt, 1955). The formation of the pigment is a useful method of cultural differentiation between Bord. parapertussis and the other species. The chemical nature of the pigment is unknown and no attempted analysis has been recorded.

4) Growth of Bord. bronchiseptica

This species grew rapidly in casein hydrolysate media but also grew well in simpler media. Glutamic acid was the main metabolite, while nicotinic acid was an essential growth factor (Ulrich and Needham, 1953; Rowatt, 1955). Amino acids were not essential for growth of this organism. Citrate and lactate were satisfactory carbon and energy sources in media containing ammonium salts (Proom, 1955).

The three species have been shown to have some similarities in their growth requirements. They all require nicotinic acid and glutamic acid is essential for Bord. pertussis and Bord. parapertussis. No carbohydrates are utilised. Despite these findings, few attempts have been made to discover common metabolic activities, the knowledge of which might aid in comprehending the activities of growth inhibitors. Although Bord. pertussis was inhibited by colloidal sulphur and peroxide, the other species were not (Rowatt, 1957b). It is difficult to understand why only one species was inhibited by peroxide, as all three organisms produced catalase (Bradford and Slavin, 1937; Rowatt, 1957b). Casein hydrolysate media appear to be most suitable for growing large quantities of the organisms, as they are cheap and easy to prepare, while supporting good growth of virulent bacteria.

ANTIGENS OF THE BORDETELLA SPECIES

Serological Investigations.

Agglutination and agglutinin-adsorption tests have been widely used in studies of the Bordetella species. Among the first workers to find differences in Bord. pertussis strains were Krumweide, Mishulow and Oldenbusch (1923), who demonstrated two serologically distinct groups. Leslie and Gardner (1931) studied a number of strains of the same species after growth on Bordet-Gengou medium. The strains, which were isolated at different times and underwent varying numbers of subcultures, formed four groups or phases. The two extreme forms were phases I and IV, while phases II and III were intermediate in their characteristics. The most recently isolated strains formed phases I and II but older strains were in the other two phases. Using cultures from single phase I colonies, it was shown that on subculture the organism passed through all phases, the changes being partially reversible. However, phase II did not occur regularly as an intermediate between phases I and III. Much of the work has been repeated by other workers (e.g. Flosdorf, Dozois and Kimball, 1941) but the existence of phase II has never been confirmed. Using lyophilised cultures to ensure stability,

Flosdorf *et al.* (1941) observed phase changes in both directions, but found that they occurred most frequently from phase I through phase III to phase IV. Several workers suggested that freshly isolated, virulent or phase I strains of *Bord. pertussis* formed a single, serologically homogeneous group (Standfast, 1951b; Ungar, Muggleton and Stevens, 1954). In contrast to this, Andersen (1952, 1953) showed differences between virulent strains. She used the term "K antigen" to denote material destroyed by heating at 120° for one hour. The agglutination of bacteria which had been heated at 100° for one hour, was attributed to "O antigens". Her results suggested that a common O antigen existed in the three *Bordetella* species. Several K antigens were found, some being type specific, while others were species specific. Any one strain of any species contained one or more of these K antigens. The exact nature and location of the O and K antigens of the *Bordetella* species has not yet been determined. Andersen (1953) showed that O agglutinating material was completely covered with K material. This is similar to results obtained in the *Enterobacteriaceae*, where the O and K nomenclature was initially used. In the genus *Klebsiella*, the K antigens are capsular or slime materials but

there is no evidence that this is so in Bordetella species. In Bord. bronchiseptica, which is motile and flagellate, the presence of a capsular structure is unlikely and it is possible that the K antigens are surface structures of the type which Wilkinson (1958) termed microcapsules. Eldering, Hornbeck and Baker (1957) confirmed the presence of a common, heat-stable antigen which was also present in avirulent strains of Bord. pertussis and of Bord. bronchiseptica. Fourteen heat-labile antigens were described, some of which were species specific although others were common to two or three species. No correlation between type specificity and protection was shown in Bord. pertussis (Andersen and Bentzon, 1958). Homologous vaccines gave no superiority over heterologous vaccines in mouse protection tests.

Phase changes are mainly gradual, occurring over a number of subcultures, but Lacey (1953, 1960) described changes in the antigenic structure of Bordetella species occurring in one or two subcultures due to the influence of growth conditions. The antigenic structure was determined by the temperature of growth and by the composition of the medium. He postulated the existence of two extreme antigenic states or modes. Using medium with sodium chloride as the main salt component and incubation at 37°, the X or xanthic

mode was obtained. The main characteristics of this were ochre-coloured growth, high haemagglutination titre, agglutinability by heavy metal salts and the possession of a surface antigen common to the three species. Sodium, potassium, halide and other ions favoured growth in the X mode. When the medium contained magnesium sulphate as the principal salt, growth was pale greenish blue. There was little haemagglutination and no agglutination with antisera prepared against other Bordetella species. This was called the C or cyanic mode and was favoured by lower incubation temperature, magnesium and sulphate ions. The influence of the ions was a function of their chemical nature and not of their concentration. The concepts of Lacey are completely novel and await confirmation by other workers. They render the antigenic patterns of the Bordetella species even more complex. It is difficult to ascertain what relationship may exist between Lacey's modes and the phases or other antigenic states described by earlier workers.

On the basis of agglutination tests, a form of phase variation was postulated in Bord. parapertussis (Kasuga, Ukishima and Nakase, 1958). Three phases were thought to exist, possessing different thermolabile and thermostable antigens.

Using precipitation in agar gel, Maclennan (1961) detected a large number of antigens which were common to all species in the genus Bordetella. Species specific antigens were also present. Virulent Bord. bronchiseptica strains possessed a thermostable antigen which was absent from avirulent strains. The latter could be differentiated into three groups each of which had a distinct thermostable antigen.

All these results indicate that the Bordetella species undergo antigenic changes during laboratory culture as do other Gram negative bacterial species which have been examined. In this thesis, the term virulent is applied to strains of Bord. pertussis which have been isolated from cases of whooping cough and preserved in the freeze-dried state. Those strains which, through prolonged subculture, have been enabled to grow on meat digest agar are termed avirulent.

Physical and chemical extraction of Bordetella species.

1) Preparation of cell wall and associated materials.

Many attempts have been made to identify particular biological activities of the bacterial cell with one of its components. Cell walls, being relatively easily prepared, have been the subject of many studies. The bacteria were broken down by grinding in a ball mill, freezing and thawing, and by ultrasonic treatment (Flosdorf and Kimball, 1940a). Small glass beads (ballotini) were used to disintegrate the bacteria and release cell-wall material (Munoz, Ribi and Larson, 1959). The use of a press similar to that of Hughes (1951) also permitted disruption of thecells and isolation of the cell walls (Billaudelle et al., 1960). Of the various methods of disintegrating the bacteria, almost all suffer from the grave disadvantage that localised heating may occur with its attendant protein denaturation and destruction of biological activities. In attempting to avoid this, Cruickshank and Freeman (1939) digested the whole bacteria with trypsin to leave an insoluble residue of cell-wall material. The long incubation period at 37^{o}, which was required, necessitated the addition of suitable

antibacterial agents to inhibit the growth of contaminants.

When very drastic procedures were used, one component of the bacterial cell wall, the lipopolysaccharide, was obtained in a soluble form while the bacterial proteins were denatured and precipitated. Bord. pertussis cells have been extracted with dilute hydrochloric acid (Felton et al., 1945; Eldering, 1942) or with trichloracetic acid (Eldering, 1941). The extraction method of Westphal, Luderitz and Bister (1952), using warm aqueous phenol solutions, was also applied to the Bordetella species (Baeva, 1957; Maclennan, 1960). Lipopolysaccharide in combination with some protein was obtained by extracting the bacteria with pyridine: water mixtures or with a solution of urea, thiourea and formamide (Akiya, Takahashi, Kuriyama and Ogawa, 1951; Maitland and Guerault, 1958).

2) Preparation of soluble antigens.

Many antigens can be obtained from Bordetella cells by treating them with dilute aqueous solutions of salts. In some instances (e.g. urea treatment) antigens from within the cell may be released as a result of increased permeability of the cell wall, while other antigens may be dissolved from the bacterial surface.

Physiological saline (0.85% w/v NaCl) and 2.0 M NaCl solutions have been used, as well as 1.0 M sodium acetate and 0.05 M calcium chloride (Warburton and Fisher, 1951; Evans and Adams, 1952; Masry, 1952; Frappier and Guerault, 1954, 1955; Maitland and Guerault, 1958; Dolby, 1958). The soluble antigens have been purified by precipitation methods involving methanol and ethanol and by isoelectric precipitation (e.g. Pillemer, Burrel and Ross, 1947; Pennel and Thiele 1951; Robbins and Pillemer, 1950; Dolby 1958). Preferential adsorption of some of the antigens onto aluminium phosphate or red cell stroma has also been used (Pillemer, 1950; Warburton and Fisher, 1951). Other methods of purification have involved salting out, or decrease of the ionic concentration, methods which have been used widely in the isolation of proteins (Warburton and Fisher 1951; Masry, 1952), but it is only recently that nucleic acids and proteins from Bord. pertussis have been separated by electrophoresis (Hammersten, Palmstierna and Meyer, 1959).

Although these and similar methods have been used in preparing Bordetella antigens, few, if any, pure antigens have been isolated,. This is due to the complexity of the bacterial cell which contains many components with similar physico-chemical properties. The isolation of any single

antigen free from other materials is very difficult and in very few cases have the products been tested for physical or immunological homogeneity. Thus any biological activity associated with such products need not be due to a single antigen. This must be considered when regarding claims for the identity of antigens with different biological activities.

Biological Activities of Antigens.

1) Haemagglutination.

Keogh, North and Warburton (1947) observed that erythrocytes of man, mouse, fowl and other animals were agglutinated by saline suspensions of the three Bordetella species and by culture supernatants. Although freshly isolated strains were rich in haemagglutinin, the content fell on subculture; no haemagglutinating activity was detected in avirulent strains. Standfast (1951b) confirmed this for Bord. pertussis and showed that the rate of fall of haemagglutinin titre with subculture varied for different strains. About 25% of freshly isolated strains of Bord. pertussis lacked haemagglutinin (Masry, 1952), but this might have been due in some cases to cultural conditions giving rise to the cyanic mode of growth described by Lacey (1960). In this mode, the cells possessed little haemagglutinin. On solid media, the

haemagglutinin was associated with the bacterial cell, as was also the case in the early stages of growth in liquid medium. Later, the haemagglutinin diffused into the medium.

Studies of the bacterial surface, using the electron microscope, have not revealed any structure which might account for haemagglutination. Thus the Bordetella species differ from various general of the Enterobacteriaceae in which haemagglutinating activity was correlated with the presence of surface appendages known as fimbriae (Duguid, Smith, Dempster and Edmunds, 1955; Duguid and Gillies, 1957). Collee (1961) described the presence of cell-bound and cell-free haemagglutinin in Clostridium welchii. This material was a product of strains subcultured in the laboratory and was absent from freshly isolated strains. In contrast, the haemagglutinin of Bord. pertussis, although existing in similar forms, is related to the freshly isolated virulent strains.

Unlike viral haemagglutinins, that of Bord. pertussis did not elute from the erythrocytes (Fisher, 1948a; Fukumi, Shimizaki, Kobayashi and Uchida, 1956) and treatment of the red blood cells with the receptor-destroying enzyme obtained from Vibrio cholerae did not render them inagglutinable, tending rather to increase the haemagglutinin titre. The titre was reduced however, when

erythrocytes treated with potassium periodate were used, suggesting that haemagglutinin receptors on the cells were destroyed (Fisher, 1948b). Solely on the grounds of differing titres for human and fowl erythrocytes, Thiele (1950) postulated the existence of two distinct haemagglutinins. Her evidence was inconclusive and she did not appear to consider that the surfaces of the two erythrocyte species would differ considerably and would in all probability require different amounts of antigen to effect their agglutination. Nor was there any investigation of the possible presence of inhibitory substances at the red cell surface.

Haemagglutinin has been found in most cell-free extracts of Bordetella cells (e.g. Masry, 1952). The material was active over the same pH and temperature range required for stability of erythrocytes (Warburton and Fisher, 1951). Although 37° was normally used for haemagglutination tests, some workers claimed that conditions were optimal at 42 - 46° (Ungar, 1949). Several workers showed that in the soluble form, the haemagglutinin of Bord. pertussis lost its activity on heating to 56° for 30 min., although it retained much of its antigenicity (Keogh and North, 1948; Masry, 1952). Extracts also lost their activity during storage, even at -10° (Masry, 1952). The rate of loss of activity was reduced by the addition

of 50% (v/v) glycerol. The stability of the haemagglutinin could be increased by adsorption onto aluminium phosphate, the activity then being unaffected by formalin, vigorous shaking, or heating at 56°, all of which were shown to inactivate the soluble material (Keogh and North, 1948; Fisher, 1950; Warburton and Fisher, 1951). Heated haemagglutinin could be adsorbed onto erythrocytes, rendering them agglutinable by antisera to the unheated antigen. During Seitz filtration, the haemagglutinin was almost completely adsorbed, but there was little loss of activity following passage through membrane filters. Masry (1952) suggested methanol precipitation as a method of purification but Fukumi and his co-workers (1953b) claimed that this resulted in complete loss of activity. Adsorption onto aluminium phosphate, followed by elution under different ionic conditions, was also suggested as a method of purification (Warburton and Fisher, 1951).

The activity of Bord. pertussis haemagglutinin could be inhibited by immune sera or by treatment of the erythrocytes with saline extracts from Haemophilus influenzae (Keogh et al., 1947). An inhibitor, removable by acetone extraction, was present in some human sera (Ungar

and James, 1949; Fisher and Keogh, 1950). Fisher (1948a) showed that haemagglutination could be inhibited by red cell stroma, the component responsible being an acetone - soluble, heat-stable lipid which could be crystallised as yellow flakes. In a later paper, the same worker (Fisher, 1949) purified the inhibitor and suggested that it was a lipid containing cholesterol. An emulsion of cholesterol in saline did not exhibit complete inhibition unless saponifiable, acetone-soluble lipids from the stroma were also present. These lipids could be replaced by lecithin or oleic acid. It seems that the haemagglutinin of Bord. pertussis is readily inhibited by lipids; this may indicate the nature of its receptor on the red cell surface.

2) Coagulation of plasma.

The presence in some virulent strains of Bord. pertussis of a factor causing coagulation of oxalated or citrated rabbit and sheep plasma was reported by Billaudelle (1955a, 1955b). No coagulase was present in avirulent strains. Bord. parapertussis strains also possessed a coagulase, but Bord. bronchiseptica was not tested. The coagulase was slow-acting, requiring 4-12 hr. incubation at 37° before the plasma was completely solidified. No attempt was made to isolate the

factor responsible or to determine its relationship to other biological activities of the Bordetella group.

3) Histamine Sensitisation.

Mice are normally resistant to anaphylactic shock and to the effects of histamine injected parenterally. Cells and certain fractions of Bord. pertussis caused anaphylactic shock in mice and also rendered them very susceptible to injected histamine (Parfentjev, Goodline and Virion, 1947a and 1947b). The antigen responsible for these effects was termed the histamine sensitising factor (H.S.F.) Mice could be sensitised following a single intra- abdominal dose of a whole cell vaccine. Different strains of mice showed marked differences in their ability to become sensitive to anaphylaxis following injection of Bord. pertussis cells (Pittman, 1951a), some strains becoming 100 - 200 times more susceptible to histamine. Female mice became more susceptible to histamine than did male mice. Rabbits and guinea pigs could not be actively sensitised (Kind, 1958). Only virulent strains of Bord. pertussis possessed H.S.F., while it was absent from Bord. parapertussis and Bord. bronchiseptica.

H.S.F. was present in saline washings of the

cells, in whole bacteria and in fractions obtained by disintegration of the bacteria (Maitland and Guerault, 1958). In cultures grown on Bordet - Gengou medium, bacteria grown on cellophane contained more H.S.F. than those grown directly on the medium. This suggests that the H.S.F. is an extracellular product which normally diffuses into the medium from the bacteria. Some workers claimed that the H.S.F. was destroyed when bacteria were shaken with glass beads or ground in the dried state (Maitland, Kohn and Macdonald, 1955). In contrast to this, Munoz et al. (1959) found that fractions from bacteria disintegrated with glass beads were rich in H.S.F. Heating of bacterial vaccines to 60° for 60 min. did not significantly affect the H.S.F. but it was destroyed when the cells were heated at 80° for 30 min. (Pittman, 1951b; Maitland et al., 1955). Heated bacteria were still capable of killing mice which had been sensitised previously with unheated bacteria. The effect of the H.S.F. was neutralised with antihistaminic drugs including quercitin and also with antibacterial sera (Thiele and Schuchardt, 1952; Maitland and Guerault, 1958; Chedid and Boyer, 1958).

As well as increasing sensitivity to histamine, pertussis vaccines rendered mice more

susceptible to the effects of vaccines or lipopolysaccharide extracts prepared from Shigella dysenteriae, Escherichia coli and other Gram negative bacteria (Kind, 1958). They also became more susceptible to infection with saprophytic bacteria (Arch and Parfentjev, 1957). This led to the theory that sensitivity of mice following the injection of Bord. pertussis might be a non-specific hypersensitivity to a number of endotoxin-like materials.

4) Labile toxins.

Bacteria-free filtrates from liquid cultures of virulent Bord. pertussis or saline washings of bacteria grown on solid media, contained an exotoxin (Katsmpes, Brooks and Bradford, 1942). The toxin was lethal for mice when given intravenously and was dermonecrotic in guinea pigs and rabbits (Kind, 1956). Wood (1940) showed that the toxicity of the bacilli could be reduced by repeated washing with saline. The soluble toxin was adsorbed during filtration through asbestos or porcelain filters and was destroyed or inactivated by heating the superanatants at 54° for 10 min., by formalin and by storage at 0° for several weeks (Wood, 1940; Frappier and Guerault, 1954; Maitland and Guerault, 1958). The addition

of glucose (50% w/v) or sucrose had a stabilising effect on the toxin (Katsampes et al., 1942). The exotoxin was poorly antigenic but adsorption onto aluminium phosphate increased its antigenicity (Katsampes et al., 1942; Andersen 1953). Thermolabile exotoxins were also demonstrated in Bord. parapertussis and Bord. bronchiseptica. Neutralisation of the toxins from all three species of Bordetella could be obtained with homologous or heterologous antitoxin.

Toxin has also been obtained from Bord. pertussis cells by freezing and thawing (Evans and Maitland, 1937) or by ultrasonic disintegration of the bacteria (Smolens and Favell, 1947). It was extracted from disintegrated bacteria by treatment with calcium chloride solutions (Robbins and Pillemer, 1950). It was highly potent but electrophoretically heterogeneous. Toxin was separated from other components by electrophoresis of ultrasonic extracts, followed by chromatography on ion exchange cellulose (Billaudelle et al., 1960). Four different fractions with dermonecrotic activity were obtained but only one of these contained lethal toxin.

Toxins extracted from the bacteria resembled the exotoxin, being lethal and dermonecrotic for mice, guinea pigs, rabbits and rats (Evans and Maitland, 1937) Violle, 1948; Robbins and

Pillemer, 1950). Both toxins were also thermolabile and were detoxified by formalin. The poor antigenicity of the toxins precluded complete immunological proof of their identity. It was shown, however, that injection of either exotoxin or extracted toxin into rabbits was followed by a secondary antibody response when the other was injected (Brown, 1949). This indicates that the exotoxin and a toxic component of the extracts are immunologically very similar and possibly identical.

5) Endotoxins.

In contrast to the exotoxins which are thermolabile proteins, most Gram negative bacteria possess thermostable lipopolysaccharide endotoxins. They are also called somatic, O or surface antigens and are materials lying at or near the bacterial surface, where they play an important role in the agglutination of the bacteria by homologous antisera. The properties of somatic antigens were reviewed by Westphal (1960). The complete antigens were lipopolysaccharide-protein - lipid complexes which were strongly antigenic, pyrogenic anc toxic. As well as causing the formation of specific agglutinins when injected into animals, the somatic antigens caused increases in the non-specific defence mechanisms of the body (Rowley,

1956; Landy and Pillemer, 1956). The complete antigen from a few bacteria was split into its component parts and the properties of the individual moeities examined. Removal of the lipid attached to the protein (lipid B) did not alter the biological properties of the complex. After mild hydrolysis to remove the protein, a toxic, pyrogenic but poorly antigenic lipopolysaccharide remained. The pyrogenic and toxic activities were due to the lipid conjugated to the polysaccharide (lipid A). When an artificial protein - lipopolysaccharide complex was formed, the polysaccharide could be removed by hydrolysis, leaving a toxic and pyrogenic lipoprotein (Luderitz, Westphal, Eichenberger and Neter, 1958).

Acid - extracted lipopolysaccharides from Bord. pertussis and Bord. parapertussis were toxic for mice and gave limited protection against experimental infection in these animals (Eldering, 1941, 1942). Lipopolysaccharide from Bord. pertussis elicited resistance to challenge with Salmonella typhi in mice, but no details were given of the method of preparation of the material or of its chemical properties (Landy, 1956). It was also reported that the lipopolysaccharide caused an increase in the properdin level of the experimental animals. Phenol extraction of Bord.

Table 2. Composition of the lipopolysaccharides from Bordetella species. (Maclennan, 1960).

Preparation.	N	P	Nucleic acid.	Aldo-heptose.	Hexosamine.	Hexose.
bronchiseptica	7.7	2.1	0	18	16,6	5
pertussis	3.9	2.5	1.4	34	20.3	6
parapertussis	8.2	2.6	0.6	18	16.5	5

All values are expressed as percentages.

pertussis cells yielded lipopolysaccharides which comprised 3.4 - 5.2% of the dried bacteria (Baeva, 1957; Maclennan, 1960). Similar materials were obtained from the other Bordetella species, but very little was extracted from avirulent strains of Bord. pertussis and Bord. bronchiseptica (Maclennan, 1960). The materials were all white powders, were sparingly soluble in water, and gave negative biuret and positive Molisch tests. Chemical analysis gave the results shown in table 2. The lipopolysaccharides were very resistant to acid hydrolysis and drastic conditions were required to obtain the constituent sugars for analysis by paper chromatography. All three preparations appeared to contain a hexosamine and an aldoheptose. The hexosamine was identified as glucosamine while the heptose spots corresponded in colour and position with D - glycero - L-mannoheptose. Although heptoses have been reported in other bacterial polysaccharides, mainly on the basis of chromatographic evidence (e.g. Davies, 1955), in only two species have they been isolated and characterised. From a strain of Escherichia coli, Weidel (1955) isolated L - glycero - D - mannoheptose and D - glycero - D - galactoheptose was isolated from Chromobacterium violaceum and characterised by Maclennan and

Davies (1957).

The lipopolysaccharide from Bord. pertussis was dermonecrotic in rabbits and lethal for mice when given intravenously or intraperitoneally (Baeva, 1957). In contrast, Maclennan (1960) found that lipopolysaccharide prepared from this organism was non - toxic although similar material from the other two Bordetella species was toxic and dermonecrotic. All three materials were pyrogenic and were non - antigenic, although they were haptenic, forming precipitates with homologous antisera prepared against whole bacteria. Precipitation tests showed that the lipopolysaccharides from each of the species were serologically distinct.

6) Capsular Antigens.

Several attempts have been made to demonstrate the presence of capsules in Bord. pertussis cultures. A staining technique using Wright's stain was employed by Lawson (1940), who claimed that it revealed the presence of capsules. Using the same method, Evans and Adams (1952) claimed that capsules were present in a large number of strains isolated from clinical cases of whooping cough. However, the proportion of capsulate bacteria in each culture was not stated,

although less capsular material was observed in older strains. Ungar and Muggleton (1949), using Lawson's method, found capsules in freshly isolated strains and in laboratory strains which were no longer agglutinable with antisera prepared against virulent strains. On the other hand, other staining methods were unsuccessful in demonstrating capsular material. However, electron microscopy revealed structures suggesting the presence of capsules (Ungar, Muggleton and Stevens, 1954). These structures could be artefacts due to the method of preparation of the specimens. There is no report of the India ink method of Duguid (1951) having been applied. Such a method reveals the presence of capsules without introduction of artefacts due to the physical and chemical processes involved in other staining procedures. Although the evidence for presence of capsules is not conclusive, it seems possible that Bord. pertussis produces some material of the slime layer or microcapsular type. This might explain the K agglutination of Andersen (1953). Moreover, solution of microcapsular material in liquid media might be responsible for the increased viscosity in such media reported by Fisher (1948b), or the "mucin - like" substances of low nitrogen content precipitated during attempts to purify the exotoxin

(Burrel, Robbins and Pillemer, 1948). Antisera prepared against washings of cells grown on solid media gave a visible modification of the outer layer of unwashed bacilli, revealing a structure of indefinite shape, which was more characteristic of a slime layer than of a true capsule (de Repentigny and Frappier, 1956).

PROTECTIVE ANTIGENS

1) Methods of Assay.

Although no suitable method of in vitro assay of the potency of Bord. pertussis vaccines exists, two methods of assay involving experimental infection of mice have been used. The first method, developed by Burnett and Timmins (1937), employed intranasal challenge of anaesthetised animals. Significant immunity could be shown following the intraperitoneal administration of formalin-treated vaccines. The infection was produced on the bronchial epithelium and has the advantage that there is some similarity to human infection, The method was studied by several workers (e.g. Miller and Silverberg, 1939; Standfast, 1951a) and was shown to lack qualitative reliability. For this reason it was superseded by other techniques. An improvement on the intranasal method was the

intratracheal challenge advocated by Te Punga and Preston (1958). The method produced a lethal pulmonary infection and higher mortality was obtained than by intranasal challenge. Specific active and passive immunity against the challenge was demonstrated. The method had the disadvantage that the mice were exposed twice to anaesthetic, the initial exposure being required to reduce their natural resistance and the second being for the operative procedure. Even in the hands of a skilled operator, some mice (1-2%) were killed by the inoculation procedure.

The method most commohly used for assay of pertussis vaccines, is the active protection test in mice challenged with a lethal intracerebral dose of a mouse - virulent strain of Bord. pertussis. This method was introduced by Kendrick, Eldering, Dixon and Misner (1947), who obtained a suitable bacterial strain, such that 50% of the mice were killed by a dose of approximately 300 organisms. When a large number of strains were tested for virulence by the intracerebral route, all "phase I" strains were virulent, although they could be divided into groups differing in the number of bacteria required to produce a lethal infection (Kendrick, Updyke and Eldering, 1949). Only 2 out of 33 Bord. parapertussis strains were

virulent, as were all the 4 Bord. bronchiseptica strains tested. Rough strains of Bord. pertussis were seldom virulent by this route.

Standfast (1958) showed that when vaccines were assayed in mice, intranasal and intracerebral challenge resulted in different estimates of their potencies and that the results of field trials corresponded very well with those obtained by the mouse potency test using intracerebral challenge, but not with intranasal challenge. He also found that vaccines heated to 100° for 1 hr. lost all their protective potency against intracerebral challenge, but showed little loss when assayed intranasally. He suggested that two different antigens were involved. Passive protection tests, using antisera prepared against whole bacteria and various fractions, also led to the belief that two separate antigens were involved (Dolby and Standfast, 1958).

Active immunity in mice tested by intracerebral challenge has the advantage of reproducibility, especially when the same challenge strain and mouse strain are used. There is always some loss of mice due to post - operative deaths and, as in other methods, the worker must be experienced in the procedure if these deaths are to be reduced to a minimum. The correlation of

results of field trials and vaccine potency assessed by intracerebral challenge in mice (Medical Research Council, 1959) tends to make this the method of choice in laboratory evaluation of vaccine.

A further complication affecting vaccine assay resulted from the finding of Evans and Perkins (1954a, 1955a) who showed that killed suspensions of Bord. pertussis gave two distinct immune responses following intraperitoneal injection in mice. The first was an interference type, developing rapidly and of brief duration, while the second was a slow antibody response reaching a high level of immunity and persisting for a long time. However, the effect of the interference immunity can be minimised by challenging the mice sufficiently long after vaccination. The period of fourteen days, usually allowed between vaccination and challenge, is the minimum permissible if interference immunity is not to influence the results significantly.

In a further method of assay, developed by Evans and Perking (1953, 1954b), the ability of vaccines to produce specific agglutinating antibody in mice was tested. This method was applied to vaccines used in field trials and some correlation was obtained between the protection

obtained in children and the agglutinin response in mice, when whole bacterial vaccines were tested (Medical Research Council, 1959).

2) Vaccines.

a) Whole cell vaccines.

Bacteria grown on solid or liquid medium have been used in Bord. pertussis whole cell vaccines for the immunisation of children against whooping cough. The results of field trials showed that vaccines grown in liquid medium did not differ significantly from those prepared on solid medium (Medical Research Council, 1959). Whole cell vaccines are not entirely satisfactory because of the occurrence of local and general reactions following their administration. These vaccines were found to deteriorate in potency during storage and, on the basis of the mouse protection test, it was estimated that they had a half - life of 5 - 6 years when stored at 4° (Ungar and Basil, 1957). Because of the instability of fluid vaccines, Armitage and Perry (1957) recommended the use of a freeze - dried standard containing 6% dextran to minimise the loss of antigenicity during drying. A freeze - dried vaccine showed no deterioration after ten years storage (Kendrick, Eldering, Hornbeck and Baker, 1955).

Several preservatives have been used in pertussis vaccines including phenol, formalin and cresol (North, Anderson and Graydon, 1941; Pittman, 1952). These chemicals all caused a fall in vaccine potency, as did heating or prolonged ultraviolet irradiation. Merthiolate was considered to be the most effective preservative, as it causes little loss in protective capacity during storage for over a year (Pittman, 1952; Billaudelle, 1960).

Using cross - protection in mice, Kendrick, Nadolski, Eldering and Baker (1953) showed that the best protection was obtained against challenge by the homologous Bordetella species, although Bord. pertussis vaccines gave some protection against Bord. bronchiseptica infection and vice versa. The protective potency of Bord. pertussis strains declined on subculture and vaccines prepared from avirulent strains afforded little protection (North et al., 1941; Standfast, 1951b). The variability of vaccines has been reduced now that freeze - dried standards are available and strains can be lyophilised until required for culture. Reactions of varying severity following the administration of whole cell vaccines to young children have led to attempts to isolate a "protective antigen".

b) Protective Fractions.

An early attempt to prepare cell - free protective material involved tryptic digestion of Bord. pertussis cells (Cruickshank and Freeman, 1937). The soluble material was precipitated with ethanol and was found to confer immunity on mice challenged by the intranasal route. The undigested residue was also protective. Dolby and Standfast (1958) were unable to confirm these results, although some protection was obtained against intracerebral infection in mice. Eldering (1942) claimed that acid - extracted polysaccharides from the three Bordetella species gave some protection against intraperitoneal challenge of mice with Bord. pertussis, but as the method of infection differed from that used by other workers, it is not possible to assess the significance of these results. It seems unlikely that the polysaccharides would be sufficiently potent antigens to induce a good antibody response and the result could be due to the interference immunity described by Evans and Perkins (1954a). Phenol extracted lipopolysaccaride from Bord. pertussis was not protective (Baeva, 1957).

The ease with which protective material could be washed off the bacteria led to the belief that it was at or near the bacterial surface (Frappier

and Guerault, 1954, 1955; Maitland and Guerault, 1958). Although bacterial washings contained protective material, the washed cells still retained their protective ability. Several workers isolated cell wall material and found that it protected against experimental infection (Yoshida et al., 1955; Munoz et al., 1955; Billaudelle et al., 1960). Some of the preparations were not examined for the presence of intact cells or cytoplasmic matter and it seems probable that much more than cell wall material was present. Electron microscopic examination of the preparations of Munoz et al., (1959) clearly showed the presence of cell membranes and cytoplasm but these were absent from the cell walls prepared by Billaudelle et al. (1960). The latter workers removed extraneous material by exhaustive washings in a series of buffer solutions, sodium chloride solutions and distilled water. The product, which corresponded to about 21% of the dry weight of the bacteria, was a good immunising agent, doses of 50 - 200 µg. per mouse being protective. The immunising potency was destroyed by pancreatic digestion or urea, was reduced by ethanol, methanol or glycerol, but was unaffected by freeze - drying. The material was only toxic for mice in doses above 4 mg. and it had little

pyrogenic activity. Chemical analysis revealed 10 - 15% polysaccharide, 20% lipid, protein and a trace of nucleic acid. From these results, it appears that the antigen or antigens responsible for protection against intracerebral challenge in mice is either part of the cell wall of Bord. pertussis or very closely associated with it. It is possible that fractions of the cell wall have no protective activity and that these could be removed without any loss of potency. It is unlikely, however, that protection is due to any single molecular species but rather that it requires the presence of a number of macromolecules which are situated at or near the bacterial surface.

Protective material has been selectively adsorbed from ultrasonic extracts of bacteria onto red cell stromata (Pillemer, 1950). The product was termed stroma protective antigen (SPA.). The material contained haemagglutinin, but no toxin was adsorbed. Although the protective power of extracts and of SPA was unaffected by heating at 56° for 1 hr., protective material could no longer be adsorbed onto the stromata from heated extracts. Further work (Pillemer, Blum and Lepow, 1954) using a strain of Bord. pertussis which produced little or no haemagglutinin, showed that the SPA could be produced on a large scale and was stable over long periods when stored at 1°, although it

was inactivated by heating at 100°. Field trials using SPA revealed that it induced a high degree of immunity in children but caused a high proportion of local reactions of varying severity in the recipients (Medical Research Council, 1959). The chemical nature of the material adsorbed onto the stromata has not been determined as it comprises a very small proportion of the total present. Thus it has not been ascertained whether it is the same protective antigen as that found in cell washings and cell walls.

3) Relation to other Antigens.

The possible relationship of the protective material to other biological activities has been considered by many workers. It was shown that virulent Bord. pertussis possessed several characteristic properties which were absent from the avirulent organisms (Ungar and Muggleton, 1949; Standfast, 1951b). Thus virulent bacilli produce an exotoxin, a haemagglutinin and an HSF., are soluble in 10% (w/v) bile salts and are precipitated by aluminium or cadmium phosphate suspensions, as well as being protective in mice. These properties are lost on subculture. The changes in some of these characteristics during subculture have been studied by Standfast (1951b) and figure 1. shows his schematic representation

of their loss in a typical strain of Bord. pertussis. Each of these characteristics varied independently and the order of their loss varied with the strain.

The protective material has for some time been thought to be associated with the antigens involved in bacterial agglutination by immune sera, as fractions having protective activity also stimulated production of antisera capable of agglutinating the bacteria (e.g. Frappier and Guerault, 1954). This was thought to have been disproved when SPA was found to produce few agglutinins (Evans and Perkins, 1955b) but recently Andersen (personal communication) has shown that the strain used for the preparation of the SPA was antigenically different from most other virulent strains and agglutinin production by the SPA could only be detected using the homologous strain of Bord. pertussis as antigen. In recent field trials there was also good agreement between agglutinin response and protection in children, the only exception being the SPA, where it is possible that a heterologous strain was used in tests for agglutinins (Medical Research Council, 1959). It seems that there is fairly good correlation between the protective capacity of the material and its ability to stimulate agglutinin production. This is not unexpected, as it has been suggested that

the site of the protective material is at or near the cell surface.

The haemagglutinin content of strains and their antigenic potency in vaccines also showed some correlation (Keogh et al., 1947; Keogh and North, 1948). Extracts with high haemagglutination titres were found to be good immunising agents in mice and were also used in babies; no details of their protective value in children were given, although there were no ractions following injection as found with whole cell vaccines or other fractions (Warburton and Fisher, 1951; Fisher, Warburton, Wettenhall and Williams, 1951). Other antigens besides haemagglutinin were almost certainly present in these extracts. This might account for the contrasting results of Masry (1952), who prepared haemagglutinating extracts free from toxin and material stimulating antibody production. The toxin - free haemagglutinin did not induce active immunity in mice. The absence of haemagglutinin from a bacterial strain giving good protection (Pillemer et al., 1954) further confirms the absence of any protective capacity in the antigen causing haemagglutination. If the haemagglutinin had a role in protection, it would also be expected that the other Bordetella species, all excellent haemagglutinin producers, would

provide protection against Bord. pertussis. This, in fact, is not the case.

The exotoxin produced by Bord. pertussis has also been shown to have little effect in immunity. As it is a poor antigen, this is to be expected and, like the haemagglutinin, it is common to all Bordetella species. The presence of exotoxin in vaccines presents a problem, as it must be inactivated without affecting the immunising potency (e.g. Billaudelle, 1960). Preparation of cell wall material free from toxin but possessing excellent immunising ability also indicates that these two antigens are not related.

The presence of an antigen unique to virulent Bord. pertussis might initially suggest that it was related to the protective material. This applies to the HSF and some workers showed that protective fractions also contained HSF. (Munoz et al., 1959). Moreover histamine - sensitising and protective properties of whole cell vaccines showed close correlation. However Dolby (1958) obtained a bacterial fraction which gave good protection in mice challenged intracerabrally, but contained little HSF.

Insufficient is yet known about some antigens such as the coagulase discovered by Billaudelle (1955a), to determine whether they are related to

Table 3. The antigens of the Bordetella species.

Antigen	Bordetella species:		
	pertussis	parapertussis	bronchiseptica
Protective antigen.	+	+	+
Haemagglutinin.	+	+	+
Labile toxin.	+	+	+
HSF.	+	-	-
Coagulase.	+	+	
Pertussis lipo-polysaccharide.	+	-	-

the protective material. At present, it seems that the only correlation is between protective ability and agglutinin production. The distribution of the various antigens among the Bordetella species is shown in table 3.

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MATERIALS AND METHODS.

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1. Organisms

The following bacteria were used :-

i) Virulent Bord.pertussis.

Strains H5,31,32,35,36,51 and 52 were obtained from Dr. B.W. Lacey, Westminster Hospital, London.

Strains L85,L91,G1/252,G1/291,G1/353 and W 2000 were vaccine strains obtained from Dr. A.F. B. Standfast, Lister Institute of Preventive Medicine, Elstree, Hertfordshire.

Strain 134 used by Pillemer (1950), was obtained from Dr. Standfast.

Strain 18/323, an intracerebral challenge strain used by Kendrick et al.(1947), was also obtained from Dr. Standfast.

Strain 4412 was obtained from Dr. H. Billaudelle, Karolinska Institute, Stockholm.

Strain 4507 was isolated from a case of whooping cough in the City Hospital, Edinburgh.

Strains NCTC/8085,8090,8189,8474 and 8631 were obtained from the National Collection of Type Cultures, Colindale, London.

ii) Avirulent Bord.pertussis.

Strains L51,L54,G1 146,G1 154 and NCTC 364 were obtained from Dr. Jean Dolby, Lister Institute, Elstree. The NCTC 364 strain was the original "phase IV" strain of Leslie and Gardner (1931).

Strains CN 1529 and 2216 were obtained from Dr. H. Proom, Wellcome Research Laboratories, Beckenham, Kent.

Strain 4507Av was obtained from the virulent strain 4507 by repeated subculture.

iii) Bord. parapertussis.

The two strains used, NCTC 7385 and 8250, were both obtained from the National Collection of Type Cultures.

iv) Bord. bronchiseptica.

Strains NCTC 454,8344,8761 and 8762 were obtained from the National Collection of Type Cultures.

Strain H 106 was obtained from Dr. B.W. Lacey.

v) Haemophilus species. All strains were obtained from the National Collection of Type Cultures. The strains were :-

Haemophilus influenzae type a 8466, type b 7279, type c 8469, type d 8470, type e 8455 and type f 7918.

Haemophilus aegyptius 8135, Haemophilus canis 8540, Haemophilus haemolyticus 8479, Haemophilus parainfluenzae 7857, and Haemophilus suis 4557 and 7440.

All strains were freeze-dried immediately on receipt. Strains obtained in the freeze-dried state were cultured and sufficient ampoules

Table 3a. Composition of charcoal agar

Casamino acids (Difco technical).....	10.0 g.
Nicotinamide.....	0.01 g.
KH_2PO_4.....	0.5 g.
$MgCl_2, 6H_2O$.....	0.4 g.
$FeSO_4, 7H_2O$.....	0.01 g.
$CaCl_2$.....	0.1 g.
$CuSO_4$.....	0.001 g.
Soluble starch (BDH).....	1.5 g.
Charcoal (BDH activated).....	2.0 g.
Agar* (Davis New Zealand).....	10.0 g.
Distilled water.....	1.0 litre.

* The agar in this and all other solid media was defatted by extraction with chloroform.

prepared to last throughout the work. When strains were resuscitated from the freeze-dried state, the contents of an ampoule were emulsified in 1% (w/v) casamino acid solution and spread over two Petri dishes of Bordet-Gengou medium. Haemophilus species were grown on blood agar.

2. Media and Cultural Methods

a) Solid media.

i) Bordet-Gengou medium was prepared as described by Cruickshank (1960). The medium contained potato extract, glycerol, agar and 50% (v/v) horse blood. Plates were not dried but were stored in the refrigerator. They were used within 2-3 days of preparation. When the medium was used for recovery of organisms from experimental animals, it was rendered selective by the addition of penicillin (0.5 units/ml.)

ii) Charcoal agar had the composition shown in table 3a.

iii) Resin agar was a similar medium containing yeast extract and anion exchange resin instead of the nicotinamide and charcoal respectively

(see p 83.)

b) Liquid media

i) For Virulent Bord.pertussis.

a) In growth experiments, the basic medium and the modification of it described in the text were

used (see p 72.)

b) Growth of large quantities of bacterial cells was carried out in the tris - resin medium described in p. 80 of this thesis. Unless specified to the contrary, it contained $CuSO_4$ (0.001% w/v) and yeast extract (5% v/v) in addition to the other components.

ii) Other Bordetella species.

All other Bordetella species were grown in the basic medium described on p 72. of this thesis. In addition to the components listed, it contained $CuSO_4$ (0.001% w/v) and yeast extract (5% v/v). No starch or resin was added.

Yeast extract was prepared by the method of Cohen and Wheeler (1946).

Ion exchange resins were treated before use with 1N NaOH in the case of anion exchange resins or with 1N HCl in the case of the cation exchange resin.

Sterilisation. All media were sterilised by autoclaving at 15lbs./ in^2 for 15 min.

c) Growth conditions.

In the growth experiments, virulent Bord. pertussis was grown in 200 ml. amounts of medium in Roux bottles. When the bacteria were grown for antigenic studies, Roux bottle cultures were also used, the bottles being incubated on their sides for 5-6 days and turned daily. Avirulent

Bord.pertussis and other Bordetella species were grown in 250 ml. amounts of medium in 500 ml. Ehrlenmeyer flasks. The flasks were incubated for 48 hr. with constant agitation on a reciprocating shaker rotating at approximately 100 r.p.m. (Distillers Company Ltd., Epsom, Surrey). All incubations of Bordetella species were carried out at 35°. Haemophilus species were grown on blood agar at 37°.

d) Harvesting of bacteria.

i) Solid medium

Cells were washed off the medium with saline from a polythene wash-bottle and recovered by centrifugation at approximately 5,000 G for 30 min. using a bowl temperature of 0°.

ii) Liquid medium.

Resin was removed from the resin medium by passing the culture through a coarse sintered glass filter. The filtrate or the medium in other cases was then centrifuged under the same conditions as were used for recovering the cells from solid media.

Saline is used to denote 0.85% (w/v) sodium chloride solution, buffered to pH 7.0 with 0.002M phosphate (KH_2PO_4 - Na_2HPO_4).

Thioglycollate saline. Sodium thioglycollate (0.02% w/v) was added to saline. The solution was

prepared immediately prior to use. The sodium thioglycollate was stored in a tightly-stoppered container at -40°.

Ringers' solution contained (g./100 ml. distilled water) : NaCl 0.9; KCl 0.046; $CaCl_2$ 0.037; Na_2HPO_4 0.29; HCl 0.4 ml. 1.0 N solution.

c) Measurements of growth.

i) Turbidities were measured by removing 2 ml. aliquots from the Roux bottles, unless stated otherwise.

ii) Total Counts were made on suitable suspensions of bacteria using a phase contrast microscope. A calibration curve relating total count to turbidity was prepared and used for routine estimation of total counts.

iii) Viable Counts were carried out on Bordet-Gengou medium by the method of Miles and Misra (1938).

Values of pH were measured on a Pye electric pH meter.

3. Immunological Methods

Experimental animals.

Rabbits. Cross-bred animals, bred in the Bacteriology Department, University of Edinburgh, were used.

Mice. Strain A2G white mice were bred in the Bacteriology Department from stock animals obtained

from the Laboratory Animals Centre, Carshalton, Surrey.

a) Preparation of antisera.

Rabbits were inoculated subcutaneously with twelve, weekly injections of 1 ml. of the antigen. The first two injections always consisted of formalised material. Thereafter, merthiolate (0.01%) was the only preservative added. Antisera for gel diffusion studies were prepared, using two injections of formalin - killed bacteria containing 10^{10} cells/ml. Ten subsequent injections contained the same amount of ultrasonically treated material (see p. 63) with merthiolate (0.01% w/v) as preservative. Antisera for agglutination and protection tests were prepared, using a similar course of six injections, the first two comprising bacteria killed with formalin.

b) Active protection tests.

Suitable groups of 4-5 weeks' old mice were injected intraperitoneally with 0.2 ml. of the protective agent. They were challenged 14 days later with an estimated 50,000 organisms of the mouse - virulent strain 18/323 (Kendrick et al., 1947). The challenge dose, taken from a 24 hr. culture on Bordet - Gengou medium, was emulsified in 1% (w/v) casamino acid solution. It was injected intracerebrally to the anaesthetised mice. Deaths during the 48 hr. following challenge were

ignored as being due to cerebral damage. Thereafter, the mice were observed until 14 days after challenge. All deaths were noted and the mouse brains were cultured on Bordet - Gengou medium. When Bord.pertussis was not isolated from the brain of a dead mouse, that animal was excluded from the experiment.

c) Passive protection tests.

Groups of 6-7 weeks' old mice were injected intraperitoneally with antiserum (0.2 ml.), diluted with saline when necessary. The mice were challenged four hours later by the intracerebral route, using the same dose and other conditions as in active protection tests.

d) Toxicity tests.

These were performed using six weeks' old mice. The animals were injected intraperitoneally with 0.2 ml. of a suspension of the material under examination. All deaths occurring until 48 hr. after the inoculation were regarded as being due to the toxic agent.

e) Histamine sensitisation tests.

These were performed in a manner similar to that employed by Dolby (1958). The mice were injected intraperitoneally with the test material and challenged four days later with 10 mg. of histamine diphosphate in 0.5 ml. sterile distilled water. All deaths within 24 hr. after challenge were recorded. The histamine was injected intraperitoneally

f) Agglutination test. The method was similar to that of Robbins and Pillemer (1950). Bacterial suspensions were standardised turbidimetrically to contain 10^{10} cells/ml. Aliquots (0.3 ml.) of suspension were added to dilutions of antiserum in saline in 75 mm x 5 mm tubes. The tubes were thoroughly shaken, incubated at 37°C for 2 hr. and left overnight at room temperature. Normal serum and saline controls were always included. Results were read at 18 hr. The titre was taken as the reciprocal of the highest dilution giving agglutination visible with the hand lens.

g) Haemagglutination tests. The method used was similar to that described by Masry (1955). Human group O cells were washed three times in saline and suspended to a concentration of 1% (v/v) in saline. Twofold dilutions in saline of the haemagglutinin preparation were made in 75mm x 10mm tubes using 0.5 ml. volumes. The erythrocyte suspension (0.5 ml.) was added to each tube. Saline controls were always included. Medium controls were included when necessary. The tubes were thoroughly shaken then incubated in a water bath at 37° for 90 min. The titre was taken as the reciprocal of the highest tube giving complete agglutination. Cell-bound haemagglutinin was measured, using serial dilutions of suspensions, standardised turbidimetrically to contain 10^{10} organisms/ml.

h) Haemagglutinin inhibition tests. Saline dilutions of the inhibitory agents (0.5 ml.) were mixed with 0.4 ml. of a haemagglutinin preparation containing 5 minimal haemagglutinating doses (the minimum amount of haemagglutinin giving complete agglutination of the erythrocytes). After 5 min., 0.1 ml. of a 5% (v/v) erythrocyte suspension was added. The tubes were shaken and incubated as in the haemagglutination tests. The titre of the inhibitor was taken as the reciprocal of the inhibitor dilution in the last tube showing complete inhibition, multiplied by five.

j) Precipitation reactions in agar gel.

i) Double diffusion in agar gel was carried out by the method of Crumpton and Davies (1956). This was a modification of the original method of Ouchterlonie (1953). The medium used was saline solidified with 1% (w/v) Difco "Bacto" agar. Merthiolate (0.001%) was added to inhibit the growth of microorganisms. The agar was dissolved by steaming at 100°. The hot solution was then filtered through a pad of Whatman cellulose powder (standard grade) prepared on a Buchner funnel. The pad was washed prior to use with a large volume of distilled water. The filtered medium was then distributed into screw-capped glass bottles. When required, the medium was melted in the steamer and sufficient agar poured into clean, sterile Petri

dishes to form a layer 3 mm deep. Petri dishes of medium were left for 24 hr. at 0°. to ensure that the medium was sufficiently solid to permit clean removal of the material from the reservoirs. These were cut with sterile cork borers. The agar was removed from the cork borers by the application of gentle suction. Suitable reservoir patterns were accurately drawn on cards and placed beneath the Petri dishes.

The antigens and antisera were added, using sterile standard Pasteur pipettes. The plates were placed in a moist chamber at room temperature for the immune reaction to develop. The lines of precipitation were observed and recorded after 7 and 14 days.

ii) Immuno-electrophoresis. The micro-technique described by Scheidegger (1955) was used. Agar (1% w/v) was dissolved in phosphate-citrate buffer at pH 7.1 (McIlvaine, 1921) by steaming at 100° for 60 min. Glass microscope slides were thoroughly washed, allowed to dry and the medium (2.5 ml.) was poured uniformly onto each slide and allowed to solidify. Antigen reservoirs were cut as in gel diffusion tests. The slides were placed in a Shandon Universal electrophoresis apparatus. Electrical contact between the slide and the buffer vessels was made by filter paper strips (Whatman No 1), coated with the buffered gel. After

application of the antigens, a current of 5 mA at 50 volts was applied for 4 hr. The antiserum reservoirs were cut, using a sharp scalpel and the agar was removed by suction. Antiserum was added and the slide placed in a Petri dish containing wet filter paper discs. The precipitation reaction was allowed to develop for 48 hr. at room temperature. For a few experiments, a phosphate-citrate buffer (0.04M:0.02M) at pH 5.7 was used.

All lines of precipitation in gel diffusion and immuno-electrophoresis were recorded by sketching. Where the results were of sufficient importance, they were recoreded photographically using the apparatus designed by Rudge (1960). To ensure the absence of surface particles of dust, the gel was washed thoroughly with distilled water. Immuno-electrophoresis slides were photographed directly. The gel diffusion agar was carefully transferred under water from the Petri dishes to optically clear glass plates. When very faint lines were present, an attempt was made to enhance them by the addition of 0.05% (w/v) cadmium sulphate solution prior to photography. This method has been shown by Crowle (1960) to intensify precipitation reactions in agar gel.

4. Physical and Chemical Methods.

a) Ultrasonic disintegration of cells.

Bacterial cells from resin agar cultures were

suspended directly into distilled water to a turbidity of 10^{10} cells/ml. The suspensions were put in 75 mm x 10 mm test tubes surrounded by a beaker of ice-water. The suspension was then treated for 10 min. with an MSE-Mullard ultrasonic disintegrator, using a ¼" diameter probe. After disintegration, the material was centrifuged at 2,500 G for 10 min. in a refrigerated centrifuge. The deposits were discarded. Supernatants used for gel diffusion were preserved with merthiolate (0.01%) or were stored at -40°. until required. Antigens for immuno-electrophoresis were similarly prepared but no preservative was added. They were dialysed against the appropriate buffer before use.

When the ultrasonic disintegrator was used to suspend materials (e.g. lipopolysaccharides) in water, 1 - 2 min. treatment was found to be sufficient.

b) Preparation of cell walls.

Cell walls of Bord.pertussis were prepared by a method developed by Salton (1952). The bacteria were suspended in cold distilled water and shaken for 15 min. with No. 12 grade ballotini glass beads in a Mickle disintegrator. The glass beads were removed by passing the suspension through a a coarse, sintered glass filter. Unbroken cells

were removed by centrifuging at 2500 G for 10 min. at 0°. The residue was discarded. The supernate was removed and centrifuged at 10,000 G for 15 min. at 0°. The deposited cell walls were washed three times with 2 M sodium chloride solution and three times with distilled water. The final product was freeze-dried.

Enzymes used for treating cell walls and other purposes.

Lipase prepared from wheat germ, was obtained from L.Light and Co., Colnbrook, Buckinghamshire.

Lysozyme was prepared from egg-white by the method of Carter (1949).

Papain and trypsin were obtained from L. Light and Co.

Receptor destroying enzyme (RDE) was a cholera filtrate preparation obtained from N-V Phillips-Roxane, Amsterdam, Holland.

c) Phenol extraction of lipopolysaccharides.

Westphal et al. (1952) found that lipopolysaccharide in a fairly pure form could be extracted from microorganisms with phenol. The method of extraction used was similar to that described by Davies (1958). Freeze-dried bacterial cells were suspended at a concentration of 10% (w/v) in distilled water. The suspension was warmed to 65°. An equal volume of phenol

(90% w/v) at the same temperature, was added. The single phase system was stirred at 65° for 30 min. Suspensions were cooled to 2° and centrifuged at this temperature to separate the aqueous and phenolic phases. The aqueous phase was carefully removed and poured into ten volumes of acetone at -10°. The precipitated material was redissolved in water and precipitated with two volumes of cold ethanol. Sodium acetate (0.1% w/v) was added to accelerate precipitation. The final precipitate was again dissolved in water, dialysed and freeze-dried.

d) Concentration of solutions of high molecular weight compounds.

In many instances it was required to concentrate solutions of high molecular weight compounds without destroying their biological properties. Two methods were used, both employing the principle of ultrafiltration.

i) The first method was a modification of that employed by Kohn (1959). The solution to be concentrated was placed in a dialysis sac which was tied tightly. The outside of the sac was then moistened slightly and the sac was placed in a beaker. Polyethylene glycol (average molecular weight 6000) was then added and the concentration allowed to proceed until sufficient reduction in volume had occurred.

ii) The second method was similar to that of Palmstierna (1960). The dialysis sac containing the solution was placed in a bed of sodium carboxymethyl cellulose and covered with a further layer of this material. (The cellulose derivative used was "Courulose" obtained from British Celanese, Ltd., Coventry, England.) All concentrations were carried out at 2-3°. Tests of both methods showed little increase in the concentration of salts in the solutions. There was no detectable leakage of the polyethylene glycol or cellulose derivative into the dialysis sacs.

e) Chromatography on ion exchange cellulose materials.

Two ion exchange cellulose materials were used in most of this work. Carboxymethyl cellulose (CM-cellulose) was prepared by the method of Sober and Peterson (1956), or was obtained commercially. The other derivative was diethylaminoethyl cellulose (DEAE-cellulose) which was obtained commercially. The cellulose material was suspended in a suitable buffer as a thick slurry, This was de-aerated by applying a suction pump to the slurry in a stoppered Buchner flask. De-aeration was complete after 100 - 120 min. at a pressure of 400 mm. of mercury. The material was carefully packed into a column of internal diameter 10 mm.,

which was surrounded by a jacket containing circulating cold water. The column was perfused with buffer for 24 hr. prior to use. Buffer gradients were obtained by pumping the final buffer through a micro-pump (LKB. Produktur A.B., Stockholm, Sweden) into a mixing vessel containing the original buffer and stirred constantly with a magnetic stirrer. Fractions were collected at timed intervals in test tubes in an automatic fraction collector.

5. Chemical estimations.

i) Nitrogen content of samples was determined by the micro-Kjeldahl method. Digestion, in triplicate, was carried out for 3 hr. on a Gallenkamp micro-digestion apparatus. After digestion, the solution was quantitatively transferred to a round-bottomed Quickfit flask and steam-distilled using a form of Markham still, The ammonia was trapped in 2% (w/v) boric acid and determined colourimetrically using Nessler's reagent. Absorptions were read at 425 mμ. in a spectrophotometer.

ii) Total phosphorus was determined by the method of Fiske and Subba Row (1925). The colour produced was read at 680 mμ. in a spectrophotometer.

iii) Nucleic acid was estimated relative to a standard of purified ribonucleic acid (British Drug Houses, Ltd., Poole) by measuring the

absorption of aqueous solutions at 260 mμ.

iv) Hexoses were determined by the method of Dische, Shettles and Osnos (1949).

v) Hexosamines were estimated by a modification of the method of Rondle and Morgan (1955). Duplicate samples were hydrolysed with 2N HCl for 12 hr. at 100°. in sealed glass ampoules (Svennerholm, 1957). The hydrolysates were neutralised with 2N NaOH using phenolphthalein as indicator, then made just acid with 0.2N HCl. Acetylacetone reagent (1 ml.), prepared by dissolving 1 ml. redistilled acetylacetone in 50 ml. 0.5N sodium carbonate solution, was added to the unknown in a pyrex boiling tube. The tube, closed by a loose glass bulb, was heated at 100°. for 20 min., then cooled to room temperature. Redistilled ethanol (5 ml.) and 1 ml. of Ehrlich's reagent (0.8g. of p-dimethylaminobenzaldehyde in 60 ml. ethanolic HCl, prepared by adding 30 ml. of ethanol to an equal volume of concentrated HCl) were added. The volume was made up to 10 ml. with redistilled ethanol. The intensity of the colour produced was measured at 530 mμ. Blanks and glucosamine standards were included in each set of determinations.

vi) Heptoses were estimated by the colourimetric method of Dische (1953) as modified by Maclennan (1960). The heptose content was calculated

relative to the values for D-glycero-L-mannoheptose given by Davies (1957). These values were found to agree with those for an authentic sample of the sugar used in one experiment. Tests were made with the method to determine whether glucose or glucosamine interfered. Neither of these sugars had any effect.

vii) Anthrone value. The procedure used was based on that described by Fairbairn (1953), using 0.1% (w/v) anthrone in 72% sulphuric acid, The polysaccharide solution, containing 200-400 μg. material, in a 150 mm. x 25 mm. boiling tube, was cooled in running water. Anthrone reagent (10 ml.) was slowly added from a burette. The tubes were then heated at 100°. for 8 min. and cooled in water. The colour was measured at 625 mμ. Blanks and glucose standards (containing 100 μg.) were included in each set of determinations. All determinations were made in duplicate.

viii) Paper chromatography of sugars.

The material under examination was hydrolysed with 96% (w/v) formic acid for 24 hr. at 100°. in a sealed glass ampoule. The acid was removed by distillation in vacuo. The residue was then hydrolysed with N HCl for 12 hr. in a sealed glass ampoule at 100°. The hydrochloric acid was removed by distillation under reduced pressure.

The residue was dissolved in a small volume of distilled water to form a syrup. The syrup was centrifuged to remove humin and stored at 0°.

Single-dimensional descending paper chromatography was used with Whatman no. 1 paper and four solvent systems.

a) Ethyl acetate:pyridine:water, 2:1:2 (Jermyn and Isherwood, 1949)

b) Ethyl acetate:acetic acid:water, 3:1:3, (Jermyn and Isherwood (1949).

c) Butyl alcohol:acetic acid:water, 4:1:5, (Partridge 1948).

d) Benzene:Butyl Alcohol:pyridine:water, 1:5:3:3 (Albon and Gross 1952).

All the solvents which were not of analytical reagent quality were re-distilled prior to use. Systems containing pyridine were always freshly prepared. Chromatograms were run for 24 hr. at room temperature in all the solvent systems except (c). In system (c) the solvent was allowed to run for 96 hr. at 25°. in a thermostatically controlled room. Sugar spots were applied using micro-pipettes prepared from capillary tubing. Standard solutions of sugars (1% w/v) were included in all runs. Solvents were allowed to evaporate from the papers at room temperature in a fume cupboard. Reducing sugar spots were detected by spraying with p-anisidine hydrochloride (Hough,

Jones and Wadman, 1950) and amino sugars were revealed with ninhydrin or with the modified Ehrlich reagent (Partridge, 1948).

g) Electron microscopy.

Specimens of cells or cell walls were fixed with excess formalin, centrifuged at 10,000 G for 10 min. and resuspended in distilled water. In some experiments, the excess fixative was removed by dialysis against distilled water. The specimens were applied to carbon-collodion membranes on copper grids with a platinum inoculating loop and allowed to dry overnight in a dessicator. The grids were shadowed at an angle of 15° with gold-palladium alloy. The specimens were examined in the Metropolitan Vickers EM 6 electron microscope and suitable fields photographed using Ilford Special Lantern plates.

EXPERIMENTAL AND RESULTS.

Chapter I.

GROWTH EXPERIMENTS.

GROWTH EXPERIMENTS WITH VIRULENT BORD. PERTUSSIS.

The aim of this work was to evaluate some casein hydrolysate media with particular regard to the yield of bacteria and the biological activities of the cells. As some of the biologically important antigens of Bord.pertussis have been shown by previous workers to be extracellular, it was foreseen that a liquid medium comprised solely of dialysable materials would be very useful. Media containing blood were considered unsuitable because of the presence of non-bacterial components of high molecular weight and because of their expense. A basic medium similar to those of Cohen and Wheeler (1946) and of Verwey et al. (1949) was initially used. It contained (g./litre of solution): Casamino acids 10g.; nicotinamide 0.02g.; KH_2PO_4 0.5g.; $MgCl_2,6H_2O$ 0.4g.; $FeSO_4,7H_2O$ 0.01g. and $CaCl_2$ 0.1g. This medium was unable to support growth unless starch and a sulphur source were added.

1. Sulphur source.

Cystine, cysteine and glutathione have all been used as the sulphur source for Bord.pertussis, but the addition of such a compound to the medium prior to autoclaving may be undesirable. Alternatively, the addition of a component after sterilisation of the bulk of the medium would increase the risk of contamination.

Table 4. Growth in media containing cysteine.

Growth time (Hr.)	Amount of growth		
	Sulphur source		
	Autoclaved in medium	Seitz filtered	Autoclaved per se.
24	0	0	0
48	0.02	0.03	0
72	0.03	0.15	0.03
96	0.08	0.33	0.05
120	0.20	0.54	0.05

Table 5. Use of different sulphur sources.

Growth time (Hr.)	Amount of growth		
	Cysteine	Cystine	Glutathione
24	0.02	0	0.01
48	0.04	0.01	0.05
72	0.10	0.02	0.27
96	0.35	0.10	0.45

Roux bottles were prepared containing the basic medium with the addition of starch (BDH soluble starch, 1g./l.). Cysteine at a final concentration of 0.01g./l., was added to the medium in various ways.

a) Autoclaved with the medium.

b) Sterilised by Seitz filtration and added to the autoclaved medium.

c) Sterilised by autoclaving and added to autoclaved medium.

Table4 shows the growth in the three media. The result confirms the view of Rowatt (1957b) that autoclaved cysteine inhibits growth. In the cysteine solution which was autoclaved, the presence of hydrogen sulphide was readily detected with lead acetate paper. Because of the more rapid growth when the sulphur source was sterilised by filtration, this method was adopted for all liquid cultures of virulent Bord.pertussis.

Three sulphur sources have been used in liquid cultures and these were compared. The basal medium containing starch was used as in the previous experiment and the filtered sulphur source (cystine, cysteine or glutathione) was added at a concentration of 0.01g./l. The growths obtained are recorded in table5 . The most rapid initial growth was obtained when glutathione was sterilised by Seitz filtration and

added to the sterile basic medium in all experiments.

2) Adsorbents.

In casein hydrolysate media, the presence of adsorbents is necessary to counteract the effect of growth inhibitors. Starch has been widely used, but its addition to the basic medium was undesirable, as a medium free from non-dialysable components was required to study the extracellular non-dialysable products of growth. The starch can be enclosed in dialysis sacs but this may affect its adsorbing ability. Another possibility is to use an adsorbent which is particulate and insoluble and can be removed after growth by passing the complete culture through a coarse filter. Charcoal can be used but, as it is only available as a very fine powder, filters able to remove it might also remove a proportion of the bacteria in the culture. The replacement of starch by resin as described by Kuwajima et al., (1957) would be advantageous as the resin is in the form of large particles which can be easily removed. A variety of media were set up to compare the effect of resin and starch in the basic medium.

a) Basic medium containing starch (1.0g./l.), dissolved in the medium.

Table 6. The effect on growth of the addition of soluble starch and an ion exchange resin to the basic medium.

Growth time (Hr.)	Amount of growth				
	Basic medium with the addition of			Basic medium with dialysis tube containing	
	Starch	Resin	Starch and Resin.	Starch	Starch and Resin.
24	0.01	0.03	0.03	0	0
48	0.02	0.06	0.18	0	0
72	0.04	0.14	0.40	0	0
96	0.07	0.29	0.53	0	0
144	0.36	0.48	0.74	0.01	0.05
240	0.93	0.93	0.98	0.12	0.36
336	0.96	0.95	0.97	0.90	0.91

b) Basic medium with the addition of the anion exchange resin Dowex 1 (1.0g./l.).

c) Basic medium with the addition of starch (1.0g./l.) and resin (1.0g./l.).

d) As (a), the starch being dissolved in 10 ml. of the basic medium and enclosed in a dialyses sac. This was added to the rest of the medium prior to sterilisation. Samples of the medium were withdrawn aseptically after sterilisation and the presence of starch tested for with iodine and by the anthrone test. Any bottles of medium which gave positive results due to leakage of starch from the sac, were rejected.

e) As (c), both the resin and the starch being in a dialysis sac.

The amount of growth obtained in the different media is shown in table 6. The medium containing starch (starch medium) gave good growth but was undesirable. When the starch was enclosed in a dialysis sac, growth occurred to the same final value, but was considerably slower than in the medium containing free starch.
This could be due to the smaller surface area available for adsorption or to the time taken for toxic substances to pass through the membrane.
The addition of the ion exchange resin to the basic medium was associated with good and relatively rapid growth which occurred in the

presence or absence of starch. The effect of the resin was much less marked when the resin was enclosed with starch in a dialysis sac. The basic medium with the addition of resin (resin medium) was thus an ideal basis for the isolation of extracellular, high-molecular weight products of Bord.pertussis, as the resin was easily removed by decantation or filtration, leaving a completely dialysable medium.

Kuwajima et al. (1957) tested several anion exchange resins and found that several types all gave similar growth-supporting effects. The few exceptions were resins which were destroyed by the heat-sterilisation of the medium. Two other anion exchange resins, Amberlite IR4B and Amberlite IRA 400 were compared with Dowex 1, and the results in the three resin media were almost identical. (Turbidities of 0.54, 0.51 and 0.52 respectively after 144 hr.).

In order to determine whether the resin medium was as efficient as the starch medium in growing cultures from minimal inocula, ten-fold dilutions of a bacterial suspension, grown on charcoal agar, were inoculated into a series of Roux bottles containing 200 ml. of each medium. Viable counts on these dilutions were performed using Bordet-Gengou medium. The results showed that both media were capable of growing the organism,

if, after inoculation, there were as few as three viable cells/ml. of medium.

Kuwajima et al. (1957) found that while most anion exchange resins were able to support growth in the absence of starch, cation exchange resins were uniformly unsuccessful. This was confirmed, as the cation exchange resin Amberlite IR 120 had no growth supporting capacity. This resin did not enhance the effect of an anion exchange resin when both were added to the basic medium.

The effect of the anion exchange resin could be due to the removal of toxic products present in the medium prior to growth, or to the adsorption of toxic factors produced during growth. The substances most commonly implicated as inhibitors of the growth of Bord.pertussis, have been unsaturated fatty acids (Pollock, 1947), and products of the heat sterilisation of organic sulphur compounds (Proom, 1955; Rowatt, 1957a). An experiment was carried out to test the ability of starch in the starch medium and resin in the resin medium to counteract such inhibition. The following inhibitors were added to the media:-

a) Casamino acids (Difco technical) were refluxed with ether and the ether-soluble fraction was evaporated to dryness. The product was a yellow, viscous, lipid-like material. It was added to the medium at a concentration of 1µg./ml.

Table 7. The effect of some inhibitors on the growth of Bord.pertussis.

Additions	Percentage inhibitions of growth in	
	starch medium	resin medium
None	0	0
Ether extract	42	10
Cysteine	100	21
Oleic acid	100	67

b) A solution of autoclaved cysteine was added to give a final concentration of 30μg./ml.

c) Oleic acid (10μg./ml.).

The inhibitory effects of the three substances after 192 hr. incubation are shown in table 7. It is evident that resin is more efficient than starch in overcoming the effects of the three inhibitors.

If the effect of the resin is to remove toxic products present in the medium before inoculation, removal of the resin after sterilisation should leave growth unaffected. Roux bottles were set up with :-

a) basic medium containing 0.1% (w/v) Dowex 1 anion exchange resin.

b) as in (a) but freed from resin by decantation of the liquid into fresh sterile Roux bottles after it had itself been sterilised.

c) resin sterilised separately then added to the sterile basic medium immediately prior to inoculation of the bacteria.

The growths in media (a) and (c) were very similar (respectively 0.66 and 0.65 after 144 hr. incubation), but no growth was obtained in medium (b). This suggests that the presence of the resin is required during growth. It is therefore most convenient to include the resin in the medium prior

Table 8. The effect of casamino acid concentration on growth.

	Amount of growth in medium containing:				
Concentration of casamino acids.(% w/v)	1.0	0.5	0.5	0.1	0.1
Concentration of glutamate.	-	-	0.315	-	0.57
Medium	(a)	(b)	(c)	(d)	(e)
Growth time (hours)					
24	0.02	0.02	0.01	0.02	0.02
48	0.05	0.04	0.04	0.04	0.05
96	0.19	0.24	0.18	0.13	0.21
144	0.46	0.53	0.43	0.15	0.46
192	0.69	0.68	0.67	0.15	0.67
240	0.69	0.68	0.68	0.15	0.68

to sterilisation and remove it after growth has ceased.

3) Casamino acid concentration.

In the basic medium with the addition of starch, it seemed that the casein hydrolysate was being poorly utilised by virulent strains of Bord. pertussis as the final growth of avirulent strains was considerable greater (0.69 and 1.24 respectively after 240 hr. growth). In an attempt to determine whether this was so, Roux bottles were set up with the basic medium containing different concentrations of casamino acids with or without the addition of sodium glutamate. The glutamate was added, as it has been shown to be one of the principal metabolites of the species and its addition to casein hydrolysate media was said to increase the final yield of bacteria (Rowatt, 1957a). The growth obtained in the different media is shown in table 8. Thus, media (a), (c) and (e) which all contain the same final concentration of casamino acids give the same yield of bacteria. This is no better however than that obtained in medium (b) which only contains half the concentration of casein hydrolysate. In medium (d) which only contains 0.1% w/v casamino acids it is assumed that growth is limited by the lack of nutrients and not by other factors. This being so, the same applies to medium (b) where the

increase in growth (five fold) is the same as the increase in nutrients. These results suggest that almost half the available nutrients are not being utilised in the normal medium, a figure which is borne out by the growth obtained for an avirulent strain.

4) pH value.

The incomplete utilisation of the medium could be due to several causes, among them accumulation of toxic factors and exhaustion of growth factors. The Bordetella species all liberate ammonia from the medium during growth and as a result there is a rapid rise in its pH value. It was thought that this accumulation of ammonia in the medium might be a cause of cessation of growth, as in the basic medium the pH rose to about 8.4 by the time the stationary phase of growth was reached. It seemed possible that growth at a lower pH value than that of 7.0 usually used, might result in better growth. Thus Rowatt (1957a) showed that although the initial growth rate of Bord.pertussis was the same between pH values of 5.9 and 7.7, the rate slowed earlier at pH 7.6 - 7.7 than at lower values. To test this in resin medium, bottles of medium were prepared and the pH values adjusted to 6.5, 7.0, 7.5 and 8.0 with 1.0 N NaOH. The average growth in ten experiments at each pH, together with the standard deviation and the

Table 9. Growth in the resin medium adjusted to various initial pH values.

Growth time (hr)	Amount of growth				Standard deviation in growth				pH of medium			
	Initial pH				Initial pH				Initial pH			
	6.5	7.0	7.5	8.0	6.5	7.0	7.5	8.0	6.5	7.0	7.5	8.0
24	0.02	0.26	0.03	0.03	0.00	0.00	0.00	0.00	6.5	7.0	7.5	8.0
48	0.03	0.05	0.06	0.06	0.00	0.01	0.02	0.01	6.5	7.0	7.6	8.1
72	0.06	0.11	0.16	0.20	0.02	0.01	0.03	0.03	6.6	7.1	7.7	8.2
96	0.06	0.23	0.30	0.32	0.02	0.03	0.04	0.03	6.6	7.4	8.0	8.4
120	0.18	0.40	0.51	0.37	0.03	0.04	0.03	0.02	6.6	7.6	8.2	8.4
144	0.30	0.55	0.53	0.37	0.04	0.02	0.03	0.02	6.8	8.0	8.3	8.4
192	0.76	0.70	0.54	0.37	0.03	0.02	0.02	0.02	7.4	8.3	8.4	8.4
240	0.82	0.70	0.54	0.38	0.02	0.01	0.01	0.00	8.1	8.3	8.4	8.4

average pH value reached, are recorded in table 9. It can be seen that the final yield of growth from lower pH values was greater than that from the higher initial pH values, while the initial growth rate was slower in the former. The standard deviations indicated that there was less variation in the results of this type of experiment than might be expected, especially considering the number of factors affecting growth of Bord. pertussis. The pH values showed that there was a rapid rise in pH associated with the increases in turbidities. No increase in turbidity was observed after the apparently growth-limiting pH 8.3 - 8.4 was reached.

It was evident that the pH characteristics for a medium for growing virulent Bord. pertussis had two requirements. The pH should be high enough to give an adequate initial rate of growth and there should be sufficient buffering capacity to prevent a decreased final yield due to the rise in pH of the medium. The medium already contains some buffering capacity due to the addition of KH_2PO_4, and amino acids and an experiment was carried out in which the buffering capacity was increased by the addition of varying amounts of pH 7.0 tris (2-amino-2-(hydroxymethyl)-1:3-propanediol) buffer to the resin medium. The growth curves obtained in this experiment are

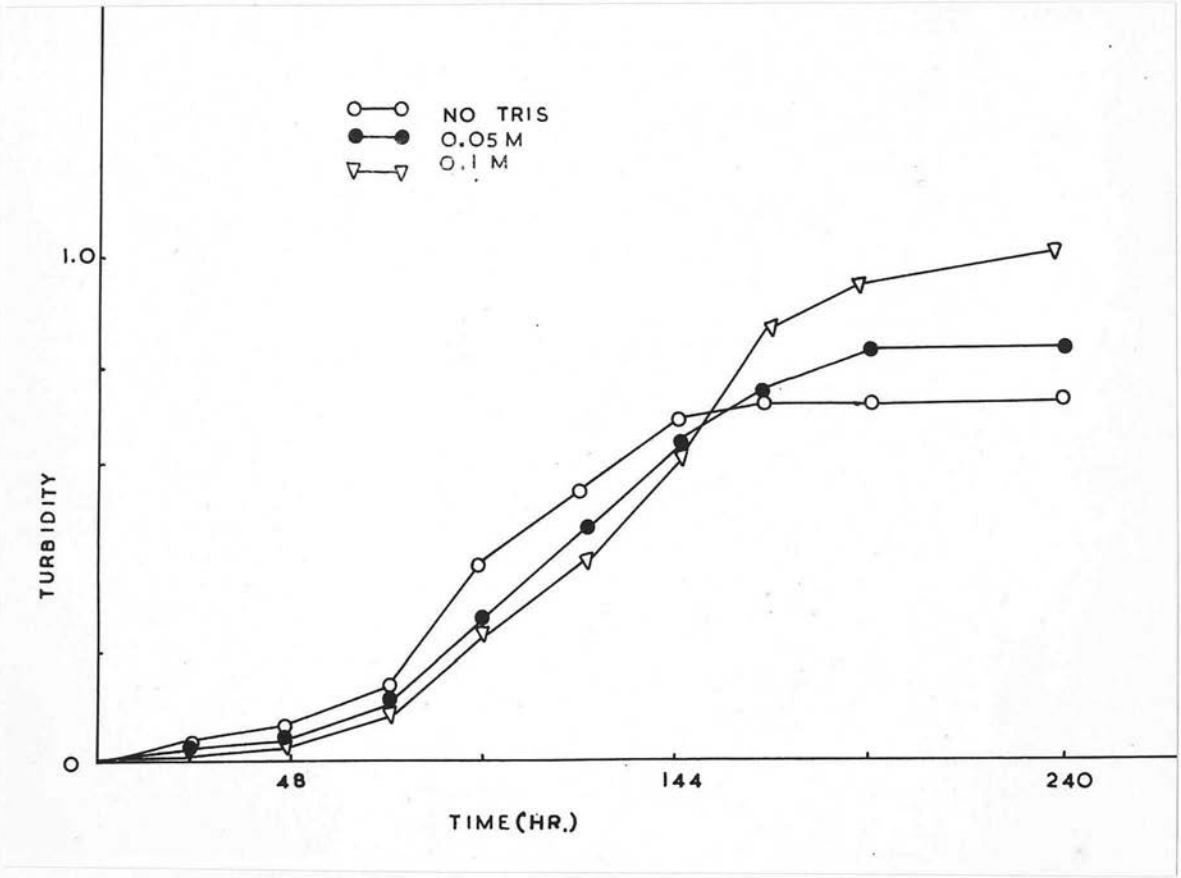

Fig. 2. Growth curves (turbidities) of Bord.pertussis strain 4507 in medium containing tris buffer.

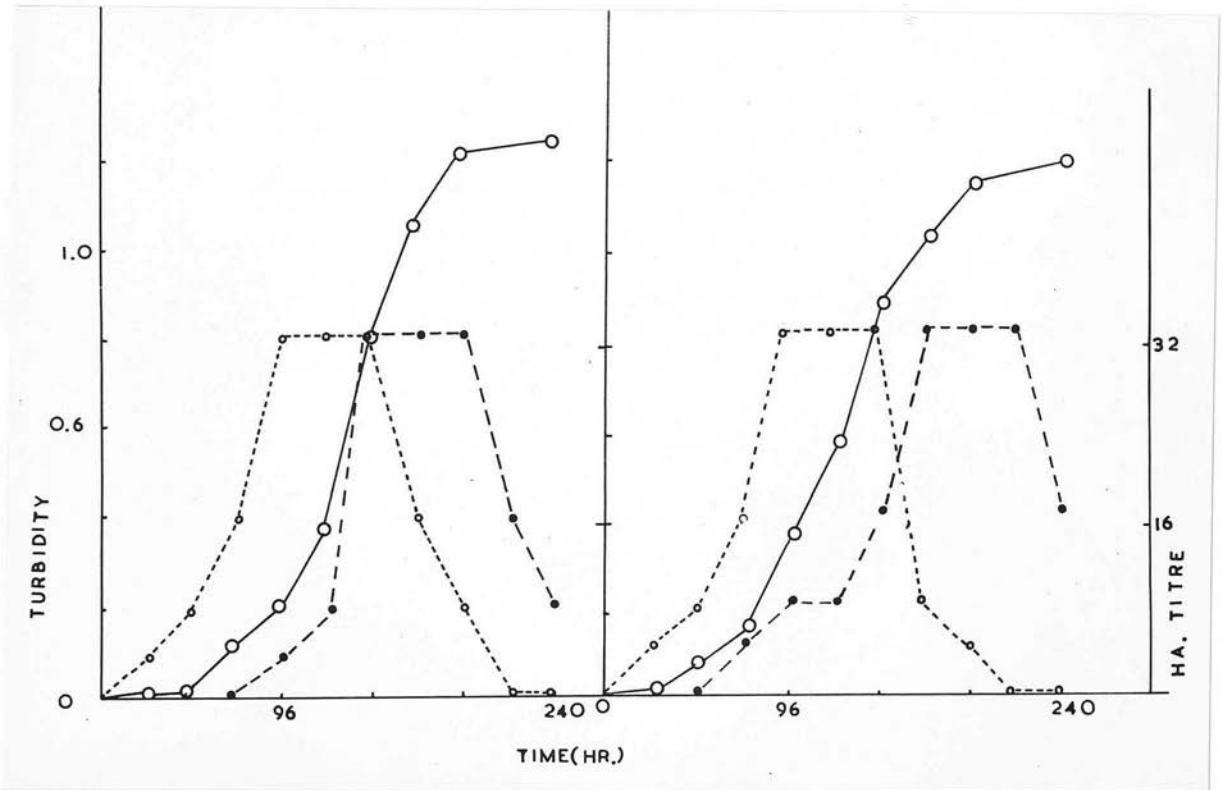

Fig. 3a. Fig. 3b.

Fig. 3 shows growth and haemagglutinin production in tris-resin medium (3a) and tris-resin-yeast medium (3b). The growth (as measured by turbidities) is shown in the continuous curves. The dotted and dashed lines indicate titres of cell-bound and cell-free haemagglutinin respectively. (Strain 4507).

shown in fig. 2. Although growth was slightly slower in the medium containing 0.1 M tris than in the medium without tris, the final yield was increased considerably. The average increase in total growth after 240 hr. for twelve experiments in the medium buffered with 0.1 M tris (tris-resin medium) compared with twenty-two experiments in the unbuffered resin medium, was 50 per cent.

5) Growth factors.

Although nicotinamide is capable of acting as the only growth factor for Bord. pertussis, various authors have claimed that other growth factors, present in yeast extract, will stimulate growth (e.g. Rowatt, 1957a; Jebb and Tomlinson, 1957). In an experiment in which one set of Roux bottles contained the tris-resin medium and the other set contained this medium with the addition of yeast extract in place of nicotinamide, there was little difference in growth, or in haemagglutinin production between the two media (fig. 3). Both media supported growth from an inoculum of two viable cells/ml. of medium.

From these results, the tris-resin medium was considered to be a very suitable liquid medium for the growth of Bord. pertussis. In its final form, it contained in addition to the basic constituents previously outlined: 0.1% (w/v) Dowex 1 anion exchange resin and 0.3% (w/v) tris. The pH of

the medium was adjusted to 7.0 before sterilisation. After growth in the medium, the resin was removed by filtration through muslin or through a coarse sintered-glass filter. Although the resin could be regenerated with sodium hydroxide and used again, this was only done when large quantities of medium were being used to grow cells for antigenic studies and the resin from the growth experiments above was discarded after use. The tris-resin liquid medium was shown to support the growth of all ten virulent strains tested: L85, L91, W 2000, H5, H36, 18/323, NCTC 8070, 8474 and 8631.

6) The tris-resin medium as the basis of a solid medium.

The starch medium was unable to support growth of virulent Bord. pertussis when solidified with agar, but the resin or tris-resin media both supported growth. The growth on several forms of solid medium was compared by determining the amount of growth obtained from identical inocula on the different media. Plates of the following media were prepared :

a) Bordet-Gengou medium;

b) Basic medium with 0.3% (w/v) activated charcoal (BDH) and 0.1 M tris;

c) Tris-resin medium;

d) As medium (c) with the addition of 0.1% (w/v) soluble starch.

Media (b), (c) and (d) were solidified by the addition of 1% (w/v) New Zealand agar. With the exception of Bordet-Gengou medium, the media were cooled to 45° after sterilisation, then poured. This temperature was used to permit rapid solidification and even distribution of the charcoal or resin throughout the medium, rather than as a sediment at the bottom of the plate. A bacterial suspension, obtained by emulsifying the growth after 48 hr. on charcoal agar into 1% (w/v) casamino acid solution, was prepared as a series of tenfold dilutions in the same diluent and used to inoculate the plates of media. A total count and viable counts on the four media showed viabilities as follows : (a) 16%. (b) 20%, (c) 18% and (d) 32%. The colonies on media (c) and (d) were larger than those on the other two media. They were transparent at first, becoming gray-white and translucent after several days. They adhered to the medium, especially after prolonged incubation. The bacterial cells were typical of virulent strains in their agglutinability by antisera to other virulent Bord. pertussis strains, haemagglutinating ability, toxicity and alum precipitability (Standfast, 1951b). After ten subcultures on the tris-resin solid medium (with or without starch), the cells retained their virulent properties. To confirm the usefulness of the tris-resin solid

Table 10. Table of media used.

Medium	Additions to the basic medium	Details on page
Resin medium	resin, glutathione.	75
Tris-resin medium	resin, glutathione, tris.	82
Tris-resin-yeast medium	tris, resin, glutathione and yeast extract (nicotinamide omitted).	82
Starch medium	starch, glutathione.	74
Charcoal agar	starch, charcoal, glutathione and agar.	53
Resin agar	tris, resin, glutathione, yeast extract and agar (nicotinamide omitted).	83

Table 11. Challenge of unvaccinated mice with Bord. pertussis (strain 18/323).

Bacteria		Mice
Total count	Viable count	Deaths/total challenged
1	1	2/10
10	7	3/10
100	73	4/9
1000	730	6/10
10000	7300	9/10
100000	73000	10/10

medium, the 22 virulent strains of Bord. pertussis listed in the methods were used for inoculation and all grew well. In all cases where the presence of blood was undesirable, a resin agar medium was used.

The different media which have been used in the growth studies are shown in table 10.

7) Cells grown in resin media as a source of protective material.

Protectivity in mice challenged by the intracerebral route (Kendrick et al., 1947) was chosen as being the most suitable and accurate method of testing Bord. pertussis cells and fractions. This method has been shown to give good agreement with the results of controlled vaccine trials in humans (Medical Research Council, 1959). The test relies on the use of a susceptible strain of mice and a bacterial challenge strain of proven virulence. The challenge strain (18/323) was originally used by Kendrick et al. (1947) and has been found to be satisfactory by most workers in this field. After preliminary tests, the A2G strain of white mice was employed. The LD_{50} for the strain was determined using tenfold dilutions of the challenge strain inoculated into groups of ten mice. The results are shown in table 11. The LD_{50} calculated from these results by the method of Reed and Muench (1938) was 137 bacteria per

Table 12. Comparison of Bordetella pertussis vaccines prepared from cells grown in various media.

Strain 4507				
Medium	Vaccine dose			
	4×10^9		4×10^8	
Resin	2/9*	8.29°	7/9*	0.74°
Tris-resin	1/7	9.75	7/7	1.03
Tris-resin-starch	2/7	7.05	5/7	1.07
Tris-starch	3/10	7.91	8/10	0.56
K 276 (Ref. vaccine)	2/9	8.29	7/9	0.74
Unvaccinated controls 10/10*				

Strain 134				
Medium	Vaccine dose			
	2×10^9		4×10^8	
Tris-resin	4/12*	10.53°	10/14*	2.63°
Tris-resin-starch	5/14	10.48	8/12	3.18
Tris-starch	3/14	16.77	8/14	5.30
Unvaccinated controls 14/14*				

* Deaths/total challenged.

° χ^2 calculated according to Bradford Hill (1942)

mouse. The mice were tested frequently throughout the work and it was found to be stable in value. In protection tests, a challenge dose of about 50,000 bacteria (approximately 350 x LD_{50}) was standardised turbidimetrically and viable counts were also performed.

a) Vaccines prepared from cells grown on different media.

Although the new tris-resin medium has been shown to give good growth of apparently virulent bacteria, it was necessary to show that such bacteria could induce protection against experimental infection. Virulent strains of Bord. pertussis (4507 and 134) were grown in a variety of media (see table 10, p.85). The bacteria were harvested, washed once with saline and suspended in saline containing merthiolate (0.01% w/v). The vaccines were kept at 0° for one month to permit detoxification. A reference vaccine (K 276) grown on Cohen Wheeler medium and kindly provided by Dr. Jean Dolby (Lister Institute, Elstree) was also included in the experiment. The results are shown in table 12. From the results obtained with the two strains tested, it appears that there is little difference in the protective capacity of the cells grown in the different media. The similar result for the reference vaccine indicates that there is not much

difference in protectivity between the vaccines grown in tris and resin media and vaccines prepared by other workers from cultures in Cohen Wheeler medium.

b) Vaccines prepared from cells grown in two types of tris-resin medium.

As the bacteria in resin-containing media adhered to the resin particles (Kuwajima et al., 1957), it seemed possible that considerable amounts of protective material might be lost by adhesion. An experiment was designed to investigate this possibility and to compare vaccines prepared from the whole culture, with washed cell vaccines. Bord. pertussis (strain 4507) was grown in tris-resin medium and in the same medium modified by putting the resin into dialysis sacs, thus preventing the adsorption of non-dialysable bacterial products onto the resin. After growth, merthiolate (0.01% w/v) was added and, after detoxification at 0^{o} for one month, the vaccines were prepared by direct turbidimetric standardisation of the whole cultures. Sterile medium, containing no resin, was used as diluent. The free resin in the tris-resin medium was removed by decantation. A portion of the culture in the modified medium was centrifuged at about 20,000 G for 30 min. to obtain the bacterial cells. The bacteria were washed twice with sterile saline,

Table 13. Protectivity of cells grown in resin media

Vaccine prepared from:	Vaccine dose					
	2 x 10^9		4 x 10^8		8 x 10^7	
Resin medium	5/14*	11.04•	7/12	7.45	10/12	0.48
Resin (sac) medium	4/14	13.29	8/14	5.67	9/12	0.21
Washed cells	4/13	12.52	8/11	2.34	9/12	0.21
Unvaccinated controls					15/15*	

* Deaths/total challenged.

• χ^2 value.

then suspended in sterile, resin-free medium. The dilutions of the vaccine were prepared as above. Groups of mice were vaccinated with one of the three preparations and challenged intracerebrally fourteen days later. Table 13 shows the results, which indicate that there is little difference in the protectivity of the three vaccines. This is further evidence that the medium containing free resin is satisfactory for growing cells for vaccine production and for studies on protection. The results for the washed cells show that although protective material may be present in the washings, as has been suggested by several workers (e.g. Maitland and Guerault, 1958), insufficient material is removed to have a significant effect on the vaccines.

A new medium for the growth of virulent Bord.pertussis has been prepared. It gives good growth of the bacteria in high yield. The bacterial cells are similar to those grown in other media, with respect to protective capacity and other characteristics. The new liquid medium has also been used as the basis of a solid medium.

Chapter II.

CELL-WALL CONSTITUENTS.

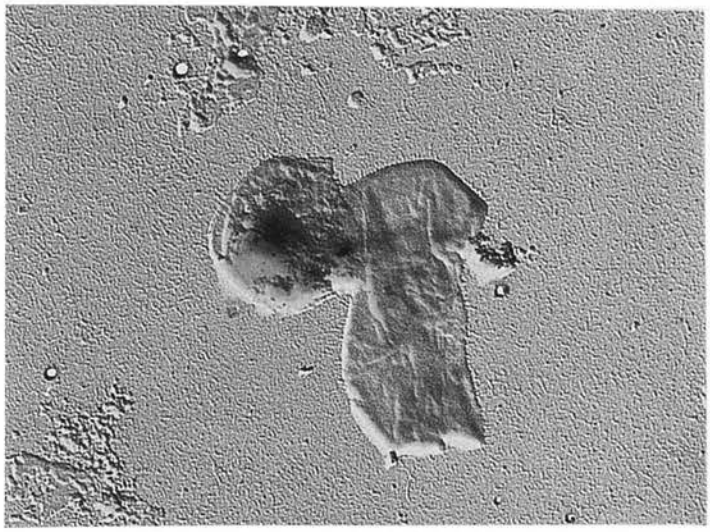

Fig. 4.

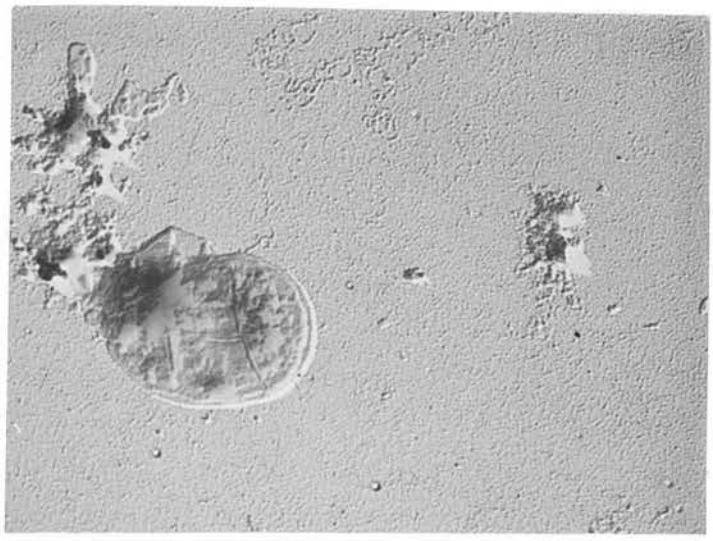

Fig. 5.

Figs. 4 and 5 show cell walls prepared from Bord. pertussis (strain 4507) disintegrated with ballotini glass beads. x 30,000.

THE CELL WALL AND RELATED ANTIGENS

Recent work has increasingly indicated that the cell wall of Bord. pertussis is a rich source of the protective material against intracerebral infection in mice (Munoz et al., 1959; Billaudelle et al., 1960). An attempt was made to confirm this and to determine whether the protection was due to any single part of the cell wall which might be designated a protective antigen.

1) Preparation.

Cell walls were prepared by disrupting the bacteria with ballotini glass beads, but as can be seen from the electron micrographs (figs. 4 and 5), even after washing with buffers and distilled water, electrondense cytoplasmic material was still present. Although the cell walls might be freed from extraneous matter by further washings as shown by Billaudelle et al., (1960), other methods were tested. These involved digestion of the cytoplasmic material with enzymes, or its removal by treatment with a detergent.

a) Trypsin - The cell walls were suspended at a concentration of 1% (w/v) in phosphate buffer (0.05M), pH 8.0. Trypsin (0.02% w/v) was added and the suspension was incubated for 3 hr. at 37°, with constant stirring. The cell wall was recovered by centrifugation, washed once with distilled water and freeze-dried. The product

comprised 45% of the initial dry weight of the cell walls.

b) Lysozyme - Cell walls were suspended at a concentration of 1.0% (w/v) in 0.05 M tris buffer at pH 7.6. Lysozyme (0.01% w/v) was added and the suspension was incubated at 37° for four hours with constant stirring. The product was recovered as in (a). The yield was 86% of the initial material.

c) Sodium lauryl sulphate - The method of treating the cell walls was similar to that of Shafa and Salton (1960). The cell walls were suspended in the detergent solution (0.2% w/v) and stirred for 30 min. at 37°. They were recovered as in (a), the yield being 42% of the original dry weight.

d) Lipase - To a 1.0% (w/v) suspension of the cell walls in phosphate buffer (0.05M, pH 7.0), the enzyme (0.05% w/v) was added and the mixture stirred at 37° for six hours. The washed, freeze-dried product comprised 40% of the dry weight of the untreated cell walls.

Electron microscopy, which was used as a control during cell wall preparation, was also used to study the effects of the different enzymes. The material which had been treated with lysozyme did not appear to have undergone any visible change, but the other three preparations differed from the original cell walls shown in figs. 4 and 5 .

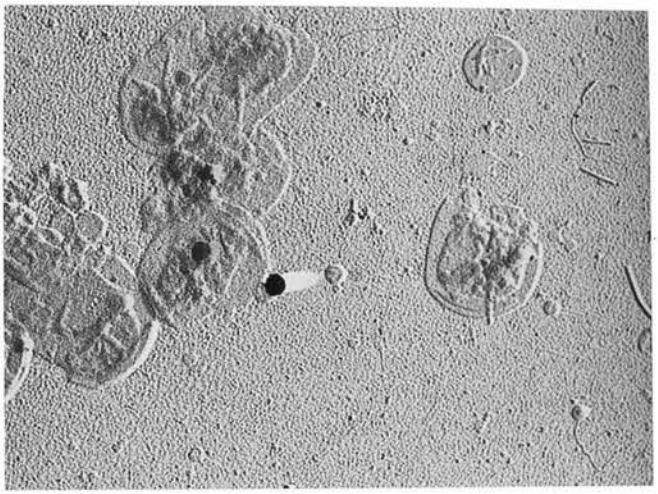

Fig. 6. x 30,000.

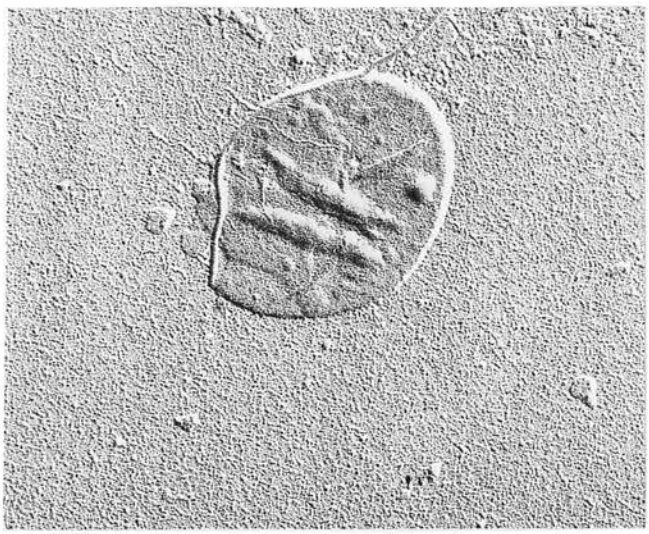

Fig. 7. x 40,000.

Figs. 6 and 7 show cell walls prepared from Bord.pertussis strain 4507 and treated with lipase and sodium lauryl sulphate respectively.

Table 14. The protectivity of cell wall preparations

Dose/mouse	Cell wall treated with									
	Untreated		Lipase		Detergent		Trypsin		Lysozyme	
200 µg.	3/12*	9.65+	2/10	10.21	2/12	12.11	----	----	----	----
100 µg.	----	----	----	----	----	----	5/12	10.94	4/12	9.87
40 µg.	6/12	4.59	8/11	1.34	5/10	4.27	----	----	----	----
10 µg.	----	----	----	----	----	----	9/10	0.02	6/12	3.90
8 µg.	8/10	0.56	7/10	1.57	9/10	0	----	----	----	----
Unvaccinated controls			10/10*					13/13*		

* Deaths/total number of mice challenged.

\+ χ^2

Although the lipase-treated material contained little electron - dense cytoplasmic matter, there still appeared to be a collapsed cytoplasmic membrane present (fig. 6). Treatment with the detergent however, removed both the electron-dense material and the membrane (fig. 7). The product of trypsin treatment appeared to be indistinguishable from the cell walls treated with sodium lauryl sulphate.

2) Active protection tests.

The cell wall preparations were also tested in mice to determine their protective ability. The results of active protection tests are shown in table 14. All the preparations induced some protection in mice, although weight for weight, the cell wall was not as effective an immunising agent as the whole bacteria. The cell wall, which in a typical experiment comprised about 20% of the dry weight of the bacteria (1.0 g. of dried bacteria yielded 205 mg. of cell walls), protected most of the mice when an injection of 100 - 200 µg. was used. This is equivalent to 500 - 1000 µg. of whole bacteria, but when whole bacterial vaccines were used, similar protection was given by 2×10^9 cells (i.e. approximately 120 µg.). It thus appears that about 75% of the protective material originally present was lost or inactivated during the preparation of the cell

walls. Examination of the results in table 14 shows that further protective material was lost during the enzymic treatments. Although lipase for example removed 60% of the original material, the same amount of the enzyme-treated cell wall was necessary for protection, as of the untreated cell wall.

Lipopolysaccharides are known to be present in the cell walls of Gram negative bacteria and they can be selectively extracted from the cell walls or from whole bacteria with phenol (Westphal et al., 1952). Phenol- extracted lipopolysaccharide from Bord. pertussis cells (strain 4507) was found to be nonprotective when mice were inoculated with doses of 100 µg., all the mice in a group of ten being killed by the intracerebral challenge. This was not unexpected on account of the poor antigenicity of the injected material.

3) Passive protection tests.

Antisera were prepared against whole bacteria and against trypsinised cell walls, rabbits being injected with six, weekly doses of 2 mg. of the material. The sera were then tested for protection by passive immunisation of mice. A portion of the antibacterial serum was also adsorbed with lipopolysaccharide, as it was thought that such adsorption might remove some of the antibodies involved in protection. The

Table 15. Protective capacity of antisera

Antiserum to	Serum dose		
	0.2 ml.	0.05 ml.	0.0125 ml.
Whole bacteria	4/12*	5/10	9/10
Whole bacteria (absorbed with lipopolysaccharide)	2/10	6/10	10/10
Cell wall	4/12	7/12	9/10
Unvaccinated control 17/17			

* Deaths/total challenged

lipopolysaccharide in a 0.5% (w/v) solution was emulsified using the ultrasonic disintegrator and added to an equal volume of antiserum. The mixture was incubated at 37° for 12 hr., centrifuged at 100,000 G for 1 hr. to remove the antigen - antibody precipitate and any excess antigen, and tested in gel diffusion for the presence of antibodies to the lipopolysaccharide. The absorption process was repeated until these antibodies could no longer be detected. The final product was lyophilised and dissolved in saline to the same volume as the original serum.

When the two antisera and the absorbed preparation were tested in mice, the results shown in table 15 were obtained. The antibacterial serum gave good protection before and after absorption, indicating that the antibodies giving a precipitation reaction with lipopolysaccharide were not involved in protection. The antiserum prepared against trypsinised cell walls also gave good protection, confirming the results obtained in active protection tests.

4) Toxicity.

Although the cell wall preparations had been shown to be good protective agents, their value as such in vaccines for human use would be limited if they still contained large amounts of labile toxin or if there was sufficient lipopolysaccharide endotoxin present to give a toxic response. When

Table 16. **Examination of cell walls for H.S.F.**

Cell wall treated with	Dose / mouse	
	1 mg.	100 µg.
Untreated	2 / 10*	0 / 10
trypsin	0 / 10	0 / 10
lipase	7 / 10	0 / 10
sodium lauryl sulphate	4 / 9	0 / 10
Unvaccinated controls	0 / 15	

* Deaths / total number of mice challenged.

the enzymically treated cell walls were tested in mice, the material digested with lipase or with trypsin was non - toxic even at a dose of 1 mg. The same result was obtained with cell walls treated with sodium lauryl sulphate. Untreated cell walls were toxic at a dose of 1 mg., killing all ten mice in the group challenged, but there was only one death in a group of ten mice challenged with 100 μ g. of the same material. The lysozyme - treated material was not tested.

5) Histamine sensitisation.

The presence of H.S.F. in cell walls of Bord. pertussis, has been reported by Munoz et al. (1959) and although the significance of this antigen in human infection was unknown, it was desirable to know whether it was present in the enzymically treated cell walls. When these fractions were tested in mice, the results shown in table 16 were obtained. A large dose (1 mg.) was needed for sensitisation of the mice to occur, suggesting that relatively little H.S.F. was present. It appeared that only the trypsin treated material lacked the antigen.

6) Chemical studies.

a) Cell Walls

As it was to be expected that enzymic treatment would have changed the chemical composition of the

Table 17. Chemical composition of cell wall preparations

Cell wall treated with	% fall in weight	N	P	Hexose	Hexosamine	Heptose	Anthrone
Untreated	-	11.8	0.3	3.5	2.7	1.9	7.5
Lysozyme	14	13.0	0.3	3.7	1.9	1.8	8.1
Lipase	60	9.5	0.1	4.2	4.6	1.7	10.2
S.L.S.	58	12.2	0.3	5.8	3.0	2.8	9.1
Trypsin	55	11.4	0.3	7.8	3.8	2.7	11.7

All values are expressed as percentages.

cell walls, they were examined to determine whether these changes could be related to any of the changes in biological activities. The results of a series of analyses are shown in table 17.

The proportion of polysaccharide, as measured by the various sugar estimations, has been increased in most of the preparations by the removal of other material. An exception is the cell wall treated with lysozyme, which has smaller amounts of sugar due to the action of the enzyme. It was thought that the enzyme trypsin would have caused a fall in the nitrogen content due to digestion of proteins, but this was not observed. In the other preparations there was little change in the nitrogen content except where lipase was used. The decrease in this case must be assumed to be due to splitting of a bond between lipid and protein, a result which is also reflected in the reduced phosphorus content. Ether extraction of untreated cell wall removed 5.3% of lipid, but insufficient material was available to permit determination of the lipid content of the enzyme-treated preparations.

b) Lipopolysaccharides.

The lipopolysaccharides were extracted from a number of Bordetella strains and submitted to chemical analysis. The results are shown in table 18 . The yield of material was of the same

Table 18. Lipopolysaccharides of Bordetella species

Virulent Bord. pertussis	Yield	Nucleic acid.	N	P	Hexose	Hexosamine	Heptose
4507	7.1	26.5	4.8	0.5	18.2	9.6	23.5
91	5.3	20.9	4.2	0.6	18.7	7.3	23.3
Avirulent strains							
4507 (Av)	7.2	17.5	6.4	0.8	6.7	11.1	20.0
2216	7.1	19.4	5.2	0.2	7.1	13.0	23.7
51	7.5	24.6	6.3	0.1	2.9	16.4	13.3
Bord. parapertussis							
7385	-	12.5	6.3	0.1	2.9	13.8	24.8
Bord. bronchiseptica							
106	-	23.3	6.8	0.5	3.2	16.9	15.6
8344	4.7	25.5	5.0	0.6	4.9	11.1	11.5
8761	-	19.5	5.8	0.5	2.9	16.7	16.2
8762	5.8	31.5	5.5	0.5	2.8	14.0	18.1

All figures are expressed as percentages.

order for most of the strains of the different species. As the lipopolysaccharides were all Biuret negative, it was assumed that the only other material present was nucleic acid. Although the composition of the material from different strains of any one species were similar, there were marked differences between virulent and avirulent strains of Bord. pertussis. The former contained less hexosamine and more hexose, but both groups contained a large proportion of heptose, as did the Bord. pertussis lipopolysaccharide. An interesting feature of the results is that the total of hexosamine and heptose in all the strains except one (Bord. bronchiseptica 8344), is approximately the same. The phosphorus is assumed to be present in the lipid and nucleic acid components, but the relatively high nitrogen values are unlikely to be solely due to the nucleic acid and hexosamine. It is possible that small amounts of protein, insufficient to affect the Biuret test, are present in some or all of the preparations.

Paper chromatography showed two main sugar components in the hydrolysed lipopolysaccharides of all strains. One spot moved slowly in all solvent systems and reacted with p-anisidine, ninhydrin and modified Ehrlich reagent. This indicated that it was due to an amino sugar and it moved at the same rate as glucosamine. Unfortunately galactosamine, which moves at almost

the same rate as glucosamine in most solvents, was not avilable for comparison. It was possible that both these amino sugars were present, but very large amounts of material would be necessary to confirm their presence. The second major spot reacted with p- anisidine but not with ninhydrin. A similar spot was reported by Maclennan (1960), who suggested that it was caused by D-glycero-L-mannoheptose. In most solvent systems the unknown spot was similar in position and colour to the authentic heptose (kindly supplied by Dr. Richtmeyer, National Institutes for Health, Bethesda, U.S.A.). In some chromatograms the true heptose gave a lighter spot when sprayed with anisidine than did the unknown. It is possible that the spot in hydrolysates was due to more than one sugar and that a hexose such as glucose was also present. This might explain the apparent absence of a major spot due to glucose in the hydrolysates from virulent strains of Bord. pertussis. A very slow-moving spot, giving reactions with ninhydrin and anisidine and seen in most hydrolysates, was probably due to an oligosaccharide. It was possible that other sugars were present in small amounts as several other very faint spots were occasionally observed in some of the materials, but their identification was precluded by lack of sufficient hydrolysed lipopolysaccharides.

In an attempt to clarify the results obtained

for hexose, the cysteine - sulphuric acid reaction used for its estimation was examined for the interfering effect of glucosamine. The amino sugar did not give any value itself, however, nor did it alter the values given by glucose or other hexoses. The same result was observed at the wavelengths used for the heptose estimations. Interference could be caused by the presence in the hydrolysed material of small amounts of cysteine liberated from protein.

Maclennan (1960) had reported that lipopolysaccharides obtained from Bordetella species were very resistant to acid hydrolysis and this also might affect the estimations made with the cysteine - sulphuric acid reaction. For this reason, Maclennan recommended a heating time of 10 min. for the interaction of the acid and the specimen. When continuous spectra of the cysteine-polysaccharide chromophores were studied, it was seen that the adsorption peaks at 405 mμ and 495 mμ due to hexose and heptose respectively, were of the same intensity when heating for 3 min. or for 10 min. was employed. This indicates that the shorter time is sufficient to permit complete hydrolysis of the polysaccharide. The liberation of hexosamine during hydrolysis was also studied. When a sample of Bordetella bronchiseptica (strain 8762) lipopolysaccharide was hydrolysed with 1.0 N HCl, the amounts of hexosamine shown in

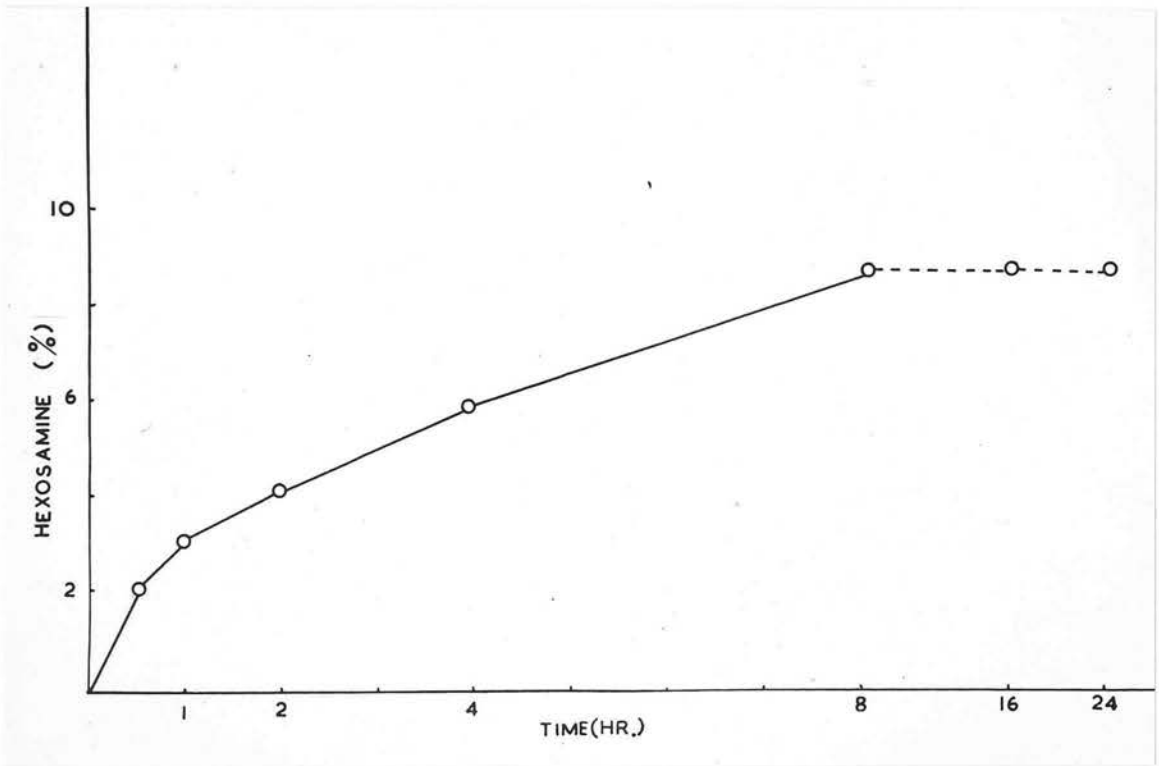

Fig. 8. Hydrolysis curve of Bord.bronchiseptica lipopolysaccharide (strain 8762), showing release of amino sugar during treatment with 1.0 N HCl.

fig.8 were released. From this, it seems that, under the conditions used, hydrolysis for 8 hr. was sufficient to liberate all the hexosamine. The results for this sample a re lower than those shown for the same strain in table 18as more nucleic acid was present. The same batch of lipopolysaccharide was used to determine the validity of the nucleic acid estimations. The material was treated for 12 hr. at 37° with ribonuclease to remove any nucleic acid present. The lipopolysaccharide was then recovered by ultracentrifugation at 100,000 G for 4 hr., washed once with distilled water, centrifuged again and freeze-dried. The final product, 65% of the weight of the original material, corresponded to 35% of nucleic acid by difference. The value for nucleic acid obtained by ultraviolet absorption was 31% which is in good agreement with the amount of material removed by enzymic action.

The confirmatory experiments suggest that most of the substances which might be expected to interfere in the chemical estimations do not in fact do so. The effect of lipids has not been investigated and it is possible that they may alter the values obtained in cysteine-sulphuric acid and other reactions. Until lipid is obtained from the lipopolysaccharide in a pure form, it will not be possible to investigate its effect on other estimations.

Cell walls of virulent Bord.pertussis have been isolated and shown to be good protective agents, even after the removal of adherant cytoplasmic material. The lipopolysaccharide present in cell walls and whole cells, had no detectable protective activity. Chemical analysis of lipopolysaccharides from all Bordetella species revealed the presence of hexose, heptose and hexosamine. The proportions of these sugars differed in the material from the various species.

Chapter III.

SEROLOGICAL STUDIES.

Table 19. Agglutination reactions of Bordetella strains

Strain	Antisera to: Virulent Bord. pertussis		Avirulent Bord. pertussis		Bord. parapertussis	Bord. bronchiseptica
	2,000(6)	134	364(3)	146	7,385	8,344
Virulent Bord. pertussis						
L85	25,600	N.T.	1,600	100	50	50
L91	25,600	800	400	50	50	50
W2000	25,600	3,200	400	100	50	100
G1252	12,800	800	200	50	50	50
G1291	12,800	800	200	100	50	50
G1353	25,600	800	200	100	100	100
18/323	12,800	3,200	800	200	50	100
4412	12,800	N.T.	200	50	50	50
4507	25,600	800	800	100	100	200
NCTC 8085	25,600	1,600	200	100	50	200
8090	12,800	N.T.	400	50	50	400
8189	12,800	1,600	200	50	50	200
8474	12,800	1,600	200	50	50	400
8631	12,800	N.T.	400	50	50	50
134	1,600	25,600	50	50	50	50
Avirulent Bord. pertussis						
L51	800	50	800	50	100	50
L54	400	50	400	50	100	100
G1146	100	50	50	12,800	50	100
G1154	100	50	50	12,800	50	50
NCTC 364	100	50	12,800	50	100	100
CN1529	100	50	400	100	50	400
CN2216	400	50	800	100	50	50
4507 Av	800	50	400	50	100	50
Bord. parapertussis						
NCTC 7385	400	200	800	50	12,800	1,600
8250	200	100	100	50	12,800	800
Bord. bronchisepticus						
NCTC 454	100	N.T.	50	50	1,600	6,400
8344	100	50	100	50	1,600	6,400
8761	200	50	200	50	800	6,400
8762	100	50	100	50	400	6,400
H106	100	50	50	50	800	6,400

Serological studies.

All the Bordetella strains used in the present studies were examined by agglutination tests, using antisera prepared against a number of bacterial strains. Antisera were prepared against virulent Bord.pertussis (seven strains), four avirulent strains and one strain each of Bord.parapertussis and Bord.bronchiseptica. The results are shown in table 19.

All the virulent strains except one formed a homogeneous group. The exception was the strain 134 which was used by Pillemer et al. (1954). It also differs from other virulent strains tested, as it does not possess haemagglutinin. The avirulent strains could be divided into two groups, the first, comprising six strains, were agglutinated by antisera to virulent strains, but the second group of two strains (146 and 154) were not agglutinated by the lowest dilution of antisera to virulent strains. The other two Bordetella species formed discrete agglutinative groups giving similar reactions with all the sera tested. Insufficient homologous sera were used with these species to permit the study of strain differences.

The value of agglutination tests is often limited by autoagglutinability of the bacterial cells, such as is found in avirulent strains and in several

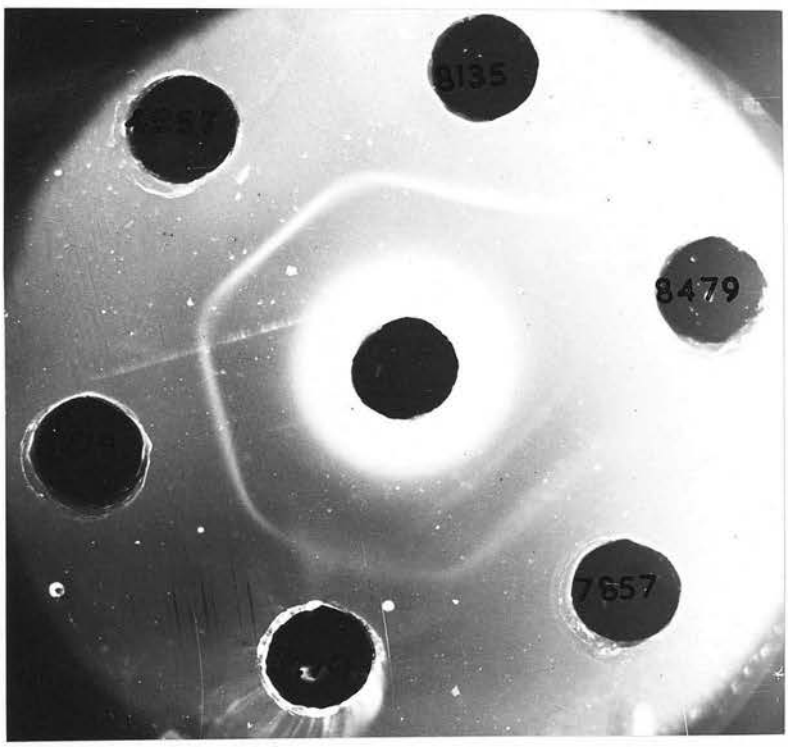

Fig. 9. Antiserum against an avirulent Bord.pertussis strain(2216) tested with Haemophilus extracts. H.parainfluenzae(7857) gives two precipitation lines while H.influenzae(7279,8470), H.suis (4557),and H.aegyptius(8135) give a single line. H.haemolyticus(8479) gave a very faint line which is not visible in the photograph.

of the Haemophilus species. For this reason, gel diffusion tests were used in further studies of Bordetella strains and in attempts to determine the antigenic relationships between the genus Bordetella and the genus Haemophilus. The antigens used in gel diffusion were ultrasonic extracts of t the bacteria. When Haemophilus strains were examined in gel diffusion tests, using antisera prepared against Haemophilus suis strains, about ten lines of precipitation were observed, but when antisera to Bordetella strains were employed, a single line of precipitation was obtained in most cases. The antigen responsible for this line was common to all the Haemophilus species tested and its presence could be shown by all Bordetella antisera except that against the avirulent strain 146. Conversely, a single precipitation line was obtained when the Bordetella antigens, except strains 146 and 154, were tested against antisera prepared against Haemophilus suis. In a few of the Haemophilus strains, a second line was obtained using heterologous sera, but no antisera to these strains were available to permit assay of the Bordetella strains. Fig. 9 shows the reaction between a Bordetella antiserum and a number of Haemophilus antigens.

When Bordetella species were tested using antisera prepared against the different species

and strains, a large number of common antigens were detected. Most sera revealed 10 - 14 antigens common to all three Bordetella species, including virulent and avirulent strains of Bord. pertussis. Two avirulent strains were exceptional, as they possessed only two antigens common to other avirulent strains and to other Bordetella species. Antiserum against strain 146 gave only two lines of precipitation with other Bordetella antigens but gave eight lines with homologous antigens or with antigens prepared from strain 154. These strains have been reported by other workers as being different in many respects from other avirulent Bord. pertussis strains (Rowatt, 1957b; Maclennan, 1960).

The number of antigens common to the Bordetella species made it very difficult to detect species specific or strain specific antigens. Many of the lines of precipitation overlapped, giving large areas of diffuse and heavy precipitation. Efforts to counteract this by altering the concentrations of reactants and the temperature at which reaction occurred were not successful. It was thought that immuno-electrophoresis might give improved separation. A number of buffer systems of varied pH and ionic concentration were tried. When the antigens used were whole bacterial extracts, the best resolution

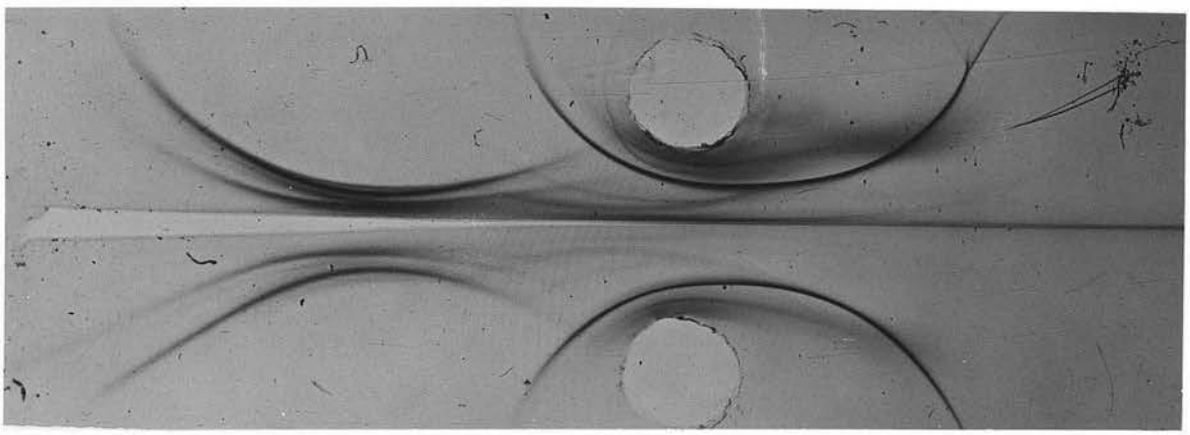

Fig. 10. Immunoelectrophoresis of virulent Bord.pertussis. The circular reservoirs both contain antigens from the virulent strain 4507, while the central reservoir contains antiserum to the virulent Bord.pertussis strain W2000. Most lines of precipitation lie on the anodic side of the reservoirs.

was obtained using phosphate: citrate buffer (pH 7.1) and a current of 5 mA at 50 V for 3 - 4 hr. Under these conditions, up to 18 lines of precipitation could be identified when bacterial extracts were tested with homologous antisera. Most of these antigens were common to all strains of the three species and it was difficult to identify species specific antigens with any certainty. Fig. 10 shows a typical reaction when a virulent Bord. pertussis strain was tested with antiserum to another virulent strain.

Only one apparently species specific antigen was observed when virulent Bord. pertussis antigens were tested with antisera to the same and other virulent strains. The line of precipitation was very diffuse and was very close to the antigen reservoir. A similar line, diffuse and close to the antigen reservoir, was noted when the same systems were examined by gel diffusion. In a few cases the line was present when antisera to virulent strains were used with antigens from avirulent strains and vice versa. The line was never present when antigens of pertussis strains were tested with antisera against heterologous species. A similar line was observed when Bord. parapertussis and Bord. bronchiseptica antigens were tested with homologous antisera. The diffuse nature of the line and its proximity

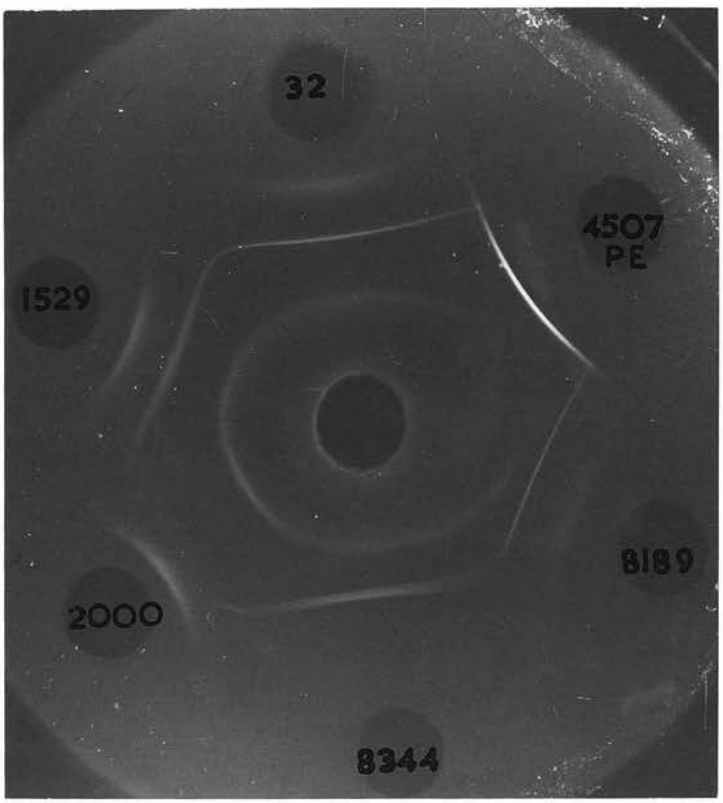

Fig. 11. Antiserum against an avirulent strain of Bord. pertussis(2216) in the central reservoir, tested against extracts from three pertussis strains (8189,32,2000), an avirulent strain (1529) and a Bord.bronchiseptica strain(8344).One reservoir contains lipopolysaccharide from the virulent Bord.pertussis strain(4507 PE).

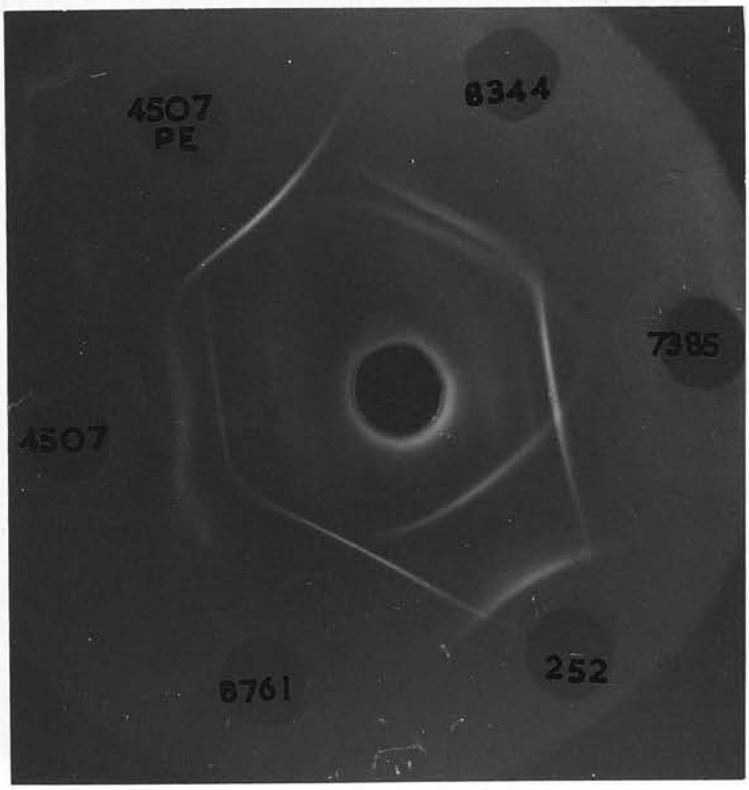

Fig. 12. Antiserum against an avirulent Bord.pertussis strain(2216) tested against extracts from two virulent strains(4507,252), the lipopolysaccharide extracted from one of them(4507 PE), a Bord. parapertussis strain(7385) and two Bord. bronchiseptica strains(8344,8761). The serum is in the central reservoir.

Lipopolysaccharide reactions in gel diffusion.

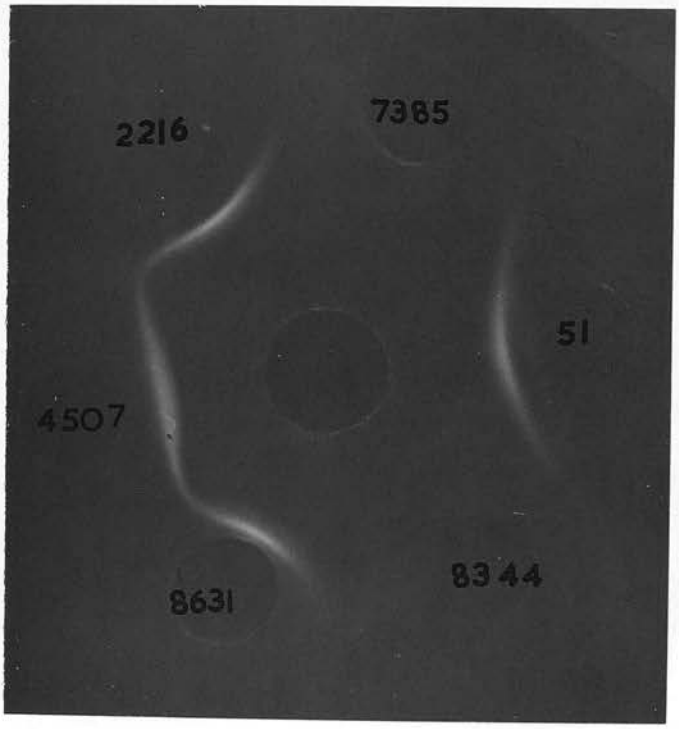

Fig. 13. The central reservoir contains antiserum to a virulent Bord.pertussis strain (85). The peripheral reservoirs contain lipopolysaccharides from two virulent strains (4507,8631), two avirulent strains (2216,51), a Bord. parapertussis strain (7385) and a Bord. bronchiseptica strain (8344).

Lipopolysaccharide reactions in gel diffusion.

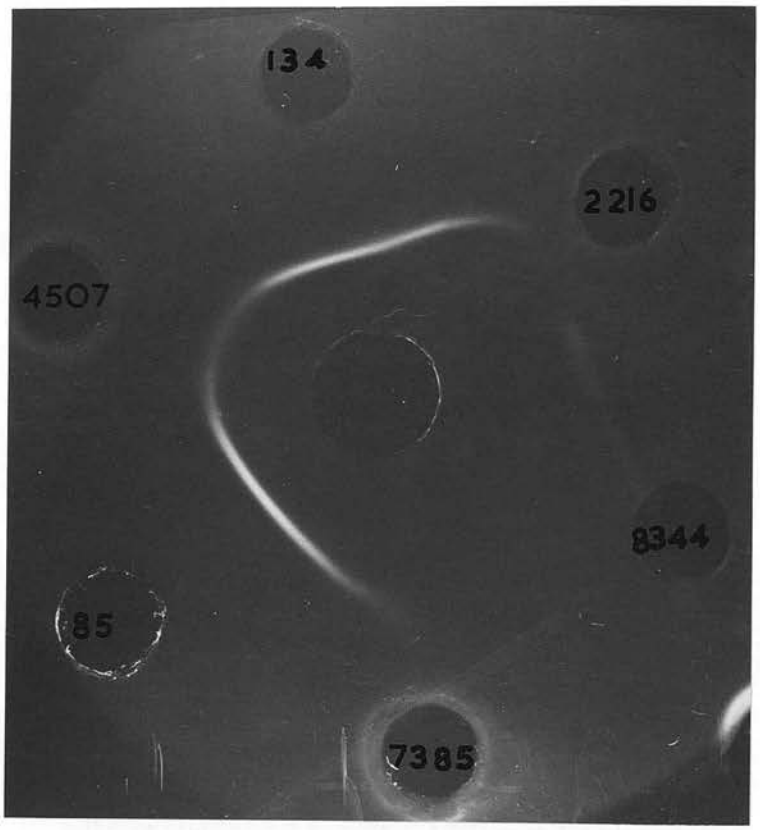

Fig. 14. The central reservoir contains lipopolysaccharide from the virulent Bord.pertussis strain 4507. The other reservoirs contain antisera to three virulent strains (85,4507,134), an avirulent strain (2216), a Bord.parapertussis strain(7385) and a Bord.bronchiseptica strain(8344).

to the antigen reservoir in gel diffusion and immuno-electrophoresis indicated that it might be caused by a lipopolysaccharide. When extracted lipopolysaccharides were tested, reactions of identity were obtained with the diffuse lines in the corresponding whole bacterial extracts. Figures 11 and 12 show typical results obtained when whole cell extracts and the lipopolysaccharides were tested with antisera. These results confirm those of Maclennan (1960), who found that the lipopolysaccharides of the three Bordetella species were species specific. The extracted antisera were tested against a number of antisera but, as they were poorly soluble in saline, they were homogenised by ultrasonic treatment prior to placing in the gel reservoirs. A single, sharp line of precipitation was obtained when the lipopolysaccharides were tested against antibacterial sera to the same species. Lipopolysaccharides from six virulent and avirulent Bord. pertussis strains were shown to be immunologically identical. Antibodies to one of these lipopolysaccharides were shown to be present in all antibacterial Bord. pertussis sera (except strain 146) but not in antisera to other Bordetella species. The lipopolysaccharides were not identical with the antigens revealed by antisera to Haemophilus suis. (figs. 13 and 14).

Lipopolysaccharide reactions in agar gel diffusion.

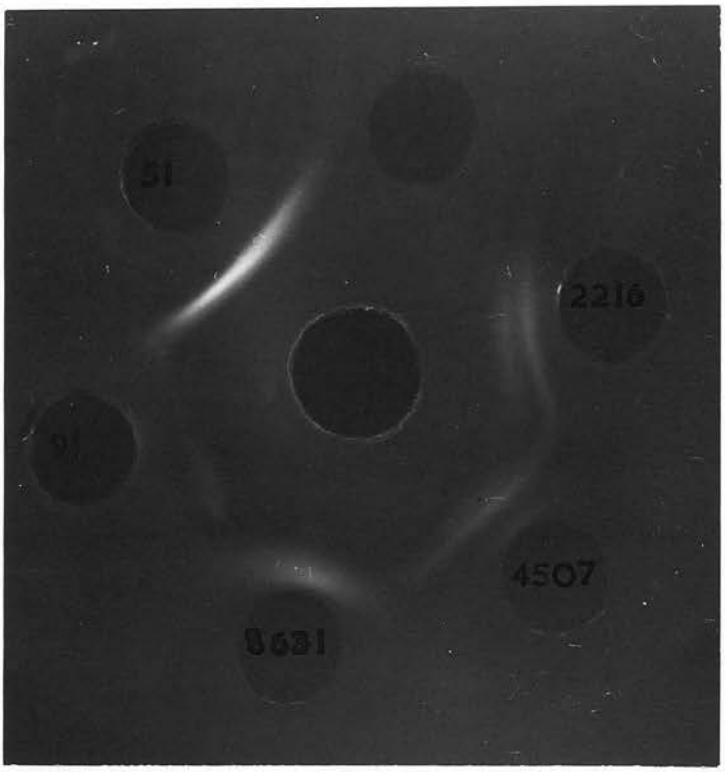

Fig. 15. Bord.pertussis antiserum (to avirulent strain 4507Av) tested against lipopolysaccharides from five pertussis strains. The preparation from strain 2216 gives two lines of precipitation.

In a few tests, two lines of precipitation were obtained when lipopolysaccharides from Bord. pertussis were examined, using antibacterial sera. Fig. 15 shows an example of this. The second line might be due to polysaccharide being liberated by the ultrasonic treatment used to bring the material into solution. As the polysaccharide would have a slightly different diffusion rate from the lipopolysaccharide, two lines of precipitation might thus be formed. Alternatively, there might be more than one specificity within the one molecule, as has been observed in the lipopolysaccharides of Salmonella species (e.g. Staub, 1960). It would require numerous tests with materials from a much larger number of strains and antisera against artificial antigens prepared from the lipopolysaccharides to confirm this. Because of the ease of isolation of the lipopolysaccharides in a form free from other antigens, they were easily identified in gel diffusion. The large number of other antigens detected could not be specifically identified or associated with any particular biological activity as no pure material was available for comparison.

Immunological relationships between the Bordetella species have been studied by the use of agglutination tests and precipitation reactions in agar gel. Although a large number of common antigens exist, the lipopolysaccharides are species specific. Both virulent and avirulent cells of Bord.pertussis possess the same lipopolysaccharide.

Chapter IV.

HAEMAGGLUTININS.

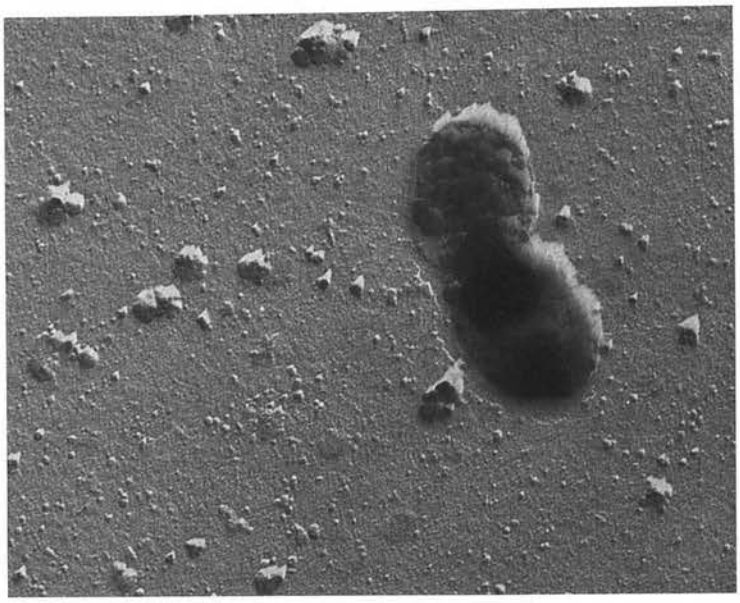

Fig. 16.

Fig. 17. Figs. 16 and 17 show Bord.pertussis cells, strain 4507, fixed with formalin. The excess fixative was removed by dialysis and washing respectively. x 30,000.

HAEMAGGLUTININS OF THE BORDETELLA SPECIES

After it had been shown that haemagglutination and protection by Bord. pertussis cells were due to different antigens (Pillemer, 1950; Masry, 1952), few further studies on the haemagglutinin have been attempted. The ease with which this antigen is lost on subculture and its instability have been reported by many workers (e.g. Standfast, 1951b). The present work is an attempt to determine the nature of the haemagglutinating material of the Bordetella species.

A number of the strains of the three species were examined by electron microscopy to ascertain whether haemagglutination was associated with surface appendages such as fimbriae (Duguid and Gillies, 1957). The bacteria from solid and liquid cultures were tested, medium constituents being removed by washing the formalin - fixed cells with distilled water or by dialysing them against a large volume of distilled water. Figures 16 and 17 show typical preparations after dialysis and washing respectively. The dialysed preparations never revealed anything resembling fibriae, but in 4% of the washed preparations, structures were seen which at first appeared to resemble fimbriae (fig.18). However, they were atypical, being scanty and irregular, and appear to

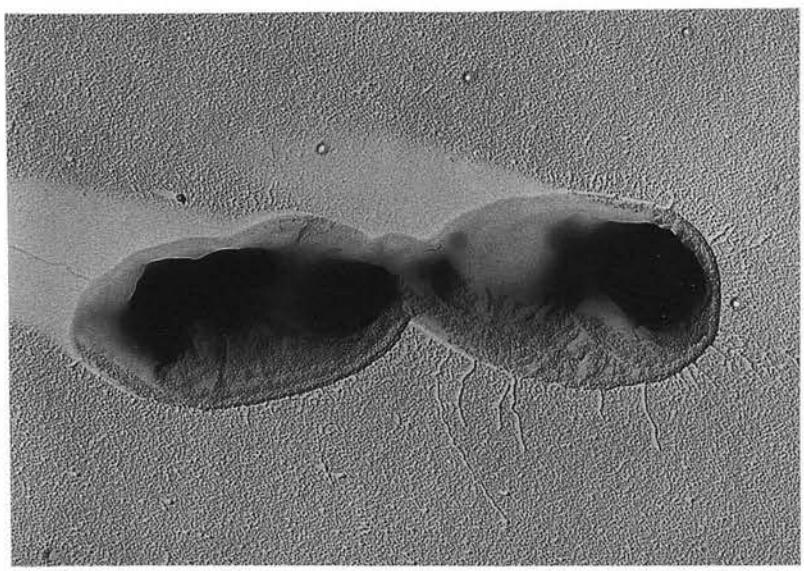

Fig. 18. Bord.pertussis cells (strain 8474). The formalin used for fixing the bacteria was removed by washing with distilled water. At the surface can be seen small filamentous appendages. (x 40,000).

be artifacts from the method of preparation of the specimen, only being found in the washed material andnot during dialysis.

A study of cell - bound haemagglutinin from 48 hr. resin - agar cultures of Bordetella species was then made. The bacteria were suspended in saline, washed briefly by centrifugation and resuspended in saline. When these suspensions were tested for haemagglutination of several erythrocyte species, the results obtained were inconsistent. It seemed that cell - free extracts would provide a better and more easily studied source of haemagglutinin.

The methods of Masry (1952) were first used to obtain soluble haemagglutinin. Extraction of the bacteria with 2.0 M sodium chloride or with 1.0 M sodium acetate at 37° for 48 hrs. yielded solutions, which, after removal of the bacteria by centrifugation at 25,000 G for 20 min., gave titres of 64 to 128. Some autolysis of the bacteria occurred during the extraction, as the soluble preparations contained 5 - 10% nucleic acid. After concentration with polyethylene glycol, the extracts were tested by gel diffusion to determine the number of antigens present. Using antisera against the extracts or against whole bacteria, five or six lines of precipitation were obtained, indicating the presence

Table 20. The effect of oxygen on the haemagglutinin of Bord. parapertussis.(strain 7385).

Time (min.)	Haemagglutination titre in	
	Saline	Thioglycollate saline
0	128	128
10	8	64
20	0	32
30	0	32
40	0	32
50	0	32
60	0	4

of at least these numbers of antigens.

Haemagglutinating extracts were normally stored at 0° in stoppered flasks, but it was observed that there was a rapid fall in titre, resulting in complete loss of activity after 2 - 4 days. This loss of activity could not be ascribed to microbial contamination, as it was unaffected by the presence of antimicrobial agents. It could have been due to autolytic enzymes from the bacteria but their activity at 0° would have been minimal. It was thought that the loss of titre might be due to autoxidation of the extracts. Thus, the haemagglutinating activity might be due to some moeity capable of existing in oxidised and reduced forms, only the latter being active in haemagglutination. A preliminary test showed that oxygenation of the extracts rapidly reduced their haemagglutinating activity. The inhibition was partially reversed by the reducing agent sodium thioglycollate. Table 20 shows the results of an experiment in which oxygen was passed through a solution of haemagglutinin. The solution (50 ml.), was contained in a large boiling tube surrounded with ice and oxygen was passed through at a rate of 15 ml./min. Samples (1 ml.) were withdrawn at varying times and titrated in saline and thioglycollate saline. The

inhibition was initially reversible following reduction, but finally the titre in thioglycollate saline also fell. It seems possible that some surface denaturation of the haemagglutinin occurred due to the agitation over a prolonged period of time. An inert gas, nitrogen, did not cause a similar loss in titre, the initial and final titres being 128 in saline when the same flow rate and other conditions were used. It was difficult to ensure that all the conditions were exactly similar and variations in the size of the gas bubbles might be sufficient to alter the amount of surface denaturation. Similar results were obtained on repeating the experiment using Bord. pertussis haemagglutinin. When de-aerated solutions were used and extracts kept in tightly stoppered vessels under nitrogen at 0^{o}, the haemagglutinating activity was retained for several weeks. Thioglycollate saline was used routinely for all haemagglutination titres henceforth.

An attempt was made to obtain higher titre extracts than those prepared using Masry's (1952) method. A suitable method was developed for extracting bacteria grown for 48 hr. on resin agar. The cells were removed from the culture plates with a sterile scalpel and were suspended in ice - cold 1.0 M sodium chloride solution which

had previously been de-aerated using a suction pump. The cuvettes of a Mickle disintegrator were then filled with the suspension, leaving no air space, and tightly stoppered. They were then shaken at the maximum amplitude for 15 min. The cells were removed from the extract by centrifugation and haemagglutination titres in saline and thioglycollate saline were determined. In both diluents the extracts gave titres up to 2048. In an attempt to determine whether all the haemagglutinin was extracted, a batch of Bord. pertussis cells (strain W 2000) was extracted four times using the above procedure. The first extract had titres of 1024 and 2048 in saline and thioglycollate saline respectively, but no haemagglutinin was detected in the subsequent extracts. It thus seems that a single extraction is sufficient to obtain all the haemagglutinin in the bacteria. No further active material was obtained when cells were submitted to ultrasonic disintegration. The extracted haemagglutinin contained more nucleic acid (10 - 20%) than that prepared by the method of Masry (1952) and this was presumed to be due to lysis of the cells. Gel diffusion indicated the presence of at least seven antigens, all of which were common to extracts from Bord. parapertussis and Bord. pertussis. This extraction procedure did not release any

haemagglutinating material from Bord. bronchiseptica strains, nor did a number of other methods. As the titre of the cells from these organisms fell after extraction, it seemed that the haemagglutinin was possibly very readily inactivated even using mild extraction procedures. Five strains of Bord. bronchiseptica were tested so that it seemed unlikely to be due to differences within the strains.

When the extracted haemagglutinin from five Bord. pertussis strains (4412, 4507, H32, L91 and W2000) and a single Bord. parapertussis strain (7385) were tested against a number of erythrocyte species, they showed the same spectrum of activity. As it was difficult to standardise the different erythrocyte suspensions, differences in titre of one tube were not considered to be significant. When the extracts were diluted to give the same titre with human blood group "O" cells, most other erythrocytes were agglutinated to the same titre (human "A" and "B", horse, sheep, pig, rabbit, mouse and guinea pig), but fowl cells were only agglutinated to one quarter of this titre and ox cells were inagglutinable. Fowl cells were seldom available in adequate amounts, but repetition of the experiment gave the same results. Because of the lack of cells, it was impossible to determine

the validity of Thiele's (1952) hypothesis that separate haemagglutinins existed for human and fowl erythrocytes. It was possible to investigate the inagglutinability of the ox cells. Initially it was thought that cells might have been insufficiently washed and that serum inhibitors might still be present. This seemed unlikely and they remained inagglutinable after ten washings in saline. Fisher (1948a) reported that treatment of fowl erythrocytes with the receptor-destroying enzyme (RDE) of Vibrio cholerae increased their haemagglutination titre. When ox cells were treated with RDE and thoroughly washed, they were agglutinated to the same titre as other erythrocyte species. No increase in titre was observed when sheep or human cells were similarly treated with the enzyme. Attempts to remove the inhibitor from ox erythrocytes by treatment with sodium metaperiodate caused lysis of the cells, and periodate treatment of human cells reduced their titre from 512 to 64. It seems that ox erythrocytes possess some surface component which inhibits the action of Bordetella haemagglutinins. As the inhibitor can be removed by RDE, which is a neuraminidase, it is probable that a neuraminic acid complex is involved.

Mannose, which was inhibitory for some non-fimbrial Escherichia coli haemagglutinins (Duguid

and Gillies, 1957), did not inhibit those of Bord. parapertussis and Bord. pertussis. The Bordetella haemagglutinins were strongly inhibited by the sterile supernatant from cooked - meat medium. Wickham (1956) cited blood group substance in cooked meat medium as an inhibitor of Clostridium welchii haemagglutinin. This seemed unlikely to be the inhibitor of haemagglutination by Bordetella species, as these agglutinated erythrocytes of all three human blood groups to the same titre. Moreover, peptone, which is rich in blood group substance, did not inhibit the haemagglutinins of Bord. pertussis and Bord. parapertussis. From these results, it seems that the inhibitor present in cooked meat medium is not the blood group substance, but it could be lipid of the cholesterol type similar to the stroma inhibitors investigated by Fisher (1948a, 1949).

In an attempt to determine the chemical nature of the haemagglutinin, high-titre extracts were treated with several enzyme preparations. Titrations were then performed at 0^{o} to minimise enzymic action on the erythrocytes and all the necessary controls were included. Lipase, lysozyme and RDE were without effect, but all activity was lost after treatment with papain or trypsin. This result could indicate one or more of three possibilities :

a) the haemagglutinin is a protein destroyed by these enzymes;

b) the enzymes destroy a protein associated with the haemagglutinin and essential for its activity;

c) substances are liberated from other components of the extracts which then inhibit haemagglutination.

The first of these possibilities was the most likely, but the others could not be excluded and it was obvious that further purification of the extracts was necessary.

Warburton and Fisher (1951) absorbed Bord. pertussis haemagglutinin onto aluminium phosphate, then eluted it with 1.0 M sodium chloride solution. Although the haemagglutinin could be removed from the extracts by adsorption onto the phosphate gel, no active material could be eluted from it so other methods of purification were examined. The haemagglutinin was not deposited by ultracentrifugation at 100,000 G for 2 hr. but it could be precipitated by addition of an equal volume of saturated ammonium sulphate solution to the extracts. Unfortunately, much of the haemagglutinating activity was lost when dialysis of the fractions was continued for more than a few hours. The reason for this inactivation could not be found. Any extract containing haemagglutinin

lost its activity on dialysis against distilled water or saline and the activity could not be regained by recombining extract and dialysate, or by the addition of a number of cations. Titres of extracts were not reduced when saline containing 0.01% (w/v) versene was used as diluent, the erythrocytes also being washed and suspended in this solution. This indicated that the loss of activity was not due to the effect of metallic ions. It seemed that the haemagglutinin might be adsorbed onto the dialysis membrane, but no loss in titre was noted when strips of cellophane were suspended in the extracts. The conditions of this experiment were, of course, different from those prevailing inside a dialysis tube where osmotic pressure is involved. When an extract was passed under pressure through a cellophane membrane, no haemagglutinating activity was detected in the eluate. Extraction of the membrane by passing 1.0 M sodium chloride through it, in the opposite direction from the extract, produced a solution with high haemagglutinin titre suggesting that it had been adsorbed onto the membrane.

An alternative method of removing the high concentration of salts present in the extracts was desirable and the use of the cross - linked dextran known as sephadex (Porath and Flodin, 1959) was investigated. Passage of the extracts through

columns of sephadex suspended in distilled water, resulted in absorption of most of the salts, as measured by tests with silver nitrate. The eluate from the column possessed all the original haemagglutinating activity. Application of the de-salted extracts to solumns of DEAE - cellulose equilibrated with 0.02 M phosphate buffer was attempted as a method of further purification, but when the columns were eluted with 1.0 M sodium chloride, thermostable haemagglutinins were eluted from the cellulose along with the thermolabile bacterial haemagglutinin. The presence of these haemagglutinins in ion-exchange celluloses has previously been reported by Schmidt, Grossgebauer and Hartmann, (1960).

The lability of the haemagglutinating extracts, the difficulty of obtaining large amounts of material, and the complications encountered during their purification made continued study of this aspect unjustifiable in terms of the amount of time and labour involved, although the methods used have proved valuable in studying other products of the Bordetella cells.

Soluble haemagglutinin has been extracted from Bord.pertussis and Bord.parapertussis. It was reversibly inactivated by oxygen, but was destroyed by proteolytic enzymes. A further type of inactivation was encountered during dialysis. Extracted haemagglutinin gave the same titre with most erythrocyte species, but ox cells were inagglutinable unless treated with neuraminidase. Haemagglutination was inhibited by a component of cooked meat medium.

Chapter V.

PIGMENTS.

PIGMENTS PRODUCED BY BORDETELLA SPECIES

1) Blue pigment.

Although it has been known for some years that a brown pigment is produced by Bord. parapertussis (Bradford and Slavin, 1937), a recent report has indicated that Bord. pertussis cells contain a blue material. (Hammersten et al., 1959). This blue pigment was found in the washings of large amounts of bacteria grown in 500 litre cultures and was thought to be blue in the oxidised form. A small quantity of these washings, obtained from the Karolinska Institute in Stockholm, was dark blue in colour. It was not feasible to obtain the material from Bord. pertussis in this laboratory, because of the difficulty in growing large amounts of the bacteria. As the other two Bordetella species can be cultured much more readily, this led to their examination to determine whether they contained an analogous blue material.

When Bord. bronchiseptica cells were harvested from casamino acid medium by centrifugation, the bacterial deposits were at first pink or buff in colour, but when they were left exposed to the air for any length of time, the surface of the deposits became grey-green. Similarly, if the cells were suspended in a small volume of saline or distilled water, the passage of air or oxygen through the suspension rapidly

rendered it greyish - green. Thus it appeared probable that this species also contained a pigment. Attempts to extract it by stirring the cells in Krebs - Ringer solution were unsuccessful, but this could have been due to the use of a different type of stirrer from that employed in Stockholm. It seemed that the use of a very high speed stirrer and also the different cultural conditions might have caused the pertussis cells to lyse and liberate their pigment. When urea was added to the Krebs - Ringer solution, the pigment was readily extracted from the bronchiseptica cells.

a) Method of extraction.

After trying several methods, the following extraction procedure was adopted:

i) the unwashed bacteria harvested from 5 litres of medium (approximately 10 g. dry weight) was frozen at - 40°.

ii) the deposit was thawed at room temperature and suspended in 200 ml. of Krebs - Ringer solution containing 0.1 M urea, and the suspension was stirred for 30 min. in the cold. It was then centrifuged at 25,000 G for 60 min. at 0°. The supernatant was retained.

iii) the bacteria were re-extracted as in (ii).

iv) the deposited cells were then extracted twice with cold distilled water.

The final deposit of cells was pink in colour and did not change colour on oxygenation. It was freeze - dried, and kept for other studies. The four extracts were pooled and formed a viscous, bluish - green solution, which was concentrated to a small volume using polyethylene glycol. This solution was very viscous and opalescent, and it was centrifuged at 50,000 G for 30 min. The deposit was discarded, and the supernatant was dialysed against 0.02 M phosphate buffer at pH 7.2.

b) Purification.

The extraction procedure was found to liberate about 1% of the dry weight of the cells and immunoelectrophoresis of the product showed that at least 15 antigens were present. The dialysed extract was now applied to a column packed with DEAE - cellulose and allowed to flow through under gravity. The blue material passed through the column while most of the other components present were retained. Elution of the column with saturated sodium chloride solution, followed by dialysis against distilled water and lyophilisation, showed that 90% of the non-dialysable material in the extract had been retained by the DEAE - cellulose. After the blue material had passed through, the columns were normally discarded. The blue solution was concentrated, dialysed against 0.02 M acetate

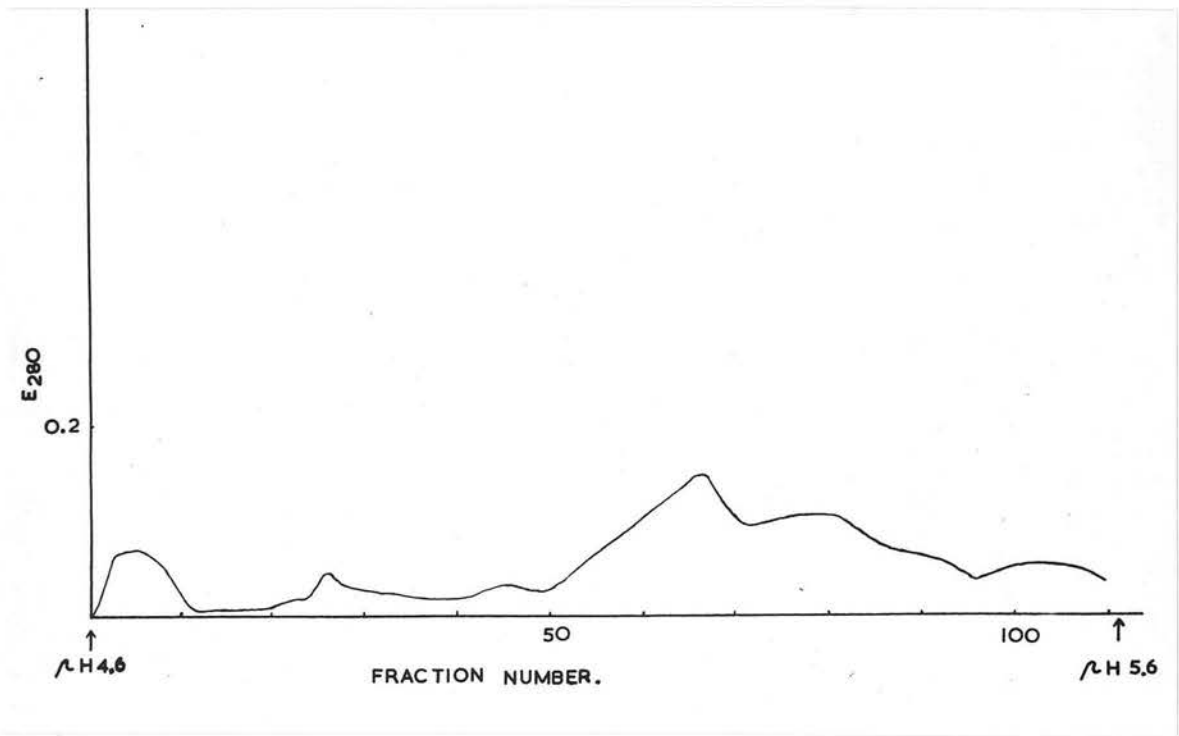

Fig. 19. The ultraviolet absorption (at 280 mμ) of material from pigment extracts, eluted from CM-cellulose under pH gradient (acetate 0.02 M, pH 4.6-5.6).

buffer for 48 hr. and centrifuged at 100,000 G for 15 min. It was then dialysed against the same buffer for a further 24 hr. and applied to a column of CM - cellulose. The blue material was retained on the column but could be eluted by using a pH gradient of acetate buffer (0.02 M) to a final pH value of 5.6. A mixing vessel of 200 ml. volume initially filled with buffer at pH 4.6 was used. The buffer was pumped through the column at a flow rate of 10 ml. per hour, and fractions of 4 ml. volume were collected. The blue material began to elute at a pH value of 5.12. The ultraviolet absorption of the material eluting from the column is shown in fig. 19. The fractions containing the blue pigment were pooled, concentrated, dialysed and re-chromatographed on a fresh column of CM - cellulose using the same conditions as above. The blue fractions were again collected, concentrated, dialysed thoroughly against ditilled water and stored at - 40°. In a typical preparation, 20 g. of bacteria yielded 20 mg. of this material.

Similar material was obtained from all five strains of Bord. bronchiseptica and purified in this way. The same extraction and purification procedure was applied to the two Bord. parapertussis strains. Ion exchange celluloses were used in exactly the same way to purify the

Bord. pertussis material and it was also applied to concentrated resin-medium culture supernatants of this organism, yielding small amounts of blue material from three strains (4412, 4507 and 8474) but not from two strains which did not produce haemagglutinin although virulent in all other characteristics (134, and a derivative of 4412), or from three avirulent strains (4507 AV, L51, and L46). One of the strains, the 4412 derivative, was grown on a 500 litre scale in Stockholm so that it was unlikely that production of the blue pigment could have been overlooked.

Immuno-electrophoresis was used to check the purity of the preparations. The eluates from the CM - cellulose columns contained three components in most cases, although some contained as many as five. However, the pertussis preparations and one of those from Bord. bronchiseptica appeared to be homogeneous. The differences in purity were thought to be due to differing numbers of components in the crude extracts, caused by varying degrees of lysis of the bacteria. Attempts to purify the pigment further, using amino-ethyl cellulose and ECTEOLA - cellulose were unsuccessful. Pigment which was apparently homogeneous could be obtained after electrophoresis on cellulose columns or in agar gel using phosphate :citrate buffer at pH 5.7, but both methods had disadvantages which prevented their use on a preparative scale. No satisfactory

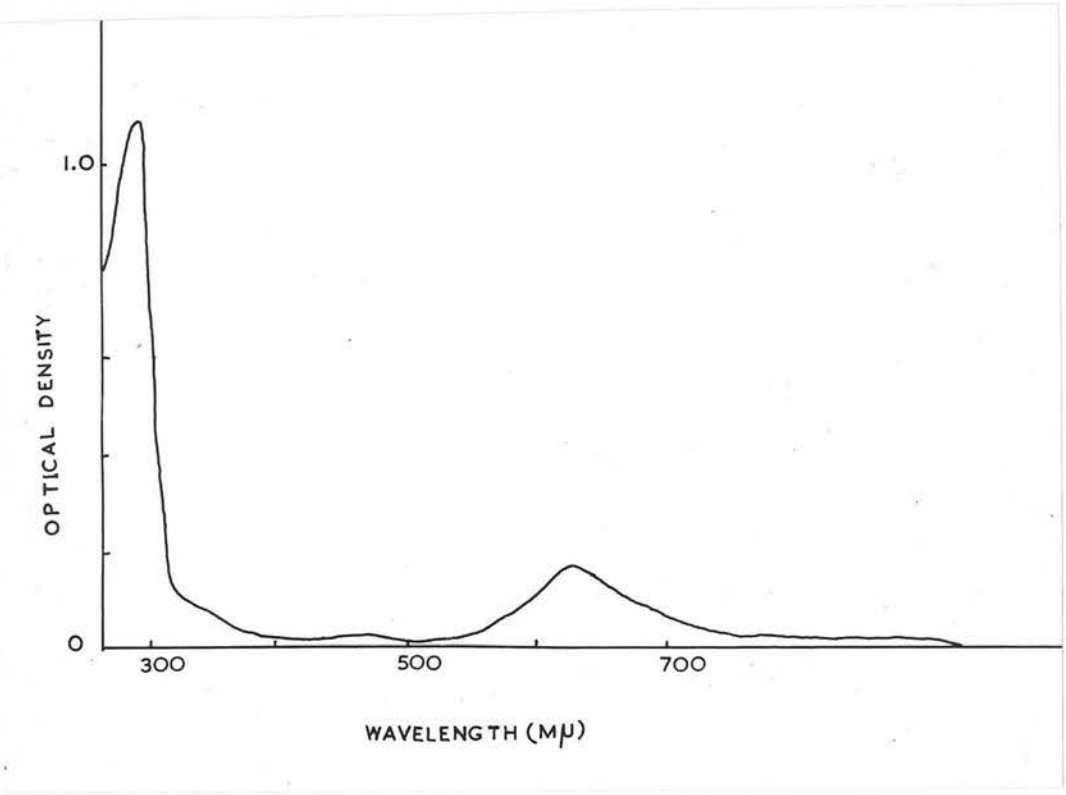

Fig. 20. Absorption spectrum of the blue pigment extracted from Bord. bronchiseptica strain 8344 (oxidised form).

electrophoresis columns were available and the pigment could only be purified on a very small scale, recovery involving the cutting of columns. Electrophoresis in agar gel separated the pigment from other components, but it could not be quantitatively eluted from the gel.

c) Properties.

i) The pigments from all three Bordetella species could be purified in the same way, suggesting that they were similar in their physical properties. In particular, they all started to elute from a CM - cellulose column at the same pH value of 5.12.

ii) Under electrophoresis in agar gel at pH 6.7, all three pigments moved towards the cathode at approximately 1 cm./hr. when a current of 50 milliamps. at 50 volts potential difference was used. This suggests that, under these conditions, the molecules posses a similar overall positive charge.

iii) Continuous spectra on all preparations showed the presence of absorption peaks at 280 mμ and 625 mμ. In the purest preparations, the ratio $E_{625} : E_{280}$ was 0.475. Fig. 20 shows a typical absorption curve for a Bord. bronchiseptica preparation. The addition of suitable reducing agents caused the peaks at 625 mμ to disappear, but no new peaks appeard.

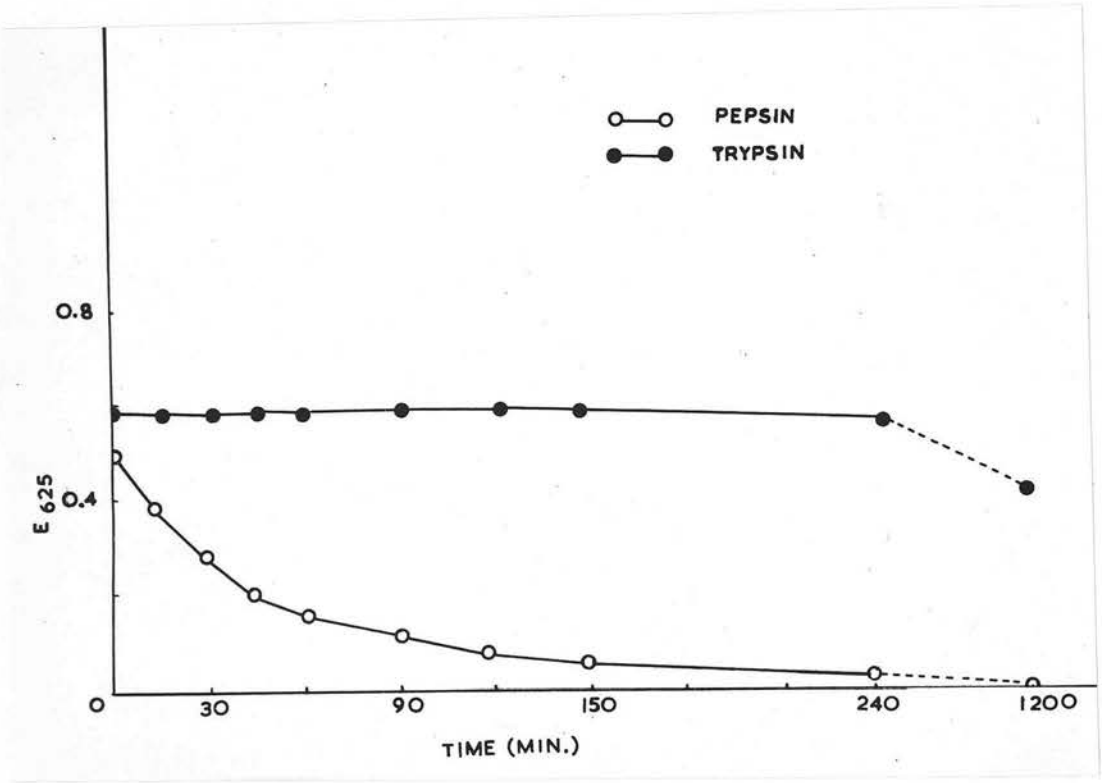

Fig. 21. Destruction of Bord.bronchiseptica blue pigment by proteolytic enzymes (as measured by reduction of absorption at 625 mμ).

iv) All pigments could be reduced by glutathione, cysteine or ascorbic acid, rendering them completely colourless. The reduction could be reversed by dialysing away the reducing agent or by oxidation with air or oxygen.

v) The presence of the absorption peak at 280 mμ suggested that the pigments might be proteins or peptides. Analysis of a pertussis preparation revealed a nitrogen content of 14.3% such as might be expected in a typical protein.

vi) The colour was rapidly destroyed when preparations were treated with the enzyme pepsin (1 mg./ml.) at pH 2.5. A control experiment showed that no change in colour occurred in the absence of the enzyme. Trypsin or chymotrypsin, at the same concentration, caused a slight fall in the absorption of a solution at 625 mμ, after prolonged incubation at pH 7.5 and 37°. The fall in the absorption with the various enzymic treatments is shown in fig. 21. The results for trypsin and chymotrypsin were almost identical and the effect of trypsin only is shown. These results indicate that the protein is rapidly digested by pepsin, but that the other two enzymes only act on it slowly. It would be necessary to confirm the presence of free amino groups to ascertain that digestion was actually occurring.

vii) The blue colour was not lost on prolonged dialysis against ethylene diamine tetracetate at pH 7.0, nor was there any apparent change following dialysis over the pH range 3.0 to 8.0.

viii) The blue colour was destroyed by boiling and could not be regained by oxidation.

ix) When incubated at 37° in the presence of washed cell suspensions of Bord. pertussis, Bord. bronchiseptica or Bacillus cereus the blue material from Bord. pertussis was rapidly reduced. The pigment was not destroyed and could be recovered from the supernatants and quantitatively reoxidised with oxygen. No reduction was observed when the cells and pigment were separated by dialysis membrane.

x) The colour of the oxidised form of the blue material and the absence of colour in the reduced form were indicative of a metal-protein complex. The position of the absorption maximum indicated that any metal present might be copper. A sample of a Bord. bronchiseptica preparation was dialysed for several days against twice-distilled water, then freeze-dried. An aliquot of the product was then homogenised in a platinum crucible with a weighed amount of "specpure" alumina and 0.5 ml. of twice-distilled water. The homogenate was gently

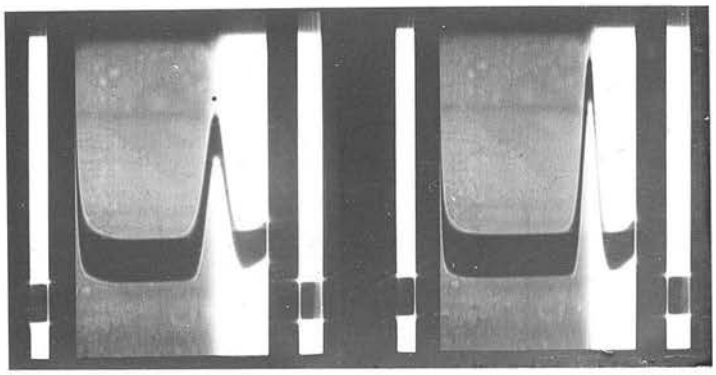

Fig. 22b. Fig. 22a.

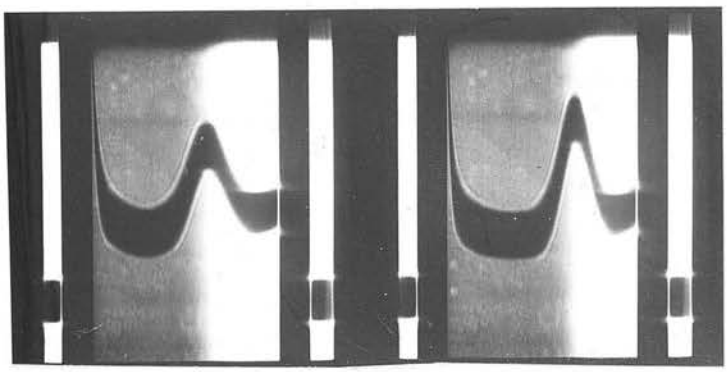

Fig. 22d. Fig. 22c.

Fig. 22 shows the sedimentation pattern of the blue pigment from Bord.bronchiseptica (strain 8344) in the Spinco model E analytical centrifuge. The pigment (10 mg./ml.) was dissolved in phosphate buffer,(0.2M, pH 7.2). Photographs were taken on reaching full speed (a), and after 16 (b), 48 (c) and 60 (d) minutes.

heated to dryness and incinerated at about 300°, until all the carbon had been volatilised. The sample was then analysed spectrographically (this was kindly performed by Dr. Purves,, Department of Agriculture, Edinburgh University). The material contained 0.156% copper and 0.013% barium. Manganese, iron and strontium were not present in detectable amounts. The presence of barium is somewhat surprising, as this metal is not known to have any biological activity or to enter into complexes with proteins. The presence of copper explains the blue colour of the metalloprotein complex.

xi) Ultracentrifuge studies were made on the blue pigment from the same strain that was used for the metal analysis. The sedimentation patterns showed only one component (fig. 22). The blue colour of the pigment (seen as a white background to the boundary) migrated with the peak and sedimentation occurred slowly. From a series of runs using different concentrations of the pigment in 0.2 M phosphate buffer (pH 7.2), the sedimentation coefficient was found to be $S_{20,w} = 1.59 \times 10^{-13}$. The diffusion coefficient calculated during a sedimentation run over 90 min. was $D_{20,w} = 9.1 \times 10^{-7}$. From

these values, the molecular weight was calculated to be 16, 500 ± 2,900. (The ultracentrifugal analysis and calculations were kindly performed by Dr. Cruft, Biochemistry Department, Edinburgh University).

The results for the copper content and the molecular weight indicate that the complex contains approximately one atom of copper / two molecules of protein.

2) Red - brown pigment.

When the blue pigment was eluted from the first CM - cellulose column, using a pH gradient, the top of the column was seen to be reddish brown in colour. This colour had obviously been masked previously by the excess of blue pigment. The red material was not eluted by the acetate buffer at pH 5.6 nor by phosphate buffer (0.02 M, pH 7.2) applied directly to the column. It could be removed by phosphate buffer containing 2.0 M sodium chloride and was partially purified by applying it to a fresh column after dialysis against acetate buffer (0.02 M, pH 5.6). It was then eluted using pH and salt gradient to 2 M sodium chloride and phosphate buffer (pH 7.2). There was only a small amount of red pigment in the extracts, but it was found in pertussis extracts and supernatants and in the Bord. bronchiseptica extracts. No red pigment was found in any of the

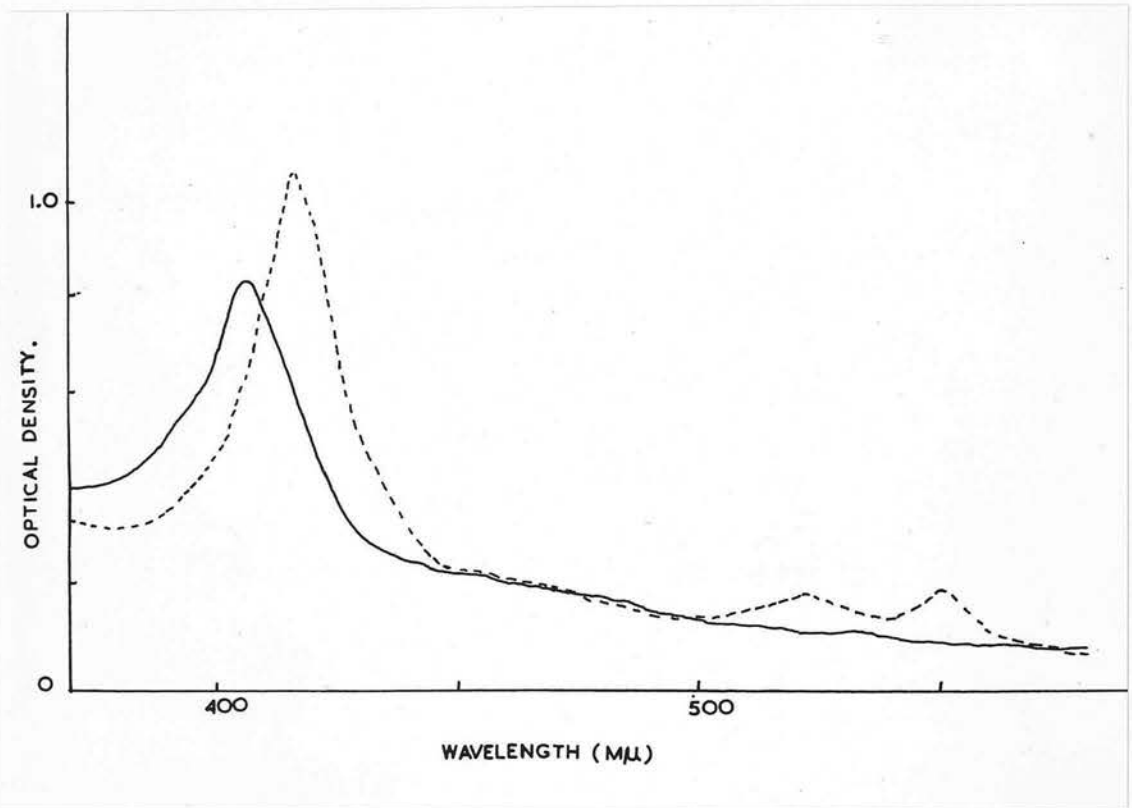

Fig. 23. Absorption spectrum of Bord.bronchiseptica(strain 8344) cytochrome. Continuous line, oxidised form; broken line, reduced form.

bacterial strains which did not contain blue pigment.

The small amounts of the material available prevented detailed analysis. The colour of the preparations suggested that they might contain cytochromes although tests with Nadi's reagent did not indicate the presence of cytochrome oxidase. Continuous spectra revealed the presence of absorption maxima at 280 mμ, and 408 mμ in the oxidised state. When the pigment was reduced with cysteine, the peak at 280 mμ remained but that at 408 mμ disappeared and was replaced by one at 416 mμ. Two new peaks appeared at 522 mμ and 550 mμ. Fig. 23 shows continuous spectra of the pigment from Bord. bronchiseptica 8344. These optical properties indicated that the pigment resembled cytochrome c. A preparation of mammalian cytochrome c (Sigma Chemicals, St. Louis, Missouri) showed an absorption maximum at 410 mμ in the oxidised form and maxima at 416 mμ, 520 mμ and 549 mμ in the reduced form. The mammalian preparation also adsorbed onto the CM-cellulose and eluted from it under the same conditions as the bacterial pigment. This suggests that the red material is possibly a bacterial form of cytochrome c. The yeild of the bacterial cytochrome was approximately 33 μg./g. of dry cells. The content of this pigment is thus considerably lower than that of the blue pigment.

Two pigments have been isolated from Bordetella species for the first time. One of these, a protein containing copper (0.156%), had an absorption maximum at 625 mμ in the oxidised form. It was extracted from virulent cells of all three species. The second pigment was a bacterial cytochrome having some similarities to mammalian cytochrome c. It was isolated from Bord.pertussis and Bord.bronchiseptica.

DISCUSSION.

Vaccines for the prevention of whooping cough are normally prepared from whole bacteria which have been killed by heating or by formaldehyde. Although these vaccines are relatively effective when administered to young children, difficulties have been encountered in obtaining adequate yields of virulent cells. During culture, Bord. pertussis readily becomes avirulent but, as avirulent cells afford little protection, it is essential that the cultures employed in vaccine production are composed solely of virulent cells. A study of several culture media was made in an attempt to obtain optimal growth conditions for the culture of virulent bacteria.

As the whole bacteria contain a number of antigens which have been shown to have little or no effect in experimental infection, it is preferable that a protective fraction should be obtained. The material used for prophylaxis should contain a minimal amount of the antigens which play no active role in protection. Few such fractions have been studied previously, and only one, the stroma protective antigen (SPA) of Pillemer (1950) has been used in human prophylaxis. The study was not extensive and further investigations of cell-free protective material arenecessary.

1) Media for Bord. pertussis.

The development of media for the growth of virulent Bord. pertussis has been complicated by the presence of substances which inhibit its growth. Two inhibitors of growth in casein hydrolysate liquid media have been identified as colloidal sulphur or sulphides (Proom, 1955) and unsaturated fatty acids (Pollock, 1947); a third inhibitor, possibly a peroxide, has been invoked by Rowatt (1957a). The origin of these inhibitors has been the source of some speculation. Colloidal sulphur and sulphides are produced by the destruction of sulphur-containing amino acids during heat sterilisation of the medium. Although the formation of these compounds is minimised by sterilising any additional organic sulphur source by filtration, small quantities of cystine and cysteine are present in casein hydrolysate. The unsaturated fatty acids are presumed to originate mainly in lipids and the use of a hydrolysate of purified casein, such as casamino acids, is desirable. Peroxides are unstable and difficult to detect and their mode of formation is unknown.

To permit growth, any inhibitors present in the medium must be removed by the addition of adsorbents such as charcoal, starch or anion exchange resins. Charcoal was not used in liquid

media, as its small particle size renders it difficult to work with and to remove from the culture vessels. Starch has been shown by many workers to permit good growth, but as it is non-dialysable, it hinders the study of extracellular, high molecular-weight products of the bacteria.

Anion exchange resins, which are obtained as large granules, are able to support good growth of virulent Bord. pertussis and they can easily be removed by coarse sintered glass filters or by decanting the medium. Poorer growth was obtained when the resin was enclosed in a dialysis sac. This could be due to the time taken by the inhibitors to pass through the membrane to the resin. Medium containing resin permitted growth in the presence of amounts of oleic acid or autoclaved cysteine which were completely inhibitory in starch medium, indicating that resin was a superior adsorbent. The necessity for the presence of the adsorbent during growth is more difficult to explain. Toxic factors may be introduced into the medium with the bacterial inoculum, but with the relatively small inocula used in the present experiments, this is unlikely. The bacteria may also elaborate toxic factors during growth and these may accumulate until the adsorptive capacity of the resin is exceeded.

The process of adsorption of materials onto the anion exchange resins is a chemical equilibrium which is subject to various conditions. Thus, the increasing alkalinity of the medium during growth may cause partial elution of growth inhibitors previously adsorbed onto the resin. It is also possible that substances formed during the growth of Bord. pertussis adsorb onto the resin more readily than some of the growth inhibitors. The growth in resin medium seems to confirm that starch is required only as an adsorbing agent and not as a matabolite, as has been suggested by James (1949).

Growth stopped at a pH value of about 8.2 in either starch or resin medium. When the buffering capacity of the medium was increased with tris, higher yields of bacteria were obtained. The pH requirements of the medium appeared to be twofold : i) the initial pH value should be high enough (7.0 - 7.5) to give an adequate rate of growth, and ii) the buffering capacity should be such that the rise in pH during growth is minimised. Vaccines prepared from a number of different casamino acid media showed similar protective capacities in mice and were in no way inferior to a standard vaccine grown in Cohen Wheeler medium. As the cells obtained were typical in their properties of protective ability,

haemagglutination and agglutinability, the tris-resin medium seemed ideal for the study of virulent Bord. pertussis. It has also been used as the basis of a solid medium which gave good growth. The tris-resin medium would seem to be ideal for vaccine production, yielding large quantities of virulent bacteria and at the same time, being cheap and easy to prepare.

2) Cell-wall material.

Following the disruption of virulent Bord. pertussis, it was possible to confirm the results of other workers who have shown that cell-wall preparations are protective (Munoz et al., 1959; Billaudelle et al., 1960). Even after washing with sodium chloride solutions and with distilled water, cytoplasmic material adhered to the cell walls and could be detected by electron microscopy. It was possible that the protection was due to the cytoplasmic material rather than to the cell wall. However, when extraneous material was liberated from the cell walls by treatment with enzymes or with a detergent (sodium lauryl sulphate), the protective capacity was retained, although no cytoplasmic material was visible by electron microscopy and the preparations were no longer toxic for mice. The cytoplasmic membranes

of bacteria which have been examined, are composed of lipoproteins (McQuillen, 1960) and trypsin, lipase or detergent solubilise them, leaving the intact cell wall. It seems from the results of protective tests, that the cell wall or some part of it contains the protective agent against intracerebral challenge in mice.

It is interesting that in a species such as Bord. pertussis, most of the protective activity should be associated with the cell wall and not with the labile toxin or other antigen such as the haemagglutinin or the HSF. The role of these antigens in human and animal infection is almost unknown. Although labile toxin and haemagglutinin were absent from the cell wall after enzymic treatment, HSF. was present in all except one preparation. The HSF. may be a protein, as it has been shown to be thermolabile by Pittman (1951b). This suggestion is strengthened by the inactivation of the HSF. following treatment with the proteolytic enzyme trypsin. As relatively large doses of material, of the order of 1 mg., were required to effect sensitisation, the amounts of HSF. present were probably small. Other workers have shown that cell-wall material contains HSF, (Munoz et al., 1959), but protective material free from HSF. has been prepared by Dolby (1958), indicating that the antigen is

not essential for protection. It thus seems that the most suitable material for use as a protective agent is tyypsinised cell wall.

Although the cell walls can be used as protective agents in experimental animals, the very stringent sterile precautions required lead to difficulties when they are used in human prophylaxis. Two methods of preparations may be used. i) All techniques may be carried out with sterile precautions. A programme of this type was used by Pillemer et al. (1954) in preparing stroma protective antigen. Many technical difficulties must be overcome if sterility is to be maintained. ii) The second type of preparation employs non-sterile techniques and the final product is sterilised by filtration, heat or chemicals. Fewer technical problems are involved, but the final sterilisation presents several difficulties. Filtration cannot be used, as the cell walls will not pass through a sterilising filter. Formalin cannot be used, as it has been shown to inactivate protective material (Billaudelle, 1960), as did heating when whole-cell vaccines were tested (Pittman, 1952). Merthiolate or some other suitable mild chemical antimicrobial agent could possibly be used or the vaccine might be irradiated with ultraviolet light.

If a suitable method of sterilisation could be evolved, there appears to be no reason why trypsin-treated cell wall should not be used to immunise young children against whooping cough.

Even after enzymic treatment, the cell wall is a complex chemical entity containing lipids and a variety of polymers including polysaccharide and protein. The role of the lipopolysaccharide in the protective activity of the cell wall appears to be a minor one, but its removal from the other components would probably involve drastic procedures which might destroy any protective capacity of the remainder. It is conceivable that although some moiety of the cell wall functions as a protective antigen, it could no longer do so when the cell wall is resolved into its molecular units. Thus, the lipopolysaccharide might be necessary as an adjuvant to stimulate the formation of the antibodies against other cell-wall material. The lipopolysaccharide is itself a hapten and it may be associated with a peptide in the natural state. Although neither of these are antigenic when separated, the complex could be antigenic and could stimulate the formation of antibodies with specificities directed against the two components. The antibodies to the lipopolysaccharide were shown to be devoid of protective activity as their removal from a serum

prepared against whole bacteria left the antiserum as potent as it was prior to adsorption. Many lipopolysaccharides from Gram negative bacteria are endotoxins, but the evidence for such activity in preparations from Bord. pertussis is conflicting. Baeva (1957) claimed that phenol-extracted lipopolysaccharide from this organism was toxic for mice, but Maclennan (1960) was unable to confirm this result. The presence of endotoxin in a vaccine is undesirable but if the cell wall of Bord. pertussis was used, the amount of lipopolysaccharide present would be insufficient to make the vaccine unduly toxic.

The protective material in the trypsin or detergent treated cell wall may be identical with the protective antigen obtained by Pillemer et al. (1954) and adsorbed onto red cell stromata. No accurate determination of the amount of protective antigen in Bord. pertussis cells could be made, but Pillemer and his co-workers estimated that it comprised less than 1% of the nitrogenous material in ultrasonic extracts of the bacteria. The ultrasonic treatment would split the cell wall into small fragments and many of these would contain lipopolysaccharide. Neter et al. (1956) showed that, under certain conditions, lipopolysaccharides could be adsorbed onto red cells. It is possible.

that fragments of Bord. pertussis cell walls, containing protective material, are adsorbed onto red cell stromata, the association being due to the presence of the lipopolysaccharide.

Treatment of the cell wall with lysozyme removed relatively little material and the only effect on the chemical composition was to reduce the hexosamine content. The substrate of this enzyme is known to be mucopeptides containing glusosamine. Much more material was removed by the action of lipase or sodium lauryl sulphate. These act by splitting lipid-protein bonds and thus solubilising the cytoplasmic membrane which was known to be associated with the crude cell walls. The proportion of sugars in the cell walls was increased due to the removal of the protein and the lipid.

Chemical analysis of the lipopolysaccharides from the different Bordetella species showed some similarities. All the materials contained hexose, hexosamine and heptose, and in each case, nucleic acid was present as a contaminant. The values obtained for the sugars differ from those of Maclennan (1960), the most marked differences being in the preparations from virulent Bord. pertussis. The contents of hexose, hexosamine and heptose were 18%, 9% and 23% respectively, whereas the

corresponding values obtained by Maclennan were 6%, 20% and 34%. It is possible that the variation in these results is due to differences between the lipopolysaccharides produced by different strains of Bord. pertussis. The smaller differences obtained for the other species could be due to the presence of differing amounts of nucleic acid, the ribose content of which might interfere in some of the estimations.

The chromophores in the cysteine-sulphuric acid reactions used for the estimation of hexose and heptose are unknown. Thus, although glucosamine was shown to have no effect on the determinations, this might not be true of small quantities of other sugars and other compounds. The presence of small amounts of peptides, not detectable by the Biuret test, could act as a source of cysteine which would alter the results. Fatty acids from hydrolysed lipids might also have some effect. It is probable that small amounts of other sugars are present, as was indicated in some chromatograms, but more material from a larger number of strains of all species would be required to confirm this.

A further paradox is seen in the results for Bord. pertussis lipopolysaccharides. These contained 18% of hexose as estimated by the cysteine-sulphuric acid reaction, but no apparent

hexose spots could be detected by paper chromatography using the anisidine spray reagent. It is possible that the hexose gives a spot which is coincident with that of heptose in all the solvent systems studied. A similar occurrence has been recently reported by Bain and Knox (1960) during chromatographic studies on hydrolysed lipopolysaccharides from Pasteurella multocida. These workers obtained spots corresponding to hexoses but none corresponding to heptose, despite a content of 19% of the latter sugar when estimated by the cysteine-sulphuric acid method. They were able to confirm the presence of a heptose spot, coincident with galactose, by applying the hydrolysates as a band and eluting the sugars from the chromatogram prior to estimation. The eluate from the galactose spot gave reactions for hexose and heptose.

The hexosamine observed in the Bordetella chromatograms appears to be glucosamine and/or galactosamine. These two sugars behave similarly in paper chromatography and no galactosamine was available for comparison. The identity of the heptose remains uncertain. The chromatographic evidence indicated that it might be D-glycero-L-mannoheptose, but isolation of the sugar and its characterisation would be necessary to confirm its

identity. The optical enantiomorph L-glycero-D-mannoheptose behaves similarly in chromatography and preparation of the heptitols or other suitable derivatives is necessary to differentiate between these two heptoses.

The high nitrogen values, over 6% in some preparations, are unusual in lipopolysaccharides. The hexosamine content of 16% would account for 0.8% of nitrogen and this element would also be present in nucleic acids. An accurate estimate of the nitrogen present in the latter source is impossible without knowing which purine and pyrimidine bases are present. The nitrogen content might also be increased by the presence of small amounts of peptides. The low phosphorus values might be due to the presence of nucleic acids, but could also be derived from phospholipids.

3) Serology.

Agglutination tests showed that the Bordetella strains tested formed six serological groups. The majority of virulent strains of Bord. pertussis formed a single group, thus confirming the results obtained by a number of other workers (Standfast, 1951b; Ungar et al., 1954). One strain (134) was exceptional, being poorly agglutinated by antisera prepared against other virulent strains. This

strain has been used as a source of protective material but possesses no haemagglutinin (Pillemer et al., 1954), and it apparently differs also in its surface properties. As it is poorly agglutinated by antisera against avirulent strains, it does not appear to be a true intermediate between the virulent and avirulent states.

The avirulent Bord. pertussis strains also formed two groups, but one of these, comprising the strains 146 and 154, has now been accepted as being unlike other avirulent strains (Rowatt, 1957b). These two strains have been subcultured in the laboratory for a very long time and are of unknown origin. It is possibly best to omit them from consideration with other avirulent strains, as they bear no resemblence to any other known strain or organism. The small number of strains and antisera used did not detect any serological differences among the Bord. parapertussis or among the Bord. bronchiseptica strains respectively.

Although the agglutination tests gave an indication of antigenic similarities between the surfaces of different bacterial strains, they were of little value in indicating the number of antigens shared by these strains. An extensive sharing of antigens between strains of the different species was indicated using the technique of

precipitation in agar gel. Even strain 134 showed no noticeable differences from other strains when examined by this method. The two avirulent strains 146 and 154, were completely different from all other strains, possessing only two antigens common to them. Nor were these two strains similar to the Haemophilus species which also possessed one or two antigens common to Bordetella strains.

The large number of common antigens in the Bordetella strains rendered the identification of species specific or strain specific antigens very difficult. No better results could be obtained using immuno-electrophoresis. The only components which could be positively identified were the lipopolysaccharides. As these materials could be obtained free from other antigens, their identification with the lines in the whole-cell extracts was relatively easy. It was possible to confirm the results of Maclennan (1960), who found that each Bordetella species possessed a specific lipopolysaccharide. As the lipopolysaccharide found in Bord. pertussis was common to virulent and avirulent strains, this explains the earlier finding that it had no significant role in protection, as it is known that avirulent strains do not protect against experimental infection

(North et al., 1941).

The occurrence of a second line of precipitation when some lipopolysaccharides were tested by gel diffusion, was similar to the result of Maclennan (1960), using the material obtained from Bord. bronchiseptica. It is possible that the ultrasonic treatment used to suspend the samples, released some polysaccharide, thus giving two substances with the same antigenic specificity but with different molecular size and diffusion rate. This might cause a second line of precipitation. In support of this theory, the second line is nearer the antibody reservoir than the main line, and so it is probably caused by a molecular species which diffuses more rapidly than the lipopolysaccharide. There are other possibilities however, as it has been suggested that one antibody-antigen system may give more than one line of precipitation under certain conditions (Grabar, 1957).

4) Haemagglutinins.

Differences in the haemagglutinins of the three Bordetella species can be observed during culture. In Bord. pertussis, this antigen is rapidly lost on subculture and it is therefore a useful criterion of the virulent state, being easily

tested, qualitatively and quantitatively. The other two species retain their haemagglutinins despite prolonged subculture.

The haemagglutinin could be extracted in a soluble form, from Bord. pertussis and Bord. parapertussis but not from Bord. bronchiseptica. The antigen is possible inactivated by the extraction procedure in the third species. The two soluble haemagglutinins were readily inactivated by oxidation, but the activity could be partially restored by the addition of reducing agents. The inactivity of the oxidised forms may explain the results of previous workers who have found that the haemagglutinin is inactivated on storgge, the deterioration being independent of temperature. A second type of inactivation occurred, but it was irreversible and was probably due to denaturation. This lability made the task of purification extremely difficult and a further complication was encountered when it was found that all activity was rapidly lost when soluble haemagglutinin was dialysed. No explanation could be found for this third type of inactivation. Evidence of the protein nature of the haemagglutinin was also obtained by its destruction by the proteolytic enzymes, papain and trypsin.

The finding that most erythrocyte species were agglutinated to the same titre indicated that the

receptor on the red cell surface is present in all species. In ox cells, the receptors were covered with a substance which could be removed by the enzyme neuraminidase. A similar substance may be present in fowl cells and would explain the results which led Thiele (1950) to speculate on the existence of distinct haemagglutinins for human and fowl cells. The inhibitory action of a compound in cooked meat medium indicated that it was a lipid. These substances have been extensively studied by Fisher (1948b, 1949), who confirmed the presence of lipid receptors on the erythrocyte surface.

5) Pigments.

a) Blue pigment.

Although it was indicated by Hammarsten et al. (1959) that Bord. pertussis contained some form of blue material, no attempt was made to identify the substance or to determine its properties. The present work has shown that a blue pigment is produced by all three Bordetella species, but it has not been found in avirulent Bord. pertussis. The physical properties of the pigment and the common method of isolation indicate that the same material is produced by each species. The substance has been purified and it appears to be a protein of low molecular weight forming a stable complex

with copper.

Similar compounds have been isolated from other bacterial species and they have been given specific names in preference to using such terms as "blue pigment". The bright blue colour of the Bordetella pigment has led to the suggestion of the name "azurin". A green pigment from Desulphovibrio desulphuricans has been named desulphoviridin by Postgate (1956), and helicorubin was applied to a red pigment extracted from snails of the genus Helix (Keilin, 1956). Although these other materials have been given more definitive names by the inclusion of the generic title, any combination of Bordetella and azurin seems unnecessarily clumsy.

Although no previous report of azurin exists, similar pigments have been isolated from other microorganisms. The material most closely resembling azurin was isolated by Horio (1958a) from Pseudomonas aeruginosa. It also had an absorption maximum at 625 mμ in the oxidised form, but was colourless when reduced. In crude extracts, it, too, was associated with red pigments of the cytochrome type. In a further paper (Horio, 1958b) the Pseudomonas blue pigment was shown to have a copper content of 0.212%. It acted as an electron acceptor in two enzymic systems, yeast lactic dehydrogenase and pig heart

cytochrome c reductase. A blue pigment found in Pseudomonas lemonnieri (Hugo and Turner, 1957) also had an absorption peak at 625 mμ but differed from azurin as it was insoluble in water.

Although no attempt has so far been made to determine the function of azurin, there seems little doubt that it is a respiratory pigment associated with oxygen uptake. Azurin is autoxidisable, unlike the pigment from Pseudomonas aeruginosa (Horio, 1958b) and so, could act as an intermediary between the non-autoxidisable cytochromes and atmospheric oxygen. It might be involved in the oxidative deamination of amino acids, as the Bordetella species are very active in this respect and this might explain the high azurin content of the cells (1 mg./ g. dry cells). However, azurin has not been obtained from cells of avirulent Bord. pertussis, suggesting that this form differs metabolically from the other species. It is intriguing that in this species the change from the virulent to the avirulent state is associated with the loss of so many factors: protective capacity, haemagglutinin, toxin, HSF., and azurin. It seems that during laboratory culture Bord. pertussis is so adapted that these properties are no longer required.

b) Red pigment.

Cytochrome compounds similar to cytochrome c

in their spectroscopic properties have been isolated from a number of bacteria (Postgate, 1956; Horio, 1958a; Gibson, 1961), but there is no previous report of the isolation of such compounds from the genus Bordetella. The cytochrome was present in much smaller amounts than the azurin and it has not yet been obtained from Bord. parapertussis. The absorption maxima are very close to those of mammalian cytochrome c, and to the figures given by Horio (1958a) for a cytochrome isolated from Pseudomonas aeruginosa, the only other pathogenic species which has been extensively investigated. Although a number of pigments from bacterial sources show some similarities to cytochrome c obtained from animals, there is no previous report of chemical similarities such as the common method of purification using CM-cellulose.

SUMMARY.

1). A liquid medium supporting growth of virulent Bordetella pertussis has been described. It contains casamino acids, nicotinamide, glutathione tris buffer and inorganic salts as well as an anion exchange resin. The medium is reproducable, giving good yields of bacteria and is also cheap and easy to prepare. After the resin has been removed by decantation or filtration, the medium contains only dialysable components. Cells grown in this medium produce haemagglutinin and protective antigen. The liquid medium can be solidified by the addition of agar and then compares favourably with Bordet-Gengou medium for the growth of Bordetella species.

2). The protective activity of Bordetella pertussis cells grown in tris-resin medium was assayed by intracerebral challenge in mice and was shown to reside in the cell wall. The activity was not destroyed by treatment of the cell wall with trypsin, lipase or detergent, although these removed adherant cytoplasmic material and trypsin destroyed the histamine-sensitising factor. The lipopolysaccharide moiety of the cell wall had no major role in protection.

3). The lipopolysaccharides from Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica showed some chemical similarities, containing hexosamine, heptose and hexose in varying proportions. The lipopolysaccharides were species specific, but that isolated from Bord.pertussis was present in virulent and avirulent strains.

4). Agglutination tests showed some similarities between strains of the same Bordetella species. Precipitation tests in agar gel revealed a large number of antigens common to all three species. The only antigens which could be identified were the species specific lipopolysaccharides. Two antigens were found to be common to the Bordetella and Haemophilus genera.

5). Soluble haemagglutinins were prepared from Bord. pertussis and Bord. parapertussis. They were reversibly inactivated by oxygen and all activity was rapidly lost on dialysis. They were destroyed by the proteolytic enzyme trypsin. An inhibitor to haemagglutination was found in cooked meat medium, and an inhibitory material, removable by treatment with neuraminidase, was found on ox erythrocytes. Most red cell species were agglutinated to the same titre.

6). Two previously unknown pigments have been isolated from Bordetella species. A blue pigment, for which the name azurin is suggested, has been obtained from virulent strains of all three species. Azurin is a protein of low molecular weight, approximately 16,500, containing copper (0.156%). It has absorption maxima at 280 mμ and 625 mμ in the oxidised form, but the peak in the visible wavelengths disappears on reduction with cysteine or thioglycollate. Azurin is autoxidisable.

A cytochrome of the c type has been isolated from Bord.pertussis and Bord. bronchiseptica. It has absorption maxima at 408 mμ in the oxidised form and at 416 mμ, 522 mμ and 550 mμ in the reduced form. The method used for the purification of the bacterial cytochrome could also be applied to mammalian cytochrome c.

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