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GENE TRANSFER IN BORDETELLA PERTUSSIS

by

SALEH FADHEL MOHSIN AL-SALLAMI

Presented for the degree of Doctor of Philosophy in the Faculty of Science, University of Glasgow.

Department of Microbiology. December, 1981.
Declaration

This thesis is the original work of the author. He wishes to acknowledge that all animal experimentation was done in collaboration with Dr. R. Parton.
ACKNOWLEDGEMENTS
ACKNOWLEDGEMENTS

I should like to express my thanks to Drs. Roger Parton and John Coote for their guidance, encouragement, conscientious supervision and inexhaustible patience throughout my research.

Thanks are also due to Professor A.C. Wardlaw for his academic guidance & readiness to solve my economical and social problems.

I express my thanks to the Ministries of Health and Higher Education in Aden, who provided my Grant. My thanks also go to Mrs. Elizabeth Berry for her willingness to help and to Mrs. Anne Mosson for her fast and efficient typing.
SUMMARY

In order to have available a procedure for gene transfer in *Bordetella pertussis*, the transformation system described originally by Branefors (1964) was re-examined and further developed. Under her original conditions, few streptomycin-resistant transformants were obtained in the present investigation when DNA prepared from the 44122/7R streptomycin-resistant strain was incubated with competent 44122/7 streptomycin-sensitive cells.

In attempts to improve the transformation frequency, conditions for DNA isolation, preparation of competent cells and exposure of recipient cells to DNA were examined in detail. Best results were obtained when bacteria (10^8 ml^-1) of strain 44122/7 taken at the end of exponential growth in modified Stainer and Scholte medium (1971) were chilled on ice for 40 min in the presence of DNA prepared by the procedure of Marmur (1961) after lysis of the cells by the method of Schwinghamer (1980).

The conditions which had a significant favourable effect on transformation were age of recipient culture, exposure of cells to DNA on solid rather than in liquid media, and chilling and exposure of recipient cells to dibutyryl cyclic AMP. Under these conditions there was obtained a 500-fold increase in transformation frequency compared with the original Branefors conditions; cAMP and CaCl₂ showed only little or no consistent beneficial value.

Another approach to gene transfer involved the use of plasmid R68.45 to mobilize a chromosomal marker in *Bordetella pertussis*. This plasmid, which confers resistance to carben-
icillin, kanamycin and tetracycline, was reproducibly transferred from Escherichia coli to the 44122/7R B. pertussis strain. This was demonstrated by the 7R strain acquiring resistance to the 3 plasmid-borne antibiotics to which it previously had been sensitive. However, there was no evidence that the streptomycin-resistance marker on the B. pertussis chromosome was mobilized by the plasmid. That the plasmid did have intrinsic mobilizing capability was shown by the chromosomal marker, nalidixic acid resistance, which was transferred from one E. coli strain to another by this vector.

In experiments to characterize the Branefors strains, it was observed that the streptomycin-resistant strain, unlike the sensitive strain, was highly virulent for mice by the intracerebral route. The average LD$_{50}$ of the Str$^R$ strain was 360 c.f.u. compared with $>7.9\times10^3$ for the Str$^S$ strain. In contrast, there was no significant difference in intranasal virulence between these strains.

In addition to the higher intracerebral virulence, the streptomycin-resistant strain possessed agglutinogen 2, was more sensitive to penicillin, erythromycin, tetracycline and ampicillin, appeared to contain more histamine-sensitizing factor and less heat-labile toxin than the parent strain. SDS-PAGE showed that there is no difference in the X-mode bands, indicating that they are not involved in the i.c. virulence.

Investigation of 4 streptomycin-resistant transformants and 3 mutants which had been freshly-isolated from the parent strain showed a strong correlation between streptomycin-resistance and intracerebral virulence. However, there was
one mutant (unfortunately lost) where this correlation did not obtain. The above Str$^R$ strains resembled the original Branefors Str$^R$ strain in having increased sensitivity to other antibiotics although they did not possess agglutinogen 2.

When the virulence of strain 44122/7R was attenuated by repeated subculture on trypticase soya agar, streptomycin-resistance was unaffected. This showed that genes for intracerebral virulence and Str$^R$ are not identical.

In contrast to the findings of Parker (1976) and Kloos et al., (1978) the addition of Dowex-1-chloride, charcoal or starch to solidified Stainer and Scholte liquid medium did not give satisfactory growth for the proposed genetical studies.

The main result of this investigation is the establishment of an efficient gene-transfer system in *B. pertussis* which should be useful in further studies of the physiology and pathogenic properties of the bacterium.

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<tr>
<td>B-G</td>
<td>Bordet-Gengou agar</td>
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<td>CAA</td>
<td>Casamino acids solution</td>
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<tr>
<td>car</td>
<td>Carbenicillin</td>
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<td>cAMP</td>
<td>Cyclic adenosine-3'-5' monophosphate</td>
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<td>cfu</td>
<td>Colony forming units</td>
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<td>Chromosomal mobilization ability</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ED$_{50}$</td>
<td>Median effective dose</td>
<td></td>
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<tr>
<td>F-HA</td>
<td>Fimbrial haemagglutinin</td>
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<td>HA</td>
<td>Haemagglutinin</td>
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<td>HSF</td>
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<td>IAP</td>
<td>Islets-activating protein</td>
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<td>in</td>
<td>Intranasal</td>
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<tr>
<td>IU</td>
<td>International unit</td>
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<td>kana</td>
<td>Kanamycin</td>
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<tr>
<td>K</td>
<td>Thousand</td>
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<tr>
<td>LD$_{50}$</td>
<td>Median lethal dose</td>
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<tr>
<td>LPF</td>
<td>Lymphocytosis or leukocytosis-promoting factor</td>
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<tr>
<td>LPF-HA</td>
<td>LPF haemagglutinin</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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MPT  Mouse protection test
MRC  Medical Research Council
NTG  N-methyl-N'-nitro-N-nitrosoguanidine
NA  nutrient agar
NaI  nalidixic acid
ou  opacity unit
PA  Protective antigen
PBS  Dulbecco A phosphate-buffered saline
PD$_{50}$  Median protective dose
PHLS  Public Health Laboratory Service
PIF  Polymorphonuclear leukocyte-inhibitory factor
RNA  Ribonucleic acid
rpm  Revolutions per minute
SDS  Sodium dodecyl sulphate
SDS-PAGE  Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Str$^R$  Streptomycin-resistant
Str$^S$  Streptomycin-sensitive
Trans  Transformant
Tris  Tris-(hydroxymethyl) aminomethane
TSA  Trypticase soya agar
X-mode  Xanthic mode: normal state of *Bordetella pertussis*
SSC  0.15M saline plus 0.015M sodium citrate
7S  Streptomycin-sensitive 44122/7S strain of Branefors
7R  Streptomycin-resistant 44122/7R strain of Branefors
INTRODUCTION
1. **Taxonomy of *Bordetella pertussis***

*Bordetella pertussis* was originally listed in the genus *Haemophilus* in an early generic arrangement of bacteria published by Bergey *et al.* (1923). The other members of the present genus *Bordetella* were also placed originally in *Haemophilus*. Andersen (1953) noted that *Haemophilus pertussis*, *H. parapertussis*, and *H. bronchiseptica* had a common 0-antigen and haemorrhagic toxin. Although Winslow (1920) suggested that the three species be classified as one group, it was Moreno-Lopez (1952) who first proposed the term *Bordetella* for this group of organisms and renamed them *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica*. Thus the genus *Bordetella*, belonging to the family *Brucellaceae* of the order *Eubacteriales*, is divided into three species. The main characters used to differentiate these species are summarized in Table 1. *B. pertussis* is non-motile and does not grow on plain nutrient agar; *B. parapertussis* is non-motile but grows on nutrient agar; *B. bronchiseptica* is motile and grows on nutrient agar. *B. pertussis* is pathogenic for man (Bordet and Gengou, 1906) as is *B. parapertussis* (Bradford and Slavin, 1937). *B. pertussis* causes whooping cough which is essentially a disease of infancy and early childhood. However, *B. parapertussis* causes about 5% and sometimes up to 20 to 30% of the reported cases of whooping cough in the United States and Europe (Lautrop, 1971; Linnemann and Perry, 1977). *B. bronchiseptica* is primarily an animal pathogen. It was first isolated from dogs by Ferry in 1911 and was isolated from a child with whooping cough by Brown in 1926 (Lautrop and Lacey, 1960). Its antigenic relatedness to
B. pertussis was shown by Ferry and Noble (1918) by means of agglutination tests and by Ferry and Flix (1918) using a complement fixation procedure.

2. Pertussis: the disease

It is surprising that pertussis remains one of the least understood of the common infectious diseases despite the recent accumulation of much data concerning the biology of Bordetella pertussis.

The first written description of the disease (according to Cone, 1970) appeared in 1578, when an epidemic occurred in the city of Paris and was recorded by Baillou. Further epidemics in England between 1670 and 1680 were described by Sydenham (1676). The cause of the disease was not known until Bordet and Gengou (1906) first isolated the causative organism. The aetiology was confirmed in 1933 by the MacDonalds who inoculated their own children with a fresh isolate of B. pertussis (MacDonald and MacDonald, 1933).

The disease was certainly both severe and dreaded in the years before the 2nd World War. It caused epidemics with large fluctuations in the number of notifications and a considerable number of deaths each year. In 1958 there was a striking fall in the numbers of both notifications and deaths, but since then the trend has been somewhat irregular (MRC, 1977).

Whooping cough is an acute, communicable disease characterized by spasmodic attacks of coughing that are accompanied by an inspiratory whoop. The disease is transmitted directly by
Table 1. Differential characteristics of the three Bordetella species.

<table>
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<th>B. bronchiseptica</th>
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<td>Motility</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>Growth on plain agar medium</td>
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<td>+</td>
<td>+</td>
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<td>Tyrosinase production</td>
<td>-</td>
<td>+</td>
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<td>Splitting of urea</td>
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<td>+</td>
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<td>Oxidase</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>Catalase</td>
<td>+ or - *</td>
<td>+</td>
<td>+</td>
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<td>Reduction of nitrates</td>
<td>-</td>
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<td>Utilization of citrate</td>
<td>-</td>
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<td>+</td>
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<td>Growth on peptone agar</td>
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<td>Factor 14</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G + C content moles %</td>
<td>61</td>
<td>61</td>
<td>66</td>
</tr>
</tbody>
</table>

** Data selected from Lautrop (1960) and Pittman (1974)

* 70% of freshly isolated strains are positive.
droplets from coughing or sneezing. The most infectious period is early in the disease. B. pertussis is non-invasive and in a typical case, there is an incubation period of seven to fourteen days followed by three main stages, the catarrhal, the paroxysmal and the convalescent (Bradford, 1959). During the catarrhal stage the cough is only occasional with rhinorrhea and tearing. In the acute or paroxysmal stage, the paroxysms may vary in number from a few to twenty or more per day. Severe paroxysms may last for 5-10 min and the child may nearly strangle in expectorating the thick, viscid mucus. Occasional cyanosis is seen and vomiting is frequent. In complicated cases secondary infection may lead to death, most frequently due to pneumonia, haemorrhagic events and occasionally toxic encephalitis; the most frequent cause of death is secondary infection. At about the fourth week, the number and severity of the paroxysms decrease and the convalescent period begins. Full recovery from the infection can take up to several months and there is no passive transfer of immunity from mother to foetus (Lorber, 1975).

The infection induces profound biochemical alterations in the blood. For example, Regan and Tolstoouhov (1936) found the average values of pH, inorganic phosphate and glucose were decreased in the blood of whooping cough patients whereas uric acid was increased. A significant inhibition of the hyperglycaemic response in the blood of patients following administration of epinephrine was reported by Badr-El-Din et al. (1976)

The disease occurs at all times of year with a peak incidence during the winter months. Pertussis has no natural
animal reservoir and no healthy carriers (Wilson and Miles, 1975).

The mortality rate from pertussis began to decline markedly in some countries, notably North America, around the beginning of this century (Bradford, 1960). This decline was thought to be the result of improved nutrition and of significant shifts in the demographic characteristics of the population (Gordon and Hood, 1951). Miller et al. (1974) suggested that vaccination which was introduced on a national scale in the U.K. in 1957, also has had a great influence on decline of incidence of pertussis. Bassili and Stewart (1976), however, considered that improvement in socio-economic conditions were more important than immunization in leading to this decline. Stewart (1977) plotted death rates from whooping cough at ages 0-15 years. He claimed that for Scotland the logarithmic regression line between 1943 and 1957 predicted very accurately the actual subsequent slope of the curve to low level reached in 1963. A similar finding was made with the data from England and Wales. However, Stewart carried out his conclusion by selecting data from the periods 1950-56; 1943-57 and 1929-57. Had he used the data for the whole of the period 1919-57 the pre-vaccination decline would have appeared slower and the decline since 1957 more marked (MRC, 1977).

Figure 1 shows the decline in the number of notifications and deaths from pertussis in England and Wales during 1940-1976.
Figure 1.

Whooping cough: Notifications and deaths in England and Wales from 1940-1976. (Joint Committee on Vaccination and Immunization, 1977).

--- notification

--- deaths
Whooping cough vaccination introduced on a national scale.

-Notifications

-Deaths
3. Pertussis vaccine and vaccination

Since proven cases of reinfection are rare, Laing and Hay (1902) postulated that the immunity to pertussis is long-lasting. Bordet (1906), investigating the nature of immunity to pertussis, demonstrated antibodies to Bordetella pertussis in the sera of children who had recovered from the disease. Since this original study, much attention has been paid to the development of vaccines capable of producing an antibacterial immunity. In general, the vaccines have consisted of whole bacilli which have been killed by various means such as heat, formaldehyde and merthiolate (Evans, 1942).

Zachariassen, 1929 (quoted by Madsen, 1933) was the first to demonstrate a degree of protection by a plain vaccine given shortly before an epidemic of whooping cough in the Faroe Islands. Only 67.5% of the vaccinated children contracted the disease compared with 98.2% in the unvaccinated group. In America and Canada, experience indicated that pertussis vaccine could produce significant protection. Kendrick and Eldering (1939) found a home exposure attack rate of 89.4% in unvaccinated children and 34.95% in those who had received Michigan vaccine. In those exposed to infection at school an even better degree of protection estimated at 90%, was found.

The earliest British trials of pertussis vaccines from 1942 to 1944 were organised by the Whooping Cough Immunization Committee of the MRC. Children attending nurseries and Welfare Clinics were immunized or left unimmunized in a controlled fashion. However, no significant difference was found between
the incidence of attack between 1948 and 1951 in children who had received three injections of one or other of different batches of British vaccines and unvaccinated children (MRC, 1977). In a later series of field trials in Britain, the difference between vaccinated and unvaccinated children was significant. The average attack rate in the home exposure for 10 trials was 18.2% in the vaccinated and 87.3% in the unvaccinated groups of children and cases in the vaccinated groups were on average, less severe and of shorter duration than those in the unvaccinated (MRC, 1951).

Prior to the studies of the Medical Research Council (1951, 1956, 1959) the only country possessing a standard pertussis vaccine was the United States (National Institute of Health, 1948). A British Reference Vaccine was established in 1957 as one giving 80% protection against home exposure. As a result of comparing potency of various vaccines in the MRC field trials and in the intracerebral mouse protection test (MPT) the mouse test of Kendrick et al. (1947) became a standard procedure in the control laboratory (MRC, 1959). By 1964, this test was recommended by the World Health Organization as the standard assay for pertussis vaccine protective potency (WHO, 1964).

The decline in 1958-1961 in the numbers of both notification and deaths due to pertussis to less than half the rates experienced by all ages in 1954-1957 was followed by a further decline in each subsequent four year period up to 1973 (MRC, 1977). However, the wave of pertussis during 1963 and 1964 had raised doubts concerning the efficacy of the vaccine used in earlier
years. Thus, in 1966, the Public Health Laboratory Service constituted a working party to investigate the field experience with whooping cough vaccine. The investigation showed that after home exposure to the disease, 56% of the vaccinated children developed a paroxysmal cough compared with 67% of unvaccinated children. This report led Preston and Evans (1963) and Preston (1965) to examine freshly isolated strains of *B. pertussis* from cases of whooping cough.

They reported a change in the prevalent serotypes from the type 1, 2 and 1, 2, 3 common before 1958 to type 1, 3 strains which were dominant in 1963-1964. These findings were confirmed during a further study of the working party (PHLS, 1973) and it was concluded that the change in serotypic composition of the prevalent bacteria might have accounted for the reduction in efficacy of the vaccine in use from 1963-1968. The British Pharmacopoeia requirements for the potency of pertussis vaccine were then altered and a better degree of protection was observed in children who received the new vaccines (Preston and Stanbridge, 1972).

Experience showed that well organized mass immunization programmes with the currently available pertussis vaccines were capable of reducing the incidence of whooping cough in children by a factor of 50 and that the incidence of serious untoward reactions was considered to be exceedingly rare. However, a number of problems are still associated with present day vaccine. Different lots of vaccine, made in the same way, from the same strains, sometimes show different properties.
According to Griffith (1978), different vaccines vary in quality, safety and efficacy according to the production strains of *B. pertussis*, the method of manufacture and quality control procedures.

There are indications that pertussis vaccine occasionally produces severe reactions in children with permanent sequelae or death. Whether all such sequelae are caused by pertussis vaccines or whether they occur by chance in relation to the date of inoculation is disputed. Stuart-Harris (1978), stated that in the United Kingdom the assessment of cases reported to the Committee on the Safety of Medicines showed that most of the illnesses temporally associated with vaccination did not appear to be clinically different from illnesses continually occurring in children of similar age irrespective of recent inoculation. It is therefore difficult to believe they are the result of a neurotoxin that Stewart (1977) described as a component of the vaccine. Hannick and Cohen (1979) noted a small but significant rise in insulin levels of infants 8 h after vaccination.

It has been reported that a full course of immunization with vaccines containing a pertussis component not only reduces the attack-rate of whooping cough, but also the severity of illness (MRC, 1977). Stewart (1978), however, re-emphasized his report of 1977 that pertussis vaccine is intrinsically toxic because potent histamine-sensitizing and other pharmacologically-active substances are inseparable from the immunizing components. He estimated the incidence of permanent brain
damage in the United Kingdom (from 1957 to 1976) to be between 1 in 10,000 and 1 in 50,000 of children vaccinated. The controversy surrounding the safety and efficacy of pertussis vaccine and the resulting publicity led to a decline in the acceptance rate of vaccination to 40% or less in 1977 compared to 70%-80% before 1974 (Stuart-Harris, 1978). Such decline was presumably responsible for the serious outbreak of pertussis which began in October 1977 in which the peak incidence was higher than it had been for more than two decades.

One of the problems with present vaccines is that the precise nature of the protective antigen is not well understood. The identification and purification of the protective antigen of B. pertussis has been attempted by numerous workers including Nakase (1978) and Robinson et al. (1981), but such attempts have not been successful. Another approach is the separation of the protective activity from toxicity in B. pertussis by the manipulation of growth conditions (Parker, 1978). The reported conversion of B. pertussis to B. parapertussis through mutagenesis (Kumazawa and Yoshikawa, 1978) also raised the possibility of the production of mutants of B. pertussis which have lost or gained particular properties. Although still at an early stage, genetic studies could lead to a better understanding of the biological activities in B. pertussis and hence contribute to solving the problems of today's vaccine.
4. Pathophysiological and immunological activities of *Bordetella pertussis*.

A variety of biologically-active components of *B. pertussis* have been described, but their exact role in the disease is not well understood. Whole *B. pertussis* cells injected into animals induce many effects that can be grouped into two fundamental actions; increased immunological response to antigens given with the cells; toxic actions and increased susceptibility to various forms of shock.

Some of the components responsible have been implicated as virulence factors in the pathogenesis of pertussis, some as protective factors in immunity to pertussis, and others as responsible for the untoward reactions of the vaccine.

4.1 Protective antigens

The protective antigens (PA) are thought to be cell wall components (Billaudelle et al. 1960). The activity of PA was destroyed by heat (80°C for 30 min), by proteolytic enzymes (trypsin) and by 45% (w/v) phenol indicating that it is protein in nature (Munoz, 1963). This was confirmed by the work of Jakus et al. (1968) who also showed that the protective activity was lost at high pH. Acetone was found to have no affect on PA indicating that lipid moieties were not essential for activity. Similarly Wardlaw and Jakus (1966) showed that protective activity was unaffected by sodium metaperiodate, a reagent widely used to degrade carbohydrates.

Sutherland (1963) investigated the antigenic relationship
of the protective antigens of *Bordetella* species and reported no cross-protection between *B. pertussis* and *B. parapertussis* or *B. bronchiseptica*. Kendrick *et al.* (1953) however, stated that there was some cross-protection in animals between *B. pertussis* and some *B. bronchiseptica* vaccines and between *B. parapertussis* and *B. bronchiseptica* but none between *B. pertussis* and *B. parapertussis*. It is surprising that *B. parapertussis* is not protective against *B. pertussis* if the conversion of *B. pertussis* to *B. parapertussis* by mutagenesis reported by Kumazawa and Yoshikawa (1978) is correct. However, it is possible that in some circumstances, in the absence of antibody to PA, an immune response to other surface components such as those shared with the other *Bordetella* species is capable of conferring resistance to *pertussis* (Olson, 1975). Preston and Stanbridge (1972) have provided evidence that the type-specific pertussis agglutinogens 2 and 3 play an important role in immunity to whooping cough in the child.

Sato *et al.* (1974) reported that immunization with a purified preparation containing haemagglutinin (HA) and leukocytosis-promoting factor (LPF) protected mice from intracerebral (i.c.) infection with a virulent strain of *B. pertussis*. Arai and Sato (1976) separated this preparation into two distinct haemagglutinins namely fimbrial haemagglutinin (F-HA) and leukocytosis-promoting factor haemagglutinin (LPF-HA). Sato *et al.* (1979) showed that F-HA was non-toxic and protected mice against i.c. challenge with *B. pertussis* whereas LPF-HA was toxic and non-protective at doses tolerated by mice. In contrast to these results, Munoz and Bergman (1977) observed that fractions
which they lacking F-HA, designated pertussigen could protect mice and were tolerated by mice at doses of at least 10 µg/mouse. LPF-HA made by the method of Arai and Sato (1976) was toxic at doses of 0.1 to 0.5 µg/mouse. Munoz et al. (1981) suggested that pertussigen (now thought to be identical with LPF-HA), was the most important antigen for the immunization of mice against i.c. infection with B. pertussis. They found that mice were protected with 1.4 µg/mouse (PD$_{50}$) of a pertussigen which was free from demonstrable F-HA, whereas purified F-HA failed to protect mice at a dose of 10 µg/mouse. Passive protection tests with antisera against purified pertussigen protected mice from i.c. infection, whereas anti-F-HA antibodies did not protect mice. Recently, Robinson et al. (1981) have suggested that there may be two distinct mouse-protective antigens, one associated with outer membrane proteins and another associated with F-HA. However, they did not rule out the possibility that a minor component common to both preparations was responsible for protection.

4.2 Histamine-sensitizing factor

This activity was first observed by Parfentjev and Goodline (1947) when intraperitoneal injection of pertussis vaccine or cell extracts into mice made the animals up to 100-fold more sensitive to a later injection of histamine or serotonin. Of the three Bordetella species, only B. pertussis possesses the component which was named by Maitland et al. (1955) as the histamine-sensitizing factor (HSF).
Purification of HSF to homogeneity has been difficult to achieve. It has been suggested that HSF owed its activity to a protein moiety since it was susceptible to heat and proteolytic enzymes (Pieroni et al., 1965). Carbohydrate was not found to any appreciable extent and studies utilizing sodium metaperiodate indicated that carbohydrate was not required for biological activity (Wardlaw and Jakus, 1966). HSF isolated from intact cells contained a considerable amount of lipid (Lehrer et al., 1974), whereas HSF from culture supernatant fluids was lipid-free (Morse and Morse, 1976). The latter workers suggested that this difference may be because HSF is closely associated with lipid when in the cell envelope and that this bond is broken on release of the HSF from the cells.

The mechanism of HSF is uncertain but various workers (Fishel et al., 1964; Bergman and Munoz, 1966; Pieroni and Levine, 1967; O'Brien and Fishel, 1971) have suggested that the action of HSF was via the autonomic system, either by inducing a hyper-reactive state of the α-type receptors or by blockade of the β-type receptors. However, Hewlett et al. (1978) have obtained evidence that *B. pertussis* vaccination does not cause β-adrenergic blockade. It has also been suggested that the primary effect of HSF is to alter cyclic-AMP metabolism in the host either by an effect on intracellular ATP concentrations or by interfering in some way with adenylate cyclase or phosphodiesterase activity (Parker and Morse, 1973).

Pittman (1970) has suggested that the apparently neurogenic nature of the paroxysmal cough of pertussis may be a manifest-
ation of HSF, or at least some toxic property of *B. pertussis*.

4.3 Leukocytosis-promoting factor

Leukocytosis in cases of whooping cough was first observed by Fröhlich (1897) cited by Morse (1965). It occurs either in clinical infections or in lung-infected or vaccinated mice and is due to an increase in the overall number of circulating leukocytes, mainly the lymphocytes (Tuta, 1937; Morse, 1965). The component of the bacterium responsible for this increase was designated as the leukocytosis or lymphocytosis-promoting factor (LPF) (Kurokawa *et al.* 1968).

LPF was reported to be a protein of molecular weight 110,000. It was insoluble in water and isotonic buffers and required an alkaline pH with the addition of 0.5 or 1M NaCl or 4M urea buffer to keep it in solution (Sato and Arai, 1972). In other experiments however, the molecular weight was estimated at 87,000 (Morse and Morse, 1976). LPF was isolated free from lipid and contained less than 1% carbohydrate and 14.5% nitrogen and thus appeared to be a protein (Morse, 1976).

LPF was thought to be the factor that caused slow weight gain and late deaths in the mouse weight-gain toxicity test for pertussis vaccine (Kurokawa *et al.* 1965). The mechanism by which lymphocytosis is produced has been elucidated mainly by Morse and his colleagues (Morse, 1965; Morse and Riester, 1967; Morse and Bray, 1967; Morse and Barron, 1970 and Taub *et al.* 1972). They concluded that the peripheral lymphocytosis was accompanied by depletion of the small lymphocytes in the lymphoid
organs such as the thymus, spleen and lymph node.

There was little or no stimulation of lymphocytosis by LPF when given subcutaneously (Finger et al. 1972). Intravenous injections however, rapidly stimulated lymphocytosis in mice and some effect was seen after intraperitoneal administration. It is not certain whether the site of action of LPF is on the lymphocyte or the post-capillary venule, or both, or whether the rate or the route of entry of lymphocytes from peripheral lymphoid tissue into the blood is normal.

Morse and Morse (1976), found that LPF, as well as being responsible for the induction in mice of leukocytosis with both lymphocytosis and granulocytosis also caused histamine sensitization, hypoglycaemia and unresponsiveness to the hyperglycaemic effects of epinephrine. A similar substance inducing lymphocytosis in mice also was found to cause late-appearing toxicities (LAT) such as late body-weight loss and late death of mice (Kurokawa et al. 1965).

4.4 Adjuvants

Killed B. pertussis cells were shown to exert an adjuvant effect on the production, in rats and mice, of homocytotropic antibodies similar in characteristics to human IgE-type reaginic antibodies (Mota, 1958). The adjuvant potency of B. pertussis was found to be extremely high when inoculated simultaneously with other antigens and in fact, was found to rival or even exceed the adjuvant action of traditional agents such as Freund's adjuvant (Munoz, 1963).
At least two distinct substances in *B. pertussis* cells have the ability to enhance antibody responses. One is a heat-labile component (destroyed at $80^\circ C$ for 30 min) which is found to stimulate IgE production in mice and rats (Mota and Peixoto, 1966) and to enhance the process of experimental hyperacute allergic encephalomyelitis in rats (Munoz and Bergman, 1977). The other substance is the heat-resistant (100$^\circ C$ for 1 h) lipopolysaccharide endotoxin (Morse, 1976). LPS from *B. pertussis* is not very active in stimulating IgE when compared to the heat-labile adjuvant. However, its action appears to be similar to endotoxins of other gram-negative bacteria (Munoz and Bergman, 1977).

The mechanism of the adjuvant activity is not well understood, but Finger et al. (1970), postulated that *B. pertussis* induce increased recruitment of antigen responding cells and proliferation of memory cells. It has also been suggested that *B. pertussis* exerts its effect directly on the precursors of antibody-forming cells by increasing their rate of cell division (Reed et al., 1972). A possible adjuvant effect on tumours is contradictory. Floersheim (1967) reported that *B. pertussis* cells enhance the growth of certain tumours whereas Likhite (1974) reported the opposite effect.
4.5 Islets-activating protein

Insulin secretion was shown to be enhanced in rats after injection with pertussis vaccine (Sumi and Ui, 1975). The factor responsible for this sustained enhancement of insulin secretion was purified from the culture fluid of *B. pertussis* by different procedures of column chromatography, and the purified substance termed Islets-activating protein (IAP). In polyacrylamide gel electrophoresis it showed a band of molecular weight 77,000 (Yajima et al. 1977). Although the factor was regarded as a protein it had a carbohydrate content of 1.0-1.5% but no lipid (Ui et al. 1978). It was rather a stable protein, and its activity was retained at pH values ranging from 3-10 and with temperatures as high as 50°C for 15 min (Yajima et al. 1977). When IAP was injected intravenously into rats at doses as low as 0.02 to 0.1 μg, insulin secretion was significantly enhanced (Yajima et al. 1978). When IAP was injected into mice at doses higher than 0.5 μg, the mice showed a marked leukocytosis and their mortality in response to histamine challenge was increased. Higher doses were also found to abolish epinephrine-induced hyperglycaemia (Yajima et al. 1977).

4.6 Agglutinogens

Agglutinogens are surface antigens that react with their corresponding antibodies to cause the cells to agglutinate. Bordet and Sleeswyk (1910) cited by Munoz and Bergman (1977) described the agglutination of cells by specific antisera and
found that all freshly isolated *B. pertussis* cultures agglutinated in the presence of these sera, while rough strains had lost this activity. Eight different agglutinogens have been described for *B. pertussis*, six (agglutinogens 1-6) are species specific and two (agglutinogens 7 and 13) are shared by other species of the genus *Bordetella* (Munoz and Bergman, 1977). Agglutinogen 1 is the dominant factor in all smooth cells of *B. pertussis* but the most important antigen combinations appear to be 1, 2; 1, 3; 1, 2, 3 because cross reactions experiments showed that vaccines prepared from types 1, 2; 1, 3 and 1, 2, 3 were highly and equally protective in mice, whereas the protection offered by type-1 strains was too poor to be of practical value (Cameron, 1967). However, factor 1 antiserum afforded equal or better protection in mice than factor 2 antiserum against 1, 2 infection and likewise better than factor 3 antiserum against 1, 3 infection (Pittman, 1970).

Serotypic changes *in vivo* have been observed after prolonged nasopharyngeal colonization of the marmoset with the serotypes of *B. pertussis* that cause natural infection in the child (types, 1, 2, 3; 1, 2; 1, 3) (Stanbridge and Preston, 1974). A similar observation was made by Preston *et al.* (1980), when they inoculated rabbits with type-1 organisms. The bacteria mutated within a few days to either type 1, 2 or type 1, 3 and then infections persisted for several months.

Agglutinogens produced specific skin reactions of induration and erythema in sensitized rabbits. Agglutinogens have also been used in skin tests to detect susceptibility to whooping cough in children and to evaluate the efficacy of vaccines.
Although Flosdorf et al. (1943) found a high rate of positive skin tests amongst children who had the disease, the skin test was later found to be unreliable and has not been used in public health practice (Munoz and Bergman, 1977).

The agglutinogen composition of strains isolated in England has changed over the last few decades. In the late 1940's to the mid-1950's the dominant serotype was 1, 2, 4 whereas in the late 1950's and the early 1960's the dominant serotype was 1, 3. In 1963 to 1964 only 15% of 132 fresh isolates had agglutinogen 2. (Preston, 1963). Changes in serotype have also been noticed in Canada (Chalvardjian, 1965), Australia (Blaskett, 1971) and the United States (Eldering et al. 1969). This has significance in the prophylaxis of pertussis since vaccines lacking agglutinogen 3 were thought to be inferior to those containing 1, 2, 3 (Preston and Stanbridge, 1972). Agglutinogens 2 and 3 may play an important role in immunity to whooping cough in the child (Preston and Stanbridge, 1972). In the rabbit, type 1, 2, 3 vaccine was more effective than type 1, 2 in preventing persistent infection with type 1, 3 organisms (Preston et al. 1980). There are numerous instances of type 1, 3 infection occurring in children who have received only antigens 1 and 2 in their vaccination (Preston, 1976). If vaccinated children have antibody against type 3, but not against type 2, then type 1, 2 infections predominate (Kuronen and Huovila, 1978). Pertussis vaccine should therefore be rich in all three of these antigens.
4.7 Haemagglutinins

The haemagglutinin HA of *B. pertussis* was first described by Keogh et al. (1947) as a substance capable of agglutinating red blood cells of chickens and other animals. Not all strains are haemagglutinating but most freshly isolated strains are found to have this property. Electron microscopic examination of the surface of phase-I *B. pertussis* cells has revealed the presence of fine filaments resembling fimbriae or pili, which become adsorbed onto erythrocytes (Morse and Morse, 1970).

HA is sensitive to heat with over 90% of the activity destroyed in 30 min at 56°C. It is also inactivated by the proteolytic enzymes, trypsin and therefore must be protein (Morse and Morse, 1976). Arai and Sato (1976) separated a purified preparation containing HA into two distinct haemagglutinins namely the leukocytosis-promoting factor haemagglutinin (LPF-HA) and the fimbrial haemagglutinin (F-HA). Both antigens agglutinated erythrocytes but the activity was much higher in F-HA than in LPF-HA (Pittman, 1979).

Electron micrographs of LPF-HA showed spherical molecules about 6nm in diameter whereas F-HA was composed of filamentous structures about 2 x 40nm in size (Sato et al. 1981). The LPF-HA was claimed to be identical with pertussigen (Munoz and Bergman, 1978). LPF-HA and F-HA are readily distinguished by the character of the erythrocyte agglutination they produce in test tubes. F-HA produces a diffuse pattern of settling of the red cells which can be dispersed by the gentlest agitation whereas LPF-HA binds red cells into a coherent sheet that can
be dispersed only by vigorous shaking (Irons and MacLennan, 1979).

Antisera against the two haemagglutinins did not cross-react in neutralization reactions, and the dominant serological specificity of LPF-HA was expressed only by phase-I *B. pertussis* organisms, whereas that of F-HA could be detected in preparations from phase-IV organisms and in other species of *Bordetella* (Irons and MacLennan, 1979).

4.8 Adenylate cyclase

Adenylate cyclase is an enzyme which catalyses the synthesis of adenosine cyclic 3',5' monophosphate (cyclic AMP) from ATP. The enzyme was first detected in *B. pertussis* vaccine by Wolff and Cooke (1973). Later Hewlett and Wolff (1976) reported the presence of adenylate cyclase in large quantities in the supernatant fluid of 24 h cultures of exponentially growing *B. pertussis* as well as in the cells themselves. These later workers suggested the presence of 4 components of enzyme activity in *B. pertussis* cultures: 1) In the supernatant culture medium which contains up to 20% of the total adenylate cyclase present during mid-exponential growth. 2) Associated with intact cells and measurable without cell disruption (20-45%). 3) Extracytoplasmic adenylate cyclase which comprises 40-60% of the total activity. 4) Either intracellular or in some manner protected from destruction during trypsin treatment of intact cells (7-9%).

The purified enzyme gave a single band on SDS-PAGE. It was heat-labile, had a temperature optimum of 30°C, a pH optimum of
7-8 and a molecular weight of approximately 70,000. The enzyme was markedly inhibited by fluoride and weakly inhibited by monovalent salts but its activity was not altered by α-keto acids or non-substrate nucleoside triphosphates (Hewlett and Wolff, 1976).

Hewlett et al. (1977) investigated the persistence of the enzyme activity in different stocks of B. pertussis vaccine. The vaccines were found to contain adenylate cyclase activity equivalent to 10% of the value measured five years previously, despite the fact that all the vaccines studied met the required limits for potency, toxicity, adjuvant content, and organism concentration.

The loss of enzyme activity in B. pertussis when grown in modified Hornibrook medium was reported by Parton and Durham (1978). They stated that this loss was accompanied by the loss of other biological properties like PA, HSF and agglutinogens associated with normal phase-I strains.

Recent studies have shown that B. pertussis adenylate cyclase activity was stimulated in vitro by a protein activator of mammalian origin which, in addition, may have an effect on its activity in vivo (Hewlett et al. 1978). The significance of the enzyme in the relationship between B. pertussis and its host is not known.

4.9 Polymorphonuclear leukocyte-inhibitory factor

This was first detected in the culture fluid of phase-I organisms of Bordetella pertussis. It could also be extracted
from bacterial cells by mild sonication suggesting that it may be associated with the surface structures of the bacteria (Utsumi et al. 1978). Partially purified polymorphonuclear leukocyte inhibitory factor (PIF) was found to be free from lipopolysaccharide (LPS), haemagglutinin and a leukocyte agglutinin, but histamine-sensitizing and cytotoxic activities were detected.

Its activity was first observed when phagocytosis of opsonized targets and chemotaxis of polymorphonuclear leukocytes (PMN) was inhibited by extracts of *B. pertussis*. Its susceptibility to heat and protease treatments suggested its protein nature. Imagawa et al. (1980) reported some discrepancies in PIF characters, for instance the activities in vitro appeared in the low molecular weight fraction on Sephadex G-25 gel filtration but it was later found that dialysis of the protease treated extract of the outer-membrane resulted in no loss of in vivo activity. However, this raised the possibility that PIF is a small, non-protein molecule firmly attached to a protein carrier (Utsumi et al. 1978).

The inhibitory effect of PIF on the host's defence mechanism: the resistance of *B. pertussis* to surface phagocytosis, the higher content of PIF in phase-I than phase-IV organisms and the close parallel of PIF production with production of other components characteristic of phase-I virulent organisms indicated that PIF may be implicated in the pathogenesis of pertussis (Imagawa et al. 1978). However in a recent report the failure to separate the PIF activity from the histamine-sensitizing activity (HSF) by DEAE-chromatography, and the similarity between
HSF and LPF suggested that a single factor may be responsible for both the blood leukocytosis and the observed failure of PMN to migrate from the blood vessels (Imagawa et al. 1980).

4.10 Lymphocyte mitogen

Kong and Morse (1975; 1976) first reported the presence of a potent mitogen for murine lymphocytes in B. pertussis culture supernatant fluids. Although mouse spleen and lymph node cells responded fully, pertussis mitogen did not stimulate normal thymocytes. The mitogen was found to be identical with LPF. Further investigations of LPF in B. pertussis showed that it was the T-lymphocytes which were the target cells for the mitogen which caused the generation of cytotoxic effector cells in cultures of lymphocytes (Kong and Morse, 1977). These cells had the electron microscopical features of T-cell lymphoblast, sedimented in the higher density fraction of a bovine serum albumin gradient, were lysed by thy 1.2 antiserum plus complement, and therefore were T-cells (Kong and Morse, 1979). It was proposed that it was the T-cells that responded, although a second cell type, probably the macrophage, was required to initiate the stimulatory events, perhaps by processing the mitogen.

4.11 Heat-stable toxin (endotoxin or lipopolysaccharide)

B. pertussis endotoxin possesses all of the usual biological properties associated with endotoxins. It is pyrogenic and toxic, it elicits the Shwartzman phenomenon and non-specific immunity in mice. It has adjuvant properties and provokes interferonemia (Ayme et al. 1980). However, its potency may differ from one
strain to another. MacLennan (1960), found that LPS from one
B. pertussis strain was not toxic for mice in 1 mg amounts
whereas endotoxin prepared from another strain was toxic to mice
in doses of 200 to 300 \( \mu g \) given intraperitoneally. The toxin
is an integral part of the cell envelope and thus not free to
diffuse so that the surface nature of pertussis infections
probably provides little opportunity for endotoxin to act.
The fact that pertussis patients usually are not febrile suggests
that endotoxin pyrogen is indeed not being absorbed (Dolby, 1965).

The chemical composition of the toxin has been studied,
and hexosamine aldoheptose, hexose 2-keto-3-deoxyoctonate (KDO),
D-glucosamine, D-glucose and lipid have been found (MacLennan,
Recent reports indicated that two non-identical polysaccharide
chains are present within B. pertussis LPS (Le Dur et al. 1978)
together with two distinct lipids, lipid A and lipid X (Ayme
et al. 1980). In lipid A pyrogenicity was reduced to a very low
level and toxicity and Shwartzman reactivity were absent,
whereas lipid X retained all the endotoxic properties of the
unfractionated endotoxin.

4.12 Heat-labile toxin

This toxin was first described in B. pertussis as a dermon-
ecrotic substance extracted from dried cells that had been
ground with crystals of sodium chloride (Bordet and Gengou, 1909)
It is a labile substance which is released upon lysis of the
cells (Wood, 1939). Some strains contain more toxin than others
but recently-isolated cultures contain most toxin (Lawson, 1933).
When *B. pertussis* cells were disrupted, they were found to be dermonecrotic and even fatal if were given intraperitoneally or intravenously to mice (Evans, 1939). Heat-labile toxin (HLT) is of cytoplasmic origin (Billaudelle, 1960) and fractionation studies indicated that it is a protein (Munoz, 1963). The toxin is pharmacologically extremely potent. The toxin was reported to be present in culture supernates during the early log phase of growth (Lane, 1968). However, Cowell et al. (1979) found that through log phase and into the stationary phase of growth the toxin was cell-associated and not detected in the culture supernate. HLT has been found in all three species of *Bordetella* (Ptak et al., 1969).

Pathologically the toxin causes a profound necrotising inflammation of the respiratory tract in mice extending through it to some depth (Banerjea and Munoz, 1962). However, HLT is not involved in establishing active immunity to pertussis since vaccine preparations free of HLT will immunize mice against intracerebral challenge with *B. pertussis* and children against whooping cough. Moreover, HLT preparations free of protective activity can also be obtained (Munoz and Bergman, 1977).

**Interrelationship between the immunological and pathophysiological activities**

It is well known that *B. pertussis* expresses various pathophysiological activities in both the child and in experimental animals.

Levine and Pieroni (1966) proposed that *B. pertussis* contains a single, unique proteinaceous component responsible
for some of these effects seen in mice including sensitization to histamine, induction of lymphocytosis, protection against i.c. challenge with virulent \textit{B. pertussis} and adjuvant activity. Lehrer \textit{et al.} (1974) and Sato \textit{et al.} (1974) obtained partially purified HSF but they found their preparations still retained PA. Parker and Morse (1973) obtained highly purified preparations of HSF/LPF and found that it had high mouse-protective activity. They also proposed that all three of these activities were found in one substance. Munoz and Bergman (1977) proposed a unifying name "pertussigen" for this component in which HSF; late-acting toxin; heat-labile adjuvant; PA and (LAT) were included.

On the other hand, Dolby (1958) and Nagel (1970) did not conclude that HSF, LPF and PA were identical. In addition Sato \textit{et al.} (1974) found that some preparations of \textit{B. pertussis} were less active in producing sensitization to histamine than in protecting mice. Cameron (1976) reported that changes in the cultural conditions for \textit{B. pertussis} caused some discrepancies between the levels of HSF and PA in the resulting culture. Nagel and Graaf (1978) reported the purification of PA which was devoid of detectable HSF and LPF activities and Nakase (1978) also reported the purification of PA which had no HSF activities.

The relationship between PA and HA was first reported by Keogh \textit{et al.} (1948) who showed that preparations containing HA induced active immunity in mice against intranasal infection with \textit{B. pertussis}. However, when the challenge was given intracerebrally no protection was obtained (Masry, 1952). Sato and Arai (1972) proposed that HA, HSF, PA and LPF were identical. It is now known that \textit{B. pertussis} produces two
distinct haemagglutinins, F-HA and LPF-HA (Arai and Sato, 1976; Irons and MacLennan, 1979). Arai and Sato (1976) showed that F-HA was non-toxic and protected mice from intracerebral challenge with *B. pertussis*. In contrast, Munoz et al. (1981) showed that pertussigen (LPF-HA), free of F-HA protected mice.

The ability of agglutinogens to function as mouse-protective antigen seems unlikely (Schuchardt et al. 1963; Eldering et al. 1966) despite the evidence for their involvement in protecting the child (MRC, 1959; Preston, 1966; Preston and Stanbridge, 1972).

IAP was shown to enhance insulin secretion (Sumi and Ui, 1975) abolish epinephrine induced hyperglycaemia, induce lymphocytosis and histamine-sensitization (Yajima et al. 1977) and so is probably identical with pertussigen. Similarly PIF could not be separated from histamine-sensitizing activity (Utsumi et al. 1978) and the lymphocyte mitogen was found to be identical with LPF (Kong and Morse, 1977). Thus it appears that a single component of *B. pertussis* is responsible for histamine-sensitizing activity and possibly for protective activity in mice.

In summary, the exact nature and range of properties of the various biologically-active components of *B. pertussis* have still to be defined. However, there seems to be at least five distinct components which may have an important role in the pathogenesis of whooping cough: pertussigen, HLT, LPS, F-HA and adenylate cyclase.
5. Variation in *Bordetella pertussis*

Four distinct types of variation have been reported for *B. pertussis*: 1) phase variation during which the organism undergoes a series of mutational changes. 2) antigenic modulation is a reversible, phenotypic change brought about by alterations of the growth conditions. 3) serotype variation, which occurs spontaneously either *in vitro* or *in vivo*. 4) mutation to *B. parapertussis*.

In 1910 Bordet and Sleeswyck (cited by Aprile, 1971) found that agglutinating sera for the identification of freshly-isolated strains of *Haemophilus pertussis* could not be made successfully by injecting animals with old stock strains of organism grown on agar.

This was because of the serological changes that occur as the result of adaptation to normal media growth. Leslie and Gardner (1931) studied these changes in detail. They analysed the agglutinogenic properties of 32 strains of *H. pertussis* and concluded that they all fell into one or another of four distinct groups, designated phases I, II, III and IV. They proposed that phases I and II corresponded to the smooth or pathogenic forms of other bacteria, whereas phases III and IV correspond to the rough, relatively harmless saprophytic forms. They also found that during prolonged cultivation there was a general tendency to pass from the smooth (phase-I) state to the rough (phase IV) state, during which cells lost their biological activities. These included PA (Kasuaga et al. 1954; Aprile, 1972), HSF (Kind, 1953; Aprile, 1972; Parton and Wardlaw, 1975), agglutinogen 1 (Elderling et al. 1962), HA (Keogh and North, 1948;
Standfast, 1951); 28k and 30k envelope polypeptides found in phase-I strains (Parton and Wardlaw, 1975) and adenylate cyclase activity (Parton and Durham, 1978).

Parker (1976) suggested that phase degradation in *B. pertussis* during serial culture resulted from mutations which were independently selected for by inhibitors present in artificial media. She proposed that intermediate strains may show any of a variety of changes, but that changes occur in succession leading finally to a degraded strain.

Recently, Bemis et al. (1977) examined the biochemical, cultural and morphological characteristics of *B. bronchiseptica* isolates from dogs and reported a type of phase variation which involved changes in colonial morphology, haemagglutination, haemolysis, flagellation and fimbriation.

Lacey (1960) described another type of variation which he called antigenic modulation when he was developing a selective medium for *B. pertussis*. He found that a crude rabbit serum against *B. parapertussis*, which agglutinated *B. pertussis* to a titre of 800, failed even at a titre of 2 to agglutinate the same strain of *B. pertussis* grown on Bordet-Gengou medium containing magnesium sulphate in place of sodium chloride. He stated that this variation which he called Antigenic Modulation was reversible and quite different to the stable mutational phase variation described by Leslie and Gardner (1931). The changes were postulated to be from X through an intermediate stage I. The X stands for "Xanthic" and describes the yellowish-ochre hue of confluent growth of freshly isolated strains on Bordet-Gengou medium containing NaCl. C stands for "Cyanic"
and describes the greyish-blue appearance of growth on B-G medium containing a high level of MgSO₄. Bacterial cells in C-mode, unlike those in X-mode, do not agglutinate red-blood cells, are not haemolytic or agglutinable by heavy metal salts, have no surface antigen related to B. parapertussis or B. bronchiseptica, and are serotypically homogeneous. Lacey (1960) postulated two possible hypotheses to explain modulation. The first was that modulation could be attributed to changes in the position of constant amounts of antigens and the second that it could be attributed to changes in position accompanying or resulting from changes in amount of antigens.

A similar phenotypic change was described by Pusztai and Joó (1967) when B. pertussis phase-I strains were grown in high levels of nicotinic acid. The cells lost PA, HSF and agglutinogens, but HLT was unaffected. This was thought to be the result of impaired cell wall synthesis, as only cell wall antigens were lost. Wardlaw et al. (1976) showed the loss of PA and HSF when B. pertussis was grown in high nicotinic acid but not in high nicotinamide-containing media.

Changes of serotype in B. pertussis were observed during colonisation after pre-nasal infection in both the marmoset (Stanbridge and Preston, 1974) and the rabbit (Preston et al. 1980). These workers observed that if type-I organisms are introduced intranasally in either animal, they immediately acquire one of the type-specific antigens (2 or 3) and the animal became colonised with either type 1, 2 or type 1, 3 organisms.
Other forms of variations however, were also observed in *B. pertussis*. Anderson (1952) demonstrated definite serological differences even in smooth strains and showed that under certain conditions, smooth strains could dissociate into variants differing in antigenic structure from the parent strain, but often identical to that found in other freshly isolated strains. Cameron (1967) demonstrated that the serological composition of *B. pertussis* suggested that the serotype 1, 2, 3 might be regarded as the parent from which the others were derived. He showed that vaccines prepared from types 1, 2; 1, 3 and 1, 2, 3 were highly and equally protective, whereas the protection offered by type-I strains was too poor to be of practical value. Preston and Stanbridge (1972) confirmed this when they had shown that the parent 1, 2, 3 and the intermediate 1, 2; 1, 3 types could each establish infection and could change to different serotype during the course of the infection, whereas type I organisms were found only at a late stage of the infection, and even then did not constitute the predominant serotype. They also stated that four main serotypes of *B. pertussis* (1, 2, 3; 1, 2; 1, 3; 1) undergo spontaneous variation involving loss or gain of antigen 2 or antigen 3.

The loss or gain of biological activities during genotypic or phenotypic changes in *B. pertussis* could have important practical applications. Variants could be produced which had unusual combinations of biological activities which might be useful for vaccine manufacture or for immunological or physiological studies.
The conversion of *B. pertussis* to *B. parapertussis* was claimed after treatment of fresh isolates of *B. pertussis* with NTG and selection for antibiotic resistance (Kumazawa and Yoshikawa, 1978). They found that some of the mutants obtained had gained the ability to split urea and utilize citrate and lost histamine sensitizing activity. Most of the mutants however, differed in some characteristics from typical *B. parapertussis*. Kloos et al. (1978) reported similar findings but no details were given.

6. Growth requirements of *Bordetella pertussis*

For many years the optimal medium for the cultivation of *B. pertussis* has been the solid medium originally recommended by Bordet and Gengou (1906). Strains grown on this medium maintained their biological properties unaltered for varying lengths of time. In the search for a suitable, chemically defined solid or liquid medium a prime consideration has been the maintenance of the antigenic properties characteristic of freshly isolated strains.

Development of a minimal liquid medium.

*B. pertussis* was originally found not to grow in liquid media without blood (Lawson, 1939), or tissue extracts (Toomey and McClelland, 1933). Initially it was thought that the reason was the fastidious characteristics of the organism, but later it was shown to have simple growth requirements and the initial problems were due to growth inhibitors.
Hornibrook (1939) was first to describe a medium suitable for the propagation of phase-I B. pertussis which did not contain blood or tissue extract. The original formula included hydrolysed casein, soluble starch, salts cysteine and yeast extract. Hornibrook (1940) modified his medium to a defined mixture of amino-acids in place of casein, inorganic salts, cysteine, starch and nicotinic acid. Since that time, many modifications for his original formulation have been published (Verwey and Sage, 1945; Wilson, 1945; Cohen and Wheeler, 1946; Verwey et al., 1949; Sutherland and Wilkinson, 1961). These liquid media have consisted of a casein hydrolysate to which were added various salts, growth factors and either starch, charcoal or anionic resins.

Stainer and Scholte (1971) were the first to describe a simple chemically defined medium for B. pertussis. It contained sodium glutamate, proline, cysteine, inorganic salts and growth factors. The development of this medium was an important step in the study of the genetics of B. pertussis, since it provides the basis for the development of a chemically defined solid medium on which may be achieved auxotroph isolation as well as isolation of mutants resistant to inhibitors. Hewlett and Wolff (1976) described a modified Stainer and Scholte liquid medium consisting of the same components except the Tris was reduced. Kloos et al. (1978) modified this medium by changing the final CaCl₂ concentration to 0.001M and adding 0.25% (w/v) casein hydrolysate (enzymatic digest). The latter workers used this medium in genetic studies in B. pertussis. They stated that it was suitable for exposure of recipient cells to donor DNA in a
transformation system in *B. pertussis*.

Development of a minimal solid medium.

Bordet and Gengou (1906) were the first to isolate *B. pertussis* on a glycerated potato-extract agar medium containing 50% blood (Rowatt, 1957). Since that time many attempts to develop a simpler medium have been made. Pollock (1947) found that blood could be replaced by serum, serum albumin or charcoal in solid media. Sutherland and Wilkinson (1961) claimed to have developed a solid medium supporting the growth of low inocula. Their medium was semi-defined and contained an anion exchange resin (Dowex-1 chloride) to remove growth inhibitors.

Parker (1976) proposed a defined solid medium based on Stainer and Scholte liquid medium with the addition of sodium acetate; Dowex-1 chloride and solidified with 1% (w/v) agarose. She stated that sodium acetate was stimulatory for *B. pertussis* strains when used in small amounts and Dowex-1 chloride removed any inhibitors remaining in the medium.

Kloos et al. (1978) recommended a semi-defined casein hydrolysate agar medium which is another modification of Stainer and Scholte medium. It contained mono-sodium glutamate, inorganic salts, casein hydrolysate, growth factors, activated charcoal and was solidified by 1.5% (w/v) Bacto-agar. They stated that on this medium colonies of most strains were just visible to the naked eye by 48 h and were 1 to 1.2 mm in diameter by 72 h, but it was not suitable for prolonged storage of cultures. They also proposed a defined solid medium in which casein hydrolysate was replaced by a mixture of amino acids, glycine,
aspartic acid, serine, methionine, alanine, lysine, tryptophan, leucine, isoleucine, valine and cysteine. They showed that this medium was suitable for the growth of recombinants following transformation in \textit{B. pertussis}.

Growth inhibitors.

It was observed that during autoclaving of media, fatty acids were extracted from cotton wool plugs of the flasks or test tubes and deposited on the glassware in a quantity sufficient to inhibit growth of \textit{B. pertussis}. Starch was found to annul this inhibition (Pollock, 1947). Nicholson (1951) using solid medium for growth of \textit{B. pertussis} found that serine inhibited growth and all peptones were inhibitory. Growth inhibition due to peroxides was first reported by Mazloum and Rowley (1955). They suggested that this inhibition was due to organic peroxides formed in media during autoclaving rather than to hydrogen peroxide.

Rowatt (1957) studied growth inhibitors in some detail. She compared her studies on the inhibitory effect of oleic acid with those of Pollock (1947), who found that growth in broth sterilized in flasks with cotton wool plugs was inhibited by 4 \( \mu \text{g ml}^{-1} \) oleic acid. She also suggested that inhibition of growth could be due to colloidal sulphur compounds formed during autoclaving of agar, casein hydrolysate or other nutrient sources. Thus for the growth of \textit{B. pertussis}, media free from inhibitors is desirable. This would be especially true in genetical experiments since recombination is reported to be very inefficient (Branefors, 1964; Kloos \textit{et al.}, 1978) and growth inhibitors would
cause further reductions in the number of the recombinants isolated.

7. Genetic studies in bacteria

7.1 Transformation

Bacterial transformation is the displacement of a segment of the bacterial chromosome by a homologous, or partly homologous, segment of free DNA present in the environment of the cell by a process called recombination. For the displacement to occur, the DNA molecule has to enter the cell (Venema, 1979).

The phenomenon was first discovered by Griffiths (1928) when he inoculated a large quantity of Rough cells of *Streptococcus pneumoniae* under the skin of a mouse together with heat-killed Smooth cells. The mouse died within a few days and blood from the animal yielded only Smooth cells. Later Dawson and Sia (1931) succeeded in carrying out such transformation in vitro. They showed that Rough pneumococci could be transformed to Smooth capsulated cells by DNA from Smooth cells. The chemical substance responsible for transformation was called transforming principle. Avery et al. (1944) succeeded in purifying pneumococcal transforming principle and identified it as DNA.

Since that time, transformation has been demonstrated in other genera of bacteria notably *Haemophilus, Neisseria, Bacillus* and *Escherichia*. However, because of the existence of different types of bacterial transformation systems, they have been divided into groups. The first group includes those systems where competence, or the ability of a cell to interact with exogenous DNA in such a fashion that a subsequent change of genotype results, is a specially evolved physiological state which may be obtained in the laboratory by growing the cells through a
defined regimen. This includes the transformation system of Streptococcus pneumoniae, Bacillus subtilis and Haemophilus influenzae. The second group of transformation systems is exemplified by E. coli and consists of those organisms where competence is artificially induced by procedures such as calcium treatment (Low and Porter, 1978).

Other differences between transformation systems are apparent. For example, Joenje and Venema (1975) stated that in B. subtilis DNA is capable of interacting in a transient way both with competent and non-competent cells, whereas in S. pneumoniae this type of interaction is restricted to competent cells only.

When transformable bacterial cells such as those of Streptococcus pneumoniae, Haemophilus influenzae and Bacillus subtilis were exposed to DNA, the DNA was found in two states. In one state, the DNA was susceptible to deoxyribonuclease (DNase) after its attachment to the cells, and in the other state it was permanently bound and not susceptible to DNase (Hayes, 1968). A short time after transformable (competent) cells bind DNA permanently, the DNA loses its biological activity in transformation. This is known as the eclipse period and is found in both Streptococcus pneumoniae and Bacillus subtilis (Venema et al. 1965) but not in Haemophilus (Voll and Goodal, 1961). The reason for this change in state of the transforming DNA is unclear. Lacks (1962) found that when extracts of competent Pneumococcus were analysed at intervals after exposure to $^{32}$P-labelled DNA one half of the bound radioactivity was found (immediately after entry) in highly polymerised single-stranded
DNA, and the other half was degraded into acid-soluble fragments. He proposed a model in which the penetrating end of the DNA duplex encounters a nuclease which breaks down one strand sequentially while pulling the other intact strand into the cell.

Lacks and Greenberg (1973) postulated that the nuclease is located at the cell surface. A surface-located nuclease activity could be induced through mechanical or enzymic damage to the cell wall or by a change in the ionic conditions in non-competent *S. pneumoniae* (Seto et al. 1975). Later, Lacks and Greenberg (1976) proposed a model for entry of DNA into competent *S. pneumoniae* and *B. subtilis* cells in which the endonuclease, situated adjacent to the DNA binding protein, cuts the strand opposite to the break produced as a consequence of binding. The bound DNA strand can then enter the cell, while the immobile endonuclease digests the opposite strand to produce oligonucleotides which are liberated into the medium external to the cell.

After its entry the transforming DNA was found to integrate with the host cell chromosome within approximately 30 min at 37°C in *S. pneumoniae, H. influenzae* and *B. subtilis* (Venema, 1979). Subsequent to its entry into *E. coli* cells, the transforming DNA was found as single-stranded DNA (Lacks et al. 1967). In *B. subtilis* transforming DNA re-extracted after entry was described as a high molecular-weight single-stranded DNA by Piechowska and Fox (1971). They observed that the physical state of the entered donor DNA was changed, presumably after becoming complexed to a cellular constituent, and became partially resistant to DNase. The constituents adhering to the single-stranded DNA were believed to originate both from the cell
wall and the plasma membrane and were endowed with autolytic and endonucleolytic DNase activity (Pieniazek et al., 1977).

The obvious mechanism for initiation of integration of transforming DNA into the chromosome is contained in the possibility of pairing of the complementary base sequences of the donor DNA and the recipient chromosome. This is concluded from the existence of single-stranded regions in the competent cells in B. subtilis (Harris and Bar, 1969) and in H. influenzae (Leclerc and Setlow, 1975). Seto and Tomasz (1977), on the other hand observed that treatment of transforming S. pneumoniae cells with a number of DNA-intercalating agents shortly after entry of donor DNA inhibited the appearance of transformed cells. They suggested that the donor-recipient complexes contain single-stranded, not base-paired segments of DNA.

7.2 Competence

This is the physiological state of the recipient cells in which they are able to bind and take up DNA in a form resistant to exogenously added DNase (McCarty and Avery, 1946). It is usually found when the logarithmic phase of growth changes into the stationary phase (Venema, 1979).

A substance which induces competence in bacteria was studied by several workers. In S. pneumoniae it was found to have a molecular weight of approximately 10,000 and a net positive charge at pH 7.8, it was heat-labile and inactivated by proteolytic enzymes such as trypsin (Tomasz, 1973). It was suggested that development of competence relates to changes in membrane
permeability, permitting two of the nucleases to appear at the outer layer of the cell membrane or in the periplasmic space, thus unmasking the DNA-binding sites, otherwise hidden by the cell wall (Starosciak et al. 1975). Crabb et al. (1977) stated that the role of the competence factor is that of a highly specialized permeability-causing agent, and further evidence for this permeability was found in _B. subtilis_ by the release of cellular DNA into the surrounding medium when competence develops. However, Leclerc and Setlow (1975) have found, unlike cells in the exponential phase of growth, competent cells of _H. influenzae_ contain a special type of DNA with single-stranded regions. Venema (1979) stated that these regions are not present in a transformation-deficient mutant incapable of associating donor DNA with the recipient chromosome. This is an indication that single-stranded regions in DNA of competent cells are important for an early step in the integration of donor DNA into recipient chromosome.

The uptake of DNA by competent cells is affected by many factors. For example, when the mean molecular weight of DNA preparations was reduced to about $5 \times 10^5$ daltons by ultrasonic treatment or by spraying through an atomiser, uptake ceased and no more transformants of _Pneumococcus_ were found (Rosenberg et al. 1959). Similarly, uptake of DNA in _E. coli_ was rapidly abolished by treatment with DNase, and also by heating to $100^\circ$C, which separated the two strands of the double helix (Marmur and Lane, 1960). If the heated, denatured DNA was cooled rapidly the strands remained separate so that a single-stranded preparation of half the initial molecular weight
was obtained. Such single-stranded DNA was virtually inactive in transformation and this correlated with loss of its ability to be taken up by competent bacteria.

An effect of adenosine 3',5'-cyclic monophosphate (cAMP) on competence in bacteria has been reported (Wise et al. 1973). Cyclic AMP added to exponentially growing cells of _H. influenzae_ increased competence for transformation by 100 to 10,000-fold. However, no effects of added cAMP have been seen in Gram-positive species (Clark and Bernlohr, 1972).

The effect of ethylene-diamine tetra-acetic acid (EDTA) was observed by Morrison (1971), who found that EDTA prevented entry of donor DNA in _B. subtilis_ without interfering with binding. Seto and Tomasz (1974) made similar findings in _S. pneumoniae_. However, Garcia et al. (1978) stated that in _B. subtilis_, EDTA irreversibly inhibited the transformability as well as the cellula binding of DNA in whole cells, protoplasts and membrane vesicles, mainly due to a permanent alteration of the DNA receptors.

CaCl₂, on the other hand enhanced transformation in _E. coli_ by inducing competence activity in the recipient cells (Oishi and Cosly, 1972). A similar action of CaCl₂ was also found in _Pseudomonas putida_ (Mylorie et al. 1978). Molhot and Doskocil (1978) showed that lysozyme and sucrose enhanced transformation efficiency synergistically in _E. coli_ in the presence of CaCl₂. They suggested that high concentrations of CaCl₂ affected the surface layers of the Gram-negative bacterium so as to allow entry of DNA.
Antibiotics acting on the cell envelope, such as penicillin and cephalosporin, stimulated competence in *B. subtilis* whereas polymyxin appeared to inhibit it (Lopez et al. 1974). However, the induction of full competence in *S. pneumoniae* by glycine (Kohoutova, 1973) suggested to the author that the competent cell may be considered as a temporary sphaeroplast. Further evidence for this was found when Lacks et al. (1975) observed that competent cells were rapidly converted to sphaeroplasts in concentrated sugar solutions.

7.3 **Linkage by transformation**

In transformation, two genes are said to be linked if they are transferred together on the same molecule of transforming DNA (Hayes, 1968). Linkage of different determinants has been found in many bacterial species. In *pneumococcus*, for example, the loci for streptomycin resistance and mannitol fermentation as well as three loci determining different degrees of resistance to sulphonamide are linked (Hotchkiss and Evans, 1958). In addition a number of loci concerned with the synthesis of capsular polysaccharides are linked (Ravin, 1961; Jackson, 1962). In *B. subtilis* a number of mutations involving histidine synthesis are linked and can be mapped (Ephrati-Elizur et al. 1961).

Linkage between determinants of certain characters is found when transforming DNA carrying them yields double transformants with high frequency. This was found in many organisms including *B. subtilis* (Ephrati-Elizur, 1968). The difference in the frequency of transfer in linked and unlinked markers can be simply calculated. For example, if two markers are carried by different molecules (unlinked), the probability that one bacteriu
will take up both molecules, and so be transformed with respect to both markers, is the product of the probabilities for the independent events (Hayes, 1968). Hayes simplified this by giving an example in which a donor strain contains unlinked genes A and B in its chromosome, whereas the recipient strain lacks them and in this case they are designated a and b. When recipient cells were exposed to donor DNA they were found to yield Ab and aB transformants with a frequency of 1%. The expected frequency of double AB transformants is therefore 0.01% or one per 10,000. A figure higher than this would indicate that both markers were carried on the same fragment of DNA.

7.4 Mutation and Mutagenesis

In living organisms genetic information is transmitted from one generation to the next, and the maintenance of the precise organisation of such organisms depends on the high degree of accuracy of that transmission. A considerable amount of heritable variation provides us with genetic markers which are essential for genetic mapping. The origin of a new genetic marker is called a mutation, which is an alteration in the nucleotide sequence at some point in the organism's DNA. This may lead to the production of a protein that has an altered amino acid sequence. There are three major types of mutations, the base-pair substitution or point mutation; the frame-shift mutation and the deletion mutation. In base-pair substitution a specific site in the wild type chromosome such as guanine-cytosine is replaced in the mutant chromosome by a different base-pair such as adenine-thymine. In frame-shift mutations,
one or a few bases are inserted into or deleted from a specific site within the gene. In large deletions a long sequence of bases representing a major segment of the gene is removed.

Mutation in bacteria can be either induced or spontaneous. Early work on spontaneous mutation in bacteria was concerned with mutation to phage or drug resistance as these mutants could easily be selected by plating large numbers of bacteria on medium containing either the appropriate phage or antibiotic. Later work concentrated on the isolation of auxotrophic mutants, only able to grow if a particular amino acid, vitamin or nucleotide was provided.

Chemical mutagens were used on *B. pertussis* to isolate different types of mutants including some that resembled *B. parapertussis* (Kumazawa and Yoshikawa, 1978) and auxotrophs for Leucine and Tryptophan (Kloos *et al*. 1978). Both groups used the replica-plating method for the inoculation of mutants onto the selective media. The mutagen was N-methyl-N'-nitro-N-nitrosoguanidine (NTG), which is a methylating agent, acting on the replication region of the chromosome (Lawley, 1968; Guerola *et al*. 1971). McCalla (1968) and Lawley (1968) found that the main product of NTG action on DNA was the formation of 7-methylguanine. They stated that this supported the suggestion that mutagenic methylnitroso compounds yield diazomethane which by loss of nitrogen give rise to a carbonium ion which is the active methylating agent.
Bacterial plasmids and chromosomal mobilization

Plasmids are extrachromosomal structures of DNA which can reproduce autonomously irrespective of whether they can or cannot integrate into the host chromosome (Lederberg, 1975). The genes which they carry are not essential for growth of the host cell; but they do confer additional properties such as resistance to antibiotics which may in some circumstances assume crucial importance (Collee, 1973). They are widespread among Gram-negative bacteria. Möller et al. (1978) have shown that most naturally occurring strains of the Enterobacteriaceae contain small circular non-conjugative plasmids ranging in size from 0.8 to 10 mega-daltons (Mdal). Their capacity for transfer of extrachromosomal DNAs was observed in an early work. Lederberg et al. (1948) showed that strains of E. coli carrying different genetic markers could produce recombinants when mixed together.

Fredericq (1950) was the first to find that the capacity to produce colicins was conferred by colicinogenic factors, which could be transmitted in infective fashion from cell to cell. The transfer of resistance (R) factor was discovered by Watanabe (1963). The R-factors confer on their host cells resistance to drugs, such as penicillin, chloramphenicol, tetracycline or streptomycin. Hayes (1968), was the first to recognise that sexual fertility depended on a special genetic factor (F), present in some bacterial strains and not in others. The F factor behaves as a free infective particle independent of the rest of the genome. This and other genetic elements such as bacteriocins and resistance transfer factors were called transmissible plasmids.
Plasmids replicate independently of the cell chromosome and their DNA contains a region which serves as a point of initiation for the action of the appropriate DNA polymerase, but this does not exclude the interaction between plasmids and the chromosome especially during replication (Novick, 1969).

The spread of plasmids through bacterial population is often greatly helped by their promoting the formation of a specific apparatus for their own cell-to-cell transfer (Fincham, 1976). Some transmissible plasmids can promote the cell-to-cell transfer of other DNA elements as well as themselves. The transfer of host chromosomal fragments by autonomous plasmids with a fully operational transfer system usually takes place at a frequency at least one thousand-fold lower than that of the plasmid (Willetts, 1970). Despite this low frequency, chromosome transfer determined by plasmids comprises a wide field of study in bacterial genetics.

Two groups of chromosome-mobilizing plasmids have been described. In one, transfer of chromosomal material is not necessarily accompanied by inheritance of the whole plasmid. In the other, which encompasses the F prime (F') and R prime (R'), a hybrid plasmid is formed which comprises both plasmid and bacterial DNA in which all or most of the plasmid functions have been preserved (Rieb et al. 1980). This author stated that the latter type of plasmids include the Incp-I plasmids (plasmids shown to have chromosome mobilization ability), which include R68, and R68.45 plasmids and others. They possess the ability to establish themselves in a wide range of Gram-negative bacteria, as well as having chromosomal mobilizing activities (cma). The widest survey of plasmids for the occurrence of
cma has been carried out in *Pseudomonas aeruginosa* (Pemberton and Holloway, 1973). From this work the plasmid R68.45 was isolated from the broad host-range plasmid R68 (Resistant to carbenicillin; tetracycline and kanamycin) (Jacoby and Shapiro, 1977). It was observed that R68 promoted chromosomal marker transfer in *P. aeruginosa* strain PAT at frequencies as high as 4 x 10^{-3}/donor cell. (Stanisich, 1972). The physical examination of R68.45 showed that it had acquired an extra piece of DNA of the order of 1.4-1.6 Mdaltons (Jacob *et al.*, 1977). There is some evidence for an 1800 base-pair insertion situated close to the kanamycin resistance marker on the R68 chromosome (Holloway, 1979). This observation is of significance because it has been observed that spontaneous loss of the kanamycin marker is usually associated with loss of cma by R68.45. A recent report has claimed that the plasmid R68.45 differs from its parent R68 only by an additional DNA segment, 2120 bp long, located close to the kanamycin resistance gene (Rieb *et al.*, 1980). The additional DNA segment of R68.45 is a duplication of a pre-existing DNA region of R68.

The intergeneric transfer of the plasmid R68.45 from *E. coli* to *Erwinia* strains was reported by Chatterjee (1980) who found the frequency of transfer was consistently higher with an *E. coli* strain than with *Erwinia* spp. as recipients, and when matings were done on solid surfaces rather than in liquid. The plasmid was also reported to be transferred into other bacterial genera including *Rhodopseudomonas* with a frequency of 10^{-4} - 10^{-8}/donor cell (Sistrion, 1977) and *Rhizobium* with a frequency of 10^{-6}/donor cell (Beringer *et al.*, 1978). Plasmid R68.45 is well known for
its chromosome mobilizing ability in many organisms including *Pseudomonas aeruginosa* (Hass and Holloway, 1976), *Rhizobium leguminosarum* (Beringer et al. 1978), *Agrobacterium tumefaciens* (Hamada et al. 1979) and *Erwinia* spp (Chatterjee, 1980).

The existence of plasmids has been demonstrated in some strains of *B. pertussis*; *B. parapertussis* and *B. bronchiseptica* (Kloos et al. 1978). In both *B. pertussis* and *B. parapertussis* strains, only a small cryptic plasmid with a mass of approximately 3 megadaltons was found. In *B. bronchiseptica* however, medium-size to large plasmids have been found in addition to a small plasmid similar in size and concentration to that found in the other species.

7.6 Genetics of bacterial virulence

The concepts and methodologies that have developed in studies of gene structure and gene expression are now being applied to the study of the genetic basis of pathogenicity. It is hoped that an understanding of pathogenic mechanisms at the molecular level will lead to new control measures for infectious diseases. Maas (1981), stated that studies on the movement and location of genes will be useful for understanding population changes and the epidemiology of bacterial infections: Studies on gene expression will give information about the biosynthesis of substances and structures associated with virulence: Studies of mutants with altered virulence properties may explain the mode of action of virulence factors and their interaction with host cells.
Genetic factors in the host also may have a profound influence on bacterial virulence. Rutter et al. (1975) stated that adhesion of pathogens to receptor sites in the host is specific and such specificity can be demonstrated by genetic evidence. For example, they showed that K88-positive E. coli did not attach to the intestinal brush-borders of certain pigs. The animals in which adhesion did not take place were resistant to infection. Gibbons et al. (1978) found that susceptibility to K88-positive E. coli was due to the presence of an intestinal receptor which was inherited in a simple Mendelian fashion with adhesion being dominant over non-adhesion.

Damage to host cells occurs in certain bacterial infections due to the release of toxins which usually attack specific targets in the host. Genes for toxin production may be located either on plasmids or on the chromosome (Warren et al. 1975; Khan and Novick, 1980). For example, in Staphylococcus aureus, the genetics of two toxins (enterotoxin B and exfoliative toxin) has been investigated recently. The former is responsible for food poisoning and the latter for a condition in children called scalded-skin syndrome. The genetic determinants for both toxins have been found on plasmids. The best example of a chromosomally determined toxin is cholera toxin which shows many similarities to the plasmid-determined heat-labile toxin of E. coli (Dallas and Falkow, 1980).

The genetic determinants for resistance to host defence may be located on plasmids. A good example is the plasmid col. v. on which resistance to the bactericidal action of serum
was located in *E. coli* (Smith, 1974).

### 7.7 Gene transfer in *B. pertussis*

Gene transfer in *B. pertussis* has been studied only by transformation. Branefors (1964) showed that after exposure of a streptomycin-sensitive strain (44122/7) of *B. pertussis* to DNA from a streptomycin resistant variant of the same strain (44122/7R), some cells became streptomycin-resistant. The recipient strain was sensitive to streptomycin concentrations over 1 μg ml⁻¹ on solid media, whereas, the resistant strain could grow in a solid medium containing more than 5000 μg ml⁻¹ of streptomycin. Branefors used no factor to enhance DNA uptake and found that cells in the late exponential phase of growth were competent. She emphasized that the highest rate of transformation was obtained only when casamino acids in the growth medium were replaced by 0.5% (w/v) casein hydrolysate (tryptic digest).

The only other report of transformation in *B. pertussis* came from Kloos *et al.* (1978), who were able to transfer the ability to utilize certain amino acids to amino acid auxotrophs of *B. pertussis*. They stated that Leu-12 and TrpE-5 mutants of the strain 185 demonstrated good competence in modified Stainer and Scholte liquid medium. They also demonstrated a high transformation frequency in modified Stainer and Scholte liquid medium solidified by 1.5% (w/v) Bacto-agar and supplemented with 0.1% (w/v) activated charcoal. Gene transfer in these media occurred within 8 to 10 h of exposure of the recipient cells to 1 to 5μg ml⁻¹.
of donor DNA in the presence of 0.001M CaCl₂.

The existence of bacteriophages which are lytic against <i>B. pertussis</i> was reported by Grant (1979). He noted that in the absence of type strains, and the difficulties of classifying <i>Bordetella</i> pathogens, the importance of this observation may be of value in epidemiology and clinical medicine, although no evidence for a transducing phage at present is available.

### 7.8 Streptomycin resistance

Streptomycin resistance was the prime selection mechanism used in the present studies on <i>B. pertussis</i>. It has been characterized as a mutation affecting the 30S ribosome subunit in bacteria (Nomura, 1970). The action of streptomycin on sensitive bacteria seems to be initially on protein synthesis, followed by an inhibition of respiration and nucleic acid synthesis, and disruption of the cytoplasmic membrane (Gould, 1973). The combination of streptomycin with the 30S component of the ribosome in sensitive cells leads to the insertion of a wrong amino acid into growing polypeptide chains, whereas in resistant cells the combination of streptomycin with the 30S component does not occur (Taip <i>et al.</i> 1978).

Wallace and Davis (1973) stated that studies on the initiation of chain-elongating ribosomes showed that streptomycin allows initiation, but blocks the initiation complex from becoming a chain-elongating ribosome. The ribosome then releases and it is no longer active in protein synthesis, though it is not inert. It recycles onto mRNA, forming a blocked initiation complex again.
This cyclic blockade explains why sensitivity to killing is dominant in Str\textsuperscript{S}/Str\textsuperscript{R} heterozygotes, despite the presence of resistant ribosomes as well as sensitive ones.

Recently Kogut and Carrier (1980) proposed that after entry into sensitive cells, streptomycin combined with specific sites on 30S ribosomal subunits which are saturated at one, or a few, molecules per ribosome. The affected subunits are then subverted to the synthesis of abnormal proteins which are rapidly degraded. The specific binding is irreversible, so that affected subunits cannot recover normal function. As more and more ribosomes combine with antibiotic, the production of functional proteins steadily declines until it is insufficient to sustain further growth.

Other mechanisms for Str\textsuperscript{R} in bacteria have also been reported. Kaji and Tanaka (1968) found that resistance to streptomycin was due to the alteration in the 30S subunits so that it does not bind to the antibiotic. Weinstein (1970) stated that resistance could be developed by inability to transport streptomycin to an intracellular site or by the induction of enzymes that metabolize the drug. Streptomycin resistance could also be developed by an acquired R-factor that conferred resistance to several antibiotics. Alper and Ames (1978) postulated that streptomycin-resistance was found in mutants in the cyclic AMP control system in Salmonella typhimurium. They proposed that antibiotic enters the bacteria through a transport system normally used for transporting fuel/carbon sources and that this was accomplished because of a structural similarity between the antibiotic and the natural substrate of the particular transport system involved.
OBJECT OF RESEARCH
OBJECT OF RESEARCH

Few genetic studies with *B. pertussis* have been reported. A procedure for gene transfer would facilitate the study of the interrelationships between the various immunological and pathophysiological activities of this organism.

The object of the research was to confirm previously reported DNA-mediated transformation and to establish optimum conditions for the process. An alternative procedure involving plasmid-mediated chromosome mobilization not so far attempted in *B. pertussis* was also considered to be worth exploring.

Characterization of the phenotypes of parent and derived strains was undertaken to determine the separation or linkage of particular properties, such as those associated with virulence and antibiotic resistance. It was the overall intention of this work to lay the foundations for further detailed studies on *B. pertussis* genetics.
MATERIALS AND METHODS
1. Bacteriological materials and methods

1.1 Strains

Several phase-I and phase-IV *B. pertussis* and two strains of *Escherichia coli* were studied.

*B. pertussis* strain 44122/7S is a phase-I, streptomycin-sensitive strain used for vaccine production in Sweden.

Strain 44122/7R is a streptomycin-resistant (>500 \( \mu g \) \( ml^{-1} \)) mutant of strain 44122/7S. Both strains were supplied as freeze-dried cultures by Dr. P. Brane dels, University of Goteborg, Institute of Medical Microbiology, Department of Bacteriology, Gulheds gatan 10, S-413 46 Goteborg, Sweden.

*B. pertussis* strains 353 phase-I, 353 phase-IV; L84 phase-IV; 18334 phase-I; 8631 phase-IV; D30042 phase-IV and Taberman phase-I were obtained from the Microbiology Department culture collection.

Strain 18323, the intracerebral challenge strain was obtained from Dr. F. Sheffield, Division of Immunological Products Control, National Institute for Medical Research (Hampstead Laboratories), Holly Hill, Hampstead, London, NW3.

*Escherichia coli* strain Jc 53-1 has a chromosomal mutation conferring resistance to nalidixic acid (\( \text{Nal}^R \)) (20 \( \mu g \) \( ml^{-1} \)) as well as auxotrophic mutations making it Pro\(^-\) Met\(^-\). It also carries the R plasmid, R68.45, which confers resistance to kanamycin (20 \( \mu g \) \( ml^{-1} \)); carbenicillin (200 \( \mu g \) \( ml^{-1} \)) and tetra-cycline (10 \( \mu g \) \( ml^{-1} \)).

*Escherichia coli* strain Jc 3272 has chromosomal mutations which confer resistance to streptomycin (200-500 \( \mu g \) \( ml^{-1} \)) and also render it His\(^-\), Trp\(^-\); and Lys\(^-\). Both strains were supplied by Dr. B.E.B. Moseley, Department of Microbiology, University of Edinburgh.
Stock cultures were maintained in the freeze-dried state or were suspended in 20% (w/v) glycerol in 1% (w/v) casamino acids solution (CAA) (Appendix 1) and stored frozen in liquid nitrogen or at -70°C. For growth, stock cultures were suspended in 1% (w/v) CAA and pipetted onto plates of solid media.

1.2 Media

The following media were used for cultivation or selection of either *B. pertussis* or *E. coli* strains:

- Bordet-Gengou medium (Gibco Bio-Cult Diagnostics, Glasgow, Scotland) containing 20% (v/v) defibrinated horse blood (Gibco).
- Stainer and Scholte defined liquid medium (Stainer and Scholte, 1971).
- Modified Stainer and Scholte minimal liquid medium as described by Kloos et al. (1978).
- Cohen and Wheeler medium, as described by Branefors (1964).
- Nutrient agar (Oxoid Ltd., Basingstoke, Hants, England).
- Trypticase soy agar (BBL, Cockeysville, Maryland, 21030, U.S.A.).

Details of media preparation are given in Appendix 1.

For the selection of variants, the required concentration of antibiotics needed to prevent the growth of the wild type was added to the appropriate medium.

a) For transformation the selective medium was B-G agar containing 200 \( \mu g \text{ ml}^{-1} \) streptomycin sulphate (Sigma Chemical Co.). Streptomycin was prepared as a 20 mg ml\(^{-1} \) solution, filter
sterilized (Microflow 25 Disposable filter assembly, 0.45 μm pore size) and added to the medium at 50°C just before pouring into the plates.

b) For plasmid transfer selection, nutrient agar or B-G agar containing 20 μg ml\(^{-1}\) kanamycin and 200 μg ml\(^{-1}\) carbenicillin (Beecham Research Laboratories, Brentford, England) were used.

c) To demonstrate chromosomal mobilization, nutrient agar and B-G plates were prepared as above (b) except that nalidixic acid (Sigma) at 20 μg ml\(^{-1}\) (for E. coli strains) or streptomycin at 200 μg ml\(^{-1}\) (for B. pertussis) was also incorporated.

1.3 Growth of cultures

Freeze-dried cultures were reconstituted with 1% (w/v) CAA and grown on B-G agar. The plates were incubated at 35°C for 3-4 days in a closed plastic box containing a beaker of water to saturate the atmosphere.

For preparation of DNA, liquid cultures were grown by inoculating 2-litre dimpled conical flasks containing 1-litre amounts of Stainer and Scholte minimal liquid medium with a loopful of cells from B-G plates. Flasks were incubated in an orbital incubator at 100 rpm and at 35°C for 48h.

For the preparation of competent cells from phase-I B. pertussis strains, 250ml dimpled conical flasks, containing 100 ml modified Stainer and Scholte liquid medium were inoculated with a loopful of cells from B-G plates. With phase-IV B. pertussis and E. coli strains nutrient broth (Oxoid) was used. All flasks were incubated in an orbital incubator at 100 rpm and at 35°C.
Purity of cultures was checked by Gram-staining and by inoculation onto nutrient agar and onto B-G plates.

Challenge cultures for virulence tests and mouse protection tests were prepared by resuspending freeze-dried stock cultures in 1% (w/v) casamino acids solution, or by thawing the frozen cultures. Cell suspensions were inoculated onto B-G plates and incubated for 48 h at 35°C. Cells were then heavily inoculated onto further B-G plates, incubated for 24 h at 35°C, and the resulting growth used to prepare the challenge suspensions.

1.4 Estimation of optical density

The optical densities of bacterial suspensions were measured at a wavelength of 540nm, or at 600nm in the Schwinghamer procedure (see below), in a Pye-Unicam model SP500 spectrophotometer using polystyrene cuvettes with a 1 cm light path.

1.5 Estimation of viable count

Serial, ten-fold dilutions of cell suspensions were made in sterile saline or 1% (w/v) CAA and 0.1 ml amounts of dilutions expected to give countable numbers of colonies were spread in duplicate onto B-G plates. These were then incubated for 3-4 days at 35°C. The average number of colonies per dilution was multiplied by the dilution factor to calculate the number of viable cells in the original suspension.
1.6 Standardization of bacterial concentration by opacity

The bacterial concentration was estimated by comparison with the International Opacity Reference Preparation (World Health Organisation International Laboratory for Biological Standards, National Institute for Biological Standards and Controls, Holly Hill, Hampstead, London), designated as having 10 International Opacity Units. The dilution factor used to match the original cell concentration with the reference preparation was calculated and multiplied by 10 to give the concentration in opacity units of the undiluted bacterial suspension. A concentration of 1 ou was considered to be equivalent to approximately $10^9$ organism per ml.

1.7 Antibiotic sensitivity

a) B-G plates were streaked with B. pertussis strains from 48 h B-G agar cultures, then Neo-sensitab antibiotic discs (Taas, Strupgaardsvij 30, 2630 Taastrup, Denmark), were transferred onto each plate. Each antibiotic disc was tested on at least two separate B-G cultures for each strain. Plates were then incubated for 4-5 days at $35^\circ$C and the diameter of the zones of inhibition were measured.

b) Various concentrations of antibiotics were also incorporated into B-G agar and the plates streaked and incubated as above. The antibiotic concentration which caused complete inhibition of growth was recorded.
2. Bio-assay method

2.1 Mice

All mice used were from a randomly-bred closed colony originally derived from the HAM/ICR strain (Charles River U.K. Ltd., Manston Road, Margate, Kent).

2.2 Protective activity

Three-week old male HAM/ICR mice were randomized in groups of ten before being injected intraperitoneally with 0.5ml of graded doses of heated (56°C for 30 min) B. pertussis cell suspensions. After fourteen days the mice were anaesthetized with ether and challenged intracerebrally with 0.03ml CAA containing approximately $10^5$ organisms (approximately 100 LD$_{50}$) of B. pertussis strain 18323. Groups of mice used as controls were unimmunized, injected with approximately $5.4 \times 10^3$; $5.4 \times 10^2$ 54. and 5.4 organisms per mouse, respectively to check the virulence of the challenge strain. Mice dying within three days of challenge were excluded from the results. Surviving mice were counted after 14 days. Mice at the point of death on day 14 were counted as dead.

2.3 Histamine-sensitizing activity

Seven to eight-week old HAM/ICR mice of either sex were injected intraperitoneally, in groups of five, with 0.5 ml of graded doses of heated (56°C for 30 min) B. pertussis cell suspensions. After five days the mice were injected intraperitoneally with 3 mg of histamine dihydrochloride (Sigma) contained
in 0.5 ml of saline.

Survivors were counted after 4 hours.

2.4 Heat-labile toxin

Toxicity was assayed in 3-4 week old male HAM/ICR mice. Challenge cultures were prepared from \textit{B. pertussis} liquid cultures harvested by centrifugation (10,000 g, 30 min, 4\(^{\circ}\)C). The cell pellets were resuspended in saline and standardized by opacity. Mice were injected intraperitoneally, in groups of four, with 0.5 ml of graded doses of these suspensions. Deaths were recorded over the next three days.

2.5 Virulence by the intracerebral route

Challenge cultures were prepared as described in section (1.3) and challenge suspensions adjusted to 10 ou. A series of ten-fold dilutions (numbered 1 — 8) of this original suspension were prepared in 1\% (w/v) CAA. Samples (0.05 ml) from dilutions 7 and 8 were inoculated onto duplicate B-G plates for viable counts. Amounts (0.03 ml) from selected dilutions (depending on the virulence of the strain) were injected intracerebrally into randomized groups of ten 3-week old male HAM/ICR mice. Dead mice were counted daily for 14 days. Mice dying within three days of challenge, presumably due to trauma, were excluded from the results. Mice at the point of death on day 14 were counted as dead.
2.6 Virulence by the intranasal route

Challenge suspensions were prepared as described for the intracerebral route. Randomized groups of five 3-week old male HAM/ICR mice were anaesthetized with ether and inoculated with graded doses of the challenge suspension. A standard dropping pipette (2 ml plastic-pak syringe Dynatech AG Switzerland) was used to deposit 0.05ml of the inoculum onto the external nares. Deaths were recorded after 14 days and surviving mice were killed and examined as follows:

a) The gross lung pathology was rated from no apparent lesion to massive involvement (0 to 4).

b) Portions of the lungs taken from the consolidated lobe were removed aseptically then smeared onto B-G plates and incubated at 35°C for 4-5 days. The amount of growth was expressed by scores from 0 to 4, which represented no growth and graded amounts of growth (< 10 c.f.u. to confluence) respectively (North, 1946).

The sum of the scores for culture and gross pathology was considered as the lung score of the mouse (Pittman et al. 1980).

From the raw data obtained in these bio-assays ED$_{50}$ values (and 95% confidence limits) and relative potency values were estimated by the Probit method either by calculation (Prof. A.C. Wardlaw, personal communication) or by use of a computer programme. The method for combining the results of several independent assays (A.C. Wardlaw, personal communication) is given in Appendix 3.

*The computer programme for analysing HSF and MPT results was developed by statisticians in the department of Epidemiology and Biometrics, School of Hygiene, University of Toronto, Canada.
3. **Immunological Materials and Methods**

3.1 **Slide agglutination**

*B. pertussis* strains were grown on B-G plates for 48 h at 35°C. Two drops of a turbid cell suspension in saline (0.85% w/v) were put onto a clean dry microscope slide. To one, a drop of pertussis agglutinogen-specific antiserum (obtained from Dr. S. Zahkarova, Institute of Epidemiology and Microbiology of the USSR Academy of Medical Sciences, Moscow, USSR) was added. Saline was added to the other drop as a control. The slide was agitated gently for a few minutes, and any agglutination was noted.

3.2 **Haemagglutination tests**

Cells from 72 h B-G plate cultures were suspended in Dulbecco A phosphate buffered saline (PBS) pH 7.3 (Oxoid Ltd.) and the opacity of the suspension adjusted to 10 ou. The test was performed in Cooke micro-titre trays (Sterilin Ltd, Sussex, England) with standard microdroppers and microdiluters (Cooke Engineering Co., Alexandria, Virginia). Two rows of wells were used for each strain. The first two wells were controls in which only PBS and red cells were added. In the remaining wells serial two-fold dilutions of the bacterial suspension were prepared. To each well, two drops (0.05 ml) of 2% (v/v) horse erythrocytes in PBS were added. The reagents were mixed and the micro-titre tray was incubated for 1 h at 4°C. The haemagglutination titre was taken as the reciprocal of the highest dilution showing complete agglutination of the erythrocytes.
4. Genetical Materials and Methods

4.1 Preparation of DNA

Strain 44122/7R was grown in Stainer and Scholte liquid medium at 80 rpm and 35°C for 48 h. Cells were harvested by centrifugation at 10,000 x g at 4°C for 20 min. The pellet was then treated in one of the following ways.

a) Modified Branefors method (after Branefors, 1964)

The pellet was disrupted after freezing in dry ice by three passages through an X-press (LKB Instruments Ltd., South Croydon, Surrey). The broken cells were suspended in 10 ml SSC (0.1M NaCl containing 0.015M sodium-citrate) and centrifuged at 25,000 x g for 20 min at 4°C. The supernate was suspended in 75 to 100 ml of 2M NaCl in order to extract the DNA and centrifuged at 25,000 x g for 20 min. The upper layer containing most of the DNA was slowly poured into 4 volumes of 95% (v/v) ethanol. The DNA precipitated as white gelatinous mass and was spooled off with a glass rod, transferred into 100 ml of 2M sodium chloride and left overnight at 4°C. The crude DNA preparation was further purified from protein by the addition of 0.1% (w/v) sodium deoxycholate. The mixture was allowed to stand for 2 h at 37°C and 1 h at 4°C and was then centrifuged at 3,900 x g for 20 min. The supernate was poured into 4 volumes of 95% (v/v) ethanol. The precipitated DNA was spooled off into 10 ml of 2M NaCl. This DNA was further purified by treatment with 50 μg ml⁻¹ RNase (Sigma) (dissolved in 0.15M NaCl, pH 5.0 and heated to 80°C for 10 min to destroy any contaminating DNase activity) at 37°C for 1 h. Further protein was
removed by the addition of 0.1% (w/v) sodium deoxycholate at 37°C for 1 to 2 h and at 4°C for another 1 to 2 h. The mixture was centrifuged at 10,000 x g for 20 min and the supernate precipitated with 95% (v/v) ethanol and dissolved in 10 ml sterile 2M NaCl.

b) Kirby's method (after Kirby, 1964)

Approximately 5 g of cells was suspended in 100 ml of 0.15M NaCl, 0.1M EDTA buffer at pH 8.0. 4 g amino salicylic acid (sodium salt) was dissolved in the suspension and then 10 ml of 25% (w/v) sodium dodecyl sulphate in 50% (v/v) ethanol was added, the suspension was heated to 60°C in a water bath and maintained at this temperature for 15 min with occasional gentle stirring. It was allowed to cool and 100 ml phenol-cresol-water solution (prepared by the addition of 70 g m-cresol, 0.5 g 8-hydroxyquinoline and 70 ml distilled water to a 500 g bottle of phenol) was added to the lysed suspension. The stopper of the flask was taped down and it was clamped on to a wrist-action shaker for 30 min to disperse the clumped material. The emulsion was centrifuged at 10,000 x g for 20 min and 4°C in 250 ml polypropylene bottles in an MSE 18 centrifuge. The upper aqueous phase was transferred to a 250 ml beaker an equal volume of cold 2-ethoxyethanol was gently layered on to it and the two layers were carefully mixed with a glass rod. Fibres of DNA were precipitated and were spooled onto the glass rod. The rod was drained against the side of the beaker and the DNA redissolved in 40 ml of diluted (1/10) SSC. RNase solution (prepared as described in the previous section) was added to the redissolved DNA to give a final concentration of 50 μg ml⁻¹ and
incubated at 37°C for 30 min. The solution was adjusted to 0.4M with NaCl solution. One volume of phenol-cresol-water and centrifuged was added and the mixture shaken to separate the aqueous layer as before. To the aqueous layer an equal volume of iso-propanol was added and the DNA collected for spooling on to a glass rod. The DNA was redissolved in 10 ml dilute (1/10) SSC and solid NaCl added to 0.5M and sodium benzoate to 20% (w/v). The DNA was precipitated by addition of one volume of 2-butoxy-ethanol and collected on a rod as before. The final DNA precipitate was redissolved in 10 ml SSC and stored at 0 - 4°C. A few drops of chloroform were added to maintain sterility if the solution was to be kept for more than 24 h.

c) Modified Marmur method (after Marmur, 1961)

The cells pellet added to 4 volumes of buffer (pH 8.0) containing 0.15M NaCl, 0.3M EDTA, 0.5% (w/v) sodium sarcosinate and 5.0% (v/v) chloroform. Cells were broken by grinding at 4°C into a slurry for 30 min with a mortar and pestle. To the slurry 5M sodium perchlorate was added to give a final concentration of 1M. The mixture was gently shaken for 30 min at room temperature. An equal volume of chloroform:iso-amyl alcohol (24:1 v/v) was added, and the mixture gently shaken for 30 min at room temperature. The emulsion was centrifuged at 10,000 x g for 10 min at 4°C in an MSE 25 centrifuge. The supernate was removed and extracted twice more with chloroform:iso-amyl alcohol. DNA was then precipitated from the supernate by gentle addition of two volumes of ethanol, and wound onto a glass rod. It was then dissolved in a small volume of dilute in normal strength (1/10) SSC. The solution was then made up in normal strength SSC and RNase.
(pre-incubated at 2 mg ml$^{-1}$ in SSC (pH 4.9) in a boiling-water bath for 10 min before plunging into ice) was added to the DNA solution to a final concentration of 0.2 mg ml$^{-1}$ and the mixture incubated at 37°C for 1 h. At this point pronase (Koch-Light Laboratories Ltd., Colnbrook, Bucks., England), a non-specific protease (pre-incubated in dilute (1/10) SSC at final concentration of 0.3 mg ml$^{-1}$ for 2 h at 37°C) was added. The DNA was then extracted as before using chloroform:iso-amyl alcohol, but with only one repeat and was then precipitated in two volumes of ethanol. The DNA was spooled off, dissolved in 10 - 15 ml of dilute (1/10) SSC, and distributed into Bijou bottles in 1 ml aliquots and frozen at -20°C.

d) A combined Schwinghamer (1980) and modified Marmur method

To avoid the possibility of DNA shearing during mechanical cell disruption, the use of lysozyme to produce spheroplasts for gentle lysis of cells was tried (Schwinghamer, 1980). A mild osmotic shock, in the presence of EDTA, is applied to force lysozyme molecules through the outer membrane and to expose the murein layer to rapid enzymatic degradation.

Figure 2 is a flow diagram to show the steps for cell lysis as outlined by Schwinghamer (1980). The cell lysate was then treated by the modified Marmur method for DNA extraction.
Figure 2.

Optimum conditions for preparation of transforming DNA.
Bacterial culture

Measure absorbance at 600 nm, use culture volume equivalent to 12 ml at $A_{600nm} = 1$

Centrifuge (10,000 x g, 20 min, 0°C), resuspend cells in cold TS (Tris 0.05M, NaCl 0.05M, pH 8.0) buffer.

Detergent wash

Add sodium Lauryl sarcosinate (Sarkosyl) to 0.1% (v/v) vortex mix 15-30 sec, centrifuge as above and drain pellets well.

Resuspend cells in 0.4 ml TES (TS + EDTA 0.01M);

Mild osmotic shock

Add 0.35 ml concentrated sucrose mix (Sucrose 1.6M, Tris 0.55M, EDTA 0.1M), keep at 5°C for 10-20 min.

Add 0.15 ml lysozyme (5 mg ml$^{-1}$ in Tris 0.05M, pH 8.0) mix, add 3.6 ml cold water.

Incubate 5 - 20 min

Add 2.5 ml of 2.5% (w/v) Sarkosyl, mix slowly to clear.

Lysis

Treat by modified Marmur procedure for DNA extraction.
Estimation of DNA.

DNA was estimated according to a modified Burton method (Burton, 1955). A standard graph of absorbance at (595 minus 700) nm against μg ml\(^{-1}\) 2-deoxy-D-ribose (Puriss. CHR-Koch-Light Laboratories Ltd., Colnbrook, Bucks., England) was plotted. A series of dilutions of extracted DNA were prepared in dilute (1/10) SSC buffer. Triplicates of 1 ml of each dilution were made. As a blank, 1 ml of dilute (1/10) SSC was added to each of three test tubes. To all tubes the following was added.

a) 0.1 ml of 20% (v/v) perchloric acid
b) 2.0 ml of 4% (w/v) diphenylamine in glacial acetic acid
c) 0.1 ml of 1.6 μg ml\(^{-1}\) acetaldehyde

All test tubes were incubated in a water bath at 30°C overnight. The absorbance of the solution was calculated by subtraction of the readings at \(E_{700}\) from the readings at \(E_{595}\) using polystyrene cuvettes with a 1 cm lightpath. The value of DNA was determined from the deoxyribose graph by converting absorbance readings obtained into μg ml\(^{-1}\), then multiplying by 4.8.

4.2 Preparation of competent cells

a) After Bratfors (1964)

A recipient strain from a freeze-dried culture was grown at 35°C for 48 h on B-G plates. One loopful of bacteria was transferred to a 100 ml Erlenmeyer flask containing 10 ml of Cohen and Wheeler liquid medium to which 1% (w/v) casein hydrolysate had been added. It was incubated at 37°C on the rotary shaker
Figure 3.

Standard curve for 2-deoxy-D-ribose,
(after Burton, 1956).
for 18 - 30 h. From this pre-culture, 1 ml portions were transferred to a number of 100 ml flasks each containing 10 ml of liquid medium to which varying amounts (0.03% to 1% (w/v)) of casein hydrolysate had been added. These cultures were incubated on the rotary shaker for varying lengths of time (10 - 36 h) for the development of competent cells.

b) Modified Kloos et al. (1978) method

A recipient strain was inoculated onto B-G plates and incubated for 48 h at 35°C. A loopful of cells culture was inoculated into 250 ml flasks containing 100 ml modified Stainer and Scholte liquid medium containing 0.25% (w/v) casein hydrolysate (enzymatic digest). The flasks were incubated in a rotary shaker (Gallenkamp, London) at 35°C for 48 h at 80 rpm.

c) Spheroplasts preparation

Spheroplasts from strain 44122/7S were prepared by growing the recipient cells in Stainer and Scholte liquid medium containing 1% (w/v) glycine for 48 h at 35°C in a rotary shaker (Gallenkamp, London) at 80 rpm.

4.3 Transformation procedures

a) Branefors method

One ml of competent cell culture was centrifuged at 4,000 x g on a bench centrifuge (MSE Superminor). The pellet was suspended in 10 ml fresh Cohen and Wheeler (C&W) liquid medium, containing 0.5% (w/v) casein hydrolysate. 2ml of the diluted cells were then treated with donor DNA at a final concentration of 5 μg ml⁻¹. The cell suspension was then incubated for
4 - 5 days at 35°C. Another 2 ml of the diluted cells were treated in the same manner except no DNA was added as a control. Figure 4 shows the steps followed in this method.

b) Modified Kloos et al. (1978) method

DNA and any other additions were added to 0.9 ml competent cells to give a final volume of 1.0 ml. The samples were chilled on ice for 40 min and then 0.05 ml portions of each sample were inoculated onto duplicate B-G plates and incubated for 48 h at 35°C. The bacteria on each plate were resuspended in 2 ml of 1% (w/v) casamino acids and 0.1 ml was spread onto duplicate B-G plates with or without 200 μg ml⁻¹ streptomycin. The plates were incubated for 4 - 5 days at 35°C. Streptomycin-resistant colonies from both test and control plates were counted.

4.4 Plasmid transfer procedure

From Jc 53-1 to Jc 3272 E. coli strains. Each of the above strains was grown overnight on nutrient agar (Oxoid) plates. A loopful of each was then inoculated into a 250 ml conical flask containing 100 ml nutrient broth (Oxoid) and incubated for 5 h in a rotary shaker of 37°C. The donor and recipient strains were then mixed at a ratio of 1:5 (v/v) and transferred onto a membrane filter (47 mm x 0.45 μm) under negative pressure. A similar volume of cells of each strain alone was treated in the same manner, as controls. A series of dilutions of each culture were made in normal saline for viable counts. Filters containing the mixed strains or the donor and the recipient were transferred onto nutrient agar
Figure 4.

Optimum conditions for transformation
(after Branefors, 1964)
Recipient from lyophilized culture to solid medium (48 h)

Pre-culture: 1 loopful to 10 ml of liquid medium containing 1% (w/v) casein hydrolysate (18-30 h).

Competence culture: 1 ml to 10 ml of liquid medium containing 0.03-1% (w/v) casein hydrolysate (10-36 h).

1 ml sample centrifuged
Pellet suspended in 10 ml fresh C & W liquid medium containing 0.03-1% (w/v) casein hydrolysate.

2 ml treated with DNA
Incorporation of DNA for 4 - 12 h at 37°C
Solid medium with 200 µg/ml of streptomycin incubated for 4 - 5 days.

2 ml without addition of DNA as control.
Incubated for 4 - 12 h at 37°C
Solid medium with 200 µg of streptomycin for 4-5 days.
(Oxoid) plates. All plates were incubated for 24 h at 37°C.
The resulting growth from the filters was resuspended in 3ml of saline and each sample was serially diluted in normal saline.

From each dilution a duplicate of 0.1 ml was transferred onto selective media for both plasmid transfer and chromosomal mobilization. All plates were incubated for 24 h at 37°C.

From *E. coli* Jc 53-1 to *B. pertussis* 44122/7R

The same procedure for gene transfer used for *E. coli* to *E. coli* strains was followed except in:

a) Recipient was grown on Stainer and Scholte liquid medium for 48 h at 35°C, instead of nutrient broth.

b) The diluent was 1% (w/v) CAA instead of normal saline.

c) Plates were incubated for 48 h instead of 24 h for gene transfer.

d) The selective medium was B-G agar containing antibiotics instead of nutrient agar.

e) Plates were incubated for 4 - 5 days before colonies were observed.

From 44122/7RP(7R + R68.45 plasmid) to D30042-IV *B. pertussis*

A similar procedure was followed but since the donor strain had lost its ability to grow in Stainer and Scholte liquid medium, both donor and recipient strains grown on solid media. The donor was grown on B-G agar whereas the recipient was grown on nutrient agar.
4.5 Mutagenesis

10 ml of cells of *B. pertussis* of strain 44122/7S grown for 48 h at 35°C in Stainer and Scholte liquid medium was inoculated into 100 ml Stainer and Scholte liquid medium and incubated in a rotary shaker for 48 h at 35°C. NTG which was freshly prepared in water was added to give either 40 µg/ml or 100 µg/ml final concentration. Samples for viable count were taken at time zero and at 15 min intervals. From these a series of dilutions were prepared and inoculated onto B-G plates, and incubated at 35°C for 4 - 5 days. Samples for mutant selection were taken at 15 min and 45 min and centrifuged. The pellets were washed with Stainer and Scholte medium and then resuspended in fresh medium and incubated for 24 h at 35°C for gene expression. A 0.1 ml sample was then inoculated onto B-G plates containing streptomycin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹) or Neomycin (50 µg ml⁻¹). Plates were incubated for 4 - 5 days at 35°C. A control culture was treated in the same way except that no mutagen was added. B-G agar without antibiotics were also used as control.

4.6 Attenuation of strains by repeated subculture

To obtain *B. pertussis* degraded strains, phase-I (44122/7S and 44122/7R) strains were first inoculated onto Trypticase soy agar (TSA); TSA + 5% (v/v), TSA + 2% (v/v) and TSA + 1% (v/v) horse blood. The plates were then incubated for 4 - 5 days at 35°C. To determine whether growth was the result of a carry-over of nutrients from the original B-G plates, each
strain was transferred again onto the same medium and incubated as above. Growth from 1% (v/v) blood plates was used for further serial passages to try to obtain a derivative of each strain that would grow on TSA without blood. Thus each strain was passed from TSA + 1% (v/v) blood to TSA + 1% (v/v) blood and then to TSA alone. Growth from TSA was then used as a heavy inoculum for further TSA plates. After growth on TSA was stabilized, all subcultures were made from TSA to TSA plates.

5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein profiles of whole cells, envelopes and cytoplasmic fractions were obtained by SDS-PAGE by Dr. R. Parton according to the method of Laemmli (1970) as modified by Ames (1974).

Envelopes fractions were obtained by disrupting the cells by 3 passes through an LKB X-press (Parton and Wardlaw, 1975). After thawing, the broken cells were centrifuged at 100,000 g for 30 min and the supernate was taken as a crude cytoplasmic fraction. The envelope fraction was obtained by repeated washing of the deposit. Samples were solubilized for electrophoresis as described by Wardlaw et al. (1976).
RESULTS
1. Development of the transformation system

The transformation procedure of Branefors (1964) was investigated using her original strains, *B. pertussis* strain 44122/7R (Str^R^) as a donor and 44122/7S (Str^S^) as a recipient. However, only a very low number of transformants was obtained (Table 2). Transformation frequency was approximately 5-fold less than that reported by Branefors and with DNA prepared by Kirby's procedure no transformants were observed. CaCl\_2, which was reported to enhance transformation by promoting binding of foreign DNA with cell receptors in other Gram-negative bacteria such as *E. coli* (see Introduction), did not show a similar effect in *B. pertussis*. The transformation frequency was barely above the mutation rate which was 0 or 2 Str^R^ colonies present in the controls (without the addition of DNA) or in cells treated with DNase. A low frequency of such colonies would be expected in *B. pertussis* or in other organisms as spontaneous mutations. The low frequency of transformation justified the consideration of other factors which might affect the efficiency of the transformation system.

1.1 Preparation of DNA and gene expression in liquid and solid media.

There is a minimum size of the transforming DNA below which no transformation takes place (see Introduction). The cell disruption procedure used for the DNA preparation in the above experiment therefore may have contributed to the low transformation frequency obtained. The procedure used by Marmur (1961) for cell disruption, however, although more gentle
Table 2. Transformation of streptomycin resistance:

Branefors Method

Competent cells of strain 44122/7S and DNA from strain 44122/7R were prepared according to Branefors (1964) as described in the Materials and Methods section. The transformation procedure is also given in the Materials and Methods section.

<table>
<thead>
<tr>
<th>Addition to Competent Cells</th>
<th>Number of streptomycin-resistant colonies from 1ml competent cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA</td>
<td>30</td>
</tr>
<tr>
<td>0.01M CaCl₂</td>
<td>10</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA + 0.01M CaCl₂</td>
<td>50</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA + 50 μg ml⁻¹ DNase (Sigma)</td>
<td>0</td>
</tr>
</tbody>
</table>
than that used by Branefors, did not improve the transformation frequency (Table 3). However, by chilling the recipient with the donor DNA the frequency was improved more than 2-fold. The use of heat shock was not as effective as reported for E. coli (Table 3).

This somewhat crude step was replaced by a more gentle lysis procedure as used by Schwinghamer (1980). DNA was then extracted by a modified Marmur method. This DNA was used for transformation with recipient cells prepared by the procedure of Branefors. Results obtained (Table 4) did not show any significant difference in the number of transformants obtained compared to that obtained when DNA was prepared by either Branefors or by Marmur procedures. Thus the DNA preparation did not seem to be the factor which caused the low level of transformation.

When competent cells were prepared by the method of Kloos et al. (1978) and were subsequently incubated on B-G plates rather than in liquid medium for gene expression (after treatment with DNA) a better yield of transformants was obtained from this preparation of DNA. Four typical experiments are shown in Table 5. Transformation frequency ranged from 4,400 to 8,000 Str^R colonies ml^-1 original recipient culture which is relatively high compared either with results obtained from following Branefors procedure or with that reported by her. In one of these experiments the number of Str^R colonies isolated from samples treated with DNase was also relatively high compared with those isolated from a similar sample in the previous experiments. In all experiments B-G plates
Table 3. Transformation of streptomycin resistance: DNA prepared by modified Marmur method.

The procedure for competent cell preparation and the transformation procedure are given in Table 2.

<table>
<thead>
<tr>
<th>Addition to</th>
<th>Number of streptomycin-resistant colonies from 1ml competent cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA</td>
<td>44</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA, chilled for 20 min</td>
<td>108</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA, chilled for 2.0 min, then heat shocked for 4 min at 37°C</td>
<td>112</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA + 50 μg ml⁻¹ DNase (Sigma)</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 4. Transformation of streptomycin resistance:
DNA prepared by combined Schwinghamer-Marmur Method

Competent cell preparation and transformation procedure were as described in Table 2.

<table>
<thead>
<tr>
<th>Addition to Competent cells</th>
<th>Number of streptomycin-resistant colonies from 1ml competent cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA,</td>
<td>30</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA, chilled 20 min</td>
<td>40</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA, chilled 20 min then heat shocked 4 min at 37°C</td>
<td>30</td>
</tr>
<tr>
<td>5 μg ml⁻¹ DNA + 50 μg ml⁻¹ DNase</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5. Transformation of streptomycin resistance: gene expression on solid medium.

DNA was prepared by a combined Schwinghamer and Marmur procedure, competent cells were prepared by a modification of the procedure of Kloos et al. (1978) as described in the Materials and Methods section. Competent cells were chilled for 40 min after addition of DNA and incubated on B-G agar for 48 h before spreading on selection plates.

<table>
<thead>
<tr>
<th>Addition to Competent cells</th>
<th>Number of streptomycin-resistant colonies from 1 ml competent cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μg ml⁻¹ DNA + 50 μg ml⁻¹ DNase</td>
<td>Expt. 1  Expt. 2  Expt. 3  Expt. 4</td>
</tr>
<tr>
<td>None</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>10 μg ml⁻¹ DNA</td>
<td>4,800 7,200 4,400 8,000</td>
</tr>
<tr>
<td>10 μg ml⁻¹ DNA + 50 μg ml⁻¹ DNase</td>
<td>0 0 0 1,600</td>
</tr>
</tbody>
</table>
inoculated with DNA alone showed no growth which indicated that there was no carry-over of streptomycin-resistant cells in the DNA. The DNA prepared by any of the above mentioned methods was sterile.

The yield of DNA extracted by the combined Schwinghamer and Marmur procedures (5.6 mg litre\(^{-1}\) cell culture) was slightly more than that prepared by Marmur procedure alone (4.8 mg litre\(^{-1}\)).

The important factors which appeared to affect transformation in *B. pertussis* were therefore, the chilling of the recipient cells for 40 min after treatment with donor DNA, and the maintenance of gene expression on solid medium rather than in liquid medium. Transformation was abolished by the treatment with 50 µg ml\(^{-1}\) DNase. The DNA preparation in all cases was sterile.

It was concluded therefore that the increased numbers of Str\(^R\) colonies on the selection plates, over and above the control plates, were true transformants.

1.2 Competence of recipient cells

The affinity of recipient cells for DNA was investigated more thoroughly.

1.2.1 Effect of age of culture: The recipient strain 44122/7S was grown in Stainer and Scholte liquid medium and samples for transformation were taken after 33 h, 42 h, 48 h, and 53 h. Gene recombination and expression were allowed to
take place on B-G plates for 48 h at 35°C in all subsequent transformation experiments before the cells were resuspended and inoculated onto the selective medium. Results obtained showed (Table 6) that the optimal competence time was at 48 h which is the late exponential phase in _B. pertussis_. However, transformation frequency was much lower than that found for experiment in Table 5.

1.2.2 Effect of CaCl₂: The addition of CaCl₂ to the transformation system appeared to have a significant effect on transformation frequency in one experiment (Table 7). Both 0.005M and 0.01M final concentrations gave an enhanced frequency of transformants. However, these findings could not be repeated in subsequent experiments and approximately 50% of these colonies were unable to agglutinate with anti-phase-1 _B. pertussis_ antiserum in slide agglutination tests. Since CaCl₂ did not show any effect in a previous experiment (Table 2) its positive effect in this experiment must remain doubtful.

1.2.3 Effect of protease and cyclic AMP: No significant effect on transformation frequency was noted by adding 50 μg ml⁻¹ pronase just before the addition of donor DNA to competence cells (Table 8). Although a two-fold increase was obtained in one experiment it was not obtained in the other. Cyclic AMP (50 μg ml⁻¹) when added to exponentially growing recipient cells did not enhance competence whereas dibutyryl cyclic AMP at the same concentration gave a significant increase in the number of streptomycin-resistant colonies (Table 8).
Table 6. Effect of age of culture on competence of *B. pertussis*.

DNA and competent cell preparations and the transformation procedure are described in Table 5.

<table>
<thead>
<tr>
<th>Age of culture</th>
<th>Number of streptomycin-resistant colonies from 1ml competent cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>33 h</td>
<td>10</td>
</tr>
<tr>
<td>42 h</td>
<td>30</td>
</tr>
<tr>
<td>48 h</td>
<td>60</td>
</tr>
<tr>
<td>53 h</td>
<td>20</td>
</tr>
<tr>
<td>Control (No DNA added)</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 7. The effect of CaCl$_2$ on transformation frequency in B. pertussis.

Competent cells, DNA and transformation procedure were as described in Table 5.

<table>
<thead>
<tr>
<th>Addition to Competent cells</th>
<th>Number of streptomycin-resistant colonies from 1ml competent cell culture containing CaCl$_2$ at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.001M</td>
</tr>
<tr>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>10 µg ml$^{-1}$ DNA</td>
<td>70</td>
</tr>
</tbody>
</table>
Table 8. Effect of dibutyryl cAMP, cAMP and protease on transformation frequency.

Competent cells, DNA and transformation procedure were as described in Table 5.

<table>
<thead>
<tr>
<th>Addition to Competent cells</th>
<th>Number of streptomycin-resistant colonies from 1ml competent cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Expt.1</td>
</tr>
<tr>
<td></td>
<td>800</td>
</tr>
<tr>
<td>10 μg ml(^{-1}) DNA</td>
<td>4,400</td>
</tr>
<tr>
<td>10 μg ml(^{-1}) DNA + 50 μg ml(^{-1}) dibutyryl cAMP</td>
<td>14,800</td>
</tr>
<tr>
<td>10 μg ml(^{-1}) DNA + 50 μg ml(^{-1}) cAMP</td>
<td>1,200</td>
</tr>
<tr>
<td>10 μg ml(^{-1}) DNA + 50 μg ml(^{-1}) pronase</td>
<td>N.T</td>
</tr>
<tr>
<td>10 μg ml(^{-1}) DNA + 100 μg DNase</td>
<td>0</td>
</tr>
</tbody>
</table>

* Not Tested
1.2.4 Effect of chilling: The effect of chilling the competent cells on ice for various times was investigated. Samples of recipient cells were taken after 20, 40 and 60 min at 0°C and used for transformation as in previous experiments. More streptomycin-resistant colonies were isolated from cultures which had been chilled for 40 min (Table 9). The number of streptomycin-resistant colonies was 14-fold greater in cells chilled for 40 min than in both 20 and 60 min chilling and 28-fold greater than in the non-chilled sample.

1.2.5 Change to spheroplasts.

Spheroplasts from strain 44122/7S were prepared by growing the recipient cells in Stainer and Scholte liquid medium containing 1% (w/v) glycine (see Materials and Methods). Samples were treated for transformation with DNA prepared by the modified Marmur method and Braneors transformation procedure. No transformants were isolated. Spheroplasts were found to be able to revert and grow on normal B-G medium.

Thus, the age of culture, chilling and dibutyryl cAMP were found to affect competence and hence the transformation frequency in B. pertussis. Cyclic AMP, protease, and spheroplast preparation had no detectable effect in competence.

The transformation to Str\textsuperscript{R} of the strain 8631-IV was attempted, using DNA from strain 44122/7R under the conditions described in Table 5. No Str\textsuperscript{R} transformants were obtained from this strain under those conditions.
Table 9. Effect of chilling on transformation frequency

Competent cells, DNA and transformation procedure were as described in Table 5.

<table>
<thead>
<tr>
<th>Addition to Competent cells</th>
<th>Number of streptomycin-resistant colonies from 1ml competent cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>$10 \mu g \text{ ml}^{-1}$ DNA, 20 min chilling</td>
<td>20</td>
</tr>
<tr>
<td>$10 \mu g \text{ ml}^{-1}$ DNA, 40 min chilling</td>
<td>280</td>
</tr>
<tr>
<td>$10 \mu g \text{ ml}^{-1}$ DNA, 60 min chilling</td>
<td>20</td>
</tr>
<tr>
<td>$10 \mu g \text{ ml}^{-1}$ DNA, no chilling</td>
<td>10</td>
</tr>
</tbody>
</table>
2. **Mutagenesis**

2.1 **Chemical mutagenesis**

At the start of this project only one phase-I strain of *B. pertussis* in the departmental culture collection namely 44122/7R, had a marker, streptomycin-resistance, which could be used as basis for selection for transformants. Other markers would have been useful for gene transfer studies and so the optimal conditions for mutagenesis were determined.

Initially, the effect of mutagen concentration and preparation, and the time of exposure to the mutagen were investigated using streptomycin-resistance as the selection mechanism.

Two concentrations of NTG (40 µg and 100 µg ml\(^{-1}\)) were used to treat cells and the cultures were sampled at intervals to determine the total viable count and the number of streptomycin-resistant cells which were present (Figure 5). With 40 and 100 µg ml\(^{-1}\) NTG, the highest number of streptomycin-resistant cells was obtained after 45 min exposure. Samples taken before or after this time gave a lower number of streptomycin mutants. With 100 µg ml\(^{-1}\) NTG the rate of killing was higher and fewer mutants were obtained. For example the percentage survivors with 100 µg ml\(^{-1}\) NTG were 58.3%; 30% and 27.7% after 15; 30 and 45 min respectively. With 40 µg ml\(^{-1}\) NTG the percentage survivors were 77%; 55.5% and 43.8% at similar time intervals.
Figure 5. Effect of NTG on cell viability and mutant isolation.

A 48 h culture of strain 44122/7S was prepared as described in the Materials and Methods section. The culture was divided into two portions. One was treated with 40 μg ml⁻¹ final concentration NTG. The other was treated with 100 μg ml⁻¹. Samples for viable counts and mutant selection were then taken. (Materials and Methods section 1.5).

Treatment with 40 μg ml⁻¹ NTG, O-cell viability, ●-streptomycin-resistant mutants.

Treatment with 100 μg ml⁻¹ NTG, Δ-cell viability, ▲-streptomycin-resistant mutants.
NTG at 40 \( \mu g \text{ ml}^{-1} \) seemed to be the optimum concentration for mutagenesis. Both lower and higher NTG concentration gave fewer \( \text{Str}^R \) mutants. For example, when the cell suspension was treated with only 4 \( \mu g \text{ ml}^{-1} \) final concentration of NTG only a few \( \text{Str}^R \) mutants were isolated (Figure 6). The concentration of 100 \( \mu g \text{ ml}^{-1} \) NTG gave a larger number but this was still lower than that obtained under optimum conditions of 40 \( \mu g \text{ ml}^{-1} \) (Figure 5).

In subsequent experiments, NTG was used to try to obtain mutants of \textit{B. pertussis} resistant to other antibiotics. Cells exposed to NTG at a final concentration of 40 \( \mu g \text{ ml}^{-1} \) for 45 min were inoculated onto B-G agar containing 200 \( \mu g \text{ ml}^{-1} \) of either kanamycin, neomycin or streptomycin. Unfortunately, no mutants resistant to either kanamycin or neomycin were isolated in several experiments in which very high frequencies of \( \text{Str}^R \) mutants were obtained. In all experiments 0.1 ml cell suspension was inoculated onto the selective medium without treatment with NTG. The number of streptomycin-resistant mutants in these controls ranged from 0 to 20 \( \text{ml}^{-1} \) of untreated cells.

It was found that NTG lost its mutagenic effect with time in solution. When the cell suspension of strain 44122/7S was treated with 40 \( \mu g \text{ ml}^{-1} \) NTG 24 h after mutagen preparation both killing and \( \text{Str}^R \) mutant recovery were drastically reduced (Figure 6). Samples inoculated onto B-G agar containing streptomycin after 15 min of treatment with 40 \( \mu g \text{ ml}^{-1} \) NTG left for 24 h at room temperature gave 34-fold less difference in \( \text{Str}^R \) mutant recovery compared with the recovery obtained from the same NTG concentration freshly prepared.
Figure 6. Effect of NTG after storage at room temperature and of fresh preparations at low concentration

Cell preparation and treatment were as described in Figure 2 except that one portion was treated with a 24 h old 40 μg ml⁻¹ NTG preparation.

The other portion was treated with 4 μg ml⁻¹ of a fresh preparation.

Treatment with stored NTG (40 μg ml⁻¹); O-cell viability, ▲-streptomycin-resistant mutants.

Treatment with fresh preparation (4 μg ml⁻¹); Δ-cell viability, ▲-streptomycin-resistant mutants.
Thus $\text{Str}^R$ mutants from \textit{B. pertussis} were easily obtained. An optimum concentration and time of treatment with NTG were found for selection of $\text{Str}^R$ mutants, but mutants resistant to other antibiotics such as kanamycin and neomycin were not obtained under these conditions.

2.2 Mutant selection by \textit{in vitro} subculture

\textit{B. pertussis} will grow on media other than B-G medium. This ability was used by Standfast (1951) to demonstrate cultural changes.

Strain 44122/7R was initially subcultured from B-G medium to TSA + 5% (v/v) blood, then to TSA + 2% (v/v) blood and finally to TSA + 1% (v/v) blood. A heavy inoculum from the latter was used to inoculate TSA plates without blood. The strain was then subcultured 34 times on TSA medium. All cultures were incubated for 4 days at 35°C. However even after 34 subcultures on TSA the strain was unable to grow on the media (TSA) as single colonies. Growth only occurred where a heavy inoculum was applied to the plates. The strain was then characterized (see section 5).

3. \textit{Plasmid transfer and chromosome mobilization}

The plasmid R68.45 was reported to have the ability to mobilize chromosomal markers within a wide range of Gram-negative bacteria (Holloway, 1979). In this study streptomycin-resistance was assumed to be a chromosomal mutation and the ability of phase-IV strains to grow on nutrient agar was also used as a marker.
The ability of the plasmid to transfer to other strains was first checked in *E. coli* strains. The plasmid was transferred from *E. coli* strain Jc 53-1 to Jc 3272 by transconjugation using the membrane method. After exposure to the donor strain carrying R68.45 the recipient strain Jc 3272 was able to express resistance to kanamycin, carbenicillin and tetracycline coded for by the plasmid (Table 10). The frequency of plasmid transfer was very high (approx. 1 in 4 donor cells). In addition resistance to nalidixic acid which is a marker mobilized from the chromosome of the donor strain was also expressed, but at a lower frequency ranging from $3.3 \times 10^{-7}$ to $6.4 \times 10^{-6}$ per donor cell.

A similar experiment was done, using *E. coli* Jc 53-1 as donor and strain 44122/7R *B. pertussis* as recipient. Plasmid transfer was achieved but, with a lower frequency, ranging from $0.95 \times 10^{-4}$ to $1.95 \times 10^{-4}$ per donor cell and no expression of the nalidixic acid marker was observed (Table 11). The strain 44122/7R was able to express resistance to the antibiotics kanamycin, carbenicillin and tetracycline coded by the plasmid, in addition to its normal resistance to streptomycin.

With the plasmid present in strain 44122/7R (44122/7RP), transfer to any other *B. pertussis* phase-I strains required markers other than Str$^R$. Due to the lack of such markers the strain D 30042 phase IV *B. pertussis* was used as a recipient, because this strain would grow as single colonies on nutrient agar whereas the donor 44122/7RP would not. Nutrient agar containing carbenicillin and kanamycin was used as the selection medium since only phase-IV *B. pertussis* cells which had inherited
Table 10.  Plasmid transfer and chromosomal mobilization in *E. coli* strains

5 h culture of donor and recipient strains were prepared and mixed as described in the Materials and Methods section.  Viable cell counts were performed on donor and recipient cultures.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Strain</th>
<th>No. of colonies per ml of original culture isolated on selective media</th>
<th>Plasmid transfer frequency for donor cell</th>
<th>Chromosomal mobilization frequency for donor cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N.A containing 200μg ml⁻¹ Str. and car. + 20μg ml⁻¹ kana.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Donor Jc 53-1</td>
<td>0</td>
<td>0</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Recipient</td>
<td>0</td>
<td>0</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Jc 3272</td>
<td>0</td>
<td>0</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Jc 57-1 + Jc 3272</td>
<td>2.0 × 10⁷</td>
<td>3 × 10³</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jc 53-1</td>
<td>0</td>
<td>0</td>
<td>0.26</td>
</tr>
<tr>
<td>2</td>
<td>Jc 3272</td>
<td>0</td>
<td>0</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>Jc 53-1 + Jc 3272</td>
<td>1.32 × 10⁷</td>
<td>2.81 × 10³</td>
<td></td>
</tr>
</tbody>
</table>
Table 11. Plasmid transfer and attempted chromosomal mobilization from E. coli to phase-I B. pertussis

Cell culture, mixing and viable counts were done as described in Table 10 except that a B. pertussis 40 h culture was used and viable counts were done on B-G plates.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Strain</th>
<th>B-G agar containing 200μg ml⁻¹ car. and Str. and 20μg ml⁻¹ kana.</th>
<th>B-G agar containing 200μg ml⁻¹ car. and Str. and 20μg ml⁻¹ kana.</th>
<th>Plasmid transfer frequency per donor cell</th>
<th>Chromosomal mobilization frequency per donor cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Jc 53-1</td>
<td>0</td>
<td>0</td>
<td>0.95 x 10⁻⁴</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>44122/7R</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Jc 53-1 + 44122/7R</td>
<td>5 x 10³</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Jc 53-1</td>
<td>0</td>
<td>N.T.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>44122/7R</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Jc 53-1 + 44122/7R</td>
<td>2 x 10⁴</td>
<td>0</td>
<td>1.95 x 10⁻⁵</td>
<td>0</td>
</tr>
</tbody>
</table>

* Not tested
the plasmid would grow on it. Results obtained from two experiments showed plasmid transfer from the phase-I to D 30042 phase-IV *B. pertussis* strain but, with a frequency ranging from $1.29 \times 10^{-5}$ to $1.29 \times 10^{-5}$ per donor cell (Table 12). No colonies which were also Str$^R$ were obtained. Str$^R$ colonies would have indicated that chromosome mobilization from the donor 44122/7RP strain had taken place. The plasmid transfer frequency was relatively low, even lower than that obtained from *E. coli* Jc 53-1 strain to *B. pertussis* 44122/7R strain, which may mean that the level of plasmid transfer was too low to detect chromosomal mobilization of the Str$^R$ marker.

Other phase-IV *B. pertussis* strains were also tested for plasmid transfer from the Str$^R$ phase-I strain (Table 12). The strains 11615-IV and L84-IV *B. pertussis* acquired the plasmid R68.45, but again with low frequencies of $1.9 \times 10^{-5}$ and $1.6 \times 10^{-5}$ respectively. No chromosomal mobilization for the Str$^R$ marker was observed in any of the strains, despite the fact that the donor and recipient concentration was increased up to 10 times in order to try to detect a lower level of Str$^R$ transconjugants (Table 12 Expt. 3).

The stability of the plasmid was studied in both *E. coli* and *B. pertussis* strains. The strains which had gained the plasmid were also kept frozen in 1% CAA at -70°C for more than six months and neither strain lost the drug resistant properties characteristic of the plasmid. This however, is expected since stability of the plasmid was reported in other organisms such as *Erwinia* and *E. coli* spp. (Chatterjee, 1980). The 44122/7RP strain was routinely subcultured on B-G agar supplemented with
Table 12. Plasmid transfer and attempted chromosomal mobilization from *B. pertussis* 44122/7RP to phase-IV strains.

Donor cells were prepared by resuspending 48 h growth from B-G plates in 1% CAA. Recipient Cells were prepared by resuspending 48 h growth from nutrient agar in 1% CAA. Transconjugation procedure was done as described in Table 10.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Strains</th>
<th>No. of colonies per ml of original recipient culture isolated on selective media</th>
<th>Plasmid transfer frequency per donor cell</th>
<th>Chromosomal mobilization frequency per donor cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N.A containing 200 µg ml⁻¹ car. and 20 µg ml⁻¹ kana.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.A containing 200 µg ml⁻¹ of car. and Str.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Donor 44122/7RP 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Recipient D30042-IV 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>44122/7RP + D30042-IV 8 x 10³</td>
<td>0</td>
<td>1.29 x 10⁻⁵</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Donor 44122/7RP 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Recipient D30042-IV 0</td>
<td>0</td>
<td>1.28 x 10⁻⁵</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>44122/7RP + D30042-IV 1.54 x 10⁴</td>
<td>0</td>
<td>1.9 x 10⁻⁵</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Donor 44122/7RP 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Recipient 11615-IV 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>44122/7RP + 11615-IV 1.08 x 10⁶</td>
<td>0</td>
<td>1.6 x 10⁻⁶</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Donor 44122/7RP 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Recipient L84-IV 0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>44122/7RP + L84-IV 8.0 x 10⁻⁶</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
carbenicillin and kanamycin. A few colonies were inoculated from B-G plates onto B-G plates supplemented with kanamycin, and carbenicillin and tetracycline were grown to demonstrate the stability of the plasmid. The strain 44122/7RP was shown to have lost its ability to grow in Stainer and Scholte liquid medium. However, other strains of B. pertussis which had gained the plasmid R68.45, such as strain D 30042-IV, did not lose their ability to grow in this medium.

The plasmid R68.45 therefore showed the ability to transfer from E. coli to E. coli strains with chromosomal mobilization of the nalidixic acid marker, and from E. coli to B. pertussis strains but, with no chromosomal mobilization of this marker. The plasmid was also transferred from B. pertussis to B. pertussis strains, but again no chromosomal mobilization of the Str^R marker was detected.

4. Development of minimal solid medium

The development of a solid minimal medium for B. pertussis would have numerous advantages for genetic studies of this organism. It would make it possible to isolate amino acid auxotrophs and to select for recombinants of various types after transformation.

In this study the minimal solid media described by Parker (1976) and Kloos et al. (1978) were tested for their ability to support of growth of phase-I B. pertussis as single colonies. Only a few colonies were observed on either medium indicating a killing effect possibly due to growth inhibitors. Attempts
were then made to develop a more efficient minimal solid medium for *B. pertussis*. The medium contained bovine serum albumin, starch or Dowex-1 chloride (Sigma), substances reported to adsorb growth inhibitors. Hornibrook (1948), Stainer and Scholte (1971) and Parker (1976) liquid media were solidified with 1% (w/v) agarose (Sigma) or purified agar (Oxoid). Dowex (1% w/v) and starch (1-5% w/v) or albumin (1-5% w/v) were added either singly or together. By streaking with various *B. pertussis* strains from a 48 h B-G culture it was found that only phase-IV strains could grow as single colonies on all of these minimal media. Growth of phase-I strains was rather scanty after 4-5 days compared with growth on B-G medium and generally occurred only at the point of initial inoculation. Only a few single colonies developed on the minimal plates, but when these colonies were subcultured onto fresh minimal medium no further growth was observed. Thus attempts to grow phase-I *B. pertussis* on minimal solid media were not successful.

5. Characterization of *B. pertussis* strains used for genetic studies.

5.1 Immunological and pathophysiological activities

5.1.1 Mouse virulence

a) Intracerebral route

The virulence for mice of the *B. pertussis* strains used for genetic studies was compared to that of a selection of strains from the departmental culture collection. The LD$_{50}$ values were determined by the probit method.
In seven independent experiments, the LD$_{50}$ values obtained for Branefors streptomycin-sensitive and resistant strains were consistently different (Table 13). Strain 44122/7R was highly virulent for mice by the intracerebral route, with LD$_{50}$ values ranging from 126 to 630 cfu per mouse. Thus, this strain is as virulent as B. pertussis strain 18323, the standard intracerebral challenge strain used in the mouse protection test (Standfast, 1958). Strain 44122/7S was much less virulent with LD$_{50}$ values ranging from $7.9 \times 10^3$ to $>1.4 \times 10^7$. Although these values showed wide variation they were significantly different from the corresponding values for strain 44122/7R in all of the seven experiments as indicated by the 95% confidence limits. The virulence of 44122/7R relative to 44122/7S also varied greatly between experiments with relative potency values ranging from $1.4$ to $1.04 \times 10^5$. This variation may be attributed, at least in part, to inaccuracy in opacity standardization of the challenge suspensions, in the viable counting procedure and in the fitting of the dose response curves for probit analysis.

In some experiments the values for LD$_{50}$ and relative potency of the two strains were also determined by Probit analysis using a computer programme. The computer print-outs for these assays are given in Appendix (2). The LD$_{50}$ and relative potency values again show that strain 44122/7R is highly virulent for mice by the intracerebral route compared with 44122/7S and the difference is significant in each experiment. When the values obtained by calculation or computation were compared (Table 14) a number of differences were noted. For example, the
Table 13. Virulence for mice by the intracerebral route: Branefors streptomycin-resistant and sensitive strains.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; in colony forming units per mouse (and 95% confidence limits)</th>
<th>Relative potency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7S</td>
<td>44122/7R</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&gt;1.8 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>199 (154,257)</td>
</tr>
<tr>
<td></td>
<td>&gt;1.7 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&gt;1.9 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>520 (276,997)</td>
</tr>
<tr>
<td></td>
<td>&gt;360</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&gt;1.4 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>134 (100,182)</td>
</tr>
<tr>
<td></td>
<td>&gt;1.04 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&gt;2.9 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>355 (83,1.5x10&lt;sup&gt;3&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>&gt;237</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.9 x 10&lt;sup&gt;3&lt;/sup&gt; (1.3x10&lt;sup&gt;3&lt;/sup&gt;,3x10&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>562 (211,1.5x10&lt;sup&gt;3&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>&gt;3.4 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>630 (423,939)</td>
</tr>
<tr>
<td></td>
<td>&gt;183</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.8 x 10&lt;sup&gt;4&lt;/sup&gt; (250, 4x10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>126 (66, 240)</td>
</tr>
<tr>
<td></td>
<td>143</td>
<td></td>
</tr>
</tbody>
</table>

* Potency of strain 44122/7R relative to that of 44122/7S.
Table 14. Virulence in mice by the intracerebral route: Comparison of \( LD_{50} \) and relative potency values obtained by calculation and computation.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Strain</th>
<th>Calculated values</th>
<th>Computed values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( LD_{50} ) (cfu/mouse)</td>
<td>Rel.pot.</td>
</tr>
<tr>
<td>2</td>
<td>44122/7S</td>
<td>&gt;1.9 x 10^5</td>
<td>1.0*</td>
</tr>
<tr>
<td></td>
<td>44122/TR</td>
<td>520</td>
<td>360</td>
</tr>
<tr>
<td>4</td>
<td>44122/7S</td>
<td>&gt;2.9 x 10^4</td>
<td>1.0*</td>
</tr>
<tr>
<td></td>
<td>44122/TR</td>
<td>355</td>
<td>&gt;237</td>
</tr>
</tbody>
</table>

* 44122/7S is taken as the standard and assigned a relative potency value of 1.0
LD$_{50}$ values of strain 7R determined with the computer programme were closer than those obtained by calculation i.e. 240 and 250 cfu per mouse compared with 520 and 355 cfu. It was not possible to calculate the LD$_{50}$ values for 7S in these two experiments because of the extremely low virulence of this strain. For the same reason, the LD$_{50}$ values for 7S and the relative potency values estimated by the computer were widely different.

By combination of the computed data for the two experiments (Appendix 2) a best-estimate value of 310 was found for the relative potency of 7R (however, see footnote on p.156)

Transformants isolated after transforming the streptomycin-sensitive strain to streptomycin resistance were examined in a similar manner. Four transformants designated Trans.A,B,E and X were compared with either the Str$^R$ or Str$^S$ Branefors strains (Table 15). The LD$_{50}$ of Trans.A ranged from 120 to 660 cfu per mouse and that of Trans.B was <94 cfu per mouse. Trans.E and Trans.X were examined only once with LD$_{50}$ values of <78 and <73 cfu respectively. Thus these transformants were as virulent as strain 44122/7R. Some of these virulence test data were also analysed by computer (Appendix 2.2). Again the transformants were found to be highly virulent compared with the Str$^S$ parent strain, with LD$_{50}$ values for Trans.E, Trans.X and 44122/7S of 22, 11 and 2487 cfu per mouse respectively. The computed relative potencies of the transformants E and X (compared with strain 44122/7S), were 115 for Trans.E and 227 (obtained by calculation)
Table 15. Virulence in mice by the intracerebral route:

LD$_{50}$ of streptomycin-resistant strains from transformation experiments (obtained by calculation).

<table>
<thead>
<tr>
<th>Expt.</th>
<th>LD$_{50}$ in colony forming units per mouse (and 95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>7.9 x 10$^3$</td>
</tr>
<tr>
<td>6</td>
<td>&gt; 3.4 x 10$^4$</td>
</tr>
<tr>
<td>7</td>
<td>1.8 x 10$^4$</td>
</tr>
<tr>
<td>8</td>
<td>5.5 x 10$^3$</td>
</tr>
</tbody>
</table>
for Trans.X. A comparison of the calculated and computed results of this experiment is shown in Table 16. The results obtained by the two methods are very similar. The computer enabled the estimation of LD$_{50}$ and relative potency values which could not be obtained by calculation because greater than 50% killing was obtained even at the lowest challenge doses.

Several mutants isolated either from the control plates of transformation experiments i.e. spontaneous mutants (Mutant-X) or by mutating the strain 44122/7S with NTG (Mutant-S, Mutant-4 and Mutant-1-1) were also tested for mouse virulence. With the exception of Mutant-4 all mutants were as virulent as other Str$^R$ strains (Table 17) with LD$_{50}$ values from 35 to 74 cfu per mouse. Mutant-4 was examined only once and showed to be avirulent (LD$_{50} > 6.6 \times 10^4$ cfu per mouse), but unfortunately the mutant was lost before further investigation of virulence or other characters. The relative potency of the Mutant-1-1 compared with 44122/7S was 19 whereas that of Mutant-S was 226 (Table 17). 

LD$_{50}$ and relative potency value for Mutant-X by computation were found to be 7 cfu per mouse and 362.7 respectively (Table 16).

The MIC of streptomycin for Mutant-4 was not investigated before its loss, thus whether its avirulent character was paralleled by a low MIC is not known.

Other B. pertussis strains and their streptomycin-resistant derivatives were also tested in order to further investigate the relationship between streptomycin-resistance and intracerebral virulence. In addition, a freshly isolated strain (B. pertussis Taberman) was tested. The phase-I strains 18334, 353-I, L84-I,
Table 16. Virulence for mice by the intracerebral route:
Comparison of LD$_{50}$ and relative potency values
obtained by calculation and computation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Calculated values</th>
<th>Computed values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD$_{50}$(cfu/mouse)</td>
<td>Rel.pot.</td>
</tr>
<tr>
<td>44122/78</td>
<td>5.5x10^3</td>
<td>1.0</td>
</tr>
<tr>
<td>Trans.X</td>
<td>&lt; 73</td>
<td>&gt; 75</td>
</tr>
<tr>
<td>Mutant-X</td>
<td>&lt; 88</td>
<td>&gt; 62.5</td>
</tr>
<tr>
<td>Trans.E</td>
<td>&lt; 78</td>
<td>&gt; 70</td>
</tr>
</tbody>
</table>
Table 17. **Virulence for mice by the intracerebral route:**

Comparison of Branefors strains with Str^R^ mutants.

(by calculation).

<table>
<thead>
<tr>
<th>Expt.</th>
<th>LD_{50} in colony forming units per mouse (and 95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44122/7S</td>
</tr>
<tr>
<td>4/5</td>
<td>7.9x10^3</td>
</tr>
<tr>
<td></td>
<td>(1.3x10^3, 3x10^4)</td>
</tr>
<tr>
<td>6</td>
<td>5.5x10^3</td>
</tr>
<tr>
<td></td>
<td>(1.5x10^3, 2.10^4)</td>
</tr>
<tr>
<td>9</td>
<td>1.4x10^3</td>
</tr>
<tr>
<td></td>
<td>(589, 3.5x10^3)</td>
</tr>
</tbody>
</table>
their streptomycin-resistant isolates designated 18334s-1, 353-1s-1 and L84-1s-1 and Taberman, were found to be avirulent for mice by the intracerebral route. Some typical results are shown in Table 18. The LD50 of the strains Taberman, 18334 and 18334s-1 were > 6.8 x 10^6; > 2.4 x 10^4 and > 2.4 x 10^3 cfu per mouse respectively. The LD50s of strains 353-I, 353-I s-1, L84-I and L84-1 s-1 were even higher and could not be calculated from available data. Department cultural collection strains and their StrR isolates were also tested for virulence by the intracerebral route (Table 19). Strains 353-IV; 353-IVs-1; L84-IV were avirulent with high challenge doses. The virulence of the strains 8631-IV and its StrR spontaneous mutant was too low to be calculated since neither strain killed any of the twenty mice injected even at doses of 1.7x10^7 and 5.3x10^4 cfu per mouse respectively.

Finally, the virulence of the strain 44122/7R after prolonged subculture on TSA (Strain 44122/7R(34)) or after acquiring the plasmid R68.45 (Strain 44122/7RP) was also investigated to determine whether any change had occurred. The subcultured strain was found to have greatly reduced virulence (LD50 - 3.1x10^4 cfu per mouse) compared with the parent strain. Acquisition of the plasmid had no significant effect on the virulence of 44122/7RP.

b) Intranasal route.

The observed difference in virulence intracerebrally of strains 44122/7S and 44122/7R prompted the investigation of their virulence in mice by the intranasal route.
Table 18. Virulence for mice by the intracerebral route:
Other phase-I B. pertussis

<table>
<thead>
<tr>
<th>Expt.</th>
<th>LD$_{50}$ in colony forming units per mouse (and 95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44122/7S</td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>4 x 10$^3$</td>
</tr>
<tr>
<td></td>
<td>(3.10$^3$, 7x10$^3$)</td>
</tr>
</tbody>
</table>

Table 19. Virulence for mice by the intracerebral route:
Comparison of phase-IV strains and strain 44122/7R.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>LD$_{50}$ in colony forming units per mouse (and 95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44122/7R</td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1 x 10$^4$</td>
</tr>
<tr>
<td></td>
<td>(1.69x10$^3$, 7.4x10$^4$)</td>
</tr>
</tbody>
</table>
Table 20. Virulence for mice by the intracerebral route:
Strain 44122/7R, before and after attenuation
and acquisition of plasmid R68.45

<table>
<thead>
<tr>
<th>Expt.</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; in colony forming units per mouse (and 95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44122/7R</td>
</tr>
<tr>
<td>13</td>
<td>3.1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>&lt; 540</td>
</tr>
<tr>
<td></td>
<td>(1.5x10&lt;sup&gt;4&lt;/sup&gt;, 8.3x10&lt;sup&gt;4&lt;/sup&gt;)</td>
</tr>
<tr>
<td>14</td>
<td>&lt; 122</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
There was no striking difference in virulence by the intranasal route. The streptomycin-resistant strain appeared to be almost twice as virulent as the streptomycin-sensitive strain as judged by lethality (Table 21) but this difference was not significant. The relative potency value for the Str$^S$ strain was within the 95% confidence limits for the value for the Str$^R$ strain (Appendix 2.3). The LD$_{50}$ was $9 \times 10^5$ cfu per mouse for the Str$^S$ strain and $4.8 \times 10^5$ for the Str$^R$ strain. These values are similar to those reported for strain 353 (also virulent intranasally) examined by Standfast (1961), and for strain 18323 which is used routinely for intranasal infection of mice in this Department (L.Q. Stevenson, personal communication).

Other parameters of intranasal infection namely the lung pathology and recovery of organisms from the lungs after 14 days of infection were also examined. Again no significant difference between the two strains was observed.

5.1.2 Histamine-sensitizing activity

HSF activities in heated ($56^\circ$C for 30 min) cell suspensions of strains 44122/7S and 44122/7R from four growth experiments are summarized in Table 22. The complete data obtained from these four experiments are given in Appendix (3).

When results from the individual experiments were combined the overall best-estimate values for the potency of 44122/7R relative to that 44122/7S was 1.96 with 95% confidence limit (Appendix 3). Thus the Str$^R$ strain appeared to contain significantly more HSF than the parent strain.
Table 21. Virulence for mice by the intranasal route:
Branefors streptomycin-resistant and sensitive strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose (cfu/mouse)</th>
<th>Deaths/injected mice</th>
<th>Lung pathology score from 0 to 4</th>
<th>Amount of growth score from 0 to 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7S</td>
<td>4.3x10^6</td>
<td>4/5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4.3x10^5</td>
<td>2/5</td>
<td>1;3;3 (Total=7)</td>
<td>2;4;4 (10)</td>
</tr>
<tr>
<td></td>
<td>4.3x10^4</td>
<td>0/5</td>
<td>2;3;3;3;3 (14)</td>
<td>0;4;4;4;4 (16)</td>
</tr>
<tr>
<td>Saline</td>
<td>0/5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose (cfu/mouse)</th>
<th>Deaths/injected mice</th>
<th>Lung pathology score from 0 to 4</th>
<th>Amount of growth score from 0 to 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7R</td>
<td>1.52x10^7</td>
<td>5/5</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.52x10^6</td>
<td>5/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.52x10^5</td>
<td>0/5</td>
<td>2;3;3;3;4 (15)</td>
<td>4;4;2;4;4 (18)</td>
</tr>
<tr>
<td></td>
<td>1.52x10^4</td>
<td>0/5</td>
<td>1;2;2;3;4 (12)</td>
<td>3;4;1;4;3 (15)</td>
</tr>
</tbody>
</table>

Computed results (Lethality)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rel. pot.</th>
<th>95% confidence limits</th>
<th>LD_{50} (cfu/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7S</td>
<td>1.0</td>
<td></td>
<td>9.0 x 10^5</td>
</tr>
<tr>
<td>44122/7R</td>
<td>1.88</td>
<td>0.31</td>
<td>11.65</td>
</tr>
</tbody>
</table>

* All died
Table 22. Histamine-sensitizing activity of Branefors strains

Cells were grown for 48 h in Stainer and Scholte medium or in Hornibrook Medium (Experiment 3).

<table>
<thead>
<tr>
<th></th>
<th>HSD&lt;sub&gt;50&lt;/sub&gt; in opacity unit (ou. ml per mouse)</th>
<th>Rel. pot. (and 95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44122/7S</td>
<td>44122/7R</td>
</tr>
<tr>
<td>1</td>
<td>3.92</td>
<td>3.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.66</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.96</td>
<td>3.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8.81</td>
<td>4.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Best-estimate value of relative potency (see Appendix 3) of 44122/7R (and 95% confidence limit) = 1.96 (1.17, 3.28).
5.1.3 Heat-labile toxicity

Heat-labile toxin is one of the pathophysiological activities found in freshly isolated \textit{B. pertussis} strains which may have a role in virulence. \textit{HLT} is lethal to mice when unheated cell suspensions are injected intraperitoneally.

Two independent HLT tests were done on Branefors Str\textsuperscript{S} and Str\textsuperscript{R} strains (Table 23). The first experiment was with groups of 10 mice per dose in which strain 44122/7S was found to be 4-fold more potent with regard to HLT activity.

In the second experiment groups of 4 mice per dose were used. Strain 44122/7S was again found to contain more HLT than 44122/7R but this difference was not significant. The best-estimate value of HLT potency of 44122/7S relative to 44122/7R from the two experiments was 2.5 (Appendix 4). Thus the Str\textsuperscript{S} strain which is low in virulence for mice by the intracerebral route appears to have a higher HLT content than the virulent, Str\textsuperscript{R} strain.

5.1.4 Haemagglutinating activity

The haemagglutinin(s) of \textit{B. pertussis} may be involved in attachment of the organism to the epithelial surfaces of the host and therefore may be important in virulence. When Branefors Str\textsuperscript{S} and Str\textsuperscript{R} strains were compared for HA content no significant difference was found although the HA titre in both strains was very low. The titres obtained from the Str\textsuperscript{R} strain were 2 and 4 compared with 2 and 2 in the Str\textsuperscript{S} strain in two separate growth experiments.
<table>
<thead>
<tr>
<th>Expt.</th>
<th>Dose (μg/ml/ mouse)</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7R</td>
<td>5.0</td>
<td>0.699</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.3979</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>0.0969</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>-0.2041</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>44122/7S</td>
<td>2.5</td>
<td>0.3979</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>0.0989</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>-0.2041</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.3125</td>
<td>-0.5051</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rel. pot.</th>
<th>95 pc limits</th>
<th>( \text{ED}_{50} )</th>
<th>( \log_10 )</th>
<th>( \log_2 )</th>
<th>( \log_{10} )</th>
<th>( \log_{2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>7R</td>
<td>4.27</td>
<td>0.63</td>
<td>23.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7S</td>
<td>4.443</td>
<td>2.614</td>
<td>8.757</td>
<td>0.6477</td>
<td>14.51</td>
<td>0.0177</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Dose (μg/ml/ mouse)</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7R</td>
<td>5.0</td>
<td>0.6990</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.3979</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>0.0969</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>-0.2041</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.3125</td>
<td>-0.5051</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>44122/7S</td>
<td>5.0</td>
<td>0.6990</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.3979</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>0.0969</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>-0.2041</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.3125</td>
<td>-0.5051</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rel. pot.</th>
<th>95 pc limits</th>
<th>( \text{ED}_{50} )</th>
<th>( \log_10 )</th>
<th>( \log_2 )</th>
<th>( \log_{10} )</th>
<th>( \log_{2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>7R</td>
<td>2.62</td>
<td>0.4186</td>
<td>22.54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7S</td>
<td>1.239</td>
<td>0.632</td>
<td>2.479</td>
<td>0.0931</td>
<td>11.34</td>
<td>0.3255</td>
</tr>
</tbody>
</table>
5.1.5 Agglutinability

The cell-surface agglutinogens of Str$^S$ and Str$^R$ Branefors strains and derived strains were examined by slide agglutination tests in case of any possible change during the acquisition of the Str$^R$ marker. Specific antisera to B. pertussis agglutinogen factors 1, 2, 3 and 4 were used.

The strain 44122/7R was found to differ from the 44122/7S strain in possessing factor 2 specificity in addition to factors 1 and 3 found in the Str$^S$ strain. None of the Str$^R$ transformants or Str$^R$ mutants tested had acquired factor 2. The attenuated strain (44122/7R (34)) retained agglutinogen 2 even after prolonged subculture on blood agar and TSA medium (Table 24). None of the strains tested gave a positive reaction with the factor 4 antiserum.

5.1.6 Protective activity

Graded doses of heated cell suspensions of strains 44122/7S and 44122/7R were injected into randomized groups of 10 3-week old mice in order to assay mouse-protective activity after challenge. Results were analysed by the probit method using a computer programme (Appendix 5) and Table 25. The relative potency value and confidence limits show that there was no significant difference in protective activity in the two strains.
Table 24. Slide agglutination tests with type-specific antisera.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Visible agglutination with antisera specific for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Factor 1</td>
</tr>
<tr>
<td>44122/7S</td>
<td>++</td>
</tr>
<tr>
<td>44122/7R</td>
<td>++</td>
</tr>
<tr>
<td>44122/7R(34)</td>
<td>++</td>
</tr>
<tr>
<td>Mutant-2</td>
<td>++</td>
</tr>
<tr>
<td>Mutant-X</td>
<td>++</td>
</tr>
<tr>
<td>Trans.X</td>
<td>++</td>
</tr>
<tr>
<td>Trans.B</td>
<td>++</td>
</tr>
</tbody>
</table>

+++ = Rapid formation of large agglutinates, complete within 3 min.

++ = Agglutination visible within 3 min and complete in 5 min.

- = No agglutination visible to naked eye within 5 min.
### Table 25. Mouse-protection test data

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose (ou.ml per mouse)</th>
<th>No. of mice tested</th>
<th>No. protected</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7S</td>
<td>5.0</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>44122/7R</td>
<td>5.0</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

### Computed results

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative potency</th>
<th>95% confidence limits</th>
<th>ED$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7S</td>
<td>1.0</td>
<td></td>
<td>2.85</td>
</tr>
<tr>
<td>44122/7R</td>
<td>1.57</td>
<td>0.332</td>
<td>9.917</td>
</tr>
</tbody>
</table>
5.2 Biochemical characterization

5.2.1 Antibiotic sensitivity tests

The observation of differences between Branefors Str\textsuperscript{R} and Str\textsuperscript{S} B. pertussis strains with respect to virulence for mice by the intracerebral route and agglutinogen content, prompted further characterization tests.

In order to determine if there was any relationship between the degree of Str\textsuperscript{R} and virulence of the various strains, the minimal inhibitory concentration (MIC) of streptomycin was investigated by streaking the organism onto B-G plates containing various amounts of antibiotic. Table 26 shows that all streptomycin-resistant mutants and transformants derived from Branefors sensitive strain were resistant up to 7000 \( \mu g \) ml\(^{-1} \) streptomycin although at this level of antibiotic growth was poor. With 6000 \( \mu g \) ml\(^{-1} \) streptomycin, good growth was obtained with all of these strains. The parent 44122/78 strain was found to be sensitive to 1 \( \mu g \) ml\(^{-1} \) streptomycin i.e. it did not grow in the presence of this level of antibiotic. Strain 18334 which is avirulent intracerebrally showed a similar sensitivity. A streptomycin-resistant mutant of the latter (18334\textsubscript{S-1}) was obtained but was also shown to be avirulent intracerebrally (section 5.1.1). However, the MIC of streptomycin for this strain was 2000 \( \mu g \) ml\(^{-1} \).

The relationship between Str\textsuperscript{R} and resistance to other antibiotics was investigated by the use of Neo-sensitab antibiotics discs. The effect of each antibiotic was tested on at least 2 plate cultures of each strain, and the average zone of
Table 26. Minimal inhibitory concentration of streptomycin of *B. pertussis* strains.

48 h cultures was streaked from B-G plates onto B-G plates containing various concentration of streptomycin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC in $\mu$g ml$^{-1}$ streptomycin</th>
<th>Strain</th>
<th>MIC in $\mu$g ml$^{-1}$ streptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7</td>
<td>1</td>
<td>Mutant-1</td>
<td>7000</td>
</tr>
<tr>
<td>18334</td>
<td>1</td>
<td>Mutant-2</td>
<td>7000</td>
</tr>
<tr>
<td>18334&lt;sup&gt;s-1&lt;/sup&gt;</td>
<td>2000</td>
<td>Mutant-X</td>
<td>7000</td>
</tr>
<tr>
<td>44122/7R</td>
<td>7000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44122/7R(34)</td>
<td>7000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44122/7RP</td>
<td>7000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans.A</td>
<td>7000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans.B</td>
<td>7000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans.E</td>
<td>7000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans.X</td>
<td>7000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
inhibition (i.e. from the edge of disc to edge of growth) was
determined (Table 27). With each of the antibiotics tested,
except streptomycin, the streptomycin-resistant strains showed
a slightly increased sensitivity compared with the parent
44122/7S strain.

5.2.2 Haemolysis production

Freshly-isolated *B. pertussis* strains characteristically
show small zones of haemolysis around their colonies on B-G
media, whereas degraded strains usually lack this character.
Since Branefors stated that strain 44122/7R was more resistant
to growth inhibitors than the parent strain it was thought that
it might also possess other features characteristic of degraded
strains. The strains were examined for haemolysin production
on B-G plates which contained 20% (v/v) horse blood and on TSA
containing 5% (v/v) horse blood. Two other phase-I strains,
353-1 and 18334, were also examined. Haemolysis was observed
with all four strains on both media and no differences were
observed in the size of zones of haemolysis.

5.2.3 Tyrosine utilization test

The apparent conversion of *B. pertussis* to *B. parapertussis*
during mutagenesis was reported by Kumazawa and Yoshikawa (1978).
Some mutants gained the ability to utilize tyrosine and citrate
and split urea. The strain 44122/7R and Str^R mutants and
transformants obtained from the Str^S 44122/7S *B. pertussis*
strain were tested for tyrosine utilization to screen for
possible conversion to *B. parapertussis*. Both the parent
Table 27. Antibiotic sensitivity tests: Disc Method.

<table>
<thead>
<tr>
<th>Antibiotic Disc</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44122/7S</td>
</tr>
<tr>
<td>Streptomycin (160 μg)</td>
<td>21</td>
</tr>
<tr>
<td>Penicillin (2 μg)</td>
<td>9</td>
</tr>
<tr>
<td>Penicillin (100 μg)</td>
<td>27</td>
</tr>
<tr>
<td>Erythromycin (10 μg)</td>
<td>18</td>
</tr>
<tr>
<td>Erythromycin (78 μg)</td>
<td>22</td>
</tr>
<tr>
<td>Tetracycline (10 μg)</td>
<td>12</td>
</tr>
<tr>
<td>Tetracycline (80 μg)</td>
<td>16</td>
</tr>
<tr>
<td>Ampicillin (10 μg)</td>
<td>24</td>
</tr>
<tr>
<td>Ampicillin (33 μg)</td>
<td>25</td>
</tr>
</tbody>
</table>
strain and one \textit{B. parapertussis} strain were tested as controls. None of the Str$^R$ mutants or transformants nor the parent strain were found to be tyrosine positive.

Another characteristic of \textit{B. parapertussis}, production of brown pigmentation on B-G agar was examined. None of the \textit{B. pertussis} variants showed this property.

5.2.4 Effect of starch on growth of \textit{B. pertussis}

Branefors (1964) had shown that the Str$^R$ strain 44122/7R had the ability to grow in Cohen and Wheeler liquid medium without starch, whereas strain 44122/7S would not.

In initial experiments it was found that neither strain would grow in Cohen and Wheeler medium without starch unless a large inoculum was given. Thus the relationship between the effect of starch and the percentage of inoculum on growth of the Branefors strains in liquid and solid Cohen and Wheeler medium was examined more closely. Growth from 48 h culture on B-G plates was streaked onto Cohen and Wheeler solid medium containing various concentrations of starch (0, 1, 2, 5\%). The two strains were also inoculated into Cohen and Wheeler liquid medium containing similar concentrations of starch. It was found that both strains would grow on solid Cohen and Wheeler medium with and without starch. In the liquid medium growth of either strain was supported only when the inoculum was greater than 2\% (v/v). Thus, contrary to the report of Branefors (1964) starch did not have a differential effect on the growth of these strains.
5.2.5 Comparison of 44122/7S and 44122/7R strains by SDS-PAGE.

The protein profiles of strains 44122/7S and 44122/7R whole cells, envelope and cytoplasmic fractions were compared by slab-gel electrophoresis in the presence of SDS. Figure 7 shows a side-by-side comparison of these profiles. No differences in whole cell or envelope protein content could be detected. Both strains showed typical phase-I envelope profiles (Wardlaw et al. 1976). However, a major difference was observed in the profiles of the cytoplasmic fractions. The strain 44122/7R showed a prominent band (arrow), with a molecular weight of approximately 29,000 dalton, which was absent or much reduced in strain 44122/7S.
Figure 7. Protein profiles of strains 44122/7S and 44122/7R. Whole cells, envelope and cytoplasmic proteins.
DISCUSSION
1. Development of a transformation system

Various cell components in *B. pertussis* including pertussigen, protective antigen; agglutinogens; heat-labile toxin and endotoxin have been described, but in most instances the genetic basis of their biological activities has not been determined. Apart from gene mapping in *B. pertussis*, genetic studies may provide an important understanding of the relationship of the various biological activities in this organism. It would be important for example to determine if the virulence-associated properties are coded for by closely linked genes or perhaps even by a single gene or operon. It would be also of interest to refer each of the various biological activities in *B. pertussis* to a particular cell component, since a great deal of work to investigate the interrelationship between these activities has yielded controversy. For instance whether activities such as HSF; PA; LPF are due to one cell component or due to several components is still not clear. It may be possible by gene transfer between strains with different biological activities to select strains possessing particular biological properties, but not others. At the moment a better understanding of the protective activity and the toxicity caused by pertussis vaccines is most urgently needed. Gene transfer of protective components from a phase-I strain to an avirulent, non-toxic phase-IV strain may lead to the isolation of a derivative of *B. pertussis* which would be useful for the production of a safer vaccine.

At the time this work started, there had been only one report describing transformation of streptomycin-resistance
by exposing Str$^S$ cells to DNA extracted from a Str$^R$ strain (Branefors, 1964). She reported the isolation of Str$^R$ transformant with a frequency higher than 800 colonies ml$^{-1}$ under optimal conditions. The concentration of caseinhydrolysate (0.5% w/v) and the time between addition of DNA and plating onto the selective medium (11 h) were reported to be crucial factors. Subsequently Kloos et al. (1978) reported transfer of auxotrophic markers by transformation in B. pertussis strains. Although the frequency of transformation of some strains was high, other strains had only very low competence or lost competence shortly after their isolation. Kloos et al. found the optimal transformation conditions to be 8-10 hours exposure to 1 to 5 µg ml$^{-1}$ of donor DNA on both liquid and solid (modified Stainer and Scholte) medium in the presence of 0.001M CaCl$_2$.

In order to demonstrate transformation and to define the optimal conditions affecting such a system the procedures of Branefors (1964) and Kloos et al. (1978) were repeated or modified in the present studies. In addition, procedures used successfully with other organisms, such as that of Cohn et al. (1972) in the transformation of E. coli, were also employed.

In these studies, repetition of Branefors work was not very successful and only a few Str$^R$ transformants were isolated under her original optimal conditions. It is not known whether this was due to slight differences in technique or in reagents or due to changes in the competence of the recipient strain. It is possible that the recipient strain had lost some of its competent ability since such changes have been reported in
other strains of *B. pertussis* (Kloos *et al.* 1978). Even under the most favourable conditions found in this study the transformation frequency was never as high as that reported by Branefors. She reported that the optimum period of competence was in cultures 20 to 27 h old in 0.25 to 0.5% (w/v) casein hydrolysate after a preliminary growth (pre-culture) of 18 to 30 h in 1% (w/v) casein hydrolysate. In the work reported here, the optimum period of competence was found to be around 48 h after inoculation which is in the late exponential phase of growth of the organism. This would be equivalent to that of Branefors if the pre-culture period is considered.

The duration of competence was relatively long since streptomycin-resistant mutants were generated over approximately ten hours. This would be expected for organisms such as *B. pertussis* which have long division times. Branefors reported a longer period of competence of at least 18 to 20 hours. It is likely that the high degree of competence found in Branefors studies enabled her to isolate Str$^R$ transformants over this longer period. In less competent cells a significant transformation frequency would only be observed during the period of optimal competence.

Prior to the present study, factors known to increase competence in other organisms had not been previously tried in *B. pertussis*. These included CaCl$_2$ (Oishi and Cosloy, 1972) chilling and heat shock (Cohn *et al.* 1972); protease (Fuchs and Dobrzanski, 1978) and cyclic AMP (Wise *et al.* 1973). The effect of CaCl$_2$ treatment in this study was inconclusive. In some experiments there was no effect on transformation
frequency (Table 2) whereas in one experiment it was apparently effective (Table 7). Since subsequent repetition of the latter experiment was unsuccessful and an enhanced effect of CaCl$_2$ on transformation frequency on B. pertussis was reported by Kloos et al. (1978) further investigation, perhaps with different strains, is needed to decide whether or not CaCl$_2$ is useful in this context.

Cosloy and Oishi (1972) reported that heat shock was essential for DNA penetration into recipient cells of E. coli. A similar importance for the chilling step with this organism was reported by Taketo and Kuno (1974). The investigation of the effect of these factors on B. pertussis indicated that chilling for 40 min significantly enhanced the transformation frequency. In contrast to E. coli heat shock of the competent cells for 4 min at 37°C after chilling abolished this enhancement. It is possible that chilling rendered the recipient cells more permeable to the DNA penetration, since the viable counts of the cell suspensions before and after chilling were unaltered. The reason for the negative effect of the heat shock step is not known. It may reverse the effect of chilling or it may have an entirely different mode of action, perhaps by reducing cell viability.

The role of proteolytic enzymes in increasing transformation frequency in Streptococcus sanguis was interpreted in terms of an unmasking model, which has been proposed to explain the mechanism of competence development in bacterial transformation by Seto et al. (1975). According to this hypothesis the external cell layer becomes loose and un masks specific receptors
for DNA binding which are present both in competent and non-competent cells. Cells remaining non-competent therefore, would not be affected by the proteolytic enzyme. Fuchs and Dobrzanski (1978), however, did not define the exact action of the proteolytic enzyme on the recipient cells, but suggested the possibility that trypsin, which they used, eliminates an unknown cellular factor which obstructs DNA-cell receptor interaction. Electron microscopy showed cell surface alteration and thus supported this hypothesis. The enzyme was effective only within a short period, i.e.: not later than 5 min after the period of cell DNA contact.

Treatment of B. pertussis with a proteolytic enzyme did not enhance the transformation frequency. However, the cell envelopes of Gram-negative bacteria have quite a different composition and molecular architecture to those of Gram-positive organisms and the enzyme would probably have quite different effects on B. pertussis and Streptococcus sanguis.

Another factor reported as enhancing competence for donor DNA uptake in other organisms was cyclic-AMP. Both cyclic-AMP and dibutyryl cAMP were tried in B. pertussis. The latter significantly improved transformation with approximately a 5-fold increase in transformation frequency. No effect was observed with cyclic AMP. This is in contrast to the results obtained with Haemophilus influenzae in which Wise et al. (1973) reported the increased transformation with cyclic AMP but not with dibutyryl cyclic AMP. Endogenous cyclic AMP is at highest concentration in bacterial strains at late exponential or stationary phase (Okabayashi et al. 1963). At this period, several bacterial strains such as H. influenzae were reported
to be competent (Leclerc and Setlow, 1975). This may explain why high concentration of exogenous cyclic AMP have some effect on competence in \textit{H. influenzae}. It is possible therefore, that a high concentration of dibutyryl cAMP in \textit{B. pertussis} has a similar effect. It is also possible that the difference between cyclic AMP and dibutyryl cyclic AMP is due to permeability differences and that \textit{B. pertussis} and \textit{H. influenzae} differ in their permeability to these agents.

Having explored the factors that influenced the efficiency of transformation, the next stage in the investigation was to characterize the donor and recipient strains, particularly from the standpoint of virulence-associated properties. In the future, it would be of interest to transform an intracerebrally avirulent \textit{B. pertussis} strain with DNA extracted from the attenuated strain which retained resistance to streptomycin, but was no longer virulent in order to see if the \textit{Str}^R marker induced virulence in the recipient. Another point of interest in the transformation of phase-IV strains with DNA from phase-I strains would be to attempt to transfer a particular biological activity and not another since phase-IV \textit{B. pertussis} lacks several biological activities characteristic of fresh isolates. This should be possible if the biological characters in question were coded for by unlinked genes. However, such work needs more markers (which will also be useful for the start of a linkage analysis in this organism) because it is difficult to select for any of the biological characters directly, although highly specific antisera might make this possible.
2. Mutagenesis

Genotypic variation in *B. pertussis* induced *in vitro* by repeated sub-culture was first noted by Leslie and Gardner (1931). The genetic basis for this process remains obscure. Parker (1976) postulated that the degradation of freshly-isolated strains of *B. pertussis* results from a stepwise process of selection by various growth inhibitors which eventually leads to strains which have irreversibly lost several of the normal range of biological activities. Parker (1978) inoculated freshly isolated strains onto TSA (trypticase soy agar) containing decreasing concentrations of horse blood. The resulting growth was heavily inoculated onto TSA plates until consistent growth was obtained. Although Parker did not fully characterize the subcultured strains with regard to their biological activities, she defined three phenotypic stages in which *B. pertussis* could be found. These are a) a fresh isolate, which is a strain that has been passaged a minimum number of times since isolation and maintained only on B-G medium without peptone; b) an intermediate strain which is one able to grow on TSA with small amounts of blood, but not on TSA alone; c) a derivative strain which exhibited stable growth on TSA medium without blood.

The procedure of Parker (1978), was repeated with strain 44122/7R and, after adapting the culture to TSA, it was subcultured 34 times on this medium. Although confluent growth could be obtained from a heavy inoculum, the passaged strain would not grow as single colonies. Thus according to Parker's
definition this strain could probably be considered as a mutated derivative strain even though it would not grow as single colonies and still contained agglutinogens. This, however, indicated that Parker's definitions should perhaps be restricted to growth characteristics rather than to biological activities.

Chemical mutagenesis has been used for the isolation of different types of *B. pertussis* mutants including its apparent conversion to *B. parapertussis*.

In this study NTG was used to mutate *B. pertussis* strain 44122/7S and to isolate antibiotic resistant mutants. The frequency of mutation to streptomycin-resistance was quite high, in contrast to neomycin and to kanamycin where no resistant mutant was isolated. It is possible that such a difference in mutation frequency between the antibiotic is due to the fact that the Str\(^R\) phenotype can be given by mutation in one of several genes (see Introduction). The higher frequency of Str\(^R\) mutants obtained at 40 \(\mu g\) ml\(^{-1}\) NTG rather than 100 \(\mu g\) ml\(^{-1}\) or 4 \(\mu g\) ml\(^{-1}\), and at 45 min rather than before or after indicates that both the concentration of NTG and time of incubation are crucial in obtaining a high number of mutants. Although Kloos et al. (1978) did not give details of incubation of their strains with NTG, the 200 \(\mu g\) ml\(^{-1}\) NTG used by them seemed high unless their strain was much more resistant to the mutagen than strain 44122/7S. Killing in strain 44122/7S was 73.3% after 45 min treatment with 100 \(\mu g\) ml\(^{-1}\) NTG and 56.2% after 45 min treatment with 40 \(\mu g\) ml\(^{-1}\). It is reasonable to assume that the lower frequency of Str\(^R\) mutants obtained from samples treated with
100 μg ml⁻¹ NTG was due to higher killing and hence less opportunity for mutants to survive.

The Str⁰ mutants obtained were investigated for possible resemblance to B. parapertussis, but none of them was positive in the tyrosine test. However, it is possible that conversion to B. parapertussis does occur in mutants other than those resistant to streptomycin.

Mutagenesis in B. pertussis could be employed in several aspects of genetic studies of this organism. For example, mutants resistant to other drugs would have been useful markers to initiate mapping studies. In addition, it would have been of interest to compare virulence in mutants resistant to drugs such as kanamycin to see if there was an increased virulence associated with drug resistance as was apparent with Str⁰ strains.

3. Plasmid transfer and chromosomal mobilization

Chromosome mobilization during plasmid transfer was another means of gene transfer attempted in order to supplement or improve upon the low level of transfer obtained by transformation. The ability to transfer the bacterial chromosome from one bacterial strain to another by conjugation is possessed by several different plasmids including the plasmid R68.45 (see Introduction). Like other Incp-I plasmids it promoted chromosomal mobilization in the strain PAT of P. aeruginosa at frequencies as high as 4 x 10⁻³ per donor cell for a range of markers (Stanisich and Holloway, 1971). The plasmid was reported to transfer from E. coli to E. coli and also to Erwinia strains (Chatterjee, 1980). The
plasmid also mobilized several chromosomal markers of *Erwinia chrysanthemi*. The frequency of transfer of the plasmid was always higher with *E. coli* than with *Erwinia* spp. as recipients.

In this study, although the plasmid transfer was very high, ranging from 0.22 to 0.26 between *E. coli* strains, the chromosomal mobilization was found to be relatively low compared with the plasmid transfer frequency. The mobilization frequency of the chromosomal marker nal\(^R\) from *E. coli* Jc 53-1 to *E. coli* Jc 3272 varied from \(3.3 \times 10^{-7}\) to \(6.4 \times 10^{-6}\) per donor cell. There has been no previous report on the transfer of this plasmid to *B. pertussis* strains. The plasmid was transferred from *E. coli* Jc 53-1 to *B. pertussis* strain 44122/7R with a frequency of \(0.95 \times 10^{-4}\) to \(1.95 \times 10^{-4}\) per donor cell, but no chromosomal mobilization of the marker nal\(^R\) was observed. Even assuming that the transfer frequency was good enough to detect nal\(^R\) recombinants on the selective medium, the recipient strain (44122/7R) would possibly not be able to express the nal\(^R\) phenotype because the piece of *E. coli* chromosome would not integrate by recombination into the *B. pertussis* chromosome. A similar finding was reported for intergeneric plasmid transfer by Chatterjee (1980). He reported that transfer of chromosomal markers such as gal\(^+\), his\(^+\), lys\(^+\) occurred in crosses between *Erwinia* spp. but not between *Erwinia* and *E. coli* strains. However, it has been reported that plasmids that have acquired fragments of bacterial chromosome can express these genes without integration into the recipient chromosome in *P. aeruginosa* (Rieb et al. 1980).
In order to assess the plasmid's ability to mobilize chromosomal DNA, plasmid transfer was tried between *B. pertussis* strains, using the strain 44122/7RP (44122/7R + R68.45) as a donor. Apart from the Str^R marker available in the strain 44122/7R no other chromosomal marker was available in phase-I strains. The only other marker available to be used for this study was the ability of phase-IV strains to grow on nutrient agar. The plasmid was transferred from phase-I *B. pertussis* (44122/7RP) to strain D30042 phase-IV. Surprisingly, the transfer frequency was low, even lower than that from *E. coli* to phase-I *B. pertussis* strains, and ranged from $1.28 \times 10^{-5}$ to $1.29 \times 10^{-5}$. No chromosomal mobilization for the Str^R marker was detected. Since this range of transfer was within the range reported to mobilize host chromosomal DNA to recipient cells in *Erwinia* spp., it is possible that the plasmid does not integrate with the host chromosome in *B. pertussis* in order to take up the Str^R marker. However, other reasons should not be ruled out since phase-IV *B. pertussis* strains showed a lower frequency of plasmid acceptance compared with phase-I strains, which suggests that the acceptance of foreign DNA by phase-IV strains is lower than in phase-I strains. If this suggestion is correct then it is possible that there is a further reduction in the acceptance of the mobilized chromosomal DNA by phase-IV strains. This hypothesis was supported by the results obtained from several phase-IV strains in which the transfer was always less than that obtained when the recipient was a phase-I strain. Further support comes from the incompetence of phase-IV 8631 *B. pertussis* to take up foreign DNA by transformation as no Str^R transformants
were obtained when this strain was used as recipient with DNA extracted from 44122/7R.

Ludwig and Johansen (1980) described a derivative of the plasmid R68.45 which mobilized the chromosome of *Rhizobium meliloti* strains more efficiently than the parent R68.45. This plasmid therefore would be used for further investigation of the ability of *B. pertussis* strains to acquire and express chromosomal markers.

4. Virulence and other characteristics of *B. pertussis* strains

Variation in the virulence of *B. pertussis* strains has been related to a number of factors. For example, some strains were able to grow in the mouse brain whereas others could not (Standfast, 1958). Infection by the intranasal route has been shown to depend on the strain's ability to grow in the lung after lodgement. Gray (1946) found that death of mice following an intraperitoneal infection was the result of toxaemia and there was no evidence of infection or multiplication of the organism in the peritoneum.

In the present study high virulence by the intracerebral route was observed in a group of streptomycin-resistant *B. pertussis* strains. Strain 44122/7R and several Str^R^ transformants and mutants showed a much higher virulence intracerebrally than their streptomycin-sensitive parent strain. Kunicki-Goldfinger (1979) emphasised that the surface structures of bacterial cells are important in determining pathogenicity
and that the variability of virulence of a bacterial population could be due to genotypic variation. These changes occur by different processes including mutation, recombination connected with transformation and loss or acquisition of new plasmids. It is therefore possible that the high virulence in the $Str^R$ strains is of genotypic origin, and results from an enhancement of the ability of $Str^R$ strains to grow faster in the brain. Another possibility is that the $Str^R$ mutation caused a certain change in the cell surface which resulted in an increase in its resistance to host defence mechanisms such as phagocytosis or complement-mediated killing. Adams and Hopewell (1970) stated that resistance to local brain macrophage may explain differences in i.c. virulence. It is also possible that $Str^R$ mutation rendered the cell envelope of these strains more permeable for the release of potent substances such as pertussigen. Support for this suggestion comes from the finding of an approximately 2-fold higher HSF level in the $Str^R$ strain than in the $Str^S$ Brænfores strains which presumably would lead to more HSF being released by these cells into the circulation. Further support could also be drawn from the increased sensitivity to several antibiotics in the $Str^R$ strains, possibly indicating cell-surface changes.

There was no apparent difference between the $Str^R$ and $Str^S$ strains in virulence for mice by the intranasal route. The $Str^S$ strain appeared to be slightly more virulent intraperitoneally than the $Str^R$ strain. This indicates that the Heat-labile toxin, which was proposed to play a part in the intranasal virulence by facilitating the primary lodgement of the organism
in the lung (Standfast, 1958) and for intraperitoneal toxaemia (Proom, 1947), was not involved in the differences in virulence found intracerebrally.

The apparent linkage between the $\text{Str}^R$ phenotype and virulence intracerebrally was considered an important observation since it was confirmed by subsequent transformation experiments in which transfer of the $\text{Str}^R$ marker was always accompanied by increased virulence by the intracerebral route in transformants. Indeed, all but one of four $\text{Str}^R$ mutants expressed high virulence by this route. The exceptional performance of the mutant designated Mutant-4 should be noted but, since it was examined in only one experiment, and subsequently lost, a firm conclusion can not be drawn. It is also interesting to note that agglutinogen factor 2 which was found in strain 44122/7R, but not 44122/7S, was not linked to the $\text{Str}^R$ marker nor to the virulence factor as neither the $\text{Str}^R$ transformants or $\text{Str}^R$ mutants possessed it. They all resembled the $\text{Str}^S$ strain in this respect.

It is probable that the $\text{Str}^R$ mutation in $B. \text{pertussis}$ in some way enhances virulence intracerebrally in mice rather than introducing it. This could be deduced from the results obtained from Branefors $\text{Str}^R$ and $\text{Str}^S$ parent strains and the $\text{Str}^R$ derivates compared with the strain 18334 and its $\text{Str}^R$ mutant. In the first case Branefor's $\text{Str}^S$ strain had a high, but measurable $LD_{50}$ which was greatly reduced in the $\text{Str}^R$ mutant. In strain 18334 there was apparently no virulence character to be enhanced by the $\text{Str}^R$ mutation. A similar observation was obtained with the strain 8631-IV and its $\text{Str}^R$ mutant. Strain 8631-IV is a degraded strain (phase-IV) and therefore it was
expected to be avirulent. Neither the parent 8631-IV strain nor its StrR mutant showed any sign of virulence intracerebrally at the doses tested.

It was thought that the virulence of the StrR Branefors strain and the other StrR derivatives of the StrS parent strain might be related to the very high degree of streptomycin-resistance in these strains. The MIC of streptomycin was 7000 µg ml\(^{-1}\) compared to approximately 2000 µg ml\(^{-1}\) found in the StrR mutant of strain 18334. However, the attenuated strain 44122/7R(34) still had the same MIC of streptomycin, but it had greatly reduced virulence intracerebrally. The LD\(_{50}\) of the attenuated strain was in the range of that of the StrS Branefors strain; whereas that of StrR parent and derived strains was as high as that of the intracerebrally virulent reference 18323 strain (Standfast, 1958). This loss of virulence indicates that as the strain becomes degraded, the effect of the StrR mutation in enhancing virulence disappears. Whether degradation by subculturing resulted in reduced ability to grow intracerebrally, in reduced immunity to the host's defence mechanism, or in a reduction in the potent substances released during infection was not investigated.

The gain of the plasmid R68.45 by the StrR Branefors strain did not affect its virulence intracerebrally, although there was expression of resistance to kanamycin, carbenicillin and tetracycline in addition to streptomycin. However, other workers have shown that gain of R-factor plasmids conferring resistance to several antibiotics including streptomycin did not increase virulence. Smith and Tuker (1979) studied the
effect of R-factor plasmids on the virulence and infectivity of *Salmonella typhimurium* and *Salmonella gallinarum* in mice. They found none of the R\(^+\) forms were more virulent than their R\(^-\) parent forms. Some R\(^+\) forms actually produced less mortality than their R-parents. Whether a *B. pertussis* strain could lose virulence after gaining R-plasmids has yet to be investigated.

The HSF content of Branefors strains estimated from four independent experiments indicated a significant difference between them. The Str\(^R\) strain contained slightly more HSF activity than the Str\(^S\) strain. However, to confirm this observation more growth experiments and HSF tests with larger numbers of mice would be needed.

The role of pertussigen (=HSF) in the establishment of, or mortality associated with pertussis infection is not clear although sensitization and lymphocytosis are evident in human and experimental infections (Pittman, 1980). However, a relationship between HSF content and high intracerebral virulence for mice seems unlikely since other intracerebrally virulent strains, such as 18323 and 353, are not noted for their elevated HSF content. Despite this, a comparison of HSF levels in other *B. pertussis* strains and their streptomycin-resistant counterparts would be of interest and would allow us to draw a firmer conclusion as to whether such a relationship exists. Further work is also required to determine if the growth medium has a significant influence on the amount of HSF contained in these strains as indicated in the present study.
Although the \( \text{Str}^S \) strain was less virulent by the intracerebral route and contained less HSF activity than the \( \text{Str}^R \) strain, its heat-labile toxin content was apparently higher. This, however, would need to be confirmed in further experiments and HLT tests with larger numbers of mice. Use of the more sensitive test for HLT in suckling mice (Katsampes et al. 1942) would also be helpful.

The two Branefors strains expressed almost an equal haemagglutination titre suggesting that they contain a similar amount of HA but it is not known whether or not they have a similar content of F-HA and LPF-HA. Differences in the HAs in the two strains might be measured if each strain was grown in static liquid culture to promote F-HA or in shaken liquid culture to promote LPF-HA and then retested for haemagglutination (Arai and Munoz, 1979).

No difference was found in the ability of vaccines prepared from these two strains to protect against intracerebral infection despite the massive difference in virulence. This is somewhat puzzling, but there is no evidence that other intracerebrally virulent strains make better vaccines for mice than intracerebrally avirulent strains. In fact, Preston and Stanbridge (1976) stated that vaccines of avirulent strains will protect mice against intracerebral challenge.

The serotype of the \( \text{Str}^S \) strain was 1, 3 whereas that of the \( \text{Str}^R \) strain was 1, 2, 3. The latter must have gained the factor 2 since it is a mutant of the \( \text{Str}^S \) strain. However, loss or gain of agglutinogens in \textit{B. pertussis} is not uncommon.
Thus the mutation from 44122/7S to 44122/7R presumably involved more than a single mutation including streptomycin-resistance agglutininogen change and possibly changes in HSF and HLT.

The possession of factor 2 by the StrR strain and the attenuated strain and the possession of intracerebral virulence characters but not factor 2 by the transformants suggests that factor 2 agglutinogen does not play an important role in virulence intracerebrally in mice and is not linked to the streptomycin-resistance marker. It is obvious therefore that agglutinogens in B. pertussis, though reported to be important in immunity in children (Preston and Stanbridge, 1972), were not significant in establishing the infection intracerebrally in mice.

There was no indication of degradation in the StrR strain such as increased resistance to growth inhibitors as reported by Branefors (1964). The StrR strain 44122/7R was described as able to grow in Cohen and Wheeler medium without starch whereas the StrS strain 44122/7S would not. When retested, these two strains as well as several other StrR strains did not show any difference in the ability to grow without starch either in solid or in liquid media. The size of the inoculum was found to be the crucial factor in determining whether growth occurred. The streptomycin-resistant mutant would not be expected to be degraded since selection for StrR mutants could be simply done by streaking a heavy inoculum onto streptomycin containing media while degradation in B. pertussis is a multi-step process (Parker, 1978). Even after 34 sub-cultures on TSA medium the attenuated strain still retained some of its phase-I characteristics such as the agglutinogens.
Another type of mutant of \emph{B. pertussis} has been described. Kumazawa and Yoshikawa (1978) described the conversion of \emph{B. pertussis} to \emph{B. parapertussis} by mutagenesis. Kloos et al. (1978) reported a similar finding. In both studies, however, the mutants isolated showed agreement with some of the \emph{B. parapertussis} criteria, but not with all.

In this study \emph{Str}^R mutants obtained either by transformation or by NTG mutagenesis showed no agreement with \emph{B. parapertussis} criteria. In fact, \emph{Str}^R mutants were found to contain more HSF than even their \emph{Str}^S parent strain whereas \emph{B. parapertussis} does not synthesize HSF.

The reason for the difference in mouse virulence intracerebrally between streptomycin-resistant and sensitive strains is not known at present. Increased resistance to host-defence mechanisms may be responsible. Factors such as the content of the antigen that induces bactericidal antibody reported in some \emph{B. pertussis} strains (Ackers and Dolby, 1972) and resistance to the phagocytic and bactericidal capabilities of alveolar macrophage (AM) of the host reported by Muse (1978) may be worthy of investigation. The two strains did not show any difference in both mouse protection test and virulence by the intranasal route. Variations in surface protein composition associated with virulence has been reported in \emph{Neisseria gonorrhoeae}. Lambden et al. (1979) showed that the heat-modifiable outer-membrane proteins are important virulence attributes of the gonococcus.
The profiles of $\text{Str}^S$ and $\text{Str}^R$ strains obtained by polyacrylamide gel electrophoresis in the presence of SDS, however, did not show any differences in envelope proteins. However, an extra major band in the cytoplasmic proteins was observed in the $\text{Str}^R$ strain which could be related either to virulence or streptomycin resistance. Investigation of the cytoplasmic protein profiles of virulent $\text{Str}^S$ strains and avirulent $\text{Str}^R$ strains might help to resolve the problem.

5. Development of a minimal solid medium

In contrast to the findings of Parker (1976) and Kloos et al. (1978) the addition of Dowex-1-chloride, charcoal or starch to solidified Stainer and Scholte liquid medium did not give satisfactory growth for the proposed genetic studies. It is possible that none of the additives were able to remove completely all growth inhibitors developed either during autoclaving of the medium, or during growth of the cells. The unsuitability of such media for genetic studies in B. pertussis is obvious since transformation frequency in this organism is very low. If such media were used, the isolation of transformed cells might be missed since only a small ratio of the cells inoculated would grow. It is interesting to note that the few colonies which were able to grow on solidified supplemented Stainer and Scholte medium failed to grow when subcultured onto the same medium. Their initial growth was presumably due to carry-over of essential growth factors from the B-G medium from which they were inoculated.
Degraded strains would grow on the minimal medium, but these strains unlike the phase-I strains also grew on nutrient agar. The development of a truly efficient and reproducible minimal solid medium for *B. pertussis* therefore, would seem to merit further investigation as it would be useful for isolation of auxotrophic strains which would facilitate gene transfer studies.
APPENDIX
APPENDIX 1

Preparation of Media

Bordet-Gengou plates

400 ml B-G agar base (Gibco Bio-Cult Diagnostics, Paisley, Scotland) melted and cooled to 45°C.

20 ml glycerol (autoclaved)
100 ml fresh citrated horse blood (Gibco) warmed to 37°C.

Add glycerol to the agar and add the blood aseptically. Mix gently by rolling and pour into 90 mm diameter, triple-vent, plastic petri-dishes. Remove surface bubbles by light flaming. Plates were stored in the cold room and used within three weeks of preparation.

Stainer and Scholte medium (Stainer and Scholte, 1971)

Ingredients:

- L-glutamate (monosodium salt) 10.72 g
- L-proline 0.24 g
- NaCl 2.5 g
- KH₂PO₄ 0.5 g
- KCl 0.2 g
- MgCl₂.6H₂O 0.1 g
- CaCl₂ 0.02 g
- Tris (hydroxymethyl-methylamine) 6.075g
- L-cysteine 0.04 g
- FeSO₄.7H₂O 0.1 g
- Ascorbic acid 0.02 g
Nicotinic acid  
Glutathione

Dissolve ingredients 1-8 in 800 ml distilled water. Adjust pH to 7.4 with 2.5N HCl. Make up volume to 990 ml with distilled water. Autoclave at 15 p.s.i. for 15 min. Immediately before use dissolve ingredients 9-13 in 10 ml distilled water, filter sterilize with Millipore filter of 0.45 μm pore size (Millipore S.A. Molshein, France), and add aseptically to bulk medium.

1% Casamino acids solution

10 g casamino acids (Difco Technical).
0.1 g MgCl₂·6H₂O
0.016 g CaCl₂ (anhydrous)
5 g NaCl

Dissolve in 1 litre of distilled water. Adjust the pH to 7.0 with 1N NaOH. Autoclave for 15 min at 15 p.s.i.

Modified Hornibrook Medium (Parton and Wardlaw, 1975)

1. Casamino acids (Difco Technical)  
2. Nicotinamide (0.1% (w/v) solution)  
3. CaCl₂ (anhydrous)  
4. NaCl  
5. MgCl₂·6H₂O, 1% (w/v) solution  
6. KCl  
7. K₂HPO₄, 10% (w/v) solution  
8. Soluble starch  
9. Distilled water  
10. Glutathione, 0.1% (w/v) solution
Prepare solutions, 2, 5 and 7 just before use. Dissolve ingredients 1, 3, 4 and 6 in 800ml distilled water and add solutions 2, 5 and 7. Suspend starch in 25 ml distilled water. Separately boil up 175 ml of distilled water and add starch suspension to it. Mix immediately and add to the bulk medium. Make volume up to 1 litre with distilled water. Bring pH to 7.0 with approximately 0.45g of Na$_2$CO$_3$. Dispense in 100 ml amounts in screw cap bottles and autoclave at 121°C for 15 min. Store at 4°C.

**Cohen and Wheeler Medium (1946)**

1. Bacto-Casamino acids (Difco-Technical)  
2. Yeast extract (Difco)  
3. Soluble starch  
4. MgCl$_2$·6H$_2$O, 25% solution  
5. CaCl$_2$, 10% solution  
6. FeSO$_4$·7H$_2$O 1% solution  
7. CuSO$_4$·5H$_2$O solution  
8. L-arginine, 0.01% solution  
9. L-histidine, 0.005% solution  
10. KH$_2$PO$_4$  

1000 ml distilled H$_2$O  

pH 7.8

Solid medium was prepared by adding 1.5% (w/v) Bacto-agar (Difco) to the liquid medium. After sterilization and cooling to 45°C, 10% (v/v) horse blood was added.
APPENDIX 2

2.1 Combination of computed results from four independent experiments with \textit{B. pertussis} strains 44122/7S and 44122/7R.

### Experiment 2

<table>
<thead>
<tr>
<th>Dose (cfu) per mouse</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9060</td>
<td>3.9571</td>
<td>10</td>
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</tr>
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<tr>
<td>90.6</td>
<td>1.9571</td>
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<tr>
<td>44122/7S</td>
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<tr>
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<tr>
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Average slope = 0.516

F-Test of non-parallelism = 2.27
F-Test of heterogeneity = 1.13

<table>
<thead>
<tr>
<th>Rel.pot.</th>
<th>95 PC limits</th>
<th>ED$_{50}$</th>
<th>LogR</th>
<th>WLogR</th>
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<tbody>
<tr>
<td>7R</td>
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<td>0.00012</td>
<td>0.000</td>
<td>0.004</td>
<td>2.1x10$^6$</td>
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## Experiment 4

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</thead>
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</tr>
<tr>
<td>540</td>
<td>2.7324</td>
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<td>4</td>
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<tr>
<td>54</td>
<td>1.7324</td>
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<tr>
<td>44122/7S</td>
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<tr>
<td>29</td>
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</tr>
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</table>

- **Average slope** = 0.939
- **F-Test of non-parallelism** = -0.00, \( F_{5PC} = 3.84 \)
- **F-Test of heterogeneity** = 0.41, \( F_{5PC} = 2.37 \)

<table>
<thead>
<tr>
<th>Rel. pot.</th>
<th>95 PC limits</th>
<th>( ED_{50} )</th>
<th>LogR</th>
<th>WlogR</th>
</tr>
</thead>
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<td>0.000</td>
<td>0.077</td>
<td>42554.8</td>
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</table>
Procedure for combining the results of two individual experiments (given on pages 154 and 155) using the values for the logarithm of the relative potency ($\log R$) and their weighted values ($W\log R$) given in the computer print-outs.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Strain</th>
<th>$\log R$</th>
<th>$W\log R$</th>
</tr>
</thead>
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<tr>
<td>2</td>
<td>7S</td>
<td>-3.9133</td>
<td>0.04</td>
</tr>
<tr>
<td>4</td>
<td>7S</td>
<td>-2.2498</td>
<td>0.23</td>
</tr>
</tbody>
</table>

The best-estimate of the logarithm of the relative potency ($\overline{\log R}$) is given by

$$\overline{\log R} = \frac{\Sigma (W\log R \cdot \log R)}{\Sigma W\log R}$$

Substituting the values given above

$$\overline{\log R} = \frac{(0.04 \times -3.9133) + (0.23 \times -2.2498)}{0.23 + 0.04}$$

$$= \frac{-0.1565 + -0.5175}{0.27}$$

$$= -2.4954$$

Taking antilogs yields

$$\overline{R} = 0.0032$$

The 95% confidence limits are given by

$$\text{Antilog} \left[ \overline{\log R} \pm \frac{1}{\Sigma W\log R} \right]$$

$$\Sigma \log R = 0.27$$

Limits = $-2.4954 \pm 1.9245$

$$= (-4.4199 , -0.5709)$$

Taking antilogs yields (0.00004 , 0.2685)

\[ \therefore \] the potency ($R$) of *B. pertussis* strain 44122/7S relative to that of strain 44122/7R in the intracerebral virulence test

$$= 0.0032 \times (0.00 , 0.27)$$

or, Strain 7R is approximately 310-fold more virulent than 7S

* Footnote: This value represents the results of only two of the experiments given in Table 13, the remainder not being suitable for the computer programme because of the extremely low virulence of strain 7S.
APPENDIX 2.2

Intracerebral virulence test data. Computed results

Str\textsuperscript{R} transformants and mutant compared with their Str\textsuperscript{S}
parent strain.

<table>
<thead>
<tr>
<th>Dose (cfu) per mouse</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
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</tr>
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<td>10</td>
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<td>2.8921</td>
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<td>9</td>
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<tr>
<td>78</td>
<td>1.8921</td>
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<td>7</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Rel. pot.</th>
<th>95pc limits</th>
<th>ED\textsubscript{50}</th>
<th>Log\textsubscript{R}</th>
<th>WLog\textsubscript{R}</th>
<th>LOED\textsubscript{90}</th>
<th>WLED\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>7S</td>
<td>227.572</td>
<td>26.480 ***</td>
<td>10.93</td>
<td>2.3571</td>
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<td>1.0387</td>
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<tr>
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<td>6.86</td>
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<tr>
<td>Mutant-X</td>
<td>115.348</td>
<td>15.588 ***</td>
<td>21.57</td>
<td>2.0620</td>
<td>0.83</td>
<td>1.3338</td>
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</tbody>
</table>
**Intracerebral virulence test data.**

**Calculated results**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7S</td>
<td>4.32x10^4</td>
<td>4.635</td>
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<tr>
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<td>4.32x10^3</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>1.1x10^3</td>
<td>3.04</td>
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</tr>
<tr>
<td></td>
<td>1.1x10^2</td>
<td>2.04</td>
<td>5</td>
</tr>
</tbody>
</table>

Rel. pot. 95 pc limits ED_{50}

| 7S         | 575, 6.9 x 10^4 | 1.6 x 10^4 |
| Mutant S   | 80            | 28.8, 138 x 10^3 | 200 |

**APPENDIX 2.3**

**Intranasal virulence test data.**

**Computed results**

<table>
<thead>
<tr>
<th>Dose per mouse (cfu x 10^4)</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>7S</td>
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<tr>
<td></td>
<td>43</td>
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</tr>
<tr>
<td></td>
<td>4.3</td>
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<tr>
<td>7R</td>
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</tr>
<tr>
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<tr>
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<td>1.5</td>
<td>0.1818</td>
<td>5</td>
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</tbody>
</table>

No. of cycles = 4
APPENDIX 3

HSF DATA

Method for combining the results of several independent assays (A.C. Wardlaw, personal communication).

To illustrate the procedure used to combine the results of two or more HSF or MPT assays, take the following sets of computed HSF results. The blocked-in figures are the values taken for combining.

Experiment 1

<table>
<thead>
<tr>
<th>Dose per mouse (cu ml)</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
</tr>
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<tbody>
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</tr>
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<td>0.9031</td>
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</tr>
<tr>
<td>2.00</td>
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<td>4</td>
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</tr>
<tr>
<td>0.500</td>
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<td>4</td>
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</tr>
<tr>
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<td>0.9031</td>
<td>4</td>
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<tr>
<td>7R</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>3</td>
</tr>
<tr>
<td>2.00</td>
<td>0.3010</td>
<td>4</td>
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<td>0.500</td>
<td>-0.3010</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>0.125</td>
<td>-0.9031</td>
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</table>

Rel. pot. 95 pc limits

<table>
<thead>
<tr>
<th>Dose per mouse (cu ml)</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>7S</td>
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<tr>
<td>8.00</td>
<td>0.9031</td>
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<td>2.00</td>
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</tr>
<tr>
<td>0.125</td>
<td>0.9031</td>
<td>4</td>
<td>0</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose per mouse (cu ml)</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
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</thead>
<tbody>
<tr>
<td>7R</td>
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<td>8.00</td>
<td>0.9031</td>
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<td>-0.3010</td>
<td>4</td>
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<tr>
<td>0.125</td>
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Rel. pot. 95 pc limits
### Experiment 3

<table>
<thead>
<tr>
<th>Dose</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
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<td>0.3010</td>
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<td>-0.3010</td>
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<td>0</td>
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<tr>
<td>0.125</td>
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<td>0</td>
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**Rel. pot. 95 pc limits**

<table>
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<tr>
<th>ED&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Log&lt;sub&gt;R&lt;/sub&gt;</th>
<th>WLog&lt;sub&gt;R&lt;/sub&gt;</th>
<th>LOED&lt;sub&gt;50&lt;/sub&gt;</th>
<th>WLED&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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</tr>
<tr>
<td>7R</td>
<td>0.508</td>
<td>0.151</td>
<td>1.733</td>
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### Experiment 2

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<th>Dose</th>
<th>Log dose</th>
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<th>Responding</th>
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<tr>
<td>7S</td>
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**Rel. pot. 95 pc limits**

<table>
<thead>
<tr>
<th>ED&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Log&lt;sub&gt;R&lt;/sub&gt;</th>
<th>WLog&lt;sub&gt;R&lt;/sub&gt;</th>
<th>LOED&lt;sub&gt;50&lt;/sub&gt;</th>
<th>WLED&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>7S</td>
<td>5.66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7R</td>
<td>2.977</td>
<td>1.492</td>
<td>5.154</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment</td>
<td>Dose</td>
<td>Log dose</td>
<td>Tested</td>
<td>Responding</td>
</tr>
<tr>
<td>------------</td>
<td>------</td>
<td>----------</td>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>7S</td>
<td>8.00</td>
<td>0.9031</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0.3010</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>-0.3010</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>-0.9031</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>7R</td>
<td>8.00</td>
<td>0.9031</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0.3010</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>-0.3010</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>-0.9031</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rel. pot.</th>
<th>95 pc limits</th>
<th>ED_{50}</th>
<th>Log_{R}</th>
<th>WLog_{R}</th>
<th>LOED_{50}</th>
<th>WLED_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>7S</td>
<td>3.92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7R</td>
<td>1.00</td>
<td>0.156</td>
<td>6.415</td>
<td>3.92</td>
<td>0.00</td>
<td>1.53</td>
</tr>
</tbody>
</table>

B. pertussis 44122/7S vaccine is taken as the standard and assigned a relative potency of 1.00 (i.e. R = 1.00)

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Strain</th>
<th>Log_{R}</th>
<th>WLog_{R}</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>7R</td>
<td>0.3381</td>
<td>1.06</td>
</tr>
<tr>
<td>3</td>
<td>7R</td>
<td>-0.2945</td>
<td>3.56</td>
</tr>
<tr>
<td>2</td>
<td>7R</td>
<td>0.4738</td>
<td>13.81</td>
</tr>
<tr>
<td>1</td>
<td>7R</td>
<td>0.00</td>
<td>1.53</td>
</tr>
</tbody>
</table>
The combined estimate of Relative potency ($\bar{R}$) of 44122/7R vaccine is given by:

$$\log R = \frac{\sum (\log R \cdot W \log R)}{\sum W \log R}$$

$$= \frac{(0.00 \times 1.53) + (0.4738 \times 13.81) + (-0.2945 \times 3.56) + (0.3381 \times 1.06)}{1.53 + 13.81 + 3.56 + 1.06}$$

$$= 0 + 6.5432 + (-1.0484 + 0.3584)$$

$$= 19.96$$

$$\log R = 0.2932$$

$$\bar{R} = 1.96$$

Limits = Antilog $\left[ \log R \pm \frac{\sqrt{\sum W \log R}}{4.4677} \right]$}

$$= \text{Antilog} \left[ 0.2932 \pm \frac{\sqrt{19.96}}{4.4677} \right]$$

$$= \text{Antilog} \left[ 0.2932 \pm 0.2238 \right]$$

$$= \text{Antilog} \left[ 0.517 \text{ and } 0.0694 \right]$$

$$= 3.288 \text{ and } 1.17$$

\(\therefore\) Combined estimate of R (and 95% confidence limits for \textit{B. pertussis} 44122/7R vaccine from 4 independent HSF experiments = 1.96 (1.17, 3.288).
APPENDIX 4

Combination of computed results from HLT Expt. 1 and 2.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Strain</th>
<th>Rel. pot.</th>
<th>$\log R$</th>
<th>W$\log R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>7S</td>
<td>1.239</td>
<td>0.0931</td>
<td>11.34</td>
</tr>
<tr>
<td>1</td>
<td>7S</td>
<td>4.443</td>
<td>0.6477</td>
<td>14.51</td>
</tr>
</tbody>
</table>

\[
\bar{R} = \frac{\sum (\log R \cdot W\log R)}{\sum W\log R} = \frac{(11.34 \times 0.0931) + (14.51 \times 0.6477)}{11.34 + 14.51} = \frac{1.055 + 9.398}{25.85} = 0.4044
\]

\[\bar{R} = 2.537\]

Confidence limits = Antilog \[\frac{\log R + \sqrt{\sum W\log R}}{2}\]

= Antilog \[\frac{0.4044 + \sqrt{25.85}}{2}\]

= Antilog \[0.4044 + 0.1967\]

= Antilog 0.2077 and 0.6011

1.613, 3.991

\[\therefore \bar{R} \text{ (combined estimate of relative potency (and 95\% confidence limit) of 7S with respect to HLT)} = 2.537 \text{ (1.613, 3.991).}\]
## APPENDIX 5

### Mouse Protection test data

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose per mouse (ou ml)</th>
<th>Log dose</th>
<th>Tested</th>
<th>Responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7S</td>
<td>5.00</td>
<td>0.699</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0.00</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>-0.699</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>44122/7R</td>
<td>5.00</td>
<td>0.699</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0.00</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>-0.699</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

No. of cycles = 3

<table>
<thead>
<tr>
<th>Doses</th>
<th>XMEAN</th>
<th>YMEAN</th>
<th>CSX2</th>
<th>CSXY</th>
<th>CSY2</th>
<th>FDEV</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7S</td>
<td>0.1375</td>
<td>4.631</td>
<td>4.2987</td>
<td>58543</td>
<td>8.6043</td>
<td>0.63</td>
<td>1.36</td>
</tr>
<tr>
<td>44122/7R</td>
<td>0.0789</td>
<td>4.791</td>
<td>4.6730</td>
<td>4.6085</td>
<td>5.0745</td>
<td>0.53</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Average slope = 1.166

\[
G = 0.315
\]

F-test of non-parallelism = 0.32  \( F_{5pc} = 3.84, DF 1/INF \)

F-test of heterogeneity = 0.58  \( F_{5pc} = 3.00, DF 2/INF \)

<table>
<thead>
<tr>
<th>Rel.pot.</th>
<th>95 pc limits</th>
<th>( ED_{50} )</th>
<th>( \log R )</th>
<th>( W\log R )</th>
<th>( LOED_{50} )</th>
<th>( WLED_{50} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>44122/7S</td>
<td>1.00</td>
<td>2.85</td>
<td>0.4542</td>
<td>2.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44122/7R</td>
<td>1.570</td>
<td>0.332 9.917</td>
<td>1.81 0.1958 1.84</td>
<td>0.2583 3.57</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


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