GENE   TRANSFER   IN   BORDETELLA PERTUSSIS

by

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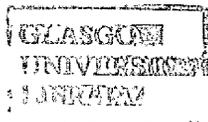
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Declaration

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.

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SUMMARY

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In order to have available a procedure for gene transfer in
Bordetella pertussis, the transformation system described origin-
ally 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, condit-
ions 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 trans-
formation frequency compared with the original Branefors conditions;
cAMP and $CaCl_2$ 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 trans-
ferred 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 strepto-
mycin-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 intra-
cerebral route. The average LD_{50} of the Str^R strain was 360
c.f.u. compared with $> 7.9 \times 10^3$ 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 intra-cerebral 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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ABBREVIATIONS

B-G	Bordet-Gengou agar
CAA	Casamino acids solution
car	carbenicillin
cAMP	cyclic adenosine-3'-5' monophosphate
cfu	colony forming units
C-mode	Cyanic mode : Variant of <u>Bordetella pertussis</u>
cma	chromosomal mobilization ability
DNA	Deoxyribonucleic acid
ED_{50}	Median effective dose
F-HA	Fimbrial haemagglutinin
HA	Haemagglutinin
HSF	Histamine-sensitizing factor
HSD_{50}	Median histamine-sensitizing dose
HLT	Heat-labile toxin
IAP	Islets-activating protein
ic	intracerebral
in	intranasal
IU	International unit
kana	kanamycin
K	thousand
LD_{50}	Median lethal dose
LPF	lymphocytosis or leukocytosis-promoting factor
LPF-HA	LPF haemagglutinin
LPS	Lipopolysaccharide

MPT	Mouse protection test
MRC	Medical Research Council
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
NA	nutrient agar
Nal	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 <u>Bordetella pertussis</u>
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 O-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. bron-
chiseptica.   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 child-
hood.   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 character-ized 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.

Characters[**]	B.pertussis	B.parapertussis	B.bronchi-septica
Motility	-	-	+
Growth on plain agar medium	-	+	+
Tyrosinase production	-	+	-
Splitting of urea	-	+	+
Oxidase	+	-	+
Catalase	+ or -[*]	+	+
Reduction of nitrates	-	-	+
Utilization of citrate	-	+	+
Growth on peptone agar	-	+	+
Sensitization of mice to histamine	+	-	-
Specific heat-labile agglutinogens :-			
Factor 1	+	-	-
Factor 12	-	-	+
Factor 14	-	+	-
G + C content moles %	61	61	66

** 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 hyper-
glycaemic response in the blood of patients following adminis-
tration 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

Notifications
Deaths

Whooping cough vaccination
introduced on a national scale
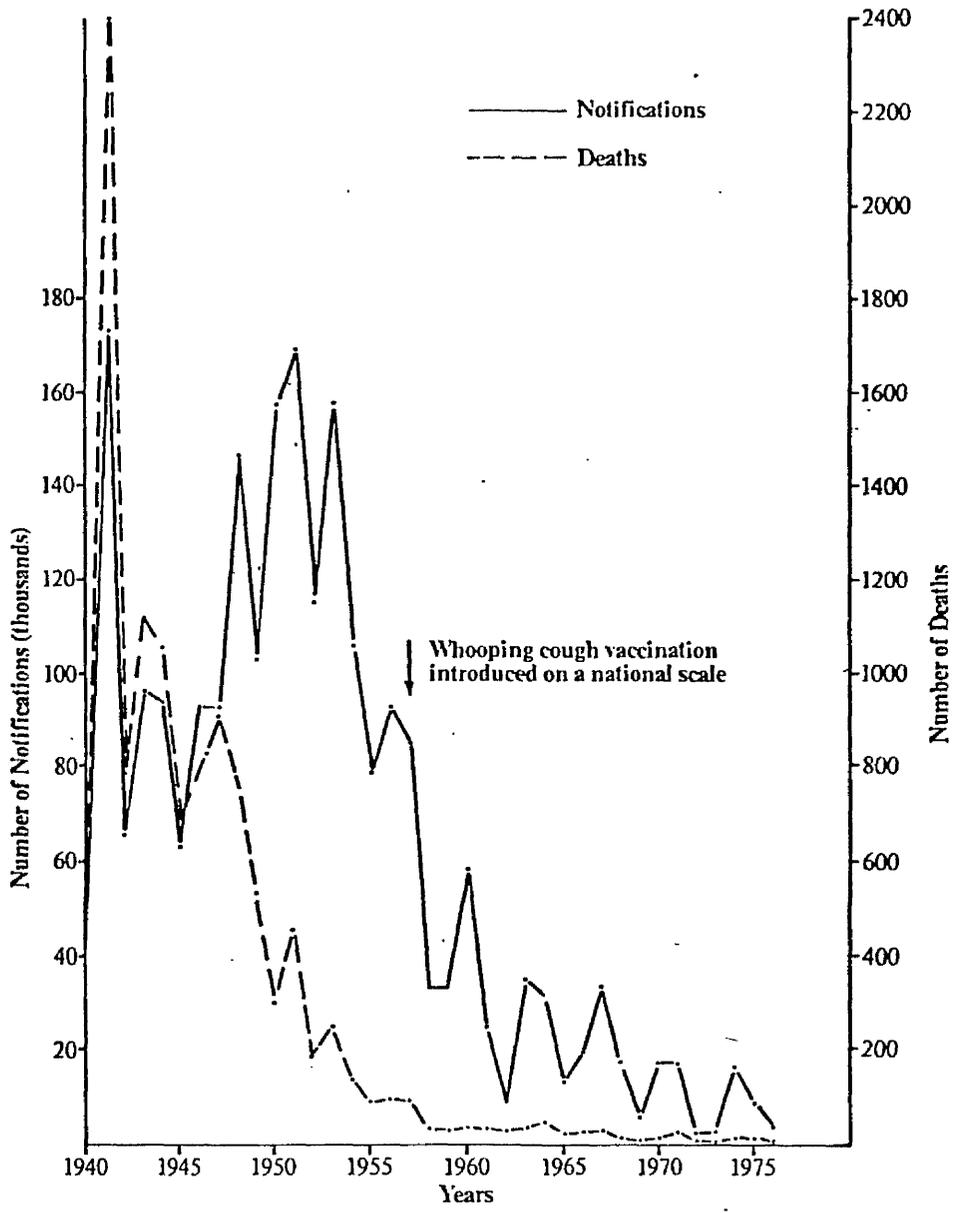
3.   Pertussis vaccine and vaccination

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 un-
vaccinated 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 experi-
enced 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 con-
firmed 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

(Parker, 1978). 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 pharma-cologically-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 funda-
mental 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 <u>Bordetella</u> species and reported
no cross-protection between <u>B. pertussis</u> and <u>B. parapertussis</u>
or <u>B. bronchiseptica</u>. Kendrick <u>et</u> <u>al</u>. (1953) however, stated
that there was some cross-protection in animals between
<u>B. pertussis</u> and some <u>B. bronchiseptica</u> vaccines and between
<u>B. parapertussis</u> and <u>B. bronchiseptica</u> but none between
<u>B. pertussis</u> and <u>B. parapertussis</u>. It is surprising that
<u>B. parapertussis</u> is not protective against <u>B. pertussis</u> if the
conversion of <u>B. pertussis</u> to <u>B. parapertussis</u> 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 <u>Bordetella</u> 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 <u>et</u> <u>al</u>. (1974) reported that immunization with a
purified preparation containing haemagglutinin (HA) and leuko-
cytosis-promoting factor (LPF) protected mice from intracerebral
(i.c.) infection with a virulent strain of <u>B. pertussis</u>.
Arai and Sato (1976) separated this preparation into two distinct
haemagglutinins namely fimbrial haemagglutinin (F-HA) and leuko-
cytosis-promoting factor haemagglutinin (LPF-HA). Sato <u>et</u> <u>al</u>.
(1979) showed that F-HA was non-toxic and protected mice against
i.c. challenge with <u>B. pertussis</u> 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 respons-
ible 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'Bryan 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 <u>B. pertussis.</u>

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 <u>et</u> <u>al</u>. 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 <u>et</u> <u>al</u>. 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 <u>et</u> <u>al</u>.
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°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°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 under-
stood, 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 agglutinoge 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 agglutin-
ated 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 super-natant 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) Extracyto-plasmic 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 agglu-

tinin, 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 discrep-

ancies 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 μg 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,
1960; Aprile and Wardlaw, 1973; Konstantinov et al. 1978).
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 pathophysio-
logical activities

It is well known that B. pertussis expresses various patho-
physiological 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 B. pertussis and adjuvant activity.
Lehrer et al. (1974) and Sato et al. (1974) obtained partially
purified HSF but they found their preparations still retained
PA. Parker and Morse (1973) obtained highly purified prepar-
ations 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;
LPF; heat-labile adjuvant; PA and (LAT) *late-acting toxin* were included.

On the other hand, Dolby (1958) and Nagel (1970) did not
conclude that HSF, LPF and PA were identical. In addition
Sato et al. (1974) found that some preparations of B. pertussis
were less active in producing sensitization to histamine than
in protecting mice. Cameron (1976) reported that changes in the
cultural conditions for 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 et al. (1948) who showed that preparations containing HA
induced active immunity in mice against intranasal infection
with 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 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 lympho-
cytosis 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
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 (Eldering 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 indepen-
dently 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⟷C 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 $MgSO_4$. 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 agglutin-
ogens, 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 physio-
logical 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 $CaCl_2$ 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.pertussi
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 hydro-
lysate 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 hydro-
lysate 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 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 B. pertussis. Starch was found to annul this inhibition (Pollock, 1947). Nicholson (1951) using solid medium for growth of 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 μg 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 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 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 Strepto-
coccus 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 trans-
formation. 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 P^{32}-
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. pneu-
moniae 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 trans-
forming 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 possib-
ility 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 proteo-
lytic 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 5×10^5 daltons by ultra-sonic 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.

$CaCl_2$, 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 $CaCl_2$ 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 $CaCl_2$. They suggested that high concentrations of $CaCl_2$ 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 herit-
able 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-methyl-guanine. 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.

7.5 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. Møller 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 specifi·
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 trans-
fer 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 Shaprio,
1977). It was observed that R68 promoted chromosomal marker
transfer in P. aeruginosa strain PAT at frequencies as high as
4×10^{-3}/donor cell. (Stanisich, 1972). The physical examin-
ation 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 (Sistron, 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^{-}

of donor DNA in the presence of 0.001M $CaCl_2$.

The existence of bacteriophages which are lytic against B. pertussis was reported by Grant (1979). He noted that in the absence of type strains, and the difficulties of classifying Bordetella 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 B. pertussis.   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 et al. 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^S/Str^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 irreversbile, 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^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 patho-
physiological 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 μg ml^{-1}) mutant of strain 44122/7S.   Both strains were supplied as freeze-dried cultures by Dr. P. Branefors, University of Göteborg, Institute of Medical Microbiology, Department of Bacteriology, Guldhedsgatan 10, S-413 46 Göteborg, 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 (Nal^R) (20 μg 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 μg ml^{-1}); carbenicillin (200 μg ml^{-1}) and tetracycline (10 μg ml^{-1}).

Escherichia coli strain Jc 3272 has chromosomal mutations which confer resistance to streptomycin (200-500 μg 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 μg 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 $35^{\circ}C$. Cells were then heavily inoculated onto further B-G plates, incubated for 24 h at $35^{\circ}C$, 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 $35^{\circ}C$. 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 pre-
paration was calculated and multiplied by 10 to give the concen-
tration 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°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 x 10^3; 5.4 x 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 B. pertussis liquid
cultures harvested by centrifugation (10,000 g, 30 min, 4°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 sus-
pension 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 intra-
cerebrally 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 confluency) 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 haema-
gglutination 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-hydroxy-quinoline 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 poly-propylene 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 re-dissolved 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
was added and the mixture shaken and centrifuged 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) con-
taining 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 concen-
tration 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
(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 concen-
tration 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

$\downarrow$

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

$\downarrow$

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

$\downarrow$

deter-
gent
wash

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

$\downarrow$

Mild

osmotic

shock

Resuspend cells in 0.4 ml TES (TS + EDTA 0.01M);
add 0.35 ml concentrated sucrose mix (Sucrose 1.6M,
Tris 0.55M, EDTA 0.1M), keep at 5°C for 10-20 min.

$\downarrow$

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

$\downarrow$

Incubate 5 - 20 min

$\downarrow$

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

$\downarrow$

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 mg 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 Branefors (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 trans-
ferred 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).

μg ml^{-1} 2-deoxyribose

Absorbance at (595 minus 700) nm
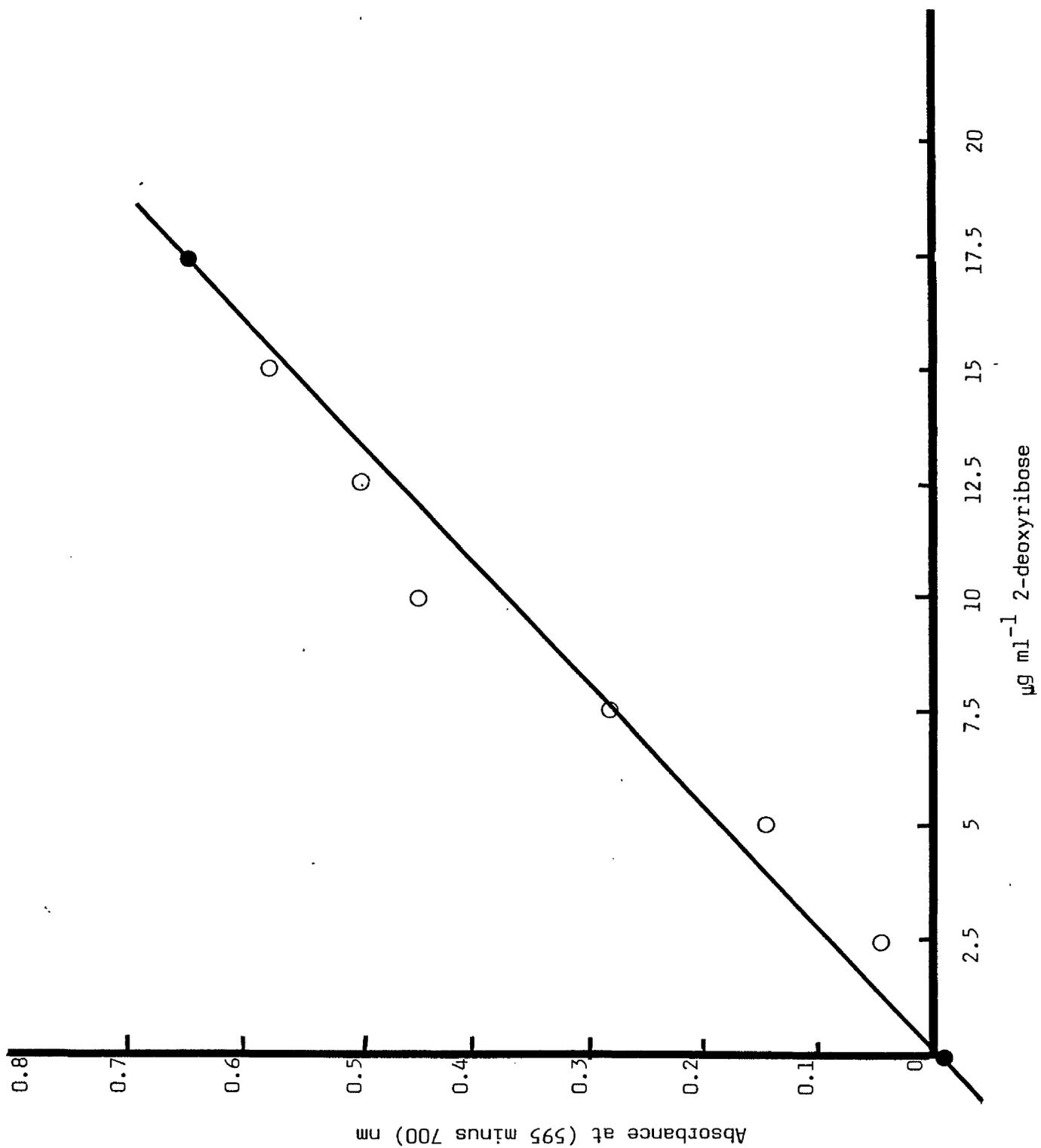
for 18 - 30 h. From this pre-culture, 1 ml portions were transferred to a number of 100 ml flasks each containing 10 ml of liquid medium to which varying amounts (0.03% to 1% (w/v)) of casein hydrolysate had been added. These cultures were incubated on the rotary shaker for varying lengths of time (10 - 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 0.05ml 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 2ml 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 contain-
ing 0.03-1% (w/v) casein hydrolysate (10-36 h).

1 ml sample   centrifuged

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

2ml 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.

2ml 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 chromo-
somal 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 electro-
     phoresis (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 electro-phoresis 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.

Addition to Competent Cells	Number of streptomycin-resistant colonies from 1ml competent cell culture
None	0
$5 \ \mu g \ ml^{-1}$ DNA	30
0.01M $CaCl_2$	10
$5 \ \mu g \ ml^{-1}$ DNA + 0.01M $CaCl_2$	50
$5 \ \mu g \ ml^{-1}$ DNA + $50 \ \mu g \ ml^{-1}$ DNase (Sigma)	0

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.

Addition to Competent cells	Number of streptomycin-resistant colonies from 1ml competent cell culture
None	8
5 μg ml^{-1} DNA	44
5 μg ml^{-1} DNA, chilled for 20 min	108
5 μg ml^{-1} DNA, chilled for 20 min then heat shocked for 4 min at 37°C	112
5 μg ml^{-1} DNA + 50 μg ml^{-1} DNase (Sigma)	10

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.

Addition to Competent cells	Number of streptomycin-resistant colonies from 1ml competent cell culture
None	0
5 μg ml^{-1} DNA,	30
5 μg ml^{-1} DNA, chilled 20 min	40
5 μg ml^{-1} DNA, chilled 20 min then heat shocked 4 min at 37°C	30
5 μg ml^{-1} DNA + 50 μg ml^{-1} DNase	0

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.

Addition to Competent cells	Number of streptomycin-resistant colonies from 1ml competent cell culture			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
None	0	0	0	0
10 μg ml^{-1} DNA	4,800	7,200	4,400	8,000
10 μg ml^{-1} DNA + 50 μg ml^{-1} DNase	0	0	0	1,600

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 trans-
formation in <u>B. pertussis</u> 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 $CaCl_2$: The addition of $CaCl_2$ 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-I B. pertussis antisera in slide agglutination tests. Since $CaCl_2$ 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.

Age of culture	Number of streptomycin-resistant colonies from 1ml competent cell culture
33 h	10
42 h	30
48 h	60
53 h	20
Control (No DNA added)	0

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.

Addition to Competent cells	Number of streptomycin-resistant colonies from 1ml competent cell culture containing $CaCl_2$ at :			
	0.001M	0.005M	0.01M	0.1M
None	2	4	5	2
10 μg ml^{-1} DNA	70	400	400	20

Table 8.   Effect of dibutyryl cAMP, cAMP and protease

on transformation frequency.

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

Addition to Competent cells	Number of streptomycin-resistant colonies from 1ml competent cell culture			
	Expt.1	Expt.2	Expt.3	Expt.4
None	800	400	800	800
10 μg ml^{-1} DNA	4,400	1,200	1,200	1,200
10 μg ml^{-1} DNA + 50 μg ml^{-1} dibutyryl cAMP	14,800	12,800	11,200	5,200
10 μg ml^{-1} DNA + 50 μg ml^{-1} cAMP	1,200	N.T*	N.T	N.T
10 μg ml^{-1} DNA + 50 μg ml^{-1} pronase	N.T	800	2,800	N.T
10 μg ml^{-1} DNA + 100 μg DNase	0	0	0	1,200

* 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 con-
taining 1% (w/v) glycine (see Materials and Methods).   Samples
were treated for transformation with DNA prepared by the
modified Marmur method and Branefors 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^{R} of the strain 8631-IV was
attempted, using DNA from strain 44122/7R under the conditions
described in Table 5.   No Str^{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.

Addition to Competent cells	Number of streptomycin-resistant colonies from 1ml competent cell culture
None	0
10 μg ml^{-1} DNA, 20 min chilling	20
10 μg ml^{-1} DNA, 40 min chilling	280
10 μg ml^{-1} DNA, 60 min chilling	20
10 μg ml^{-1} DNA, no chilling	10

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.

<u>Figure 5.</u>   <u>Effect of NTG on cell viability and mutant</u>
         <u>isolation.</u>

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 con-
centration 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.

No. of streptomycin-resistant colony forming units per ml.

Time (h) after addition of NTG
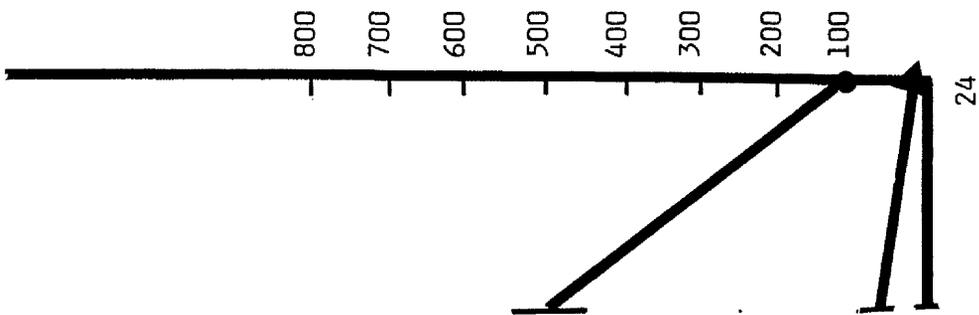
No. of colony forming units per ml ($\times 10^6$)
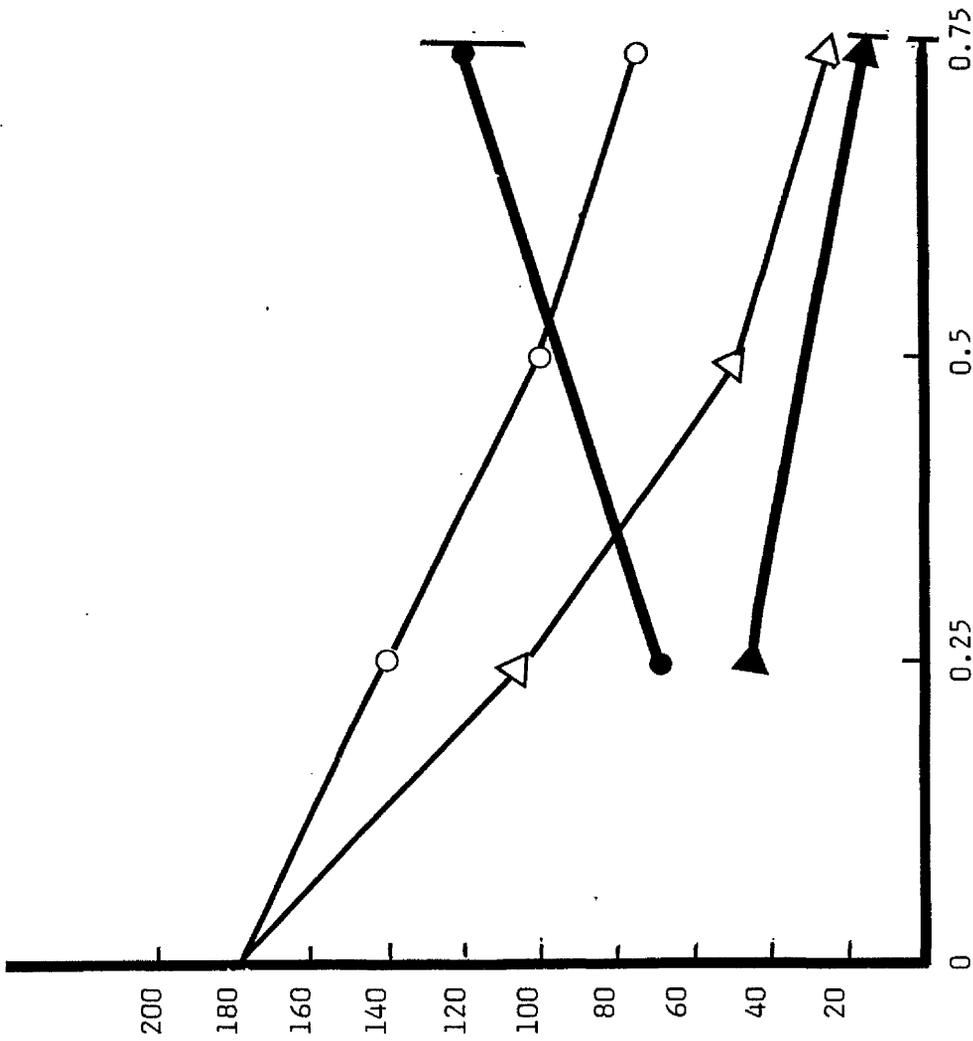
NTG at 40 μg ml^{-1} seemed to be the optimum concentration for mutagenesis. Both lower and higher NTG concentration gave fewer Str^R mutants. For example, when the cell suspension was treated with only 4 μg ml^{-1} final concentration of NTG only a few Str^R mutants were isolated (Figure 6). The concentration of 100 μg ml^{-1} NTG gave a larger number but this was still lower than that obtained under optimum conditions of 40 μg ml^{-1} (Figure 5).

In subsequent experiments, NTG was used to try to obtain mutants of B. pertussis resistant to other antibiotics. Cells exposed to NTG at a final concentration of 40 μg ml^{-1} for 45 min were inoculated onto B-G agar containing 200 μg 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 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 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 μg ml^{-1} NTG 24 h after mutagen preparation both killing and Str^R mutant recovery were drastically reduced (Figure 6). Samples inoculated onto B-G agar containing streptomycin after 15 min of treatment with 40 μg ml^{-1} NTG left for 24 h at room temperature gave 34-fold less difference in 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}); $\bigcirc$-cell viability,
$\bullet$-streptomycin-resistant mutants.

Treatment with fresh preparation (4 μg ml^{-1}); $\triangle$-cell
viability, $\blacktriangle$-streptomycin-resistant mutants.

No. of colony forming units (Str^R) per ml.

100 90 80 70 60 50 40 30 20 10

.75

.5

.25

Time (h) after addition of NTG

100 90 80 70 60 50 40 30 20 10

No. of colony forming units (x 10^7)
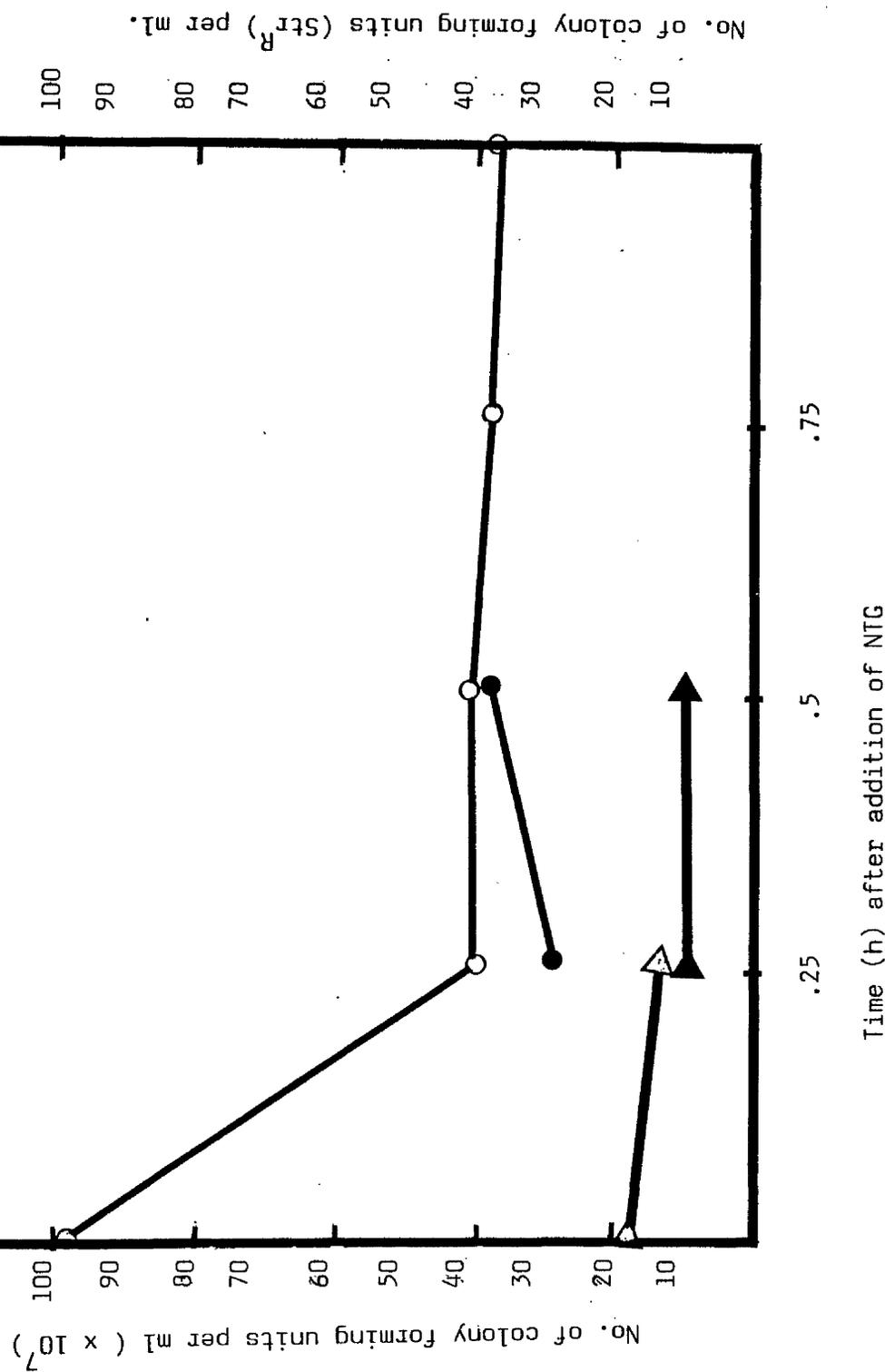
Thus Str^R mutants from <u>B. pertussis</u> were easily obtained.
An optimum concentration and time of treatment with NTG were
found for selection of Str^R mutants, but mutants resistant to
other antibiotics such as kanamycin and neomycin were not
obtained under these conditions.

2.2   Mutant selection by <u>in vitro</u> subculture

<u>B. pertussis</u> 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.   <u>Plasmid transfer and chromosome mobilization</u>

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 3.3×10^{-7} to 6.4×10^{-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 0.95×10^{-4} to 1.95×10^{-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 inherite

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.

Expt.	Strain	No. of colonies per ml of original culture isolated on selective media		Plasmid transfer frequency for donor cell	Chromosomal mobilization frequency for donor cell
		N.A containing 200μg ml^{-1} Str. and car. + 20μg ml^{-1} kana.	N.A containing 200μg ml^{-1} Str. and car. + 20μg ml^{-1} kana. and nal.		
1	Donor Jc 53-1	0	0		
	Recipient Jc 3272	0	0		
	Jc 57-1 + Jc 3272	2.0×10^{7}	3×10^{3}	0.22	3.3×10^{-7}
2	Jc 53-1	0	0		
	Jc 3272	0	0		
	Jc 53-1 + Jc 3272	1.32×10^{7}	2.81×10^{3}	0.26	6.4×10^{-6}

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.

Expt.	Strain	No. of colonies per ml original recipient culture isolated on selective media		Plasmid transfer frequency per donor cell	Chromosomal mobilization frequency per donor cell
		B-G agar containing 200μg ml⁻¹ car. and Str. and 20μg ml⁻¹ kana.	B-G agar containing 200μg ml⁻¹ car. and Str. and 20μg ml⁻¹ kana. and nal.		
1	Jc 53-1	0	0		
	44122/7R	0	0		
	Jc 53-1 + 44122/7R	5×10^3	0	0.95×10^{-4}	0
2	Jc 53-1	0	N.T*		
	44122/7R	0	0		
	Jc 53-1 + 44122/7R	2×10^4	0	1.95×10^{-5}	0

* 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 x 10^{-5} to 1.29 x 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 x 10^{-5} and 1.6 x 10^{-}

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 $-70^{\circ}C$ 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.

Expt.	Strains	No. of colonies per ml of original recipient culture isolated on selective media		Plasmid transfer frequency per donor cell	Chromosomal mobilization frequency per donor cell
		N.A containing 200 μg ml^{-1} car. and 20 μg ml^{-1} kana.	N.A containing 200 μg ml^{-1} of car. and Str.		
1	Donor 44122/7RP	0	0		
	Recipient D30042-IV	0	0		
	44122/7RP + D30042-IV	8×10^3	0	1.29×10^{-5}	0
2	Donor 44122/7RP	0	0		
	Recipient D30042-IV	0	0		
	44122/7RP + D30042 -IV	1.54×10^4	0	1.28×10^{-5}	0
3	Donor 44122/7RP	0	0		
	Recipient 11615-IV	0	0		
	44122/7RP + 11615-IV	1.08×10^6	0	1.9×10^{-5}	0
4	Donor 44122/7RP	0	0		
	Recipient L84-IV	0	0		
	44122/7RP + L84-IV	8.0×10^{-6}	0	1.6×10^{-6}	0

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 intra-cerebral challenge strain used in the mouse protection test (Standfast, 1958). Strain 44122/7S was much less virulent with LD_{50} values ranging from 7.9×10^3 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 14 to 1.04×10^5 . This variation may be attri-buted, 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.

Expt.	LD_{50} in colony forming units per mouse (and 95% confidence limits)		Relative potency*
	44122/7S	44122/7R	
1	$> 1.8 \times 10^6$	199 (154,257)	$> 1.7 \times 10^4$
2	$> 1.9 \times 10^5$	520 (276,997)	> 360
3	$> 1.4 \times 10^7$	134 (100,182)	$> 1.04 \times 10^5$
4	$> 2.9 \times 10^4$	355 ($83,1.5 \times 10^3$)	> 237
5	7.9×10^3 ($1.3 \times 10^3, 3 \times 10^4$)	562 ($211,1.5 \times 10^3$)	14
6	$> 3.4 \times 10^4$	630 (423,939)	> 183
7	1.8×10^4 (250, 4×10^5)	126 (66, 240)	143

* 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.

Expt.	Strain	Calculated values		Computed values	
		LD_{50} (cfu/mouse)	Rel.pot.	LD_{50} (cfu/mouse)	Rel.pot
2	44122/7S	$>1.9 \times 10^5$	1.0^*	2.1×10^6	1.0^*
	44122/7R	520	360	250	8.3×10^3
4	44122/7S	$> 2.9 \times 10^4$	1.0^*	4.2×10^4	1.0^*
	44122/7R	355	> 237	240	167

*   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 (Appendix2.2). 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).

Expt.	LD_{50} in colony forming units per mouse (and 95% confidence limits)					
	44122/7S	44122/7R	Trans.A	Trans.B	Trans.E	Trans.X
5	7.9×10^3 $(1.3\times10^3, 3\times10^4)$	562 $(211, 1.5\times10^3)$	120 $(49, 295)$			
6	$> 3.4 \times 10^4$	630 $(423, 939)$	660 $(29, 1.4\times10^3)$			
7	1.8×10^4 $(250, 4\times10^5)$	126 $(66, 240)$		< 94		
8	5.5×10^3 $(1.5\times10^3, 2\times10^4)$				< 78	< 73

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 x 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 (from 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.

Strain	Calculated values		Computed values	
	LD_{50}(cfu/mouse)	Rel.pot.	LD_{50}(cfu/mouse)	Rel.pot.
44122/7S	5.5×10^3	1.0	2.5×10^3	1.0
Trans.X	< 73	> 75	11	227.6
Mutant-X	< 88	> 62.5	7	362.7
Trans.E	< 78	> 70	22	115.3

Table 17.   Virulence for mice by the intracerebral route :

Comparison of Branefors strains with Str^R mutants.

(by calculation).

| Expt. | LD$_{50}$ in colony forming units per mouse (and 95% confidence limits) | | | | |
	44122/7S	44122/7R	Mutant-X	Mutant-S	Mutant-1-1
/5	7.9×10^3 (1.3×10^3, 3×10^4)	562 (211, 1.5×10^3)		35 (9, 138)	
18	5.5×10^3 (1.5×10^3, 2.10^4)		< 88		
19	1.4×10^3 (589, 3.5×10^3)				74 (55, 100)

their streptomycin-resistant isolates designated 18334^{s-1}, $353-I^{s-1}$ and $L84-I^{s-1}$ and Taberman, were found to be avirulent for mice by the intracerebral route. Some typical results are shown in Table 18. The LD_{50} of the strains Taberman, 18334 and 18334^{s-1} were > 6.8×10^4; > 2.4×10^4 and > 2.4×10^3 cfu per mouse respectively. The LD_{50}s of strains 353-I, $353-I^{s-1}$, L84-I and $L84-I^{s-1}$ were even higher and could not be calculated from available data. Phase IV Department cultural collection strains and their Str^R isolates were also tested for virulence by the intracerebral route (Table 19). Strains 353-IV; $353-IV^{s-1}$; L84-IV were avirulent with high challenge doses. The virulence of the strains 8631-IV and its Str^R spontaneous mutant was too low to be calculated since neither strain killed any of the twenty mice injected even at doses of 1.7×10^7 and 5.3×10^4 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 (LD_{50} - 3.1×10^4 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

Expt.	LD_{50} in colony forming units per mouse (and 95% confidence limits)				
	44122/7S	44122/7R	18334	18334^{s-1}	Taberman
10		56 (3.5,912)	$> 2.4 \times 10^4$	$> 2.4 \times 10^3$	
11	4×10^3 $(3.10^3, 7 \times 10^3)$		$> 7.9 \times 10^3$		$> 6.8 \times 10^4$

Table 19.   Virulence for mice by the intracerebral route :

Comparison of phase-IV strains and strain 44122/7R.

Expt.	LD_{50} in colony forming units per mouse (and 95% confidence limits)			
	44122/7R	353-IV	353-IV^{s-1}	L84-IV
10	56 (3.5, 912)	$> 4.3 \times 10^4$	$> 1.3 \times 10^4$	
12	1×10^4 $(1.69 \times 10^3, 7.4 \times 10^4)$	$> 2.7 \times 10^5$		$> 5.7 \times 10^5$

Table 20.   Virulence for mice by the intracerebral route :

Strain 44122/7R, before and after attenuation

and acquisition of plasmid R68.45

Expt.	LD$_{50}$ in colony forming units per mouse (and 95% confidence limits)		
	44122/7R	44122/7R(34)	44122/7RP
13	< 540	3.1 x 10^4 (1.5x10^4, 8.3x10^4)	
14	< 122		140 (95,208)

There was no striking difference in virulence by the intra-
nasal 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 $9x10^5$ cfu per mouse for
the Str^S strain and $4.8x10^5$ 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 ($56^{o}C$ 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 signif-
ically more HSF than the parent strain.

Table 21. Virulence for mice by the intranasal route :
Branefors streptomycin-resistant and sensitive
strains

Strain	Dose (cfu/ mouse)	Deaths/ injected mice	Lung pathology score from 0 to 4	Amount of growth score from 0 to 4
44122/7S	4.3×10^{6}	4/5	2	2
	4.3×10^{5}	2/5	1;3;3 (Total=7)	2;4;4 (10)
	4.3×10^{4}	0/5	2;3;3;3;3 (14)	0;4;4;4;4 (16)
	Saline only	0/5	0	0
44122/7R	1.52×10^{7}	5/5	-*	-
	1.52×10^{6}	5/5	-	-
	1.52×10^{5}	0/5	2;3;3;3;4 (15)	4;4;2;4;4 (18)
	1.52×10^{4}	0/5	1;2;2;3;4 (12)	3;4;1;4;3 (15)

Computed results (Lethality)

Strain	Rel. pot.	95% confidence limits		LD_{50} (cfu/mouse
44122/7S	1.0			9.0×10^{5}
44122/7R	1.88	0.31	11.65	4.8×10^{5}

* 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).

	HSD_{50} in opacity unit (ou. ml per mouse)		Rel. pot. (and 95% confidence limits)	
	44122/7S	44122/7R	44122/7S	44122/7R
1	3.92	3.92	1.0	1.0
				(0.15, 6.41)
2	5.66	1.9	1.0	2.97
				(1.49, 5.15)
3	1.96	3.86	1.0	0.5
				(0.15, 1.73)
4	8.81	4.04	1.0	2.17
				(0.45, 39.5)

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 B. pertussis strains which may have a role in virulence. HLT is lethal to mice when unheated cell suspensions are injected intraperitoneally.

Two independent HLT tests were done on Branefor Str^S and Str^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^S strain which is low in virulence for mice by the intra-cerebral route appears to have a higher HLT content than the virulent, Str^R strain.

5.1.4 Haemagglutinating activity

The haemagglutinin(s) of 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^S and Str^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^R strain were 2 and 4 compared with 2 and 2 in the Str^S strain in two separate growth experiments.

Table 23. Heat-labile toxicity in Branefors streptomycin-sensitive and streptomycin-resistant strains.

Computed Results

Expt.1	Dose (ou.ml/mouse)	Log dose	Tested	Responding
44122/7R	5.0	0.699	10	6
	2.5	0.3979	10	3
	1.25	0.0969	10	0
	0.625	-0.2041	10	0
44122/7S	2.5	0.3979	10	8
	1.25	0.0989	10	8
	0.625	-0.2041	10	2
	0.312	-0.5051	10	1

	Rel.pot.	95 pc limits	ED_{50}	Log_R	$W\text{-}log_R$	$LogED_{50}$	$W\text{-}logED_{50}$
7R			4.27			0.63	23.73
7S	4.443	2.614 8.757	0.96	0.6477	14.51	0.0177	35.75

Expt.2	Dose (ou.ml/mouse)	Log dose	Tested	Responding
44122/7R	5.0	0.6990	4	3
	2.5	0.3979	4	3
	1.25	0.0969	4	0
	0.625	-0.2041	4	0
	0.3125	-0.5051	4	0
44122/7S	5.0	0.6990	4	4
	2.5	0.3979	4	3
	1.25	0.0969	4	0
	0.6250	-0.2041	4	0
	0.3125	-0.5051	4	0

	Rel.pot.	95 pc limits	ED_{50}	Log_R	$W\text{-}log_R$	$LogED_{50}$	$W\text{-}logED_{50}$
7R			2.62			0.4186	22.54
7S	1.239	0.632 2.479	2.12	0.0931	11.34	0.3255	22.81

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.

| Strain | Visible agglutination with antisera specific for :- | | | |
	Factor 1	Factor 2	Factor 3	Factor 4
44122/7S	++	-	+++	-
44122/7R	++	++	+++	-
44122/7R(34)	++	++	+++	-
Mutant-2	++	-	+++	-
Mutant-X	++	-	+++	-
Trans.X	++	-	+++	-
Trans.B	++	-	+++	-

+++ = 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.

C

Table 25.   Mouse-protection test data

Strain	Dose (ou. ml per mouse)	No. of mice tested	No. protected
	5.0	10	7
44122/7S	1.0	10	2
	0.20	10	1
	5.0	10	7
44122/7R	1.0	10	3
	0.20	10	1

Computed results

Strain	Relative potency	95% confidence limits		ED_{50}
44122/7S	1.0			2.85
44122/7R	1.57	0.332	9.917	1.81

5.2   Biochemical characterization

5.2.1 Antibiotic sensitivity tests

The observation of differences between Branefors Str^R and Str^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^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 μg ml^{-1} streptomycin although at this level of antibiotic growth was poor. With 6000 μg ml^{-1} streptomycin, good growth was obtained with all of these strains. The parent 44122/7S strain was found to be sensitive to 1 μg 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^{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 μg ml^{-1}.

The relationship between Str^R and resistance to other anti-biotics 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.

Strain	MIC in μg ml^{-1} streptomycin	Strain	MIC in μg ml^{-1} streptomycin
44122/7	1	Mutant-1	7000
18334	1	Mutant-2	7000
18334^{s-1}	2000	Mutant-X	7000
44122/7R	·7000		
44122/7R(34)	7000		
44122/7RP	7000		
Trans.A	7000		
Trans.B	7000		
Trans.E	7000		
Trans.X	7000		

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-I 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.

Antibiotic Disc	Zone of inhibition (mm)					
	44122/7S	44122/7R	Trans. A	Trans. B	Mutant -1	Mutant -2
Streptomycin (160 μg)	21	0	0	0	0	0
Penicillin (2 μg)	9	9	10	10	9	9
Penicillin (100 μg)	27	30	31	32	32	33
Erythromycin (10 μg)	18	22	22	25	22	24
Erythromycin (78 μg)	22	21	28	30	30	30
Tetracycline (10 μg)	12	13	15	16	15	16
Tetracycline (80 μg)	16	23	26	24	24	24
Ampicillin (10 μg)	24	30	30	31	30	30
Ampicillin (33 μg)	25	32	32	32	32	31

strain and one 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 B. parapertussis, production of brown pigmentation on B-G agar was examined. None of the B. pertussis variants showed this property.

5.2.4 Effect of starch on growth of 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.
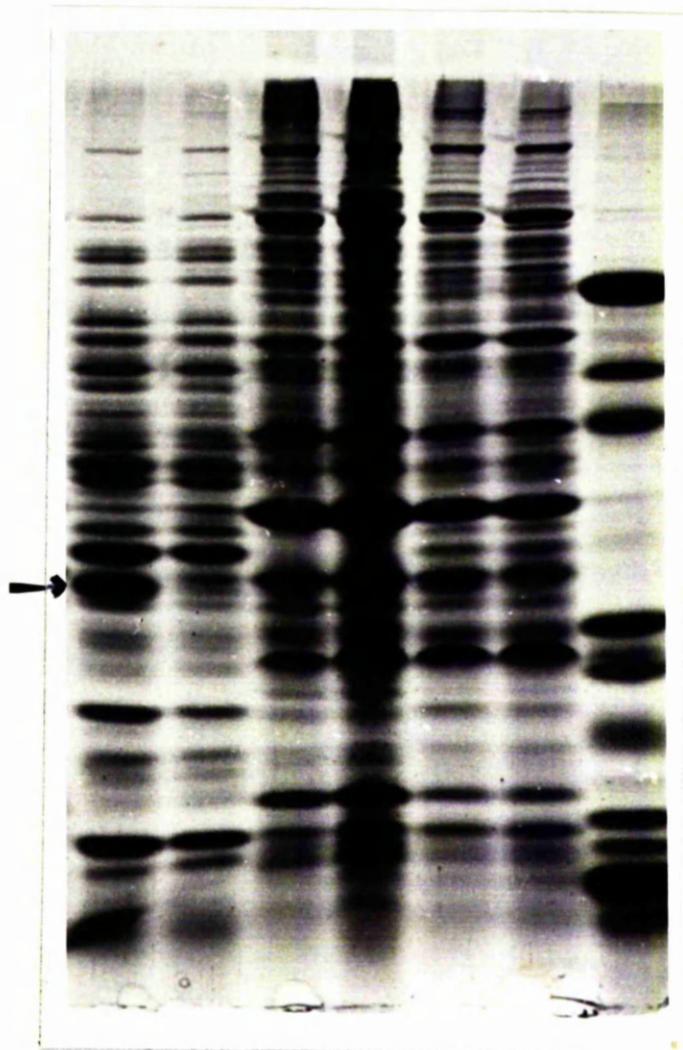
Cytoplasmic   Envelopes   Whole   Mol.wt
fractions   Cells   markers

7R   7S   7R   7S   7R   7S

DISCUSSION

1. <u>Development of a transformation system</u>

Various cell components in <u>B. pertussis</u> 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 <u>B. pertussis</u>, genetic
studies may provide an important understanding of the relation-
ship 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
<u>B. pertussis</u> 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 bio-
logical 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 <u>B. pertussis</u> which would be useful for the product-
ion 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 $\mu g\ ml^{-1}$ 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 trans-
formation 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 trans-
formation frequency would only be observed during the period
of optimal competence.

Prior to the present study, factors known to increase com-
petence 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 $37^{\circ}C$ after chilling abolished this enhance-

ment.   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 unmasks 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 H. influenzae.   It is possible therefore,
that a high concentration of dibutyryl cAMP in B. pertussis has
a similar effect.   It is also possible that the difference
between cyclic AMP and dibutyryl cyclic AMP is due to permea-
bility differences and that B. pertussis and 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
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 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 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 sub-cultured 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 μg ml^{-1} NTG rather than 100 μg ml^{-1} or 4 μg 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 μg 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 μg ml^{-1} NTG and 56.2% after 45 min treatment with 40 μg 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 Str^R 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 Str^R 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 3.3×10^{-7} to 6.4×10^{-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 0.95×10^{-4} to 1.95×10^{-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 plasmids 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 x 10^{-5} to 1.29 x 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
Branefors 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 intraperit-
oneally 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 Str^R phenotype and
virulence intracerebrally was considered an important observation
since it was confirmed by subsequent transformation experiments
in which transfer of the Str^R marker was always accompanied by
increased virulence by the intracerebral route in transformants.
Indeed, all but one of four Str^R mutants expressed high viru-
lence 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 agglutin-
ogen factor 2 which was found in strain 44122/7R, but not
44122/7S, was not linked to the Str^R marker nor to the virulence
factor as neither the Str^R transformants or Str^R mutants
possessed it. They all resembled the Str^S strain in this respect.

It is probable that the Str^R mutation in B. pertussis in
some way enhances virulence intracerebrally in mice rather than
introducing it. This could be deduced from the results obtained
from Branefors Str^R and Str^S parent strains and the Str^R
derivates compared with the strain 18334 and its Str^R mutant.
In the first case Branefor's Str^S strain had a high, but measur-
able LD_{50} which was greatly reduced in the Str^R mutant. In
strain 18334 there was apparently no virulence character to be
enhanced by the Str^R mutation. A similar observation was
obtained with the strain 8631-IV and its 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 Str^R mutant showed any sign of virulence intracerebrally at the doses tested.

It was thought that the virulence of the Str^R Branefors strain and the other Str^R derivatives of the Str^S 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 Str^R 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 Str^S. Branefors strain, whereas that of Str^R 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 Str^R 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 Str^R 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 invest-

igated.

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 ($\equiv$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 Str^S strain was less virulent by the intra-
cerebral route and contained less HSF activity than the 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 haema-
gglutination 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 intra-
cerebrally avirulent strains.   In fact, Preston and Stanbridge
(1976) stated that vaccines of avirulent strains will protect
mice against intracerebral challenge.

The serotype of the Str^S strain was 1, 3 whereas that of
the Str^R strain was 1, 2, 3.   The latter must have gained the
factor 2 since it is a mutant of the Str^S strain.   However, loss
or gain of agglutinogens in B. pertussis is not uncommon.

Thus the mutation from 44122/7S to 44122/7R presumably involved more than a single mutation including streptomycin-resistance agglutinogen change and possibly changes in HSF and HLT.

The possession of factor 2 by the Str^R 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 Str^R strain such as increased resistance to growth inhibitors as reported by Branefors (1964). The Str^R strain 44122/7R was described as able to grow in Cohen and Wheeler medium without starch whereas the Str^S strain 44122/7S would not. When retested, these two strains as well as several other Str^R 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 Str^R 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 character- istics such as the agglutinogens.

Another type of mutant of B. pertussis has been described. Kumazawa and Yoshikawa (1978) described the conversion of B. pertussis to 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 B. parapertussis criteria, but not with all.

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

The reason for the difference in mouse virulence intra-cerebrally 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 B. pertussis strains (Ackers and Dolby, 1972) and resistance to the phagocytic and bactericidal capabilities of alveolar macro-phage (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 Neisseria gonorrhoeae. Lambden et al. (1979) showed that the heat-modifiable outer-membrane proteins are important virulence attributes of the gonococcus.

The profiles of Str^S and Str^R strains obtained by poly-
acrylamide 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 Str^R strain which could be related either to virulence or
streptomycin resistance. Investigation of the cytoplasmic
protein profiles of virulent Str^S strains and avirulent 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 $45^{\circ}C$.

> 20 ml glycerol (autoclaved)
>
> 100 ml fresh citrated horse blood (Gibco) warmed to $37^{\circ}C$.

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
KH_2PO_4	0.5 g
KCl	0.2 g
$MgCl_2.6H_2O$	0.1 g
$CaCl_2$	0.02 g
Tris (hydroxymethyl-methylamine)	6.075 g
L-cysteine	0.04 g
$FeSO_4.7H_2O$	0.1 g
Ascorbic acid	0.02 g

Nicotinic acid	0.004g
Glutathione	0.1 g

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).

0.1g $MgCl_2.6H_2O$

0.016 g $CaCl_2$ (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)	10g
2.	Nicotinamide (0.1% (w/v) solution)	1ml
3.	$CaCl_2$ (anhydrous)	0.002g
4.	NaCl	5g
5.	$MgCl_2.6H_2O$, 1% (w/v) solution	2.5ml
6.	KCl	0.2g
7.	K_2HPO_4, 10% (w/v) solution	2.5ml
8.	Soluble starch	1g
9.	Distilled water	800ml
10.	Glutathione, 0.1% (w/v) solution	1ml

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_2CO_3. Dispense in 100 ml amounts in screw cap bottles and autoclave at $121^\circ C$ for 15 min. Store at $4^\circ C$.

Cohen and Wheeler Medium (1946)

1.	Bacto-Casamino acids (Difco-Technical)	10 g
2.	Yeast extract (Difco)	2.5 g
3.	Soluble starch	1.5 g
4.	$MgCl_2.6H_2O$, 25% solution	1.6 ml
5.	$CaCl_2$, 10% solution	0.1 ml
6.	$FeSO_4.7H_2O$ 1% solution	1.0 ml
7.	$CuSO_4.5H_2O$ solution	0.05ml
8.	L-arginine, 0.01% solution	1.0 ml
9.	L-histidine, 0.005% solution	1.0 ml
10.	KH_2PO_4	0.5 g

1000 ml distilled H_2O

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 $45^\circ C$, 10% (v/v) horse blood was added.

APPENDIX 2

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

Experiment 2

	Dose (cfu) per mouse	Log dose	Tested	Responding
44122/7R	9060	3.9571	10	9
	906	2.9571	10	6
	90.6	1.9571	10	3
44122/7S	198000	5.2967	10	2
	19800	4.2967	10	1
	1980	3.2967	10	2
	198	2.2967	10	0

Average slope = 0.516

F-Test of non-parallelism = 2.27   F 5PC = 3.84

F-Test of heterogeneity = 1.13   F 5PC = 2.61

	Rel.pot.	95 PC limits		ED_{50}	LogR	WLogR
7R				254.7		
7S	0.00012	0.000	0.004	2.1×10^6	-3.9133	0.04

Experiment 4

	Dose (cfu) per mouse	Log dose	Tested	Responding
44122/7R	54000	4.7324	5	5
	5400	3.7324	5	4
	540	2.7324	5	4
	54	1.7324	5	1
44122/7S	29000	4.4624	5	2
	2900	3.4624	5	1
	290	2.4624	5	0
	29	1.4624	5	0

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

	Rel.pot.	95 PC limits		ED_{50}	LogR	WLogR
7R				239.4		
7S	0.0006	0.000	0.077	42554.8	-2.2498	0.23

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 (LogR) and their weighted values (WLogR) given in the computer print-outs.

Experiment	Strain	LogR	WLogR
2	7S	-3.9133	0.04
4	7S	-2.2498	0.23

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

$$\overline{\text{LogR}} = \frac{\Sigma \, (\text{WLogR} \, . \, \text{LogR})}{\Sigma \, \text{WLogR}}$$

Substituting the values given above

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

$$\overline{\text{LogR}} = \frac{-0.1565 + -0.5175}{0.27}$$

$$= -2.4954$$

Taking antilogs yields

$$\overline{R} = .0032$$

The 95% confidence limits are given by

$$\text{Antilog} \left[\overline{\text{LogR}} \pm \sqrt[1]{\Sigma \, \text{WLogR}} \right]$$

$$\Sigma \, \text{Log R} = 0.27$$

$$\text{Limits} = -2.4954 \pm 1.9245$$

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

Taking antilogs yields (0.00004 , 0.2685)

∴ the potency ($\overline{R}$) of <u>B. pertussis</u> strain 44122/7S relative to that of strain 44122/7R in the intracerebral virulence test

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

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^R transformants and mutant compared with their Str^S
parent strain.

	Dose (cfu) per mouse	Log dose	Tested	Responding
7S	75000	4.8751	10	8
	7500	3.8751	10	8
	750	2.8751	10	3
	75	1.8751	10	1
Trans.X	73200	4.8645	10	10
	7320	3.8645	10	10
	732	2.8645	10	10
	73.2	1.8645	10	7
Mutant-X	88000	4.9445	10	10
	8800	3.9445	10	10
	880	2.9445	10	10
	88	1.9445	10	8
Trans.E	78000	4.8921	10	10
	7800	3.8921	10	10
	780	2.8921	10	9
	78	1.8921	10	7

	Rel. pot.	95pc limits	ED_{50}	Log_R	$WLog_R$	$LOED_{50}$	$WLED_{50}$
7S			2487.71			3.3987	3.17
Trans.X	227.572	26.480 ***	10.93	2.3571	0.69	1.0387	0.88
Mutant-X	362.670	34.077 ***	6.86	2.5595	0.55	0.8363	0.67
Trans.E	115.348	15.588 ***	21.57	2.0620	0.83	1.3338	1.13

Intracerebral virulence test data.

Calculated results

	Dose	Log dose	Tested	Responding
44122/7S	4.32×10^4	4.635	5	4
	4.32×10^3	3.635	5	2
	4.32×10^2	2.635	5	1
	43.2	1.635	5	0
Mutant S	1.1×10^5	5.04	5	5
	1.1×10^4	4.04	5	4
	1.1×10^3	3.04	5	4
	1.1×10^2	2.04	5	2

	Rel. pot.	95 pc limits	ED_{50}
7S		575, 6.9×10^4	1.6×10^4
Mutant ,S	80	28.8, 1.38×10^3	200

APPENDIX 2.3

Intranasal virulence test data.

Computed results

	Dose per mouse (cfu x 10^4)	Log dose	Tested	Responding
7S	430	2.6335	5	4
	43	1.6335	5	2
	4.3	0.6335	5	0
7R	1520	3.1818	5	5
	152	2.1818	5	5
	15.2	1.1818	5	0
	1.5	0.1818	5	0

No. of cycles = 4

Doses		XMEAN	YMEAN	CSx2	CSxX	CSY_2	FDEV	Slope
7S	3	1.9477	4.977	1.0750	1.0388	1.4148	0.41	0.97
7R	4	1.6818	5.000	1.1384	3.7870	12.7024	0.05	3.33

Average slope = 2.180

G = 0.365

F-test of non parallelism = 3.08   F 5 pc   3.84, DF = 1/INF
F-test of heterogeneity   = 0.17   F 5 pc   2.61, DF = 3/INF

	Rel. pot.	95 pc limits		ED_{50}	Log_R	$WLog_R$	$LOED_{50}$	$WLED_{50}$
7S				90.79			1.9581	3.25
7R	1.889	0.315	11.652	48.07	0.2762	1.63	1.6818	3.25

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 4

	Dose per mouse (ou ml)	Log dose	Tested	Responding
7S	8.00	0.9031	4	2
	2.00	0.3010	4	0
	0.500	0.3010	4	0
	0.125	0.9031	4	0
7R	8.00	0.9031	4	3
	2.00	0.3010	4	1
	0.500	-0.3010	4	0
	0.125	-0.9031	4	0

	Rel. pot.	95 pc limits		ED_{50}	Log_R	$WLog_R$	$LOED_{50}$	$WLED_{50}$
7S				8.81			0.9449	1.52
7R	2.178	0.454	39.564	4.04	0.3381	1.06	0.6068	2.67

Experiment 3

	Dose	Log dose	Tested	Responding
7S	8.00	0.9031	5	4
	2.00	0.3010	5	4
	0.500	-0.3010	6	0
	0.125	-0.9031	4	0
7R	8.00	0.9031	6	5
	2.00	0.3010	5	1
	0.500	-0.3010	5	0
	0.125	-0.9031	4	0

	Rel. pot.	95 pc limits		ED_{50}	Log_R	$WLog_R$	$LOED_{50}$	$WLED_{50}$
7S				1.96			0.2915	7.11
7R	0.508	0.151	1.733	3.86	-0.2945	3.56	0.5860	7.05

Experiment 2

	Dose	Log dose	Tested	Responding
7S	8.00	0.9031	5	4
	4.00	0.6021	5	1
	2.00	0.3010	5	0
	1.00	0.00	5	0
7R	2.00	0.3010	5	3
	1.00	0.00	5	0
	0.500	-0.3010	5	0
	0.250	-0.6021	5	0

	Rel. pot.	95 pc limits		ED_{50}	Log_R	$WLog_R$	$LOED_{50}$	$WLED_{50}$
7S				5.66			0.7531	0.07
7R	2.977	1.492	5.154	1.90	0.4738	13.81	0.2792	24.34

Experiment 1

	Dose	Log dose	Tested	Responding
7S	8.00	0.9031	5	5
	2.00	0.3010	5	0
	0.500	-0.3010	5	0
	0.125	-0.9031	5	0
7R	8.00	0.9031	5	5
	2.00	0.3010	5	0
	0.500	-0.3010	5	0
	0.125	-0.9031	5	0

	Rel. pot.	95 pc limits		ED_{50}	Log_R	$WLog_R$	$LOED_{50}$	$WLED_{50}$
7S				3.92			0.5931	2.89
7R	1.00	0.156	6.415	3.92	0.00	1.53	0.5931	2.89

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

Expt.	Strain	Log_R	$WLog_R$
4	7R	0.3381	1.06
3	7R	-0.2945	3.56
2	7R	0.4738	13.81
1	7R	0.00	1.53

The combined estimate of Relative potency ($\bar{R}$) of 44122/7R vaccine is given by :

$$\overline{Log_R} = \frac{\Sigma(Log_R . WLog_R)}{\Sigma WLog_R}$$

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

$$= \frac{0 + 6.5432 + -1.0484 + 0.3584}{19.96}$$

$$\overline{Log_R} = 0.2932$$

$$\bar{R} = 1.96$$

Limits $= $ Antilog $\left[\overline{Log_R} \pm \sqrt[1]{\Sigma WLog_R}\right]$

$= $ Antilog $\left[0.2932 \pm \sqrt[1]{19.96}\right]$

$= $ Antilog $\left[0.2932 \pm 1/4.4677\right]$

$= $ Antilog $\left[0.2932 \pm 0.2238\right]$

$= $ Antilog 0.517 and 0.0694

$= 3.288$ and 1.17

∴ Combined estimate of R (and 95% confidence limits for 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.

Expt.	Strain	Rel.pot.	Log_R	WLog_R
2	7S	1.239	0.0931	11.34
1	7S	4.443	0.6477	14.51

$$\overline{\text{Log}_R} = \frac{\Sigma\ (\text{Log}_R \cdot \text{WLog}_R)}{\Sigma\ \text{WLog}_R} = \frac{(11.34 \times 0.0931)\ +\ (14.51 \times 0.6477)}{11.34\ +\ 14.51}$$

$$= \frac{1.055\ +\ 9.398}{25.85} = 4.044$$

$\bar{R}\ =\ 2.537$

$$\text{Confidence limits} = \text{Antilog} \left[\overline{\text{Log}_R} \overset{+}{-} \sqrt[1]{\Sigma\text{WLog}_R}\right]$$

$$= \text{Antilog} \left[0.4044 \overset{+}{-} \sqrt[1]{25.85}\right]$$

$$= \text{Antilog} \left[0.4044 \overset{+}{-} 0.1967\right]$$

$$= \text{Antilog}\ 0.2077\ \text{and}\ 0.6011$$

$$1.613 \qquad 3.991$$

∴ $\bar{R}$ (combined estimate of relative potency (and 95% confidence

limit) of 7S with respect to HLT)

= 2.537 (1.613, 3.991).

APPENDIX 5

Mouse Protection test data

Sample	Dose per mouse (ou ml)	Log dose	Tested	Responding
44122/7S	5.00	0.699	10	7
	1.00	0.00	10	2
	0.20	-0.699	10	1
44122/7R	5.00	0.699	10	7
	1.00	0.00	10	3
	0.20	-0.699	10	1

No. of cycles = 3

	Doses	XMEAN	YMEAN	CSX2	CSXY	CSY2	FDEV	Slope
44122/7S	3	0.1375	4.631	4.2987	5.8543	8.6043	0.63	1.36
44122/7R	3	0.0789	4.791	4.6730	4.6085	5.0745	0.53	0.99

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

	Rel.pot.	95 pc limits		ED_{50}	Log_R	$WLog_R$	$LOED_{50}$	$WLED_{50}$
44122/7S	1.00			2.85			0.4542	2.94
44122/7R	1.570	0.332	9.917	1.81	0.1958	1.84	0.2583	3.57

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ABSTRACT