THE HEAT-LABILE TOXIN OF *BORDETELLA PERTUSSIS*

by

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I dedicate this work to my wife, Ann.
Throughout the course of my studies
she provided me with every support and
encouragement.

SUMMARY

This work was aimed at advancing knowledge of the heat-labile toxin (HLT) of Bordetella pertussis along several lines, ultimately directed at gaining a better understanding of the possible roles of the toxin in the pathogenesis of B. pertussis infections and in the development of immunity. The following observations were made:

1 Assay and stability of the toxin

1.1 When assaying the lethal toxicity of HLT in the mouse, neither the sex nor the weight of the animal had a significant influence on the result.

1.2 The assay of HLT by its haemorrhagic activity in 4 d old mice was at least 10 times as sensitive as the LD_{50} estimation in 3-4 wk old mice.

1.3 Contrary to the report of Violle (1950, cited by Muñoz, J.J., 1971. In : Kadis, S., Montie, T.C. and Ajl, S.J. (editors). Microbial toxins. Vol.2A. p.271-300. Academic Press. New York and London) adult mice (strain Hr/Hr) were found to be susceptible to the dermonecrotic activity of HLT.

1.4 HLT was not haemolytic for sheep, rabbit, horse, human or cod red cells, even in doses as large as 2.5 mouse LD_{50} per ml. There was no hot-cold lysis.

1.5 Circumstantial evidence is presented that HLT may exist in an inactive form which can be converted by proteases into an active form.

1.6 For storing HLT, freezing (-20°C) was found to be satisfactory. The mean rate of inactivation of 4 preparations tested over periods up to 3 yr was 10% per annum.

2 Production of HLT

2.1 HLT production was suppressed during growth of cultures in a high magnesium medium that yielded antigenically modulated (C-mode) cells.

These cells contained only 4-5% of the toxicity of X-mode cells, ie, cells that had been grown in normal medium. The lowered toxicity of the C-mode cells was not due to excretion of HLT into the culture medium. In X-mode cells, it was confirmed that HLT was mainly cytoplasmic but with some evidence of exposure on the bacterial surface. By affecting HLT, antigenic modulation causes changes in at least one component of the organism which, unlike the other reported changes associated with the X- to C-mode transition, is not principally a component or feature of the cell-surface or envelope. However, this may not be unique since C-mode colonies lack the zone of haemolysis shown by X-mode colonies (Lacey, B.W., 1960, J.Hyg.(Camb.). 58, 57-93), which is presumably due to a diffusible haemolysin.

2.2 Contrary to published results (Pusztai, Z. and Joó, I., 1967, Ann.Immunol.Hung. 10, 63-67) there was a significant reduction in the toxicity of cells modulated by growth in media containing high levels of nicotinic acid, although the reduction was less than that observed by growth in a high magnesium medium (60% reduction cf 95%).

2.3 Fourteen B. pertussis strains, some of which had been pre-selected as potentially highly toxigenic, were compared for toxicity. There was only about a 3-fold difference between the least and most toxic of these strains. The Pillemer strain 134 was chosen for routine production of cells rich in HLT.

2.4 Bordet-Gengou medium, charcoal agar, modified Hornibrook medium, and Stainer and Schölte (12G) medium differed little in their ability to support toxin production by B. pertussis strains 134, 18334 and 77/18319. The time of harvesting the cells was more important. On the solid media, cells were more toxic after 24 h growth than 72 h, whilst the converse was true with cells grown in the liquid media.

2.5 Although the level of iron in the medium is critical for the production of several bacterial toxins, HLT yields were independent of the iron content of the medium over a range of concentrations which with other bacterial species causes marked effects on toxin production.

3 Purification of HLT

3.1 Little success was achieved in attempting to purify HLT by the method used to obtain the purest HLT preparation to date (Nakase, Y., Takatsu, K., Tateishi, M., Sekiya, K. and Kasuga, T., 1969, Jpn.J. Microbiol. 13, 359-366). However it was demonstrated that the HLT in lysates of B. pertussis could potentially be purified 20-fold by the following steps: preparative ultracentrifugation, fractional ammonium sulphate precipitation and gel filtration on Ultrogel AcA 44. These procedures in combination had the potential to give an HLT preparation containing 1000 LD_{50} per mg protein which is roughly one-quarter as pure as the HLT which was reported by Nakase et al (1969) to be completely homogeneous.

3.2 For the first time a molecular weight value is reported for HLT, namely 89,000 daltons ($\pm$ 10%) as estimated by gel filtration on Ultrogel AcA 44.

4 Toxoiding HLT

4.1 HLT in lysate supernatant fluids was toxoided by 15 d incubation at $4^{o}C$ with 0.1% (w/v) formaldehyde. Sterile, control toxin without formaldehyde kept 15 d at $4^{o}C$ did not lose any toxicity (a necessary control not reported by previous investigators).

4.2 Toxoiding with formaldehyde had the unexpected effect of appearing to increase the histamine-sensitizing (HSF) and lymphocytosis-promoting (LPF) activities of B. pertussis lysate supernatant fluids

by about 2-fold. However to make this comparison required the supernatant fluid with no added formaldehyde to be heated 30 min at 56°C before injection into mice. It was suggested that formaldehyde treatment made the HSF/LPF more resistant to degradation by tissue enzymes in the mouse.

5 Role of HLT in immunity

5.1 Toxoided HLT was immunogenic in rabbits, giving rise to antitoxin that neutralized the haemorrhagic activity of HLT in suckling mice. The same toxoid was not immunogenic in mice although the animals responded by producing precipitins to other antigens in the crude preparation. This confirms the previously reported lack of immunogenicity of HLT toxoid in mice and the responsiveness of rabbits.

5.2 Mice passively immunized with crude, rabbit antitoxin were protected against intracerebral challenges with B. pertussis but only if the antitoxin was given before, and not with, the challenge dose. Against intranasal challenges, antitoxin was also protective if given before the challenge but was even more effective if given with the challenge organisms (ie, IN/mix test). However, once the crude antitoxin was absorbed with live B. pertussis cells it lost its protective activity as measured in the IN/mix test. It seems unlikely that anti-HLT was acting, in this or the other infection tests, as a protective antibody. Nevertheless, it is still possible that HLT is involved in the pathogenesis of B. pertussis infections and that antibodies directed against HLT could be protective. For instance, the possibility should not be discounted that the toxin may have a ciliostatic, or local necrotic, or permeability-altering activity, and that local secretory antibodies (IgA or IgE) may be significant in immunity to B. pertussis infections of the respiratory tract, both in mouse and in man.

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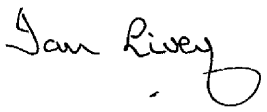
DECLARATION

This thesis is the original work of the author



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ABBREVIATIONS LIST

cfu	Colony forming units
CM-cellulose	Carboxymethyl-cellulose (cation-exchange resin)
DEAE-cellulose	Diethylaminoethyl-cellulose (anion-exchange resin)
EAE	(hyperacute) Experimental allergic encephalomyelitis
ED_{50}	Effective dose, fifty. The dose producing a specified effect in 50% of the test subjects
FCA	Freund's complete adjuvant
F-HA	Fimbrial haemagglutinin
FIA	Freund's incomplete adjuvant
HA	Haemagglutinin
HLT	Heat-labile toxin
HSD_{50}	Histamine-sensitizing dose, fifty. The dose of *B. pertussis* vaccine sensitizing 50% of mice to the lethal effects of 3 mg of histamine dihydrochloride
HSF	Histamine-sensitizing factor
IAP	Islet-activating protein
IC	Intracerebral
IC/mix test	Passive mouse protection test in which challenge *B. pertussis* organisms are mixed with serum and given intracerebrally
IM	Intramuscular
IN	Intranasal
IN/mix test	Passive mouse protection test in which challenge *B. pertussis* organisms are mixed with serum and given intranasally
IP	Intraperitoneal
IP/IC test	Passive mouse protection test in which serum is given intraperitoneally before an intracerebral challenge with *B. pertussis* organisms

IP/IN test	Passive mouse protection test in which serum is given intraperitoneally before B. pertussis organisms are given intranasally
IV	Intravenous
JCVI	Joint Committee on Vaccination and Immunization
LD_{50}	Lethal dose, fifty. The dose (eg, of heat-labile toxin) lethal to 50% of the animals to which it is administered
LPF	Lymphocytosis-promoting factor
LPF-HA	Lymphocytosis-promoting factor haemagglutinin
LPS	Lipopolysaccharide
MLD	Minimum lethal dose
MRC	Medical Research Council
MRD	Minimum reactive dose (eg, of heat-labile toxin injected subcutaneously into suckling mice)
N	Nitrogen
ou	Opacity unit. A measurement of bacterial concentration based upon the opacity of a cell suspension relative to the 5th International Opacity Reference Preparation (designated as having an opacity of 10 international opacity units)
oue	Opacity unit equivalents. A measurement of the concentration of bacterial cell-lysates based upon the opacity of the cell suspension from which the lysate was derived
PA	(mouse) Protective antigen, as measured in the intracerebral mouse protection test
PBS	Phosphate buffered saline
PHLS	Public Health Laboratory Service
ppt	Precipitate

pI	Isoelectric point
S	Svedberg unit
SC	Subcütaneous
SDS-PAGE	Sodium dodecyl sulphate, polyacrylamide gel electrophoresis
Tris	tris-(hydroxymethyl)aminomethane
Ve	Elution volume
Vo	Void volume
WHO	World Health Organization

INTRODUCTION

SECTION I BORDETELLA PERTUSSIS

1 Taxonomy of the genus Bordetella

The genus Bordetella, proposed by López (1952) and accepted in the 7th and 8th editions of Bergey's Manual of Determinative Bacteriology (Pittman, 1957 and 1974), consists of B. pertussis, B. parapertussis and B. bronchiseptica. The salient features of the members of this genus, of uncertain family affiliation, have been described by Pittman (1974). All 3 species are minute Gram-negative coccobacilli (0.2-0.3 μm by 0.5-1.0 μm) found arranged singly, in pairs or more rarely short chains. Colonies as formed on Bordet-Gengou medium are "smooth, convex, pearly, glistening, nearly transparent, surrounded by a zone of haemolysis without a definite periphery". The organisms are strict aerobes with a temperature optimum of 35-37°C. Carbohydrates are not utilized but certain amino acids are oxidatively metabolized. Nicotinic acid (or nicotinamide) and a sulphur source (eg, cysteine or methionine) are necessary growth factors. All species possess a common, heat-stable O antigen, heat-labile agglutinogen (no.7) and heat-labile, dermonecrotic toxin (HLT).

Features for differentiating the species within the genus are outlined in table 1. Recently however the validity of considering the 3 members of the genus Bordetella as distinct species has been questioned, in particular the distinction made between B. pertussis and B. parapertussis (Kumazawa and Yoshikawa, 1978; Kloos et al, 1979). By treatment with N-methyl-N'-nitro-N-nitrosoguanidine B. pertussis strains have been converted into mutants resembling B. parapertussis.

2 Pertussis - the disease

The terms pertussis and parapertussis have been proposed to

Table 1 Differential characteristics of *Bordetella* species

Differential characteristics	*B.pertussis*	*B.parapertussis*	*B.bronchi-septica*
Appearance of colonies on Bordet-Gengou agar (days)	≈4	2-3	1-2
Growth on MacConkey agar	-	+	+
Growth/browning on peptone agar	-/-	+/+	+/-
Urease activity	-	+	+
Citrate utilization	-	+	+
Oxidase activity	+,-	-	+
Nitrate reduction	-	-	+
Flagella	-	-	+
Specific heat-labile agglutinogen	1	14	12
Ability to sensitize mice to histamine	+	-	-
G + C content, moles %	61	61	66

Adapted from Pittman (1974) and Pittman and Wardlaw (personal communication).

describe the infections produced by B. pertussis and B. parapertussis (Lautrop, 1960). These infections are usually recognized when they give rise to the clinical syndrome of whooping cough with parapertussis typically being a milder and less frequently diagnosed form of the disease than pertussis (Lautrop, 1960; Linnemann and Perry, 1977). B. bronchiseptica may also on rare occasions give rise to whooping cough although it is primarily an animal parasite and pathogen (Lautrop, 1960). Adenoviruses have also been implicated as a causative agent of whooping cough (Olson, 1975; Linnemann, 1979).

Pertussis, as described by Pittman (1970) and Olson (1975), is an acute respiratory tract infection most common in infants and young children. It is sometimes accompanied by pulmonary and/or neurological complications and may be fatal. In its typical form it is characterized by episodes of paroxysmal coughing and a marked lymphocytosis. After an average incubation period of 7 d (limits 6-20 d) the disease enters the catarrhal stage. There is rhinorrhea, some conjunctival injection and tearing, occasional sneezing and a mild cough, all of which may be accompanied by a low-grade fever. After 1-2 wk of the catarrhal stage the characteristic symptom of paroxysmal coughing may appear. Initially the episodes of paroxysmal coughing increase in severity with increasing numbers of coughing bouts and coughs per bout. These episodes may end in an insipatory "whoop" and vomiting may occur. The symptoms gradually subside during the convalescent stage of the disease but it may be months before the bouts of coughing disappear.

A characteristic feature of the disease is the confinement of bacterial multiplication to the ciliated epithelium of the respiratory tract without invasion of the underlying tissues (Mallory and Hornor, 1912). Furthermore it is only possible to isolate the organism during the

catarrhal and early paroxysmal stages of the disease even although the symptoms persist much longer (Pittman, 1970).

Antibiotic therapy is generally of little value in the treatment of pertussis although it may modify the course of the disease if given early enough. The main benefits seem to be in cutting the risk to those in contact with the patient by shortening the infectious period and in reducing complications from secondary infections (Pittman, 1970; Olson, 1975). Erythromycin is the antibiotic of choice (Olson, 1975; Altemeier and Ayoub, 1977). Antipertussis serum has been used both prophylactically and therapeutically but its effectiveness is doubtful (Pittman, 1970 cf. Olson, 1975). In the absence of completely effective treatment for pertussis, preventative measures are of paramount importance, viz. vaccination and improvements in socio-economic conditions.

Man is the only known reservoir for B. pertussis. The organisms are spread by symptomatic patients, there being no proven chronic carrier state, although subclinical and clinically unrecognized infections do occur (Linnemann, 1979). Transmission is primarily by infectious droplets expelled during coughing (Linnemann, 1979) and communicability is very high for home exposures, having been measured at 87% in unvaccinated children (MRC Report, 1951). Although adults may contract pertussis (Linnemann and Nasenbeny, 1977) the incidence and severity of pertussis is greatest in infants and young children. In the USA in the 8 yr period between 1960 and 1967, 72% of reported pertussis deaths were in children in their first year of life (Brooks and Buchanan, 1970). Unlike other infectious childhood diseases there is an apparent lack of transfer of maternal protective substances to the newborn baby (JCVI Report, 1977). Also notable is the slightly higher incidence, 55% of cases, among females (Olson, 1975).

Pertussis has a worldwide distribution but is particularly serious in the developing world (World Health Statistics Report, 1977). Although there is no seasonal influence on its incidence (Olson, 1975), pertussis does follow a cyclic pattern peaking every 3-4 yr (Brooks and Buchanan, 1970; figure 1).

3 Intracerebral and respiratory tract infections of mice

Although not a natural pathogen for mice B. pertussis may grow in the lungs and brain after inoculation. At first these might appear to be very different environments but both provide ciliated cells among which the bacteria multiply.

3.1 The intracerebral infection

High intracerebral mouse-virulence is not a property of freshly isolated B. pertussis. Standfast (1958) obtained LD_{50} values ranging from 10^4-10^8 organisms for 14 freshly isolated strains. In contrast, a few strains have been selected which are extremely virulent intracerebrally (Pittman, 1970) and with which very small infecting doses, perhaps even a single cell, are sufficient to initiate a fatal infection (Standfast and Dolby, 1961). The ability of vaccines to protect mice injected intracerebrally with such organisms (eg, strain 18323) is widely used to assay the protective potency of pertussis vaccines for human immunization.

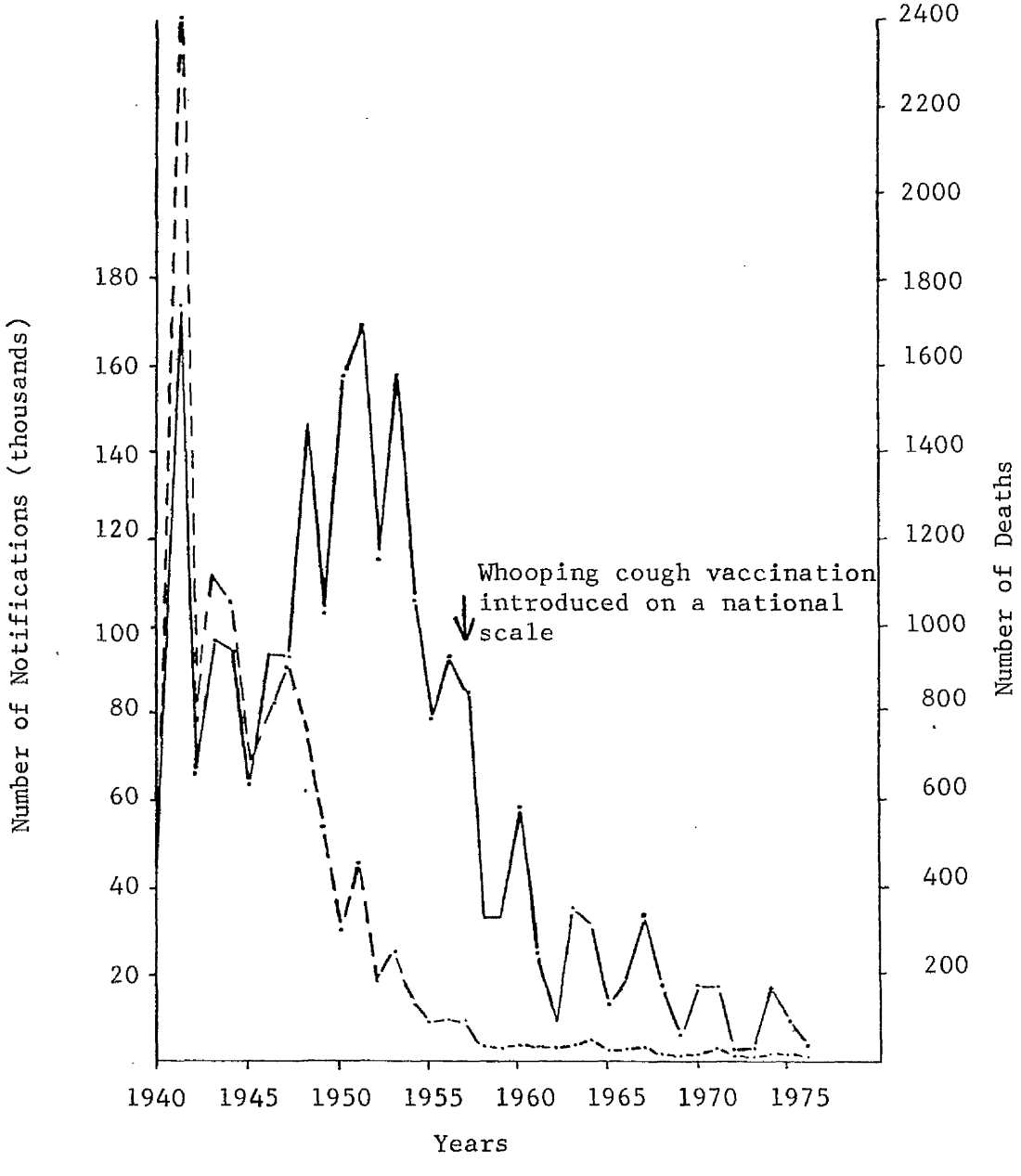
On injecting mice intracerebrally the deposited dose is only about 10% of that injected (Dolby and Standfast, 1961), the greater part being lost into the circulation (Cairns, 1950) through a temporary breakdown in the blood-brain barrier (Dolby and Standfast, 1961). The remaining bacteria multiply among the cells of the ciliated layer on the ependymal lining of the ventricle and apparently nowhere else (Berenbaum, Ungar and Stevens, 1960; Iida et al, 1962). When the

Figure 1 Whooping cough notifications and deaths, England and Wales, 1940-1976.

Data from the Report of the Joint Committee on Vaccination and Immunization (1977).

——— Notifications

------- Deaths


Number of Notifications (thousands)
180
160
140
120
100
80
60
40
20
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
Number of Deaths
Whooping cough vaccination introduced on a national scale
1940
1945
1950
1955
1960
1965
1970
1975
Years

infecting bacteria reach 10^5 or 10^6 cfu the blood-brain barrier breaks down, becoming permeable to human serum albumin, horse gamma globulin and the dye Pontamine sky blue (Berenbaum, Ungar and Stevens, 1960; Holt et al, 1961; Iida et al, 1966). The breakdown in the blood-brain barrier is closely correlated in passively and actively protected mice with a dramatic fall in the numbers of viable B. pertussis (Dolby and Standfast, 1961).

The pattern of growth for the highly intracerebrally virulent strain 18323 has been characterized by Dolby and Standfast (1961). The rate of growth, which is independent of the size of the infecting dose, steadily decreases as the infection progresses, but nevertheless a critical level is attained and the mouse dies. The lethal level falls steadily from 10^8 cells for early (day 5) deaths to 10^7 for late (day 14) deaths. Doses much above the critical level are toxic and deaths occur within hours.

3.2 The respiratory tract infection

Respiratory tract infections can be initiated in mice by instilling B. pertussis intranasally as described by Burnet and Timmins (1937), intratracheally (Bradford, 1938; Te Punga and Preston, 1958) or, as reported recently, by aerosol inhalation (Sato et al, 1980). Intranasal instillation has been the most widely used of these techniques but the other methods are considered to give more uniform and reproducible results (Te Punga and Preston, 1958; Sato et al, 1980).

The number of organisms needed to initiate a pulmonary infection varies with the strain but 100 or fewer viable organisms may be all that is required (North and Anderson, 1941; Andersen and Bentzon, 1958; Dolby, Thow and Standfast, 1961). Pulmonary infections do not spread between mice within a cage (Dolby, Thow and Standfast, 1961). The growth

rate in the first few days of the infection is dependent upon the size of the inoculum. In a study in which doses of 10^2-10^9 viable cells per mouse were given, it was noted that the larger the inoculum the slower the growth rate, until at doses of ca. 10^8 cells there was no net growth. With larger doses there was a decline in the numbers of viable cells (Dolby, Thow and Standfast, 1961). These findings are in general agreement with observations made earlier by Fisher (1955). With sub-lethal doses the bacteria typically achieve a maximum count between 7-14 d, depending on the strain (Proom, 1947; Cooper, 1952; Andersen and Bentzon, 1958; Fisher, 1955; Dolby, Thow and Standfast, 1961; Sato et al, 1980). Thereafter their numbers gradually decline. Their complete clearance from the lungs takes about 1-2 mth from the time of inoculation (Proom, 1947; Pittman, 1951; Andersen, 1953a; Geller and Pittman, 1973; Pittman, Furman and Wardlaw, 1980) although they have been detected in small numbers as late as 3 mth (Dolby, Thow and Standfast, 1961). The latter workers cultured B. pertussis from homogenized lung preparations and not from the cut surface of one area of the lung, thereby presumably increasing their chances of recovering the organism. When the infecting dose is large (eg, $\geq 10^6$ cfu for strain C2621; Dolby, Thow and Standfast, 1961) the organisms multiply only until they reach a critical level of ca. 10^8 cells, which is more or less independent of the initial dose. When this occurs the mouse dies. In mice dying early in the course of the infection (eg, day 4) the lethal level tends to be slightly lower (eg, not more than 1 log unit) than for mice which die later (eg, day 10; Dolby, Thow and Standfast, 1961). The margin between recovery and death is a narrow one with often $\leq$1 log unit between the highest sub-lethal dose and the MLD (Dolby, Thow and Standfast, 1961). In lethal infections mice seldom die before day 5 unless the dose is so

large that it in itself is sufficiently toxic to kill or contribute to the killing of the mouse within 1-2 d of the challenge (Standfast and Dolby, 1961).

Various pathophysiological reactions have been observed in mice with B. pertussis respiratory tract infections. Sato et al (1980) reported on the changes following the infection of mice by aerosol inhalation of B. pertussis at a dose producing 10-20% mortality within 14 d. In infected as compared to non-infected mice, the rate of body weight gain was significantly reduced, and there were significant increases in lung and spleen weights and leukocyte numbers which corresponded to increases in the number of bacteria in the lungs. Spleen weights were greatest slightly before the peak of the infection at day 14 but thereafter decreased in size, although by the 21st day they were still heavier than those of control mice. Anderson and North (1943) had observed atrophy of the spleen in mice sacrificed 26 d after a comparable challenge in which 61% of the mice died. Pittman, Furman and Wardlaw (1980) classified their observations into those pertaining to lethal and sub-lethal infections. Lethally infected mice showed a loss of body weight, spleen atrophy, a pronounced hypothermia and hypoglycaemia, and highly elevated levels of leukocytes and serum immunoreactive insulin. Sub-lethally infected mice showed normal weight gain, practically normal temperature, spleen enlargement, lesser pronounced hypoglycaemia, lower but significantly elevated levels of leukocytes and serum immunoreactive insulin, and histamine-sensitization. The intensity of each reaction was related to the degree of lung involvement. Hypothermia and leukocytosis were highly correlated and the concentration of serum immunoreactive insulin was closely related to the level of leukocytosis but not to the level of glucose.

The B. pertussis infection in man is in many ways similar to the pulmonary infection in the mouse. Both are typified by a localized, non-invasive colonization of the ciliated epithelium of the respiratory tract (Mallory and Hornor, 1912 cf. Sato et al, 1980), a marked leukocytosis (Bradford, 1938; Cooper, 1952; Pittman, Furman and Wardlaw, 1980; Sato et al, 1980), hypoglycaemia (Regan and Tolstoouhov, 1936 cited by Pittman, Furman and Wardlaw, 1980 cf. Pittman, Furman and Wardlaw, 1980) and in that the young are the most susceptible (Pittman, Furman and Wardlaw, 1980; Sato et al, 1980). Moreover the onset and duration of the paroxysmal cough in the human infection, although it has no direct equivalent in the mouse, parallels the development of histamine-sensitization in the infected mouse (Pittman, 1951; Geller and Pittman, 1973; Pittman, Furman and Wardlaw, 1980). Both persist after the bacteria can no longer be cultured from the lungs. The factor responsible for histamine-sensitization has been implicated as being responsible for the harmful effects of pertussis (Pittman, 1979). Finally the pathophysiological changes observed in fatal human infections (Mallory and Hornor, 1912) are not unlike those seen in lethal respiratory infections of mice (Burnet and Timmins, 1937). The B. pertussis respiratory tract infection of the mouse is considered an appropriate animal model for studying the host-parasite interaction and in evaluating prospective protective antigens and antibodies (Pittman, Furman and Wardlaw, 1980; Sato et al, 1980). However, B. pertussis respiratory tract infections in rabbits (Preston, Timewell and Carter, 1980) and marmosets (Stanbridge and Preston, 1974) can also be used.

4 B. pertussis vaccines and vaccination

The use of B. pertussis vaccine, usually combined with diphtheria and tetanus toxoids as a triple vaccine, has been practised on a national

scale in the UK from 1957(JCVI Report, 1977). Vaccination against pertussis was introduced in response to an extensive series of carefully controlled field trials from 1946 onwards in which different sorts of B. pertussis vaccines were assessed. It was shown that vaccines could be made which gave a high degree of protection (90%) even in the face of intense infection as encountered during home exposure (MRC Report, 1951, 1956 and 1959). The intracerebral mouse protection test was also introduced as a way of measuring the protective potency of B. pertussis vaccines. There was a high degree of correlation between the potency of vaccines as measured in the field and by their performance in this test (MRC Report , 1956 and 1959).

After the introduction of widespread vaccination the incidence of pertussis declined (figure 1). However the wave of pertussis during 1963 and 1964 raised sufficient doubts concerning the efficacy of the vaccines in use for this to be re-assessed.(PHLS Report, 1969). The protective potency of the vaccines examined in this survey has been estimated as less than 20% (JCVI Report, 1977). Preston (1963 and 1965) had shown that the dominant serotype of fresh isolates of B. pertussis had changed by 1963 from the type 1,2 and less frequent type 1,2,3 strains prevalent prior to 1958 to type 1,3 strains. These findings were confirmed by the PHLS (1973) and it was concluded that the change in the serotypic composition of the prevalent bacteria might have accounted for the reduction in the efficacy of vaccines in use from 1963-1968. Indeed when vaccines including the type 3 antigen were compared to earlier vaccines deficient in this component they were found to give a better degree of protection (Preston and Stanbridge, 1972). It was also shown that the British reference vaccine contained 2.1 international units per single human dose and therefore many vaccines

in use would have failed the international potency requirement for at least 4 IU per dose (Perkins, 1969). Although not all vaccines distributed in this period exhibited these qualitative and quantitative defects (Griffith, 1978), from 1966 all vaccines marketed in Britain have contained antigen 3 and had a minimum potency of 4 IU (Perkins, 1969). Alum adjuvant was also added once more to some vaccines.

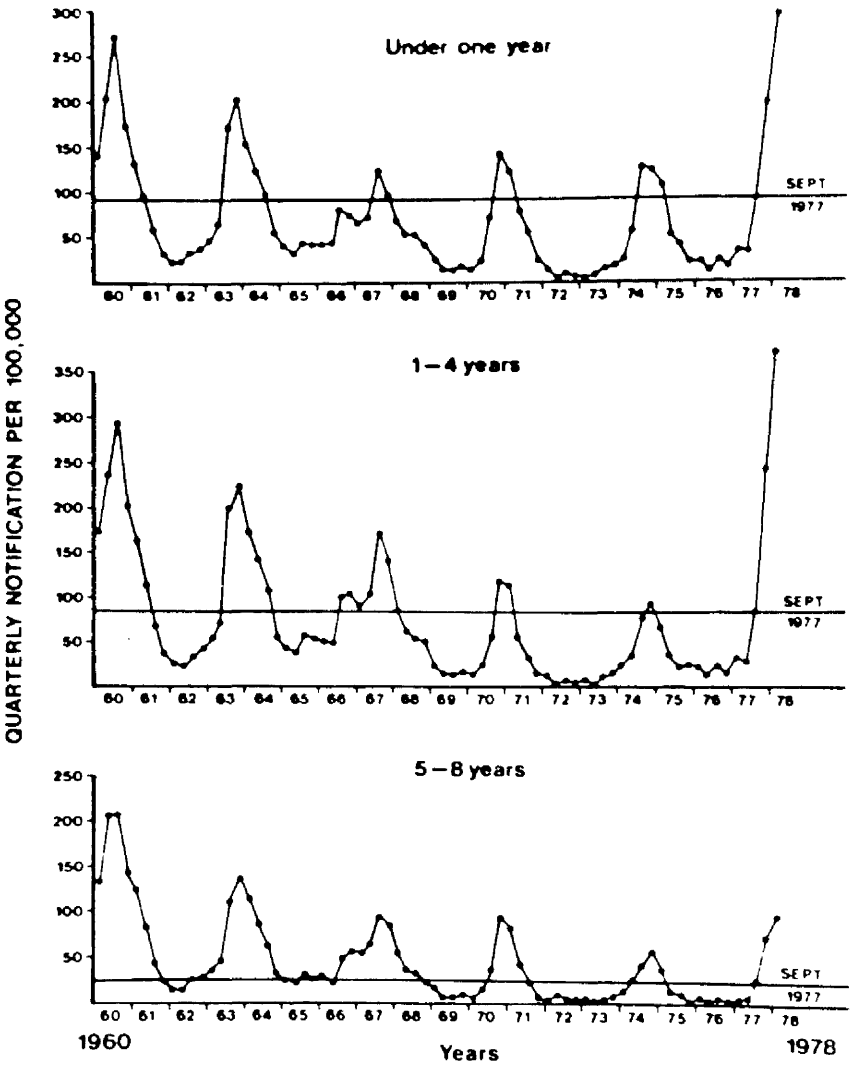
By the mid 1970's there was renewed and widely publicized concern in the UK over vaccination against pertussis. In addition to the mild reactions which are associated with many vaccines, vaccination against pertussis may occasionally cause serious neurological complications. Stewart (1977) considered the risks of serious complications too high to justify continued vaccination, especially as he considered changing socio-economic conditions to be more important than vaccination in reducing the level of pertussis (Bassili and Stewart, 1976; Stewart, 1977). When the evidence available was reviewed by the JCVI (1977) a recommendation was made for the continued use of B. pertussis vaccines on the grounds that the benefits to be accrued outweighed the risks. Although difficult to assess, the frequency of "brain damage" was estimated from data collected by the Committee on the Safety of Medicines at 1 case per 300,000 children inoculated (JCVI Report, 1977). However the National Childhood Encephalopathy Study was instigated to collect more data on this point. In the meantime it was recommended that vaccination of infants should begin at 3 and not 6 mth of age and that it was not to be used if there were medical contraindications or parental objections (JCVI Report, 1977). As a consequence of the allegations that the vaccine was unsafe there was a reduction in vaccine acceptance rates from 70-80% before 1974 to less than 40% by 1976 although it varied in different areas from 11-60% (Stuart-Harris, 1979). The very

large epidemic of 1978 (figure 2), the highest in England and Wales since before 1960, upholds the view that the vaccine in use was fully protective (Stuart-Harris, 1979). However this has been disputed (Stewart, 1979).

Benefit-risk analyses of B. pertussis vaccination in the USA have also led to the conclusion that the benefits outweigh the risks (Koplan et al, 1979; Mortimer and Jones, 1979). Nevertheless the situation is far from being satisfactory and the need for a less reactogenic vaccine is recognized. Towards this end it is important that there is a better understanding of the immunity and pathogenesis of pertussis. For such investigations respiratory tract infections of mice (Pittman, Furman and Wardlaw, 1980; Sato et al, 1980), rabbits (Preston, Timewell and Carter, 1980) and marmosets (Stanbridge and Preston, 1974) have been advocated as alternatives to the intracerebral infection in mice. Although the potency of vaccines are measured by their ability to protect mice against an intracerebral infection the test may be inappropriate (Preston and Stanbridge, 1976). It is certainly a troublesome assay to perform and subject to much variation and therefore an alternative test would be desirable for these reasons alone (Cameron, 1976). It has been proposed that vaccines be evaluated by their agglutinogen content (Preston, 1975a and b). Another quality control problem is the testing of vaccines for their toxicity. How suitable is the mouse weight-gain test (Pittman and Cox, 1965) which has never been correlated with reactogenicity of vaccines in children (Perkins, 1979)? At present attempts are being made to develop less reactogenic sub-unit vaccines, eg, Nagel and S. de Graaf, 1979; Helting and Blackkolb, 1979; Irons and MacLennan, 1979a and b; Nakase and Doi, 1979. New potency and toxicity tests may be required for monitoring a sub-unit vaccine.

Figure 2 Pertussis quarterly notification rates, England and Wales, 1960-1978.

Data from Dr. P. Lambert, Office of Population Censuses and Surveys, London.


Under one year
1–4 years
5–8 years
SEPT 1977
QUARTERLY NOTIFICATION PER 100,000
60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78
1960
Years
1978

5 Pathophysiological activities and antigens

In experimental animals B. pertussis has been demonstrated to give rise to a variety of pathophysiological responses. The factors to which many of these effects can be attributed are described briefly in this section as are a few non-pathophysiologically active components. Detailed accounts of most of these substances and their properties are given by Pittman (1970 and 1979), Morse (1976 and 1977), Olson (1975), Muñoz (1971), and Muñoz and Bergman (1977).

5.1 Heat-labile, dermonecrotic toxin (HLT)

HLT is a heat-labile, cytoplasmic protein with lethal and dermonecrotic properties. Its role in B. pertussis infections remains unresolved and is discussed more fully later (Introduction; section II).

5.2 Endotoxin (lipopolysaccharide, LPS)

Endotoxin possessing a structure different from that elaborated by the enterobacteria has been extracted from B. pertussis (Le Dur et al, 1978). The LPS is made up of 2 polysaccharide (PS1 and PS2) and 2 lipid (lipid A and lipid X) moieties. Both PS1 and PS2 are linked to lipid A but the position of lipid X within the endotoxic complex is unknown (Le Dur et al, 1978; Chaby et al, 1979). All the usual biological activities associated with enterobacterial endotoxins are produced by B. pertussis LPS. It is toxic, possesses non-specific antibacterial and antiviral activity, gives a positive local Shwartzman reaction, has adjuvant activity and is pyrogenic but less so than enterobacterial endotoxins (Chaby et al, 1979). All these activities are carried by the lipid fractions but only lipid X is toxic, pyrogenic and elicits the Shwartzman reaction.

5.3 Agglutinogens

Andersen (1953b) showed that freshly isolated B. pertussis

exist in serologically different forms according to which heat-labile K agglutinogens they express. Eldering, Hornbeck and Baker (1957) proposed an antigenic scheme for the genus Bordetella based upon the 14 recognized types of heat-labile agglutinogens. Types 1-7 and 13 are found on B. pertussis with types 1-6 being species specific but only agglutinogen 1 occurs on all strains. There are grounds for believing that the agglutinogens are important in protecting children against pertussis (Preston, 1975a and b) but how important they are in relation to other factors is not clear.

5.4 Haemagglutinin (HA)

B. pertussis produces 2 distinct HA's (Arai and Sato, 1976). There is a fimbrial HA (F-HA) which is identical with the filamentous fimbriae of B. pertussis (Sato et al, 1979) and the expression of which is greatly favoured by growth in static, poorly aerated cultures (Arai and Muñoz, 1979b). Fimbrial fragments are asymmetrical. Only one end attaches to the red cell surface and haemagglutination results from the interaction of the free ends of fimbriae attached to adjacent surfaces (Sato, personal communication cited by Jones, 1977). Components with molecular weights of 130,000 (Arai and Sato, 1976), 127,000 and 95,000 daltons (Irons and MacLennan, 1979b) have been identified with the F-HA. There is evidence that the F-HA acts as a protective antigen in the intracerebral mouse protection test (Sato et al, 1979; Irons and MacLennan, 1979b) and against B. pertussis infections of the lung in suckling mice (Sato et al, 1979) and rabbits (Irons and MacLennan, 1979b). Sato et al (1979) found the adhesion of organisms to tissue culture cells was prevented by antipertussis-HA serum. The F-HA has recently been purified and crystallized (Arai and Muñoz, 1979a). The second HA, which may have lower HA activity (Arai and Sato, 1976), is

identical with the cell-envelope component which induces lymphocytosis (LPF) and is described more fully later in this section (Introduction; section I,5,6). It should be noted however, that Morse and Morse (1976) detected no HA activity in their LPF preparation.

Characteristics by which the 2 haemagglutinins can be differentiated are listed in table 2.

5.5 Islet-activating protein (IAP)

Rats injected with IAP exhibit an enhanced hyperinsulinemia in response to insulin secretagogues such as glucose (Yajima et al, 1978a and b). IAP interacts directly with the cell membrane of the islet B-cells of the pancreas and produces a sustained and progressive activation of calcium ionophores located in the membrane, making more calcium available in an intracellular pool. This triggers insulin secretion and also activates adenylate cyclase and phosphodiesterase (Ui, Katada and Yajima, 1979; Katada and Ui, 1979).

5.6 Lymphocytosis-promoting factor (LPF)

LPF is the term used to describe the B. pertussis cell-envelope component which induces lymphocytosis in mice and presumably in humans. The lymphocytosis is due to the failure of circulating lymphocytes to leave the circulation and return to lymphoid tissues rather than being attributable to a sudden increase in lymphocyte proliferation, although LPF is a potent T-cell mitogen in vitro (Morse, 1976 and 1977 ; Morse, Kong and Ho, 1979). It is not clear whether emigration of lymphocytes from the blood is inhibited by an effect of LPF on the lymphocytes or on the vessels, or whether the effect is direct or mediated by a host effector substance.

5.7 Histamine-sensitizing factor (HSF)

HSF is the term used to describe the B. pertussis cell-

Table 2 Differential characteristics of B. pertussis haemagglutinins

Differential characteristic	Haemagglutinin (HA)	
	F-HA	LPF-HA
Description of haemagglutination	agglutinated red cells[5] dispersed by gentle flicking	red cells firmly[5] bound into coherent sheets and do not disperse on flicking
Promotion of lymphocytosis	-[1]	+[1-5]
Sensitization to histamine	-[1]	+[1-5]
Inhibited by cholesterol	+[5]	-[5]
Inhibited by haptoglobin	-[4]	+[4,5]
Susceptibility to papain	sensitive[1]	resistant[1]
Molecular form	filaments 2 x 40 nm[1] 2-2.5 x 60-100 nm[3] 2 x 30-50 nm[5]	spherical 6 nm. diameter[1,4,5] 2.8 nm diameter[3]

1 Arai and Sato, 1976

2 Sato et al, 1979

3 Morse and Morse, 1976

4 Irons and MacLennan, 1979a

5 Irons and MacLennan, 1979b

envelope component which sensitizes mice to the lethal effects of histamine. The mechanism by which this effect is produced is poorly understood but epinephrine is involved (Muñoz and Bergman, 1979a).

5.8 Interrelationship of the pathophysiological activities

Considerable evidence has now accumulated to support the hypothesis that IAP, LPF and HSF are one and the same component (Morse, 1976; Muñoz and Bergman, 1977 and 1979b; Pittman, 1979). In addition to the activities after which these factors were named this component must be considered to have HA activity (Introduction; section I,5.4), T-cell mitogenicity (Morse, Kong and Ho, 1979), the capacity to accelerate hyperacute experimental allergic encephalomyelitis (EAE) and IgE adjuvanticity (Morse, 1976; Muñoz and Bergman, 1977 and 1979b). This component termed PERTUSSIGEN by Muñoz (1976) and PERTUSSIS TOXIN by Pittman (1979) is claimed to have protective activity in the intracerebral mouse protection test (Muñoz and Bergman, 1977 and 1979b). On the other hand the highly purified preparations of Morse and Morse (1976), Irons and MacLennan (1979b) and Sato et al (1979) have all lacked protective activity as demonstrated by this test (Pittman, 1979 cited Kong, personal communication, as the source of the information on the Morse LPF).

The physiochemical and biological properties of highly purified preparations of pertussis toxin are listed in table 3. The relationship between these preparations is clearly illustrated although discrepancies do exist. LPF, HSF and IAP activities appear to be located on the F3 subunit described by Ui, Katada and Yajima (1979), although this has to be associated with either an F1 or F2 sub-unit for expression.

Pertussis toxin, as defined by Pittman (1979), is distinct

Table 3 Physiochemical and biological properties of highly purified pertussis toxin (as defined by Pittman, 1979).

Reference	Composition (%)			Molecular form	Molecular weight	Polypeptide constituents			Activity expressed
	Protein	Carbohydrate	Lipid			Number	Molec. weight	Ratio	
Morse and Morse (1976)	100	<1	<0.5	4-5 round, 2.8 nm diameter sub-units forming 7.5-8.0 nm ring structures	73,600 or 87,000	4	23,500 19,300 17,400 13,400	1 1 1 2(?)	1,2,4,5 but no HA activity
Arai and Sato (1976)	47.7	25	23.9	6 nm diameter round sub-units. Tendency to aggregate	105,000	4	26,000 20,000 13,000 10,000	major major minor minor	1,2,3
Irons and MacLennan (1979a and b)	+	No data given		6 nm diameter round sub-units. Tendency to aggregate.	No data given	4	27,200 22,400 21,000 12,600	1 1 1 1	1,2,3,6
Yajima et al (1978a and b) Ui, Katada and Yajima (1979)	100	-	-		77,000	4	24,000 20,000 20,000 11,000	F1 1 F1 1 F2 1 F3 2	1,2,4,7

1 Lymphocytosis, 2 histamine-sensitization, 3 HA activity, 4 epinephrine refractory hypoglycaemia, 5 T-cell mitogen, 6 adjuvant, 7 potentiation of insulin secretion.

from HLT, endotoxin, the agglutinogens and the fimbrial haemagglutinin.

5.9 Involvement of B. pertussis components in pathogenesis/protection

The factors involved in the pathogenesis and the development of immunity to pertussis have not been clearly defined. Protection could be afforded if the infection could be prevented or controlled by curtailing the attachment and/or multiplication of the organisms. The fimbrial haemagglutinin and agglutinogens must be prime candidates for being involved in these processes. An additional or alternative form of protection might be gained by the development of immunity to pertussis toxin. If this component is as suggested responsible for the characteristic symptoms of pertussis, the paroxysmal cough and lymphocytosis, then by blocking its effect the clinical syndrome of pertussis could be prevented (Pittman, 1979). Less well characterized is the involvement of HLT, endotoxin, an extracytoplasmic adenylate cyclase (Hewlett et al, 1976; Hewlett and Wolff, 1976), polymorphonuclear leukocyte inhibitory factor (Utsumi et al, 1978; Imagawa et al, 1979) and the 28K and 30K polypeptides, the presence or absence of which is closely correlated to the PA, HSF, LPF, EAE adjuvant and IgE adjuvant activities (Wardlaw and Parton, 1979). The 28K and 30K polypeptides are distinct from the LPF-HA and F-HA (Irons and MacLennan, 1979b).

6 Antigenic variation

Two distinct types of variation are recognised in B. pertussis but both give rise to antigenic variants which are similar in that they are deficient in a variety of components normally associated with freshly isolated organisms:

i) Phenotypic variation in which the loss of cellular constituents is freely reversible and does not involve mutational changes. These

variants arise in response to certain modifications which can be made to the growth environment.

ii) Phase variation in which the losses are a consequence of mutation and selection during repeated subculture of B. pertussis.

6.1 Phenotypic variation

Two types of phenotypic variation have been described in B. pertussis:

i) Antigenic modulation (Lacey, 1951 and 1960)

ii) Nicotinic acid induced modulation (Pusztai and Joó, 1967).

6.1.1 Antigenic modulation

Lacey (1951 and 1960) found with every 1 of over 2,000 strains of B. pertussis that they underwent marked phenotypic changes when grown on a series of solid media in which the inorganic salts were varied. When NaCl was the principal salt the cultures had the characteristics of freshly isolated B. pertussis on Bordet-Gengou medium. However, when NaCl was replaced by $MgSO_4$ the colonies were no longer haemolytic, the ability to agglutinate red blood cells was lost, there were changes in agglutinogens, agglutinability by $AuCl_3$ and $HgCl_2$ was reduced, autolysis occurred more readily, mouse-virulence decreased and the colour of confluent growth changed from yellowish-ochre to pale greenish-blue. Lacey introduced the term "antigenic modulation" to describe the phenomenon and he designated the NaCl grown cells as X-mode (X = xanthic or ochre) and the $MgSO_4$ grown cells as C-mode (C = cyanic or bluish). In addition to changes in the ionic composition of the medium, C-mode growth can be induced by growth at low temperatures (eg, 25°C) or in the presence of tellurite, fatty acids or old blood. Lacey (1960) also described an intermediate form of cell (I-mode) which was induced within a narrow range of intermediate temperatures and ion ratios.

Lacey (1960) concluded from his extensive study with over 100 different salts that the influence of ions is a function of their species and ratios and not their absolute concentrations or total ionic strength. Furthermore the influence of ions and temperature were shown to be independent since their combined influence was proportional to the sum of their separate influences. The action of the salts was, according to Lacey (1960), dependent upon growth with 7-15 cell divisions being required for completion of the process. Suspension in a salt solution was not sufficient (Lacey 1951 and 1960). Recent studies by Idigbe (1979) indicated that modulation was much more rapid than suggested by Lacey, being complete within 1-2 cell divisions.

A variety of factors in addition to these identified by Lacey (1951 and 1960) are now known to be lost during antigenic modulation. These are the PA, HSF (Holt and Spasojević, 1968; Parton and Wardlaw, 1975; Wardlaw, Parton and Hooker, 1976), LPF (Idigbe, 1979), the adjuvant(s) for reaginic antibody and for hyperacute EAE (Wardlaw et al, 1979), adenylate cyclase (Parton and Durham, 1978) and cytochrome d-629 (Dobrogosz et al, 1979).

Analysis of the protein profiles of X- and C-mode cell-envelopes has revealed the loss of at least 2 polypeptides from the C-mode cells (Parton and Wardlaw, 1975; Wardlaw, Parton and Hooker, 1976). The loss of these 28K and 30K polypeptides has been confirmed by Idigbe (1979) and Dobrogosz et al (1979). However Idigbe (1979) and Dobrogosz et al (1979) recognized the loss of 3 (28K, 30K, 100K) and 4 (27.5K, 30K, 88K, 98K) bands from whole cells and outer membranes respectively. The 28K and 30K polypeptides are also apparent in B. parapertussis and B. bronchi-septica and their levels are reduced by growth in C-mode conditions (Dobrogosz et al, 1979).

It should be noted that all the components lost are either constituents of the cell-envelope or closely associated with it in the case of the extracytoplasmic adenylate cyclase. One exception may be the loss of a diffusible haemolysin as Lacey (1960) noted that C-mode colonies of B. pertussis were not haemolytic. In the face of all these losses altered antibiotic sensitivities have been recorded. Dobrogosz et al (1979) found C-mode growth to have enhanced sensitivity to penicillin and ampicillin but increased resistance to streptomycin, erythromycin and tetracycline.

6.1.2 Nicotinic acid induced modulation

Pusztai and Joó (1967) observed a phenomenon similar to, but in their view not identical with, antigenic modulation. Growth of B. pertussis in a medium with a high nicotinic acid content (eg, 500 μg/ml cf. 1 μg/ml in the normal medium) resulted in reduced levels of PA and HSF. Also lost, in 5 of the 7 strains examined, was the capacity to be agglutinated by a phase 1 specific immune serum. Immunoelectrophoretic analysis of extracts from disrupted variant cells revealed the loss of 4 precipitin bands. Unaltered by growth in a medium with increased levels of nicotinic acid was the germ-count, pH, oxidation-reduction potential, bacterial morphology and HLT content. Subsequent studies have indicated that the amount and antigenic specificity of LPS from variant cells is also unchanged (Ackers and Dolby, 1972).

During nicotinic acid induced modulation the 28K and 30K cell-envelope polypeptides (Wardlaw, Parton and Hooker, 1976) and adenylate cyclase are lost (McPheat, 1980). The variant cells are therefore like C-mode cells in this respect in addition to lacking the PA and HSF, the loss of which have been confirmed by Ackers and Dolby (1972) and Wardlaw, Parton and Hooker (1976). However this phenomenon is claimed to differ

from antigenic modulation in that the agglutinogen specificity does not alter, the agglutinogen content merely decreases (Pusztai and Joó, 1967). On the other hand McPheat (1980) has shown agglutinogen factors 2 and 3 are lost under these conditions whilst the content of factor 1 increases.

Nicotinic acid induced modulation is not reproduced when nicotinamide is used to substitute for the nicotinic acid (Wardlaw, Parton and Hooker, 1976). Nicotinic acid modulated cells have recently been shown to have a reduced uptake capacity for both nicotinic acid and nicotinamide. Uptake is not appreciably altered by growth in a medium rich in nicotinamide (McPheat, 1980).

6.2 Phase variation

Leslie and Gardner (1931) grouped *B. pertussis* on the basis of serological and cultural characteristics into 4 groups which they designated phases I, II, III and IV. By continued subculture the virulent phase I form could be transformed through the intermediate phase II/III forms to the degraded, avirulent phase IV form which had acquired the capacity to grow on nutrient agar. However Standfast (1951b) found that freshly isolated (phase I) *B. pertussis* were not the homogeneous group suggested by Leslie and Gardner (1931). Not all freshly isolated strains possessed the full complement of characteristics which are usually associated with them. Moreover when he examined the effect of repeated subculture on a number of properties (such as growth requirements, agglutination, virulence, PA and HA) the order in which they degenerated or were lost and the rate at which they were lost differed from strain to strain. Field and Parker (1979) recorded a similar random loss of properties leading to degraded forms of *B. pertussis*. It is not possible therefore to categorize *B. pertussis* into 4 distinct phases but the terms phase I and IV can be used to represent the two extreme forms.

In addition to deficiencies in agglutinogens a variety of other components are missing from phase IV variants. Table 4 lists factors typically present in phase I organisms but absent from the degraded phase IV organisms. Other changes that occur as a result of phase variation include increased resistance to certain antibiotics and fatty acids (Dobrogosz et al, 1979; Field and Parker, 1979).

Parker (1976 and 1979) has proposed a hypothesis to rationalize phase variation in B. pertussis. It is assumed that B. pertussis is in some way metabolically abnormal, a feature which in vivo is not detrimental and may even be helpful. However in vitro a manifestation of this peculiarity may be the sensitivity of freshly isolated B. pertussis to inhibitory substances in normal growth media. There would be strong selective pressures for mutations which reduced this sensitivity. Mutations to relieve the metabolic lesion(s) could occur in any of a small number of genes, in any order and be cumulative. Therefore a large number of phenotypic forms of intermediate type strains could be formed leading to the fully degraded form. The effect of multiple mutations would be to make the process irreversible.

Table 4 Comparison of properties of phase I and phase IV *B. pertussis*

Property	Phase I	Phase IV	Reference
Protective antigen (PA)	+	-	1,2,4
Histamine-sensitizing factor (HSF)	+	-	2,3,4
Lymphocytosis-promoting factor (LPF)	+	-	4
27.5K or 28K, 30K, 88K and 98K envelope polypeptides	+	-	5,6
Cytochrome d-629	+	-	6
Adenylate cyclase activity	+	-	7,8,9
Heat-labile toxin (HLT)	+	-	(Introduction; section II, 1)

1 Kasuga *et al*, 1954
2 Aprile, 1972
3 Kind, 1953
4 Field and Parker, 1979
5 Parton and Wardlaw, 1975
6 Dobrogosz *et al*, 1979
7 Parton and Durham, 1978
8 Hewlett *et al*, 1979
9 Endoh, Takezawa and Nakase, 1980.

SECTION II THE HEAT-LABILE TOXIN OF BORDETELLA PERTUSSIS

1 Discovery

As early as 1909 Bordet and Gengou reported in a paper entitled "L'endotoxine coquelucheuse" that B. pertussis produced a heat-labile toxic substance. They described several of the salient features of the toxin which have been amply confirmed by later workers viz. it is mainly cell-associated; is released in soluble form in saline extracts from ground bacteria; is lethal to guinea pigs and rabbits; is dermonecrotic in small doses and is inactivated by heating at 55°C or by treatment with certain organic solvents. Implicit in this work was the idea that HLT was an important contributor to the pathogenesis of pertussis.

2 HLT - pathohistological changes produced and significance in the pathogenesis of pertussis

The parenteral administration of HLT gives rise to widespread pathological symptoms and death may result as has been noted in mice, guinea pigs and rabbits. Wood (1940) gave one of the most detailed accounts of the pathological effects of the toxin. In mice dying after being injected intraperitoneally with a toxic filtrate there was marked congestion of subcutaneous blood vessels and there were small haemorrhages, particularly in the abdominal musculature. The inguinal lymph glands were dark and haemorrhagic, the reproductive organs markedly congested and in the liver there were occasional small areas of necrosis. Where mice were challenged with a sub-lethal dose of HLT these acute reactions were not observed any later than the 3rd week. However in such mice and for a period of at least 3 mth the spleen was very small, bloodless and frequently no more than one-quarter the weight of a normal spleen.

The liver was also very much smaller than normal and was irregular and scarred in appearance. The abdomen was often distended with ascitic fluid and the testicles were generally small and atrophic. Microscopically there was thickening of the peritoneum, atrophy of the spleen with almost no pulp cells to be seen and the spaces between the few remaining Malpighian bodies were filled with connective tissue. There was also evidence in the spleen of previous haemorrhaging. The testicles were atrophied with degeneration of the cells lining the tubules and diminished spermatogenesis. No specific lesion was demonstrable in the liver other than slight cellular atrophy. Heart, lungs and kidneys appeared normal.

Except for the absence of the haemorrhages in the abdominal musculature and peritonitis the same changes, but with a much more rapid onset, were produced by intravenously injected toxic filtrates. Even mice dying within 48 h exhibited small pale spleens and considerably shrunken livers. Atrophied spleens were also evident, to a lesser extent, when HLT was injected subcutaneously but no hepatic damage was apparent. It should be stressed that the systemic effects attributed to HLT must be regarded with caution until verified with pure preparations of toxin. However it is likely that the atrophy of the spleen which has been observed by various workers (Wood, 1940; Katsampes, Brooks and Bradford, 1942; Anderson and North, 1943; Muñoz and Bergman, 1977) including some who used highly purified HLT (Nakase et al, 1969; Iida and Okonogi, 1971) is produced by HLT and not by other factors. Iida and Okonogi (1971) who followed the development of the pathological changes induced in the spleen suggested HLT is selectively toxic for splenic tissue ie, lienotoxic (Introduction; section II, 3.3). However the highly purified toxin of Nakase et al (1969) gave rise to significant necrosis, haemorrhage, congestion and degeneration in spleen, liver and kidney. Renal damage

was also attributed to HLT by Bordet and Gengou (1909). There may also be damage to the adrenal glands (Bordet and Gengou, 1909; Evans and Maitland, 1937), lungs (Asada, 1953c; Hatsuda, 1960) and nerve tissue (Hatsuda, 1960).

A dermonecrotic/haemorrhagic reaction is elicited when HLT is given sub- or intra-cutaneously to sheep, pigs, chickens (Violle, 1950 cited by Muñoz, 1971), guinea pigs, rabbits (eg, Bordet and Gengou, 1909; Evans and Maitland, 1937) and suckling mice (eg, Katsampes, Brooks and Bradford, 1942). Violle (1950, cited by Muñoz, 1971) found mice to be resistant to the dermonecrotic action of HLT. The development of the skin reaction in the rabbit has been fully described by Evans and Maitland (1937): "There is in 18-24 h a pale flat indurated area that may be 20-25 mm across. In the centre of this is an irregular mottled area of necrosis made up of isolated or irregularly coalescing patches, reddish- to blackish-purple, dry and slightly depressed. These cover an area from a few mm to 15 mm or more in diameter and often there is a yellowish-white base. Pus however does not form. Within the next 48-72 h the smaller patches of necrosis coalesce, the area spreading slightly and becoming darker and drier. The induration usually disappears after a few days and the necrotic plaques finally separate leaving a shallow ulcer." Similar macroscopic observations have been made by Silverthorne and Cameron (1942), Kurokawa, Ishida and Asakawa (1969) and, for the response in the guinea pig, Bordet and Gengou (1909). However in guinea pigs and suckling mice the haemorrhagic component is a much more prominent feature of the reaction. Furthermore the pale spot is much more transient in the guinea pig and absent from the suckling mouse (Kurokawa, Ishida and Asakawa, 1969). The microscopical features of the reaction as described for the rabbit reveal that the pale spot is associated with

fluid accumulation in the subcutaneous tissue and a poor blood supply in the tissue overlying the fluid. The petechial haemorrhages are the consequence of haemorrhages in capillaries in the connective tissue. Necrosis was noted in the epidermis only when these haemorrhages were severe (Kurokawa, Ishida and Asakawa, 1969).

HLT has also been demonstrated to be harmful to tissue cultures (Angela, Rosso and Giuliani, 1962a and b; Střižova and Trlifajova, 1964). However in contrast to the findings of Angela, Rosso and Giuliani (1962a and b), Střižova and Trlifajova (1964) did not find HeLa cells to be sensitive to HLT. Instead, of the 14 types of tissue culture examined the most sensitive to the cytotoxic effect of HLT were the KB cell line and primary cultures of mouse embryonic tissue.

The aforementioned toxic effects of HLT are of limited relevance to any involvement the toxin may have in B. pertussis infections in children. Nevertheless it has been suggested that the dermonecrotic HLT is responsible for the necrosis which has been observed in the respiratory tract epithelial tissue in cases of pertussis (Bordet and Gengou, 1909). Similarly Muñoz (1971) speculated that the toxic action of HLT on the spleen and lymph nodes may interfere with the development of immunity. There is in fact no conclusive evidence for HLT being a significant component in the pathogenesis of pertussis, merely implications; For instance the parallels which have been drawn between the pathohistological changes observed in the lungs of children that have died from pertussis and in animals given crude HLT preparations (Sprunt and Martin, 1943; Asada, 1953a, b and c). In B. pertussis infections of the mouse lung, HLT has been considered to act as an aggressin (Kobayashi, 1961). However Anderson and North (1943) found intranasal instillation of sub-lethal (5×10^3) and lethal (5×10^7) doses of B. pertussis in 2.5

mouse MLD of HLT increased neither the killing power nor the intensity of infection as assessed from data on groups of 5 mice. In contrast, Standfast (1958) noted that the toxin (10 mouse MLD) may enhance the virulence of B. pertussis strains in mouse lung infections, but only under certain conditions. The HLT when mixed with strains of medium virulence, as estimated by LD_{50} values, markedly increased the death rate in the smaller (10^5, 10^6) but not the larger (10^7, 10^8) dose groups. He considered that since the addition of HLT did not automatically increase virulence it was not the only virulence factor. The addition of HLT would not therefore aid the organism where the conditions for full virulence already existed (ie, high virulence strains and very large doses of medium virulence strains), nor where the organisms were defective in some other aspect of virulence (ie, low virulence strains). Virulence would only be enhanced if HLT was mixed with the challenge and was the only limiting factor. When given up to 4 h before or after the intranasal instillation of the organisms it was ineffective. Standfast (1958) concluded that HLT played a part in the primary lodgement of the organism. Furthermore he provided evidence of a mechanism by which this could occur, namely by paralysis of cilia in ciliated epithelia. This was demonstrated through the failure of india ink particles to migrate in toxin treated sheep-lung preparations but relatively large amounts of toxin had to be used. It was not established whether this effect, which was probably irreversible, was mediated by HLT acting on the cilia directly or on the ciliated cell. Ciliostasis was not evident with heated toxin ($100^{\circ}C$, 1 h) or toxin mixed with absorbed antitoxic sera free from agglutinins. In contrast to the findings of Standfast (1958), evidence gathered from experimental B. pertussis infections on rabbit tracheal mucous membrane (Matsuyama, 1977) and hamster tracheal organ cultures (Collier, Peterson and Baseman, 1977) show that ciliostasis is not necessary fo the establishment of infections on these ciliated epithelia. Matsuyama (1977

did not observe any ciliostatic effect and the earliest that Collier, Petersor and Baseman (1977) observed ciliostasis was 48 h after the initiation of the infection. Furthermore the capacity to lodge at the point of inoculation could not be conferred upon cells unable to resist mucociliary clearance by co-inoculation with cells able to do so (Matsuyama, 1977). This might have been expected if a ciliostatic toxin was in action. Moreover sterile culture fluids from infected tissues at various stages during infection had no effect on the ciliary activity, or amino acid uptake, of the respiratory tissue (Collier, Peterson and Baseman, 1977). Matsuyama (1977) did not however rule out the possibility that HLT may have some role after the infection is established, perhaps in the spread of the infection to the lower respiratory tract. Serological evidence on the involvement of HLT in B. pertussis infections is unfortunately also rather scanty and inconclusive (Introduction; section II, 8).

In addition to the possible involvement of HLT at the site of the infection in the upper respiratory tract, it has also been implicated in producing damage in the central nervous system. The pathological changes which occur when HLT is injected intracerebrally into guinea pigs have been compared to the neurological damage evident in children who died of encephalitis complicating pertussis (Fonteyne and Dagnelie, 1932 cited by Muñoz, 1971; Gabrielesco et al, 1958 cited by Muñoz, 1971). It has also been proposed that the toxin by acting directly on the respiratory centres can provoke disturbances in the nervous control of respiration (Abrosimov, 1961). Pathological changes in the hypothalamic region have been recorded after intramuscular and intravenous injection of HLT into rabbits (Hatsuda, 1960). Whether these effects on the central nervous system can be brought about by toxin released in the respiratory tract remains to be seen, but Anderson and North (1943) noted that damage to the

spleen was less prominent when HLT was given intranasally compared to intravenously, even when 80 times as much toxin was given by the former route. The implication is that very little toxin finds its way into the systemic compartment of the body.

Heat-labile toxin and mouse virulence may be lost together as in phase variation (Introduction; section I, 6.2) but it is not surprising to find that they may vary independently (Standfast; 1951a, b and 1958). Pittman (1970) even found the toxin content of organisms varied inversely with their intracerebral virulence. Further evidence that would suggest HLT has at most a limited role in B. pertussis intracerebral infections of mice was the finding by Standfast (1958) that when 0.3 mouse MLD of toxin was given with the challenge organisms there was no enhancement of their virulence.

Finally, although the pathohistological damage produced by HLT and the role of HLT in B. pertussis infections is far from being clearly defined, knowledge on its mode of action at the molecular level is almost non-existent. It has been suggested however that the phenomena observed when isolated hearts (rabbit, cat and hen) are perfused with HLT are due to impaired oxidative phosphorylation (Raškova, Střižova and Vaněček, 1961 cited by Raškova and Mašek, 1970).

3 Assay of toxicity

3.1 Lethality

Mice, guinea pigs and rabbits are all susceptible to the lethal effect of HLT but mice are the most widely used to assay lethality. There are few data permitting direct comparison of inter-species susceptibility but rabbits and guinea pigs are estimated from the results of Ospeck and Roberts (1944) and Anderson and North (1943) to be 4 and

2.5 times respectively more sensitive than mice on an equal body weight basis. The injection route also influences susceptibility to HLT. Guinea pigs and mice survived larger doses of toxin when it was given subcutaneously (SC), rather than intravenously (IV) or intraperitoneally (IP) (Bordet and Gengou, 1909; Wood, 1940). The IV route has been found to be marginally (1.5 times) more sensitive than the IP route for administering HLT to mice (Anderson and North, 1943), although Wood (1940) found no difference. Similarly, guinea pigs have been observed to die sooner when the toxin is given intravenously rather than intraperitoneally (Evans and Maitland, 1937). In a 3 way comparison of the intranasal (IN), intraperitoneal (IP) and intracerebral (IC) routes for injecting HLT, the ratios for the sensitivities of the respective methods were in order of increasing sensitivity 1:10:100 (Standfast and Dolby, 1961; Dolby and Standfast, 1961). Anderson and North (1943) had previously recorded the ineffectiveness of HLT given intranasally, ie, mice dosed with 10 MLD, as measured by intraperitoneal injection, showed no obvious signs of ill health.

In none of the aforementioned comparisons has the limit on the volume of injectable material been taken into consideration. This could for instance effectively nullify the apparently superior sensitivity of the IC route relative to the IP route.

When assaying HLT by the IP route of injection into mice a 3 d post-injection observation period has been recommended (Ishida, 1968). Most investigators have taken 2 or 3 d as a suitable time.

The IP route has been used to assay the toxicity of live B. pertussis cells as well as HLT extracts. Bordet and Gengou (1909) established that B. pertussis does not multiply in the peritoneal cavity of guinea pigs and attributed the death of such animals to the toxicity

of the injected dose rather than to active infection. Their observation has been confirmed in both guinea pigs (Leslie and Gardner, 1931) and mice (Anderson and North, 1943; Gray, 1946 cited by Standfast, 1958; Proom, 1947; Geller and Pittman, 1973). Further support for the IP route as a measure of the organism's toxin content rather than their virulence was provided by Anderson and North (1943), Ospeck and Roberts (1944) and Proom (1947). Passively administered antitoxin afforded good protection to mice against an IP challenge with live B. pertussis whereas antibacterial sera had little or no protective activity. In assaying the toxicity of B. pertussis cells in this manner the weights of the mice employed do not appear to be of much importance (Standfast, 1958). There is less evidence on the suitability of the IV route as a method for assaying the toxin content of live B. pertussis. Bordet and Gengou (1909) reported the failure of IV injected organisms to multiply in rabbits and Ospeck and Roberts (1944) showed rabbits with high antitoxin titres were protected against a lethal IV challenge.

3.2 Dermonecrotic/haemorrhagic activity

Although the skin reaction which develops in response to sub- or intra-cutaneously injected HLT is commonly described as "dermonecrotic" the type of response varies from species to species (Kurokawa, Ishida and Asakawa, 1969). With rabbits and guinea pigs the feature of the response chosen by most investigators for measurement is the necrotic lesion itself. However, Kurokawa, Ishida and Asakawa (1969) found the most suitable features were a pale ischemic-like spot in rabbits and the haemorrhagic reaction in guinea pigs. With suckling mice it is the purple-black haemorrhagic spot which is used (Katsampes, Brooks and Bradford, 1942; Andersen, 1952 and 1953b; Kurokawa, Ishida and Asakawa, 1969; Cowell, Hewlett and Manclark, 1979; Field et al, 1979).

Contaminating constituents in an HLT preparation may influence the type of reaction produced; induration as well as necrosis being produced by whole bacilli (Evans and Maitland, 1937) but being virtually absent from toxic culture filtrates (Wood, 1940). These authors contended that "bacterial antigen" was responsible for the induration. Kurokawa, Ishida and Asakawa (1969) noted that LPS increased the haemorrhagic reaction in guinea pigs and suckling mice by 2.8 and 1.6 times respectively (the effect seemed to be independent of the relative concentrations of LPS and HLT). Lipopolysaccharide had little, if any, effect on the response in rabbits and LPF had no effect on the response in any of the 3 species of animal.

Individual rabbits vary in their skin reactivity to HLT (Flosdorf, Bondi and Dozois, 1941; Anderson and North, 1943; Roberts and Ospeck, 1942; Yamamoto, Zenyoji and Kato, 1952a) by a factor of 2-or 3-fold (Roberts and Ospeck, 1942; Yamamoto, Zenyoji and Kato, 1952a). Differences between guinea pigs are even less than those between rabbits (Yamamoto, Zenyoji and Kato, 1952a) with the variation between animals usually being within 50% (Onoue, Kitagawa and Yamamura, 1963).

Skin reactions are usually measured within 24-48 h of injection of the toxin.

3.3 Lienotoxicity

The term lienotoxicity was coined by Iida and Okonogi (1971) to describe the atrophy of the spleen in mice injected intravenously with HLT preparations. The extent of the atrophy 7 d after the injection was taken as a measure of the lienotoxicity of a preparation.

3.4 Body weight loss

HLT can be assayed by following the body weight loss in mice over a 2 d period subsequent to intraperitoneal injection with HLT

(Ishida, 1968). However, weight loss may also be affected by the presence of LPS and/or LPF.

3.5 Toxicity to tissue cultures

Střižova and Trlifajova (1964) screened 14 different tissue culture lines to establish their possible susceptibility to killing by HLT. The KB line and primary cultures from mouse embryonic tissue were the most sensitive but the former tended to undergo spontaneous degeneration. Therefore primary cultures of mouse embryonic tissue were used to assay the cytotoxic effect of the toxin.

3.6 Relative merits of the assay methods

The various methods for assaying HLT have their advantages and disadvantages. For instance, the assays differ in their sensitivity ie, how little toxin they can detect. The most sensitive method is reported as being the haemorrhagic assay for HLT using suckling mice (Gardner, cited by Pittman, 1970). However the difference in sensitivity between this assay and that in which the dermonecrotic activity of HLT is measured in adult rabbits is only marginal, disregarding differences in body weight (Kurokawa, Ishida and Asakawa, 1969). Guinea pigs are less sensitive than either rabbits or suckling mice (Evans, 1947; Kurokawa, Ishida and Asakawa, 1969) eg, suckling mice have been found to be 40 times more sensitive than guinea pigs (Ishida, 1968). The measurement of dermonecrosis/haemorrhage in rabbits (Strean, 1940; Strean and Grant, 1940; Roberts and Ospeck, 1942; Evans, 1947; Yamamoto, Zenyoji and Kato, 1952a), guinea pigs (Kuwajima, Iwamura and Hirai, 1951; Yamamoto, Zenyoji and Kato, 1952a; Onoue, Kitagawa and Yamamura, 1963; Ishida, 1968) or suckling mice (Ishida, 1968) is more sensitive than the other principal method of assaying HLT viz. by recording lethality in mice. In relation

to the aforementioned assays, the assay based on body weight loss in mice is of intermediate sensitivity. It is 17.5 times less sensitive than the haemorrhagic assay in suckling mice, but 2-3 times more sensitive than either the haemorrhagic assay in guinea pigs or the assay of lethality in mice (Ishida, 1968). Finally, the cytotoxic assay of HLT, using mouse embryonic tissue culture cells, is about twice as sensitive as the assay of lethality in mice (Střižova and Trlifajova, 1964).

When assaying low levels of HLT it has to be remembered that in actual practise the most sensitive assay need not necessarily be the most appropriate; using a less sensitive assay it may be possible to detect as low or lower levels of HLT in solution. This apparent contradiction can be illustrated by the following: suppose that for a given toxin preparation the MRD is 0.1 μg protein in suckling mice whilst the MLD for adult mice is 1.0 μg, the first of these assays is the most sensitive. However, if the limit on the volume injectable in each assay is 0.05 ml and 1.0 ml respectively, it follows that in the first instance HLT can be detected at a level of 2 μg/ml whereas in the latter example 1 μg/ml can be detected.

Criteria other than sensitivity may have to be considered when choosing the way in which to assay HLT. For instance although the IV route may be marginally more sensitive than the IP route for measuring HLT by its lethal effect in mice most workers have preferred the more convenient IP route (eg, Billaudelle et al, 1960). There again Anderson and North (1943) preferred the mouse lethal (IV) assay to the dermonecrotic assay in rabbits due to less variation in the responses with the former. With the dermonecrotic/haemorrhagic assays there is not much difference

in precision between the 3 species but assays with guinea pigs and suckling mice are sometimes not so accurate, according to Kurokawa, Ishida and Asakawa (1969). Evans and Maitland (1937) found the reaction end point easier to read in rabbits than guinea pigs.

4 Antigenic variation and HLT production

4.1 Antigenic modulation

Lacey (1960) noted that when X- and C-mode cells of B. pertussis H5 were injected IP into mice LD_{50} values were about 2×10^8 and 2×10^9 organisms respectively. From this it can be inferred that the X- to C-mode transition, induced by substituting $MgSO_4$ for NaCl as the chief salt in a growth medium, produces a reduction in the toxicity of B. pertussis cells. This has not been confirmed.

4.2 Nicotinic acid induced modulation

Most strains of B. pertussis grown in media with high levels of nicotinic acid lose several of the properties normally associated with freshly isolated cells (Pusztai and Joó, 1967; Wardlaw, Parton and Hooker, 1976). They are reported however as retaining their full toxicity (Pusztai and Joó, 1967).

4.3 Phase variation

HLT production seems to be a property of freshly isolated B. pertussis that is lost, along with other properties, during the process of phase variation. Leslie and Gardner (1931) tested the toxicity of 11 phase I, 8 phase III and 4 phase IV strains by intraperitoneal injection into guinea pigs. Phase III and IV organisms could be tolerated in doses 20-30 times that required with phase I strains. Similarly strains in phases III and IV when tested for their ability to

produce necrosis were only one-tenth as toxic as a phase I strain (Flosdorf, Bondi and Dozois, 1941). This loss of toxicity has been reported for the toxin content of both cell and culture fluid (Lawson, 1933; Wood, 1940; Shibley and Hoelscher, 1934 cited by Wood, 1940; Ungar and Muggleton, 1949a and b). Japanese workers who have followed a different classification of the phase variants have nevertheless recorded results consistent with those already reported (Kasuga et al, 1954; Nakase and Kasuga, 1962).

Roberts and Ospeck (1942) who recognized phase IV strains to be less toxic (dermonecrotic) than phase I strains did however, after screening many strains for their ability to produce strongly toxic culture filtrates, identify the 2 best producers as being in the intermediate phase (either II or III). When the toxicity of the cells of the more potent strains were titrated these were as toxic as phase I organisms.

5 Other aspects of HLT production

5.1 Organism

With some toxigenic bacteria there are classical strains which yield high levels of toxin eg, the PW8 strain of Corynebacterium diphtheriae or the Staphylococcus aureus strain Wood 46 for α-toxin production (Pappenheimer, 1965, cited by Zabriskie, 1970; Bernheimer, 1970). So far no B. pertussis strain appears to have been identified as an outstanding producer of toxin. Kuwajima et al (1958) described an intermediate strain of B. pertussis No. 33 as a classical toxin-producer but there is little to substantiate such a claim. Since the strain was selected by Roberts and Ospeck (1942) for producing strongly toxic culture filtrates it has been used by relatively few authors (Kuwajima et al, 1958; Nakase and Kasuga, 1962; Střížova and Trlifajova, 1964). Nakase and Kasuga

(1962) found it only produced as much toxin as ordinary phase I organisms. Nevertheless some B. pertussis strains contain more toxin than others (table 5). Most strains are rather similar in their toxicity but there was a 13-14 fold difference in toxicity between the least and most toxic strains in the extensive survey of 201 strains by Spasojević (1977). Her most toxic strain was B-1593/57.

Others have examined strains for their ability to release a soluble toxin into the culture medium eg, Joslin and Christensen, 1940; Weichsel et al, 1940; Roberts and Ospeck, 1942. The latter workers found culture filtrates from B. pertussis strains to range from being atoxic to being lethal at dilutions of 1 in 20 and perhaps higher ie, filtrates from cultures of the intermediate strain No. 33.

On repeated subculture many toxigenic bacteria lose their ability to produce toxin (van Heyningen, 1970). This phenomenon has been noted, for instance, with Clostridium septicum and Staphylococcus aureus (α toxin production) (Bernheimer, 1944; Arbuthnott, 1970). Likewise with B. pertussis the levels of HLT are considerably reduced in the process of phase variation which is induced by repeated subculturing (Introduction; section II, 4.3). However reduced levels of HLT have also been noted in a strain which had been under cultivation for 18 mth but was still serologically phase I (Leslie and Gardner, 1931). It is not clear how rapid or permanent is the loss of HLT although Standfast (1951a) cited Evans as finding toxin production to be one of the more stable characteristics of B. pertussis. Field and Parker (1979) subcultured 3 fresh isolates on tryptic soy agar with decreasing amounts of blood until they would grow without added blood. Two of the 3 variants when injected subcutaneously into suckling mice were no longer dermonecrotic. The toxic variant at the time of testing had undergone 15

Table 5 Variation in the toxicity of B. pertussis strains

Reference	Mouse LD_{50}[a] (organisms x 10^9)	Number of strains
Cruickshank and Freeman, 1937	6.0-9.0	17
	≥ 9.0	4
Standfast, 1951a	2.0-5.4	5
Standfast, 1951b	0.3-0.8	9
Standfast, 1958	0.3-1.8	12
Spasojević, 1977	1.1-1.9	35
	2.0-4.8	92
	5.3-7.8	46
	9.0-14.1	28

[a]For all experiments except those of Cruickshank and Freeman (1937) where the results are expressed in MLD. Standfast (1951a) used the IV route of injection but on all other occasions the IP route was used.

subcultures since its isolation and the atoxic variants 16 and 32. By the 43rd subculture there was preliminary evidence that the first of the 2 atoxic strains had recovered its toxicity.

The levels of HLT in culture supernatant fluids have also been examined. They are reported as being erratic during the 1st subculture of Bordet-Gengou grown cells in a liquid medium (Kuwajima, Iwamura and Hirai, 1951) and of being reduced after as little as 5 subcultures (Imamura, 1952).

Finally Andersen (1952) noticed that small and large colony variants were produced on intracerebral passage of B. pertussis in mice. The small colony variants were invariably toxic and the large colony variants atoxic.

5.2 Culture medium

With some bacterial toxins the composition of the culture medium is critical for toxin production. For example, with at least 6 bacterial toxins (diphtheria, tetanus, Shigella dysenteriae neurotoxin, Streptococcus pyogenes erythrogenic toxin, Clostridium perfringens α toxin and Pseudomonas aeruginosa exotoxin A) toxin production is inhibited if there is more than a low level of iron (0.1-1.0 mg/l) present (van Heyningen, 1970; Bjorn et al, 1978). With the HLT of B. pertussis there are few pertinent reports on the influence of the culture medium on toxin production. There is, for instance, no preferred medium for HLT production. Most workers appear simply to have taken a medium which supported satisfactory growth. However, a few authors have indicated a preference for a particular medium (table 6). Wood (1940) experimented with a variety of media. For instance, she modified the medium recommended by Lawson replacing the serum with laked blood, tried different kinds of peptone,

Table 6 Preferred culture media for HLT production

Reference	Medium	Source of HLT
Bordet and Gengou, 1909	Bordet and Gengou agar	cell
Joslin and Christensen, 1940	Beef-heart infusion broth	culture filtrate
Wood, 1940 - recommended by Lawson	2% peptone water solution (pH 7.0) containing 10-15% fresh rabbit serum	culture filtrate
Roberts and Ospeck, 1942	Beef-heart infusion broth containing 2% peptone and 0.1% soluble starch	culture filtrate
Robbins and Pillemer, 1950	Liver-peptone broth	cell

tried semi-solid starch agar made from soluble or potato starch and potato-extract serum broth. However the toxin was not produced any more rapidly or in greater concentrations than in the medium of Lawson. Finally, Imamura (1952) referred to a synthetic liquid medium in which superior yields of HLT were achieved in the culture supernatant fluid than with a modification of Verwey's medium.

Little is known of the components in a medium which influence toxin yields but the amino acid content may be important. Imamura (1952) found that by increasing the concentration of the 7 amino acids in a synthetic medium growth improved and toxin became detectable in the culture supernatant fluids. Whether this was merely the consequence of better growth was not specified. Supplementing the synthetic medium with any one of 8 additional amino acids did not however affect growth or toxin production. More recently Stainer and Schölte (1971) devised a synthetic medium with only 3 amino acids; glutamate, proline and cystine. By increasing the levels of either glutamate or proline between 12-32 times those given in their basic 'GP' medium (ie, 670 mg and 240 mg per litre respectively) the HLT content of cultures was increased (Stainer 1977, personal communication). Yet when the casein hydrolysate in Cohen and Wheeler medium was replaced with increasing amounts of glutamate the toxicity of the cells was reduced (Lane, 1970). In broth in which 60% of the carbon was supplied as glutamate the effect was not alleviated by the addition of any one of serine, proline, glycine, aspartate or alanine to a concentration of 0.01M.

The possible significance of the salts present in a medium on HLT production has already been discussed (Introduction; section II, 4.1).

5.3 Cultural conditions

There have been a few reports on the effect of aeration on HLT

production. Wood (1940), who was interested in obtaining highly toxic culture filtrates, distributed medium in shallow layers for optimizing toxin production. However it has also been stated that the toxicity of organisms is not promoted by improving the aeration of cultures (Akama et al, 1953). Indeed the combined influence of vigorous aeration and elevated pH may be responsible for the detoxification of sparge- and vortex- aerated cultures, whereas shake-flask cultures are invariably toxic (Lane, 1968a).

Incubation in an atmosphere of 80% O_2 and 20% CO_2 has been used in the production of toxic filtrates (Weichsel et al, 1940; Roberts and Ospeck, 1942) but Wood (1940) found incubation with 30% CO_2 did not enhance the toxicity of filtrates.

Kuwajima, Iwamura and Hirai (1951) followed growth and the production of toxin in culture supernatant fluids at a variety of temperatures. Toxin was produced just as effectively at 32°C as at 37°C but was poor at 30°C as was the growth of the organisms. Most workers have incubated at temperatures between 35°C and 38°C but 32°C was used by Roberts and Ospeck (1942) and Weichsel et al (1940).

5.4 Time characteristics of HLT production

Very little is known about the time characteristics of HLT production. Lane (1968b) followed the toxicity of whole cultures. During very early exponential growth high levels of HLT were already apparent and peak toxicity had been reached before the end of exponential growth. Heat-labile toxin levels remained fairly constant or declined slowly during the stationary and decline phases of growth. Stainer (1977, personal communication) observed that higher levels of HLT could be obtained per cell when cultures were harvested prior to reaching peak opacity.

5.5 Intracellular versus extracellular location

Heat-labile toxin was first identified in extracts of *B. pertussis* cells after a failure to detect the toxin in culture supernatant fluids (Bordet and Gengou, 1909). This is consistent with HLT being predominantly cell-associated. It has been suggested that HLT is a capsular, cell-surface or envelope component (Wood, 1940; Katsampes, Brooks and Bradford, 1942; Yaguchi, 1954 cited by Yamamoto *et al*, 1957; Yoshida *et al*, 1955 cited by Billaudelle *et al*, 1960) and it can be obtained from washings or extracts from cells (appendix 1). However it has now been established that HLT is predominantly associated with the cytoplasm of *B. pertussis* cells.

When *B. pertussis* cells are lysed there is a marked increase in their toxicity (Evans and Maitland, 1937; Smolens and Flavell, 1947; Muñoz, Schuchardt and Verwey cited by Muñoz, Ribi and Larson, 1959; Novotny, 1977, personal communication; Cowell, Hewlett and Manclark, 1979). This was interpreted by Muñoz, Ribi and Larson (1959) as indicating that HLT is an internal substance which when released is more effective than when contained in the intact cell. They found support for the idea that HLT is a cytoplasmic constituent by disrupting washed cells and separating the cell-envelopes from the cytoplasmic fraction in which the HLT was almost exclusively found. The slight toxicity of the washed cell-envelopes was attributed to contamination with cytoplasmic material. These results agreed with those later obtained by Billaudelle *et al* (1960) and Cowell, Hewlett and Manclark (1979). These latter workers went one step further and established that the HLT in these cytoplasmic fractions was truly cytoplasmic in origin and did not arise from the periplasmic space. The HLT was not inactivated by trypsin treatment of whole cells nor was it released

when cells were subjected to osmotic shock treatment (Cowell, Hewlett and Manclark, 1979). It has been suggested that intracellular HLT may exist, as a precursor which is not toxic until activated (Anderson and North, 1943; Morse, 1976).

HLT can also be derived from culture filtrates and supernatant fluids (appendix 1). It has been proposed that such toxin arises, in the main, as the result of autolysis of the organisms (Pittman and Cox, 1965) and conversely as a product of actively growing cells (Wood, 1940; Roberts and Ospeck, 1942; Lane, 1968b). It has even been suggested that small vesicles seen on the outer surface of B. pertussis cells are involved in the release of HLT (Lane, 1968b). Golubeva and Kushnarev (1974) isolated HLT from such vesicles. An indication that yields in the culture fluids are considerably lower than those obtained from extracts of disrupted cells can be gathered from the observation that most workers have used cell extracts as their source of HLT (appendix 1). Culture supernatant fluids may even have to be concentrated before the toxin can be detected eg, Cowell, Hewlett and Manclark (1979) who could detect less than 0.01% of the HLT associated with the cell-lysates in the corresponding culture supernatant fluids.

5.6 Production by B. parapertussis and B. bronchiseptica

B. parapertussis and B. bronchiseptica produce toxins indistinguishable from the HLT of B. pertussis. All 3 are lethal, dermonecrotic/haemorrhagic toxins (Evans and Maitland, 1939; Bruckner and Evans, 1939) which are cytotoxic for mouse embryo tissue cultures (Střížova and Trlifajova, 1964). They are similarly affected by heat, formalin and filtration (Evans and Maitland, 1939; Bruckner and Evans, 1939), and antisera raised to one will neutralize the dermonecrotic or lethal effects of all 3 (Evans, 1940; Andersen, 1953b).

B. parapertussis would seem to produce less toxin than B. pertussis (Bruckner and Evans, 1939; Flosdorf, Bondi and Dozois, 1941; Andersen, 1953b) although the strain tested by Roberts and Ospeck (1942; Ospeck and Roberts, 1944) for its ability to produce toxic filtrates was of intermediate toxicity compared to B. pertussis strains. B. bronchiseptica strains have been found to be less or similar in toxicity to B. pertussis (Evans and Maitland, 1939; Andersen, 1953b). However too few strains of each species have been compared for the aforementioned differences to be any more than indications as to the relative toxin producing capacities of these species. Moreover the levels of toxin produced can vary as is illustrated by the observation that B. parapertussis and B. bronchiseptica grown on Bordet-Gengou medium are markedly more toxic than their nutrient agar grown counterparts (Evans and Maitland, 1939; Bruckner and Evans, 1939).

6 Purification

6.1 Disruption of bacteria and release of HLT

B. pertussis cells as opposed to culture fluids have been used most often as a source of HLT for which purpose they are invariably disrupted by one of several methods. For instance, in an extensive review of the literature in which 59 references are cited (sometimes more than once giving 67 citations in all), the cell was taken as the source of HLT 87% of the time. In 88% of these cases the cells were lysed (appendix 1). The 3 main methods which have been used to lyse the bacteria are grinding, freeze-thawing and sonication which account for 73% of these citations. Grinding and freeze-thawing although once widely used have now been replaced, to a large extent, by more convenient/effective methods (appendix 1).

When B. pertussis cells are lysed there is an increase in their toxicity (Evans and Maitland, 1937; Smolens and Flavell, 1947; Muñoz, Schuchardt and Verwey cited by Muñoz, Ribi and Larson, 1959; Novotny 1977, personal communication; Cowell, Hewlett and Manclark, 1979). The values given for the size of this increase vary viz. a 4-fold increase by grinding (Evans and Maitland, 1937); a 20-fold increase by sonication compared to that (unspecified) achieved by freezing and thawing (Smolens and Flavell, 1947) and a 20-25 fold increase by the lysozyme freeze-thaw technique of Cowell, Hewlett and Manclark (1979).

HLT can also be obtained from cells without actively disrupting them but only part of the toxin available is recovered (Roberts and Ospeck, 1942). For instance, when extracts were made from live and freeze-thawed, ground B. pertussis preparations the former extract contained only 10% of the toxicity detectable in the latter (Evans and Maitland, 1937). Similarly, Robbins and Pillemer (1950) recovered in a water extract from freeze-dried B. pertussis only 21% of the HLT obtained from the cells when sonicated.

6.2 Purification procedures

The first serious attempted purification seems to be that of Robbins and Pillemer (1950). They extracted the HLT from cells with $CaCl_2$ and fractionated this by selectively precipitating out the toxin under 2 different sets of conditions (figure 3). In the final fraction (P11) 66% of the HLT from the $CaCl_2$ extract had been recovered whilst achieving a 13.2 fold increase in purity but the material was still heterogeneous on analysis by electrophoresis. The LD_{50} of P11 to 12-15 g mice was 0.19 µg N.

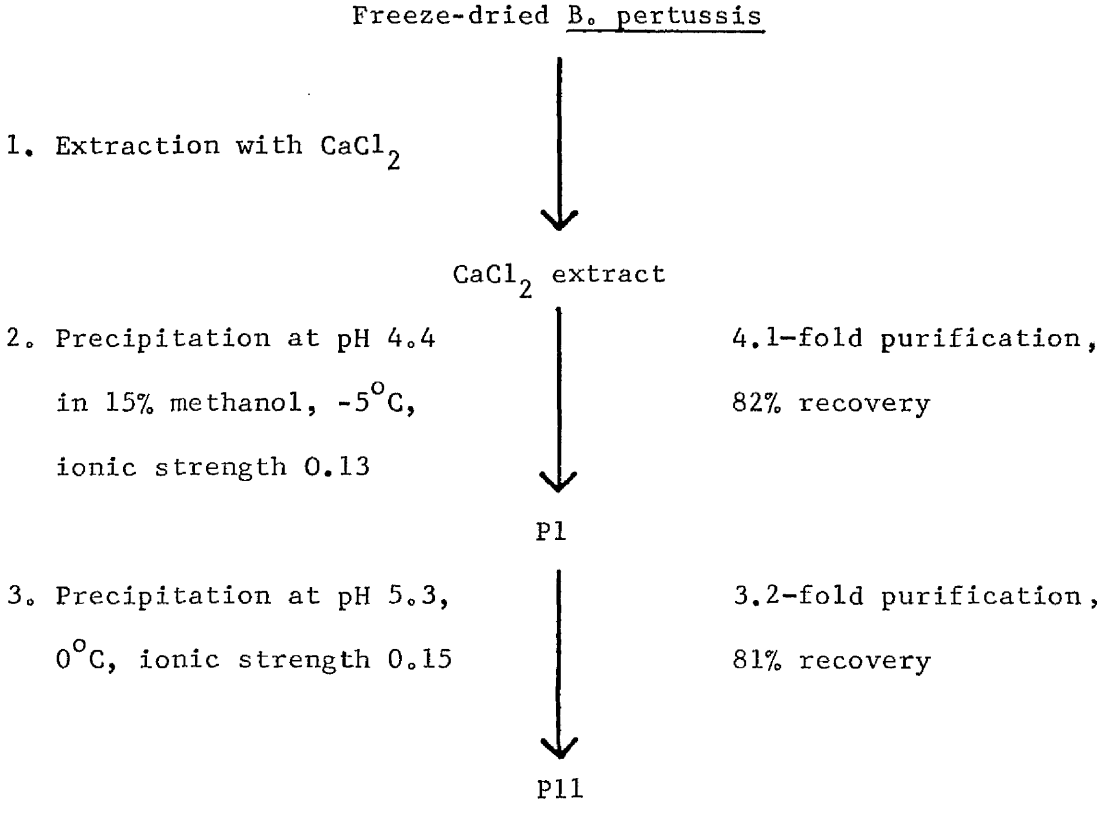
Pennel and Thiele (1951) fractionated extracts of sonically disrupted B. pertussis with ethanol at -5°C but lost much of the toxic

Figure 3 Purification procedure of Robbins and Pillemer (1950).

1 A 2.5% suspension of freeze-dried B. pertussis was prepared in 0.05M $CaCl_2$ at pH 6.5 and then centrifuged to remove bacterial debris and a mucin like material.

2 The precipitate formed from the $CaCl_2$ extract under the conditions specified was dissolved in 0.3M sodium acetate at pH 7.0 and this solution clarified by centrifugation.

3 HLT was precipitated from P1 as indicated, the precipitate dissolved in 0.15M sodium acetate at pH 7.0 and this solution clarified by centrifugation.


Freeze-dried B. pertussis
1. Extraction with $CaCl_2$
$CaCl_2$ extract
2. Precipitation at pH 4.4
in 15% methanol, -5°C,
ionic strength 0.13
4.1-fold purification,
82% recovery
P1
3. Precipitation at pH 5.3,
0°C, ionic strength 0.15
3.2-fold purification,
81% recovery
P11

activity. This inactivation was more pronounced if the pH or ionic strength was lowered, if the mixture was diluted, or had been clarified with $CaCl_2$.

Yamamoto and coworkers began their purification studies with extracts from freeze-thawed cells (Yamamoto, Zenyoji and Kato, 1952a; Yamamoto et al, 1952, 1953 and 1957) or washings from cells (Yamamoto, Zenyoji and Kato, 1952c). They used combinations of the following techniques: extraction with $CaCl_2$ (Yamamoto, Zenyoji and Kato, 1952c; Yamamoto et al, 1952 and 1953), precipitation with acetic acid (Yamamoto Zenyoji and Kato, 1952a and c; Yamamoto et al, 1952; Yamamoto et al, 1957), precipitation with 20% methanol at $-5^{o}C$ (Yamamoto et al, 1952 and 1953) or with ammonium sulphate (Yamamoto et al, 1957). In their most successful attempt an extract from freeze-thawed cells was fractionated by precipitations with ammonium sulphate and by the addition of acetic acid (figure 4). The final fraction F-0.4 was 60 times more active than the original toxic extract ie, 1 rabbit MRD = 50 ngN (cf. 3 μgN). However this mainly protein fraction (F-0.4) was still heterogeneous when analysed electrophoretically and contained agglutinogen. Subsequently Yamamoto and his coworkers managed to prepare a marginally more active preparation with an MRD of 20 ngN (Yamamoto et al, 1959 cited by Onoue, Kitagawa and Yamamura, 1963).

Billaudelle et al (1960) experimented with the use of ion-exchange chromatography on DEAE-cellulose and preparative electrophoresis for purifying HLT from cell-lysates or cell-washings but had problems with inactivation of the toxin. They did however report being able to separate the dermonecrotic activity from the lethal activity by electrophoresis although the lethal effect, which was largely destroyed during the process, did contain dermonecrotic activity.

Figure 4 Purification procedure of Yamamoto *et al* (1957).

The starting material was a filtered, supernatant fluid obtained from *B. pertussis* suspended in 0.15M sodium acetate (280 g wet weight in 2 l) which had been freeze-thawed 15 times.

1 The toxin was precipitated out of the filtrate by the addition of ammonium sulphate to 40% saturation (pH 7.0, -5°C). This procedure was repeated 3 times and the final precipitate suspended in and dialysed against saline at pH 7.0.

2 The pH of the ammonium sulphate fraction was brought to 4.4 with 1N acetic acid, the precipitate collected, dissolved and dialyzed with saline at pH 7.0.

Filtered supernatant fluid from a suspension of freeze-thawed B. pertussis

1. Fractional precipitation with ammonium sulphate

fraction 1

2. Precipitation at pH 4.4 by the addition of acetic acid

F - 0.4

DEAE-cellulose was also used by Banerjea and Muñoz (1962) to separate HLT from other constituents in cell lysates. The toxin was adsorbed onto the column and then eluted using a continuous NaCl gradient (0-1M) in pH 7.1, 0.15M phosphate buffer. Due to the instability of the eluted toxin its specific activity was not determined. Toxic fractions were mainly protein but traces of RNA, DNA and carbohydrates were also present in most fractions. In 1963 Onoue, Kitagawa and Yamamura described a method for obtaining HLT in a highly active form. An extract from B. pertussis cells disrupted by grinding, was purified successively by calcium phosphate gel treatment, ammonium sulphate fractionation, precipitation with potassium phosphate, and chromatography on a DEAE-cellulose column (figure 5). The calcium phosphate gel step was essentially preparative, to reduce the viscosity of the extract for subsequent purification. The precipitation with potassium phosphate produced no net purification in terms of μg dry weight or nitrogen but nucleic acids were eliminated. The overall process resulted in a 60-80 fold purification of the crude extract with a 5-10% recovery of toxicity. The final F2 fraction was a highly active protein solution (1 guinea pig MRD = 1 - 1.3 ngN and 1 mouse LD_{50} = 44 ngN) but was still heterogeneous containing agglutinogen and exhibiting 3 lines in gel diffusion tests.

A modification of the method of Onoue, Kitagawa and Yamamura (1963) was employed by Iida and Okonogi (1971; figure 6). The final fraction was highly active with a guinea pig MRD of 0.9 ngN and a mouse MLD of 50 - 100 ngN. Compared to the starting material or sonicate these figures represent purifications of about 90 and 6-12 times in terms of dermonecrotic and lethal activities respectively. Recovery of HLT, assuming 100% recovery in the supernatant fluid from the sonicate,

Figure 5 Purification procedure of Onoue, Kitagawa and Yamamura (1963).

1 HLT was extracted from the disrupted cells by mixing with 0.15M NaCl at pH 8.0 and centrifuging out the cellular debris.

2 Crude extract diluted 1 in 1.5 with distilled water was mixed with calcium phosphate gel. This was filtered, the filter cake washed with 0.15M NaCl and the HLT eluted with 0.15M, pH 8.0 phosphate buffer. The supernatant fluid was collected and combined with that obtained on re-extraction of the gel.

3 Ammonium sulphate was added to the gel eluate (9.12 g/100 ml). The precipitate was removed and further ammonium sulphate added (15.2 g/100 ml). The resultant precipitate was collected, dissolved and dialysed with 0.005M, pH 8.0 phosphate buffer, clarified and brought to one-tenth the volume of the original crude extract.

4 To the ammonium sulphate fraction was added potassium phosphate (18% (w/v)). The precipitate formed was collected, dissolved and dialysed with distilled water, and freeze-dried.

5 KP18 was reconstituted in pH 7.4, 0.2M phosphate buffer + 0.01M NaCl and adsorbed to a column of DEAE-cellulose equilibrated in the same buffer. After elution of unadsorbed material HLT was eluted with pH 7.4, 0.04M phosphate buffer + 0.05M NaCl.

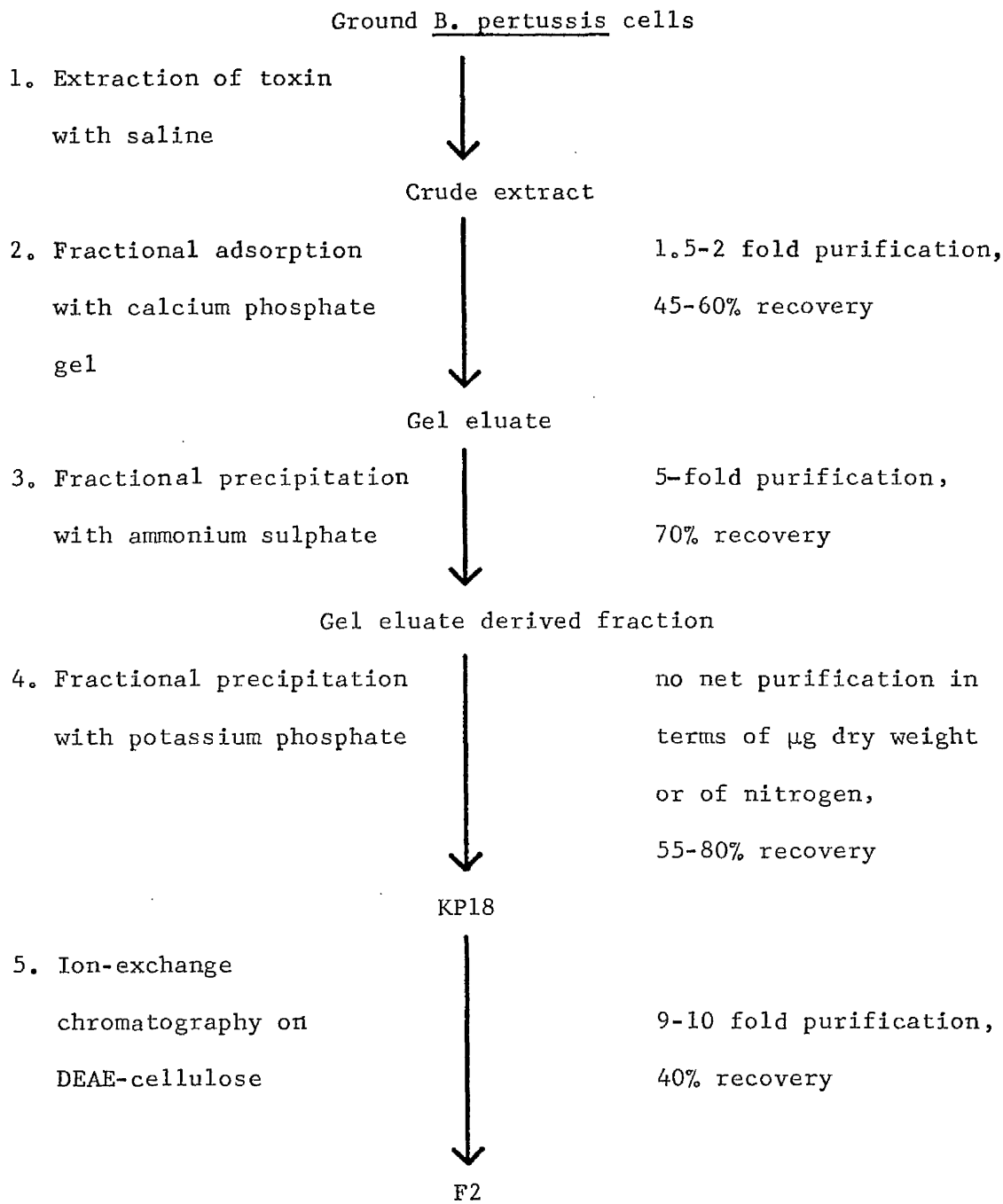

Ground B. pertussis cells
1. Extraction of toxin with saline
Crude extract
2. Fractional adsorption with calcium phosphate gel
1.5-2 fold purification, 45-60% recovery
Gel eluate
3. Fractional precipitation with ammonium sulphate
5-fold purification, 70% recovery
Gel eluate derived fraction
4. Fractional precipitation with potassium phosphate
no net purification in terms of μg dry weight or of nitrogen, 55-80% recovery
KP18
5. Ion-exchange chromatography on DEAE-cellulose
9-10 fold purification, 40% recovery
F2

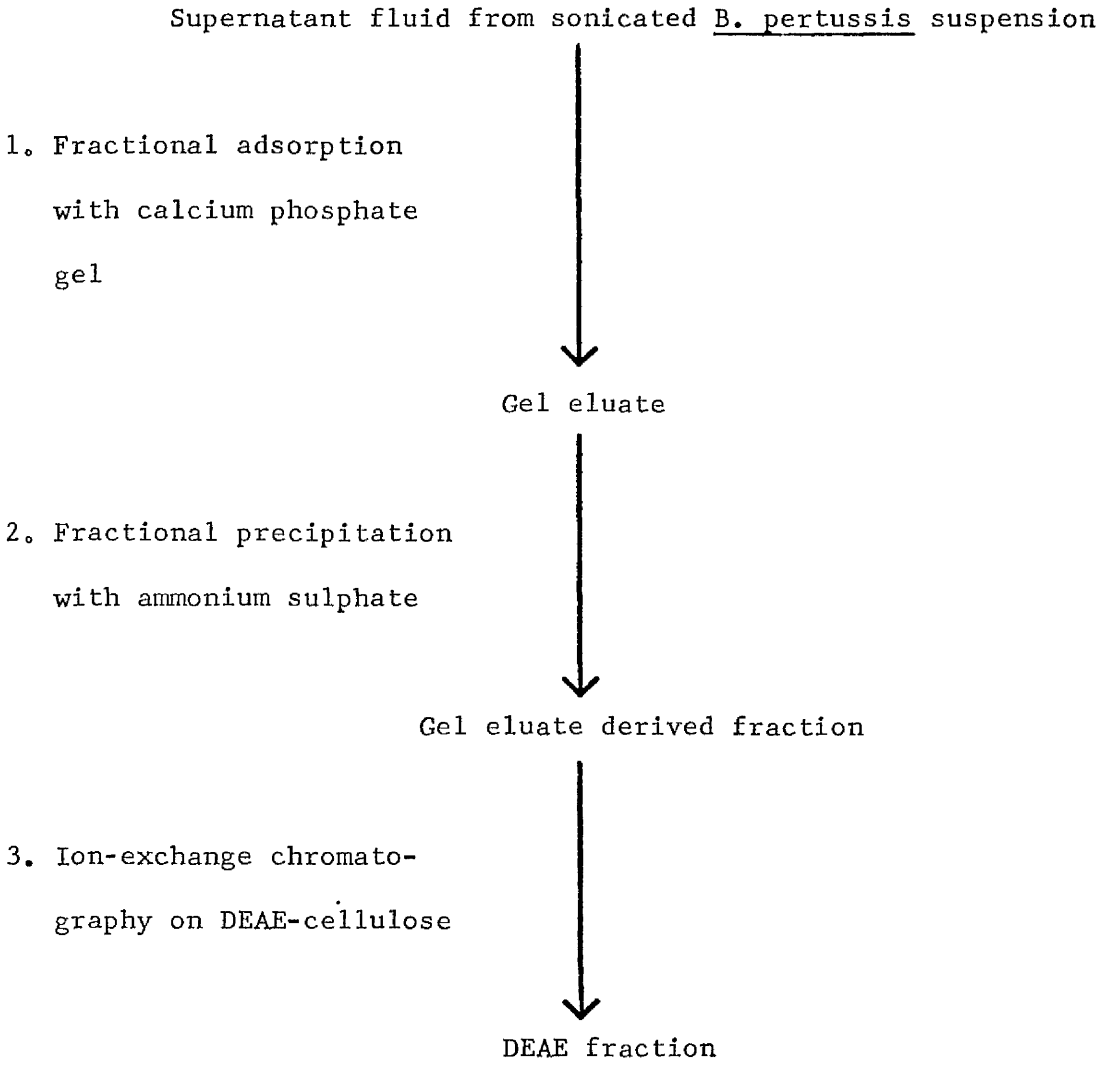
Figure 6 Purification procedure of Iida and Okonogi (1971).

The starting material was the supernatant fluid from a sonicated B. pertussis suspension (100-250 mg cells/ml distilled water).

1 The supernatant fluid was diluted 1 in 1.5 in distilled water and mixed with calcium phosphate gel. The gel was collected by centrifugation and extracted with 0.1M, pH 8.0 phosphate buffer. The gel eluate was collected by centrifugation.

2 A saturated solution of ammonium sulphate was added to the gel eluate to a final concentration of 31%. The precipitate was collected by centrifugation, dissolved in distilled water and dialysed against 0.001M, pH 7.4 phosphate buffer.

3 The ammonium sulphate fraction was fed onto a DEAE-cellulose column equilibrated in 0.001M, pH 7.4 phosphate buffer. This was followed by stepwise elution with the following buffers: 0.005M, pH 7.4 phosphate buffer + 0.01M NaCl: 0.02M, pH 7.4 phosphate buffer + 0.01M NaCl: 0.04M, pH 7.4 phosphate buffer + 0.05M NaCl. The fractions eluted with the final buffer were pooled, precipitated with ammonium sulphate (50% saturation), dissolved in distilled water and dialysed against 0.005M, pH 7.4 phosphate buffer. This material was further purified by rechromatography on a smaller column and then reconcentrated with ammonium sulphate (50% saturation).


Supernatant fluid from sonicated B. pertussis suspension
1. Fractional adsorption with calcium phosphate gel
Gel eluate
2. Fractional precipitation with ammonium sulphate
Gel eluate derived fraction
3. Ion-exchange chromatography on DEAE-cellulose
DEAE fraction

was in the region of 11% and 1% according to whether calculations were based on dermonecrotic or lethal activity. The purified material was still heterogeneous as shown by the formation of more than 1 precipitin line on Ouchterlony gel diffusion and immuno-electrophoresis. On intravenous injection of the purified toxin into mice a marked leukocytosis developed but this peaked at day 1 and not between days 3 and 5 as is characteristic for that induced by LPF (Morse and Morse, 1976).

Nakase et al (1969) also obtained a highly active preparation by fractionating the supernatant fluid from a sonicated B. pertussis cell suspension by chromatography on a DEAE-cellulose column, precipitation with ammonium sulphate and preparative acrylamide gel electrophoresis (figure 7). This was a development of earlier work (Nakase and Kasuga, 1962). The purified material had an MRD for guinea pigs of 1.6 ngN, an increase in purity over the sonic extract of 80 times. This preparation was shown to be homogeneous by ultracentrifugation, thin layer acrylamide electrophoresis, electron microscopy and in agar gel diffusion tests with antitoxin or an antitoxin containing antisera against phase 1 B. pertussis. It was free of the intracerebral mouse protective antigen, HSF and haemagglutinin (1 mg amounts tested), but agglutinins could be detected in some antitoxic sera made to the purified HLT. An increased leukocytosis was produced in mice injected intravenously with the purified toxin but the peak of leukocytosis was at day 1 and not between days 3 and 5 as is found with LPF (Morse and Morse, 1976). This purified HLT is capable of stimulating interferon production in rabbits and rabbit cell cultures (Kojima, Yoshida and Nakase, 1973).

Table 7 summarizes the pertinent points from these attempted purifications of HLT. The 3 most promising methods are those of Onoue,

Figure 7 Purification procedure of Nakase et al (1969).

The starting material was the supernatant fluid from a sonicated suspension of B. pertussis (1.5×10^9 cells/ml distilled water).

1 The HLT was adsorbed onto a column of DEAE-cellulose equilibrated with 0.005M, pH 8.0 phosphate buffer and eluted in a stepwise manner with increasing concentrations of pH 7.6 phosphate buffer ie, 0.01M, 0.02M, 0.05M. Selected toxic fractions eluted with the 0.05M buffer were concentrated by ultrafiltration (ie, F2).

2 Ammonium sulphate was added to F2 to give a 10% (w/v) solution. The precipitate formed was removed and further ammonium sulphate added to give a 20% (w/v) solution. The precipitate formed in this solution was collected and this salt fractionation step repeated twice more. The final precipitate was dialysed against 0.01M phosphate buffered saline and then freeze-dried.

3 The above product was dissolved in 0.3M, pH 8.2 borate buffer (50 mg in 3.3 ml), mixed with a small amount of sepharose 4B gel and electrophoresed in borate buffer on an acrylamide gel column (60-70V, 40mA, 35-48h, 1-2^{o}C). Selected toxic fractions were pooled, dialysed and freeze-dried.

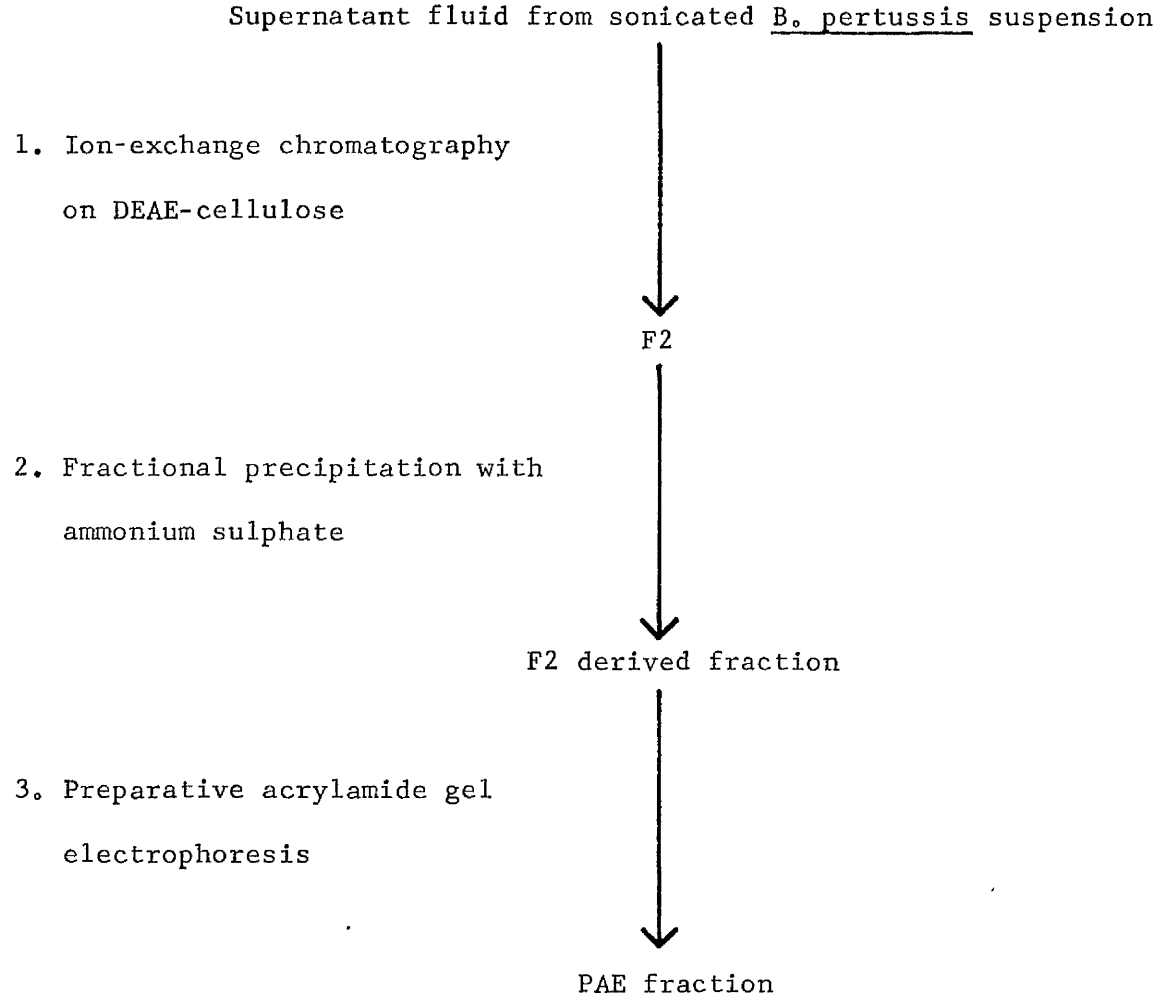

Supernatant fluid from sonicated B. pertussis suspension
1. Ion-exchange chromatography on DEAE-cellulose
F2
2. Fractional precipitation with ammonium sulphate
F2 derived fraction
3. Preparative acrylamide gel electrophoresis
PAE fraction

Table 7 Summary of the results of HLT purification studies

Reference	Toxic Activity (ngN)		Increase in purity	HLT yield (%)	Indices of purity
	Guinea Pig MRD	Mouse LD_{50}			
Robbins and Pillemer, 1950	ND	190	13.2	66	Heterogeneous on electrophoresis
Yamamoto et al, 1957	50[a]	ND	60	ND	Heterogeneous on electrophoresis, agglutinogen present
Onoue, Kitagawa and Yamamura, 1963	1.0 - 1.3	44	60-80	5-10	3 lines in gel diffusion tests, agglutinogen present
Nakase et al, 1969	1.5	ND	80	ND	Homogeneous by thin layer acrylamide electrophoresis, ultracentrifugation, agar gel diffusion tests, electron microscopy. Free of PA, HSF, HA. Agglutinins detected in some antitoxic sera to the purified toxin.
Iida and Okonogi, 1971	0.9	50-100[b]	90[c] 6-12[d]	11[c] 1[d]	More than 1 line in gel diffusion tests and immuno-electrophoresis.

ND No data given

a MRD in rabbit b MLD

c,d calculated from dermonecrotic and lethal activities respectively.

Kitagawa and Yamamura (1963), the modification of this by Iida and Okonogi (1971) and the method of Nakase et al (1969). All produced HLT preparations of a similar high activity. The method of Nakase et al (1969) seems to be best in that their material was essentially free from impurities.

7 Nature

7.1 Relationship between lethal and dermonecrotic activity

Bordet and Gengou (1909) discovered that extracts prepared from B. pertussis cells had dermonecrotic and lethal activity, an observation which has been amply confirmed. These activities are regarded, almost without exception, as manifestations of the same heat-labile toxin. Both properties were inactivated together after treatment with antitoxin or by heating (Katsampes, Brooks and Bradford, 1942; Roberts and Ospeck, 1942 and 1944). Furthermore determinations of the relative potencies of toxic extracts or antitoxic sera have given the same results irrespective of the activity monitored (Evans and Maitland, 1937; Evans, 1940; Anderson and North, 1943; Roberts and Ospeck, 1944). Finally, Onoue, Kitagawa and Yamamura (1963) have demonstrated the presence of both activities in their highly active semi-purified HLT preparation.

In contrast to the aforementioned view Billaudelle et al (1960) considered the lethal and dermonecrotic activities of B. pertussis to be attributable to 2 distinct factors. They separated the dermonecrotic and lethal activities although the fraction which contained the more readily inactivated lethal activity also expressed dermonecrotic activity. Further data which could be interpreted as supporting the view of Billaudelle et al (1960) was provided by Iida and Okonogi (1971) ie, during their purification of HLT there were 90 and 6-12 fold

purifications in terms of dermonecrotic and lethal activities respectively. However the expressions of the lethal and dermonecrotic activities may be affected by other constituents of B. pertussis (Ishida, 1968; Kurokawa, Ishida and Asakawa, 1969) and therefore not always run hand in hand. Consequently when Cravitz and Williams (1946) observed that per MLD the dermonecrosis produced by toxin extracted from cells was greater than that of toxic culture filtrates they were probably witnessing this effect. This could also account for Sauer vaccinated mice being partially protected against extracted toxin but not toxic filtrates. An alternative interpretation of these data proposed by Cravitz and Williams (1946) was the existence of a dermonecrotic factor in addition to HLT.

7.2 Chemical nature

Nakase et al (1969) obtained HLT in a highly active and purified form. They showed it to be homogeneous and free of several of the other pathophysiological activities expressed by B. pertussis (table 7). On chemical analysis positive tests for protein, amino acids and sugars were obtained. The UV absorption spectrum for the toxin was also characteristic of a protein. Tests for nucleic acids, lipids, and phosphorus were negative. Their HLT preparation was 14-16% nitrogen, 1.4% reducing sugar, and contained at least 13 different amino acids, mannose and an additional unidentified reducing substance. In short HLT appears to be a protein with a sugar moiety.

The findings of Nakase et al (1969) confirmed earlier reports, based on less highly purified material, that protein was a major constituent of HLT (Strean and Grant, 1940; Yamamoto et al, 1957; Kuroya et al, 1959 cited by Nakase et al, 1969; Nakase and Kasuga, 1962; Banerjea and Muñoz, 1962; Onoue, Kitagawa and Yamamura, 1963; Nakase

et al, 1965). In addition to physiochemical data on the chemical nature of HLT the toxin has been shown to be destroyed by the proteolytic enzyme trypsin (Banerjea and Muñoz, 1962; Cowell, Hewlett and Manclark, 1979) but not by DNase or RNase (Banerjea and Muñoz, 1962). In contrast toxin preparations prepared by Yamamoto et al (Yamamoto, Zenyoji and Kato, 1952a and c; Yamamoto et al, 1952 and 1953) were considered to be nucleoproteins with DNA as the active principle since DNase destroyed toxic activity (Yamamoto et al, 1953). However the purity of the DNase was suspect and in a subsequent study HLT was considered to be a simple protein (Yamamoto et al, 1957).

7.3 Parameters defining the physical nature of HLT

No estimations have been made of the molecular weight of HLT although values have been given for the sedimentation coefficient. The latter is a function of molecular weight but is also dependent upon shape and density. Results so far are inconclusive and range from 1.4S (Nakase et al, 1969), to 13S (Sato et al, unpublished, cited by Nakase et al, 1969), to 20S (Kuroya et al, 1959 cited by Nakase et al, 1969). It has been suggested by Nakase et al (1969), who obtained the low S value with their purified HLT preparation, that the higher values reported may be due to aggregation of HLT with other constituents of B. pertussis cells.

The isoelectric point of the toxin appears to be within the pH range of 4.0-4.8, as between these values it precipitates out of solution (Burrell, Robbins and Pillemer, 1948). Several workers have taken advantage of this property in attempts to purify the toxin by adjusting the pH to between 4.0 and 4.5 (Evans, 1940; Flosdorf and Kimball, 1940; Strean and Grant, 1940; Pennell and Thiele, 1951; Yamamoto, Zenyoji and Kato, 1952a and c; Yamamoto et al, 1952 and 1957).

Further confirmation that the pI is in the acidic pH range is the adsorption of HLT onto the anion-exchange resin, DEAE-cellulose at pH values near neutrality (Billaudelle et al, 1960; Banerjea and Muñoz, 1962; Nakase and Kasuga, 1962; Onoue, Kitagawa and Yamamoto, 1963; Nakase et al, 1969; Iida and Okonogi, 1971) but not onto the cation-exchange resin, CM-cellulose (Onoue, Kitagawa and Yamamura, 1963). Similarly on electrophoresis at pH 7.4, 7.8 and 8.2 the toxin migrated to the anode (Billaudelle et al, 1960; Onoue, Kitagawa and Yamamura, 1963; Nakase et al, 1969).

7.4 Stability

Strean and Grant (1940) reported HLT as being stable at 4°C for several months. Indeed it may retain its full potency for 4 d (Kuwajima, Iwamura and Hirai, 1951) to a week (Wood, 1940), possibly even 10 d (Katsampes, Brooks and Bradford, 1942) at 3-5°C. By 1 mth there may be a 50% loss of activity, it taking 1 yr for full inactivation (Roberts and Ospeck, 1942). In contrast to all these results, Evans and Maitland (1937) measured a 75% loss in toxic activity in 2 d and by 2-4 wk almost all activity had gone. They also reported the following losses in activity at higher temperatures: 80% after 2 d at 22°C, 95% after 2 d at 37°C or 9 h at 40°C, until at 55°C virtually all activity is destroyed in 10 min. These figures illustrate the increasing lability with increases in temperature but different values for the amount of inactivation at any one temperature have been obtained eg, at room temperature, full inactivation in 1 mth (Roberts and Ospeck, 1942), at 40°C no significant reduction in toxicity after 3 h (Onoue, Kitagawa and Yamamura, 1963).

Since the first report that the toxin's activity was much reduced by heating at 55°C for 30 min (Bordet and Gengou, 1909) there

have been many references to the stability of the toxin at temperatures in this region ie, that toxic activity is destroyed in 5-10 min at temperatures between 50-55°C (Wood, 1940; Evans and Maitland, 1937; Katsampes, Brooks and Bradford, 1942; Roberts and Ospeck, 1942; Kuwajima, Iwamura and Hirai, 1951). Most results have been recorded at 56°C and not all of these are in agreement with the aforementioned results obtained at 50-55°C (table 8). However 30 min at 56°C seems adequate in most instances to inactivate HLT whilst in solution.

In view of the heat lability of the toxin undue generation of heat should be avoided eg, by excessive grinding whilst disrupting B. pertussis cells (Evans and Maitland, 1937).

Other physical factors may influence how active the toxin will remain. For instance, at pH 9.5 toxicity is destroyed (Yamamoto et al, 1953) and it is also unstable at acidic pH values (Onoue, Kitagawa and Yamamura, 1963) being least labile at pH 7.4 (Billaudelle et al, 1960). Yet HLT may be precipitated out, in an active form, at its isoelectric point which is well into the acidic pH range (4.0-4.8; Burrell, Robbins and Pillemer, 1948). The oxidative effect of ultrasonic treatment may also inactivate the toxin although this can be avoided if an atmosphere of hydrogen is used (Tournier 1948, M.D. Thesis, cited by Grabar, 1951). Kasahara et al (1938, cited by Billaudelle et al, 1960) and Smolens and Flavell (1947) also found the toxic activity could be destroyed during ultrasonic treatment. Whether the degeneration in toxicity was due to the oxidative effect or excessive heating was not specified. Finally a combination of factors may result in detoxification although individually not sufficient to produce this effect eg, the combined influence of elevated pH and vigorous aeration in vortex-aerated cultures of B. pertussis (Lane, 1968a).

Table 8 Inactivation of HLT at 56°C

≤ 30 min	30 min	30-60 min
Lawson, 1933	Strean, 1940 and 1942	Flosdorf, Bondi and Dozois, 1941
Anderson and North, 1943	Evans, 1940	Robbins and Pillemer, 1950
Pittman, 1970	Ehrich et al, 1942	Střižova and Trlifajova, 1964
Iida and Okonogi, 1971	Flosdorf and McGuinness, 1942	
Muñoz, 1971	Yamamoto, Zenyoji and Kato, 1952b and c	
	Billaudelle et al, 1960	
	Onoue, Kitagawa and Yamamura, 1963	

A variety of chemical agents have deleterious effects on the toxic activity of HLT: organic solvents; acetone, chloroform, cresol, ethanol, ether, methanol, phenol, thymol and toluene (Bordet and Gengou, 1909; Evans, 1942; Anderson and North, 1943; Pennell and Thiele, 1951; Billaudelle et al, 1960; Onoue, Kitagawa and Yamamura, 1963); the preservatives merthiolate (Evans, 1942; Anderson and North, 1943; Lane, 1968a) and formalin (Introduction; section II, 8.1); the surface active agent sodium deoxycholate (Barta, 1963; Nakase et al, 1965). Sensitivity to inactivation by ethanol has been shown to be accentuated by the use of $CaCl_2$ to clarify toxic extracts, lowering the pH or ionic strength, or by dilution (Pennell and Thiele, 1951).

HLT seems to be relatively stable while inside the cell and protected from injury, thus the toxicity of cresol or merthiolated cells persists longer than that of toxin in solution (Anderson and North, 1943; Muñoz, 1963). On releasing HLT from the cell it may be difficult to keep it in an active state (Muñoz, 1963), which makes its purification all the more difficult eg, Billaudelle et al (1960). Nevertheless HLT can be maintained in an active form eg, by storage in a dried form (Bordet and Gengou, 1909). Dried toxic culture filtrates have been kept at chillroom temperatures for as long as 18 mth without loss of potency (Roberts and Ospeck, 1942). Anderson and North (1943) kept toxin for 12 mth without loss of activity by sealing in vacuo and storing at $2^{o}C$. Evans and Maitland (1937) also preserved freeze-dried toxin for long periods of time. Heat-labile toxin has also been stored frozen eg, 2 of 3 toxin solutions were relatively stable whereas the 3rd was partially inactivated within a short period of time (Kurokawa, Ishida and Asakawa, 1969). An alternative method of storing the toxin is in 50% glycerine or sucrose. Preparations stabilized in this manner were kept over a period of 17 mth without loss

of potency (Roberts and Ospeck, 1942 and 1944). Toxin reconstituted in a 50:50 mixture of glycerol and broth with subsequent storage at 10°C proved after 1 mth to be just as potent as freshly reconstituted toxin (Cravitz and Williams, 1946).

8 Immunology

8.1 Production and measurement of antitoxin in animals

For many years after its discovery by Bordet and Gengou (1909) HLT was considered to be non-immunogenic. Various attempts to raise antitoxin to HLT in animals had failed eg, Bordet and Gengou (1909); Miller (1934); Evans and Maitland (1937); Wood (1940). However, in 1929 Teissier et al had demonstrated the production of neutralizing antibodies to HLT in a sheep, a donkey and guinea pigs. Yet, aside from this isolated report, it was not until the 1940's that the immunogenicity of HLT was firmly established (appendix 2).

Although antitoxin has been produced in a sheep, a donkey, guinea pigs (Teissier et al, 1929) and horses (Demnitz, Schlüter and Schmidt, 1936 cited by Weichsel et al, 1940; Proom, 1947) rabbits have been the preferred species. Rabbits were employed in at least 28 of 31 reports which referred to antitoxin production (appendix 2). On the other hand the active immunization of mice against HLT with either native or formaldehyde-detoxified toxin has not met with success (Wood, 1940; Anderson and North, 1943; Ospeck and Roberts, 1944). Neither subcutaneous nor intraperitoneal injections of toxoid, identical to that effective in rabbits, were productive in spite of intensive immunization. The mice received in a course of 5 injections about 10 times as much toxoid, on an equal body weight basis, as the rabbits (Anderson and North, 1943).

A review of the literature has identified 24 references on the production of antitoxin in animals by immunization with B. pertussis cells

(5 citations), preparations of native or formaldehyde-detoxified HLT (9 and 17 citations respectively) or combinations of these materials (3 citations). In a further 7 references details of the material used were absent or ambiguous (appendix 2). It is difficult to assess which of B. pertussis cells, native or formaldehyde-detoxified HLT is superior in stimulating antitoxin production from the data available (appendix 2). The validity of comparing the antitoxin titres to each of these immunogens is questionable as the influence of other factors must be considered eg, the route, frequency and dosage of injections, differences in the responsiveness of animals etc. However it must be noted that whilst the immune response to B. pertussis cells may produce antitoxic antibodies it often does not, even though a strong response has been made to other B. pertussis components (Bordet and Gengou, 1909; Evans and Maitland, 1937; Evans, 1942 and 1944; Verwey and Thiele, 1949). Moreover, except for one report of success with formaldehyde-killed cells (Flosdorf, Bondi and Dozois, 1941) antitoxic responses to B. pertussis cells have been achieved only with live organisms. For instance although immunizations with live organisms of strain No. 33 yielded antitoxin (Roberts and Ospeck, 1944; Kuwajima et al, 1958) when killed cells were used (formaldehyde, phenol, merthiolate or heat-killed; 56 or 100°C for 30 min) no antitoxin was detected ie, a difference in titre of at least 16-fold (Kuwajima et al, 1958). Yet 30 times as many killed as live cells were used and substantial agglutinin titres were attained in response to the former. Therefore in conclusion B. pertussis cells would not seem to be as suitable in this context as native or formaldehyde-detoxified HLT. Of the 3, the toxoid is superior in eliciting an antitoxic response, according to Roberts and Ospeck (1944). The conditions used to make these toxoids are given in table 9. The concentrations of formaldehyde used ranged from 0.032-0.16% (w/v), averaging 0.1% (w/v) and the temp-

Table 9 Conditions used to convert HLT with formaldehyde into toxoid

Reference	% (w/v) formaldehyde[a]	temperature (°C)	length of incubation
Teissier et al, 1929	0.12	38	1 mth
Evans, 1940	0.08	37 0	1 wk 3 wk
Strean and Grant, 1940	0.16	37	10 d
Evans, 1942	0.08	37	2 wk
Roberts and Ospeck, 1942 and 1944; Ospeck and Roberts, 1944	ND	40	ND
Silverthorne and Cameron, 1942	0.12	37	10 d
Anderson and North, 1943	0.08	37	7 d
Cravitz and Williams, 1946	0.1[b] 0.15[c]	40 40	4 d ≦8 d
Yamamoto, Zenyoji and Kato, 1952b	0.16	37	25 h
Dolby and Standfast, 1958 and 1961	0.032	ND	ND

ND No data

a Assuming formalin is 40% (w/v) formaldehyde

b,c Production of toxoid as described by Joslin and Christensen, 1940 and Weichsel et al, 1940 respectively.

eratures from 37-40°C, although Evans (1942) followed an initial period of incubation at 37°C by storage at 0°C. These conditions are typical of those used in toxoiding other bacterial, protein toxins. The duration of the incubation period varies greatly from 25 h to 1 mth but detoxification may be very rapid under these conditions being complete within 20-25 h (Evans and Maitland, 1937; Yamamoto, Zenyoji and Kato, 1952b).

Typically the immunization schedules followed to obtain antitoxin in rabbits have consisted of a series of injections at 3-7 d intervals with increases in dosage as the immunization progresses. Data, from the references already reviewed (appendix 2), reveals that the number of injections has ranged from as few as 5 (Anderson and North, 1943) to as many as 27 (Andersen, 1953b). An analysis of the injection routes employed (19 references, 23 citations) yields the following; the subcutaneous route 13 times, the intravenous route 6 times (including figures from 2 studies in which both routes were used; Flosdorf, Bondi and Dozois, 1941; Evans, 1942), both subcutaneous and intravenous routes in conjunction 3 times and the intramuscular route once (Silverthorne and Cameron, 1942). Strean and Grant (1940) and Evans (1942) have found subcutaneous injections to be superior to intravenous ones in eliciting an antitoxic response eg, 4-32 times higher titres could be obtained (Evans, 1942). Flosdorf, Bondi and Dozois (1941) made no distinction between the 2 routes merely commenting that antitoxin could be raised by either method. Adjuvants have only been used by Andersen (1953b) and Nakase et al (1969) who used Al $(OH)_3$ and Freund's adjuvant respectively. One of the most promising immunization schedules seems to be that followed by Iida and Okonogi (1971) with which antitoxin titres of 40,960 and 6,400 units/ml were attained, reflecting the numbers of MRD's and MLD's neutralized in guinea pigs and mice respectively. It is possible however that their success may be more closely associated with the use of

their purified HLT as an immunizing agent. Unfortunately no details are provided which could clarify this point.

The development of antitoxic immunity to HLT has most often been measured by testing sera in toxin-antitoxin neutralization tests rather than challenging immunized animals with HLT (appendix 2). Rabbit antisera have been assayed for their capacity to neutralize the dermonecrotic property of HLT with rabbits (12 references), guinea pigs (3) and suckling mice (Andersen, 1952 and 1953b) as the indicator species. Likewise guinea pig, donkey and sheep sera have been tested in guinea pigs (Teissier et al, 1929) and horse sera in rabbits (Proom, 1947). Measurement of the neutralization of the lethal effect of HLT in vitro has been done in mice (12 references), suckling mice (Andersen, 1953b) and guinea pigs (Evans, 1940). The development of resistance to dermonecrosis has been measured in rabbits (8 references) and guinea pigs (Teissier et al, 1929). Lawson (1933) also adopted this assay method but did not specify the animal species used. Other ways of demonstrating antitoxin include the in vitro neutralization of the lienotoxicity of HLT for mice (Iida and Okonogi, 1971) or its cytotoxicity for tissue cultures (Stržova and Trlifajova, 1964). It has also been assayed by the passive immunization of guinea pigs (Evans, 1940) and mice (Ospeck and Roberts, 1944) to resist the lethal effect of HLT, and rabbits the lethal and dermonecrotic activities (Ospeck and Roberts, 1944). Actively immunized rabbits have also been shown to be resistant to killing by HLT (Evans, 1942; Ospeck and Roberts, 1944).

To determine antitoxin titres by neutralization of HLT in vitro a constant dose of toxin may be titrated against graded amounts of antitoxin or constant serum with varying doses of toxin. Of the references listed in appendix 2 the constant toxin-varying serum technique was used 16 times and the constant serum-varying toxin technique 9 times (3 of which were the publications by Roberts and Ospeck). Included in these

figures were 4 references where both techniques were employed, in 2 of which were indicated a preference for the constant toxin-varying serum technique (Teissier et al, 1929; Evans, 1940 cf. Flosdorf, Bondi and Dozois, 1941; Smolens and Flavell, 1947). Irrespective of the technique used, neutralization is achieved by incubation at either 37°C for between 1-2.5 h (5 references) or at room temperature for between 30 min to 3 or 4 h (9 references), although Teissier et al (1929) held the mixtures at the latter temperature for 24 h. The only other conditions used have been 1 h at 32°C (Roberts and Ospeck, 1942 and 1944; Ospeck and Roberts, 1944) and overnight at 4°C (Nakase et al, 1969). HLT is neutralized in vitro according to the rule of multiple proportions (Evans, 1940; Proom, 1947) eg, if 10 MRD is inactivated by a 1 in 50 serum dilution then 100 MRD would be inactivated by a 1 in 5 dilution of serum.

The relationship between the resistance of immunized rabbits to the dermonecrotic effect of HLT and the titres of antitoxin developed as assayed by in vitro neutralization of dermonecrosis has been investigated by Ospeck and Roberts (1944). One hundred and five paired observations were made recording the titres of antitoxin and whether a dermonecrotic response was made to 3 mouse MLD of HLT. Where titres were $\leq$ 10 units/ml (1 unit neutralizing 1 mouse MLD) only 10% of the rabbits were resistant to the skin reaction, at titres between 10 and 50 units/ml 80% were resistant, and at titres of over 100 units/ml 90% were resistant. Therefore, resistance to dermonecrosis in vivo whilst an index of antitoxic immunity does not necessarily go hand in hand with the antitoxin titres measured in vitro. Hence results such as those obtained by Silverthorne and Cameron (1942) where antitoxin was detected in the sera of rabbits which had given negative skin tests are possible. The relationship between these tests is also borne out in the results obtained by Evans (1942). In contrast, the correlation between antitoxic immunity as measured by in vitro neutral-

ization of the dermonecrotic and lethal effects of HLT in rabbits and guinea pigs respectively, and by passive protection of guinea pigs from being killed with HLT, were good ie, 3 sera were arranged in the same order of potency by these methods (Evans, 1942). However such assay procedures do differ in their sensitivity in detecting antitoxin. The tests involving neutralization of dermonecrosis are ultimately more sensitive than those tests in which lethality is measured and also have the advantage of only requiring small amounts of serum.

8.2 Antitoxic immunity and protection in intracerebral and respiratory tract infections of mice

To establish whether HLT could act as a protective antigen against an intracerebral infection with B. pertussis as in the intracerebral mouse protection test, mice have been injected with native and modified HLT preparations ie, native toxin (Verwey and Thiele, 1949; Yamamoto et al, 1952 and 1957), heat-inactivated toxin either by 30 min at 56°C or 5 min at 50-55°C (Yamamoto, Zenyoji and Kato, 1952c and Muñoz, Ribi and Larson, 1959 respectively), formaldehyde-detoxified HLT (Onoue, Kitagawa and Yamamura, 1963) and alum-precipitated formaldehyde-detoxified HLT (Nakase and Kasuga, 1962; Nakase et al, 1969). Except for Nakase et al (1969), all of these workers have demonstrated varying degrees of protection in response to injections with the aforementioned preparations. However, protective and toxic properties can, to a large extent, be separated (Verwey and Thiele, 1949; Muñoz, Ribi and Larson, 1959; Nakase and Kasuga, 1962). Indeed Nakase et al (1969) found their purified HLT to lack protective activity. Overall the data so far are inconclusive: where protection has been demonstrated other B. pertussis components could be implicated and where it was absent the development of antitoxic immunity was not confirmed. The use of passive protection tests to determine the

role of antitoxic immunity has likewise not been conclusive. The protection afforded by serum given before an intracerebral challenge with the intracerebrally virulent strain 18323 has been shown to be independent of the antitoxic content of the serum (Dolby and Standfast, 1958). Consequently sera with high titres of antitoxin may lack protective activity (Kuwajima et al, 1958). However whether protection could be afforded by antitoxin if serum and challenge were given mixed together has not been fully investigated. Yamamoto et al (1955) found sera with very low titres of antitoxin protective in such IC/mix experiments but protection might have been due to antibodies other than those to HLT. Antitoxic serum given intracerebrally 2-5 d after the intracerebral challenge is without effect (Dolby and Standfast, 1961).

Antitoxin may be protective when given before an intracerebral challenge with the toxigenic intermediate phase strain No. 33 (Ospeck and Roberts, 1944; Kuwajima et al, 1958). Whether the antibodies responsible were directed against HLT or other B. pertussis components is unresolved, as is the relationship between the protective effect being measured here and in the standard mouse protection test.

The importance of antitoxic immunity to B. pertussis respiratory tract infections in mice is also unresolved. Mice immunized with formaldehyde-detoxified HLT were protected against a lethal intranasal challenge with B. pertussis (Anderson and North, 1943). However, mice immunized intensively with this material failed to exhibit any antitoxic immunity as measured by the development of resistance to the lethal effect of HLT. Mice have also been passively protected against lethal and sub-lethal infections by the parenteral administration of antitoxic sera before or very shortly after an intranasal challenge with B. pertussis (Anderson and North, 1943; Evans, 1944; Proom, 1947). In 2 of these instances once the sera were absorbed with B. pertussis cells,

until all traces of agglutinins were gone, the protective activity was also lost although the antitoxic properties were retained (Anderson and North, 1943; Evans, 1944). In the 3rd instance some protection was claimed for an absorbed antitoxic serum but the data were not very convincing (Proom, 1947). Antitoxic serum instilled intranasally up to 24 h before (North, 1946) or with the challenge (Evans, 1944; North, 1946; Dolby and Standfast, 1958) were protective against lethal and sub-lethal infections even after absorption (Evans, 1944; North, 1946). However, North (1946) also demonstrated protection with an absorbed adult contact serum in which there had been no antitoxin. Therefore credit for the protection afforded by the absorbed antitoxic sera clearly cannot be, without reservations, attributed to anti-HLT antibodies.

8.3 Antitoxic immunity in humans

Little or no antitoxin, as assessed by assaying sera for neutralizing antibodies to HLT, is produced in response to pertussis. When Evans and Maitland (1939) and Evans (1947) tested 63 sera (23 and 40 respectively) taken during the course of the illness, and Flosdorf, Bondi and Dozois (1941) pooled convalescent serum, no antitoxin was detected. However Weichsel, Katona and Liu (1942) detected antitoxin, albeit in low titres, in some of the sera taken from 16 children during and whilst convalescing from pertussis. Low titres of antitoxin have also been demonstrated in children following recovery from the disease. Strean, Lapointe and Dechene (1941) recorded positive results with 7 of 10 sera and Cravitz and Williams (1946) with 3 of 24 sera. In contrast, Weichsel, Katona and Liu (1942) found sera from children with and without a history of pertussis to be indistinguishable. Sera taken from adults, including contact sera and sera from persons with a history of pertussis, have shown little (Weichsel, Katona and Liu, 1942) or no antitoxin (North *et al*, 1939; Weichsel, Katona and Liu, 1942; Evans, 1947).

The response to HLT injected into the skin may also be indicative of the absence or presence of antitoxic immunity to HLT. Antitoxin has been demonstrated in a small number of children who gave negative skin responses (7 and 3) but not in those in whom a skin reaction developed (3 and 2) (Strean, Lapointe and Dechene, 1941 and Cravitz and Williams, 1946 respectively). Also by the passive administration of antitoxin, positive responders have been converted into negative responders (Strean, Lapointe and Dechene, 1941). Furthermore skin test results have correlated with the data on the histories of individuals as regards pertussis (Strean, 1940; Strean, Lapointe and Dechene, 1941; Kunstler, 1945; Cravitz and Williams, 1946) and with immunity as measured by opsonizing and agglutinating anti-pertussis antibodies (Cravitz and Williams, 1946). Mainly on the basis of skin test results it has been suggested by these workers that the newborn can possess passively acquired maternal antibodies to HLT. These results suggest that the development of antitoxic immunity is far more common than indicated in the aforementioned studies in which sera were assayed for neutralizing antibodies to HLT. On the other hand, it has been reported that the skin test may fail to differentiate between children with and without a history of pertussis (eg, Weichsel et al, 1940; Silverthorne, Fraser and Brown, 1944 cited by Evans, 1947). Moreover, the skin test was of no value in distinguishing between vaccinated and un-vaccinated children (Cravitz and Williams, 1946).

In response to active immunization with toxoided HLT or a vaccine with a toxoid component antitoxin has been produced in children (Bullowa et al, 1942; Weichsel, Katona and Liu, 1942; Cravitz and Williams, 1946) and protection against pertussis demonstrated (Joslin and Christensen, 1940; Brereton, 1946; Lapointe, 1946). Likewise

passively administered antitoxin has been claimed to be of value therapeutically, especially if given early in the disease (Strean, Lapointe and Dechene, 1941; Strean, 1942 and 1943), and prophylactically (Bullowa et al, 1942).

OBJECT OF RESEARCH

The heat-labile toxin (HLT) of Bordetella pertussis has been relatively neglected by most recent investigators studying this organism. Yet this is the principal toxin of B. pertussis as judged by the lethal toxicity test in mice. Its mode of action is undefined, its possible role in pathogenicity and immunity unclear, and only one group of investigators claim to have obtained it in a pure state. The present limited study does not attempt to deal with all of these aspects where knowledge is lacking. Instead attention has been directed at the following questions:-

a) Is HLT production suppressed during antigenic variation?

b) Is there any uniquely suitable strain of B. pertussis for maximal HLT production?

c) Is there a particular medium which favours HLT production and what is the preferred time of harvest?

d) Does iron in the culture medium affect toxin production?

e) Can previously published methods for purifying HLT be repeated or adapted?

f) What is the molecular weight of HLT?

g) Can HLT be toxoided?

h) Is HLT toxoid immunogenic?

i) Does anti-HLT protect mice against experimental infection?

Some of these questions appear not to have been studied previously, others represent attempts to confirm earlier work.

Before investigating any of the above, the following questions relating to the assay and storage of HLT were answered:-

i) Do mouse weight and sex affect the responsiveness of mice to the lethal toxicity of HLT?

ii) Has HLT haemolytic activity?

iii) How stable is HLT on storage at -20^{o}C?

MATERIALS AND METHODS

SECTION I BACTERIOLOGICAL MATERIALS AND METHODS

1 Organisms

Strain 18334, a vaccine strain, was obtained from Connaught Laboratories Ltd., Toronto, Canada.

Strain 18323, the intracerebral challenge strain for the mouse protection test, was obtained from 3 sources, viz.,

i) Strain 18323 (BOB) from Dr. M. Pittman, Bureau of Biologics (BOB), Food and Drug Administration, Bethesda, Maryland 20014, USA.

ii) Strain 18323 (L6d H2) from Dr. F.W. Sheffield, Division of Immunological Products Control, National Institute for Medical Research, London, England.

iii) Strain 18323 (L4a) from Dr. P. Kendrick, Michigan Department of Public Health, Michigan 48909, USA.

Strain 134, used by Pillemer (1950) in the preparation of his protective antigen (adsorbed onto human red cell stromata).

Strain MAENO, supplied by Professor Y. Nakase, Kitasato Institute, 5-9-1 Shirokane, Minato-Ku, Tokyo 108, Japan.

Strain No. 28, provided by Dr. P. Novotny, Wellcome Research Laboratories, Beckenham, England.

Strain B-1593/57 from Dr. V. Spasojević, Institute of Immunobiology and Virology, Torlak, Vojvode Stepe 458, Belgrade, Yugoslavia.

Strain 44122/7R, a streptomycin-resistant mutant derived from a vaccine strain 44122/7 and used at the State Bacteriological Laboratory, Stockholm, Sweden. Obtained from Dr. P. Branefors, University of Göteborg, Institute of Medical Microbiology, Department of Bacteriology, Guldhedsgatan 10, S-413 46 Göteborg, Sweden.

Strain 18904 L4, from Dr. P. Kendrick, Michigan Department of Health, Grand Rapids, Michigan, USA.

Strains 77/18319 and 77/24833 were obtained as fresh isolates from Dr. R. Fallon, Department of Bacteriology and Immunology, Ruchill Hospital, Glasgow, Scotland.

Strains GL353Z, L84 I, 3865, D30042 I, D30042 IV, D3148 I and D3148 IV were originally supplied by Dr. J. Dolby, Clinical Research Centre, Harrow, England. The designation IV indicates phase IV variants.

2 Culture media

2.1 Bordet-Gengou medium

Twenty grams of dried Bordet-Gengou agar base (Gibco Bio-Cult Diagnostics Ltd., Paisley, Scotland) was added to 500 ml of a 1% (v/v) solution of glycerol (BDH, Poole, England) in distilled water. The agar base was dissolved by being heated to 100°C with frequent agitation. It was then sterilized by autoclaving at 121°C (15 psi) for 15 min. After cooling to 45-50°C, 100 ml of sterile, 37°C defibrinated horse blood (Gibco Europe) was added aseptically, mixed, and the completed medium poured into sterile, 50 or 90 mm diameter plastic petri dishes (Sterilin Ltd., Teddington, Middlesex, England).

After the medium had solidified the plates were returned to the sterile plastic sleeves in which the empty dishes are supplied and the sleeves were sealed with tape. Plates were stored at 4°C and used within 2 wk.

2.2 Charcoal agar

Dehydrated charcoal agar (Difco Laboratories, Detroit 1, Michigan, USA) was suspended in distilled water to a concentration of 62.5 g/l, heated to boiling with frequent stirring, and sterilized by autoclaving at 121°C (15 psi) for 15 min. After cooling to 45-50°C, the medium was swirled to obtain a uniform suspension of the charcoal and dispensed into sterile plastic petri dishes or universal containers to be

slanted. The medium was stored at 4°C, the plates being wrapped as for Bordet-Gengou plates.

Both Bordet-Gengou medium and charcoal agar were prepared on occasions with nicotinic acid (BDH, Poole, England) added to a final concentration of 500 μg/ml.

2.3 Modified Hornibrook medium

This medium is a modification of the recipe of Hornibrook (1939) as described by Parton and Wardlaw (1975).

	Materials	per 10 l
i	Casamino acids, Difco technical	100 g
ii	$CaCl_2$ (anhydrous)	0.02 g
iii	NaCl	50 g
iv	KCl	2 g
v	Nicotinamide (BDH, Poole, England), 0.1% (w/v) solution	10 ml
vi	$MgCl_2.6H_2O$, 1% (w/v) solution	25 ml
vii	K_2HPO_4, 10% (w/v) solution	25 ml
viii	Soluble starch (BDH, Poole, England)	10 g
ix	Glutathione (Sigma Chemical Company, St. Louis, MO, USA), 0.1% (w/v) solution	100 ml

The ingredients i-iv were dissolved in 800 ml of distilled water and the freshly prepared solutions v, vi and vii added. The starch was suspended in 25 ml of distilled water and this added, with immediate mixing, to 175 ml of boiling distilled water. This mixture was brought back to the boil and added to the rest of the medium. The volume was made up to 1 l and the pH adjusted to 7.0 with approximately 0.45 g of Na_2CO_3. This 10 times concentrate was dispensed in 100 ml amounts in

screw cap bottles, sterilized by autoclaving at 121°C (15 psi) for 15 min, cooled and stored at 4°C.

Prior to use 100 ml of concentrate was added to 890 ml of distilled water in a 2 l dimpled conical flask and autoclaved at 121°C (15 psi) for 15 min. Immediately before use 10 ml of Seitz filter sterilized glutathione solution (0.1% (w/v)) was aseptically added to give Hornibrook X-medium. Hornibrook C-medium was prepared by replacing the 50 g of NaCl with 50 g of $MgSO_4 \cdot 7H_2O$, other ingredients being the same.

2.4 Stainer and Schölte (12G) medium

This is the chemically defined medium described by Stainer and Schölte (1971) except that the amount of Tris buffer has been reduced to one-quarter of that in the original formulation (a modification suggested by Stainer, 1977; personal communication) and L-cysteine replaces cystine.

	Materials	g/l
i	L-glutamate, monosodium salt, (BDH, Poole, England)	8.04
ii	NaCl	2.5
iii	KH_2PO_4	0.5
iv	KCl	0.2
v	$MgCl_2 \cdot 6H_2O$	0.1
vi	$CaCl_2$	0.02
vii	Tris (hydroxymethyl) amino methane, (Sigma Chemical Company, St. Louis, MO, USA)	1.52
viii	L-cysteine (BDH, Poole, England)	0.04
ix	$Fe_2SO_4 \cdot 7H_2O$	0.01
x	Ascorbic acid (BDH, Poole, England)	0.02
xi	Nicotinic acid (BDH, Poole, England)	0.004
xii	Glutathione (Sigma Chemical Company, St. Louis, MO, USA)	0.1

Ingredients i-vii were dissolved in 800 ml of distilled water and the pH adjusted to 7.6 with 2.5N HCl. The volume was made up to 990 ml with distilled water and the medium sterilized by autoclaving at 121°C (15 psi) for 15 min.

Immediately before use ingredients viii-xii were dissolved in distilled water, sterilized by membrane filtration (0.45 μm pore size; Millipore SA, Molsheim, France) and aseptically added to the remainder of the ingredients. To allow for losses during filtration more of the solution was made than the 10 ml required per litre of medium.

3 Growth of cultures

3.1 Stock cultures

Freeze-dried cultures were reconstituted with sterile 1% (w/v) casamino acids solution (see below) and plated onto Bordet-Gengou medium. Plates were incubated at 35°C for 3 or 4 d in a closed plastic box containing a beaker of water to saturate the atmosphere. From this growth stock cultures were prepared on Bordet-Gengou plates or charcoal agar slopes. Cultures in sealed Bordet-Gengou plates or charcoal agar slopes remained viable at 4°C for 4-6 wk.

Casamino acids solution, 1% (w/v), was prepared as follows:-

Materials	g/l
Casein hydrolysate acid, peptone No. 5 (Gibco Bio-Cult Diagnostics Ltd., Paisley, Scotland)	10.0
$MgCl_2 \cdot 6H_2O$	0.1
$CaCl_2$	0.016
NaCl	5.0

The ingredients were dissolved in 950 ml of distilled water, the pH adjusted to 7.1 with 1N NaOH and the volume was made up to 1 l. After dispensing into suitable volumes the solution was autoclaved at 121°C (15 psi) for 15 min.

3.2 Intracerebral and intranasal challenge cultures

Challenge cultures were prepared from stock suspensions of B. pertussis in casamino acids solution (see above) containing 20% (v/v) glycerol. These were stored frozen in sealed, sterile, plastic ampoules (Sterilin Ltd., Teddington, Middlesex, England) in liquid nitrogen. The frozen suspension was thawed at room temperature, plated onto 2 or 3 Bordet-Gengou plates and incubated at 35°C for 3 d in a water saturated atmosphere. "Puddle plate" cultures were prepared from this growth as follows:- a heavy inoculum was emulsified in 3 or 4 drops of 1% (w/v) casamino acids solution on the surface of a Bordet-Gengou plate to give a confluent inoculum in the centre of the plate. Incubation proceeded as already described for 24 h and the growth used to prepare challenge suspensions.

3.3 Shake-flask cultures

For larger scale growth 500 ml or 1 l volumes of medium in 2 l conical shake-flasks were used. These were inoculated with 24-48 h seed cultures either in the form of a generous loopful of Bordet-Gengou grown cells or as a liquid seed culture (1-5% (v/v) inoculum). The flasks were then incubated at 35°C in an orbital shaker set at 80 rpm. Cultures were most commonly harvested after 48 or 72 h of growth. The purity of each culture was checked by Gram-staining and by inoculation onto Bordet-Gengou medium.

3.4 Roux bottle cultures

These were used on only one occasion. One hundred millilitre volumes of medium in Roux bottles were inoculated with a loopful of Bordet-Gengou grown cells and incubated laid flat at 35°C for 4 d. Bottles were gently rocked once or twice a day to suspend any sedimented growth. Purity was determined as for shake-flask cultures.

4 Harvest of cultures

Liquid cultures were harvested by centrifugation for 30 min at 10,000 x g and 4°C. The supernatant fluid was carefully decanted and the cell pellets were evenly suspended, unless otherwise stated, in Dulbecco "A" PBS (Oxoid Ltd., England).

Cultures were harvested from solid medium by scraping the cells off with a loop or by suspending them in Dulbecco "A" PBS and sucking them off with a Pasteur pipette. If required, cells were washed with Dulbecco "A" PBS to remove constituents of the medium before re-suspension.

5 Standardization of bacterial concentration by opacity

The concentration of bacterial suspensions was estimated by comparison with the 5th International Opacity Reference Preparation (designated as having an opacity of 10 international opacity units). The opacity reference was supplied by the World Health Organization International Laboratory for Biological Standards (National Institute for Biological Standards and Control, London, England).

The dilution factor necessary to obtain matching of the cell suspension with the reference preparation was multiplied by 10 to give the concentration of the undiluted bacterial suspension in opacity units (ou). The matching was done visually with the 2 tubes held in front of a card bearing rows of bold print.

When a cell suspension was disrupted the concentration was still described in terms of opacity ie, opacity(unit equivalents (oue)): the concentration of the lysate being directly equivalent to the cell suspension from which it was derived.

6 Estimation of viable cell numbers

Serial dilutions of cultures were made in a 1% (w/v) casamino acids solution (for its preparation see Materials and Methods; section I, 3.1), and duplicate 0.05 ml amounts of appropriate dilutions spread on

Bordet-Gengou plates and incubated at 35°C for 4 d in a water saturated atmosphere. Plates with countable numbers of colonies were selected and from these an estimate made on the number of colony forming units per ml of the original suspension.

SECTION II BIO-ASSAY METHODS

1 Mice

Mice were from a randomly-bred closed colony, originally derived from the Ham/1CR strain (Charles River, UK Ltd.). For the following bio-assays mice of either or both sexes were used. Where both sexes were used they were divided equally between test preparations to avoid possible bias.

2 Toxicity tests

2.1 Mouse lethal toxicity test

Mice of 3-4 wk of age were injected intraperitoneally with 0.5 ml volumes of graded doses of B. pertussis samples. Groups of mice were arranged such that the weight distribution of the mice in each group was similar. Deaths of mice were recorded in the 3 d post-injection period.

2.2 Haemorrhagic activity in suckling mice

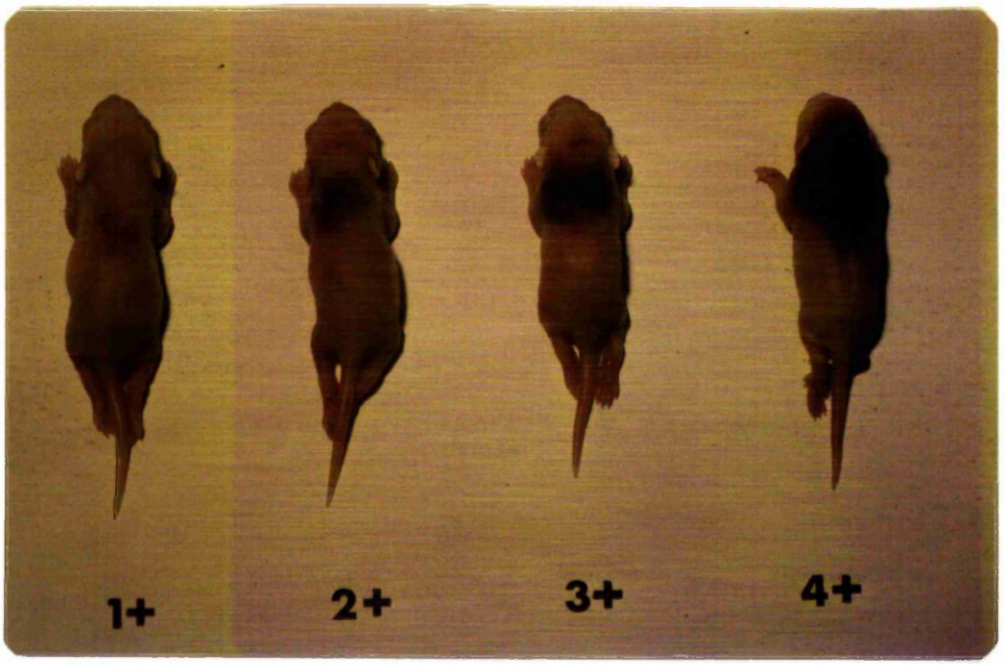
Four day old mice were injected subcutaneously in the back of the neck with 0.05 ml volumes of graded doses of B. pertussis samples. Twenty-four hours later, any deaths were recorded and haemorrhagic reactions were scored on a 1+ to 4+ scale (plate 8).

Neutralization of the haemorrhagic reaction was used as an index of the antitoxin content of antisera. Dilutions of antiserum were mixed with equal volumes of toxin giving a final concentration of 1 minimum reactive dose (MRD) of toxin per 0.05 ml (ie, toxin producing a 1+ haemorrhagic reaction; see plate 8). As a control, normal rabbit serum was titrated in parallel with the antiserum under test. Mixtures were

Plate 8 Haemorrhagic effect of HLT in suckling mice

Haemorrhagic reactions are graded on a 1+ to 4+ scale.

One minimum haemorrhagic dose of toxin produces a 1+ reaction.


1+
2+
3+
4+

incubated at room temperature (20-24°C) for 2 h and then assayed for toxin as already described. One unit of antitoxin was taken as the smallest amount of antiserum producing complete neutralization of 1 minimum haemorrhagic dose of toxin.

2.3 Haemolytic activity

HLT was assayed for its ability to lyse sheep, rabbit, horse, human (O, rhesus +) and cod red blood cells suspended in either Dulbecco "A" PBS (Oxoid Ltd., England) or citrate dextrose saline (cod red cells only). To doubling dilutions of HLT made in the U-shaped wells of a WHO tray were added equal volumes (0.5 ml) of washed (4 times) red cells at a concentration of 1% (v/v). Control wells without toxin were included. After gentle mixing the cell suspensions were incubated at 35°C for 30 min before being examined for the development of haemolysis.

3 HSF assay

Mice of 7-8 wk of age were injected intraperitoneally with 0.5 ml of graded doses of vaccine, ie, heat-detoxified (56°C/30 min) B. pertussis cells or cell fraction. After heating, thiomersal (BDH, Poole, England) was added to a final concentration of 0.1 mg/ml and the preparation stored at 4°C.

Five days after vaccination the mice were challenged intraperitoneally with 3 mg of histamine dihydrochloride (Sigma Chemical Company, St. Louis, MO, USA) contained in 0.5 ml of Dulbecco "A" PBS (Oxoid Ltd., England). Four hours later a count was made of the number of mice dead.

4 LPF assay

Groups of 5 mice of 4-5 wk of age were injected intravenously with 0.2 ml of graded doses of samples. Control mice were inoculated

with Dulbecco "A" PBS (Oxoid Ltd., England), ie, the diluent in which the 4-fold dilutions of samples were made. Five days after inoculation mice were anaesthetized with ether and bled from the orbital plexus with heparinized capillary tubes (Harshaw Chemicals Ltd., Daventry, England). The blood samples (40 µl) were immediately transferred with a Coulter pipette into plastic vials containing 20 ml of Isoton II diluent (Coulter Electronics Ltd., Harpenden, England).

Immediately before counting, 6 drops of Zap-O-Globin (Coulter Electronics Ltd., Harpenden, England) were added to each vial to lyse the red blood cells. Enumeration of leukocytes was done using a Coulter Counter, model FN (Coulter Electronics Ltd., Harpenden, England). Duplicate or triplicate counts of each diluted blood sample were made and the mean value calculated for each sample. To correct for coincidence losses during counting, mean counts above 10,000 were adjusted by consulting a coincidence correction chart. Mean background counts, from diluent alone, were subtracted from each corrected count to obtain the final corrected mean count for each sample.

5 Passive intracerebral mouse protection test

Rabbit antisera were tested for their ability to protect groups of 10, 3-4 wk old mice against a lethal intracerebral challenge with B. pertussis, viz. 10^4 cfu of the intracerebrally virulent strain 18323 L6d H2. This was notionally equivalent to 10^5 organisms on the basis that a suspension of 10 ou equals 10^{10} organisms/ml. The virulence of the challenge culture was checked by injecting groups of mice with 10^4, 2×10^3 and 4×10^2 organisms per mouse in addition to the challenge controls which received 10^5 organisms. Mice were distributed so that each cage contained animals representing each type of treatment. Intracerebral injections (0.03 ml/ether anaesthetized mouse) were always

completed within 3 h of harvesting the cells after which the viability of the organisms in the challenge suspensions was rechecked.

Antisera were assayed for their protective capacity either by giving 0.2 ml of undiluted serum intraperitoneally 1-4 h before the challenge (IP/IC) or by mixing equal volumes of heat-treated antiserum with the challenge organisms suspended in 1% (w/v) casamino acid solution (IC/mix). The heating of these sera (56^{o}C/10 min) was to eliminate the possibility of complement mediated killing of the challenge. Cells were suspended in 1% (w/v) casamino acids solution for all IP/IC challenges and for IC/mix challenge controls. However cells suspended in normal rabbit serum were usually also included as controls for IC/mix experiments.

For 14 d after the intracerebral injections mouse deaths were recorded. Mice dying within 2 d of the challenge were excluded from the final count, not being considered "infection deaths". Differences between the results from control and test groups were tested for their statistical significance by consulting 2 x 2 contingency tables (Finney et al, 1963).

6 Passive intranasal mouse protection test

Rabbit antisera were tested for their ability to protect 3-4 wk old mice challenged with a sub-lethal intranasal dose of B. pertussis 18323 (L6d H2 or L4a). The number of organisms instilled intranasally was 10^{5} cfu (or 10^{6} organisms on the assumption that a suspension of 10 ou equals 10^{10} organisms/ml). The challenge was given to the ether anaesthetized mice in 2 (0.025 ml) drops from a dropping pipette (Cooke Engineering Company, Medical Research Division, Virginia, USA). Mice were caged such that each cage contained animals representing each type of treatment. All challenges were completed within 3 h of harvesting the cells after which their viability was rechecked.

Antisera were also tested for their protective capacity when

mixed with the challenge organisms (1N/mix). Equal volumes of cells suspended in 1% (w/v) casamino acids solution and heated antisera were mixed. Sera were heated (56°C/10 min) to prevent complement mediated killing of the challenge. Controls included cells suspended in 1% (w/v) casamino acids solution and, in most instances, cells mixed with heated normal rabbit serum. One antiserum (B) was also tested by injecting 0.2 ml intraperitoneally into mice 1-3 h before a challenge with cells suspended in 1% (w/v) casamino acids solution.

The mice were inspected daily for 14 d and deaths recorded. On day 14 the remaining mice were killed and the lungs inspected for signs of consolidation ie, greyish discoloured areas. Healthy lungs were scored as 0 and infected lungs from 1+ signifying a few small areas of consolidation to 4+ for complete or almost complete consolidation in all lobes of the lungs. A snippet of lung from the smallest of the right hand lobes was also taken and the cut surface smeared onto a Bordet-Gengou plate. After 4-5 d of incubation at 35°C scores were allocated for the resultant *B. pertussis* growth viz. 0, no growth; 1+, 1-9 colonies; 2+, 10-99; 3+, 100-semi confluent; 4+, confluent. The scores for lung pathology and culture were combined, giving totals ranging from 0 to 8, and the statistical significance of differences in the results from control and test groups assessed in the Mann-Whitney U-test. The arithmetic mean of the combined scores was also calculated as an index of the severity of the infections. Differences in the numbers of mice infected in the control and test groups were tested for their statistical significance by consulting 2 x 2 contingency tables (Finney *et al*, 1963). Mice were considered to be infected if they had positive lung pathology and/or culture.

SECTION III BIOCHEMICAL MATERIALS AND METHODS

1 Analytical

1.1 Protein estimations

For estimation of soluble proteins the method of Lowry et al (1951) was used. Where the protein content of cell-lysates or crude cell-envelope preparations was to be determined a slightly modified Lowry method was used (Herbert, Phipps and Strange, 1971). Bovine serum albumin (Fraction V, Sigma Chemical Company, St. Louis, MO, USA) was used as a standard.

The protein content of fractions from chromatographic columns was monitored by measuring the absorbance at 280 nm in a Pye Unicam SP500 spectrophotometer with a quartz 1 cm flow through cell linked to a SP40 P automatic sample changer and a SP22 chart recorder.

1.2 Iron estimations

For the determination of iron in culture media the method of Herbert, Phipps and Strange (1971) was used.

Reagents (all from BDH, Poole, England)

2,2'-Bipyridyl : 1 g was dissolved in 100 ml of 1% (w/v) hydrochloric acid

Reducing reagent : 3.5 g of hydroxyammonium chloride was dissolved in 100 ml of distilled water

Acetate solution : 355 g of AR grade ammonium acetate was dissolved in distilled water and diluted to 500 ml

6M Perchloric acid : 265 ml of 72% AR grade perchloric acid was diluted to 500 ml with distilled water

Stock standard iron solution : 0.4318 g of AR grade ferric ammonium sulphate was

dissolved and diluted to 100 ml with 0.1N hydrochloric acid to give a solution containing 0.5 mg iron/ml

Working iron standard: 10 ml of stock iron solution was diluted to 250 ml with distilled water to give a solution containing 20 µg iron/ml. This was prepared freshly for each day of testing

Culture medium (10 ml, containing not more than 2 µg iron/ml) was measured into a 6 x $^{5}/_{8}$ in. test tube and mixed with 1 ml of 6M perchloric acid. This was held at 4°C for 30 min after which the following additions were made with mixing at each step; ammonium acetate solution (1 ml), hydroxylamine solution (1 ml) and bipyridyl solution (1 ml). The mixture was then diluted to 15 ml. After standing 15 min the extinction of the solution was measured at 525 mm against a blank solution containing distilled water in the place of the sample but all the other reagents added. Standards containing 5, 10, 15 and 20 µg of iron were treated in parallel with test samples and blanks.

1.3 Sodium dodecyl sulphate, polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide slab gel electrophoresis in the presence of SDS (sodium dodecyl sulphate) was done using a discontinuous buffer system. The method used was based on those of Laemmli (1970) and Ames (1974).

Separating and stacking gels contained 11% and 5% (w/v) acrylamide (BDH, Poole, England) respectively. Both the gels and the electrode buffers contained 0.1% (w/v) SDS. Gels were prepared in glass moulds 8.0 x 7.0 x 0.3 cm, the separating gels being 5.5 x 7.0 x 0.3 cm.

Samples were adjusted to 2 mg protein/ml and 0.5 ml was added to 0.5 ml of solubilizing buffer and heated at 100°C for 5 min. Samples

of 25-50 µl were applied to the wells in the stacking gel and electrophoresed in a Uniscil slab gel electrophoresis unit (Universal Scientific Ltd., London) at a constant current of 15 mA/gel for 2-3 h. For staining and destaining, the method of Weber and Osborn (1969) was used.

For molecular weight determinations marker proteins were run alongside the samples. The marker proteins included bovine serum albumin (mwt 67,000 Sigma Chemical Company, St. Louis, MO, USA), ovalbumin (mwt 44,000 Sigma Chemical Company, St. Louis, MO, USA), chymotrypsinogen (mwt 25,700 Miles-Serravac, Maidenhead, England), trypsin (mwt 23,300 Armour Pharmaceuticals, Eastbourne, England) and horse heart cytochrome c (mwt 11,700 Koch-Light, Colnbrook, England).

For further details refer to appendix 3.

1.4 Molecular weight determination by gel filtration

The molecular weight of HLT was estimated from its movement on a column of Ultrogel AcA 44 (LKB-Produkter AB, Sweden).

The column (1.5 x 84 cm) was equilibrated with 4°C, 0.15M, pH 7.4 potassium phosphate buffer (ie, 19 parts 0.15M KH_2PO_4 to 81 parts 0.15M K_2HPO_4). The void volume (Vo) was determined from the elution position of dextran blue 2000 (0.75 ml of 5 mg/ml buffer; Pharmacia Fine Chemicals Ltd., Uppsala, Sweden). Then the elution volumes (Ve) for 3 standard proteins (Sigma Chemical Company, St. Louis, MO, USA) of known molecular weights were found viz. lysozyme (grade 1 reagent, mwt 14,400), ovalbumin (grade V reagent, mwt 44,000) and human serum albumin (fraction V, mwt 66,500). Samples (0.75 ml of 5 mg protein/ml buffer) were run individually. For each standard protein the ratio of elution volume (Ve) to void volume (Vo) was calculated and plotted against molecular weight to give a straight line. Finally the toxin, the 30-50% ammonium sulphate fraction from a B. pertussis lysate supernatant fluid, was applied to the

calibrated column. As before a 0.75 ml sample was used (25 mg protein/ml buffer). As for all other samples the flow rate was 2-3 $ml.cm^{-2}.h^{-1}$ and 1 ml fractions were collected. From the ratio of Ve/Vo for the toxin a molecular weight value was obtained from the standard curve.

The elution of samples was monitored spectroscopically as described earlier (Materials and methods; section III, 1.1) with the wavelength set at 280 nm for protein samples and 620 nm for dextran blue. The position of the HLT was established by scanning fractions for their haemorrhagic activity in suckling mice.

2 Preparative

2.1 Use of the X-press and ultracentrifugation

B. pertussis cells suspended in Dulbecco "A" PBS (Oxoid Ltd., England), unless specified otherwise, were lysed by 2 or 3 presses in an X-press (LKB Instruments Ltd., South Croydon, England). Lysates were centrifuged at 100,000 x g, for 1 h at $4^{o}C$ to separate the crude cell-envelope material from the lysate supernatant fluid.

2.2 Ion-exchange chromatography

The suitability of ion-exchange chromatography as a method of purifying HLT was investigated. The anion-exchange resin diethylaminoethyl (DEAE) cellulose (grade DE 11; Whatman Biochemicals Ltd., Maidstone, England) was chosen and a column (2.6 x 22 cm) was prepared at $4^{o}C$ in pH 8.0, 0.005M phosphate buffer (ie, 5.3 parts 0.005M KH_2PO_4 to 94.7 parts 0.005M K_2HPO_4). Onto this was fed 26 ml of toxic lysate supernatant fluid (5.7 mg protein/ml; 645 LD_{50}/ml) derived from cells suspended in the 0.005M phosphate buffer used to equilibrate the column. This was eluted, at a rate of 7 $ml.cm^{-2}.h^{-1}$, in a stepwise fashion using a series of phosphate buffers of increasing ionic strength (0.02M, 0.05M or 0.1M, and 0.2M, all pH 7.6 viz. 19 volumes KH_2PO_4 to 81 volumes K_2HPO_4 at the

appropriate molarities) and finally by lowering the pH of the 0.2M buffer. Buffers were changed after the proteins discharged by the previous buffer had been eluted. The elution of the 6 ml samples was monitored at 280 nm (Materials and methods; section III, 1). The presence of HLT in fractions was determined by injecting 0.5 ml into 3-4 wk old mice and recording the deaths over a 3 d period.

2.3 Ammonium sulphate fractionation

In preliminary experiments lysate supernatant fluids were fractionated by the addition of ammonium sulphate in the quantities used by Onoue, Kitagawa and Yamamura (1963). Ammonium sulphate was dialysed overnight (4°C) into the sample to a final concentration of 16% saturation. The precipitate (16% ppt) was collected by centrifugation (17,300 x g, 25 min, 4°C) and more ammonium sulphate was added to the supernatant fluid as already described. Again the precipitate (16-40% ppt) was separated from the supernatant fluid (40% S). During these "salting out" steps the pH was kept at 7.4, where necessary, by the addition of ammonium hydroxide. Finally precipitates were suspended and dialysed, as was the 40% S fraction, against Dulbecco "A" PBS (Oxoid Ltd., England) unless otherwise specified.

The figures given for the percentage saturation were determined from a nomogram for ammonium sulphate solutions (Dixon, 1953). These percentages (16% and 40%) do not however agree with those given by Onoue, Kitagawa and Yamamura (1963) ie, 12% and 32% respectively.

In subsequent experiments the lysate supernatant fluids were treated with DNase (DNase 1. EC 3.1.4.5; Beef pancreas, noncrystalline; Sigma Chemical Company, St. Louis, MO, USA) or DNase and RNase (RNase A; Bovine pancreas, type 1-A, protease free; Sigma Chemical Company, St. Louis, MO, USA). These were added to a final concentration of 50 µg/ml

and allowed to act at 30°C for 15 min. Alterations were also made to the amount of ammonium sulphate added (eg, 30%, 50% and 70% saturation, as calculated for the percentage saturation at 0°C). Otherwise the method was kept the same.

2.4 Gel filtration

Gel filtration was used in the purification of HLT from the ammonium sulphate 30-50% precipitates. Ultrogel AcA44 (LKB-Produkter AB, Sweden), an acrylamide/agarose gel filtration medium with an effective fractionation range of 10,000-130,000 daltons (for globular proteins), was prepared as a 2.6 x 40 cm column in pH 7.4, 0.15M potassium phosphate buffer at 4°C (ie, 19 parts 0.15M KH_2PO_4 to 81 parts 0.15M K_2HPO_4).

In the first experiment a 6 ml sample (2.8% loading) was applied, run through the column at 4.4 $ml.cm^{-2}.h^{-1}$ and collected in 5 ml fractions. In later experiments the sample size was decreased (to ≈ 2.5 ml a 1.2% loading) as was the flow rate (eg, 3.2 $ml.cm^{-2}.h^{-1}$) and the size of the fractions collected (ie, 3.1 ml). The samples applied to the column contained between 20-30 mg protein/ml.

The eluant was monitored at 280 nm as already described (Materials and methods; section III, 1.1) and the toxicity of the fractions determined by testing them in suckling mice for haemorrhagic activity.

SECTION IV IMMUNOLOGICAL MATERIALS AND METHODS

1 Formaldehyde detoxification of HLT

Three experiments were completed to establish the time required to detoxify HLT held at either 4°C or 37°C with or without the addition of 1% (w/v) formaldehyde (BDH, Poole, England) to a final concentration of 0.1% (w/v). The HLT preparation used was a membrane

filter (Millipore SA, Molsheim, France) sterilized lysate supernatant fluid adjusted to a final concentration of 2 mg protein/ml (ie, 65 LD_{50}/ml). Dulbecco "A" PBS was used for all dilutions. Samples were taken at different times throughout the treatment of the HLT under the 4 test conditions eg, 1, 3, 7, 9 and 14 d. Formaldehyde was removed by overnight dialysis of the samples at 4°C, in sterilized (121°C, 15 psi, 15 min) dialysis tubing, against Dulbecco "A" PBS. The sterility of samples was then checked by plating out onto Bordet-Gengou medium. Samples were stored frozen (-20°C) until required for toxicity testing. Mice were injected intraperitoneally with 0.5 ml volumes of neat and 1 in 10 dilutions of samples. Deaths were recorded over a 3 d period.

In subsequent experiments detoxification of lysate supernatant fluids (final concentrations 2 mg protein/ml) was achieved by incubation for 15 d at 4°C in the presence of 0.1% (w/v) formaldehyde. After dialysis to remove the formaldehyde the purity of the solutions was checked and samples were stored frozen (-20°C) for toxicity testing to confirm that detoxification had been achieved. The remainder of the solutions were stored at 4°C with thiomersal (0.01% (w/v) final concentration; BDH, Poole, England).

2 Immunization of mice

Groups of 3-4 wk old mice were immunized with toxoided lysate supernatant fluid (15 d, 4°C, 0.1%(w/v) formaldehyde). A variety of immunization procedures were adopted viz. use of adjuvants ($Al(OH)_3$, Freund's incomplete and complete adjuvants: see appendix 4 for their preparation), injection route (intraperitoneal, subcutaneous, intramuscular), number of injections. How these variables were used can be seen in table 10.

In the first 2 experiments some mice received a single

Table 10 Mouse immunization regimes

Experiment	Injection mixture, route and dosage (μg protein)			Total Dosage	
	Primary injection	2nd injection	3rd injection	μg protein	mouse LD_{50} equivalents
1 and 2	Plain toxoid; IP,100 SC,100	None or	None	100 or	5.6 or
	Toxoid/$Al(OH)_3$; IP,100 SC,100	Plain toxoid; IP, 100		200	11.2
	Toxoid/FIA; IP,100 SC,100				
	Toxoid/FCA; IP,100 IM,100				
3	Plain toxoid, SC,50	Plain toxoid,SC,50	Plain toxoid	250	14
	Toxoid/$Al(OH)_3$,SC,50	Toxoid/$Al(OH)_3$,SC,50	IP,		
	Toxoid/FIA, SC,50	Toxoid/FIA, SC,50	150		
	Toxoid/FCA, IM,50	Toxoid/FCA,SC,50			

FIA/FCA Freund's incomplete and complete adjuvants respectively

injection whilst the others received a booster injection after 4 wk. These mice were challenged with 5 or 3 LD_{50} of toxin 3 wk after the 1st injection or 2 wk after the 2nd. Where possible survivors were bled out and serum collected.

In the 3rd experiment, injections were given at 1 and 3 wk intervals respectively. Two to 3 wk after the final injection half the mice were challenged with 3 LD_{50} of toxin and half bled out for their serum. Survivors of the challenge were also bled out.

In all experiments unimmunized mice were included to check the challenge was of the correct potency. One group of control mice received the lethal challenge dose (5 or 3 LD_{50}) and another 1 LD_{50} of HLT.

Sera were produced by allowing the mouse blood to clot at room temperature for 1-2 h before being left overnight at $4^{o}C$. The serum could then be drawn off.

3 Immunization of rabbits

Four New Zealand White rabbits were immunized against the same batch of toxoid used to immunize the mice (ie, toxoided lysate supernatant fluid; detoxification by incubation for 15 d at $4^{o}C$ in the presence of 0.1% (w/v) formaldehyde). Rabbits A and B were injected according to the immunization schedule outlined in the top half of table 11. In total they received 560 mouse LD_{50} equivalents of toxin in 10 mg protein. Rabbits C and D received a larger amount of material ie, 896 mouse LD_{50} equivalents of HLT in 16 μg of protein.

Sera were produced by allowing blood to clot for 1-2 h at room temperature. If necessary the clot was freed from the side of the universal to allow it to contract. It was then left overnight at $4^{o}C$ before the serum was removed.

Table 11 Rabbit immunization regimes

Frequency of injections (days)	Injection mixture	Injection route	Dosage		
			volume (ml)	protein content (mg)	mouse LD_{50} equivalents of HLT
0	Toxoid/FCA	IM	4	4	224
31	Toxoid/FIA	IP	2	2	112
60	Toxoid/FIA	IP	2	2	112
109	Toxoid/FIA	IP	2	2	112
				Total	560
0	Toxoid/FCA	IM	4	4	224
28	Toxoid/FIA	IP	4	4	224
56	Toxoid/FIA	IP	4	4	224
84	Toxoid/FIA	IP	4	4	224
				Total	896

FIA/FCA Freund's incomplete and complete adjuvants respectively

4 Absorption of rabbit antisera

Two rabbit antisera (B,D) were absorbed with B. pertussis 134 ie, the strain used to produce the lysate supernatant fluid to which the antisera were raised. The cells were harvested from 48 h cultures of B. pertussis 134 grown in Stainer and Schölte (12G) medium.

Each absorption step involved the addition of live cells (1 g wet weight) to 10 ml of heated (56°C/10 min) antiserum. An even cell suspension was formed by shaking with glass beads, and left at room temperature (20-24°C) for 1 h. The suspension was then centrifuged (15,000 x g, 20 min, 20°C) and the serum decanted off the top of the pellet. This procedure was followed through 3 and 4 times for sera D and B respectively. After each step the sera were monitored for the presence of agglutinins against Bordet-Gengou grown B. pertussis 134. Residual B. pertussis were removed from absorbed sera by membrane filtration (Millipore SA, Molsheim, France). The sterility of these sera was confirmed by plating out onto Bordet-Gengou medium.

5 Bacterial agglutination tests

Antisera raised to a lysate supernatant fluid from B. pertussis 134 were tested for the presence of agglutinins to that organism. The method used was based on that described by Preston (1970).

Doubling dilutions of sera were made in plastic disposable trays (Cooke Microtiter System, Sussex, England) and an equal volume of bacterial suspension (30 ou) was added to each dilution. Dulbecco "A" PBS (Oxoid Ltd., England) was used both to dilute antisera and to suspend the freshly harvested cells from 24 h, Bordet-Gengou grown cultures. Cells and sera were mixed at room temperature for 5 min at 200 rpm (fastened to an orbital shaker) before being incubated in a humidified, 55°C incubator for 30 min. However agglutination titres were only determined

after overnight storage of the mixtures at 4°C. The agglutination end-point was determined using a plate microscope and the reciprocal of the dilution corresponding to this point taken as the titre.

6 Immunodiffusion and immunoelectrophoresis

6.1 Comparative double diffusion

Molten gel diffusion medium (20 ml; see below for details of its preparation) was poured into 90 mm diameter plastic petri dishes (Sterilin Ltd., Teddington, England) and allowed to set. Wells were cut either with a no. 2 cork borer (Griffin and George Ltd., Wembley, England) or with gel cutters arranged to give 4 sets of 6 wells around a central well. The cork borer was used to cut 5 wells about a central well with a 3 mm gap between the edges of the central and surrounding wells. After the wells were filled with antigens and antisera to a prearranged design diffusion was allowed to take place for 2 or 3 d at 4°C.

Preparation of the gel diffusion medium:

Constituents	quantities
Ion agar no. 2 (Oxoid Ltd., England)	15 g
NaCl	16 g
Dissolved in 1 l of distilled water by steaming for 45 min before the addition of:-	
Phenol-saline, 10% (w/v)	50 ml
Methyl orange (BDH, Poole, England)	0.12 g

Finally the medium was filtered through Whatman filter paper (grade 113) and dispensed in 20 ml amounts into universal containers.

6.2 Crossed-over electrophoresis

Molten barbitone agar (see below for its preparation) was evenly spread onto clean glass slides (14 ml of agar per 80 x 80 mm slide)

sitting on a level surface. After solidification wells were cut with a 5 mm gap between wells for antigen and the corresponding antiserum. When the wells had been filled the slides were placed in the electrophoresis chamber (Shandon electrophoresis apparatus). The slides were orientated such that antigen wells were on the cathode side of the wells containing the corresponding antiserum. To complete the electric circuit the slides were connected to the barbitone buffer (pH 8.3; see below for its preparation) at each electrode by buffer soaked gauze. A constant voltage of 100V was applied to the slides for 30 min. Slides were then inspected and precipitin lines recorded before being left overnight in barbitone buffer (pH 8.3) to remove unreacted protein. After 2 washes of 15 min in distilled water the precipitin lines were stained for 10 min with a saturated solution of amido black (Edward Gurr Ltd., Michrome Laboratories, High Wycombe, Bucks) in 2% (v/v) acetic acid. Finally the slides were washed in 2% (v/v) acetic acid/1% (v/v) glycerol and dried at 37^{o}C.

Preparation of barbitone buffer and agar:

The buffer comprised 8.5 g of sodium barbitone dissolved in distilled water to a final volume of 500 ml after the pH had been adjusted with 1N HCl to 8.3. To prepare barbitone agar, Ion agar (Oxoid Ltd., England) was dissolved, by heating, to a final concentration of 0.75% (w/v) in barbitone buffer (pH 8.3).

SECTION V STATISTICAL METHODS

1 Probit analysis

For analysis of dose-response relationships (eg, HSF and the lethal HLT assay data) where the response variable was a binomial proportion the probit transformation was used. Data were processed by the probit method (Finney, 1971) using a computer programme developed in the Department of Epidemiology and Biometrics, School of Hygiene, University of Toronto, Canada. The University of Glasgow ICL 2976 computer or Newcastle-upon-Tyne IBM 370/168 multiple access computer were used in this context. The programme yielded ED_{50} values and the potencies of samples, with 95% confidence limits, relative to a standard.

1.1 Combining the results of data processed by probit analysis

Toxicity or HSF data from independent tests on the same set of samples can be combined to give a better estimate of the relative potencies of the samples. To illustrate the procedure used, it is shown how results presented in table 18 (experiment 1) were derived from 2 sets of toxicity test data processed by probit analysis (tables 12 and 13).

1.1.1 Combined estimate of relative potency ($\bar{R}$)

The blocked-in figures in tables 12 and 13 are substituted into the following equation to give combined estimates of relative potency ($\bar{R}$) for C_1 and C_2 against X_1.

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

ie, for C_1

$$\overline{\log R} = \frac{(5.73x - 0.8324) + (44.4x - 1.2603)}{5.73 + 44.4}$$

$$= -1.211$$

$$\therefore \quad \bar{R} = 0.062$$

Likewise for C_2, where $\bar{R} = 0.040$.

Table 12 Combining the results of data processed by probit analysis - toxicity test data 1

Preparation	Dose	Log_{10} dose	Tested	Responding
X_1 (std.)	2.5000	0.3979	4	4
	1.2500	0.0969	4	4
	0.6250	-0.2041	4	3
	0.3125	-0.5051	4	0
	0.1562	-0.8063	4	0
C_1	20.000	1.3010	4	4
	10.000	1.0000	4	4
	5.000	0.6990	4	3
C_2	20.000	1.3010	4	3
	10.000	1.0000	4	4
	5.000	0.6990	3	0

	Rel.pot.	95% limits		ED_{50}	log R	WlogR	log ED_{50}	$WlogED_{50}$
X_1				0.53			-0.2765	20.46
C_1	0.147	0.072	0.490	3.60	-0.8324	5.73	0.5559	7.97
C_2	0.060	0.029	0.130	8.89	-1.225	9.64	0.9487	18.22

Table 13 Combining the results of data processed by probit analysis - toxicity test data 2

Preparation	Dose	Log_{10} dose	Tested	Responding
X (std.)	2.5000	0.3979	10	10
	1.2500	0.0969	10	10
	0.6250	-0.2041	10	10
	0.3125	-0.5051	10	6
	0.1562	-0.8063	10	0
C_1	20.000	1.3010	10	10
	10.000	1.000	10	10
	5.000	0.6990	10	4
	2.500	0.3979	10	0
	1.250	0.0969	10	0
C_2	20.000	1.3010	10	9
	10.000	1.0000	10	9
	5.000	0.6990	10	1
	2.500	0.3979	10	0
	1.250	0.0969	10	0

	Rel.pot.	95% limits		ED_{50}	logR	WlogR	$logED_{50}$	$WlogED_{50}$
X_1				0.39			-0.5333	88.74
C_1	0.055	0.039	0.078	5.33	-1.2603	44.40	0.7270	88.85
C_2	0.037	0.026	0.052	7.93	-1.4326	44.40	0.8993	88.87

Note the effect of the higher weightings for the second test (table 13) is to bring $\overline{R}$ (0.062 and 0.040, for C_1 and C_2 respectively) closer to the R values of test 2 (0.055 and 0.037) than those of test 1 (0.147 and 0.060).

1.1.2 Estimation of 95% confidence limits

As the computer print out for "WlogR" incorporates the student term "t" ie, WlogR = 1 / $(ts)^2$ there is no t factor in the expression of the limits.

$$\text{Limits} = \text{antilog}\ (\log R \pm 1 / \sqrt{\Sigma\, \text{WlogR}})$$

For C_1 the limits $= \text{antilog}\ (-1.211 \pm 1 / \sqrt{5.73 + 44.4})$

$= \text{antilog}\ (-1.211 \pm 0.141)$

$= \text{antilog}\ -1.352$ and $\text{antilog}\ -1.07$

$= 0.044$ and 0.85

Likewise for C_2, limits = 0.029 and 0.055.

2 Four-point parallel-line assay

The LPF activities of samples were compared by the 4-point parallel-line assay. This involved testing low and high doses of each preparation and analysing the data using a programme developed for use with a Programma 101 Electronic desk-top computer (British Olivetti Ltd., London). The results of an analysis of variance were provided, and the potency and 95% confidence limits for one sample relative to the other. In table 14 are the particulars relating to a comparison of the LPF contents of a heat-detoxified (56°C/30 min) toxin preparation and its formaldehyde-detoxified equivalent viz. the raw data of white cell counts (table 14a), details of the analysis of variance (table 14b) and the relative potency values with 95% confidence limits (table 14c, and already presented in table 37, experiment 1).

Table 14 Four-point parallel-line assay of LPF test data

14a Log_{10} individual white blood cell counts in relation to dose

Formaldehyde-detoxified HLT		Heat-detoxified HLT	
Low dose	High dose	Low dose	High dose
4.390	4.790	4.035	4.672
4.310	4.820	4.177	4.412
4.284	5.029	4.088	4.559
3.319	4.800	4.094	4.457
4.205	4.708	4.249	4.478

14b Analysis of variance

Source of variation	Degrees of freedom	F-ratio values	Probability
Between preparations	1	32.9	$\leq$ 0.01
Slope	1	116.2	$\leq$ 0.01
Parallelism	1	2.8	$\geq$ 0.05
Residual	16	-	-

The tabulated F-values at 1, 16 degrees of freedom are 4.44 (P = 0.05) and 8.53 (P = 0.01). Therefore there is a highly significant slope but no significant difference in parallelism, ie, the test was statistically valid.

14c Relative potency and 95% confidence limits

Formaldehyde-detoxified product	1.00
Heat-detoxified product	0.48 (0.34 - 0.64).

3 Mann-Whitney U-test

The Mann-Whitney U-test was used to compare certain types of data eg, scores from haemorrhagic toxicity assays, or lung pathology and culture scores for B. pertussis infected mice. The procedure followed was as described by Campbell (1974).

4 Use of 2 x 2 contingency tables

For the comparison of certain data in the form of proportions (eg, 9 out of 10 mice dead compared to 2 out of 10) 2 x 2 contingency tables were consulted (Finney et al, 1963). These were used to test the significance of differences in response for groups of mice given different treatments eg, passive protection afforded to groups of mice against an intracerebral or intranasal challenge with B. pertussis.

RESULTS

1 Assaying the toxicity of HLT

1.1 Measurement of the lethal effect in mice

The effects of mouse sex and weight on their susceptibility to the lethal effect of HLT were investigated. Any differences could be a source of error if not recognized whereas if detected they could be used to increase the sensitivity of the assay.

1.1.1 Mouse sex

Groups of 3-4 wk old HAM/1CR male and female mice were injected intraperitoneally with 0.5 ml volumes of graded doses of B. pertussis 18334 cell-lysates. The range and distribution of weights in each group were similar. Deaths were recorded over the 3 d post-injection period.

The results of 5 independent experiments are outlined in table 15. The toxicity test values for the female mice relative to the male mice, which have been given a rating of 1.0, ranged from 0.8-1.6. There is no indication that the sexes responded differently to the lethal toxicity of the lysates.

1.1.2 Body weight

Three to 4 wk old HAM/1CR mice were arranged into groups according to their body weight; low weight groups of 10.0-13.5 g and high weight groups of 18.0-23.0 g. Mice from both groups were injected intraperitoneally with 0.5 ml volumes of graded doses of B. pertussis 18334 cell-lysates. Doses were not weight adjusted ie, in any given experiment the same dilutions of lysate were used for both large and small mice. Deaths were recorded during the 3 d post-injection period.

The toxicity of the first lysate was estimated to be, in 2 separate experiments, about 50% less when tested in the large rather than the small mice indicating that the lighter mice were the more susceptible (table 16, experiments 1 and 2). However the measurements made on the

Table 15 The susceptibility of male and female mice to killing by B. pertussis cell-lysates

Dose of lysate/mouse (oue of 0.5 ml injected)[a]	Number of deaths/number of mice injected									
	Expt 1		Expt 2		Expt 3		Expt 4		Expt 5	
	M	F	M	F	M	F	M	F	M	F
10	NT	NT	NT	NT	NT	NT	5/5	5/5		
2.5	5/5	4/4	NT	NT	5/5	5/5	4/5	4/5		
1.25	5/5	4/5	4/5	3/5	5/5	5/5	NT	NT		
0.625	4/5	5/5	4/5	2/5	5/5	5/5	3/5	2/5		
0.312	0/5	1/5	0/5	1/5	3/5	3/5	NT	NT		
0.156	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5		
18									3/3	3/3
6									3/3	3/3
2									3/3	3/3
0.67									0/3	1/3
0.22									0/3	0/3
LD_{50}	0.51	0.47	0.61	0.80	0.30	0.30	0.75	0.96	1.16	0.74
Relative toxicity and	1.0	1.1	1.0	0.8	1.0	1.0	1.0	0.8	1.0	1.6
95% confidence limits		(0.6-2.0)		(0.3-1.7)		(0.1-8.1)		(0.2-2.7)		(0.1-5.1)

a Opacity unit equivalents (oue) is a measure of the lysate's concentration (See Materials and methods; Section I, 5).

M and F Male and female mice respectively

NT Not tested

Table 16 The susceptibility of mice differing in body weight to killing by B. pertussis cell-lysates

Dose of lysate/mouse (oue of 0.5 ml injected)	Number of deaths/number of mice injected							
	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	LW	HW	LW	HW	LW	HW	LW	HW
1.25	7/10	2/10	4/5	4/5			4/5	4/5
0.625	6/10	3/10	4/5	3/5			5/5	4/5
0.312	1/10	0/10	3/5	0/5			0/5	0/5
0.156	0/10	0/10	1/5	0/5			0/5	0/5
2					9/9	10/10		
1					9/9	10/10		
0.5					6/9	5/10		
0.25					1/9	0/10		
LD_{50}	0.72	1.67	0.34	0.60	0.41	0.50	0.54	0.61
Relative toxicity and	1.0	0.4	1.0	0.6	1.0	0.8	1.0	0.9
95% confidence limits		(0.2-0.8)		-[a]		(0.6-1.2)		(0.5-1.7)

a No 95% confidence limits given as the test was not statistically valid (F-test for non-parallelism failed)

LW and HW Low weight (10.0-13.5 g) and high weight (18.0-23.0 g) groups of mice respectively

toxicity of the second lysate were rather similar irrespective of whether large or small mice were used in the determinations. The conclusion is that although weight is not a major determinant of mouse susceptibility it cannot be disregarded.

1.2 Measurement of the haemorrhagic activity of HLT, with or without added rabbit serum, in suckling mice

This series of experiments stemmed from an observation made whilst testing antisera for their ability to neutralize the haemorrhagic activity of HLT. A cell-lysate supernatant fluid (100,000 x g, 1 h) was chosen as a source of HLT. To 0.5 ml of a suitable dilution of this toxic solution was added an equal volume of either PBS or normal rabbit serum. After mixing and 2 h incubation at room temperature 0.05 ml volumes of the mixtures were injected subcutaneously into suckling mice (4 d old) at a point behind the head. Twenty-four hours later the mice were inspected for the development of haemorrhagic reactions which were scored on a 1+ to 4+ scale (plate 8) or for death 5+. The Mann-Whitney U-test was adopted to analyse these data to assess if differences between the mixtures were significant.

Marginally higher scores, reflecting more severe haemorrhagic reactions, were recorded where serum had been added to the toxic lysate supernatant fluid. In 4 out of 5 experiments these scores were significantly higher (table 17).

1.3 Testing HLT for haemolytic activity

To see if HLT might be responsible for the haemorrhagic zone surrounding colonies of B. pertussis on Bordet-Gengou medium, an HLT preparation was tested for haemolytic activity. To 0.5 ml volumes of doubling dilutions of a lysate supernatant fluid (100,000 x g, 1 h) were added equal volumes of 1% (v/v) suspensions of red blood cells from

Table 17 Comparison of haemorrhagic activity in the presence and absence of rabbit serum

Experiment	Serum	Number of mice with score					Arithmetic mean score for haemorrhagic activity		Probability
		5+	4+	3+	2+	1+	Serum	PBS	
1	+	1	1	6	3		3.0 (11)[a]	2.0 (10)	≤ 0.05
	-				10				
2	+			6	1		2.9 (7)	2.0 (7)	≤ 0.05
	-				7				
3	+			10	2		2.8 (12)	1.9 (13)	≤ 0.05
	-			2	8	3			
4	+		1		11	2	2.0 (14)	1.8 (14)	> 0.05
	-			3	5	6			
5	+			6		1	2.7 (7)	1.0 (10)	≤ 0.05
	-					10			

a Figures in parentheses indicate the number of suckling mice tested ie, the number in the litter.

several species. After 30 min incubation at 35°C there was no evidence of lysis of sheep, rabbit, horse, human (O, rhesus +) or cod red cells, even in the presence of 2.5 mouse LD_{50} per ml of HLT. Subsequent transfer of the cells into the cold room to test for hot-cold lysis did not result in haemolysis of any of the red cell species. The heat-labile toxin would not appear to be a haemolysin.

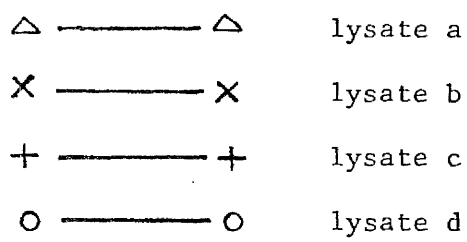
2 Storage-stability of HLT

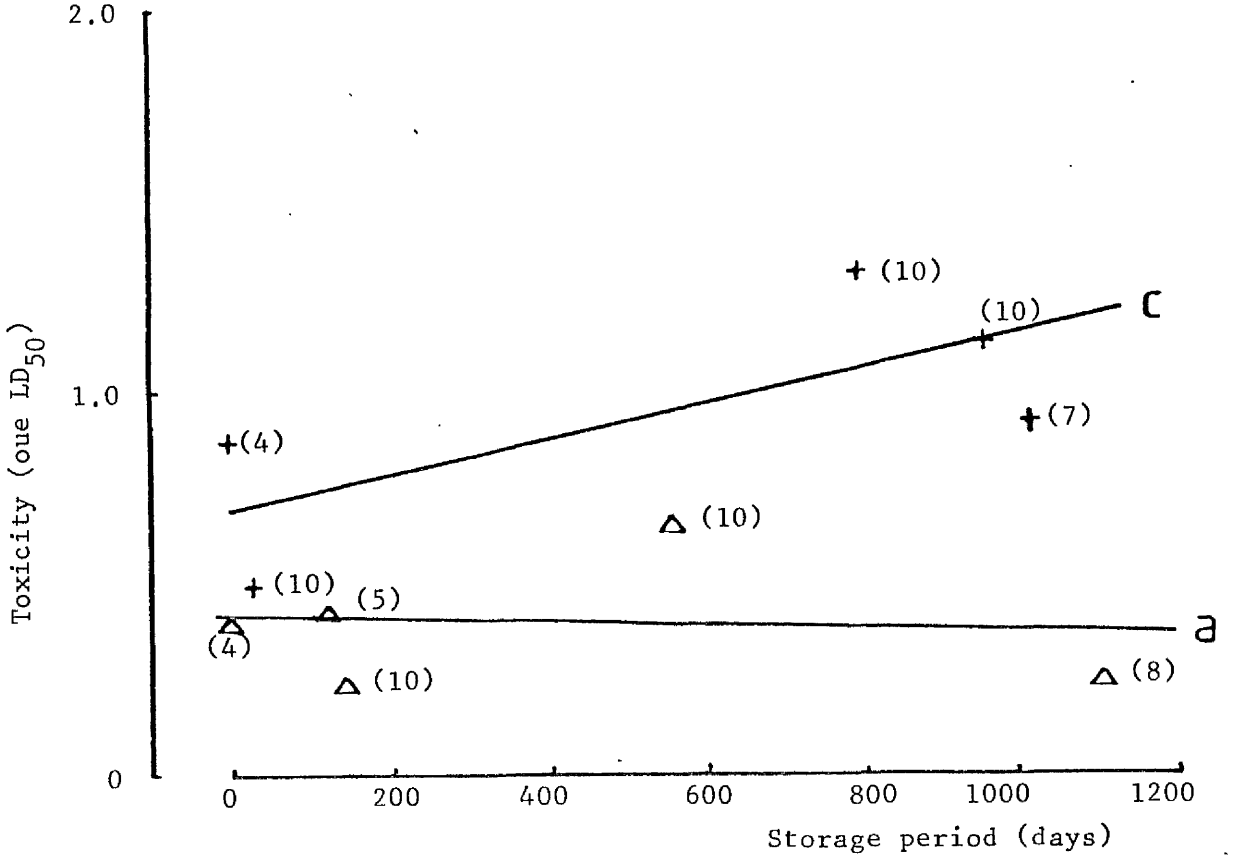
In preparation for a series of fractionation experiments, the stability of HLT to storage in the freezer (-20°C) was investigated. Four samples of toxin, cell-lysates of B. pertussis 18334 which had been grown in modified Hornibrook medium, were assayed for their mouse lethal toxicity several times during their storage at -20°C. The lysates (a,b,c and d) were prepared from cell suspensions of 70, 70, 80 and 125 ou respectively which had been stored overnight (a, b and c) or for 10 d (d) at -20°C prior to X-pressing. Thereafter 5-17 d elapsed before they were used in the first toxicity test. Test samples were re-frozen after each toxicity assay for re-testing at a later date.

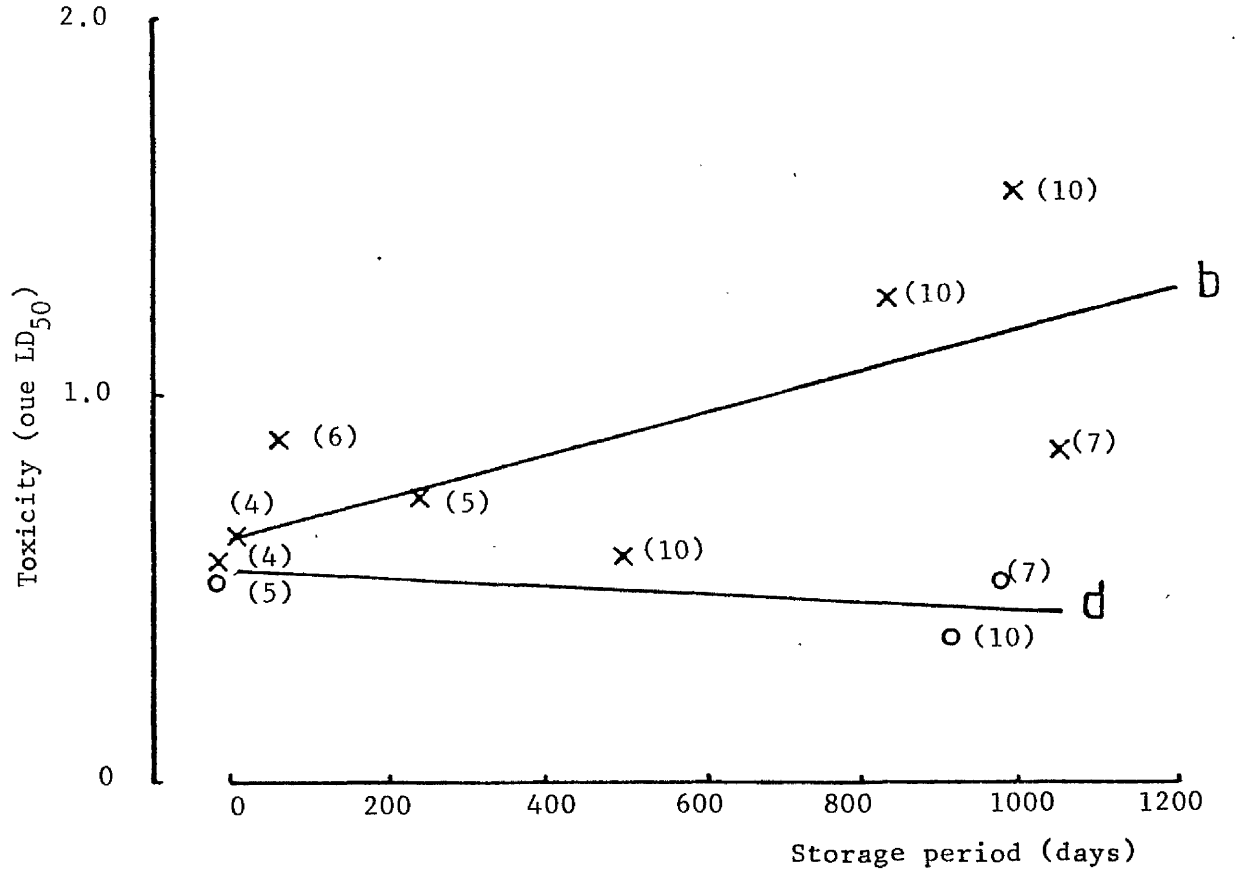
Figures 9a and b record the toxicity of the lysates during 2.5-3 yr storage at -20°C. With 2 of the lysates (b and c) there was some loss of activity during this period as indicated by the increasing amounts of lysate required to kill the same number of mice. This reduction in toxicity is estimated at 19% and 24% per annum for preparations c and b respectively, as calculated from the slopes of the regression lines fitted to the toxicity data. The correlation coefficients for the lines b and c are 0.70 and 0.73 respectively. Lysates a and d showed no signs of having lost activity and indeed the very shallow slopes on these regression lines indicates a very slight increase in toxicity. However

Figure 9 Storage-stability of HLT

The toxicity of the lysates are expressed in terms of the oue of lysate which in a 0.5 ml volume is equivalent to 1 LD_{50}. The figures in parentheses indicate the numbers of mice used per dose of lysate in each toxicity determination.




2.0
1.0
0
Toxicity (oue LD_{50})
+ (10)
(10)
c
+(4)
+(7)
△ (10)
+ (10)
(5)
(4)
a
△ (10)
△ (8)
0
200
400
600
800
1000
1200
Storage period (days)


2.0
1.0
0
Toxicity (oue LD_{50})
× (10)
×(10)
b
× (6)
×(7)
(4)
× (5)
(4)
× (10)
o(7)
(5)
d
o (10)
0
200
400
600
800
1000
1200
Storage period (days)

this is almost certainly not meaningful. Taken collectively the data indicate an annual loss of toxic activity of about 10% during storage at $-20^{o}C$.

3 Antigenic variation and HLT production

3.1 Antigenic modulation

B. pertussis cultures were grown in parallel in modified Hornibrook medium with either NaCl as the principal salt (X-medium) or $MgSO_4$ (C-medium) to produce X-and C-mode cells respectively. These were then tested for their toxicity to see if HLT, like several other B. pertussis components, would be lost during antigenic modulation (Introduction; section I, 6.1.1).

Cell suspensions of X-and C-mode cells were prepared in PBS and standardized by opacity before being divided into 3 portions; one for conversion into lysate for subsequent toxicity testing, another for SDS-PAGE analysis for the presence or absence of the 28K or 30K cell-envelope polypeptides with the final sample being made into vaccine for HSF determinations. The levels of both the 28K and 30K cell-envelope polypeptides and the HSF were monitored to ensure modulation had been achieved.

A summary of 7 independent growth experiments, 4 with B. pertussis 18334, 2 with strain 18323 (BOB) and 1 with strain 134 is presented in table 18. The figures for the C-mode preparations are given as a percentage of their X-mode counterpart. The toxicity test results prior to statistical evaluation appear in appendix 5. It is clear that growth in C-medium is accompanied by a substantial reduction in the toxicity of X-mode B. pertussis cells. Moreover this phenomenon is reproducible in 2 senses. Firstly, it has been shown to apply to more than just one strain of B. pertussis ie, strains 18334, 18323 and 134. Secondly, with

Table 18 A comparison of the toxicity and HSF content of X- and C-mode cells

B. pertussis strain	Experiment	Relative potency and 95% confidence limits of C-mode preparations (X = 100%)	
		Toxicity	HSF
18334	1[a,b]	6.2 (4.4-8.5)	0.3 (0.0-1.5)
		4.0 (2.9-5.5)	0.2 (0.0-1.1)
		6.8 (4.8-9.6)	0.7 (0.0-2.5)
		6.9 (4.9-9.6)	0.5 (0.0-1.8)
	2[a]	4.2 (2.5-6.3)	1.9 (0.6-2.7)
	3	3.2 (0.6-8.9)	$\leq$ 6.0[d]
		3.7 (0.8-12.5)	$\leq$ 6.0[d]
	4	3.7[c]	0.4 (0.0-1.2)
18323	5	4.0 (2.3-6.8)	3.4 (0.4-9.8)
	6	3.8 (2.0-6.9)	0.8 (0.1-2.4)
134	7	$\leq$ 2.4[c,d,e]	3.1 (1.8-4.4)

a Best estimates from 2 toxicity tests

b In this experiment 4 preparations were compared ie, X_1 vs C_1, C_2 and X_2 vs C_1, C_2

c No 95% confidence limits are given as the test was not statistically valid (F-tests for heterogeneity failed)

d No mice became sensitized (died) at the highest dose of C-mode vaccine (cells) tested

e Cells and not cell-lysates were tested for their toxicity

strain 18334 in particular it has been demonstrated that this result can be obtained consistently. Overall the relative toxicities for the C-mode samples, as measured by their lethality in 3-4 wk old mice, ranged from 2.4-6.9% (average 4.4%). The toxicity detected in X-mode samples was heat-labile. With one particular lysate the LD_{50} was 0.5 ml of 0.43 oue. After 20 min heating at 56°C doses as large as 0.5 ml of 30 oue failed to kill mice.

The culture supernatant fluids were in general atoxic. Only those collected from X-mode cultures of strain 18323 contained toxin, but less than that associated with the cells. C-mode culture supernatant fluids from all 3 strains were atoxic.

The HSF data show, in conformity with previous work (Parton and Wardlaw, 1975; Wardlaw, Parton and Hooker, 1976), that the C-mode vaccines made from each harvest were also much reduced in this component. Taking the results from all 3 strains into consideration the relative potencies of C-mode vaccines, in assays which permitted a value to be determined, ranged from 0.2-3.4%. Likewise analysis of cells by SDS-PAGE revealed in all cases loss of the 28K and 30K cell-envelope polypeptides from C-mode cells as reported previously (Parton and Wardlaw, 1974 and 1975; Wardlaw, Parton and Hooker, 1976). Typical electrophoretic profiles of X- and C-mode cells are shown for B. pertussis 18334 in plate 10.

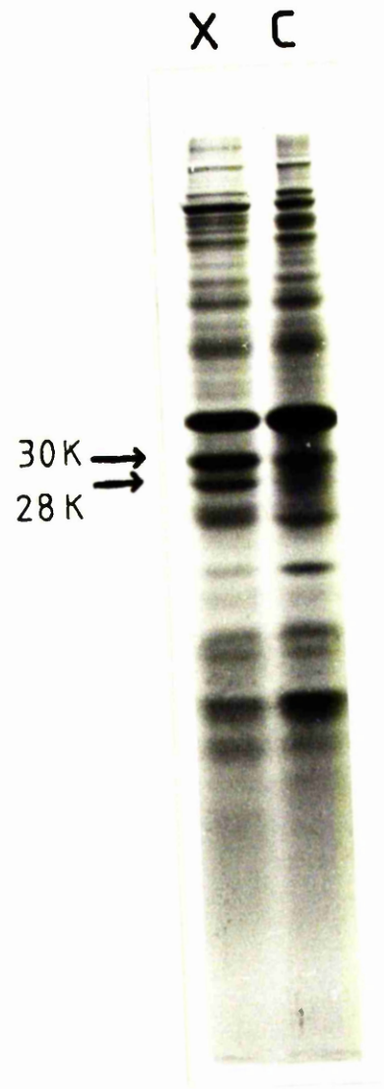
3.2 Nicotinic acid induced modulation

B. pertussis 134 was exposed to modulation by growth on either Bordet-Gengou medium or charcoal agar to which nicotinic acid had been added to a concentration of 500 μg/ml. This was to establish if, as reported by Pusztai and Joó (1967), HLT levels would remain unchanged.

Cells were cultured for 24 h on both Bordet-Gengou medium and charcoal agar with and without the added nicotinic acid. They were

Plate 10 Electrophoretic profiles (SDS-PAGE) of B. pertussis 18334 grown in X- and C-media

28K, 30K The 28,000 and 30,000 dalton cell-envelope polypeptides


X
C
30K
28K

then harvested, washed and re-suspended in PBS, and standardized by opacity. A portion of the suspension was stored for SDS-PAGE analysis for the presence or absence of the 28K and 30K cell-envelope polypeptides whilst a further portion was converted into vaccine for HSF determinations. The remainder was stored frozen for toxicity testing viz. lethality to 3-4 wk old mice.

Growth on either medium supplemented with nicotinic acid resulted in a significant reduction of about 60% in the toxicity of the cells (table 19). The loss of the 28K and 30K cell-envelope polypeptides and, in at least the Bordet-Gengou grown cells, the HSF (table 20) verified that modulation had occurred. If there had been sufficient material it might have been possible to show that there was a significant reduction in the HSF content of the cells grown on the nicotinic acid supplemented charcoal agar, even if less of a reduction that that obtained with the Bordet-Gengou grown cells.

3.3 Phase variation

The phase IV forms of strains D30042 and D3148 had $\leq$20% and 5.1% respectively of the toxicity of their phase I counterparts, as observed with cells from 48 h Bordet-Gengou grown cultures (table 21). This confirms the work of others (Introduction; section II, 4.3).

4 HLT production and purification

4.1 Production

As a prerequisite for the purification of HLT it was decided to examine the factors most likely to influence toxin production so that starting material of the highest potency would be available.

Table 19 The effect of growth on a high nicotinic acid containing medium on the toxicity of cells of B. pertussis 134

Dose/mouse (ou of 0.5 ml injected)	Number of deaths / number of mice injected			
	BG	BG-NA500	CA	CA-NA500
5	5/5	5/5	4/5	3/5
2.5	5/5	0/5	5/5	0/5
1.25	2/5	0/5	0/5	0/5
0.625	0/5	0/5	0/5	0/5
0.312	0/5	0/5	0/5	0/5
LD_{50}	1.29	3.52	2.16	4.94
Relative toxicity and	100	36.8	100	43.7
95% confidence limits		(23.4-59.9)		(19.1-79.4)

BG, BG-NA500 Bordet-Gengou medium without and with added nicotinic acid respectively.

CA, CA-NA500 Charcoal agar without and with added nicotinic acid respectively.

Table 20 The effect of growth on a high nicotinic acid containing medium on the HSF content of cells of *B. pertussis* 134

Dose of vaccine/mouse (ou of 0.5 ml injected)	Number of deaths / number of mice injected			
	BG	BG-NA500	CA	CA-NA500
32		0/5		5/5
8		0/5		4/5
2		0/5		0/5
0.5		0/5		0/5
2.4	4/5		5/5	
1.2	0/5		1/5	
0.6	0/5		0/5	
0.3	0/5		0/5	
HSD_{50}	1.99	> 32	1.42	6.77
Relative potency and 95% confidence limits	100	< 6	100	21 (15-696)

BG, BG-NA500 Bordet-Gengou medium without or with added nicotinic acid respectively.

CA, CA-NA500 Charcoal agar without or with added nicotinic acid respectively.

Table 21 A comparison of the toxicity of the phase I and IV variants of *B. pertussis* D30042 and D3148

Dose/mouse (ou of 0.5 ml injected)	Number of deaths / number of mice injected			
	D30042 I	IV	D 3148 I	IV
20	NT	NT	NT	3/4
10	NT	NT	NT	2/4
5	3/3	0/3	3/3	0/4
2.5	3/4	0/4	3/3	0/4
1.25	3/4	0/4	4/4	0/4
0.625	1/3	0/3	2/4	NT
0.312	0/3	0/3	0/3	NT
0.516	0/3	0/3	0/3	NT
LD_{50}	1.0	> 5.0	0.62	12.15
Relative toxicity and 95% confidence limits	100	< 20	100	5.1 (2.3-10.2)

NT Not tested

4.1.1 Strains

Different strains of toxigenic bacteria may vary widely in their ability to produce toxin (van Heyningen, 1970). Hence it was considered worthwhile to screen B. pertussis strains for their ability to produce HLT. Altogether 14 strains were tested, several of which had been pre-selected from larger assemblages of strains as being potentially of high toxigenicity eg,

Strain MAENO, used by Nakase et al (1969) as a source of HLT for their purification studies

Strain No. 28, discarded as a vaccine production strain because of its toxicity (Novotny, 1977 personal communication)

Strain B-1593/57, selected by Spasojević (1977) as the most toxic of 201 strains in her collection

Strains 77/18319 and 77/2483 were recent isolates and might therefore be expected to yield high levels of HLT as bacterial toxin production often declines with repeated subculture (van Heyningen, 1970; Bernheimer, 1970).

In most experiments (table 22, table 23 experiments 1-3) small groups of B. pertussis strains were grown and toxicity tested in parallel with strain 18334. After 48 h growth on Bordet-Gengou medium the strains were harvested, standardized by opacity and stored frozen until assayed in the mouse lethal toxicity test. In the first series of 3 experiments 9 strains in addition to reference strain 18334 were tested in small groups of mice (table 22; for the toxicity data prior to statistical evaluation see appendix 5). The differences between strains were small when compared on an equal opacity basis. With strain 18334 being arbitrarily assigned a toxicity of 1.0 the most toxic strain rated 2.6 and the least toxic 0.8, a modest 3-fold difference. Strains 134, No. 28 and MAENO were retested using larger numbers of mice as they promised to give the

Table 22 Toxicity test data for several strains of B. pertussis grown on Bordet-Gengou medium

Experiment	B. pertussis strain	Relative toxicity and 95% confidence limits of B. pertussis strains (strain 18334 = 1.0)
1	77/18319	1.2 (0.6-2.4)
	77/24833	0.8[a]
	18904 L4	1.1[a]
2	D30042 I	0.9 (0.3-2.2)
	3865	0.9 (0.3-2.2)
	134	2.4 (0.9-6.7)
3	D3148 I	1.4 (0.8-2.4)
	No. 28	2.6 (1.5-4.6)
	MAENO	2.0 (1.2-3.6)
4	134	2.3 (1.3-4.2)
	No. 28	2.1 (1.1-3.7)
	MAENO	2.7 (1.5-5.1)
5	134	3.1[a]/2.5[a]
	No. 28	2.3 (1.3-4.1)[b]
	MAENO	2.8 (1.5-5.2)[b]

a No 95% confidence limits are given as the results were not analysed together with those obtained for reference strain 18334 as such joint analyses proved to be statistically invalid (ie, F-test invalidity). The relative toxicity values given are based upon the LD_{50} values obtained by analysing the results for each strain independently.

b Best estimates from 2 toxicity tests.

highest yields of toxin (table 22, experiment 4 and appendix 5). Again these samples proved 2-3 times as toxic as reference samples of 18334. In each of the two comparisons made per strain with 18334 the differences in toxicity were, with one exception (experiment 2, strain 134), significant. Further confirmation of these findings was obtained in a separate growth experiment (table 22, experiment 5 and appendix 5). Although significant differences were apparent between strains 134, No. 28, MAENO and 18334 there were no appreciable differences among the 3 selected strains. However strain 134 was preferred as it gives good yields of cells.

Four other strains were tested subsequently in conjunction with some of the aforementioned strains (table 23 and appendix 5). They were either grown on Bordet-Gengou medium as already described or for 4 d in Roux bottles of modified Hornibrook medium. Only strain 44122/7R appeared to be similar in toxicity to B. pertussis 134 which was yet again shown to be more toxic than strain 18334. B. pertussis strains GL353Z and L84 I although not directly compared to strain 134 would appear to be among the least toxic strains tested as judged from their toxicity relative to strains D3148 I and D30042 I.

4.1.2 Culture medium and time of harvest

As culture media have a considerable influence on the yields of bacterial toxins (van Heyningen, 1970) B. pertussis were grown on 4 media and the HLT levels monitored. In 3 separate experiments strains 18334, 77/18319 and 134 were grown on Bordet-Gengou medium, charcoal agar and in modified Hornibrook medium and the chemically defined Stainer and Schölte (12G) medium. The latter 2 media were dispensed in 500 ml volumes in 2-litre shake flasks and inoculated with 5 ml of a seed culture grown in modified Hornibrook medium. Seed cultures for the solid media were

Table 23 Toxicity test data for Bordet-Gengou and Hornibrook grown *B. pertussis* strains

Experiment	Growth medium	*B. pertussis* strain	Number of mice per dose	Relative toxicity and 95% confidence limits
1	B-G	18334	14	1.0
		134	7	2.0 (1.2-3.1)
		B-1593/57	7	1.0 (0.5-1.7)
2	B-G	18334	15	1.0
		44122/7R	10	1.6 (1.0-2.6)
3	B-G	18334	15	1.0
		134	10	1.5 (1.0-2.0)
		44122/7R	10	1.9 (1.5-3.1)
4	H	D3148 I	4	1.0
		D30042 I	4	1.0 (0.4-2.8)
		GL353Z	4	0.7 (0.2-1.9)
		L84 I	4	0.3 (0.1-0.9)

B-G Bordet-Gengou medium

H modified Hornibrook medium

prepared by growth on the same type of medium onto which they were to be inoculated. Cells were harvested from all 4 media after 24 h and 72 h, standardized by opacity and frozen until required for toxicity testing.

It is immediately obvious from table 24 (see appendix 5 for the toxicity data prior to statistical evaluation) that no one medium is, in comparison with the other media tested, especially suitable for providing highly toxigenic B. pertussis. Relative to cells grown on Bordet-Gengou medium for either 24 h or 72 h the equivalent cells from charcoal agar, modified Hornibrook medium and Stainer and Schölte (12G) medium had toxicity ratings ranging from 1.0-1.6, 0.7-2.7 and 0.9-2.3 respectively. However on charcoal agar cells of strains 134 and 77/18319 appeared very marginally, but not significantly, more toxic than their Bordet-Gengou grown counterparts whereas strain 18334 yielded equally toxic cells on either medium. The strains did not always respond uniformly, strain 18334 gave 1.5-and 2.5-fold better yields in Stainer and Schölte (12G) medium than in modified Hornibrook medium whilst the converse held for strain 134 (1.5-fold differences in yields).

The effect of the time of harvesting cells on their toxicity is better defined. In table 25 where the data has been re-arranged so that the toxicity of the 72 h harvests are expressed relative to the 24 h harvests the effect is more apparent. With the solid media the 24 h samples were generally (5 out of 6) more toxic than the 72 h samples. With the liquid media the converse applied, 5 of the 6 72 h samples were more toxic than their 24 h equivalents and the 6th was of equal toxicity. Note that these differences were not large, never more than 1.9-fold, and individually were not statistically significant. However they occurred consistently and it seems reasonable to conclude that with solid media, 24 h cultures were more toxic than 72 h cultures whereas in liquid media the converse held.

Table 24 The toxicity of 3 strains of *B. pertussis* grown under different cultural conditions

Experiment	*B. pertussis* strain	Age of culture (hours)	Relative toxicity and 95% confidence limits (Bordet-Gengou cultures = 1.0)		
			Charcoal agar	Modified Hornibrook medium	Stainer and Schölte (12G) medium
1	18334	24	1.0 (0.4-2.5)	0.7 (0.3-1.8)	1.7 (0.7-4.1)
		72	1.0[a]	1.6 (0.6-4.4)	2.3 (0.8-6.3)
2	134	24	1.6 (0.8-3.3)	1.4 (0.7-2.9)	0.9 (0.4-1.8)
		72	1.3[a]	2.7 (1.5-6.0)	1.9 (1.0-4.1)
3	77/18319	24	1.5 (0.9-3.0)	0.8 (0.4-1.6)	0.9 (0.4-1.6)
		72	1.2 (0.6-2.4)	1.4 (0.7-2.9)	1.2 (0.6-2.4)

a Data were analysed separately due to incompatability with the other test data resulting in F-test failure. The relative toxicity values given were based upon the LD_{50} values thus obtained.

Table 25 The toxicity of *B. pertussis* harvested after 24 h and 72 h growth on various media

B. pertussis strain	Relative toxicity and 95% confidence limits of 72 h cultures (24 h cultures = 1.0)			
	Bordet-Gengou medium	Charcoal agar	Modified Hornibrook medium	Stainer and Schölte (12G) medium
18334	0.6 (0.2-7.1)	0.6 (0.2-7.1)	1.7 (0.5-6.7)	1.0 (0.1-10.9)
134	0.6 (0.2-1.4)	0.6 (0.3-1.0)	1.3 (0.6-3.0)	1.3 (0.8-2.3)
77/18319	1.2 (0.6-2.9)	0.9 (0.4-1.8)	1.9 (1.0-5.7)	1.7 (0.8-8.3)

During growth in the liquid media culture supernatant fluids were taken and tested for their toxicity. Only with the 72 h samples from cultures of strains 18334 and 134 grown in modified Hornibrook medium was there any HLT detected and even then only at low levels.

4.1.3 Iron content of medium

High concentrations of iron in culture media inhibit the production of diphtheria toxin (Pappenheimer and Johnson, 1936), tetanus toxin (Mueller and Miller, 1945), Clostridium perfringens α-toxin (Pappenheimer and Shaskan, 1944), Shigella dysenteriae neurotoxin (van Heyningen and Gladstone, 1953), Streptococcus pyogenes erythrogenic toxin (cited by van Heyningen, 1970) and Pseudomonas aeruginosa exotoxin A (Bjorn et al, 1978). It therefore seemed advisable to investigate the role, if any, of iron in the production of B. pertussis HLT. This was done in Stainer and Schölte (12G) medium modified with 6 different concentrations of $FeSO_4$ (A-F), including the usual formulation (B). The chemically defined Stainer and Schölte (12G) medium was chosen in preference to modified Hornibrook medium because with the former the iron content could be altered merely by changing the amount of iron to be added. The medium would not require to be de-ferrated to achieve very low concentrations of iron. The quantity of iron in each medium was checked by taking 10 ml samples for a colorimetric assessment of their iron content. Culture supernatant fluid from the seed culture was also assayed in this manner and was found to be effectively iron free. A 2% (v/v) inoculum of a 48 h Stainer and Schölte (12G) culture of B. pertussis 134 was used to seed each 500 ml of medium which was then incubated for 72 h. Cells were then harvested, suspended in PBS, standardized by opacity and stored frozen until required for toxicity testing. Culture supernatant fluids were also stored frozen.

From the results obtained in table 26 it can be seen that there

Table 26 Iron content of medium in relation to toxin production by B. pertussis 134

Medium	Iron content (mg/l)		Number of deaths/number of mice injected				LD_{50}	Toxicity and 95% confidence limits relative to growth from medium B
	Amount of Fe added	Measured level of Fe	Dose of cells (ou of 0.5 ml injected)/mouse 10	2.5	0.625	0.156		
A	6.3	NT	3/5	2/5	0/5	0/5	5.0	1.0 (0.4-2.6)
B	2.1	1.8	5/5	0/5	0/5	0/5	5.0	1.0
C	0.7	0.63	2/5	0/5	0/5	0/5	12.1	0.4 (0.1-1.1)
D	0.23	0.21	4/5	0/5	0/5	0/5	6.8	0.7 (0.3-1.9)
E	0.08	0.16	4/5	1/5	0/5	0/5	5.0	1.0 (0.4-2.6)
F	0.00	0.03	5/5	1/5	0/5	0/5	3.7	1.4 (0.5-3.5)

NT Not tested

is a fairly good correlation between the known amount of iron added to the medium and that assayed colorimetrically with 2,2'bipyridyl. The toxicity test only involved 4 groups of 5 mice per sample and a 4-fold dilution series in expectation of possible large differences in the toxicities of the samples. However it is immediately apparent from the results that iron has not the pronounced effect on HLT production in B. pertussis 134 as it has on, for instance, the production of diphtheria toxin which is maximal at iron levels of 0.1 mg/l (Pappenheimer, 1947).

All the culture supernatant fluids were atoxic except for that of medium A which was toxic when tested undiluted but not at 1 in 4.

4.2 Purification

Because of the intracellular location of HLT it was necessary to release it in soluble form to provide starting material for purification.

4.2.1 Disruption of B. pertussis and release of HLT

Cells were disrupted by passage through an X-press and the toxicity of the lysates compared with that of the intact bacteria.

In the first series of experiments lysates were prepared from freshly harvested, Stainer and Schölte (12G) grown cells of B. pertussis 134 by 3 passages through an X-press. These lysates were significantly more toxic than the cell suspensions from which they were derived, of the order of 8 times as toxic (range, 4-14 fold) (table 27). The smallest difference (4.1-fold, experiment 3) was observed with cells and lysates, prepared from 72 h not 48 h cultures, which had been stored for 5.5 mth at -20°C prior to toxicity testing instead of being tested on the day of their preparation. In a further series of experiments where lysates were prepared from Bordet-Gengou grown cells which had been stored overnight at -20°C prior to X-pressing there was only a slight enhancement in

Table 27 The effect of X-pressing on the toxicity of freshly harvested B. pertussis 134

Dose/mouse (ou or oue of 0.5 ml injected)	Number of deaths/number of mice injected						
	Experiment 1		Experiment 2			Experiment 3	
	cells	lysate	cells	lysates[a]		cells	lysate
10	4/4	3/3	3/5	5/5	5/5		
3.3	1/4	4/4	4/5	4/5	4/5		
1.1	1/4	4/4	0/5	5/6	4/5		
0.37	0/4	4/4	0/5	3/5	4/5		
0.12	NT	0/4	NT	0/5	0/5		
0.04	NT	0/4	NT	0/5	0/5		
20						6/6	NT
10						7/7	NT
5						5/7	NT
2.5						0/7	6/6
1.25						NT	4/7
0.625						NT	1/7
0.312						NT	0/6
LD_{50}	3.04	0.21	4.17	0.52	0.45	4.35	1.1
Relative toxicity and	1.0	14.2	1.0	8.0	9.3	1.0	4.1
95% confidence limits		(4.8-42.2)		(2.5-28.5)	(2.9-33.1)		(2.6-6.4)

a Two portions of lysates were prepared in 2 independent X-pressings of cells from the same cell suspension

NT Not tested.

toxicity (table 28). In only 2 of the 4 determinations, 1 of which used B. pertussis 134, were there significant differences (range, 1.7-3.3 fold). All samples were stored at -20°C until the toxicity tests were performed, 2 d after the cells had been harvested, 1 d after the preparation of the lysates. In one final experiment lysates prepared from frozen cells of Hornibrook grown B. pertussis were actually no more toxic than the cells from which they were made.

B. pertussis cells appear to be relatively resistant to lysis or even killing by freezing (-20°C) and thawing (room temperature). Portions of the cell suspensions from experiments 1 and 2 (table 27) were subjected to 5 cycles of freezing and thawing, yet there was no reduction in viability relative to cells held at 4°C for the same length of time. Nor was their toxicity appreciably altered (table 29).

4.2.2 Ultracentrifugation as a preparative step

Ultracentrifugation (100,000 x g, 1 h, 4°C) was used to sediment debris from the cell-lysates produced by X-pressing. The distribution of the toxicity between the pellet and the supernatant fluid is shown in table 30 and the toxicity test data upon which these figures were based in appendix 5. Also included in table 30 is the percentage of protein recovered in the supernatant fluids. The combined figures for the amount of HLT in the supernatant fluids and pellets ranged from 64-123%. When the percentages obtained from each of the 4 experiments are averaged a recovery figure of 96% is obtained, with 71% being in the supernatant fluid and 25% in the pellet. Thus there is about 2.8 times as much HLT (range, 1.9-3.3) in the lysate supernatant fluids as in the pellets. In contrast most of the protein was associated with the pellet and only 33-43% in the supernatant fluid. Consequently in experiments 1, 2 and 3 the ultra-centrifugation step produces apparent purifications of 1.3, 1.6 and 2.2-

Table 28 The effect of X-pressing on the toxicity of *B. pertussis* cells that had been stored frozen

Dose/mouse (ou or oue of 0.5 ml injected)	Number of deaths/number of mice injected with strain							
	134		No. 28		MAENO		18334	
	cells	lysate	cells	lysate	cells	lysate	cells	lysate
2.5	3/4	NT	3/4	NT	3/4	NT	4/4	NT
1.25	4/4	4/4	3/3	4/4	2/4	3/4	2/4	4/4
0.625	3/4	4/4	2/4	3/4	4/4	4/4	1/4	4/4
0.312	3/4	3/4	0/4	3/4	0/4	1/4	0/4	2/4
0.156	0/4	2/4	0/4	0/4	0/4	0/4	0/4	0/4
0.078	NT	0/4	NT	0/4	NT	0/4	NT	0/4
LD_{50}	0.39	0.18	0.32	0.83	0.79	0.47	1.03	0.31
Relative toxicity and	1.0	2.2	1.0	2.6	1.0	1.7	1.0	3.3
95% confidence limits		(0.8-6.6)		(1.1-6.2)		(0.6-4.5)		(1.7-6.5)

Table 29 A comparison of the toxicity of *B. pertussis* cells before and after 5 cycles of freezing (-20°C) and thawing (room temperature)

Dose/mouse (ou of 0.5 ml injected)	Number of deaths/number of mice injected			
	Experiment 1		Experiment 2	
	cells	F/T cells	cells	F/T cells
10	4/4	3/3	3/5	2/4
3.3	1/4	2/4	4/5	2/5
1.1	1/4	0/4	0/5	0/5
0.37	0/4	0/4	0/5	0/5
0.123	NT	0/4	NT	NT
LD_{50}	3.11	3.42	4.1	7.29
Relative toxicity and 95% confidence limits	1.0	0.9 (0.3-2.8)	1.0	0.6 (0.1-2.1)

F/T Freeze/thawed

NT Not tested

Table 30 Ultracentrifugation of B. pertussis cell-lysates: distribution of HLT and protein between the supernatant fluid and pellet

Experiment	Relative toxicity and 95% confidence limits (cell-lysates = 100%)		Percentage of cell-lysate protein in the supernatant fluid
	Supernatant fluid	Pellet	
1[a]	42 (24-74)	22 (13-38)	33
2	70 (36-131)	20 (9-38)	43
3	83 (50-135)	24 (13-39)	37
4[a]	89 (63-125)	34 (24-50)	NT
Average	71	25	38

a Best estimates from 2 toxicity tests

NT Not tested

fold respectively. However these figures are slightly misleading since there is some justification for adjusting the estimated potencies to give totals of exactly 100% with deviations from this being attributable to the imprecision of the toxicity test. If this is done for the 3 experiments the degrees of purification become 2.0, 1.8 and 2.1 respectively, an average of almost 2.0.

Typically 10 μg of lysate supernatant fluid protein represented 1 LD_{50} in 3-4 wk old HAM/1CR mice, but occasionally values as low as 5 μg were obtained.

4.2.3 Ion-exchange chromatography

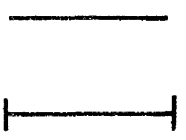
Column chromatography using diethylaminoethyl (DEAE) cellulose ion-exchange resin was used in the successful purification of HLT by Nakase et al (1969). This method was therefore explored.

B. pertussis cells were X-pressed in low ionic strength (0.005M) phosphate buffer, the lysate ultracentrifuged, the 100,000 x g, supernatant fluid adsorbed onto a column of DEAE-cellulose and eluted with a series of phosphate buffers of increasing ionic strength although the final material was eluted by lowering the pH.

Figure 11 shows the elution profile from such an experiment. Where there was sufficient HLT in fractions for 0.5 ml to be lethal to mice this has been indicated. The toxin was found spread over 3 of the 5 major peaks (c, d and e) but only the toxic fractions from c and the first half of peak d were pooled as this was the area within which most of the toxicity was supposed to reside (Nakase et al, 1969). However, only 4% of the original HLT was recovered from these fractions and 13% of the protein. The other toxic fractions only accounted for a further 9% of the HLT.

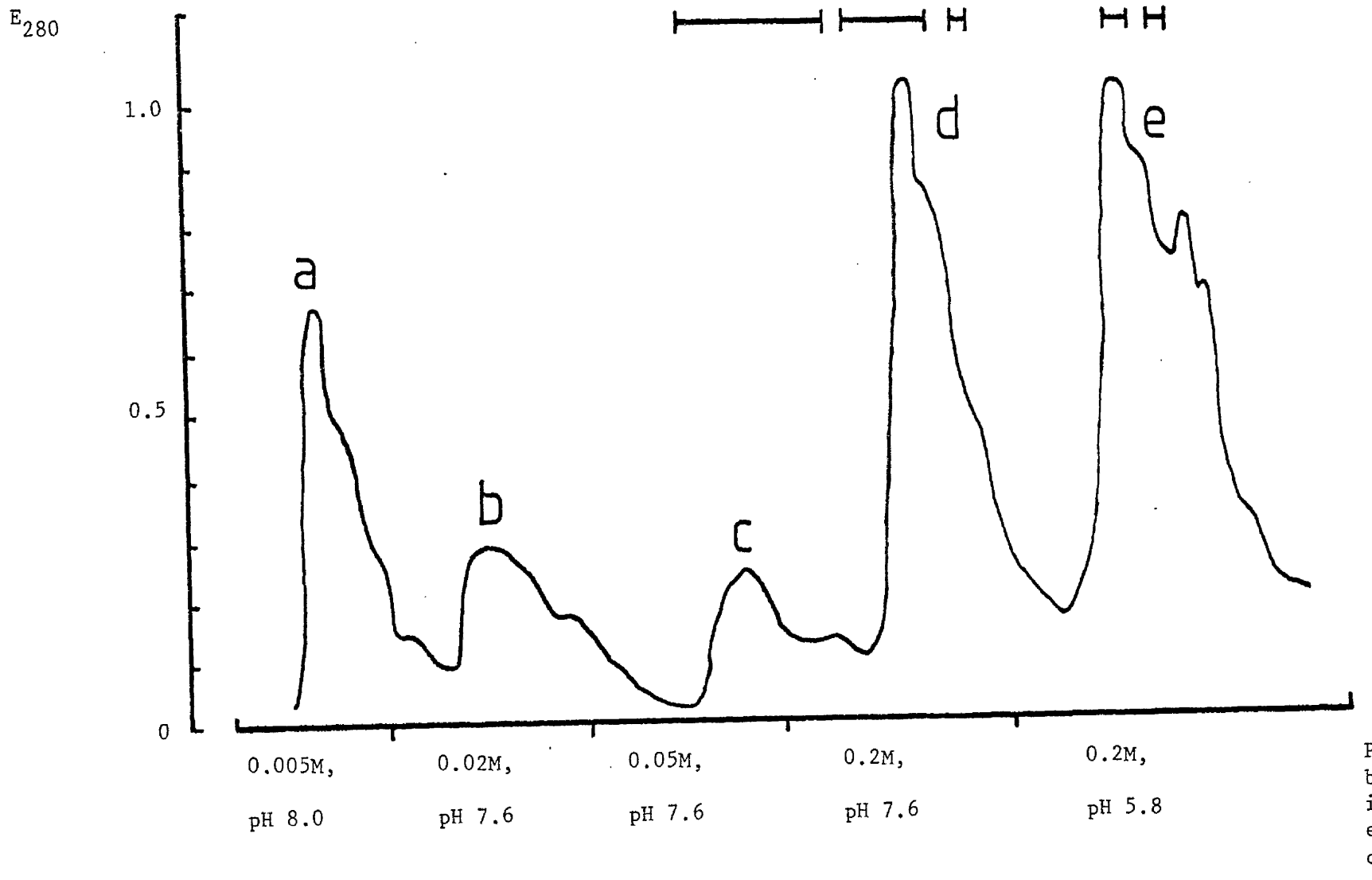
In a further experiment the 0.05M phosphate buffer was replaced

Figure 11 DEAE-cellulose column chromatography of lysate supernatant fluid



E_{280} elution profile

Areas within which fractions were toxic


E_{280}
1.0
0.5
0
a
b
c
d
e
0.005M, pH 8.0
0.02M, pH 7.6
0.05M, pH 7.6
0.2M, pH 7.6
0.2M, pH 5.8
Phosphate buffers used in stepwise elution of column

with 0.1M phosphate buffer in an attempt to elute the toxic fractions associated with peaks c and d of figure 11 in a single peak. In fact 24% of the HLT was recovered in this peak with 15% of the protein. The other toxic fractions only contributed a further 1% of the original HLT applied to the column. As the overall recovery of HLT was considered to be too low for the purification achieved (1.6-fold) this procedure was abandoned.

4.2.4 Fractionation with ammonium sulphate

With the failure of the attempt to follow the method of Nakase et al (1969) to purify HLT, fractionation with ammonium sulphate was investigated. Initially the method of Onoue, Kitagawa and Yamamura (1963) was used to fractionate cell-lysate, 100,000 x g supernatant fluids. Ammonium sulphate was added to the toxin solution in 2 stages and the precipitates formed after each addition were collected as was the final supernatant fluid ie, a 16% ppt, 16-40% ppt and a 40% S fraction (supernatant fluid). In the first 2 experiments more than 95% of the toxicity recovered was in the 16-40% precipitate with only 37% and 42% of the protein. Moreover an ultraviolet scan of the 16-40% precipitate revealed a marked reduction in the absorbance at 260 nm compared to the lysate supernatant fluid. The method looked promising and was therefore explored.

In the first of the subsequent experiments it was decided to pre-treat the lysate supernatant fluids with DNase. Although the ammonium sulphate fractionation on its own apparently separated most of the DNA from the toxin its complete removal early in the purification was desirable to prevent it from interfering in later precipitation steps. DNase (DNase 1, Beef pancreas, noncrystalline) was added to a final concentration of 50 µg/ml and the mixture incubated for 15 min at 30°C. The DNase had no effect on HLT activity (table 31) but it altered the way

Table 31 The effect of DNase on the toxicity of lysate supernatant fluid

Dose (µl)/mouse	Number of deaths/number of mice injected with supernatant fluid	
	before treatment	after DNase treatment
346	15/19	
173	10/15	
86	1/12	
43	0/16	
500		14/16
250		12/16
125		3/16
62		3/16
LD_{50}	175	175
Relative toxicity and 95% confidence limits	1.0	1.0 (0.7-1.5)

proteins in general and HLT in particular were precipitated with ammonium sulphate. In the 16-40% precipitate there was now only half as much protein (19%) and HLT as before. The other 50% of the original HLT was recovered in the 40% supernatant fluid (table 32).

As the salting out procedure adopted originally was no longer appropriate, information on the behaviour of HLT on fractional precipitation of DNase/RNase treated lysate supernatant fluids was gathered. Unfortunately, although the results of the fractionation were favourable the initial incubation of the toxin with 50 μg/ml of RNase (RNase, type 1-A, Bovine pancreas, protease free) and DNase (as before) led to a significant reduction in toxic activity of about 50% (table 33). Of the residual toxicity, most (70%) was recovered in the 30-50% precipitate as was 47% of the protein (a 1.5-fold purification). Considerably less HLT (11%) was recovered in the 30% fraction which contained 10% of the protein, and none in the other 2 fractions. The toxicity test data upon which the results given in table 33 are based are presented in appendix 5.

4.2.5 Gel filtration

Ultrogel AcA 44 was chosen as a gel filtration medium likely to be of value in separating HLT from other B. pertussis components as it had an effective fractionation range of 10,000-130,000 daltons, a range within which it was reasonable to assume HLT would fall. On this gel filtration medium 30-50% ammonium sulphate fractions yielded 4 major peaks, with HLT being eluted as a single peak on the front shoulder of the second and largest protein peak (figure 12).

In the initial run there was a recovery of 58% of the original toxicity (table 34) and 37% of the protein, a 1.6-fold purification. However on the next run (figure 12) the resolution was improved by decreasing the size of the sample applied (from a 2.8% to a 1.2% loading)

Table 32 Distribution of HLT in ammonium sulphate fractions of DNase-treated lysate supernatant fluid

Dose (µl)/mouse	Number of deaths/number of mice injected with		
	DNase-treated lysate supernatant fluid	16-40% precipitate	40% supernatant fluid
68	14/16		
34	12/16		
17	3/16		
8.5	3/16		
140		8/8	
70		7/8	
35.5		3/8	
17.8		0/8	
500			7/8
250			8/8
125			8/8
62.5			8/8
31.2			0/7
LD_{50}	23.6	42.5	40.7
Distribution of HLT (%)	100	56	58

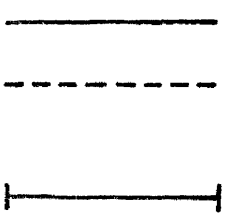
Table 33 The effect of DNase/RNase on the toxicity of lysate supernatant fluid and on the distribution of HLT and protein after ammonium sulphate fractionation

Fraction	Relative toxicity (%) and 95% confidence limits	Protein Content (%)
Lysate supernatant fluid	100	100
DNase/RNase treated lysate supernatant fluid	47 (32-67)	100
DNase/RNase treated lysate supernatant fluid	100	100
30% ppt	11 (7-18)	10
30-50% ppt	70 (44-117)	47
50-70% ppt	≤ 2	24
70% supernatant fluid	≤ 3	16

Table 34 Fractionation of ammonium sulphate 30-50% precipitates by gel filtration on Ultrogel AcA 44 : yields of HLT

Experiment	Sample	Dose (µl)/mouse	Number of deaths/number of mice injected	LD_{50} (µl)	Relative toxicity (%) and 95% confidence limits
1	30-50% precipitate	163	4/5	90.4	100
		81.5	3/5		
		40.8	0/5		
		20.4	0/5		
	Pooled gel filtration fraction	289	5/5	154.6	58 (32-105)
		144.5	2/5		
		72.2	0/5		
		36.1	0/5		
2	30-50% precipitate	328	10/10	43.3	100
		164	8/10		
		82	9/10		
		41	5/10		
	Pooled gel filtration fraction	500	9/10	127.7	34 (16-60)
		250	10/10		
		125	4/10		
		62.5	1/10		

Figure 12 The elution profile for ammonium sulphate 30-50% precipitates fractionated on Ultrogel AcA 44

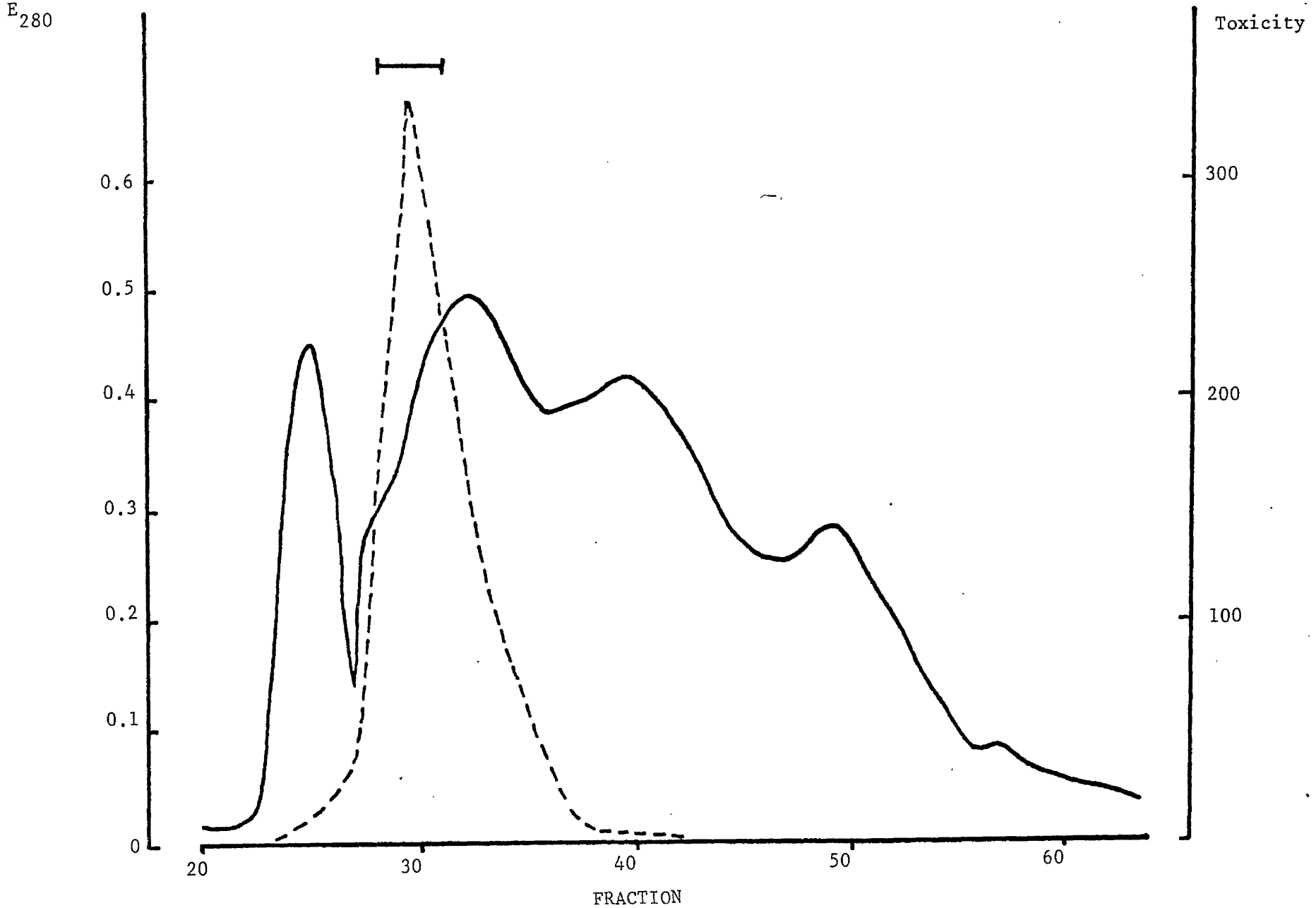


Absorbance at 280 nm

Toxicity. Reciprocal of dilution giving a 2+ haemorrhagic response

Fractions pooled

Bed dimensions	:	2.6 x 40 cm
Eluant	:	0.15M potassium phosphate buffer, pH 7.4
Flow rate	:	3.2 $ml.cm^{-2}.h^{-1}$.
Sample volume	:	2.5 ml
Fraction volume	:	3.1 ml


E_{280}
Toxicity
0.6
0.5
0.4
0.3
0.2
0.1
0
300
200
100
20
30
40
50
60
FRACTION

and the flow rate (from 4.4 $ml.cm^{-2}.h^{-1}$. to 3.2 $ml.cm^{-2}.h^{-1}$). The fractions to be pooled were also selected more carefully with only those from the front edge of the very large 2nd peak being included (ie, 28-31 inclusive). By leaving out the toxic fractions which had a high protein content there was some reduction in yield (from 58% to 34%; experiment 1 cf. 2, table 34) but this was more than compensated for by an even larger reduction in protein (from 37% to 7-8%) to give a 4-5 fold purification

5 Molecular weight estimation by gel filtration

The molecular weight of HLT was determined by gel filtration of a partially purified preparation (a 30-50% ammonium sulphate precipitate) on a column of Ultrogel AcA 44 previously calibrated with human serum albumin (m.wt 66,500), ovalbumin (m.wt 44,000) and lysozyme (m.wt 14,400), as marker proteins, and dextran blue to measure the void volume. The point at which the toxin was eluted was located by monitoring the fractions for haemorrhagic activity in suckling mice. The peak of toxicity lay within the area between fractions 69-76. This gives Ve/Vo coefficients of 1.17 and 1.29 which corresponds to a molecular weight of 89,000 daltons ($\pm$ 10%), as can be seen in figure 13.

6 Production and characterization of toxoided HLT

6.1 Detoxification of HLT - the effect of temperature and formaldehyde

The detoxification of HLT by formaldehyde was investigated as a method for the production of a toxoid suitable for immunizing mice or rabbits. Most previous investigators have incubated the toxin at 37°C with 0.1% (w/v) formaldehyde. Under these conditions detoxification is very rapid and is complete within 1 d. It was suspected that heat inactivation of the toxin may have had some part to play in the rapidity of this process. As heated toxin (56°C, 30 min) is ineffective for stimulating the production

Figure 13 Estimation of the molecular weight of HLT by chromatography on Ultrogel AcA 44

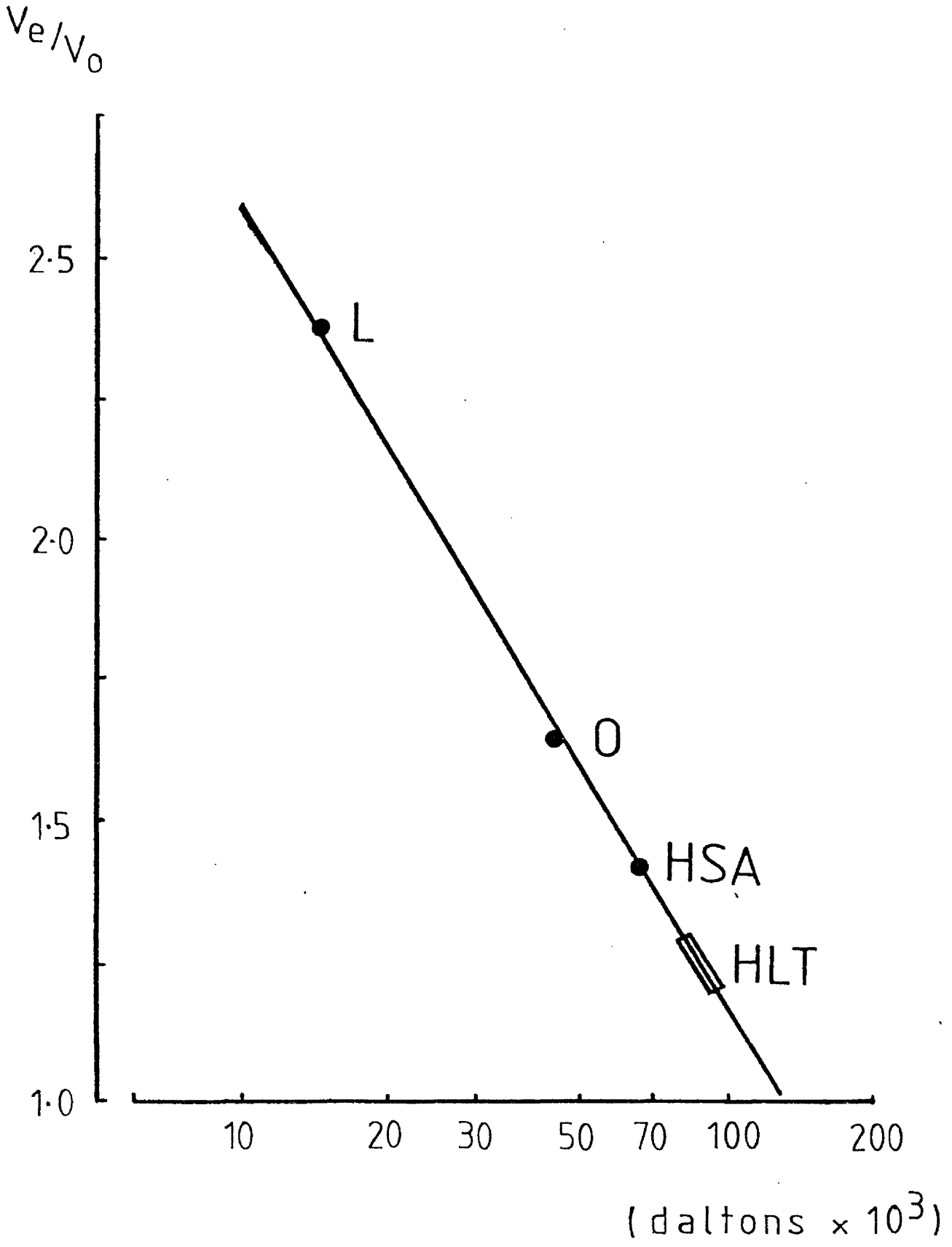
A plot of the logarithm of the molecular weight of standard proteins against the ratio of elution volume (Ve) to void volume (Vo).

HSA = Human serum albumin (mwt = 66,500)

O = ovalbumin (mwt = 44,000)

L = lysozyme (mwt = 14,400)

elution behaviour of HLT as measured by the haemorrhagic activity of fractions in suckling mice


Ve/Vo
2·5
2·0
1·5
1·0
L
O
HSA
HLT
10
20
30
50
70
100
200
(daltons × 10³)

of neutralizing antibodies to HLT (Evans, 1942) the use of the relatively high temperature of 37°C for the toxoiding of HLT might be inadvisable. Consequently, the effect of temperature in the detoxification at 37°C was investigated by comparing the rates of detoxification at this temperature with and without 0.1% (w/v) formaldehyde. This was complemented by doing a similar test at 4°C. Altogether 3 separate experiments were performed but using the one batch of toxin throughout, a 100,000 x g Millipore-filtered lysate supernatant fluid. In each experiment a portion of this toxin was taken and divided into the 4 samples required to test the toxin under each condition. Each sample was adjusted to a final concentration of 2 mg protein per ml, which was equivalent to 65 mouse LD_{50} per ml, as determined from 4 independent estimations on the stock toxin solution (table 35). Portions of each sample were removed at intervals throughout a 14 d period to assess their toxicity but before this could be done they had to be dialysed to remove formaldehyde (if present), and part taken to confirm they were bacteriologically sterile.

The combined results of the 3 experiments performed are depicted in figure 14. At 4°C there was no appreciable loss of toxicity over the 14 d test period, even 1 in 10 dilutions of the 14 d samples were, in a dose of 0.5 ml, still toxic despite initially containing only about 3 LD_{50}. However there was a gradual detoxification during storage at 4°C with formaldehyde. By the 3rd day samples diluted 1 in 10 were no longer toxic and by day 9 all toxicity was lost. At 37°C detoxification with formaldehyde was very rapid, all activity being destroyed within 1 d. The control sample held at 37°C without added formaldehyde showed a different pattern of loss: although 1 in 10 dilutions were atoxic by day 3, the undiluted samples still had residual toxicity at day 14.

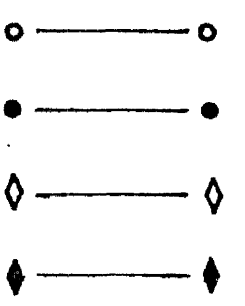
Thus it was confirmed that HLT was detoxified fairly quickly at 37°C and, when formaldehyde was added, very quickly. Whereas HLT held at

Table 35 The toxicity of the HLT preparation used to study the effect of temperature and formaldehyde on HLT detoxification

Dose (μl)/ml	Number of deaths/number of mice injected, in experiment			
	1	2	3	4
40	NT	NT	11/15	8/9
20	7/10	6/9	9/15	9/10
10	3/10	2/10	7/15	2/10
5	0/10	0/10	0/15	0/10
2.5	0/10	0/10	NT	NT
LD_{50}/ml, individual test results	69	63	59	68
geometric mean value		65		

NT Not tested

Figure 14 Detoxification of HLT : the effect of temperature and formaldehyde



$4^{\circ}C$, undiluted toxin

$4^{\circ}C$, 1 in 10 diluted toxin

$4^{\circ}C$ + formaldehyde, undiluted toxin

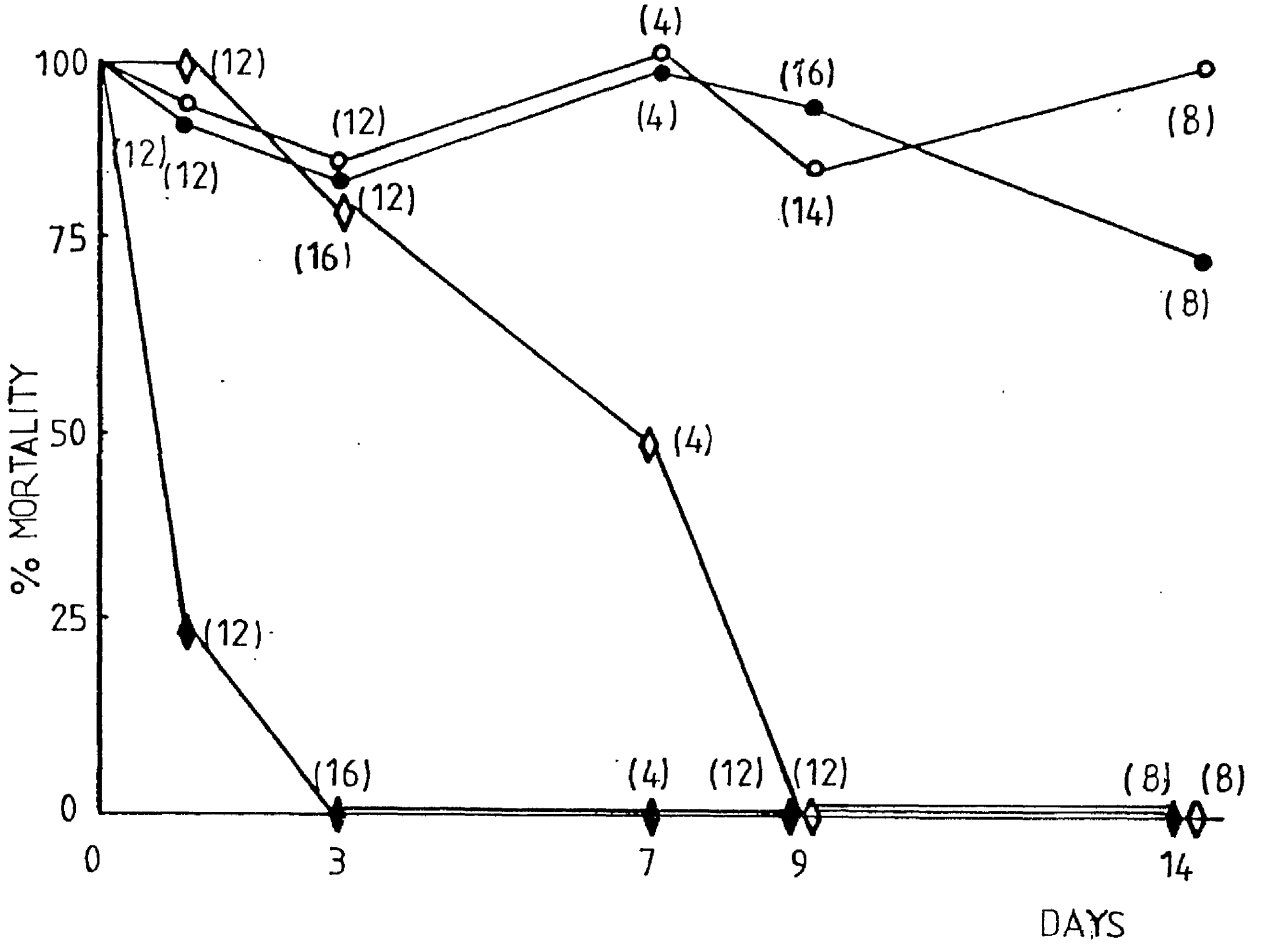
$4^{\circ}C$ + formaldehyde, 1 in 10 diluted toxin

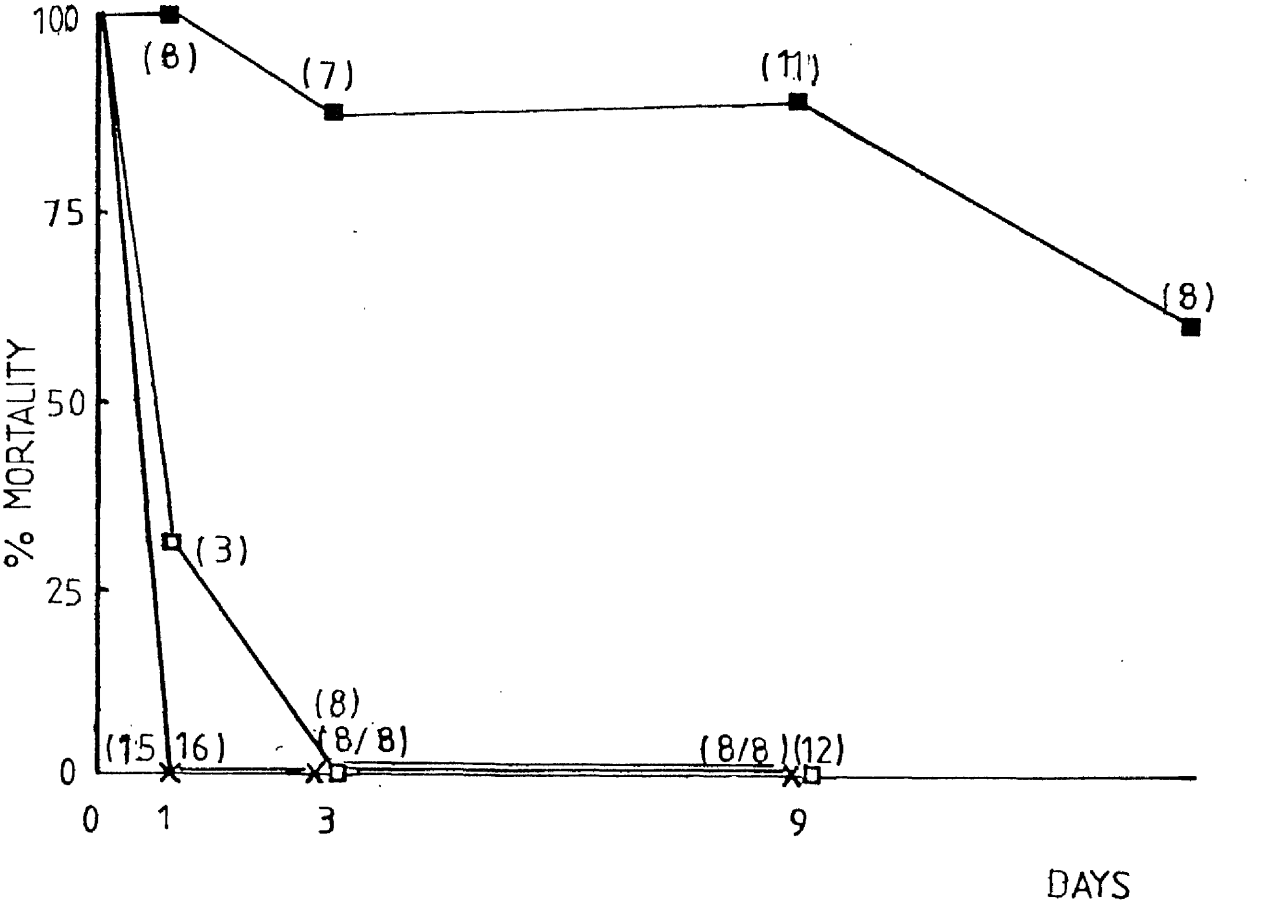
$37^{\circ}C$, undiluted toxin

$37^{\circ}C$, 1 in 10 diluted toxin

$37^{\circ}C$ + formaldehyde, undiluted and 1 in 10 diluted toxin

The figures in parentheses show the number of mice tested per point


100
75
50
25
0
% MORTALITY
0
3
7
9
14
DAYS
(12)
(12)
(12)
(12)
(16)
(4)
(4)
(16)
(14)
(8)
(8)
(4)
(12)
(16)
(4)
(12)
(12)
(8)
(8)


100
75
50
25
0
% MORTALITY
0
1
3
9
DAYS
(8)
(7)
(11)
(8)
(3)
(8)
(15)
(16)
(8/8)
(8/8)
(12)

4°C was relatively stable, unless formaldehyde was present, and even then the detoxification proceeded relatively gradually. Subsequently, a further two batches of toxin (lysate supernatant fluids) were treated with 0.1% (w/v) formaldehyde at 4°C, since this latter temperature seemed to be potentially better than 37°C for producing toxoided HLT. After 15 d the toxicity of both preparations had fallen to 1-3% of that originally present (table 36; see appendix 5 for the data before statistical evaluation), a level of activity which would not perhaps have been detected in the initial detoxification experiments which used less potent starting material. The residual toxic activity would appear to be due to HLT since when the first of these latter mentioned batches of toxoid, which were free from formaldehyde, was tested in suckling mice it elicited a haemorrhagic response. However when this material was heated (56°C, 15 min) there was no reduction in its lethal toxicity for 3-4 wk old mice, as if HLT had acquired heat-stability.

6.2 HSF and LPF activity in formaldehyde-detoxified HLT preparations

Having established that 15 d incubation at 4°C with 0.1% (w/v) formaldehyde inactivated HLT, it was decided to see what effect this treatment had on the activity of the HSF and LPF present in lysate supernatant fluids. To do this, both batches of the above mentioned toxoid were assayed against the toxin from which they were prepared, although as native toxin was too toxic for use in HSF and LPF assays heated toxin (56°C, 30 min) was used instead. Unexpectedly the heated preparations were only about one-half as active as their formaldehyde-detoxified counterparts (table 37; see appendix 5 for the HSF data prior to statistical evaluation). If HSF and LPF are heat-stable, requiring treatment at 80°C for inactivation, these results suggest formaldehyde treatment enhances their activity.

Table 36 Reduction in HLT activity after 15 d incubation at 4°C with 0.1% (w/v) formaldehyde

Expt.	Sample	Test	Mouse LD_{50}/ml	
			Individual test result	Sample geometric mean
1	Untreated HLT	1	125	111
		2	98	
		3	113	
	Formaldehyde treated HLT	1	2.5	1.7
		2	1.4	
		3	1.5	
2	Untreated HLT	1	294	285
		2	322	
		3	245	
	Formaldehyde treated HLT	1	3.6	3.6

Table 37 HSF and LPF activities in formaldehyde-and heat-($56^{o}C$, 30 min) detoxified lysate supernatant fluids

Pathophysiological activity	Relative potency and 95% confidence limits of heat-detoxified sample (formaldehyde treated samples = 100%)	
	Expt. 1	Expt. 2
HSF	40 (27-61)	76 (27-213)
LPF	48 (34-64)	36 (15-61)

6.3 Immunogenicity of formaldehyde-detoxified HLT in mice

Mice were actively immunized with the formaldehyde-detoxified HLT described above (experiment 1, tables 36 and 37), in an attempt to produce animals with demonstrable levels of anti-HLT, and thereby confirm that in detoxifying HLT a toxoid had been made, and provide animals for assessing whether antitoxin might have protective activity in experimental B. pertussis infections of mice.

In the initial two experiments a variety of immunization conditions were tested, viz. the use of adjuvants (Freund's complete and incomplete, $AlOH_3$), injection routes (intraperitoneal, subcutaneous, intramuscular) and number of injections (1 or 2). These mice received either 100 or 200 μg of protein, which corresponded to 5.6 or 11.2 mouse LD_{50} equivalents of HLT. However, with a few exceptions, these mice were unable to resist an intraperitoneal challenge with 5 or 3 mouse LD_{50} of HLT (tables 38 and 39). Moreover, sera taken from those mice which survived the challenge did not show any sign of toxin-neutralizing capacity when mixed with 5 suckling mouse MRD of HLT. However most of the sera contained precipitins to the toxoid as demonstrated by crossed-over electrophoresis, and therefore had at least made an immune response to the "toxoid", if not to HLT itself.

In a further experiment the number of immunizing injections was increased to 3 and the dosage of toxoid to 14 mouse LD_{50} equivalents. Four different immunization protocols were followed, using 80 mice in all, 4 of which died during the course of the immunization. Forty of these mice, 10 from each type of immunization followed, were challenged with 3 LD_{50} of a previously titrated toxin preparation, although this was checked by including unimmunized mice in the test and giving them undiluted or 1 in 3 diluted challenge toxin (table 40). Five of the 40 immunized mice

Table 38 Response of mice immunized with a single dose of formaldehyde-detoxified lysate supernatant fluid (100 μg protein = 5.6 mouse LD_{50} equivalents) to an intraperitoneal challenge with 5 LD_{50} of HLT

Adjuvant	Injection route	Number of deaths/number of mice challenged with 5 LD_{50} of HLT	
		Expt. 1	Expt. 2
None	intraperitoneal (IP)	4/4	5/5
"	subcutaneous (SC)	5/5	5/5
$AlOH_3$	IP	3/4	3/5
"	SC	5/5	5/5
Freund's incomplete	IP	2/5	5/5
" "	SC	5/5	5/5
Freund's complete	IP	1/4	4/4
" "	intramuscular (IM)	4/5	5/5
Response of unimmunized mice to,			
i) the challenge toxin		10/10	10/10
ii) 1 in 5 diluted challenge toxin		5/10	0/10

Table 39 Response of mice immunized with two doses of formaldehyde-detoxified lysate supernatant fluid (each of, 100 μg protein = 5.6 mouse LD_{50} equivalents) to a lethal intraperitoneal challenge with HLT

Primary immunization		Secondary	Number of deaths/number of mice challenged with HLT[a]	
Adjuvant	Injection route	immunization	Expt. 1	Expt. 2
None	intraperitoneal (IP)	Plain toxoid (IP)	5/5	5/5
"	subcutaneous (SC)	"	5/5	5/5
$AlOH_3$	IP	"	5/5	5/5
"	SC	"	4/5	5/5
Freund's incomplete	IP	"	5/5	4/4
" "	SC	"	4/4	5/5
Freund's complete	IP	"	4/4	4/4
" "	intramuscular (IM)	"	4/4	3/4
Response of unimmunized mice to,				
i) the challenge toxin			10/10	10/10
ii) 1 in 5 or 1 in 3 diluted challenge toxin respectively			3/10	2/10

a In experiment 1 the challenge was 5 LD_{50} of HLT, in experiment 2 only 3 LD_{50}

Table 40 Response of mice during immunization with formaldehyde-detoxified lysate supernatant fluid (3 doses, 14 mouse LD_{50} equivalents), and when challenged intraperitoneally with 3 LD_{50} of HLT

Group	Immunization mixtures, dosage (μg protein) and routes	Proportion of mice that died during immunization	Proportion of mice that died after challenge with toxin	
			anaphylactic deaths	toxic deaths
A	Plain toxoid (50 + 50 + 150, IP)	3/20	2/10	6/10
B	Toxoid/$AlOH_3$ (50 + 50, SC) + plain toxoid (50, IP)	0/20	2/10	8/10
C	Toxoid/Freund's incomplete adjuvant (50 + 50, SC) + plain toxoid (150, IP)	0/20	0/10	10/10
D	Toxoid/Freund's complete adjuvant (50, IM) + toxoid/Freund's incomplete adjuvant (50, SC) + plain toxoid (50, IP)	1/20	1/10	9/10
Control	None			10/10 (9/10)[a]

a Response of mice given a 1 in 3 dilution of the challenge dose.

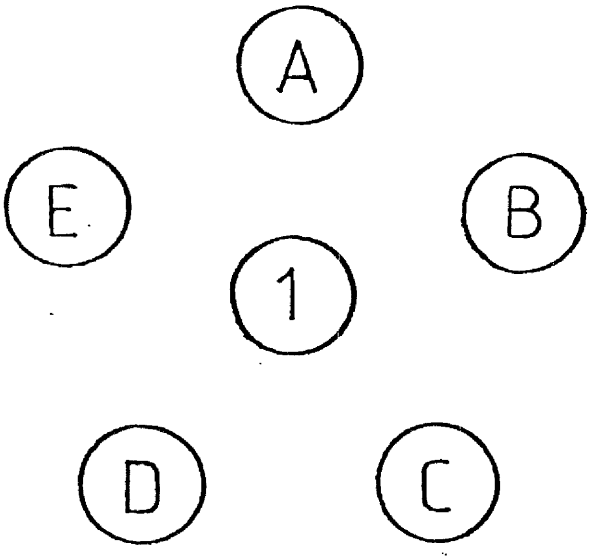
The following abbreviations were used to denote the immunization route used; intraperitoneal (IP), subcutaneous (SC) and intramuscular (IM)

challenged died within a few hours of the challenge, too soon to be toxicity deaths, probably anaphylactic deaths. This further verifies the intensity of the immunization procedures used. However the immunization was still insufficient to protect mice from a challenge with HLT, only 2 survived. These mice were bled and their serum pooled (serum E) and tested for antitoxin. There was some indication that when serum E was mixed with one suckling mouse MRD of HLT there was neutralization, but this was unconfirmed as there was insufficient material for re-testing. Pooled sera were also prepared from the remaining 35 mice which had been immunized but not challenged with HLT; there was a serum for each of the 4 immunization protocols followed (ie, A, B, C and D). However all 4 lacked antitoxin, being unable to neutralize even one suckling mouse MRD of HLT. Nevertheless, the mice had made a stronger immune response than in the initial two experiments. On crossed-over electrophoresis of sera A, B, C and D with toxoid, precipitin lines were both more numerous and stronger than before. That precipitins had been produced can also be shown by comparative immunodiffusion (plate 15) although fewer lines can be resolved.

6.4 Immunogenicity of formaldehyde-detoxified HLT in rabbits

Having failed to produce mice with demonstrable levels of circulating antitoxin by active immunization, it was decided to do so by passive immunization, provided antitoxin could be raised to the formaldehyde-inactivated HLT viz. that the latter was a true toxoid. Consequently, the same "toxoid" used with the mice was given to rabbits, a species known to be capable of producing antitoxin. Two rabbits (A and B) received 560 mouse LD_{50} equivalents of "toxoid" or 10 mg of protein, whilst two others (C and D) were given 914 mouse LD_{50} equivalents or 16.3 mg of protein. All 4 rabbits were then bled, and serum from each tested for its ability to neutralize the haemorrhagic activity of HLT.

Plate 15 Comparative immunodiffusion analysis of sera from mice injected with toxoided HLT



1 Toxoided HLT, 1 mg protein per ml

Sera raised to:-

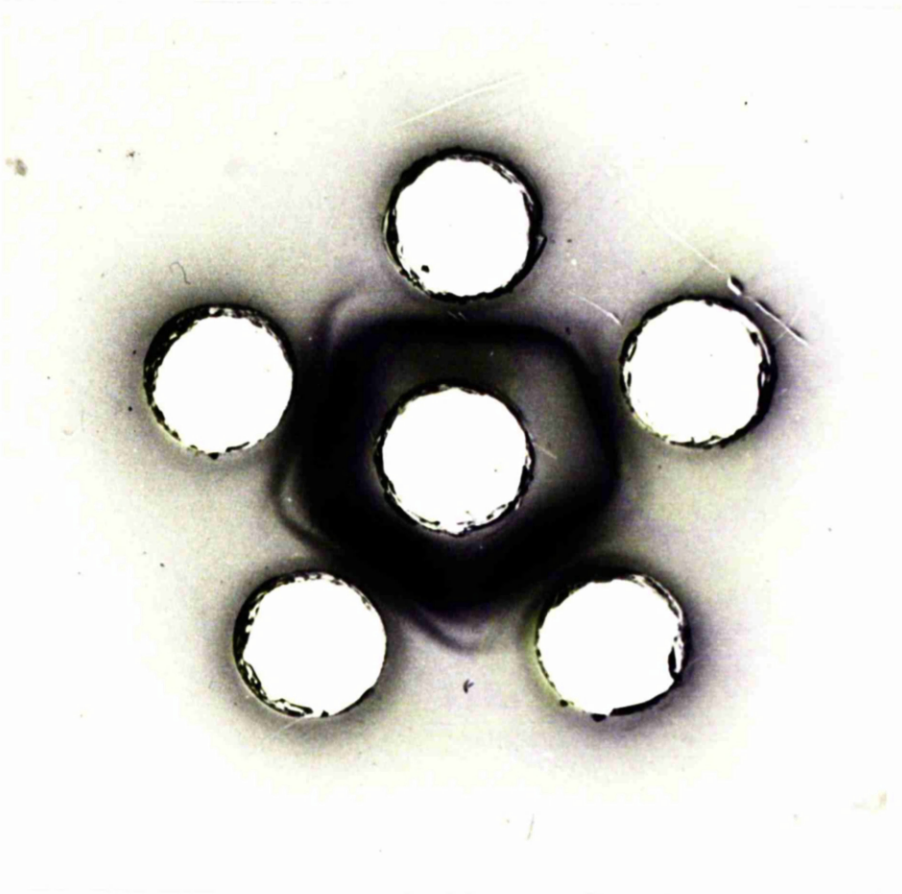
A Plain toxoid (3 doses)

B Toxoid/$AlOH_3$ (2 doses) + plain toxoid (1 dose)

C Toxoid/Freund's incomplete adjuvant (2 doses) + plain toxoid (1 dose)

D Toxoid/Freund's complete adjuvant (1 dose) + Toxoid/ Freund's incomplete adjuvant (1 dose) + plain toxoid (1 dose)

E Plain toxoid (3 doses) + an intraperitoneal challenge with 3 LD_{50} of toxin



On discovering that at least two of the rabbit antisera contained antitoxin, portions of each were absorbed with live B. pertussis cells to leave as few antibodies as possible to B. pertussis components other than HLT. This was of great importance if anti-HLT antibodies were to be assessed in experimental B. pertussis infections for protective activity.

Altogether 7 sera were titrated for their antitoxin content; the 4 rabbit antisera described above (A, B, C and D), their absorbed derivatives (B^{ab} and D^{ab}) and finally the US standard antipertussis serum which was to be included as a reference preparation in the passive mouse protection tests. In the final analysis it was found that antisera from 3 of the 4 rabbits contained antitoxin (tables 41 and 42). Antisera B, C and D had mean HLT neutralizing titres of 1,000, 120 and 100 units per ml respectively, whilst antiserum A lacked antitoxic activity ie, $\leq$ 40 units per ml (40 units per ml corresponds to 0.025 ml of undiluted serum in the 0.05 ml injection mixture). With the exception of antiserum B the antitoxic response was poor, especially when it is considered that the development of the haemorrhagic reaction in suckling mice is one of the most sensitive ways of assaying HLT. The size of the response was not apparently determined by the amount of toxoid given. Rabbit B received 40% less toxoid than either C or D, yet antiserum B had about 10 times the antitoxic activity of antisera C and D. Somewhat surprisingly, after repeated absorption with live cells of B. pertussis 134 antiserum B (1,000 units of antitoxin/ml) lost 75% of its antitoxic activity and antiserum D (100 units of antitoxin/ml) actually became toxic. The US standard antipertussis serum lacked antitoxic activity.

The agglutination titres of all 7 antisera were also determined, since agglutinin might also exert a protective effect in the passive mouse protection tests. Three separate paired titrations were completed for

Table 41 Neutralization of one MRD of toxin in suckling mice by rabbit antisera B and B^{ab}

Antiserum	Amount of serum (µl) in the 0.05 ml injection mixture	Number of haemorrhagic responses/number of mice injected		Antitoxin titre (units/ml)[a]
		Antiserum	Normal serum	
B	1.25	0/4	3/3	800
	0.625	4/4	3/3	
	1.11	0/3	3/3	900
	0.74	2/3	4/4	
	0.49	3/3	5/5	
	1.00	0/3	3/3	1410
	0.71	0/3	NT	
	0.50	2/3	NT	
B^{ab}	6.25	0/3	3/3	160
	3.12	1/3	2/2	
	1.56	3/3	2/2	
	4.17	0/3	2/2	240
	2.08	2/3	2/2	
	1.04	2/2	2/2	
	4.17	0/4	4/4	360
	2.78	0/5	4/4	
	1.85	1/4	4/4	

a Where a unit is the smallest amount of antitoxin required to neutralize 1 MRD of toxin in suckling mice

NT Not tested

Table 42 Neutralization of one MRD of toxin in suckling mice by rabbit antisera C and D

Antiserum	Amount of serum (µl) in the 0.05 ml injection mixture	Number of haemorrhagic responses/number of mice injected		Antitoxin titre (units/ml)[a]
		Antiserum	Normal serum	
C	12.5	0/4	3/3	120
	8.33	0/3	3/3	
	5.56	1/4	3/3	
	8.33	0/2	2/2	120
	5.56	1/2	2/2	
	3.70	1/1	3/3	
D	12.5	0/3	3/3	80
	6.25	1/3	2/3	
	3.12	3/3	3/3	
	8.33	0/3	3/3	120
	5.56	3/3	3/3	
	3.70	3/3	3/3	

a Where a unit is the smallest amount of antitoxin required to neutralize 1 MRD of toxin in suckling mice

each antiserum using the method of Preston (1970) with B. pertussis 134 as the indicator organism. This was the strain, from which the toxoid used to immunize rabbits A-D had been prepared, and with which antisera B and D were absorbed. As can be seen from table 43, the antisera raised to the toxoid had fairly low agglutination titres (600-1,200), much lower than the titre of about 8,000 for the US standard antipertussis serum. As expected there was no detectable agglutinin in the absorbed sera. However as another index of how successful the absorption had been, these antisera were compared with the others by immunodiffusion (plate 16). The large numbers of antigens present in the crude toxoid and the extent of the immune response made to them is clearly shown. The maximum number of precipitin lines which could be resolved for the interaction between toxoid and each antiserum were as follows: A, 8; B, 6; B^{ab}, 3; C, 7; D, 5; D^{ab}, 3; the US standard antipertussis serum, 5. Absorption of antisera B and D resulted in the loss of at least 3 and 2 precipitin lines respectively. Additional precipitin lines did form however, between these absorbed sera and neighbouring sera, indicating the acquisition during absorption of B. pertussis components.

In plate 17 the antisera have been allowed to interact with both toxoid and the toxin from which it was derived. It is obvious that the toxoiding process has not grossly altered the antigens in the crude HLT starting material.

7 Antitoxic immunity and protection of mice against intracerebral and intranasal challenges with B. pertussis

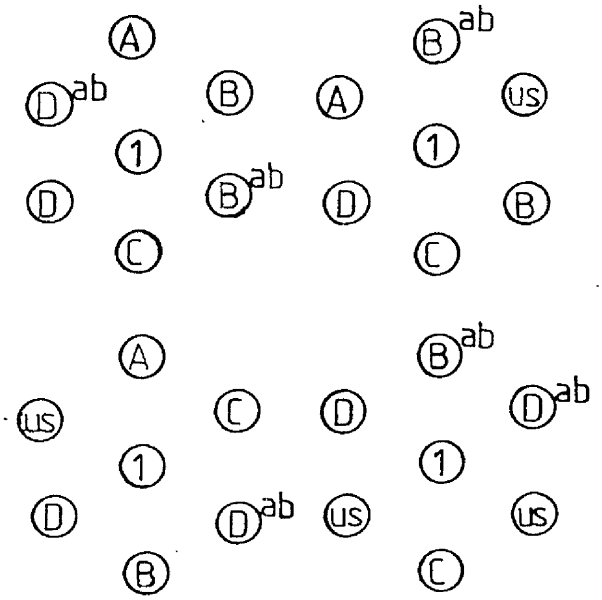
The role of antitoxic immunity in B. pertussis infections was investigated by assaying the antisera described in the preceding sections for their ability to protect mice against lethal intracerebral (IC) and

Table 43 Characterization of rabbit antisera : antitoxin and agglutinin titres

Antiserum	Mean antitoxin titre[1] (units/ml)	Mean agglutinin titre
A	< 40	1280
B	1000	570
B^{ab}	240	< 1
C	120	640
D	100	800
D^{ab}	Toxic after absorption	< 1
US	< 40	8130

1 Where a unit is the smallest amount of antitoxin required to neutralize one MRD of toxin in suckling mice. The geometric mean values presented are based on the data given in tables 41 and 42

Plate 16 Comparative immunodiffusion analysis of rabbit sera against crude toxoided HLT



1 Formaldehyde-detoxified HLT preparation (2 mg protein/ml)

A,B,C and D Undiluted rabbit sera raised to 1

B^{ab} and D^{ab} Undiluted rabbit sera B and D after absorption with B. pertussis 134

US US standard antipertussis serum

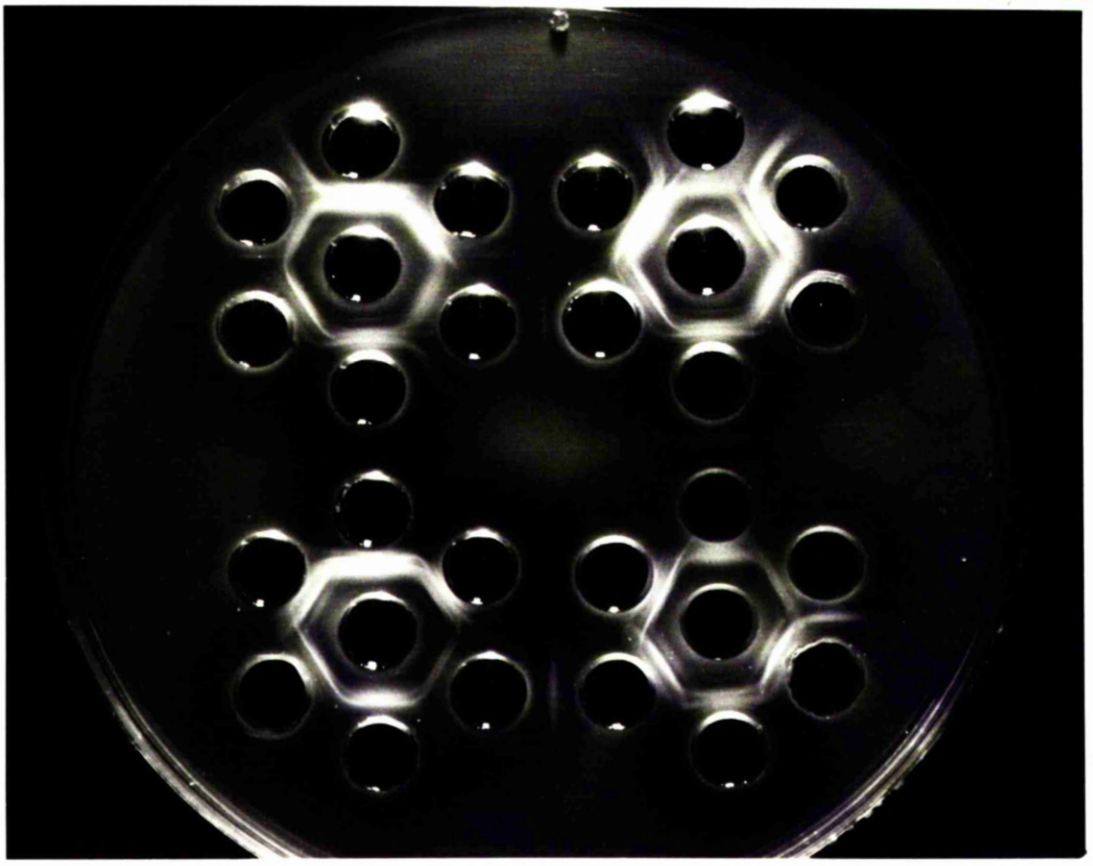
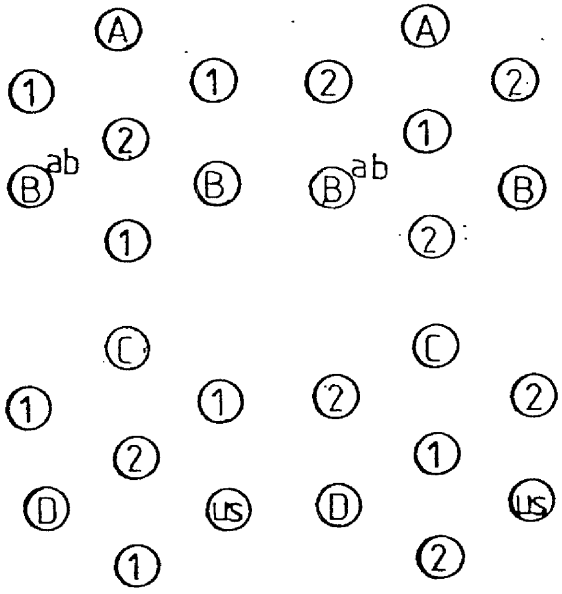


Plate 17 Comparative immunodiffusion analysis of rabbit sera against crude toxoid and the toxin from which it was made



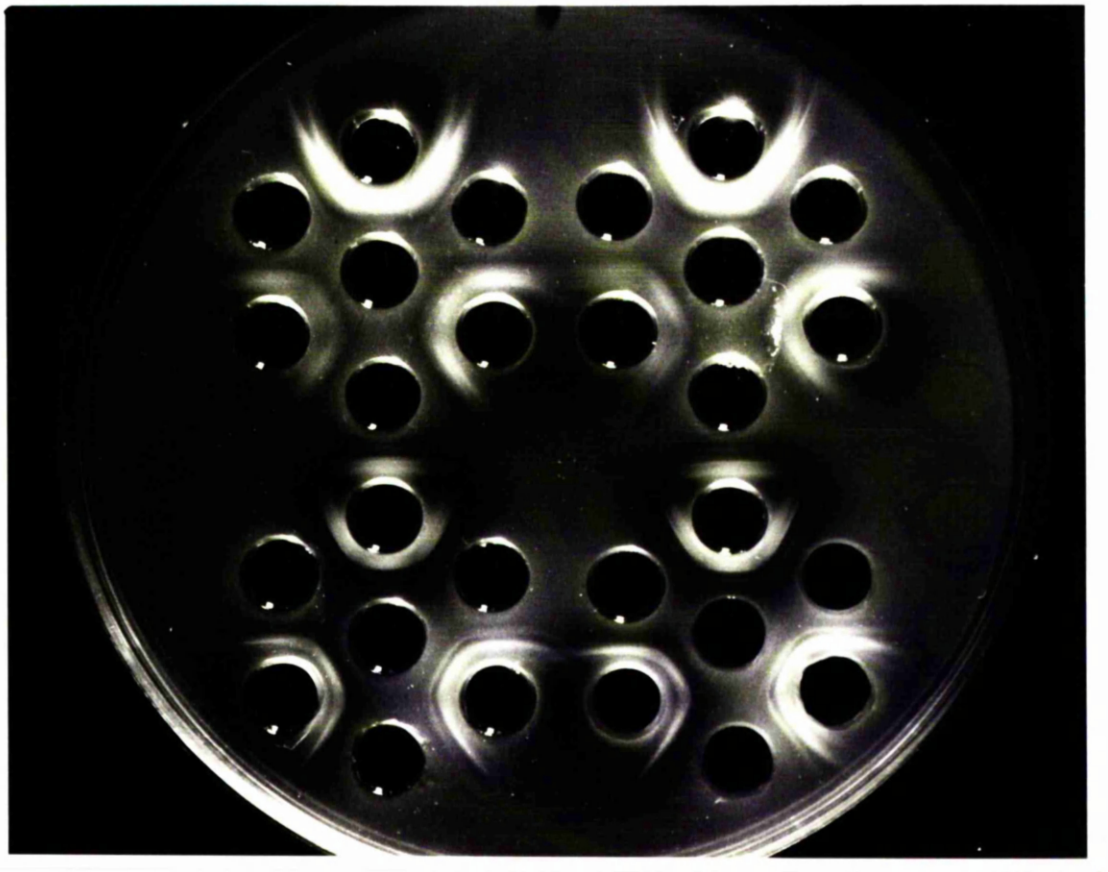
1 Formaldehyde-detoxified HLT preparation (2 mg protein/ml)

2 Active HLT preparation (2 mg protein/ml)

A,B,C and D Undiluted rabbit sera raised to 1

B^{ab} Undiluted rabbit serum B after absorption with *B. pertussis* 134

US US standard antipertussis serum



sub-lethal intranasal (IN) challenges with B. pertussis 18323. The antisera were given either diluted 1 in 2 with the challenge organisms (IC/mix or IN/mix) or undiluted in a 0.2 ml volume, intraperitoneally 1-4 h before the challenge (IP/IC or IP/IN). In the mix tests heat-treated antisera (56°C, 10 min) were used, to prevent complement mediated killing of the challenge.

7.1 Passive protective activity of rabbit antisera in B. pertussis intracerebral infections of mice

Of the 5 antisera (A, B, C, D and the US standard antipertussis serum) tested in the IC/mix experiments only the US standard antipertussis serum gave substantial protection as shown by a highly significant ($P \leq 0.005$) reduction in mortality. When mixed with 250 LD_{50} of the challenge organism only 1 of 30 mice died during the 14 d test period as compared to the 100% mortality experienced in the groups of control mice (table 44). In sharp contrast to the high protective potency of the standard antiserum, which contained no antitoxin, was the lack of protective activity (35 deaths out of 40 mice tested) associated with antiserum B, which had an antitoxin titre of 1,000 units per ml. Antibodies against HLT would seem therefore to have no protective effect in the IC/mix test. Even the few mice which did survive when given antitoxic serum B are unlikely to have done so because of anti-HLT antibodies, since this effect was also evident with antisera which contained less than 15% of the antitoxin present in B but which were raised to the same toxoid as B ie, A,C and D. The type of protective antibody which these latter mentioned antisera lack but which the standard antiserum so obviously contains could be agglutinin. Against B. pertussis 134 the standard antiserum had a titre of 8,130 as compared to 1,280 for antiserum A, the antiserum with the next highest titre. However antibodies directed at other components of B. pertussis cannot be ruled out.

Table 44 Passive protection of mice against a lethal intracerebral challenge with B. pertussis 18323 (L6d H2), by rabbit antisera

Test	Serum	Number of deaths/number of mice challenged, for experiments 1-6						
		1	2	3	4	5	6	Total
IC/mix	A	9/10	10/10	NT	NT	NT	NT	19/20
	B	8/10	10/10	8/10	NT	9/10	NT	35/40
	C	9/10	9/10	NT	NT	NT	NT	18/20
	D	9/10	10/10	NT	8/10	NT	10/10	37/40
	US	1/10	0/10	NT	NT	NT	0/10	1/30
IP/IC	B	NT	NT	5/10	NT	8/10	NT	13/20
	D	NT	NT	NT	6/9	NT	9/10	15/19
IC/mix	Normal	10/10	10/10	10/10	10/10	10/10	10/10	60/60
Control	None	10/10	10/10	10/10	10/10	10/10	10/10	60/60
1/10[a]	None	10/10	10/10	9/10	9/10	9/10	10/10	57/60
1/50[a]	None	8/10	10/10	10/10	9/10	10/10	9/10	56/60
1/250[a]	None	3/10	6/10	4/10	7/10	6/10	4/10	30/60

a Dilutions of the challenge culture used to confirm that a 250 LD_{50} challenge was being used

Only two antisera (B and D) were tested for protective activity in the IP/IC test. With antitoxic serum B (1,000 units of antitoxin/ml) there was a highly significant ($P < 0.005$) reduction in mortality ie, only 13, instead of 20 out of 20 dead. With antiserum D, which contained only 10% of the antitoxin present in B but had about the same agglutinin content, there was also protection ($P < 0.05$). Unfortunately further experimentation which may have clarified the relative protective properties of these two antisera was prohibited by the fact it was a rather extravagant use of scarce antisera. For the same reason, the experiments with antiserum B could not be followed up using its absorbed but antitoxic derivative, antiserum B^{ab}.

7.2 Passive protective activity of rabbit antisera in sub-lethal, *B. pertussis* respiratory tract infections of mice

The antitoxic antiserum B was also tested for its protective properties in IN/mix and IP/IN tests in mice. On the 14th d after the intranasal administration of 10^5 cfu of *B. pertussis* 18323 ($\pm$ antiserum) mice were sacrificed, and their lungs cultured and examined for signs of consolidation. Both these features were scored on a 0-4 basis and the combined scores taken as an index of the severity of the infection (0 = no infection, 8 = very severe infection). In table 45 the protective properties of antiserum B are assessed by comparing the mean lung scores of the animals in the test and control groups of mice. Antiserum B exerted a significant ($P \leq 0.05$) protective effect in the IN/mix tests and to a lesser extent in the IP/IN tests. When the same data is interpreted taking into consideration only whether mice were infected (ie, with lung scores ≥ 1) or not, and disregarding the grade of infection the results shown in table 46 are obtained. Again, in this a more severe test of protective activity, it can clearly be seen that

Table 45 Passive protection by rabbit antiserum B in the sub-lethal IN/mix and IP/IN tests in mice.

A comparison of mean lung scores (maximum score = 8.0)

Serum	Test	Expt. 1		Expt. 2		Expt. 3		Expt. 4	
		Score	P	Score	P	Score	P	Score	P
B	IN/mix	0.8	≤ 0.05	1.2	≤ 0.05	0.1	≤ 0.05	1.3	≤ 0.05
B	IP/IN	4.0	≤ 0.05	NT		0.1	≤ 0.05	3.8	> 0.05
Normal	IN/mix	6.2		4.2		NT		NT	
None	Challenge controls	7.2		5.0		4.4		6.0	

P The probability of the difference between the results for the test and control mice arising by chance as judged in the Mann-Whitney U-test.

NT Not tested

Table 46 Passive protection by rabbit antiserum B in the sub-lethal IN/mix and IP/IN tests in mice. Protection was assessed by the combined lung culture and pathology score, and animals with any score ≥ 1 were counted as infected.

Serum	Test	Proportion of mice with infected lungs at day 14				Proportion totals		
		Expt.1	Expt.2	Expt.3	Expt.4	All expts.	Expts. 1 + 2	Expts. 1 + 3 + 4
B	IN/mix	4/10	4/10	0/9	4/7	12/36	8/20	
B	IP/IN	9/10	NT	1/10	8/10			18/30
Normal	IN/mix	10/10	7/10	NT	NT		17/20	
None	Challenge controls	10/10	8/10	6/8	10/10	34/38	18/20	26/28

NT Not tested

antiserum B was effective. In IN/mix tests, it gave substantial and statistically, highly significant protection ($P \leq 0.005$). The combined data for experiments 1-4 show that there was a reduction in the numbers of mice infected, from 90% (34 infected out of 38) for the challenge control mice which received no serum, to 33% (12 infected out of 36). Even if only the experiments (1 + 2) which included controls given normal serum are considered, there was still a marked reduction in the number of mice infected viz. from 85% (17 out of 20 infected) for the normal serum mix controls, to 40% (8 out of 20 infected). In the IP/IN tests, it was found once more that antiserum B was less effective than in the IN/mix tests, but it still gave significant protection ($P \leq 0.05$) viz. only 60% of the mice given antiserum B were infected (18 out of 30), compared to 93% (26 out of 28) in the corresponding control mice.

However, although antitoxic antiserum B was protective, it was by no means certain that this effect was mediated by anti-HLT. To try and clarify the relationship between antitoxin and protection, several antisera with varying degrees of antitoxic activity were tested for protective activity in the IN/mix test. It was of particular interest to see how antiserum B^{ab}, the absorbed but still antitoxic derivative of antiserum B, performed. Once more antiserum B was shown to be protective, although on this occasion there was only a significant reduction ($P \leq 0.05$) in the severity of the infection and not in the actual numbers of mice infected (table 47). However, antiserum B^{ab} completely lacked protective activity, which suggests that antibody to some component other than HLT was conferring the protection given by antiserum B. This interpretation is supported by the finding that antiserum A was protective, whilst lacking antitoxin and having been raised to the same toxoid as used to produce antiserum B.

Table 47 Passive protection of mice by various antisera in the sub-lethal IN/mix test

Antiserum	Proportion of mice infected	P	Mean lung scores	P
A	4/10	≤ 0.01	0.6	≤ 0.05
B	6/9	≥ 0.05	0.8	≤ 0.05
B^{ab}	10/10	≥ 0.05	6.2	≥ 0.05
C	4/10	≤ 0.01	0.4	≤ 0.05
D	6/9	≥ 0.01	1.9	≤ 0.05
D^{ab}	10/10	≥ 0.05	5.1	≥ 0.05
US std	1/10	≤ 0.01	0.2	≤ 0.05
Normal	10/10		5.2	
None	8/9		5.1	

Protection was assessed by the combined lung culture and pathology scores (maximum score = 8.0), and animals with any score ≥ 1 were counted as infected

DISCUSSION

1 Assaying the toxicity of HLT

1.1 Measurement of the lethal effect in mice

There are two well established ways of assaying HLT, namely by its mouse lethality and its dermonecrotic/haemorrhagic activity. Although not as sensitive as the dermonecrotic assays, the mouse lethal toxicity test was chosen here as the standard procedure. It is simple and gives clear-cut results that are amenable to statistical analysis. Moreover the local facilities were geared to providing mice rather than the guinea pigs or rabbits normally used for the assay of dermonecrosis.

Although the choice of injection route and observation period were based upon the experiences of others, few data were available on the influence of mouse sex or weight upon the response to HLT. Therefore experiments were performed to provide information on these points. *B. pertussis* cell-lysates were used as a source of HLT. That HLT was the principal toxin in these lysates was shown by the fact that 98% or more of the lethal toxicity was destroyed by heating at $56^{o}C$ for 20 min.

Both male and female, 3-4 wk old HAM/1CR mice were equally sensitive to the lethal toxicity of *B. pertussis* cell-lysates ie, HLT. Nevertheless to err on the side of caution mice of one sex were used where possible in subsequent work or, when both sexes had to be used, they were distributed evenly between preparations.

When non-weight adjusted doses of lysates were given to groups of 10.0-13.5 g and 18.0-23.0 g, 3-4 wk old HAM/1CR mice, two somewhat conflicting results were obtained. With one sample of HLT there was about a 50% lower toxicity in the large mice, but with another sample this could not be confirmed. It is possible that the two HLT preparations both of which were derived from Hornibrook-grown cultures of *B. pertussis* 18334 were qualitatively different in some way, or the result may have arisen

purely by statistical fluctuation. In any case the difference was not large. The matter was not therefore explored any further, but in subsequent tests, mice of different weights were always distributed evenly among the various test preparations. It may be noted that there is not necessarily a simple proportional relationship between susceptibility to a toxin and body weight. For instance, no systematic proportionality exists between body weight and the response of mice to botulinal toxins (Lamanna, Jensen and Bross, 1955).

1.2 Measurement of the haemorrhagic activity in suckling mice

Where circumstances dictated that only small amounts of HLT be utilized, the toxin was assayed by its haemorrhagic activity in suckling mice (eg, Katsampes, Brooks and Bradford, 1942). This assay was at least 10 times more economical than the lethality test in 3-4 wk old mice; 0.05 ml of a toxin solution containing 1 mouse LD_{50} in 0.5 ml, as measured in weaned mice, gave a definite haemorrhagic reaction in suckling mice. Applications of the haemorrhagic assay included the screening of fractions for HLT activity during purification by column chromatography and for testing antisera for HLT-neutralizing antibodies. This latter category of experiment led to an unexpected finding. In 4 out of 5 experiments, HLT elicited a slightly, but significantly, more severe haemorrhagic response when diluted 1 in 2 with normal rabbit serum instead of PBS. However, because this enhancement effect was relatively small, it was not explored further. Nevertheless the possibility was considered that HLT may exist as a precursor or zymogen which must be activated for toxicity to be manifest, as suggested by Anderson and North (1943). Alternatively, the serum may have prevented HLT being lost by adsorption onto glassware. Results presented by Nakase et al (1969) could be viewed as supporting this latter interpretation since the rabbit dermonecrotic activity of their purified

HLT was 2-4 fold greater when any one of normal rabbit serum, bovine serum albumin, B. pertussis agglutinogen or heated sonic extract was included in the injection mixture. It should be possible to avoid the loss of HLT by adsorption onto glassware by using gelatin containing buffer as diluent. However, although the addition of extraneous proteins to purified HLT may be important for this reason it seems less plausible as an explanation where a crude HLT fraction is in use ie, a lysate supernatant fluid containing 50 μg protein per ml. Irrespective of the mechanism behind the enhancement phenomenon, it necessitates the use of dilutions of normal serum in parallel with dilutions of antiserum when titrating the latter for HLT-neutralizing antibodies.

Prior to the adoption of the haemorrhagic suckling mouse assay, a few adult hairless mice (Hr/Hr) which were available in the department were injected intradermally with 0.05 ml of a toxic 100,000 x g lysate supernatant fluid. A dermonecrotic response developed. The most prominent feature of the reaction in the first few days was a raised pale spot. With the larger doses small pinpoints of necrosis were evident within this area and they gradually developed and coalesced to form necrotic plaques. These irregular depressed necrotic lesions did not lend themselves to being measured. Likewise the pale spots although well defined blebs at low doses were not well defined at high doses. Note that this is in contrast to the report that mice are resistant to the dermonecrotic effect of HLT (Violle, 1950 cited by Muñoz, 1971), although it is not clear whether this refers to adult or suckling mice. Primarily for reasons of convenience, and the distinctiveness and rapidity of appearance of the haemorrhagic lesion in 4 d old mice, the latter were used.

1.3 Testing HLT for haemolytic activity

One of the major drawbacks with investigations on HLT is the

dependence on animal experimentation for titrations of the toxin. If the haemolytic activity of B. pertussis was a property of HLT it would be possible to develop a simple, rapid and inexpensive assay. However it was found that neither sheep, rabbit, horse, human (O, rhesus +) nor cod red cells lysed in the presence of as much as 2.5 mouse LD_{50} per ml of toxin (100,000 x g, lysate supernatant fluid). Neither was there a hot-cold lysis. It therefore does not seem likely that HLT has haemolytic activity. With hindsight it might have been useful to have looked for haemolysis with HLT in a cysteine-containing buffer, in case the haemolysin was oxygen-labile.

Another possible assay for HLT that was considered was the lethality test in intravenously injected embryonated eggs, which according to Novotny (1977, personal communication) are particularly sensitive to killing by this toxin. However such an assay would be less convenient than the 3-4 wk old mouse lethality test or the haemorrhagic assay in 4 d old mice. Another more promising possibility would be to assay HLT by its cytotoxicity for tissue culture cells. Although perhaps not as convenient or inexpensive as a haemolytic assay, it would enable experimentation on a scale unlikely to be realized by animal-based tests. Some groundwork has already been done in showing that certain mammalian cell-lines are susceptible to HLT (eg, HeLa and primary cultures of mouse embryonic tissue : Angela, Rosso and Giuliani, 1962a and b; Střížova and Trlifajava, 1964) but these reports require confirmation.

2 Storage-stability of HLT

The stability of HLT (cell-lysates) to storage at -20°C for periods up to 3 yr was investigated. Of the 4 different samples tested, 2 showed no decrease in activity whilst 2 lost activity at rates of about 20% per annum. Taken collectively the data indicate an annual loss

in toxicity of about 10% under these conditions, which included repeated freezing and thawing of the samples. Alternatively, the results could be said to show that genetic drift in the test mice had made them more resistant to HLT but this explanation seems improbable since it would predict that all 4 lysates would respond as one and this was not the case. In conclusion, storage at -20°C is satisfactory, as the rate of decay was slow enough to have little appreciable effect over a year or two, but storage after freeze-drying or in 50% glycerine or sucrose may be equally good.

3 Antigenic variation and HLT production

3.1 Antigenic modulation

It is generally accepted that B. pertussis cells injected intraperitoneally into mice do not multiply and that death is due to a toxaemia (eg, Anderson and North, 1943; Proom, 1947). On this basis we can interpret the results of Lacey (1960) who reported that the intraperitoneal mouse LD_{50} of strain H5 B. pertussis when in the X-mode was "ca. 2×10^8" and while in the C-mode "ca. 2×10^9". One part of the present investigation was to demonstrate more directly that growth of B. pertussis in the high magnesium sulphate C-medium results in markedly reduced levels of HLT.

The toxicity being measured when X-mode B. pertussis cell-lysates were injected intraperitoneally into mice was heat-labile (56°C, 20 min), at least to the extent of 98%. Presumably the residual 2% toxicity in 56°C heated preparations is due to endotoxin and/or pertussigen. The work done in this investigation showed that during antigenic modulation the heat-labile toxin was quite definitely lost. In the 3 strains of B. pertussis examined, in a series of 7 independent growth experiments, there was a reduction in the toxicity of the C-mode samples to 2.4 – 6.9% (mean 4.4%) of their X-mode counterparts. This loss of HLT was not due to excretion into the surrounding culture medium since the C-mode culture

supernatant fluids were atoxic. The residual 4-5% toxicity in C-mode cells was not tested for 56°C stability and therefore it is not possible to say whether the loss of HLT was absolutely complete, or whether the C-mode cells contained endotoxic (LPS) activity plus 2-3% of the original HLT of the X-mode cells.

Hitherto most of the changes associated with the X- to C-mode transition have involved components or features of the cell surface or envelope of B. pertussis ie, the agglutinogens and haemagglutinin, PA, HSF, LPF, adjuvant(s) for reaginic antibody and hyperacute EAE, adenylate cyclase, cytochrome d-629 and the 28K and 30K polypeptides. Also, C-mode cells autolyse more rapidly and are less readily agglutinated by $AuCl_3$ and $HgCl_2$ which again indicates changes in the exterior regions of the cell. The present work shows that HLT which is a cytoplasmic component (Cowell, Hewlett and Manclark, 1979) is also lost during antigenic modulation. Therefore the changes which take place during antigenic modulation are not restricted to the cell-envelope. It may also be noted that C-mode colonies on blood containing medium are non-haemolytic, which suggests that a diffusible haemolysin may also be affected by antigenic modulation.

3.2 Nicotinic acid induced modulation

During growth in media with high levels of nicotinic acid, changes may be induced in B. pertussis which are similar to those seen during growth in high-Mg induced (antigenic) modulation (Pusztai and Joó, 1967; Wardlaw, Parton and Hooker, 1976). However there are differences between high-Mg and high-nicotinic acid grown cells. According to Pusztai and Joó (1967) the latter retain their full toxicity.

In the present investigation B. pertussis 134 was grown, in two separate experiments, in media with high levels of nicotinic acid (500 μg/ml). Cells grown in these media showed loss of the 28K and 30K

cell-envelope polypeptides and in one instance at least the loss of HSF, as is characteristic for modulated cells. In the other experiment it was not clearly established whether HSF was lost or not. There was certainly not the 16 or more fold decrease that accompanied modulation in the first experiment but a more modest reduction seems to have occurred. However levels of HSF often fail to decline as markedly during nicotinic acid induced modulation as compared to antigenic modulation (Pusztai and Joó, 1967; Wardlaw, Parton and Hooker, 1976). But to come to the main point, it was found that the high nicotinic acid grown cells were 60% less toxic than their normally grown counterparts. These differences were statistically significant. This observation should be investigated more fully with strain 134 and with other strains. The differences between the observations made here and those of Pusztai and Joó (1967) might be explained by the different strains used. It would be of particular interest to discover the response of strain 18323 which is specifically reported as not modulating during growth in media with high concentrations of nicotinic acid (Pusztai and Joó, 1967).

The control mechanisms which regulate magnesium- and nicotinic acid-induced modulation are not known. However it appears that HLT production is controlled to some extent independently from the production of HSF and the 28K and 30K cell-envelope polypeptides.

4 HLT production and purification

4.1 Production

4.1.1 Strains

Before embarking on the purification of HLT it was considered worthwhile to screen a selection of B. pertussis strains to choose the best producer of HLT. With certain other toxigenic bacteria outstandingly good toxin producers exist such as Wood 46 for α toxin production by

Staphylococcus aureus (Bernheimer, 1970) and PW8 for toxin production by Corynebacterium diphtheriae (Zabriskie, 1970). At present, no universally accepted HLT-producing strain of B. pertussis has come to the fore.

In the initial screening, 10 strains of B. pertussis were tested in a series of small trials that permitted rapid assessment of their HLT producing potential. Among these strains were some selected as being potentially useful, including two recently isolated strains, a strain used in a purification study (Maeno) and another discarded as a vaccine strain because of its toxigenicity (No. 28). However all the strains tested were very similar in their toxicity with only a 3-fold difference between the least and most toxic. It is of interest that the two recently isolated strains were unremarkable in their toxicity relative to the established laboratory strains and despite the possibility that repeated subculture may lead to reduced toxigenicity (van Heyningen, 1970). However, of the 3 strains which looked promising (ie, Maeno, No. 28 and 134) there were 2 which had been pre-selected as being potentially good toxin producers. On re-assessing the toxicity of the strains Maeno, No. 28 and 134 by re-testing the original samples and twice testing samples from a further growth experiment, the original findings were confirmed. These strains were 2-3 times more toxic than the arbitrarily chosen reference strain 18334. More often than not this 2-3 fold difference was statistically significant. However the 3 strains themselves could not be put in any rank order and this led to B. pertussis 134, which grew well, being chosen.

A further 4 strains were tested at various times after this initial selection process. There were no indications that any of these contained more HLT than strain 134, although strain 44122/7R appeared to be of similar toxicity. Included in one of these later tests was strain B-1593/57 which Spasojević (1977) found to be the most toxic of the 201 strains which she had examined.

The assumption is made throughout, from evidence already presented, that the toxicity measured here by intraperitoneal injection of mice with B. pertussis cells is due, essentially completely, to HLT.

4.1.2 Culture medium and time of harvest

Since no particular medium has been proved superior for the cultivation of B. pertussis, 4 media were investigated since it is known that medium composition influences HLT production viz. when the levels of either glutamate or proline in the IG + IP medium of Stainer and Schölte (1971) were increased by 12-32 fold the amount of HLT produced was increased (Stainer, 1977 personal communication). In this study Stainer and Schölte (12G) medium, modified Hornibrook medium, Bordet-Gengou medium and charcoal agar were compared. B. pertussis cells were harvested after 24 h and 72 h growth as it had been reported that greater yields of toxin are obtained per cell when cultures are harvested prior to reaching peak opacity (Stainer, 1977 personal communication). In this appraisal of the different cultural conditions on HLT production 3 strains were used to confirm that the findings were applicable to more than just one strain of B. pertussis. These strains were B. pertussis 134, selected from 14 strains by its toxigenicity, the recent isolate 77/18319 and strain 18334, a routine vaccine strain from Connaught Laboratories, Toronto.

None of the media tested was outstanding in its ability to promote HLT production. All yielded cells of similar toxicity. There were indications however that strain 18334 gave 1.5-2.5 times more toxin in Stainer and Schölte (12G) medium than in modified Hornibrook medium, whereas a reversal of this trend was apparent with strain 134 (1.5 fold-differences in yields). Similarly if choosing between charcoal agar and Bordet-Gengou medium for producing toxigenic organisms of strain 134 or 77/18319 charcoal agar would possibly be a marginally better choice. However with such small differences, it would have taken extensive toxicity

tests to obtain fully conclusive data and this was not done. Nevertheless on solid media, cells collected after 24 h growth were generally more toxic than at 72 h, whereas the opposite occurred in the two liquid media. Recently Cowell, Hewlett and Manclark (1979) related toxin production in Stainer and Schölte (16G + IP) liquid medium to the growth cycle of batch grown B. pertussis. Cells were most toxic during the mid-exponential growth phase and varied as much as 10-fold in specific activity.

In all experiments the toxicity of the culture supernatant fluids was examined also. There was little sign that HLT was excreted into the surrounding medium in any of the cultures. Only in B. pertussis 18334 and 134, 72 h Hornibrook-grown cultures was there evidence of HLT in the supernatant fluids. This may reflect release by autolysis as opposed to active excretion. The recently isolated B. pertussis strain 77/18319 showed no signs of HLT release either by lysis or excretion. Of the 3 strains just mentioned, this latter might have been anticipated to be the most likely to do so, for if HLT is involved in the pathogenesis of pertussis it would seem necessary for toxin to be released. Of course, this may occur readily in vivo but not under the conditions used here.

4.1.3 Iron content of medium

With at least 6 toxigenic bacterial species, the iron content of the growth medium is critical for toxin production, with high levels suppressing the amount of toxin made (page 44). With all 6 of these species the optimal concentrations of iron lie between 0.1 and 1.0 mg per litre of medium. For instance, with diphtheria toxin the optimal concentration has been given as 0.14 mg/l. However if the iron level is increased to 0.5 mg/l there is complete inhibition of toxin production (Pappenheimer and Johnson, 1936). The production of HLT by B. pertussis

appears to be independent of the amount of iron in the medium over a wide range of concentrations, from 0.03 to 6.3 mg per litre. None of the cells or the culture supernatant fluids from any one of the 6 test media were appreciably more toxic than the others. However there is a case for further investigation of other than just B. pertussis 134 to which this study has been confined since this lack of responsiveness may be a strain peculiarity. Mueller and Miller (1945) identified a strain of Clostridium tetani which did not manifest the sharp dependence on a low and definite iron concentration for toxin production as is typical for this species.

However the iron content of the medium did influence B. pertussis 134 in other ways. Growth in the iron deficient medium yielded fewer cells, and when pelleted, these cells were very pale especially in comparison to cells from the iron rich medium which when pelleted were rusty brown. As the colouration was uniform throughout the pellet this indicates changes in the bacteria, perhaps in their cytochrome content, and not merely precipitated ferric hydroxide.

4.2 Purification

4.2.1 Disruption of B. pertussis and release of HLT

The first step in most purifications of HLT is disruption of the bacteria. Several methods have been used by other investigators to lyse B. pertussis cells to release the toxin (appendix 1) but an X-press was used throughout this work. Features of the X-press which make it suitable include:-

i) No heat generation or frothing during disruption

ii) very effective disruption eg, 3 pressings of B. pertussis cell-paste provides complete ($\geq$ 99%) disintegration

iii) large quantities of cells can be processed ie, 10-20 ml of a

concentrated cell suspension or even a cell-paste.

When freshly harvested B. pertussis was lysed by 3 passages through the X-press there was a marked increase in toxicity. With 3 samples tested on the day of X-pressing this increase ranged from 8-14 fold. With a 4th sample which was stored frozen prior to toxicity testing a 4.1-fold increase was noted but it is not possible to conclude from the data whether this value is significantly smaller. On several other occasions when X-pressing was done to cells which had been already stored overnight at -20°C, there was little (1.7-3.3 fold) increase in toxicity. There were no indications that these latter cells had lysed appreciably after being stored frozen overnight; cell suspensions after X-pressing became very viscous and the turbidity decreased considerably but there was no evidence for either of these events having occurred. Moreover no difference was found in the viability or toxicity of cells stored at 4°C for a few hours and those subjected to 5 cycles of freezing (-20°C) and thawing (room temperature). Evans and Maitland (1937) found no difference in toxicity even after 25-30 cycles of freezing and thawing. Perhaps when freshly harvested cells are lysed, HLT is converted by proteolytic action from an inactive to an active form, but when cells are stored frozen the proteolytic activity is impaired. Although conjectural, this would agree with the suggestion made earlier that serum protease may be responsible for enhancing the haemorrhagic activity of HLT (Discussion, 1.2). This hypothesis could be tested by adding freshly lysed cells to a lysate which had been stored frozen and looking for activation of the latter. Alternatively the size of the increase in toxicity on X-pressing may be related to the cultural origin of the cells eg, growth in Stainer and Schölte (12G) medium compared to Bordet-Gengou agar. Whatever the explanation it is apparent that differences in the enhancement of toxicity after X-pressing cannot simply be due to the efficiency of disruption or

the degree of inactivation of HLT during disruption. Nevertheless increases of 20-fold or more have been reported after sonication and by the use of a lysozyme/freeze-thaw technique (Cowell, Hewlett and Manclark, 1979).

4.2.2 Ultracentrifugation as a preparative step

Ultracentrifugation was introduced to remove insoluble material from lysates preparatory to subsequent purification. The data from 4 different lysates showed that about 71% of the HLT was recovered in the 100,000 x g supernatant fluid. This association of HLT with the cytoplasmic fraction is in conformity with the results obtained by Muñoz, Ribi and Larson (1959), Billaudelle et al (1960) and Cowell, Hewlett and Manclark (1979). These latter workers gave a figure of 73% as being typical of the amount of dermonecrotic activity recovered in 75,000 x g (30 min, 5°C) lysate supernatant fluids. As only about 38% of the total protein ends up in the soluble fraction, the ultracentrifugation step provides a 2-fold purification.

One anomaly in these results is the 25% of the original HLT in the ultracentrifugation pellet if cellular disruption is almost 100% effective and if the toxin is indeed a cytoplasmic component. The two most obvious explanations are that HLT although mainly cytoplasmic may be partly localized or trapped in the cell-envelope or that it becomes bound to the cell debris.

4.2.3 Ion-exchange chromatography

Nakase et al (1969) fractionated supernatant fluids from sonically disrupted B. pertussis on a column of DEAE-cellulose, by stepwise elution with increasing concentrations of phosphate buffer, as the first step in their purification of HLT. This ion-exchange chromatography step was claimed to produce a 10-fold purification with 80% recovery of toxicity

(Nakase and Kasuga, 1962). However in two separate attempts to reproduce these results most of the HLT activity was lost, presumably by destruction or retention on the column. In the most successful attempt, only one-quarter of the total HLT activity was recovered and the purity was enhanced only 1.6-fold. In both experiments HLT activity could be found, in varying amounts, in each of the last 3 of the 5 peaks eluted. Nakase and Kasuga (1962) observed this also. From this it could be interpreted that HLT exists in several molecular forms, but these results may simply show that HLT has a tendency to form aggregates or become associated with other cellular components. This latter suggestion is consistent with the proposal that when B. pertussis cells are lysed a disproportionate percentage of the HLT becomes associated with the particulate material which can be spun down by ultracentrifugation. Perhaps the successful recovery of HLT from DEAE-cellulose is dependent upon the proportion of the toxin in association with B. pertussis components with a strong affinity for DEAE-cellulose viz. the higher the percentage of un-associated HLT the higher the yields. It is noteworthy that other investigators have obtained variable results in the purification of HLT on DEAE-cellulose. Thus in addition to Nakase et al (1969), Onoue, Kitagawa and Yamamura (1963) and Iida and Okonogi (1971) used DEAE-cellulose successfully but Billaudelle et al (1960) and Banerjea and Muñoz (1962) had disappointing results. The lack of success experienced here led to alternative methods for purifying HLT being explored.

4.2.4 Fractionation with ammonium sulphate

Ammonium sulphate fractionation has previously been used in the purification of HLT. Onoue, Kitagawa and Yamamura (1963) increased the purity of their HLT 4-5 fold by fractional precipitation with ammonium sulphate whilst recovering 70% of the toxicity. In the present invest-

igation the concentrations of ammonium sulphate specified by these workers were used in the fractionation of a 100,000 x g, lysate supernatant fluid. Two preliminary experiments gave promising results with essentially all the toxicity being recovered in the 16-40% saturation precipitate as had been reported. This represents an effective purification of 2-2.5 fold, as about 40% of the original protein was recovered in this fraction. The fact that Onoue, Kitagawa and Yamamura (1963) achieved a 4-5 fold purification may be related to the pre-treatment of their sample with calcium phosphate gel. Nevertheless even a modest 2-2.5 fold purification is worthwhile if the yields, as here, are excellent especially with such a technically simple method. Furthermore an ultraviolet scan of one of the toxic fractions suggested that DNA was being removed. However to ensure a more complete removal of DNA the next lysate supernatant fluid was pre-incubated with DNase. This was done to eliminate interference from DNA in later purification steps. Unfortunately this altered the amount of ammonium sulphate required to salt out the HLT and a further experiment had to be completed to re-define the conditions required. With the introduction of RNase into the pre-incubation step of this experiment, the toxicity was halved. As the conditions were otherwise identical the RNase would seem to have been the culprit. As HLT is a protein the obvious conclusion must be that the RNase (from bovine pancreas) was contaminated with protease(s) in spite of being described by the manufacturer as a "protease free" RNase preparation. The fractionation step did however provide the information sought. Relative to the nuclease-treated starting material, 11% of the HLT was precipitated out at 30% saturation of ammonium sulphate and 70% in the 30-50% precipitate. In these, the only toxic fractions, there was 10% and 47% of the protein respectively. Consequently when fractionating a nuclease-treated sample it would be advisable simply to take a "50%" precipitate, as the purification achieved

would not be appreciably different from the figure of 1.5 for the 30-50% precipitate yet the yield would be slightly better.

Thus as an early step in the purification of HLT, ammonium sulphate fractionation is of limited but definite value. The purity is increased about 2-fold without any evidence of any overall loss of toxic activity, since the yields ranged from 81-114%. As marginally higher purifications were achieved when samples were fractionated directly, and not after treatment with nucleases, there may be a slight advantage in not using the nucleases until after the salting out step, but the problem encountered with the RNase would have to be resolved first.

4.2.5 Gel filtration

Although not a particularly difficult or troublesome technique, gel filtration had not hitherto been used in the purification of HLT. To assess its potential, samples of ammonium sulphate fractionated lysate supernatant fluid (30-50% ppt.) were run through a column of Ultrogel AcA 44 (effective fractionation range 10,000-130,000 daltons). On elution of the column, the toxin appeared as a single peak on the front shoulder of the first peak after the void volume material. In the second of two runs a purification of 4-5 fold was obtained with 34% recovery of the HLT. Higher yields were possible but with less purification. The resolution could possibly be increased somewhat by re-chromatography of the sample.

The potential increase in purity expected from using the various purification steps in succession was unfortunately never achieved in practise. However in table 48 is given a set of figures for such a purification based upon actual figures obtained for the individual purification steps. Under ideal conditions the end product would be expected to have a specific activity of 1,000 mouse LD_{50} per mg protein. Expressed differently 1 LD_{50} would be equivalent to 160 ng N. How this

Table 48 The purification of HLT : the potential maximum yields and increase in purity achieved by the methods used in this study

Fraction	Specific activity mouse LD_{50}/mg protein	Increase in purity		Yield (%)	
		in that step	cumulative	in that step	cumulative
cell-lysate	50				
100,000 x g lysate supernatant fluid	100	2	2	70	70
ammonium sulphate fraction	200	2	4	100	70
gel filtration fraction	1,000	5	20	35	25

would compare with some of the most active fractions reported in the literature is shown in table 49. It will be seen that the purified HLT that is potentially available by the methods used in this investigation would have a specific activity of about one-quarter of that of Nakase's material, assuming that a specific activity of 1.5 guinea pig MRD per ng N is equivalent to 44 mouse LD_{50} per ng N. Nakase's material was claimed to be 100% pure.

The introduction of a step to separate the various components by their charge might be productive. Perhaps chromatography on DEAE-cellulose would now work at this stage in the purification. Alternatively preparative acrylamide gel electrophoresis might be useful. Also a substantial increase in the purity of the starting material may be possible simply by harvesting the cells at the correct point in their growth cycle (Cowell, Hewlett and Manclark, 1979).

5 Molecular weight estimation by gel filtration

The molecular weight of HLT was estimated by gel filtration on Ultrogel AcA 44 (effective fractionation range 10,000-130,000 daltons) to be 89,000 daltons $\pm$ 10%. Unfortunately because of this relatively high molecular weight it was not possible to include, at short notice, a protein within the narrow molecular weight range of 100,000-130,000 daltons as a standard. Ideally for such determinations the protein of interest should be intermediate in molecular weight between two or more standards. In this instance the use of a gel with a higher overall fractionation range would have more readily enabled the inclusion of the required high molecular weight standards. A molecular weight estimate has never been given for HLT. The only pointer to its molecular weight until now was from sedimentation coefficients. Nakase et al (1969) gave a 1.4S value to their purified HLT although they cited values of 13S and 20S as being obtained by others (Sato et al, unpublished data; Kuroya et al, 1959). This

Table 49 The purification of HLT : a comparison of several protocols

Reference	Specific activity (ng N)		Increase in purity	Yield (%)
	Guinea pig MRD	Mouse LD_{50}		
Robbins and Pillemer, 1950	NA	190	13.2	66
Onoue, Kitagawa and Yamamura, 1963	1.0-1.3	44	60-80	5-10
Nakase et al, 1969	1.5	NA	80	NA
Iida and Okonogi, 1971	0.9	50-100	90 (6-12)[a,b]	11 (1)[a,b]
Livey	NA	160	20[b]	25[b]

a The figures given are based upon the dermonecrotic and lethal activities (in parentheses) respectively.

b Calculated with lysate as the starting material and not extracts from lysates or whole cells as used by others

NA Not available

suggests yet again that the toxin has a tendency to form associations with other B. pertussis components during fractionation. The value obtained by Nakase et al (1969) does not appear to be compatible with HLT being a protein of around 90,000 daltons. Perhaps the toxin examined here was also associated with other material but this seems unlikely as the toxicity would surely in such an instance be eluted over a wide range of fractions due to the heterogeneity of the associated proteins. Alternatively HLT may have a sub-unit construction and Nakase et al (1969) may have purified a sub-unit. Such a component would have to be able to function independently of the other sub-unit(s). Another possibility would be that HLT may have a peculiar shape or contain lipid, although the chemical analyses of Nakase et al (1969) would seem to discount the latter possibility. Either of these properties might give to a large protein a low sedimentation coefficient. Pure HLT will be required for the molecular weight estimation made here to be confirmed eg, again by gel filtration and by polyacrylamide gel electrophoresis, where the inclusion of sodium dodecyl sulphate would provide information on the polypeptide construction.

6 Production and characterization of toxoided HLT

6.1 Detoxification of HLT - the effect of temperature and formaldehyde

Toxoided HLT is a better immunizing agent for stimulating antitoxin production than native toxin (Roberts and Ospeck, 1944). To produce toxoided HLT most investigators have incubated HLT at 37°C with around 0.1% (w/v) formaldehyde for up to 1 mth although detoxification can be complete within 1 d (Evans and Maitland, 1937; Yamamoto, Zenyoji and Kato, 1952b). There seems to have been little or no experimental manipulation of the conditions eg, using different concentrations of formaldehyde or other temperatures. As the temperature of 37°C seemed inappropriate

for a heat-labile toxin and the rate of detoxification unusually rapid, it is possible that heat inactivation of the toxin could be occurring under the conditions of toxoiding used by previous investigators. As Evans (1942) had demonstrated that heat-inactivated toxin (56°C, 30 min) did not give rise to HLT-neutralizing antibodies, it was decided to follow the inactivation of HLT with and without 0.1% (w/v) formaldehyde at both 37°C and 4°C. The samples without formaldehyde were Millipore-filtered and checked for bacteriological sterility to avoid any complication from the growth of contaminants.

At 37°C with formaldehyde, toxicity was completely lost within 1 d as reported previously (Evans and Maitland, 1937; Yamamoto, Zenyoji and Kato, 1952b). Without formaldehyde the samples containing 3.25 LD_{50} per mouse dose (0.5 ml) became atoxic (ie, $<$ 1 LD_{50} per 0.5 ml) within 3 d at 37°C. Yet the undiluted (32.5 LD_{50} per 0.5 ml) samples were still toxic (ie $\geq$ 1 LD_{50} per 0.5 ml) at 14 d. At 4°C there was no detectable loss of activity during the 14 d (ie, $\geq$ 1 LD_{50} per 0.5 ml) even for the sample initially with only 3.25 LD_{50} in 0.5 ml. This is in agreement with previously published information on the stability of HLT at 4°C (page 65). The toxicity of the samples held at 4°C with formaldehyde decayed in the first 3 d at a rate similar to that observed with the 3.25 LD_{50}, 37°C samples but in this instance there was complete detoxification within 9d. One interpretation of these data is that there are two toxins which are inactivated by formaldehyde at both 4°C and 37°C but which differ in their heat-stability. The major toxin, HLT, is heat-labile and masks the presence of the minor toxin unless HLT is selectively inactivated (by incubation at 37°C, for instance). The minor toxin cannot be endotoxin (lipopolysaccharide) as this would be expected to be resistant to the formaldehyde treatment but it could be pertussigen (Muñoz, 1976) or "pertussis toxin" ie, HSF-LPF-IAP (Pittman, 1979). Alternatively, the

higher protein content of the undiluted (32.5 LD_{50} per 0.5 ml) 37°C sample could have had a stabilizing effect on the HLT and enable toxicity to persist longer than predicted from the rate of inactivation of the 1 in 10 diluted sample. This effect would not seem to be applicable to formaldehyde detoxification of HLT at 4°C.

Mere incubation at 37°C gave a fairly rapid detoxification of HLT and, when formaldehyde was present, detoxification was extremely quick. In comparison, detoxification with formaldehyde at 4°C was much slower and perhaps therefore a gentler process. Consequently it was decided to use incubation at 4°C with formaldehyde to make toxoid from HLT in preference to the higher temperature treatment. Therefore a further 2 batches of toxin were treated at 4°C with 0.1% (w/v) formaldehyde (final concentration) for 15 d to ensure complete detoxification. However it was discovered that 1-3% of the original toxicity still persisted. This was probably not detected in the earlier tests because of the low activity of the starting material (32.5 mouse LD_{50} per 0.5 ml injection volume). When one of the samples was tested in suckling mice for haemorrhagic activity it elicited a positive response. The residual toxic activity was therefore attributable to HLT except that heating the sample (56°C, 15 min) gave no reduction in lethal toxicity. As the formaldehyde had been dialysed from the sample on day 15 to stop the reaction it was not responsible for the haemorrhagic response. Perhaps the residual HLT had been partially stabilized against heat-inactivation by changes induced by the formaldehyde.

6.2 HSF and LPF activity in formaldehyde-detoxified preparations of crude HLT

The two batches of toxin which had been incubated for 15 d at 4°C with formaldehyde were examined to determine what effect this treatment had had on the HSF and LPF in those samples. As the untreated

samples were toxic, they could not be used directly to assess the original HSF/LPF activity. Therefore heated preparations (56°C, 30 min) of the same material were substituted. Histamine-sensitizing factor and LPF are generally considered to be heat-stable, requiring treatment at 80°C for inactivation. Therefore the discovery that the heated samples were less active than the formaldehyde-treated material was unexpected and does not appear to have been noted in the literature. In one experiment the figures of 48% and 40% were returned for the LPF and HSF contents respectively for the heated relative to the formaldehyde-treated sample. In the other experiment the HSF content was not determined accurately but the LPF content of the heated sample was, at 36%, significantly less than its formaldehyde-detoxified counterpart.

There are at least two possible explanations for these results. At its simplest, it would appear that HSF/LPF is inactivated more completely at 56°C for 30 min, than at 4°C with 0.1% (w/v) formaldehyde for 15 d. Although as suggested in the preceding section HSF/LPF may be even more stable at 37°C than under either of these two circumstances. On the other hand the HSF and LPF may not be inactivated at all by 30 min at 56°C nor by the formaldehyde treatment. What is perhaps being observed is an enhancement of HSF/LPF activity after exposure to formaldehyde due to a reduced susceptibility to proteolytic destruction by tissue enzymes in the mouse. Which of these suggestions is likely to be correct could be assessed by comparing the relative HSF/LPF activities of both samples with a further preparation which had been heated then formaldehyde treated. If the latter sample was, like the heated sample, less active than the formaldehyde-treated sample then this would be consistent with the first proposal. If only the heated sample was of reduced activity this could indicate that the second suggestion was applicable. It is assumed that

heating would not interfere in the way the HSF/LPF interacted with formaldehyde. The correctness of the second proposal could also be checked by testing the susceptibility of both heated and formaldehyde-treated preparations to degradation by proteases such as trypsin. It would be expected that a heated sample's susceptibility would be increased relative to that of a formaldehyde-treated sample.

6.3 The immunogenicity of formaldehyde-detoxified preparations of crude HLT

The initial aim of this study was to examine, more critically than has been done in the past, the question of whether anti-HLT might have protective activity in experimental pertussis infections of mice. In particular, whether animals actively immunized with HLT-toxoid and with demonstrable levels of circulating anti-HLT might be protected against an intracerebral or intranasal challenge. To do this required the production of mice with demonstrable levels of circulating antitoxin. Unfortunately as has been the experience of others (Wood, 1940; Anderson and North, 1943; Ospeck and Roberts, 1944), there was a singular failure to raise anti-HLT neutralizing antibodies in mice. Nevertheless other antibodies were produced, as shown by the development of precipitins. Attention was turned next to passive immunization experiments in mice with anti-HLT raised in rabbits - a species that is known to respond to HLT toxoid (appendix 2). The same batch of toxoid that had been unsuccessful in the mice was therefore used to immuniz e rabbits. Out of the 4 rabbits subjected to immunization with crude HLT toxoid in Freund's adjuvants, 1 gave a fairly good response (1,000 units of antitoxin per ml), 2 responded with low titres and 1 failed to respond. Yet each rabbit had received only about one-half as much toxoid as the mice on an equal body weight basis ie, the mice which were given the maximum dosage of 250 μg of protein had a toxoid (μg protein)

to body weight (g) ratio of 10:1 whereas for the 2.5 Kg rabbits this was nearer to 5:1. It would appear that the mouse is the much less responsive of the two species, although the need to test pooled rather than individual mouse sera might have hidden the occasional responding animal. Anderson and North (1943) likewise raised antitoxin in rabbits to a batch of toxoid which was ineffective in mice in spite of the mice having received, on an equal body weight basis, about 10 times as much toxoid..

The rabbits immunized with the crude toxoid gave variable responses in terms of the relative proportions of antitoxic antibodies and agglutinins. Interestingly, the only rabbit that failed to make a demonstrable amount of circulating antitoxin produced the antiserum which gave the highest titre of agglutinin (1,280), whilst the antiserum with the highest antitoxic activity was consistently tested as having only half this level of agglutinin. Neither were the responses related to the dosage of toxoid given. The highest antitoxin and agglutinin titres were obtained with antisera from the two rabbits which received the lower dosage ie, 40% less toxoid than the others. These results illustrate the well known variability in the way individuals within a species may respond to a given immunogen.

When the rabbit antisera were tested for the development of precipitins, both to the toxoid to which they were raised and the native toxin from which the toxoid was prepared, reactions of complete identity were evident between the two antigen preparations. Obviously the formaldehyde detoxification did not grossly alter the major antigenic components in the toxin preparation. However although it is desirable to retain a high percentage of native determinants the amount of polymerization of proteins by formaldehyde can be an even more important factor in deciding how effective an immunogen a toxoid will be (Warren, Spero and Metzger, 1973; Warren et al, 1975). Future investigators

may wish to study the effects of pH and the time that the formaldehyde is left in contact with HLT on the formation of these polymers and their immunogenicity.

7 Absorption of antitoxic sera

Two of the antitoxic rabbit sera prepared for use in the bacterial infection experiments were absorbed with live, whole cells of B. pertussis 134, the strain from which the toxoid used to immunize the rabbits was derived. It was hoped to remove as many antibodies as possible to B. pertussis components other than HLT, especially agglutinins. However antiserum B (1,000 antitoxin units/ml) lost 75% of its antitoxic activity in this process of absorption and the less active antiserum D (100 antitoxin units/ml) actually acquired haemorrhagic activity as demonstrated in suckling mice. This raises the question of why, if HLT is cytoplasmic, whole-cell absorption should remove HLT-neutralizing antibodies. It is improbable that there is leakage of HLT from the cells through complement-mediated cytolysis. The antisera were heated at 56°C prior to absorption to prevent just such complement-mediated activity. It is therefore possible that HLT occurs to some extent on the cell surface although having a mainly cytoplasmic location. This would make sense from a pathogenicity point of view if the toxin produces ciliary paralysis as has been suggested (Standfast, 1958). Moreover the toxin has been recovered in cell washings (appendix 1) and antitoxin has been raised on a few occasions in response to immunization with B. pertussis cells (appendix 2). However it is not possible from the data to differentiate between whether HLT occurs as an integral component of the cell-envelope or just becomes associated with the outer surfaces of some of the bacteria after being released by lysis of others.

In the experiments outlined here the absorptions were done with fairly large quantities of bacteria ie, 3 or 4 g wet weight of cells being

used to absorb 10 ml of undiluted serum. Indeed in the immunodiffusion tests between these absorbed sera and neighbouring antipertussis sera, precipitin lines developed demonstrating that B. pertussis antigens had been acquired by the serum during the absorptions and in sufficient quantities to be visualized in this test. If fewer cells had been used in the absorption it might have been possible to retain most of the antitoxin whilst still removing just as effectively the other antibodies shown to be lost by immunodiffusion and bacterial agglutination tests. Alternatively heated or formaldehyde killed cells might not have absorbed out HLT-neutralizing antibodies.

8 Antitoxic immunity and protection of mice against intracerebral and intranasal challenges with B. pertussis

Protective antigens and antibodies can be assessed in the intracerebral mouse protection test and in experimental respiratory tract infections of mice (page 5). In this investigation, antisera, particularly antitoxic antisera, were assessed in both these tests viz. the lethal intracerebral and the sub-lethal intranasal mouse protection tests. Implicit in the testing of antitoxic sera is the assumption that by neutralizing HLT, the initiation or development of these experimental B. pertussis infections might be prevented or ameliorated. Although the involvement of HLT in the pathogenesis of pertussis is unclear there are some ways in which it could aid in the establishment of the infecting dose and its subsequent multiplication (page 28). For example, HLT may play a part in the lodgement of the organisms among the cells of ciliated epithelia by paralysing the cilia (Standfast, 1958). Although there has been no direct confirmation of this last mentioned report it should not prove too difficult to test crude HLT and a heat-inactivated control sample for ciliostatic activity eg, on the ciliary activity of tracheal organ cultures or even in mussels

(Mytilus edulis) which are used by cell-biologists to study the action of cilia. Another possible mechanism by which HLT could act would be by interference with the development of immunity through toxic action on the spleen and lymph nodes (Muñoz, 1971).

When B. pertussis is injected intracerebrally into mice about 10% of the challenge remains in the brain to multiply among the ciliated cells on the ependymal lining of the ventricle (Berenbaum, Ungar and Stevens, 1960; Iida et al, 1962). When the infecting bacteria reach 10^5 or 10^6 cfu, the blood-brain barrier breaks down. Apparently, it is only then that circulating antibodies to B. pertussis gain access to the infecting bacteria (Dolby and Standfast, 1961). However the effect of antipertussis antibodies on the early stages of such infections can be tested by administering antiserum mixed with the challenge organisms (ie, the IC/mix test). The rabbit antisera discussed earlier and the US standard antipertussis serum were tested in this way. Only the latter serum afforded a significant ($P < 0.005$) degree of protection as measured by a reduction in mortality at the end of the 14 d test period. This standard antiserum contained no detectable antitoxin. All 3 of the rabbit antisera which had demonstrable levels of antitoxin were essentially non-protective against an intracerebral challenge, even that antiserum containing 1,000 units of antitoxin per ml as measured in suckling mice. The only other report in which an antitoxin-containing antiserum was tested in this type of experiment was performed by Yamamoto et al (1955). They found an antiserum from a rabbit whose skin was resistant to 8 MRD of toxin was protective when given mixed with the challenge organisms to mice. From the results presented here it would appear that antibodies other than those to HLT were responsible for the protection measured by Yamamoto et al, and that antitoxin is an unlikely candidate as a protective antibody in this system. Moreover anti-HLT is not protective when given intracerebrally

2-5 d after an intracerebral challenge (Dolby and Standfast, 1961). This is not to say that it is theoretically impossible for antitoxin to be protective, but merely that the conditions used were not right for this to be demonstrated. When antiserum B and D (1,000 and 100 antitoxin units per ml respectively) were given intraperitoneally in 0.2 ml volumes to mice a few hours before the intracerebral challenge both gave significant levels of protection. Unfortunately there were insufficient quantities of antiserum B after it had been absorbed for this to be tested. As it contained antitoxin it would have been interesting to see how it performed in this test since it was devoid of antipertussis components found in B. However the results of Dolby and Standfast (1958) and Kuwajima et al (1958) indicate that passively administered antitoxin is not protective against an intracerebral infection. For antitoxin to be effective in these IP/IC tests it would have to be capable of clearing an already established infection as the site of the infection is sealed off until the blood-brain barrier breaks down.

Turning to the intranasal (sub-lethal) challenge test, antiserum B gave significant protection when 0.2 ml was given intraperitoneally a few hours before the intranasal instillation of a sub-lethal dose of B. pertussis. Moreover this antiserum was even more effective if given mixed with the challenge organisms, and perhaps this allowed it to be present at the site of the infection in relatively high concentrations from the very start. This IN/mix protective activity was lost after the antiserum had been absorbed with live B. pertussis. Perhaps the 4-fold reduction in the titre of antitoxin caused by absorption was the reason for the loss of protective activity, but more likely it was due to the loss of antibodies to other B. pertussis components. Antibodies raised to the same batch of toxoid used to produce antiserum B were present in antiserum A which contained no detectable antitoxin and this latter antiserum was protective in IN/mix experiments.

The question of whether high levels of antitoxin might confer protection in either of these experimental infections of mice, and by inference in man, is therefore still open. The major limiting factors in these studies have been, and still are, the lack of a purified HLT toxoid and the apparent inability of mice to respond effectively to this particular antigen. Nakase et al (1969) reported, without giving data, that mice were not protected against intracerebral challenge by previous injection of purified, alum-precipitated HLT-toxoid. However their failure could have been due to the mice not making anti-HLT antibodies rather than to the intrinsic lack of protective activity of anti-HLT. Perhaps the only practical resolution of this question would be to give mice large quantities of pure anti-HLT raised in rabbits since this species does at least respond.

A further possibility is that anti-HLT might be unable to prevent multiplication of the bacteria in B. pertussis infections but still be able to prevent some of the manifestations of the disease. Let us suppose that the toxin produces local tissue damage in the respiratory tract and that this is a pre-condition for the entry of the HSF-LPF-IAP complex into the systemic compartment to produce lymphocytosis and other changes. Anti-HLT if available locally might thus prevent some of the clinical signs without interfering with the growth of the bacteria. This idea could be tested as follows: the development of histamine-sensitization and lymphocytosis could be monitored in mice actively or passively immunized to purified HLT toxoid and in an untreated group of control animals. If the test mice failed to respond to the infection by not becoming sensitized to histamine or developing a lymphocytosis, this would be compatible with HLT acting in the manner described.

In none of the above discussion has any particular attention been paid to the class of immunoglobulin to which the anti-HLT belongs. Yet this might be crucial in determining its potential protective activity.

In particular, antibodies of the IgE class specific for HLT might be protective by binding to tissue cells and neutralizing the postulated ciliary-paralysis effect of HLT, which as discussed above may be located on the surface of B. pertussis. Likewise, secretory IgA specific for HLT and continuously present in the mucous secretionsof the respiratory tract might exercise a protective function.

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APPENDICES

Appendix 1

Literature review on the use of culture fluids and cells as a source of HLT

1 Culture filtrates and supernatant fluids (9 citations)

Joslin and Christensen, 1940; Weichsel et al, 1940; Wood, 1940; Roberts and Ospeck, 1942; Weichsel, Katona and Liu, 1942; Ungar and Muggleton, 1949b; Kuwajima, Iwamura and Hirai, 1951; Imamura, 1952; Kuwajima et al, 1958.

2 Extracts from intact organisms (7 citations)

Evans and Maitland, 1937; Katsampes, Brooks and Bradford, 1942; Roberts and Ospeck, 1942; Burrell, Robbins and Pillemer, 1948; Robbins and Pillemer, 1950; Yamamoto, Zenyoji and Kato, 1952c; Billaudelle et al, 1960.

3 Extracts from cells disrupted by

3.1 Grinding (14 citations)

Bordet and Gengou, 1909; Teissier et al, 1929; Lawson, 1933; Miller, 1934; Evans and Maitland, 1937; Evans, 1940[a], 1942[a], 1947[a]; Silverthorne and Cameron, 1942[a]; Anderson and North, 1943[a]; Proom, 1947[a]; Abrosimov, 1961; Onoue, Kitagawa and Yamamura, 1963; Střížova and Trlifajova, 1964.

a involved freeze-thawing prior to grinding

3.2 Freeze-thawing (9 citations)

Evans and Maitland, 1937; Strean and Grant, 1940; Smolens and Flavell, 1947; Yamamoto, Zenyoji and Kato, 1952a; Yamamoto et al, 1952, 1953, 1957; Kasuga et al, 1954; Uchida et al, 1957.

3.3 Sonication (14 citations)

Flosdorf and Kimball, 1940; Flosdorf, Bondi and Dozois, 1941; Ehrich et al, 1942; Smolens and Flavell, 1947; Verwey and Thiele, 1949;

Robbins and Pillemer, 1950; Pennel and Thiele, 1951; Kuwajima et al, 1958; Muñoz, Schuchardt and Verwey cited by Muñoz, Ribi and Larson, 1959; Nakase and Kasuga, 1962; Nakase et al, 1965; Sato and Nagase, 1965; Nakase et al, 1969; Iida and Okonogi, 1971.

3.4 Hughes press (6 citations)

Frappier, Guerault and Repentigy, 1955 cited by Billaudelle et al, 1960; Billaudelle et al, 1960; Dolby and Standfast, 1958 and 1961; Dolby, Thow and Standfast, 1961; Standfast and Dolby, 1961.

3.5 X-press (1 citation)

Billaudelle et al, 1960.

3.6 Mickle disintegrator (4 citations)

Andersen 1952 and 1953b; Muñoz, Ribi and Larson, 1959; Banerjea and Muñoz, 1962.

3.7 Rotary disintegrator (1 citation)

Novotny, 1977 personal communication

3.8 Pressure cell (1 citation)

Muñoz and Ribi cited by Muñoz, 1971

3.9 Lysozyme freeze-thaw technique (1 citation)

Cowell, Hewlett and Manclark, 1979

Appendix 2

The production of antitoxin to HLT in animals

Live B. pertussis, native or toxoided HLT preparations, or combinations of these factors have been used as immunizing agents to raise HLT neutralizing antibodies. In tables 50 - 52 are cited references in which each of these immunogens or combinations of immunogens have been used. Antitoxin titres are given where possible, either in the form presented by the author(s) or as estimated from their raw data assuming where necessary that antitoxin neutralizes in multiple proportions (Evans, 1940; Proom, 1947). Only the highest antitoxin titres obtained are cited. Titres are presented as units of antitoxin per ml where a unit of antitoxin is defined as the smallest amount required to neutralize, unless stated otherwise,

i 1 MRD in rabbits or, if in parenthesis, in guinea pigs

ii 1 MLD in mice or, if in parenthesis, LD_{50} in mice

or alternatively

iii as the number of MRD of HLT to which immunized rabbits were resistant

In the absence of quantitative data the symbols + and - are used to indicate whether antitoxin was or was not produced. Where antitoxin was not assayed by a particular method the symbol ND (not determined) is used.

Table 50 A review of the literature on the production of HLT-neutralizing antibodies in rabbits immunized with either live *B. pertussis* or native HLT

Immunogen	Reference	Antitoxin titre (units/ml serum)		Resistance to HLT in skin test
		i Rabbit (guinea pig) MRD neutralized	ii Mouse MLD (LD_{50}) neutralized	iii Number of MRD neutralized
Live *B.pertussis*	Flosdorf, Bondi and Dozois, 1941	620	ND	+
	Silverthorne and Cameron, 1942	+	ND	-
	Roberts and Ospeck, 1944	ND	40	ND
	Kuwajima *et al*, 1958	(+)	(2048)	ND
	Standfast, 1958	Antitoxin produced but assay method not given		
Native HLT	Flosdorf, Bondi and Dozois, 1941	≻5120	ND	+
	Roberts and Ospeck, 1942	ND	+	+
	Roberts and Ospeck, 1944	+	≻200	ND
	Proom, 1947	2000	40	ND
	Verwey and Thiele, 1949	ND	(86)	ND
	Yamamoto, Zenyoji and Kato, 1952b	107	ND	≽10
	Yamamoto *et al*, 1955	ND	ND	8
	Kuwajima *et al*, 1958	(+)	(8192)	ND
	Střižova and Trlifajova, 1964	1000	(240)	ND

Table 51 A review of the literature on the production of HLT-neutralizing antibodies in rabbits immunized with formaldehyde-detoxified HLT

Reference	Antitoxin titre (units/ml serum)		Resistance to HLT in skin test
	i Rabbit (guinea pig) MRD neutralized	ii Mouse MLD (LD_{50}) neutralized	iii Number of MRD neutralized
Teissier et al, 1929	(300)[a,b]	ND	(30)[a]
Evans, 1940	5120	80[c]	128 or 256
Strean and Grant, 1940	5120	125	ND
Strean, Lapointe and Dechene, 1941	ND	+	ND
Evans, 1942	10240	$\geqslant 50$[d]	10240-20480
Roberts and Ospeck, 1942	ND	+	+
Silverthorne and Cameron, 1942	+	ND	-
Anderson and North, 1943	+	400	ND
Evans, 1944	400-450	ND	ND
Ospeck and Roberts, 1944	+	ND	+
Roberts and Ospeck, 1944	+	+	ND
Cravitz and Williams, 1946	ND	ND	80
Yamamoto, Zenyoji and Kato, 1952b	213	ND	$\geqslant 10$
Andersen, 1953b	ND	3200[e]	ND
Dolby and Standfast, 1958	ND	3500-13500	ND
Dolby and Standfast, 1961		Antitoxin assay method not given	ND
Nakase et al, 1969	($\geqslant$160)	ND	ND

a,b guinea pig and sheep serum respectively

c,d and e guinea pig, rabbit and suckling mouse MLD respectively

Table 52 A review of the literature on the production of HLT-neutralizing antibodies in species other than rabbits, or where data on the immunizing agents is lacking, or combinations of immunizing agents were used

Reference	Animal species immunized	Immunizing agent(s)	Level of antitoxin production
Teissier et al, 1929	Donkey	Formaldehyde-detoxified HLT, then native HLT	One ml of serum neutralized 18000 guinea pig MRD of HLT
Lawson, 1933	No data given	No data given	Animal resistant to 5 MRD of toxin in skin test
Demnitz, Schlüter and Schmidt, 1936 (cited by Muñoz, 1971)	Horse	"Toxoid"	No data given
Cravitz and Williams, 1946	Rabbit	Strean's combined endo-toxoid vaccine	No data given
Proom, 1947	Horse	Formaldehyde-detoxified HLT, then alum-precipitated native HLT	One ml of serum neutralized 10000 rabbit MRD of HLT
Smolens and Flavell, 1947	Rabbit	"Toxoid"	One ml of serum neutralized 100 mouse LD_{50} of HLT
Brown, 1949	Rabbit	Native HLT	No data given
Andersen, 1952	Rabbit	No data given	? ml of serum neutralized 2560 suckling mouse MRD of toxin
Iida and Okonogi, 1971	Rabbit	No data given	One ml of serum neutralized 40960 guinea pig MRD and 6400 mouse MLD of HLT

Appendix 3

Sodium dodecyl sulphate, polyacrylamide gel electrophoresis (SDS-PAGE)

1 Chemicals

BDH, Poole, England: acrylamide, NN'methylenebisacrylamide, glycine, sodium dodecyl sulphate (SDS), bromophenol blue, B-mercaptoethanol, glycerol, N',N',N',N'tetramethylethylene-diamine.

Sigma Chemical Company, St. Louis, MO, USA: Tris (hydroxymethyl) amino methane, ammonium persulphate.

Hopkins and Williams, Chadwell Heath, England: HCl, glacial acetic acid, ethanol, methanol.

Searle Diagnostics, High Wycombe, England: Coomassie blue R250.

2 Stock solutions

Acrylamide solution

Acrylamide	30 g
NN'methylenebisacrylamide	0.8 g

Dissolved in 100 ml of distilled water, filtered, and stored at $4^{o}C$ in a dark brown bottle.

1M Tris/HCl buffer, pH 8.8

2M Tris (hydroxymethyl) amino methane		50 ml
1N HCl	approx	16.2 ml

Made up to 100 ml with distilled water.

0.5M Tris/HCl buffer, pH 6.8

1M Tris (hydroxymethyl) amino methane		50 ml
1N HCl	approx	45 ml

Made up to 100 ml with distilled water.

Tris/Glycine running buffer, pH 8.3 (10 x concentrated)

Glycine	144.13 g
Tris (hydroxylmethyl) amino methane	30.28 g

Made up to 1000 ml with distilled water.

Ammonium persulphate: 0.8% (w/v) in distilled water, made up fresh each time.

SDS: 20% (w/v) in distilled water.

Bromophenol blue: 0.1% (w/v) in distilled water.

Overlay solution

Ethanol	5 ml
Distilled water	95 ml

Solubilizing buffer

0.5M Tris/HCl buffer, pH 6.8	25 ml
SDS, 20% (w/v)	20 ml
B-mercaptoethanol	10 ml
Glycerol	20 ml
Bromophenol blue, 0.1% (w/v)	2 ml
Distilled water	23 ml

Fixing-staining solution (Weber and Osborn, 1969)

Coomassie blue R250	1.25 g
50% (v/v) methanol	454 ml
Glacial acetic acid	46 ml

Destaining solution

Methanol	50 ml
Glacial acetic acid	75 ml
Distilled water	875 ml

3 Preparation of gels

The glass moulds for the gels were prepared as follows:- 2 glass plates (8 x 7 cm) separated by spacers were secured on 3 sides with special adhesive tape. The bottom corners were then sealed by dipping in molten paraffin wax.

The separating gel solution was prepared next:-

Acrylamide solution	36.7 ml
1M Tris/HCl buffer, pH 8.8	37.5 ml
20% (w/v) SDS	0.5 ml
N',N',N',N'tetramethyletheylenediamine	25 µl
0.8% (w/v) ammonium persulphate	10 ml
Distilled water	15.3 ml

The ingredients were mixed thoroughly but without aeration and the gel plates were filled up to a height of 5.5 cm. The gel was then overlaid with 5% (v/v) ethanol so a perfectly flat gel surface was obtained. Whilst the separating gel polymerized the stacking gel was prepared:-

Acrylamide solution	16.7 ml
0.5M Tris/HCl buffer, pH 6.8	25 ml
20% (w/v) SDS	0.5 ml
N',N',N',N'tetramethylethylenediamine	25 µl
0.8% (w/v) ammonium persulphate	10 ml
Distilled water	47.8 ml

The ingredients were mixed without aeration. Then the overlay solution was poured off the fully polymerized separating gel. This was blotted dry if necessary. The stacking gel was then poured over the separating gel, to within 3-4 mm from the top of the plate. Sample "combs" were suspended in the solution and any spaces which remained were filled with more gel solution. After polymerization the combs were carefully removed under the running buffer and the sealing tape was removed from the base of the gel plates.

4 Conditions of electrophoresis

The stock Tris/glycine running buffer was diluted 1 in 10 and 20% (w/v) SDS added to a final concentration of 0.1% (w/v). The lower electrode vessel was filled with approximately 2.5 l of this buffer. The

gel plates were then inserted into the top electrode, using a liberal amount of grease around the gasket to prevent leakage. Then the top electrode vessel was put into position and filled with the running buffer. Samples of 25-50 µl were pipetted into the wells in the stacking gel. Finally the electrode unit was connected to the power supply (lower vessel was the anode) and run at constant current (15 mA/gel) for 2-3 h ie, until the tracking dye reached the end of the gel.

5 Fixing-staining/destaining

Once removed from the glass moulds the gels were immersed in fixing-staining solution for 60-90 min. They were destained by soaking in several changes of destaining solution.

Appendix 4

Adjuvants

1 Aluminium hydroxide, $Al(OH)_3$

The method described by Weir (1978) was used.

Aluminium sulphate 5% (w/v)	25 ml
Sodium hydroxide 5% (w/v)	10 ml

The aluminium sulphate solution was added to the sodium hydroxide solution whilst stirring vigorously and the resultant precipitate washed twice by centrifugation and decantation, resuspended in Dulbecco "A" PBS (Oxoid Ltd., England) and sterilized by autoclaving (121°C, 15 psi, 15 min). The immunization mixtures were prepared by mixing equal volumes of antigen and $Al(OH)_3$.

2 Freund's incomplete adjuvant

Five volumes of mineral oil (Bayol F; Esso Petroleum Company Ltd., Purfleet, England) were mixed with 1 volume of emulsifying agent (Aracel A; Atlas Powder Company, Wilkington, Delaware, USA). Only then were 5 volumes of antigen solution introduced into the mixture. A water-in-oil emulsion was obtained by sonicating the mixture (100 Watt ultrasonic disintegrator; Measuring and Scientific Equipment Ltd., London) until a thick, creamy fluid was formed. Before use the mixture was checked to ensure that it was a true water-in-oil emulsion. This was done by injecting some of the mixture into or onto cold water to show that discrete drops developed.

3 Freund's complete adjuvant

This was prepared as for Freund's incomplete adjuvant except that the mineral oil contained the tubercle bacillus. A stock suspension of Mycobacterium tuberculosis was prepared in Bayol F (Esso Petroleum Company Ltd., Purfleet, England). Mixing was achieved by sonication (100 Watt ultrasonic disintegrator; Measuring and Scientific Equipment Ltd., London). For use the stock suspension was diluted 1 in 10 with Bayol F (Esso Petroleum Company Ltd., Purfleet, England) to give 200 μg mycobacteria/ml.

Appendix 5

Animal test results upon which the statistically evaluated data presented in tables; 18, 22, 23, 24, 30, 33, 36 and 37 were based

Table 53 Toxicity test results for the data presented in table 18, experiment 1 : the toxicity of X- and C-mode cells

Dose of lysate/mouse	Experiment 1a				Experiment 1b			
(oue of 0.5 ml injected)	X_1	X_2	C_1	C_2	X_1	X_2	C_1	C_2
20			4/4	3/4			10/10	9/10
10			4/4	4/4			10/10	9/10
5			3/4	0/3			4/10	1/10
2.5	4/4[a]	4/4			10/10	9/9	0/10	0/10
1.25	4/4	4/4			10/10	9/10	0/10	0/10
0.625	3/4	0/4			10/10	9/10		
0.312	0/4	0/4			6/10	1/10		
0.156	0/4	0/4			0/10	0/10		

a Number of deaths/number of mice injected

Table 54 Toxicity test results for the data presented in table 18, experiment 2 : the toxicity of X- and C-mode cells

Experiment 2a			Experiment 2b		
Dose of lysate/mouse (oue of 0.5 ml injected)	Number of deaths/ number of mice injected		Dose of lysate/mouse (oue of 0.5 ml injected)	Number of deaths/ number of mice injected	
	X	C		X	C
16		4/4	20		9/10
8		0/4	10		7/10
4		0/4	5		4/10
2	4/4	0/4	2.5	10/10	
1	4/4	0/4	1.25	9/10	
0.5	3/4		0.625	10/10	
0.25	0/4		0.312	6/10	
0.125	0/4		0.156	2/10	

Table 55 Toxicity test results for the data presented in table 18, experiments 3-7 : the toxicity of X- and C-mode cells

Dose of lysate[a]/mouse	Expt 3			Expt 4		Expt 5		Expt 6		Expt 7	
(oue of 0.5 ml injected)	X	C_1	C_2	X	C	X	C	X	C	X	C
40									5/5		
20		3/5	4/5		9/10		4/5		3/5		0/5
10		1/5	0/5		6/10		3/5		1/5		0/5
5		0/5					0/5		0/5		0/5
2.5	3/5			9/10				5/5			
1.25	4/5			10/10		5/5		4/5		6/10	
0.625	3/5			10/10		5/5		4/5		10/15	
0.312	2/5			7/10		0/5		0/5		1/15	
0.156	0/5			0/10		0/5		0/5		0/15	
0.078										0/10	

a In experiment 7, cells not cell-lysates were used

Table 56 Toxicity test results for the data presented in table 22, experiments 1-3 : the toxicity of B. pertussis strains

Experiment	B.pertussis strain	Dose of cells (ou of 0.5 ml injected / mouse)					
		5.0	2.5	1.25	0.625	0.3125	0.156
1	18334	4/4[a]	4/4	3/4	0/4	0/4	NT
	18904 L4	2/3	3/3	4/4	1/4	0/3	0/3
	77/18319	3/3	3/3	3/4	1/4	0/3	0/3
	77/24833	2/3	3/3	3/4	0/4	0/3	0/3
2	18334	4/4	4/4	4/4	0/4	0/4	NT
	134	3/3	3/4	4/4	3/3	1/3	1/3
	3865	2/3	4/4	2/3	2/4	0/3	0/3
	D30042 I	3/3	3/4	3/4	1/3	0/3	0/3
3	18334	4/4	4/4	4/4	0/4	0/4	NT
	D3148 I	3/3	3/3	4/4	2/4	0/3	0/3
	No. 28	3/3	3/3	4/4	4/4	1/3	0/3
	MAENO	3/3	3/3	4/4	3/4	1/3	0/3

a Number of deaths/number of mice injected

NT Not tested

Table 57 Toxicity test results for the data presented in table 22, experiments 4 and 5 : the toxicity of *B. pertussis* strains

Experiment	*B. pertussis* strain	Dose of cells (ou of 0.5 ml injected/mouse)				
		2.5	1.25	0.625	0.312	0.156
4	18334	13/15[a]	14/15	8/15	4/15	NT
	MAENO	NT	10/10	9/10	6/10	4/10
	134	NT	8/10	8/10	8/10	4/10
	No. 28	NT	10/10	9/10	6/10	1/10
5a	18334	4/4	2/4	1/4	0/4	0/4
	MAENO	3/4	2/4	4/4	0/4	0/4
	134	3/4	4/4	3/4	3/4	0/4
	No. 28	3/4	3/3	2/4	0/4	0/4
5b	18334	8/10	9/10	5/10	2/10	NT
	MAENO	NT	9/10	10/10	8/10	7/10
	134	NT	7/10	9/10	6/10	3/10
	No. 28	NT	10/10	10/10	5/10	4/10

a Number of deaths/number of mice injected

NT Not tested

Table 58 Toxicity test results for the data presented in table 23 : the toxicity of *B. pertussis* strains

Dose/mouse (ou of 0.5 ml injected)	Experiment number and *B. pertussis* strains tested											
	1			2		3			4			
	18334	134	B-1593/57	18334	44122/7R	18334	134	44122/7R	D3148	D30042	GL353Z	L84
20	13/14[a]								4/4	4/4	4/4	4/4
10	9/14											
5	6/14	7/7	2/7	6/15	6/10		3/10	7/10	4/4	4/4	4/4	3/4
2.5	0/14	0/7	0/7	0/15	3/10		0/10	0/10				
1.25		0/7	0/7	0/15	0/10		0/10	0/10	4/4	4/4	1/4	2/4
0.625		0/7	0/7	0/15	0/10		0/10	0/9				
0.312									0/4	0/4	0/4	0/4
0.078									0/4	0/4	0/4	0/4
8						6/15						
4						0/15						
2						0/15						
1						0/16						

a Number of deaths/number of mice injected

Table 59 Toxicity test results for the data presented in table 24 : the toxicity of B. pertussis grown under different cultural conditions

B.pertussis strain	Dose/mouse (ou of 0.5 ml injected	Bordet-Gengou medium		Charcoal agar		Modified Hornibrook medium		Stainer and Schölte (12G) medium	
		24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
18334	5	5/5[a]	5/5	5/5	5/5	4/5	4/5	5/5	5/5
	1.25	1/5	0/5	1/5	0/5	1/5	3/5	3/5	3/5
	0.312	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	0.078	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
134	2.5	2/5	3/5	5/5	5/5	4/5	4/5	4/5	5/5
	1.25	4/5	0/5	3/5	0/5	2/5	4/5	1/5	2/5
	0.625	0/5	0/5	1/5	0/5	2/5	2/5	0/5	0/5
	0.312	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	0.156	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
77/18319	2.5	3/5	3/5	4/5	3/5	2/5	4/5	2/5	3/5
	1.25	0/5	1/5	2/5	2/5	0/5	2/5	0/5	2/5
	0.625	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	0.312	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

a Number of deaths/number of mice injected

Table 60 Toxicity test results for the data presented in table 30 : the distribution of HLT after ultra-centrifugation of B. pertussis cell-lysates

Dose (µl)/ mouse	Expt 1a			Expt 1b			Expt 2			Expt 3			Expt 4a			Expt 4b		
	L	S	P	L	S	P	L	S	P	L	S	P	L	S	P	L	S	P
100			5/8[a]						2/4				4/5	5/5	5/5			8/10
50			5/8			7/7	4/4	3/4	0/4			8/10				10/10	10/15	1/10
25		5/7	7/8			7/8	3/4	2/4	0/4	12/15	7/10	2/10	4/5	3/5	0/5	5/10	10/15	0/10
12.5		6/7	5/8		5/7	4/8	0/4	0/4	0/4	10/15	7/10	0/10				0/10	0/14	
6.25		5/7			4/7	0/7	0/4	0/4	0/4	6/15	3/10	0/10	0/5	0/5	0/5	0/10	0/16	
3.12		1/7			0/7		0/4	0/4		1/15	0/10							
1.56													0/5	0/5	0/5			
16.75	6/10																	
8.37	9/10			6/7														
4.18	6/10			6/7														
2.09	1/10			2/7														

a Number of deaths/number of mice injected

L, S and P Lysate, supernatant fluid and pellet respectively

Table 61 Toxicity test results for the data presented in table 33 : the toxicity of lysate supernatant fluids before and after DNase/RNase treatment and ammonium sulphate fractionation

Dose (µl)/ mouse	Number of deaths/number of mice injected			
	Lysate supernatant fluid	DNase/RNase treated lysate supernatant fluid	30% ppt	30-50% ppt
60	16/16	8/10		
30	15/17	2/10		
15	5/15	1/10		
7.5	0/16	0/10		
500			6/10	
250			5/11	
125			0/10	
62.5			0/10	
142.8				8/10
71.4				8/9
35.7				2/10
17.9				0/10

Table 62 Toxicity test results for the data presented in table 36, experiment 1 : the detoxification of HLT with formaldehyde

Dose (µl)/ mouse	Untreated HLT			Formaldehyde-treated HLT		
	test 1	test 2	test 3	test 1	test 2	test 3
13.1	3/4[a]					
6.6	2/4					
3.3	0/4					
1.6	0/4					
0.8	0/4					
16.4		9/10				
8.2		3/10				
4.1		0/10				
2.1		0/10				
28.9			16/16			
14.4			15/17			
7.2			5/15			
3.6			0/16			
1000				NT	NT	8/10
500				9/10	2/10	3/10
250				0/10	0/10	NT
125				0/10	0/10	NT

a Number of deaths/number of mice injected

NT Not tested

Table 63 Toxicity test results for the data presented in table 36, experiment 2 : the detoxification of HLT with formaldehyde

Dose (µl)/mouse	Untreated HLT			Formaldehyde-treated HLT
	test 1	test 2	test 3	test 1
10.9	4/5[a]			
5.4	4/5			
2.7	3/5			
1.4	0/5			
6.8		7/10		
3.4		7/10		
1.7		3/10		
0.8		0/10		
4.5			7/10	
2.2			0/10	
1.1			0/10	
0.6			0/10	
500				7/9
250				6/10
125				0/10
62.5				0/10

a Number of deaths/number of mice injected

Table 64 HSF test results for the data presented in table 37 : HSF activity in heat- and formaldehyde-detoxified lysate supernatant fluids

Dose (µl)/ mouse	Experiment 1a		Experiment 1b		Experiment 2	
	Formaldehyde-detoxified sample	Heat-detoxified sample	Formaldehyde-detoxified sample	Heat-detoxified sample	Formaldehyde-detoxified sample	Heat-detoxified sample
500	5/5[a]	5/5	NT	NT	NT	NT
125	5/5	5/5	NT	NT	6/6	5/5
62.5	NT	NT	NT	10/10	NT	NT
31.25	5/5	4/5	NT	8/10	6/6	6/6
15.62	NT	NT	8/10	1/10	NT	NT
7.81	3/5	0/5	3/10	0/10	4/6	4/6
3.91	NT	NT	1/10	NT	NT	NT
1.95	NT	NT	0/10	NT	1/5	0/5
HSD_{50}/ml	200	40	105.3	42.2	200	161.3

a Number of deaths/number of vaccinated mice challenged with histamine

NT Not tested