ACELLULAR PERTUSSIS VACCINE TOXOIDED WITH CARBODIIMIDE

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DECLARATION

This thesis is the original work of the author.

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ABBREVIATIONS

$AbUml^{-1}$	antibody unit ml^{-1}
AP	antigen preparation(s)
(AP-n) Tn	toxoid preparation
cfu	colony forming unit
CNS	central nervous system
DCCD	dicyclohexylcarbodiimide
DTP	diphtheria-tetanus-pertussis
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.HCl
EDAC-AP	carbodiimide-toxoided antigen preparation
ELISA	enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
Fig.	figure
fMLP	N-formylmethionyl-leucyl-phenylalanine
GA	glutaraldehyde
HA	haemagglutinating activity
HSA	histamine-sensitizing activity
HSD_{50}	histamine-sensitizing $dose_{50}$; the dose/mouse which sensitizes 50% of mice to the lethal effect of a challenge of histamine dihydrochloride
IAP	islet-activating protein
ICMPT	intracerebral mouse-protection test
ip	intraperitoneal(ly)
IRI	immunoreactive insulin
iv	intravenous(ly)
kDal	kilo Dalton
kPa	kilo Pascal

LAL	*Limulus* amoebocyte lysate
LD_{50}	lethal $dose_{50}$; the dose/mouse which kills 50% of mice
LPA	leucocytosis-promoting activity
M	molar
MeβCD	heptakis (2,6-O-dimethyl)β-cyclodextrin
mol.wt	molecular weight
MRC	Medical Research Council
MWGT	mouse-weight-gain test
N	normal
95% CL	95% confidence limits
ou	opacity unit
PBS	phosphate-buffered saline
PBST	PBS - 0.05% Tween 20 (v/v)
PD_{50}	protective $dose_{50}$; the immunizing dose/mouse which protects 50% of mice from intracerebral challenge with live, virulent *B. pertussis*
PFS	pyrogen-free saline
RA	reference antiserum
rpm	revolutions per minute
WHO	World Health Organization

SUMMARY

Pertussis toxin (PT) and filamentous haemagglutinin (FHa) were extracted from the culture fluid of B. pertussis grown in (1) modified Stainer and Scholte liquid medium (SS-X), static, (2) SS-X medium plus cyclodextrin, shaken, (3) cyclodextrin-liquid (CL) medium, shaken, by a single-step procedure using dye-ligand affinity chromatography. The addition of cyclodextrin to SS-X medium and aeration greatly enhanced the amount of PT and FHa extracted with Blue Sepharose, and the resulting PT and FHA ratio in the antigen preparation depended on the cultural conditions. With cyclodextrin-supplemented media (SS-X, CL) and aeration, it appeared that the total protein extracted was accounted for as PT and FHa, whereas with SS-X (static), only approximately 50% (w/w) was accounted for as both antigens.

Culture of B. pertussis in SS-X medium supplemented with cyclodextrin (shaken), yielded the most PT, whereas growth in the CL medium (shaken) yielded the most FHa.

Minor protein components were detected in many of the PT-FHa preparations, and endotoxin was present to 1-2% (w/w) of total protein in antigen preparations AP-16 and AP-17.

The mixture of PT and FHa was toxoided with a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.HCl (EDAC). The toxoiding conditions most suitable for elimination of the pathophysiological activities of the A-protomer and B-oligomer of PT were reaction of EDAC with protein at a ratio of 80:1 by weight, at 37°C, pH 5.0 for 24 h. These toxoids had less than 0.5% of the original histamine-sensitizing activity of the untoxoided preparation, did not induce leucocytosis, hyperinsulinaemia or hypoglycaemia or affect normal weight-gain in mice. The toxoid also enhanced the in vitro chemiluminescence of rabbit peritoneal

neutrophils in response to a chemotactic peptide. Thus, it was demonstrated that activities known to be associated with PT had been eliminated.

The carbodiimide-toxoided antigen preparations were stable at $4^{o}C$. When stored at $37^{o}C$, toxoids showed partial reversion after 14-28 days: 1.0% of the original histamine-sensitizing activity associated with the B-oligomer of PT was detected, and there was approximately a 5% reversion to hyperinsulinaemia activity of the A-protomer.

Enzyme-linked immunosorbent assays (ELISA) were developed for the detection and quantitation of pertussis antigens and for specific IgG antibodies raised against these antigens.

Toxoiding with carbodiimide enhanced the antigenicity of PT and FHa as detected by antibody-ELISAs. The untoxoided antigen preparations did not stimulate IgG anti-PT and anti-FHa antibody responses in mice. Toxoids stimulated highly significant IgG anti-PT and anti-FHa antibody responses in mice, and were antigenically stable on storage at $4^{o}C$ or $37^{o}C$.

In addition to enhanced antigenicity, an important finding was the associated immunogenicity of the toxoids. Toxoids protected mice from intracerebral challenge with live, virulent B. pertussis, with protective $dose_{50}$ values from 0.8-1.11 μg/mouse. The untoxoided preparation gave partial protection, but only at doses non-lethal for mice. The toxoid also protected mice against sublethal intranasal infection and lung colonization. Mice immunized with toxoid rapidly cleared the infecting organisms from their respiratory tracts, whereas the lungs of control mice showed extensive and persistent colonization.

In summary, the carbodiimide-toxoided acellular pertussis vaccine was (i) easily produced, (ii) stable for long periods at $4^{o}C$,

(iii) greatly reduced in A-protomer and B-oligomer pathophysiological activities associated with PT, (iv) non-toxic, highly antigenic and highly immunogenic in mice.

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INTRODUCTION

SECTION 1. B. PERTUSSIS, THE CAUSATIVE ORGANISM OF WHOOPING COUGH

Until very recently, the genus Bordetella has been accepted as containing three species, B. pertussis, B. parapertussis and B. bronchiseptica (Pittman and Wardlaw, 1981). B. pertussis is the aetiological agent of whooping cough or human pertussis (per = severe, tussis = cough), a disease of the upper respiratory tract of importance in childhood; B. parapertussis is responsible for a mild pertussis-like disease in humans, and B. bronchiseptica is primarily an animal pathogen that causes such diseases as canine "kennel cough" and atrophic rhinitis in swine. However, Kersters et al. (1984) proposed B. avium as a new species responsible for coryza (rhinotracheitis) in turkey poults.

B. pertussis was first isolated in 1906 by Bordet and Gengou on a glycerol-potato-blood agar medium, which is still used, with and without modification, for the primary isolation of the organism. When grown aerobically on Bordet Gengou (BG) agar at 35°C-37°C, B. pertussis produces a punctiform, high convex, glistening, translucent colony with an entire margin and hazy zone of haemolysis (Pittman, 1974). The organism is Gram-negative and coccobacillary in stained smears, and pleomorphic forms, filaments, short chains and a capsule are occasionally seen. The metabolism of B. pertussis is oxidative and never fermentative; carbohydrates, lactate, pyruvate, acetate and intermediates of the Embden-Meyerhoff pathway are not utilized, and the organism shows a requirement for nicotinic acid, cysteine and methionine. B. pertussis also utilizes alanine, asparagine, glutamine, proline and serine (Jebb and Tomlinson, 1957; Rowatt, 1957; Parker, 1976).

In vitro, typical growth from small inocula can be obtained in

a medium containing nicotinamide, glutathione, ascorbic acid, salts, cysteine, glutamate and/or proline (Stainer and Scholte, 1971).

Abundant circumstantial evidence has pointed towards B. pertussis as the causative agent of whooping cough, although the rigorous application of Koch's postulates appears to have been made only by MacDonald and MacDonald (1933). Viruses - especially respiratory synchtial viruses and adenoviruses - and Mycoplasma pneumoniae have also been implicated as agents causative, or contributary to the pertussis syndrome (Olson, 1975; Linnemann, 1979). Concurrent virus infections during pertussis may intensify the severity of the symptoms, and the violent cough in pertussis may help to spread respiratory viruses, so that there may be epidemiological clustering of virus isolates and whooping cough cases (Linnemann, 1979).

B. pertussis is transmitted by aerosol droplets from an active case, and there is still believed to be no natural reservoir other than an infected person with clinical or subclinical disease (Kendrick, 1975). There is no good evidence for the existence of chronic carriers, although a suggestion has been made that the reservoir of infection in the United States may have shifted from children with typical disease to children or adults with atypical disease (Linnemann, 1979). In a Glasgow study reported in 1981 (Walker et al.), the diagnostic criteria of pertussis were a 'paroxysmal cough' associated with at least one or more of the following: (1) a 'whoop' with a duration of 21 days or more, (2) a 'whoop' and a pronounced lymphocytosis, (3) a positive pernasal swab for B. pertussis and (4) a four-fold rise in antibody titre to B. pertussis.

SECTION 2. PERTUSSIS WHOLE-CELL VACCINE

Pertussis whole-cell vaccine is defined as a 'saline suspension of killed *B. pertussis* usually made from a mixture of several strains so as to include agglutinogens 1, 2 and 3' (Wardlaw and Parton, 1983a). At the bulk stage pertussis whole-cell vaccine is usually mixed with diphtheria and tetanus toxoids which have already been adsorbed onto aluminium hydroxide or phosphate, or calcium phosphate, as adjuvant. Vaccines from different manufacturers are demonstrably not identical (Cameron and Stainer, 1977; Griffith, 1978) since whole-cell vaccine is not a defined substance; and although the WHO (1979) has laid down general recommendations for vaccine production, national control authorities of individual countries may introduce modifications. A flow chart for the production of whole-cell vaccine according to the WHO (1979) is shown in Fig. 1. The final batch material is subject to the appropriate tests for purity, sterility and shelf-life, the intracerebral mouse-protection test (Kendrick *et al.*, 1947) is used to assay the protective efficacy, and the mouse-weight-gain test is used to test for acute toxicity.

2.1 Toxicity of pertussis whole-cell vaccine

A standard whole-cell pertussis vaccine contains active pertussis toxin (PT), LPS endotoxin, heat-labile agglutinogens and sometimes adenylate cyclase (Hewlett *et al.*, 1977), but lacks heat-labile toxin (HLT) and often lacks filamentous haemagglutinin (FHa). The toxicity of at least two of these components, the pertussis toxin and endotoxin, has been demonstrated in laboratory animals and, therefore, it has to be expected that they are also active in the infant.

Figure 1: Flow chart for the production of pertussis vaccine according to the WHO (1979) specifications (Wardlaw and Parton, 1983a)

Stage of manufacture	Processing steps	Tests and records
Seed culture	Maintain by freeze-drying or storage in liquid N_2; grow in suitable medium to inoculate bulk.	Strains must be of known origin and history; final vaccine must contain agglutinogens 1, 2, 3.
↓		
Bulk culture	Check for normality of growth by observing growth rate, pH and agglutinogen content.	Culture purity; opacity.
↓		
Killed suspension	Treat with formaldehyde and/or thiomersal under controlled conditions, or heat at 56°C for 30 min.	Killing checked by culturing a sample.
↓		
Final bulk	Pool several killed suspensions so as to have a sufficient volume of the right mixture of agglutinogenic types: dilute in pH 7.0 buffered saline to the appropriate opacity; add preservative and adjuvant (if required).	pH, sterility, toxicity, potency and (on demonstration batches) shelf life.
↓		
Final containers	Inspect each container visually and discard those showing abnormalities such as leaks, particles or clumps.	Identity, sterility, innocuity, pH and preservative content; also toxicity and potency if not already done on Final bulk.

Reactions to pertussis whole-cell vaccine in the child may be considered under two headings; those that are mild or moderate and those which are in some degree severe (Wardlaw and Parton, 1983a).

(a) Mild or moderate reactions.

The minor, transient reactions to pertussis whole-cell vaccine include fever and irritability, and redness, swelling and tenderness at the injection site. Barkin and Pichichero (1979), in a survey of 1,232 DTP-vaccinated children, reported that only 7% of the parental respondants reported no reaction whatsoever, whereas 27% assessed reactions as 'mild' and 59% as 'moderate'. In 7% of the cases, parents judged high fever and persistent screaming as severe reactions. Cody et al.(1981) reported that 64% of 15,742 DTP-vaccinated children had local reactions during the first 48 hours post-vaccination, and 50% had minor systemic reactions. (These rates were higher than those observed in 784 children given DT vaccine only.) Of the DTP-vaccinated children, 9 developed convulsions and a further 9 suffered 'hypotonic-hyporesponsive' episodes: no evidence of encephalopathy or permanent brain damage was reported in the study. In a British study (Miller et al.,1974) of 897 vaccinated children, 12% had trivial reactions, 4% had moderate reactions and 2.6% cried excessively. Feery et al.(1985) noted that the most common reactions to either plain or adsorbed DTP vaccine were irritability and fever. Pollock et al.(1985) reported the results of a study of post-vaccination symptoms in two groups of infants, one given adsorbed DTP vaccine and the other adsorbed DT vaccine. Around 10,000 children entered the study, 6,000 receiving primary immunization with DTP and 4,000 with DT. It was found that crying, screaming and fever were slightly more frequent after adsorbed DTP than adsorbed DT, but the

frequency of more serious episodes of high-pitched screaming, pallor, convulsions, neurological disorders and sudden infant death were similar in both groups.

(b) Severe reactions.

Wardlaw and Parton (1983a) assessed that several complicating factors had to be considered when discussing 'severe reactions' following pertussis whole-cell vaccine injection. These were that (a) 'the reactions had an incidence which were between 'very uncommon' and 'very rare', and that the same batches of vaccine were likely to have been given to several thousand individuals without unacceptable effects, (b) there was no single 'severe reaction' syndrome after vaccination, (c) some of the reactions attributed to pertussis whole-cell vaccine might have arisen from other causes, and became implicated with the vaccine quite spuriously because of a coincidence of timing, and (d) most of the reports consisted of uncontrolled, retrospective observations and not of epidemiologically acceptable data'.

According to Griffith (1978) the shock, collapse, extreme pallor and persistent screaming which have been occasionally seen as rare responses occurring between 2-12 hours post-vaccination, were, with reasonable certainty, due to the vaccine. Eleven such cases were reported from 15 million doses of Wellcome pertussis vaccine injected between 1964 and mid-1977 (Griffith, 1978). Pertussis-containing vaccines from other sources (Haire et al.,1967; Hannik, 1970) were associated with much higher rates of such reactions.

With spasms, convulsions and seizures, the position remains less clear, due to the relatively high background incidence of such conditions occurring spontaneously, or for other reasons, in the infant population (Griffith, 1978). About 5% of children have a convulsion by

the age of 5 years, and 2-4% have a febrile convulsion, most commonly between the ages of 6 and 24 months; epilepsy follows a febrile convulsion in about 2% of the children. Griffith (1974) estimated that the incidence of first convulsions in children aged between 6 and 24 months was around 3 per 100,000 children per day. Since most children receive vaccination in that age group, there is a chance of about 3 per 100,000 of having a coincidental convulsion within 24 hours of vaccination. In the MRC field trials of the 1950s (Reports 1951, 1956, 1959), when 56,000 doses of vaccine were administered, 6 children had convulsions within 72 hours of vaccination, a rate of about 4 per 100,000 children per day. Pollock (1977) noted 5 cases of convulsions during the first 24 hours after injection of 79,807 doses of DTP, whereas no convulsions occurred after 73,373 doses of DT, and Griffith (1978) cited the individual case of a child who had a severe convulsion 15 minutes before vaccination!

Of all the reactions associated with infantile administration of pertussis vaccine, the most serious and alarming are those in which permanent brain damage or death may occur. There was much variation in the reported incidence of these 'severe reactions'. Strŏm (1960) suggested that 1 in 6,000 children given pertussis-containing vaccine in Sweden was left with permanent brain damage: this figure was later amended to 1 in 50,000 after recalculation of the data (Malmgren et al., 1960). Griffith (1978) estimated that Wellcome pertussis-containing vaccines were implicated in 1.7 instances of neurological reactions per 1 million doses in Britain during 1964-1977. The article of Kulenkampff et al. (1974) described the cases of 36 children who, over an eleven-year period, were admitted to a London hospital with acute neurological illness

which was noted to have started within 28 days of vaccination. However, these authors did not claim that the association was necessarily causal. Subsequently Bassili and Stewart (1976) and Stewart (1977) argued that pertussis whole-cell vaccine was 'neurotoxic' and estimated rates for neurological damage ranging from 1 in 10,000 to 1 in 60,000 vaccinated children (Stewart, 1977). However, Grist (1977) interpreted Stewart's data as showing that the risk of severe brain damage was 1 in 135,000.

In Britain, from mid-1976 to mid-1979, a nationwide enquiry into all cases of serious neurological disease in early childhood was carried out (Alderslade et al., 1981; Miller et al., 1981). This enquiry by the National Childhood Encephalopathy Study, cited 1,180 cases of serious neurological disorders in a population of 1.8 million children aged from 2-36 months. Of these cases, the first 1,000 were subjected to detailed epidemiological analysis. The main points drawn from the study were,

(1) over 95% of the cases of serious neurological disorders reported were not associated with recent (within 28 days) vaccination with pertussis-containing vaccines,

(2) the overall modal age of onset of disease with permanent sequelae was in the range of 4-10 months, coincident with the age for primary immunization with pertussis vaccine,

(3) the use of DTP, but not DT vaccine, was associated with a greater frequency of acute neurological illness than would have been expected by chance,

(4) the estimated attributable risk of a serious neurological disorder within 7 days after DTP vaccination in previously 'normal' children was

1 in 110,000 immunizations (95% CL 1 in 44,000, 1 in 360,000). For permanent (one year later) brain damage, the figure was 1 in 310,000 (95% CL 1 in 54,000, 1 in 5,310,000) immunizations.

It has to be stressed that the occurrence of severe reactions to vaccination such as encephalopathy and permanent brain damage are rare. Also, the Joint Committee on Vaccination and Immunization (1977) stated that several contra-indications had to be assessed before infantile administration of pertussis vaccine. These were: 'A history of seizures, convulsions or cerebral irritation in the neonatal period. A family history of epilepsy or other diseases of the CNS. Children with neurological defects. Any febrile illness, particularly respiratory, until the patient is recovered. Any severe, local or general reaction to a previous dose.' The high incidence of contra-indication positive cases in the studies of Kulenkampff et al.(1974) and Stewart (1979) supported the idea that some children were predisposed to having a severe adverse reaction to pertussis vaccine.

In summary, many complications of infancy, including 'sudden infant death syndrome', occur most commonly during the first six months of life, a period concomitant with many vaccinations. (It has even been suggested (Baraff et al., 1983; Fulginitti et al., 1983) that the vaccination procedure itself may provide sufficient stress to a 'predisposed' child to precipitate a reaction !). There is no doubt that pertussis-containing vaccines are implicated in some of the rare cases of permanent neurological illness after administration. It is difficult to avoid believing in the contribution of an idiosyncratic component - genetical, developmental or environmental - in the child which predisposes it to some form of reaction. What needs to be established

is the exact component (or components) of the whole-cell vaccine which may be responsible for precipitating these reactions, so that they can be rigorously controlled in current and future acellular vaccines.

2.2 Protective efficacy of pertussis whole-cell vaccine

Numerous clinical trials with pertussis vaccine have been made and have varied greatly in their design and conduct. In 1933, Madsen reported on the results of clinical trials in the Faroe Isles during the pertussis epidemic of 1923-1924. It was concluded that the disease was less severe in the vaccinated subjects, and there were fewer deaths. Sauer (1933, 1937) obtained similar results to Madsen (1933). Reference to the earliest trials may be found in Pittman (1970), the Joint Committee on Vaccination and Immunization (1977) and Miller et al. (1982).

In the 1950s, the MRC (Reports 1951, 1956, 1959) carried out a series of double-blind field trials in nearly 50,000 children in the UK. The main results were;

(1) the 25 pertussis vaccines tested varied greatly in their ability to give protection against whooping cough. The best vaccines reduced the home exposure rate to approximately 4%, whilst the least effective allowed an attack rate of 87%,

(2) vaccines containing aluminium hydroxide or phosphate adjuvants were on average no more potent than vaccines without adjuvant,

(3) the most potently protective antigen, the acellular 'Stromata Protective Antigen' (SPA) of Pillemer et al.(1954) was the most reactogenic (MRC Report, 1959),

(4) there was a good (but not perfect) degree of correlation between the protective efficacy of the vaccines in children and in the intracerebral mouse-protection test (ICMPT), but not in the intranasal mouse-protection

test (Standfast, 1958).

As a consequence of these trials the ICMPT was adopted as the laboratory procedure for assaying the potency of any pertussis whole-cell vaccine. Also, a British Reference vaccine was established from one of the field-trial batches which had given 80% protection; however, when the first International Standard Pertussis Vaccine became available in 1964, this British Reference was found to contain only 2.1 International Units (IU) per dose, well below the recommended minimum of 4.0 IU per dose (Perkins, 1969). The current reference vaccine, established in 1980, is the Second International Standard for Pertussis Vaccine, and consists of lyophilized material dispersed in ampoules, each of which is defined as containing 46 IU of protective potency.

Assessments of vaccine efficacy since 1957 have had to be made on statistically much less satisfactory data, for no further double-blind field trials have been made since those of the MRC. Extensive surveillance of pertussis case rate and severity, in relation to vaccination status, has been made in the UK over the past decade, when concern about vaccine efficacy and toxicity became a matter for public debate (Noah, 1976; Miller and Fletcher, 1976; Grob et al.,1981; Report from the Swansea Research Unit of the Royal College of General Practitioners, 1981). Similar data have been reported in the USA (Broome and Fraser, 1981) and Japan (Kanai, 1980). All these studies supported the view that pertussis vaccines in current use provided a worthwhile, though not fully satisfactory, level of protection against the disease. In the epidemic of 1977-1979 in the UK, an inverse correlation between pertussis notification rate and vaccine acceptance rate in different areas of England was shown, where attack rates per 100,000 children

aged 1-4 years decreased as the percent vaccine acceptance rate increased (Joint Committee on Vaccination and Immunization, 1981). In the same epidemic, a comparison was made of pertussis incidence in children who had received 3 doses of DTP compared to those who had 3 doses of DT vaccine (Report from the PHLS Epidemiological Research Laboratory and 21 area health authorities, 1982). The main conclusion was that the degree of attributable efficacy depended strongly on which criterion was used. For example, the relative frequency of bacteriologically-proven pertussis was approximately 14 times higher in the DT than in the DTP group, giving an efficacy of 93% as calculated from,

$$\text{Efficacy} = \frac{(\text{rate per 1,000 in DT group}) - (\text{rate per 1,000 in DTP group})}{(\text{rate per 1,000 in DT group})} \times 100.$$

However, if the more stringent criterion of reduction in attack rate in home contacts was used, the efficacy ranged from 30% to 59%. The overall conclusion was that pertussis vaccine was reasonably effective in reducing infection and the disease but did not invariably prevent severe whooping cough in vaccinated children.

The importance of agglutinogens, or serotype antigens, in pertussis vaccine has been a central problem for many years. Agglutinin responses have played a prominent role in the evaluation of pertussis vaccine efficacy. During the MRC field trials of the 1950s, Evans and Perkins (1955) showed that there was a good correlation between the protective activity of pertussis vaccines in children and their ability to induce anti-B. pertussis agglutinins in mice. This led to the suggestion that an agglutinin-production test in mice could be used to assay the protective potency of vaccines. However, a serious discrepancy emerged in that the Pillemer vaccine, which was highly effective in the

ICMPT and in protecting children, was poorly active in the Evans and Perkins test (MRC Report, 1959). Later work showed that the reason why the Pillemer vaccine had been a poor antigen in the agglutinin test was because the B. pertussis strain 134 from which it was made was of a different serotype to the Evans and Perkins test strain (Pittman, 1970).

Preston (1963, 1965) suggested that the failure of some children to acquire immunity to B. pertussis after vaccination might be due to a difference in serotype between the prevalent epidemic strains and those used in vaccine manufacture. This may not necessarily have been a correct interpretation, because of the separate complicating factor that the reference British Standard Pertussis Vaccine used in the early 1960s was lower in mouse-protective potency than it should have been. However, the belief that agglutinogens are important in the prevention of pertussis is reflected in the WHO recommendation (1979) that vaccines should contain representative strains with serotype antigens 1, 2 and 3.

2.3 Immune responses to vaccination and disease

A variety of procedures has been employed to determine the serological response following pertussis disease or immunization with pertussis vaccine; these included agglutination assays (Manclark, 1976), bactericidal assays (Aftandelians and Conner, 1973) and enzyme-linked immunosorbent assay (ELISA). Using ELISA, Granström et al. (1982a,b) reported increases in IgG anti-filamentous haemagglutinin (FHa) titres during the disease. Positive IgM and high IgG titres to FHa indicated recent infection, but the IgM and/or IgA anti-FHa response was absent in some individuals. Also, IgM titres were higher after infection than

after vaccination, and the reverse applied to IgG titres (Macauley, 1981).

Using whole-cells as the antigenic phase in ELISA, Ruuskanen et al.(1980) reported an IgG response after immunization with DTP, but low or undetectable IgM and IgA titres. Viljanen et al.(1982) using the same ELISA and antigen, demonstrated IgG, IgM and IgA responses in pertussis patients, and they proposed that the presence of an IgM and/or IgG response was evidence of recent disease. Goodman et al.(1981) detected IgA in nasopharyngeal secretions during and after natural infection but not after vaccination.

Burstyn et al.(1983) studied the immune response of vaccinees and patients with pertussis with an ELISA which employed highly purified pertussis toxin (PT) and filamentous haemagglutinin (FHa) as antigens. In children vaccinated with DTP, IgG and IgM antibodies to both antigens were detected but, with a few exceptions, no serum IgA to either. An anti-FHa IgA response was detected only from patients with disease and seldom from vaccinees. Winsnes et al.(1985) using ELISA found that (i) the IgG responses to FHa and lipopolysaccharide (LPS) were often persistent both after infection and vaccination, (ii) IgG antibodies to PT were frequently detected in high titres in patients but in low titres in vaccinees, (iii) an IgM response to FHa and LPS was present to almost 100% after vaccination and disease, whereas IgM antibodies to PT were detected in 23% of the vaccinated and 83% of the patients, (iv) none of the vaccinated children, and only 2% of the patients, gave a serum IgA antibody response to PT, and (v) there were no pronounced differences in the IgA responses to FHa and LPS in vaccinees and patients.

It was proposed that it could be possible to protect the newborn infant by ensuring that the mother had been immunized, and was able to transmit immunity *in utero* and/or through colostrum (Manclark, 1981). Burstyn *et al.*(1983) observed that infants with high placental cord-blood anti-PT IgG titres did not produce anti-PT IgG antibodies when immunized with whole-cell vaccine, whereas children with low cord titres gave good anti-PT IgG responses when vaccinated.

SECTION 3. ANTIGENIC AND BIOLOGICALLY-ACTIVE SUBSTANCES OF B. PERTUSSIS

Bordetella pertussis produces an array of biologically-active components that individually or collectively may have important roles in the host-parasite interactions in pertussis. The biological properties of these components are discussed in detail below.

3.1 Agglutinogens

In B. pertussis, agglutinogens can be operationally defined as surface antigens which stimulate the production of antibodies which cause bacterial cell agglutination (Robinson et al., 1985a). Based on agglutination and agglutinin adsorption, the genus Bordetella was differentiated by agglutinogens unique to each species (Anderson, 1953; Eldering et al., 1957). Agglutinogens 1 and 7 are common to all B. pertussis strains, with agglutinogen 7 being common to all the species of the genus Bordetella. Agglutinogens 2 to 6 vary in individual B. pertussis isolates, and agglutinogens 8, 9 and 10 are found in B. parapertussis, with number 14 being species-specific for the latter. All strains of B. bronchiseptica have agglutinogen 12 with individual isolates containing 8, 9, 10 and/or 11. It is now believed that there are three major agglutinogens in B. pertussis (1, 2 and 3), whilst 4, 5 and 6 are probably minor antigens (Preston et al., 1982). Ashworth et al. (1985) provided evidence that agglutinogens 2 and 3 were both fimbrial antigens, whereas agglutinogen 1 was not.

The role of serotype antigens in experimental B. pertussis infection in the mouse has been well studied. In the ICMPT there was little evidence that they played a significant role in immunity (Eldering et al., 1966; 1967). A marginal protective effect of factor 2

and 3 antisera against intracerebral challenge was demonstrated (Preston and Garrity, 1967) and Robinson and Irons (1983) found that fimbriae (agglutinogens) were protective in the ICMPT only in the presence of a trace of active PT.

3.2 Filamentous haemagglutinin (FHa)

B. pertussis possesses two distinct haemagglutinins with different chemical and biological properties. The filamentous haemagglutinin (FHa) appeared as fine filaments 2nm in diameter and 40-100nm in length when viewed in the electron microscope (Arai and Sato, 1976). FHa has been purified from both static liquid culture supernates (Arai and Sato, 1976; Arai and Munoz, 1979; Cowell et al.,1982) and from cell extracts (Irons and MacLennan, 1979a,b; Askelöf et al., 1982; Irons et al.,1983; Nakase et al.,1975). The separation of FHa from trace amounts of PT has proved difficult, but has been achieved through sequential column chromatography (Cowell et al.,1982; Sato et al.,1983a). The molecular weight of FHa from B. pertussis Tohama strain was estimated at 133,000 by sucrose density gradient ultracentrifugation (Arai and Sato, 1976), and FHa purified from liquid culture supernates was found to be heterogenous when examined by SDS-PAGE. Major polypeptides of molecular weight 127k and 95k (Irons and MacLennan, 1979b), 126k (Arai and Sato, 1976), and 160k, 115k and 90k (Cowell et al.,1982) were revealed. These various fragments probably arose from the polypeptide of the highest molecular weight, 220k (Irons et al.,1983).

It was originally thought that FHa was derived from bacterial cell surface fimbriae (Sato et al.,1974; Morse and Morse, 1976). Sato

et al. (1979) suggested that B. pertussis fimbriae, or haemagglutinin, played a role in attachment; they also showed that antibody specific for FHa reacted with B. pertussis fimbriae. In contradiction, Ashworth et al. (1982a) concluded that FHa was not fimbrial in origin and suggested that the labelling of fimbriae with anti-FHa antibody was due to the presence of serotype-specific agglutinins in the antisera. The precise origin of FHa is still a matter of disagreement. In the study of Blom et al.(1983), on the morphology of B. pertussis cells, fimbriae and FHa by electron microscopy, the authors concluded that FHa was derived from cell surface fimbriae.

Whether or not FHa is fimbrial in origin does not preclude its mediating attachment. Antibodies to FHa inhibited attachment of B. pertussis to mammalian cells (Sato et al., 1981), although agglutinin contamination might again have been a problem. FHa is one of the most likely candidates for inclusion in any acellular pertussis vaccine because of its lack of toxicity and its possible protective activity against animal infections.

3.3 Lipopolysaccharide (LPS)

The LPS of B. pertussis possesses many properties in common with LPS from other Gram-negative bacteria in being heat-stable, antigenic, pyrogenic and toxic. As reviewed by Wardlaw and Parton (1983b), the LPS of B. pertussis has some unusual chemical properties. Le Dur et al. (1980) found that B. pertussis endotoxin contained two chemically distinct LPS moieties which were designated LPS-1 and LPS-2, on the basis of the presence of two low molecular weight polysaccharides: PS-1, which contained non-phosphorylated KDO (3-deoxy-2-octulosonic acid),

and PS-2, which contained phosphorylated KDO. These LPS molecules also contained two lipids, lipid X and lipid A.

Robinson and Irons (1983) found that LPS was not protective in mice, and no specific protective role for LPS has been established in any model of pertussis infection. The role of LPS in the pathogenesis of, and immunity to, B. pertussis remains uncertain; and whatever its role, it appears unlikely that it will be found to be an important enough antigen, in its native state, to alter the current goal to reduce or eliminate it from current pertussis vaccines (Manclark and Cowell, 1984).

3.4 Heat-labile toxin (HLT)

Heat-labile toxin, also known as dermonecrotizing toxin (DNT) or lienotoxin, was first detected by Bordet and Gengou (1909), but very little has been learned of its chemistry, immunology and role in pathogenesis (Wardlaw and Parton, 1983b). HLT was reported to be intracellular (Cowell et al.,1979) and lost from B. pertussis cultures during X- to C-mode antigenic modulation (Livey et al.,1978).

HLT has been notably difficult to purify (Livey and Wardlaw, 1984), and the best preparations have been obtained by Onoue et al.(1963), Nakase et al.(1969), Iida and Okonogi (1971) and Sekiya et al.(1982). The pure toxin or crude cell lysate was lethal for mice and produced significant dermonecrosis, and all biological activities were lost on heating at 56^{o}C for 30 min. Crude extracts of B. pertussis damaged tissue cells in vitro (Felton et al.,1954; Angela et al.,1962), and elicited changes in the bronchial epithelium and peribronchial and perivascular connective tissue of mice after nasal instillation (Asada, 1953). Sekiya et al.(1982) and Nakase and Endoh (1985) showed that purified HLT induced spleen atrophy.

For many years after its discovery in 1909, HLT was considered to be non-antigenic. However, in the 1940s several investigators showed that crude, detoxified-HLT stimulates, both in children and experimental animals, antibodies that neutralized the dermonecrotizing and lethal activities (Munoz, 1971). Native HLT appeared not to function as a protective antigen in experimental intracerebral B. pertussis infections of mice (Munoz, 1971); however, most of this work was done with impure HLT and was not definitive. Toxoided-HLT appeared to be non-antigenic in mice but antigenic in rabbits (Livey and Wardlaw, 1984). However, Nakase et al. (1969) reported that 1.0 mg of purified formalin-detoxified, alum-precipitated HLT failed to protect mice against intracerebral challenge with live B. pertussis. These authors did not state whether the sera of immunized mice had detectable levels of anti-HLT antibody: therefore, the failure of HLT to act as a protective antigen in the mouse might have been due to the absence of antibody production under the conditions of immunization. The question of HLT being able to function as a protective antigen in the mouse or child should be regarded as unanswered (Wardlaw and Parton, 1983b).

The mechanism of action of HLT remains largely unknown. Nakase and Endoh (1985) thought that spleen atrophy was the result of HLT acting on the smooth muscles of spleen arterioles to cause vaso-constriction, and Parton (1986) showed that two anti-inflammatory agents inhibited the haemorrhagic effect produced by HLT in mouse skin.

3.5 Tracheal cytotoxin (TCT)

Tracheal cytotoxin was isolated from culture supernates of B. pertussis as a small glycopeptide containing diaminopimelic and

muramic acids (Goldman et al.,1982). The presence of these components suggested that TCT was derived from the peptidoglycan of the cell envelope. Goldman et al.(1982) also showed that partially-purified TCT caused cellular damage and ciliostasis to hamster tracheal epithelial cells. TCT may be responsible for the primary lesion in pertussis, ie: cytopathology in the respiratory tract, and therefore merits further investigation as a virulence factor.

3.6 Adenylate cyclase

In addition to the ability of pertussis toxin to activate host-cell adenylate cyclase, B. pertussis produces a heat-stable calmodulin-activated adenylate cyclase. Hewlett et al.(1977) found the enzyme present in culture supernates, and when purified was a monomeric protein of molecular weight around 70,000. Hewlett et al.(1985) indicated the presence of two forms of the adenylate cyclase toxin: one possessed only enzymatic adenylate cyclase activity but had no effect on intact target cells, and the other was both enzymatic and intoxicating in its activity.

Confer and Eaton (1982) noticed that high levels of adenylate cyclase inhibited the activity of macrophages, and suggested that the enzyme contributed to the pathogenicity of B. pertussis by interfering with the host's defence mechanisms. Van Heyningen (1982) suggested that the enzyme exerted non-specific effects on the host so that there was a diminished febrile and inflammatory response, with an enhancement of the primary infection and complications of secondary bacterial and/or viral infections. Hewlett et al.(1985) indicated that B. pertussis adenylate cyclase affected several cell types including neutrophils, macrophages, monocytes, lymphocytes, lymphoma cells and pituitary cells.

These workers confirmed the hypothesis that the adenylate cyclase was a toxin which promoted cAMP accumulation in target cells.

Confer and Eaton (1982) found that the enzyme entered phagocytic cells to catalyze the formation of cAMP, but the mechanism of entry has not been elucidated. Bacillus anthracis also produces a heat-stable, calmodulin-dependent adenylate cyclase which has been termed an 'oedema factor'. This factor was postulated to require specific interaction with the 'protective antigen' protein before it could enter mammalian cells. Leppla (1982) proposed that this 'protective antigen' of B. anthracis interacted with mammalian cells to provide a receptor for the 'oedema factor', and suggested that a similar receptor protein and mechanism permitted the entry of B. pertussis adenylate cyclase into the host cell.

3.7 Pertussis toxin (PT)

Pertussis toxin is the component of B. pertussis which has been most extensively studied and which appears unique to this bacterial species. The notion that the Histamine-Sensitizing Factor (HSF), Mouse-Protective Antigen (MPA) and Heat-Labile Adjuvant (HLAd) represented different activities of the same component was propounded in the 'Unitarian Hypothesis' of Levine and Pieroni (1966). Munoz (1976) proposed the term 'pertussigen' for a substance from B. pertussis which sensitized mice to histamine, induced leucocytosis, stimulated IgE production, induced hyperacute experimental allergic encephalomyelitis (HEAE) and protected mice against intracerebral challenge with live B. pertussis.

Pittman (1979, 1984) advanced the hypothesis that pertussis should be regarded as an exotoxinosis, in which the substance with

histamine-sensitizing, leucocytosis-promoting and islet-activating properties was responsible for the harmful effects of the bacterium and for the induction of the prolonged immunity that followed infection. This primary pathogenic component was called 'pertussis toxin' (PT), and this designation will be used throughout this review.

3.7.1 Purification of pertussis toxin and elucidation of subunit structure

Most methods for purifying pertussis toxin have used culture fluid rather than whole-cells as starting material, and there has been no uniquely favoured strain of *B. pertussis*, or growth medium (with the possible exception of the liquid medium of Stainer and Scholte (1971) and the CL medium of Imaizumi *et al.*, 1983). Examples of the methods for the purification of PT are summarized in Table 1.

Tamura *et al.*(1982, 1983) demonstrated that the molecular structure and mode of catalytic activity of PT was in accord with the A-B model of toxins (Gill, 1978). Analysis of PT by SDS-gel electrophoresis showed that the molecule contained as few as three (Arai and Sato, 1976; Kanbayashi *et al.*,1978) and as many as five different polypeptide chains (Tamura *et al.*,1982). The islet-activating protein (IAP-PT) of Yajima *et al.* (1978a) was composed of three types of polypeptide chain of molecular weight 12,000, 30,000 and 25,000 (present in the molar ratio of 1:2:1), and Sekura *et al.* (1983) found only four polypeptide chains by SDS-gel electrophoresis: their model was of a toxin composed of six subunits (S1, S2, S3, 3 x S4). Perera *et al.*(1985) reported that PT from *B. pertussis* strain 18334 consisted of five subunits, which included an additional subunit termed S1a. By peptide mapping, subunits S1, S1a and S2 showed extensive structural homology, indicating that the

Table 1. Purification of pertussis toxin

Authors	Method	Author's designation of substance; and mol.wt.
Sato and Arai (1972)	Culture fluid precipitated with $(NH_4)_2SO_4$, extracted with 1M NaCl, and purified by electrophoresis and sucrose gradient density ultracentrifugation.	LPF; 108,000
Arai and Sato (1976)	As above.	LPF; 107,000, 103,000 and 30,000 (estimated by different methods)
Lehrer et al.(1974)	Sucrose gradient density ultracentrifugation, Sephadex G-10, Biogel-A 10% agarose sieving of cell extract.	HSF; 90,000
Morse and Morse (1976)	Culture fluid precipitation, CsCl gradient and Sephadex G-150 gel filtration.	LPF; 67,000-72,000
Yajima et al.(1978a,b)	Column chromatography on hydroxyapatite, Sepharose CL-6B, con-A Sepharose, Biogel P-100.	IAP; 77,000
Irons and MacLennan (1979a,b)	Cell-extract precipitate applied to haptoglobin-Sepharose 4B.	LPF-Ha; mol.wt. similar to Morse and Morse (1976)
Munoz et al.(1981a)	A $ZnCl_2$ culture fluid precipitate extracted with Na_2HPO_4, sodium pyrophosphate, followed by Sepharose CL-6B and Biogel-A5.	Pertussigen; (mol.wt. not reported)
Cowell et al.(1982)	Application of hydroxylapatite, haptoglobin-Sepharose 4B and Sepharose CL-6B chromatographies.	LPF-Ha; 170,000 and 70,000 (major and minor bands)
Askelof and Gillenius (1982)	Cell-extract precipitate applied to fetuin-Sepharose 4B.	LPF (mol.wt. not reported)
Tamura et al.(1982)	According to the method of Yajima et al.(1978 a,b).	IAP; 117,000
Sekura et al.(1983)	Sequential chromatography of culture fluid with Affigel Blue and fetuin-Sepharose 4B.	ADP-ribosyltransferase, pertussis toxin; 113,000
Perera et al.(1985)	Cytoplasmic extract partially purified by fetuin-Sepharose 4B chromatography.	Pertussis toxin; 116,000

latter two might have arisen from proteolytic cleavage of the largest subunit; the subunits S2 and an undifferentiated S4/S5 showed no peptide map homology with S1.

The most generally accepted model for PT structure is that of Tamura et al. (1982). These authors deduced that PT was composed of five polypeptides; S1, S2, S3, S4 and S5, with molecular weights of 28,000, 23,000, 22,000, 11,700 and 9,300 respectively. The S2, S3, S4 and S5 combined to form a pentamer (termed the binding or B-oligomer) which could interact with the S1 subunit (termed the active or A-protomer) to form the intact molecule (Fig. 2). The A-protomer was shown to be an ADP-ribosyltransferase, catalyzing the transfer of ADP-ribose from intracellular NAD to a 41,000 mol.wt protein in cell membranes (Tamura et al., 1982; Hilderbrandt et al.,1983; Sekura et al.,1983). The B-oligomer was responsible for binding to the cell surface (Tamura et al.,1983).

Table 1 summarizes the molecular weight determinations for PT by independent investigators: the marked disagreements may be due to the ability of PT to interact with the carbohydrate gel filtration matrices used for molecular weight estimations. However, the amino acid compositions of highly purified leucocytosis-promoting factor, LPF (Morse and Morse, 1976), islet-activating protein, IAP (Yajima et al.,1978a; Ui et al.,1979) and pertussis toxin, PT (Sekura et al.,1983) were similar, although lysine was absent from the S1 subunit (Nicosia et al.,1986).

3.7.2 Biological properties of pertussis toxin

Some of the biological properties of PT relevant to this thesis are shown in Table 2 and discussed in detail below. Apart from these, mention will also be made of the other activities attributable to PT.

Figure 2. Structure of IAP-PT (after Tamura et al., 1982)

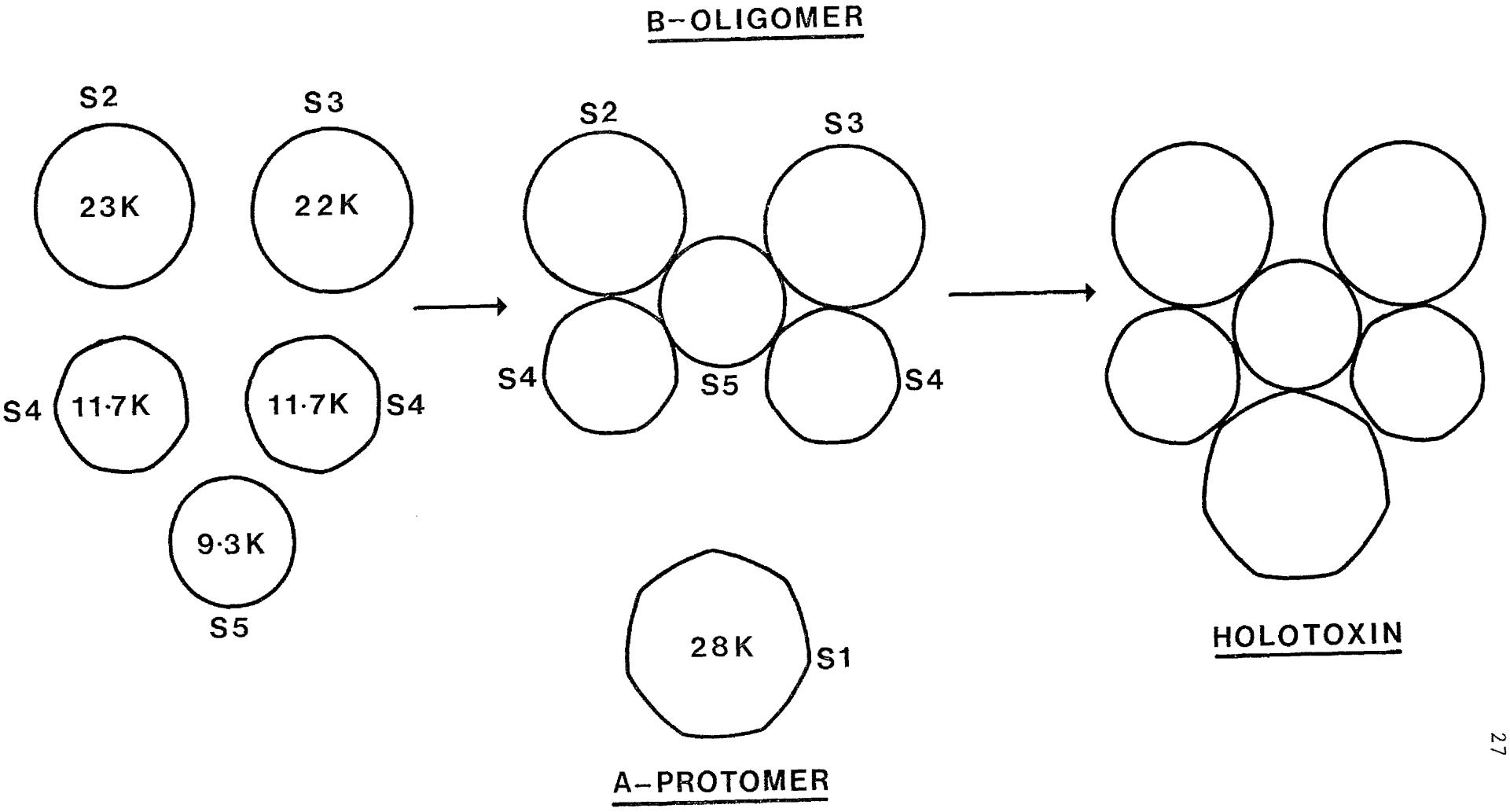


Table 2. Biological properties of pertussis toxin

Reference	Histamine-sensitization HSD_{50},[a] μg/mouse	Leucocytosis production[b] μg/mouse	Mouse protection, PD_{50},[c] μg/mouse
Lehrer et al.(1974)	0.06	NR	NR
Sato et al.(1974)	NR	NR	2.4-5.4 (F-T)
Arai and Sato (1976)	0.02	0.025	NR
Morse and Morse (1976)	0.01-0.33	0.02	NR
Yajima et al.(1978b)	0.8	0.5	NR
Irons and MacLennan (1979a)	0.03-0.05	0.02	NR
" " " (1979b)	NR	NR	maximum 4 μg[d]
Munoz et al.(1981a,b)	0.0027	NR	1.7 (G-T)
Munoz and Arai (1982)	0.0005	0.1	1.7 (G-T)
Cowell et al.(1982)	0.002	0.043	9.4 (G-T)
Robinson and Irons (1983)	NR	NR	maximum 1 μg[d]
			2.94 (G-T)
Sewell et al.(1983)	0.013	0.14	NR
Oda et al.(1984)	NR	NR	8.5 (G-T)
Sato and Sato (1984)	NR	NR	0.93 (F-T)
			0.17[e] (F-T)
Watanabe (1984)	0.0009-0.0095	0.128-0.19	12.8-24.4 (G-T)

a, HSD_{50}; the dose which sensitized 50% of the mice to the lethal effect of a challenge of histamine.

b, different authors used different criteria for leucocytosis: the data given were in μg/mouse doses which induced a significant, or highly significant, elevation in the white-cell count above baseline.

c, PD_{50}; the dose which protected 50% of the mice from intracerebral infection.

NR, not reported.

F-T, formaldehyde-toxoid.

G-T, glutaraldehyde-toxoid.

d, the maximum dose of PT which could be tested in the IC MPT because of inherent toxicity. These doses were not protective.

e, the PD_{50} of pertussis toxin-toxoid was improved by incorporation of FHa in the immunizing mixture.

(a) Histamine-sensitization.

Parfentjev and Goodline (1948) were the first to show that pertussis vaccine injected into mice made the animals hypersensitive to a subsequent injection of histamine. Normally, mice are relatively insusceptible to challenge with histamine (LD_{50} dose in the range of 344-844 mg base kg^{-1} body weight; Munoz and Bergman, 1968), but if the animals received pertussis vaccine a few days previously the LD_{50} of histamine decreased some fifty-fold. The factor in B. pertussis cells responsible for this effect was termed the histamine-sensitizing factor, HSF (Maitland, 1955).

HSF was present only in phase I, X-mode B. pertussis and was lost, or greatly diminished, during phase variation and antigenic modulation (Wardlaw et al.,1976). Histamine-sensitization is a cardinal property of PT and animal species vary greatly in the degree to which they are sensitized. Most of the work has been done with the mouse whose sensitivity to HSF was shown to be strain dependent (Munoz and Bergman, 1968). Age and sex of the animal, environmental stress and route of injection may also affect the mouse response (Munoz and Bergman, 1968). Histamine-sensitization was also induced by respiratory infection of mice with B. pertussis (Pittman, 1951; Pittman et al.,1980), or by intracerebral injection of vaccine (Munoz and Bergman, 1968). PT also sensitized mice to challenge with serotonin, bradykinin, methacholine, anoxia, cold shock, X-irradiation and endotoxin (Munoz and Bergman, 1968).

Munoz and Bergman (1977) and Munoz (1985) concluded that death after histamine challenge was due to loss of blood volume. It was considered that PT interfered with a necessary function of epinephrine (a hormone produced by the adrenal medulla which maintained blood volume).

Table 2 summarizes the HSD_{50} values for PT obtained by various investigators. A direct comparison of these data is difficult, since a variety of mouse strains was used and in only a few instances were confidence limits provided for the bioassay.

(b) Leucocytosis-promotion.

As early as 1897 Frölich (cited by Wardlaw and Parton, 1983b) observed that pertussis (whooping cough) patients frequently exhibited a marked lymphocytosis. In extreme cases the peripheral white blood cell count can rise to 175,000 per mm^3, compared to normal basal levels of 7,000-11,000 WBC/mm^3 (Munoz and Bergman, 1977). Sauer (1933) noted lymphocytosis in children injected with pertussis vaccine, and Tuta (1937) detected a similar response in rabbits. The substance responsible for this effect was designated 'leucocytosis-promoting factor' or 'lymphocytosis-promoting factor' (LPF) since the blood counts of both lymphocytes and polymorphonuclear leucocytes were substantially increased.

A response to LPF has been demonstrated in a broad range of vertebrate species including man (Olson, 1975), and for convenience the mouse has been by far the most extensively used and studied. The marked effects of mouse age and strain seen with HSF, did not seem to affect LPF-responsiveness.

Induction of leucocytosis in mice occurs most rapidly and fully if the material is injected intravenously in preference to the intraperitoneal or subcutaneous routes. Peak leucocytosis occurs between the second and fourth day after injection and declines to normal values after 2-3 weeks: sub-lethal pulmonary infection with B. pertussis induced a leucocytosis that lasted for 5 weeks (Pittman et al., 1980), but this prolonged stimulus was associated with carriage of the organisms for 2-3 weeks.

At the height of the leucocytosis response in mice, approximately 60-70% of the cells were mature, small lymphocytes of both the T- and B-cell type, with the T-cell type predominating (Morse and Morse, 1976). Most of the rest were polymorphonuclear leucocytes, but there was also a relative increase in large lymphocytes and monocytes. Eosinophils and basophils were rare, although a slight eosinophilia in rats and mice given pertussis vaccine was reported by Terpstra et al.(1979).

Morse and Riester (1967) investigated the mechanism of leucocytosis by injecting mice with ^{3}H-thymidine, and concluded that;
(i) despite the ten-fold increase in blood lymphocyte count, the new lymphocytes had not arisen from recent cell division but had been released into the blood from extra-vascular sites to which they were unable to return.

The mechanism of retention of lymphocytes in the blood stream of pertussis-treated mice was investigated by transfusion of lymphocytes labelled in vitro with ^{3}H-uridine. This showed that lymphocytosis was due to a blockade in the normal traffic of these cells between blood and the lymphatic compartments: lymphocytes that entered the blood stream had a diminished capacity to leave the post-capillary venules in lymph nodes.
(ii) there was a release of mature polymorphonuclear leucocytes from tissue reserves, as well as an increase arising from active proliferation of myeloid elements (the latter evident from the high percentage of tritium-labelled cells).

Morse and Barron (1970) postulated that lymphocytosis appeared to be due to a change in the lymphocyte cells rather than in the vascular endothelium. In support of this hypothesis, Sugimoto et al.(1983) showed that LPA appeared to result from direct interaction of PT with lymphoid cells. Also, when lymphocytes were treated in vitro with PT,

their migration to lymph nodes *in vivo* was prevented (Spangrude *et al.*, 1984). This toxin-induced alteration of lymphocytes was not lost after extended cultivation of these cells in medium free of PT, and the toxin had no effect on the viability or the ability of the lymphocytes to respond to mitogenic agents.

The assay of LPF activity of pertussis vaccine or toxin preparations is complicated by the presence of endotoxin. Peak leucocytosis with endotoxin occurs 24 hours after intraperitoneal injection, and with endotoxin-free PT at 3-4 days (Kurokawa *et al.*,1978). Therefore, by making white cell counts at 3-4 days post-injection, the interference from endotoxin can be minimized.

(c) Mouse-protection.

The potency of pertussis whole-cell vaccines has been evaluated using the intracerebral mouse-protection test (ICMPT) of Kendrick *et al.* (1947). The whooping cough immunization trials conducted by the MRC (Reports 1951, 1956, 1959) in Great Britain yielded two important results:

(i) pertussis vaccine in use at that time varied greatly in protective immunizing potency,

(ii) there was a strong correlation between the protective efficacy of a vaccine in children and its protective potency in the ICMPT.

With respect to the possible vaccine antigens responsible, examples of the mouse-protective activity of PT preparations are shown in Table 2. Unfortunately, not all the purified preparations were tested for mouse-protective activity, eg: the islet-activating protein (IAP-PT) of Yajima *et al.*(1978a,b). However, PT might not be the only component of the bacillus that protected mice against intracerebral infection. For example, FHa has been found to be protective using a single (Irons and MacLennan, 1979a; Sato *et al.*,1979; Robinson *et al.*,1981) or double-

immunization schedule (Sato et al.,1982), although FHa rigorously purified of PT was not protective (Munoz et al.,1981a; Oda et al.,1984).

Pertussis toxin was claimed to be the major mouse-protective antigen, and antisera against PT passively protected mice (Munoz et al., 1981a). However, purified native PT alone was non-protective in the ICMPT unless toxicity was removed (Table 2). Robinson and Irons (1983) showed that the dominant role of very low levels of active PT, in the ICMPT, was to enhance the protective activities of other antigens, eg: FHa and fimbriae. However, the mechanism by which PT produces this synergistic effect remains vague. Robinson et al.(1985a) considered that for any vaccine to protect mice against intracerebral challenge it must contain, firstly, antigens to stimulate the development of a protective immune response and, secondly, native PT to allow this response to occur at the site of infection. A possible mechanism of active PT would be to alter the permeability of the blood-brain barrier of the mouse allowing access of antigens and/or antibodies and immune cells into the brain (Munoz et al.,1981a). Consequently, the good correlation found between intracerebral protection in the mouse and protection in the child with whole-cell vaccines was fortuitous, in that active PT was required for a synergistic effect in the mouse but as a major protective antigen in the child (Robinson et al.,1985a). Therefore, a truly non-toxic acellular pertussis vaccine, devoid of active PT, may not pass the ICMPT (since detoxified PT has no synergistic effect) but still protect against respiratory infection because of the presence of fully toxoided PT and other antigens.

The development of respiratory models in mice was delayed because of a lack of correlation with protection in the child (Standfast, 1958). Recent studies (Pittman et al.,1980) indicated that sublethal

respiratory infection of mice might be a more relevant model for the study of pertussis in children, as the disease has similar characteristics in both hosts. FHa and toxoided PT protected mice against respiratory infection (Oda et al., 1984). Also, preparations that were devoid of active PT and non-protective in the ICMPT, eg: FHa and agglutinogens, did protect against intranasal infection (Robinson et al.,1985b). The protection against respiratory infections may depend on anti-adhesin mechanisms, hence the strong protective effect of FHa and agglutinogens. Consequently, purified bacterial adhesins may be essential components in acellular pertussis vaccines.

(d) Adjuvancy.

B. pertussis cells and/or products clearly modify a variety of immunological responses, and most interest has centered on adjuvant activity.

Whole-cell pertussis vaccine has been noted for several distinct immunopotentiating activities (Table 3). Some of the adjuvant activity was due to LPS and to a heat-labile component (Levine and Pieroni, 1966). More direct evidence that PT acted as an adjuvant was provided by studies with X- and C-mode B. pertussis vaccines, both of which contained LPS but the latter lacked PT. In two different systems - induction of experimental allergic encephalomyelitis (EAE) to guinea-pig spinal cord in Lewis rats and reaginic IgE antibodies to ovalbumin in mice - the C-mode vaccine had only slight adjuvant activity (due to LPS) whilst the X-mode vaccine had pronounced dose-dependent adjuvant effects (Wardlaw et al., 1979). However, under certain conditions pertussis vaccine exerted immunosuppressive effects, eg: enhancement of tumour growth in mice (Hirano et al., 1967).

Since the adjuvanticity of B. pertussis is not of primary importance to the studies described in this thesis, further information

Table 3. Adjuvant activities of B. pertussis and pertussis toxin

Reference	Activity
	Adjuvant activity of B. pertussis whole organism on...
Weiner et al.(1959)	production of EAE
Munoz (1963)	increased serum antibody production
Chang and Gottshall (1972)	augmentation of sensitization of mice to inhaled antigens
Likhite (1974)	immunotherapeutic application for the treatment of mammary tumours
Athanassiades (1974), Fish et al.(1984)	cell-mediated immunity
	Adjuvant activity of pertussis toxin on...
Lehrer et al.(1976)	stimulation of IgE antibody to ovalbumin in mice
Bergman et al. (1978)	induction of EAE with guinea pig spinal cord in rats
Kohno et al.(1983)	induction of experimental allergic orchitis in mice
Munoz et al. (1984)	induction of EAE in mice
Munoz and Sewell (1984)	increased inflammatory response produced by Freund's complete or incomplete adjuvant in mouse footpads

can be obtained from the reviews of Finger (1975), Morse (1976) and Wardlaw and Parton (1983b).

Pertussis toxin has also been shown to have adjuvant activity (Table 3). Although many studies have attempted to unravel the mechanism of adjuvant activity of whole-cell vaccine (Finger, 1975), few have been done with PT. In the induction of experimental auto-immune diseases, eg: EAE, PT appeared to be responsible for increasing vascular permeability in the CNS as well as having an adjuvant effect on the encephalitogenic antigen (Bergman et al.,1978; Linthicum et al.,1982). In fact, PT was the critical constituent of B. pertussis responsible for a type of encephalopathy in mice (Steinman et al.,1985).

(e) Other biological activities of PT.

A variety of other biological activities has been attributed to pertussis toxin. These included,

(i) a hypoproteinaemia in mice (Bergman and Munoz, 1969),

(ii) an in vitro mitogenicity for mouse lymphocytes (Kong and Morse, 1977a,b; Suzuki et al.,1978). The target cells for the B. pertussis mitogen were the T-lymphocytes of the spleen or lymph node, but not of the thymus whose cells were unresponsive. The mitogenic response of the T-cells required the presence of B-lymphocytes in the culture (Ho et al.,1979) and this requirement was unique and not exhibited by other mitogens,

(iii) a release of glycerol from in vitro suspensions of adipocytes from rat epididymal fat pads (Endoh et al.,1980). This stimulated release formed the basis of an in vitro assay for PT,

(iv) an activation of pancreatic islet cells. Regan and Tolstoouhov (1936) observed that children with pertussis had lower blood-glucose concentrations than normal, and that this hypoglycaemia extended into

convalescence. Oddy and Evans (1940) noticed that a pertussis extract induced hypoglycaemia; and in 1968, Gulbenkian et al.,on injecting pertussis vaccine intraperitoneally into rats and mice, induced a marked hyperinsulinaemia. Changes similar to these were observed with purified islet-activating protein, IAP (Yajima et al.,1978b). In order to elicit hyperinsulinaemia in the IAP-treated rat, glucose was injected intraperitoneally shortly before withdrawal of blood for insulin analysis; this procedure formed the basis for the assay of IAP (PT). Yajima et al. (1978b) also showed that IAP, like pertussis vaccine (Gulbenkian et al., 1968), attenuated the hyperglycaemia induced by the hormone epinephrine. The conclusion drawn from animal studies was that the hypoglycaemia associated with pertussis infection was due to a PT-induced change in the pancreatic β-cells which led to hypersecretion of insulin in response to various insulin-secreting stimuli. This conclusion was strengthened by experiments on the rate of insulin secretion by the isolated perfused organs in vitro (Katada and Ui, 1977).

Nogimori et al.(1984a) dissociated the biological activities of PT into those dependent on the ADP-ribosyltransferase activity of the A-protomer and those dependent on the divalent attachment of the B-oligomer to the target cell surface. Those activities of the former, and relevant to the present investigation, included potentiation of hyper-insulinaemia in vivo, and those dependent on the latter included promotion of leucocytosis, histamine-sensitization and adjuvant activity. Many other in vivo and in vitro activities have been attributed to PT, and these can be found in the review of Munoz (1985).

3.7.3 Molecular basis of activity of PT

Detailed investigations of the mode of action of islet-activating

protein, IAP (PT) at the molecular level have been made by Ui and Katada (1978) and Katada and Ui (1979; 1980; 1981a,b) with pancreatic islets *in vitro*. In islets from IAP-treated rats, marked changes in cAMP turnover and calcium movements across the cell membrane and within the cell were demonstrated. These authors proposed the mechanism of IAP action on pancreatic β-cells which follows:

(i) IAP bound rapidly to islet receptor cells, after which it was gradually inserted into the cell membrane and was no longer neutralized by antibody; binding was effected by the B-oligomer via its two constituent dimers, S2-S4 and S3-S4 (Fig. 2; Ui *et al.*,1985). *In vitro* insertion of the A-protomer took 30-60 minutes and was not affected by inhibition of protein synthesis, or by agents capable of disrupting microtubular-microfilament function;

(ii) as a result of insertion, the native divalent cation ionophore on the islet β-cell membrane was altered so that calcium (Ca^{2+})-flux occurred;

(iii) the inward movement of Ca^{2+} stimulated adenylate cyclase to produce more cAMP;

(iv) insulin release was stimulated by the increased levels of Ca^{2+} and cAMP.

Katada *et al.*(1982) observed that PT enhanced adenylate cyclase activity of rat C_6 glioma cells leading to increased cAMP levels. This was subsequently found to be a result of the ADP-ribosylation of a specific membrane protein (mol.wt 41,000) which was distinct from the protein ADP-ribosylated by cholera toxin (Katada and Ui, 1982a,b). Using cell-free membrane systems from C_6 glioma cells, the A-protomer was found to be the active ADP-ribosylating unit of PT (Katada *et al.*,1983), which resulted in enhanced GTP-dependent adenylate cyclase activity (Hilderbrandt *et al.*,1983).

Adenylate cyclases are under positive and negative control by guanine nucleotides and hormones. Receptor cells contain a guanine-nucleotide regulatory component (N_S) which mediates stimulatory responses. Inhibitory responses were hypothesized to be mediated by an analogous regulatory component (N_i) distinct from N_S (Hilderbrandt et al., 1983). Wong et al. (1985) suggested that the undissociated, GDP-bound conformation of N_i (the inhibitory GTP-binding protein of adenylate cyclase) was the preferred substrate for ADP-ribosylation by PT. Ui et al. (1985) postulated that the function of N_i was lost by PT-catalyzed ADP-ribosylation of its α-subunit. A consequence of this loss would be enhancement of membrane adenylate cyclase and an accumulation of intracellular cAMP. Since cAMP is a 'second messenger' of a variety of cell stimuli, it is not surprising that interaction of the A-protomer of IAP with N_i resulted in the development of diverse biological activities including hyperinsulinaemia (Nogimori et al., 1984a).

SECTION 4. MECHANISM OF THE DISEASE

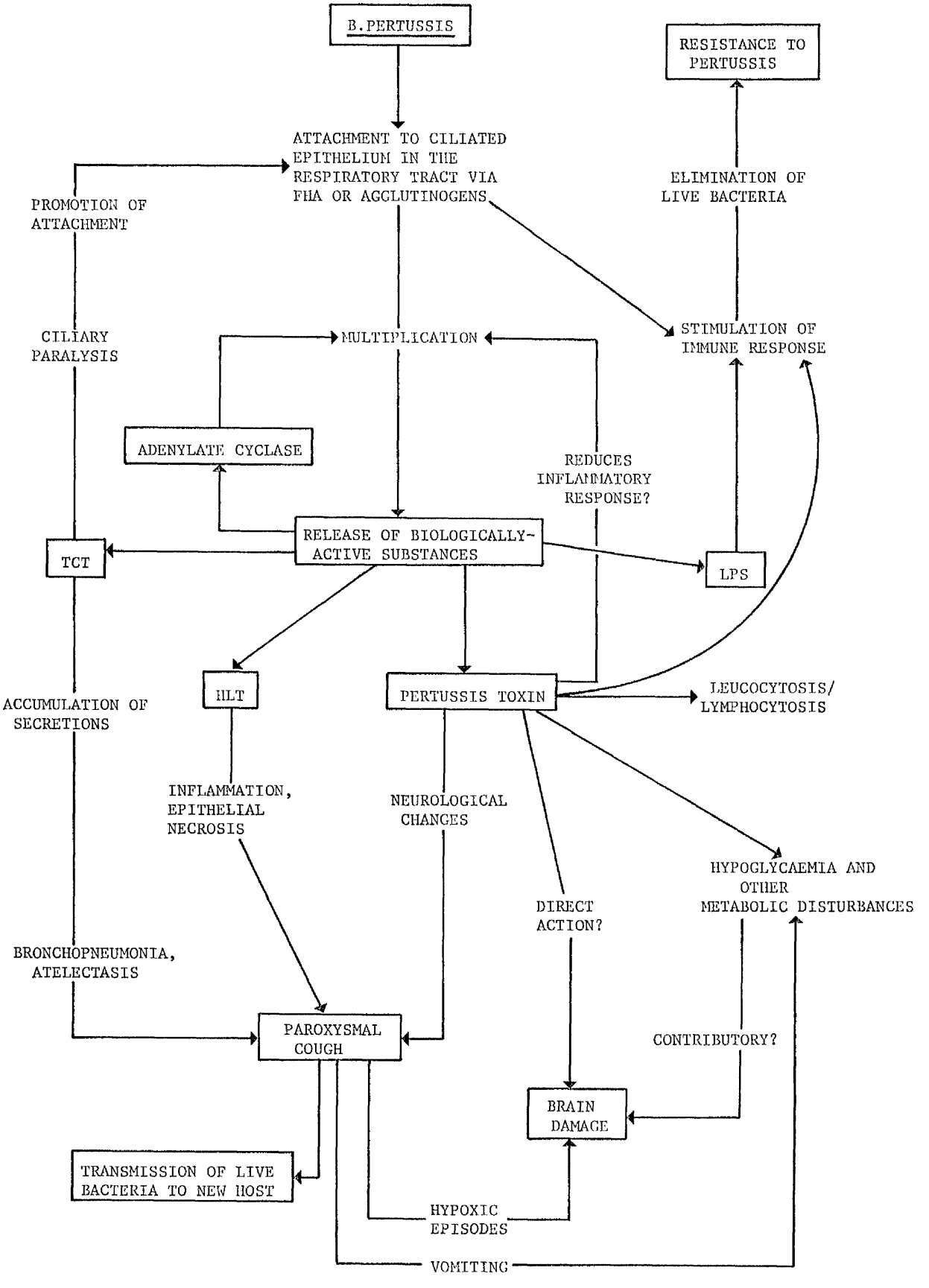
The underlying pathogenic mechanisms in pertussis are still far from clear, in particular the pathophysiological changes that give rise to the 'paroxysmal coughing' syndrome. From the current knowledge of the biologically-active components of *B. pertussis* produced *in vitro* (SECTION 3), a hypothetical scheme of events occurring during infection was constructed (Fig. 3).

The first step was believed to be lodgement of the bacteria, possibly with the assistance of FHa and fimbriae, amid the cilia of the epithelium of the upper respiratory passages. Affinity of the organism for cilia was inferred from a number of studies involving human cells (Tuomanen *et al.*,1983; Tuomanen and Hendley, 1983) and animal organ cultures (Collier *et al.*,1977; Matsuyama, 1977; Muse *et al.*,1977; Opremack *et al.*,1983). FHa was also believed to be involved in the attachment of *B. pertussis* to an epithelium-like cell line from a human intestinal carcinoma (Urisu *et al.*,1985). Tuomanen *et al.*(1985) postulated that both FHa and PT were critical to the process of adherence of *B. pertussis* to the cilia of respiratory epithelial cells. These workers suggested that PT and FHa formed an association (possibly through hydrophobic interactions) and then acted in a concerted fashion as a molecular bridge(s) between *B. pertussis* cells and mammalian cilia.

After attachment, the organisms multiplied and released a plethora of biologically-active substances.

The HLT and TCT were thought to contribute to the disease by inducing ciliary paralysis, localised inflammation and epithelial necrosis. Standfast (1958) proposed that ependymal cell cilia were paralyzed by HLT, and Goldman *et al.*(1982) and Goldman and Herwaldt (1985) reported that

FIGURE 3. SCHEME SHOWING A NETWORK OF POSSIBLE PATHOPHYSIOLOGICAL AND IMMUNOLOGICAL PATHWAYS IN HUMAN PERTUSSIS (WARDLAW AND PARTON, 1983a, BASED ON OLSON, 1975).



TCT caused ciliostasis and morphological changes in hamster tracheal rings. Such activity would lead to a reduction in the effectiveness of the mucociliary clearance mechanisms of the upper respiratory tract. However, LPS might contribute to the inflammatory response, and through its antigenic activity stimulate antibacterial antibodies which play a role in the eventual clearance of the organisms.

Pertussis toxin is seen as the factor responsible for the cardinal symptoms of the disease, although the mechanism of this exotoxinosis remains unclear. PT may be responsible for the initial alteration of host function. Meade et al.(1985) postulated from their studies that a possible role for PT in pathogenesis was the inhibition of macrophage migration to the site of B. pertussis infection. Also, the adenylate cyclase of B. pertussis could affect several mammalian cell types (Hewlett et al.,1985). Consequently, a working hypothesis was that PT and adenylate cyclase acted in a concerted fashion to alter essential cell-functions of the host's immune system required to clear the infecting organism. In effect, the respiratory tract would be isolated immunologically.

In experimental animals PT had diverse effects, some of which were demonstrable in man, eg: pronounced lymphocytosis and hypoglycaemia (Regan and Tolstoouhov, 1936; Olson, 1975) and elevated plasma insulin levels after vaccination (Hannik and Cohen, 1979). Badr-el-Din et al. (1976) reported an attenuation of the hyperglycaemic response to epinephrine in children with pertussis, a similar effect seen in rats and mice injected with PT (Yajima et al.,1978b).

SECTION 5. DEVELOPMENT OF ACELLULAR PERTUSSIS VACCINES

The earliest acellular pertussis vaccine was the 'stromata-protective antigen' (SPA) of Pillemer *et al.*(1947, 1954), which was prepared from extracts of sonically disrupted *B. pertussis* cells adsorbed onto human erythrocytes. The vaccine was protective in the ICMPT and showed clinical efficacy in the MRC field trials (MRC Reports 1951, 1956) but failed the Evans and Perkins agglutinin test (MRC Report, 1959). It was also the most reactogenic of all the vaccines tested and was not licensed for clinical use.

An acellular vaccine prepared from a trisodium phosphate extract of *B. pertussis* cells (Weihl *et al.*, 1963) was sold from 1962-1977 as 'Tri-Solgen' by the Eli Lilly company as a component of their DTP vaccine. In the initial investigation by Weihl *et al.* (1963), the extracted pertussis vaccine was antigenic and produced a markedly lower incidence of systemic and local reactions in children, compared to a whole-cell vaccine.

In the early 1960s, Millman *et al.* (1962) developed a soluble pertussis extract which was potent in the ICMPT but not tested for human efficacy. More recently, Schwick *et al.* (1980) reported that a vaccine developed at Behringwerke from urea extracts of *B. pertussis*, was potent in the ICMPT and had clinical use in children. The vaccine was reported to induce fewer local reactions in children of 3-4 months of age.

Most of the recent studies to develop acellular vaccines have concentrated on the isolation and purification of proteins from *B. pertussis*, including PT and FHa. When the investigation described in this thesis was begun (October, 1983), the only acellular vaccine licensed for clinical use was manufactured and marketed in Japan. Therefore, all published work on acellular pertussis vaccine(s) since 1983 will be discussed later.

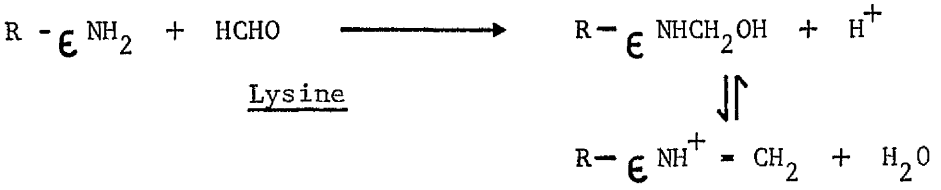
SECTION 6. CHEMICAL MODIFICATION OF BACTERIAL EXOTOXINS

Many chemical reagents have been used to modify the biological toxicity of bacterial toxins without compromising antigenicity and immunogenicity: these modified preparations are called 'toxoids'.

The first animals used for the production of diphtheria and tetanus antitoxins by von Behring and Kitasato (1890; cited Pappenheimer, 1984) were immunized with toxins that had been treated with trichloro-iodine (ICl_3) so as to reduce their toxicity. To the present day, the most extensively used reagent for chemical detoxification has been formaldehyde, although numerous other reagents have been employed for the general modification of proteins and enzymes. In this major section on toxoiding, emphasis is primarily on detoxification with formaldehyde and glutaraldehyde.

6.1 Modification of bacterial exotoxins with formaldehyde and glutaraldehyde

The reaction of formaldehyde (HCHO) with proteins is complex and can lead to a variety of heterogeneous products depending on conditions. The general subject of formaldehyde reactivity with amino acids and proteins was reviewed by French and Edsall (1945), and subsequent studies by Fraenkel-Conrat *et al.* (1945, 1947), Fraenkel-Conrat and Olcott (1946, 1948a,b) and Fraenkel-Conrat and Mecham (1949) extended the investigation of formaldehyde as a cross-linking reagent. The first step is probably a reaction with the free, uncharged ϵ-amino groups of lysine to yield aminomethylol derivatives, which can then become protonated and lose water to form Schiff bases, [equation 1].

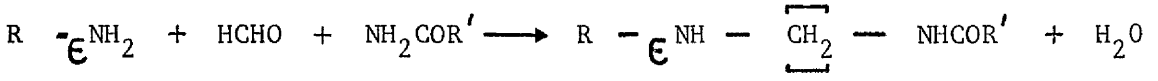


Lysine

Schiff base

... [equation 1].

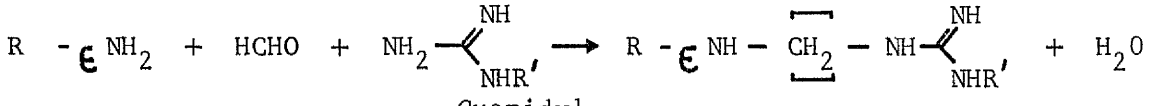
The Schiff base can finally enter into stable cross-linkages via methylene bridges, which involves the condensation of this group with an active hydrogen on primary amides [equation 2] ,



Amide

... [equation 2].

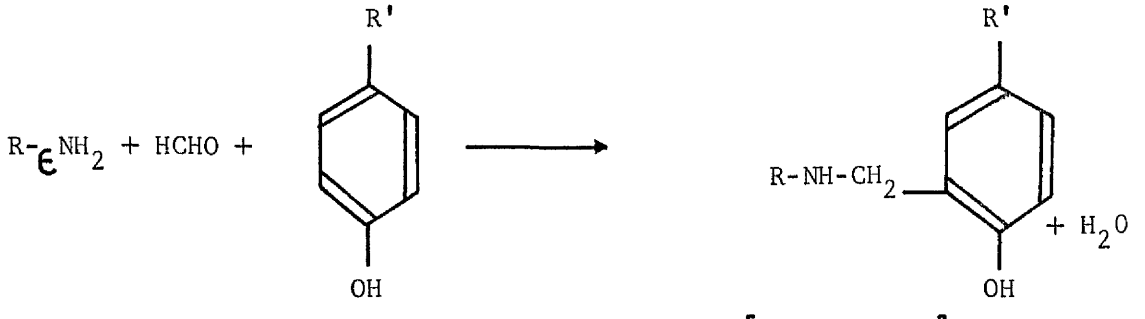
guanidyl [equation 3] ,



Guanidyl

... [equation 3].

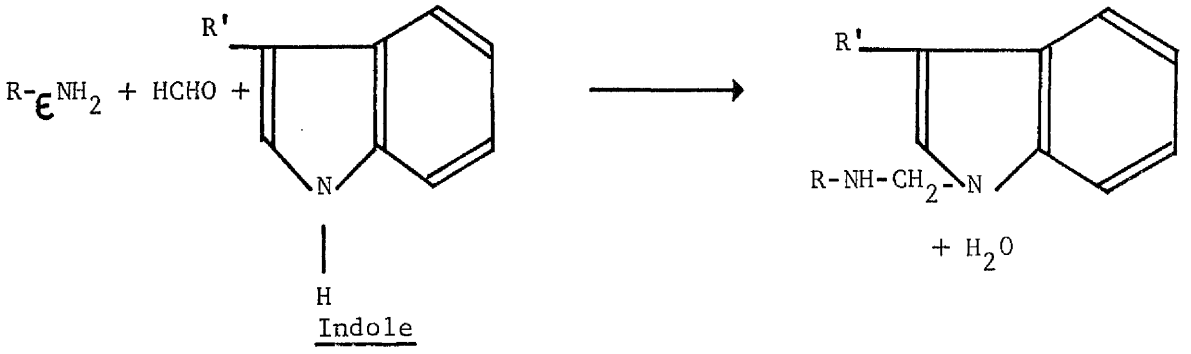
phenolic [equation 4],



Phenolic

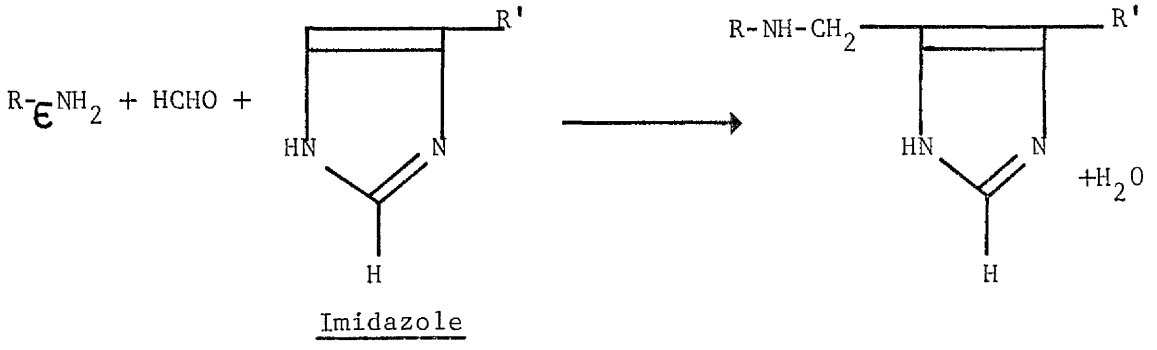
... [equation 4].

indole [equation 5],



... [equation 5].

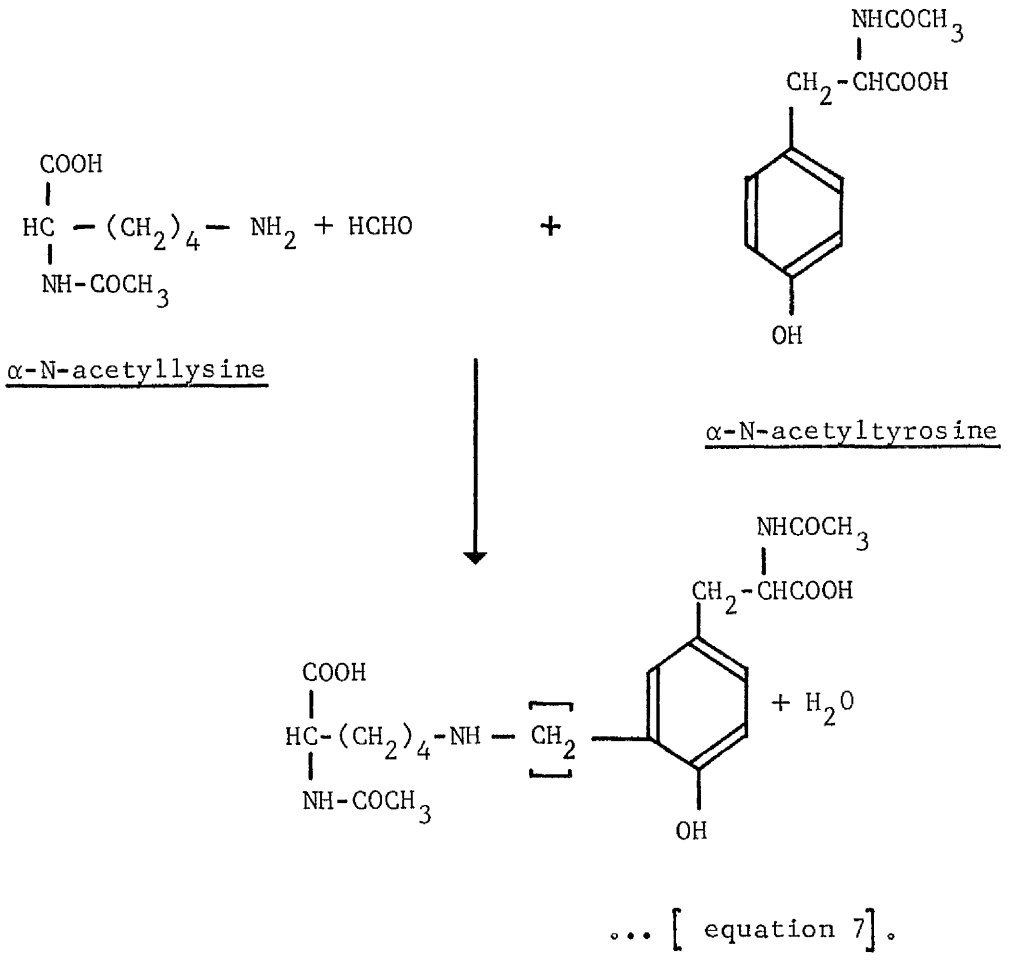
and imidazole groups [equation 6], (Blass, 1964; Blass et al.,1967).



... [equation 6].

Formaldehyde treatment, under conditions used to produce toxoids, resulted in the formation of both intra- and intermolecular methylenic bonds. From acid hydrolysis of diphtheria toxoid and formaldehyde-treated bovine serum albumin, Blass et al. (1967) isolated and identified a compound of α-N-acetyllysine and α-N-acetyltyrosine, in which the ε- amino group was linked by a methylene bridge to a

position ortho to the phenolic hydroxyl of tyrosine [equation 7].



... [equation 7].

Intramolecular bond formation between lysine and tyrosine residues was responsible for the appearance, in acid hydrolysates of diphtheria toxoid, of new acid-resistant compounds (Blass et al.,1969), and intermolecular bond formation accounted for the appearance of polymerized forms of tetanus toxoid (Raynaud et al.,1971).

Various factors influenced methylene bridge formation and the subsequent polymerization of toxoids. The concentration of formaldehyde and toxin in the reaction mixture influenced both the size and number of polymers obtained (Murphy, 1967). Also, cross-linking between toxin molecules to form toxoid polymers appeared to be random. The pH was important in toxoiding with formaldehyde (French and Edsall, 1945), although

methylene bridge formation occurred over a range of pH 3-9 (Fraenkel-Conrat and Mecham, 1949). Formaldehyde treatment did not appear to induce extensive conformational alterations in bovine serum albumin (Habeeb, 1969) or tetanus toxin (Robinson et al., 1975), and rendered proteins relatively inert to tryptic or other proteolytic enzyme digestion (French and Edsall, 1945) and acid hydrolysis (Fraenkel-Conrat and Olcott, 1948b). The increased stability of diphtheria toxoid to proteolysis was attributed to the cross-linking effect of formaldehyde (Pappenheimer et al., 1972; Bazaral et al.,1973).

Formaldehyde treatment was reported to produce little or no change in the antigenicity of proteins (Habeeb, 1969), but high concentrations did lead to a marked decrease in toxoid antigenicity (French and Edsall, 1945). Cryz et al.(1981, 1982) also noted that formaldehyde-mediated detoxification of Pseudomonas aeruginosa exotoxin A was a time-dependent process, and that the rate and extent of detoxification was increased markedly by the addition of L-lysine to the reaction mixtures. The addition of L-lysine resulted in increased antigenic alteration but yielded a toxoid which did not undergo toxic reversion on storage. However, the formaldehyde-lysine toxoid was a poor immunogen, as determined by the ability of antibody raised against it to neutralize the CHO cell cytotoxic activity of native toxin (Cryz et al.,1982). Pollack (1982) and Pollack and Prestcott (1982) also observed that apart from reducing toxicity and retaining good antigenicity, treatment with formaldehyde alone enhanced the ADP-ribosyltransferase activity of exotoxin A, whereas treatment with formaldehyde plus L-lysine reduced it. The toxoid was also immunogenic in protecting mice against challenge with large doses of exotoxin A administered intravenously (Pollack and Prestcott, 1982) or intraperitoneally (Abe et al.,1978). Pavlovskis

et al.(1981) presented data which indicated that active immunization with formalinized exotoxin A-toxoid and adjuvant stimulated protective immunity, of various degrees, against Ps. aeruginosa infections in mice.

There was evidence that formaldehyde-detoxified toxins reverted both in vitro and in vivo. This was shown with diphtheria toxin in vitro (Wadsworth et al., 1937; Linggood et al.,1963; Scheibel and Christensen, 1965; Stainer, 1968; Akama et al.,1971a), tetanus toxin in vitro (Akama et al.,1971b), Ps. aeruginosa exotoxin A in vitro (Cryz et al.,1981) and cholera toxin in vivo (Northrup and Chisari, 1972). With cholera toxin, reversion could be inhibited by heating and molecula to form the polymer 'procholeragenoid'. The residual toxicity of this polymer was inactivated with formaldehyde and the resulting toxoid did not revert in vivo or in vitro (Germanier et al., 1976). Alternatively, the addition of amino acids, especially L-lysine, to the formaldehyde-toxin mixtures, markedly improved the stability of the toxoid (Linggood et al., 1963; Cryz et al.,1981, 1982). The reaction between L-lysine, formaldehyde and protein was believed to be essentially irreversible, thus tending to stabilize the reaction product. Linggood et al.(1963) postulated that when formaldehyde alone was added to highly purified diphtheria toxin protein (represented in [equation 8] as D-H, where H is the hydrogen atom of amino or other reactive groups), toxin cross-linked polymers were produced.

$$\underset{\text{Toxin}}{D_1 - H} + HCHO + \underset{\text{Toxin}}{D_2 - H} \longrightarrow \underset{\text{Polymer}}{D_1 - CH_2 - D_2} + H_2O$$

... [equation 8].

If toxoiding was conducted in the presence of an appropriate concentration

of L-lysine, the reaction in [equation 9] could occur.

$$D_1 - H + HCHO + NH_2\,CH(COOH)(CH_2)_4\,NH_2$$

Toxin ↓ Lysine

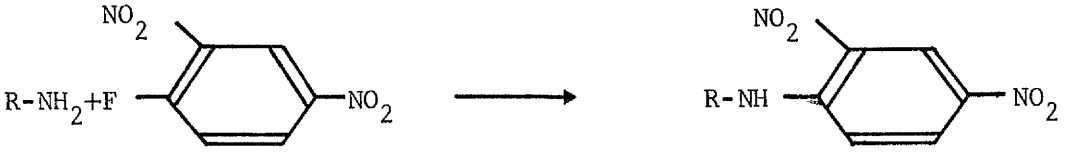
$$D_1 - \underbrace{CH_2} - NHCH\,(COOH)(CH_2)_4\,NH_2 + H_2O$$

Toxin - Lysine side chain

... [equation 9].

Therefore, the antigenicity and relative stability of the product of [equation 9] might be associated with the presence of side-chains derived in this way from the added amino acid.

Almost any reagent that reacted with the free ϵ-amino groups of lysine in purified diphtheria or tetanus toxin greatly reduced their toxicity, and, in addition to formaldehyde, such reagents included (i) ketene (Pappenheimer, 1938) which reacted with amino groups, sulphydryl groups and phenolic hydroxyl groups, (ii) 1-fluoro-2,4-dinitrobenzene (Raynaud et al.,1957; equation 10)



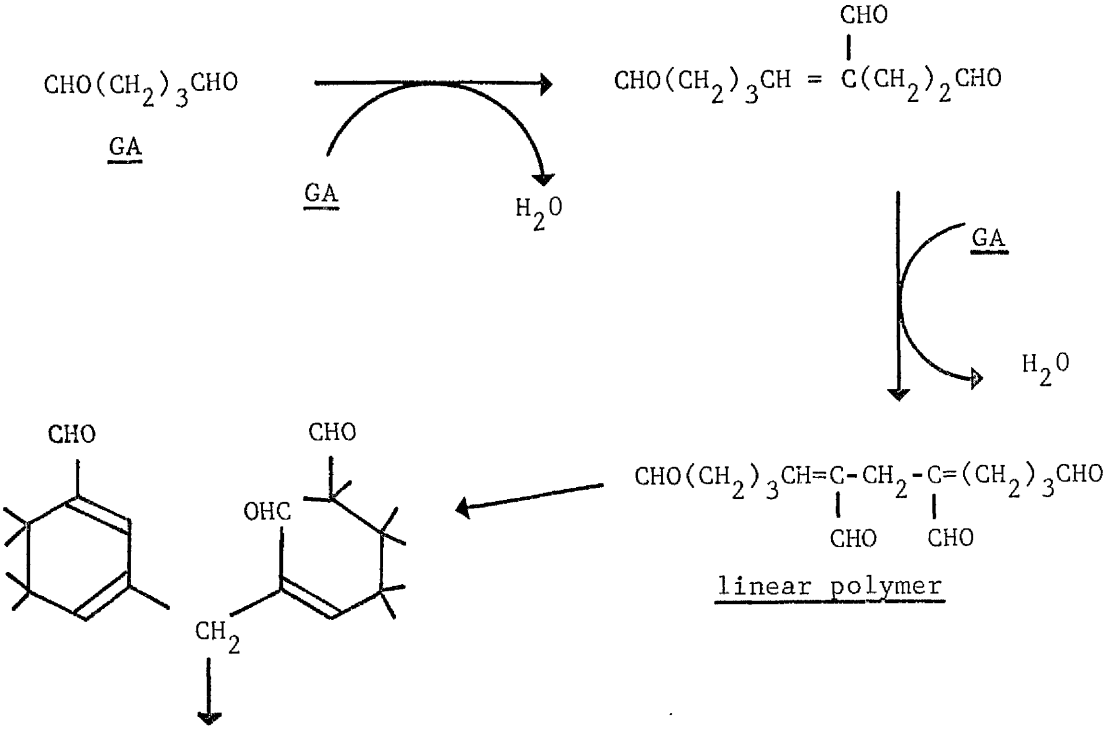
1-fluoro-2,4-dinitrobenzene

... [equation 10].

and (iii) glutaraldehyde (Relyveld and Ben-Efraim, 1983).

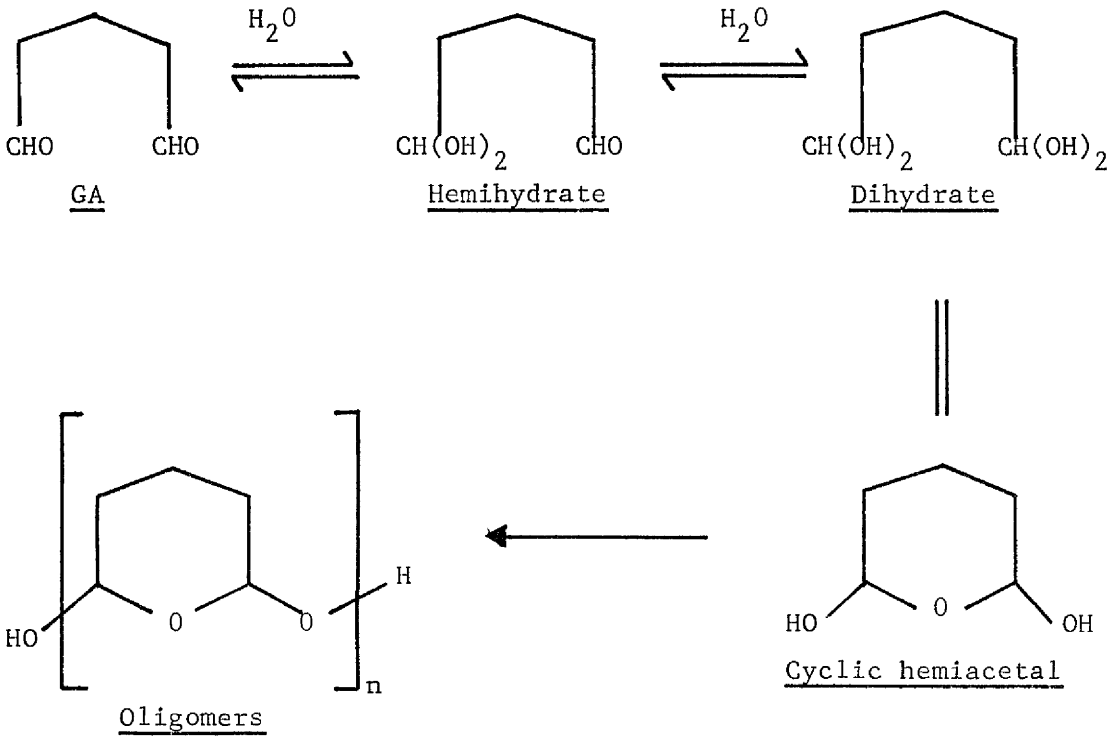
Glutaraldehyde (abbreviated to GA) has been employed as a substitute for formaldehyde for toxoiding cholera toxin (Rappaport et al.,1974), diphtheria and tetanus toxins (reviewed by Relyveld and Ben-Efraim, 1983) and pertussis toxin (Table 2). In fact, attempts were made to copolymerize diphtheria and tetanus toxins for incorporation into vaccines (Relyveld and Ben-Efraim, 1983).

The reaction of GA with proteins and the resulting products were dependent on knowledge of the chemical properties of the reagent. Richards and Knowles (1968) observed that the existence of aldol-condensed polyglutaraldehyde, either in a linear or cyclic form, was predominant in aqueous solutions of GA [equation 11].



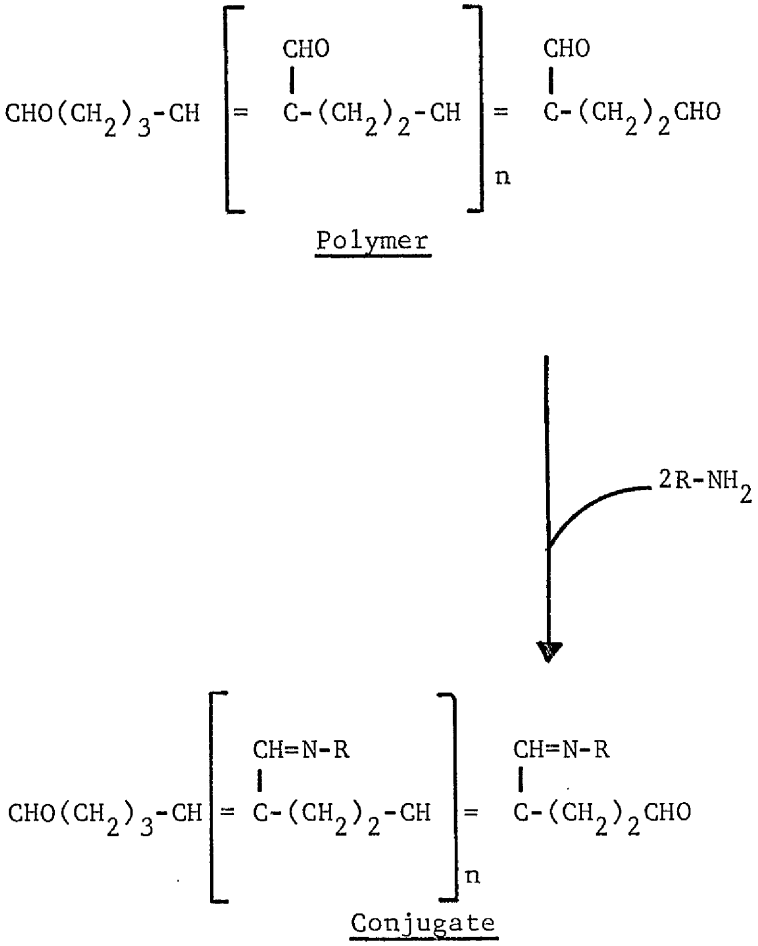
... [equation 11].

They postulated that the polymers arranged with amines by an addition reaction which gave stable products, resistant particularly to acid hydrolysis. However, aqueous solutions of GA used for the chemical modification and stabilization of proteins, consisted of free GA, the cyclic hemiacetal of its hydrate and oligomers of this in equilibrium with each other (Korn et al., 1972; equation 12).



... [equation 12].

Whipple and Ruta (1974) attributed a minimal role to the oligomers postulated by Korn et al. (1972) and a predominant role for the cyclic hemiacetal which existed in equilibrium with the free aldehyde. Monsan et al. (1975) confirmed the results of Korn et al. (1972) and Whipple and Ruta (1974) and, contrary to Richards and Knowles (1968), found no preponderance of the aldol-condensed polyglutaraldehyde but unstable hydrates which liberated GA. Only an increase in pH led to aldol condensation, and GA did not react with protein amino groups when in its free form but as an unsaturated polymer, giving imino bonds stabilized by conjugation [equation 13].



... [equation 13].

Tomimatsu et al. (1971) reported that only ϵ-amino groups of lysyl residues reacted with GA, and in the relative amounts of 4 moles of GA to one mole of lysine (Korn et al.,1972). Cheung and Nimni (1982a) concluded that a process of polymerization was induced by the initial reaction of GA with amines, and that the GA-polymer-amine complex was self-limiting in size and could undergo internal rearrangement to become chemically inert. The complexity of the reaction therefore reflected a condensation-polymerization by GA which occurred concomittantly with the production of Schiff base-derived products (Fig. 4). Hardy et al. (1976), in studying the interaction of GA with 6-aminohexanoic acid and α-N-acetyllysine, postulated the conversion of lysine side-chain amino groups into quaternary pyridinium groups as the major type of cross-link (Fig. 4).

Explanation of Figure 4

Compound A was a Schiff base formed between GA and an amine.

Compound B was the α,β-unsaturated (conjugated) Schiff base.

Compound C was the subsequent addition product of Compound B.

Compound D was derived from the reaction between Compound B and another amine.

Compounds E and F represented the GA-polymer adducts or cross-links which were also derived from Compound B via Schiff base-catalyzed polymerization.

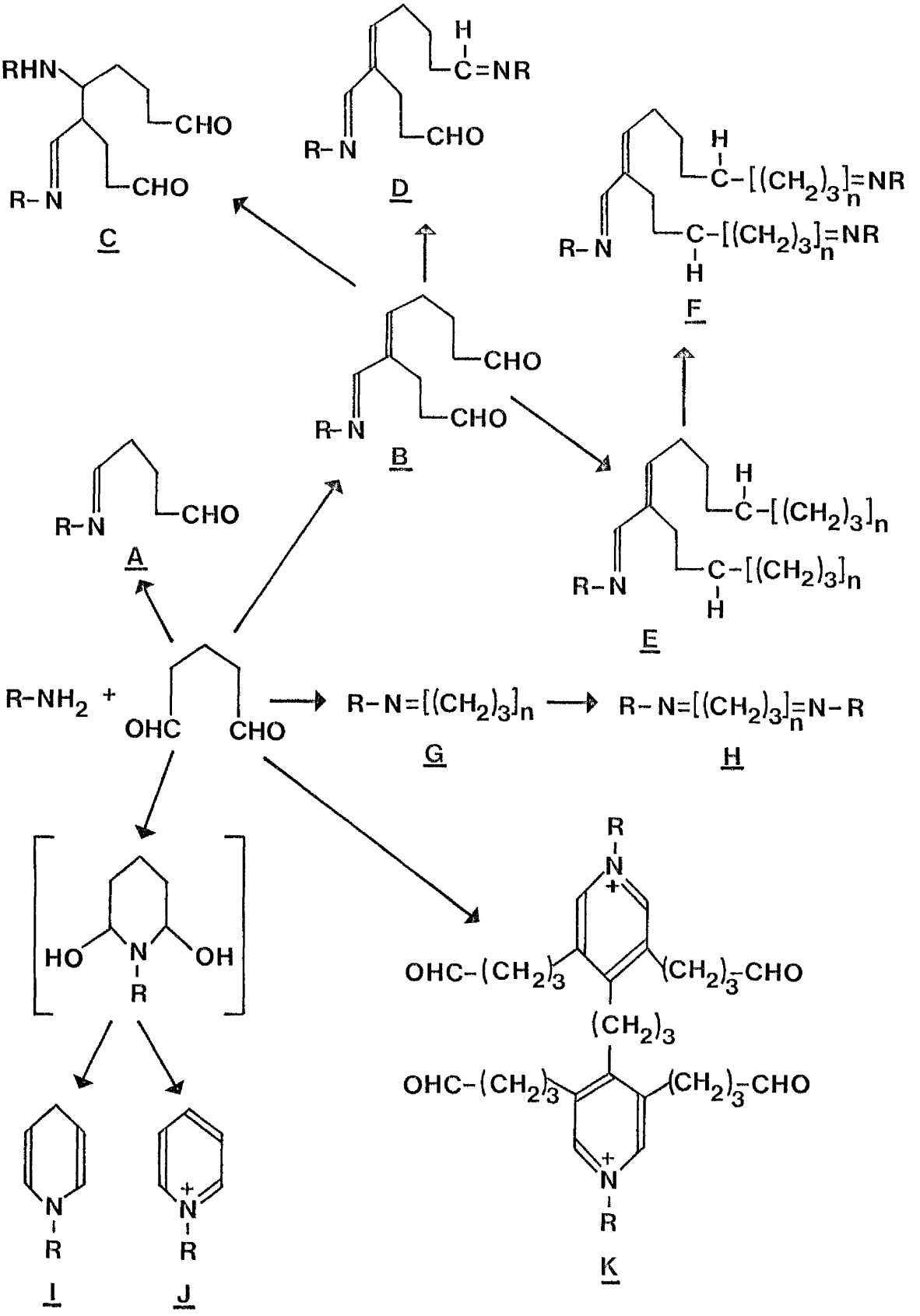
Compounds G and H may be structures similar to E and F except through some other unknown reaction mechanisms.

Compounds I and J were possible dead-end dihydropyridine or dihydropyridinium products which followed the ring closure of an intermediate.

Compound K was the cross-link proposed by Hardy et al.(1976).

Only structures C, D, F and G represent actual cross-links formed between peptide chains.

Figure 4. Schematic representation of possible reactions of ϵ-NH_2 groups of peptide bound lysyl residues with glutaraldehyde (after Cheung and Nimni, 1982a)



Habeeb and Hiramoto (1968) found that GA reacted with the α-amino groups of amino acids, the N-terminal amino groups of some peptides and the sulphydryl group of cysteine. The phenolic and imidazole rings of tyrosine and histidine were partially reactive. Also, with proteins such as BSA, human gamma globulin and ovalbumin, GA reacted predominantly with lysine amino groups to form mainly intermolecular cross-linkages. When dilute concentrations of collagen were reacted with low concentrations of GA, intramolecular cross-links were formed, and as the concentration of the reagent was increased, intermolecular cross-links were formed as collagen became more insoluble (Cheung and Nimni, 1982b). Glutaraldehyde was also capable of cross-linking BSA and BSA to ovalbumin, both of which were antigenic in rabbits (Habeeb, 1969).

The efficiency of GA as a toxoiding agent has been attributed to the stability of the reaction products. Crude cholera toxoids prepared with GA were non-toxic and antigenic in rabbits, whereas purified toxoids lost their capacity to stimulate antibody production (Saletti and Ricci, 1974). Pavlovskis et al. (1981) found that a GA-toxoid of Ps. aeruginosa exotoxin A was not as efficacious as a formaldehyde-toxoid in protecting mice against experimental bacterial infection. Walker et al. (1979) also demonstrated no significant protection with GA-toxoid against Pseudomonas infections.

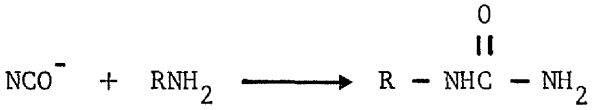
Vaccines prepared by the inactivation of tetanus and diphtheria toxins with formaldehyde have proved to be safe and effective immunogens in humans, and immunization with these two toxoids is almost universal in every developed country. The effectiveness of diphtheria toxoid in eradicating disease was reviewed by Pappenheimer (1984) and the perform-

ance of tetanus toxoid by Bizzini (1984).

6.2 Modification of bacterial exotoxins with other reagents

Other reagents have been used for toxoiding some bacterial exotoxins:

1. Carbamylation of lysyl residues with potassium cyanate [equation 14] completely destroyed the toxicity of tetanus toxin (Robinson et al., 1975).

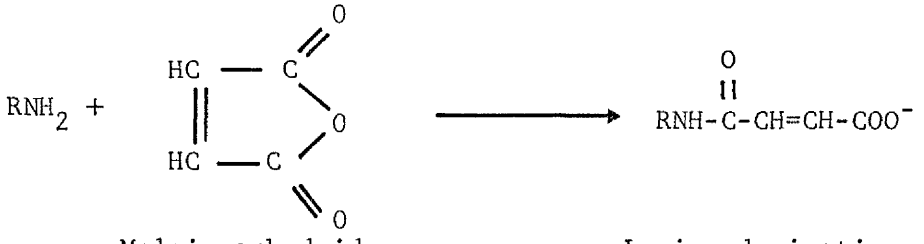


Cyanate Derivative

... [equation 14].

Although atoxic, this derivative failed to stimulate an antibody response in rabbits.

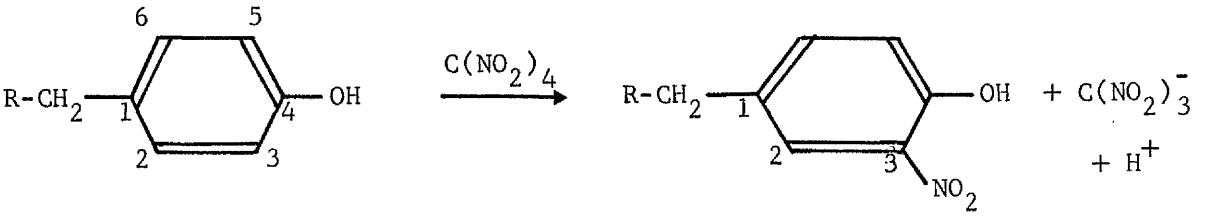
2. Bizzini et al. (1973a) reported that reaction of the lysyl residues in tetanus toxin with maleic anhydride [equation 15] produced an atoxic, non-immunogenic derivative.



Maleic anhydride Lysine-derivative

... [equation 15].

3. Bizzini et al. (1973b) modified the tyrosyl residues of tetanus toxin by highly selective nitration with tetranitromethane [equation 16].

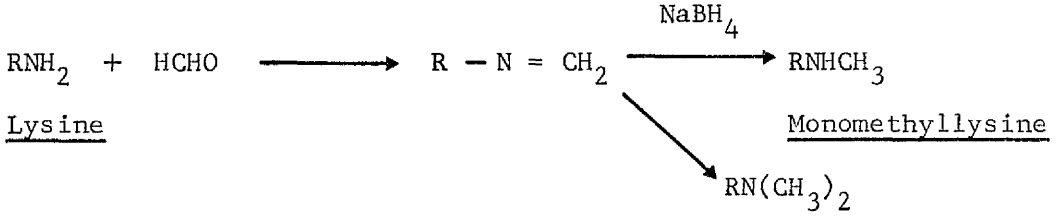


R-Tyrosine 3-Nitrotyrosine

... [equation 16].

The properties of the product of this reaction were dependent on the extent of nitration. When only a few nitro groups were introduced, the molecule was rendered atoxic but remained antigenic. With sufficient nitration, ie: the introduction of more than 33 nitro groups per molecule, antigenicity was compromized.

4. Robinson et al.(1975) used reductive methylation of tetanus toxin to yield a high degree of conversion of lysine to mono- and dimethyl-lysine [equation 17].



Lysine Monomethyllysine

Dimethyllysine

... [equation 17].

The toxicity, albeit greatly reduced, was never completely eliminated, thus negating an examination of the antigenicity of the toxoid.

5. Beugnier and Zanen (1977) also methylated diphtheria toxin to produce a toxoid with reduced ADP-ribosyltransferase activity. Interestingly, both reductive methylation or nitration modified only the

A-fragment, leaving the membrane-binding activity of the B-fragment intact.

6. Matuhasi *et al.* (1981) and Mitani *et al.* (1982) detoxified tetanus toxin by modification and conjugation with pullulan (a linear copolymer of maltotriose) with cyanuric acid. The conjugate was a good antigen for IgG production in mice. These authors considered that when the toxin was bound to pullulan it could not manifest its toxicity, since the binding site of the toxin to a cell surface receptor was stereochemically hindered.

7. A new approach to the detoxification of *Ps. aeruginosa* exotoxin A by Callahan *et al.* (1984) involved the inactivation of the enzymatic region by the specific process of photo-affinity labelling. The enzymatic activity and toxicity were irreversibly inactivated by the specific covalent binding of a substrate analogue to the enzymatically-active site. However, the physiochemical and antigenic properties of the toxoids were similar to those of native toxin.

SECTION 7. CARBODIIMIDES IN BIOLOGICAL CHEMISTRY

The carbodiimides are one of the several classes of unsaturated compounds (the heterocumulenes) based on the allene structure $-\overset{|}{C}=C=\overset{|}{C}-$, and their general formula is $R-N = C = N-R'$, where R and R' are either aliphatic or aromatic groups. General use of this group of reagents was generated by Khorana's pioneering investigations (1953) of their action in peptide and nucleotide synthesis. At the time of the second comprehensive review (Kurzer and Douraghi-Zadeh, 1967) the only major use of carbodiimides was in peptide and nucleotide coupling reactions. Since then, the synthesis and chemical reactivity of carbodiimides have been extensively studied. These general properties can be found in the excellent reviews of Khorana (1953), Kurzer and Douraghi-Zadeh (1967) and Williams and Ibrahim (1981).

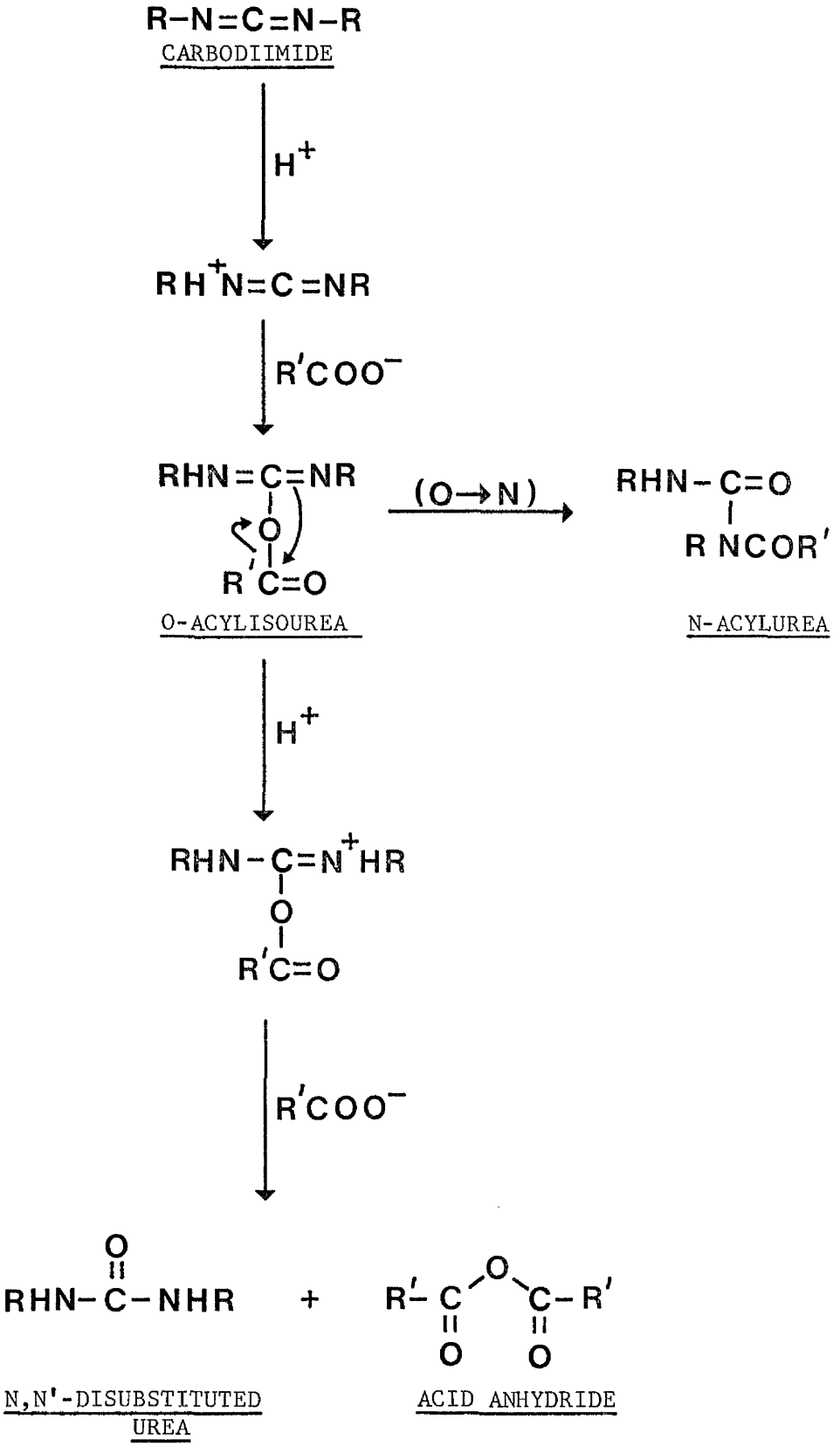
7.1 Carbodiimide-modification of carboxyl groups in proteins

The reaction of carboxylic acids with carbodiimides is a very important precursor to the synthesis of peptide links, as well as being central to the modification of reactive groups in proteins. The reaction is believed to be initiated by the protonation of the carbodiimide, the cation next being attacked by the acid anion to form the O-acylisourea (Hoare and Koshland, 1967; Fig. 5). This can either,

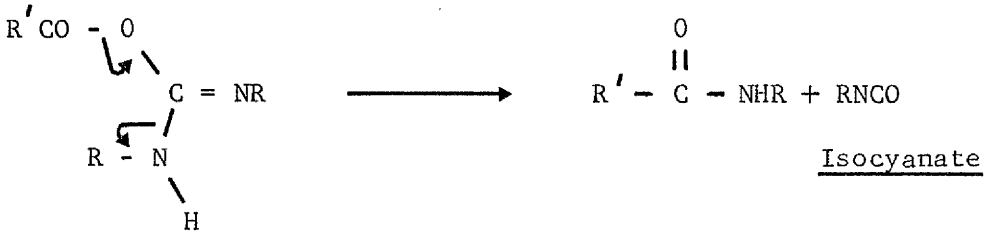
(1) rearrange by way of a cyclic electronic displacement to the stable N-acylurea (Fig. 5), or

(2) be protonated to the cation, which is subsequently converted by attack of a second anion into the N,N'-disubstituted urea and the acid anhydride (Fig. 5).

Figure 5. Mechanism of carboxyl group modification by carbodiimide



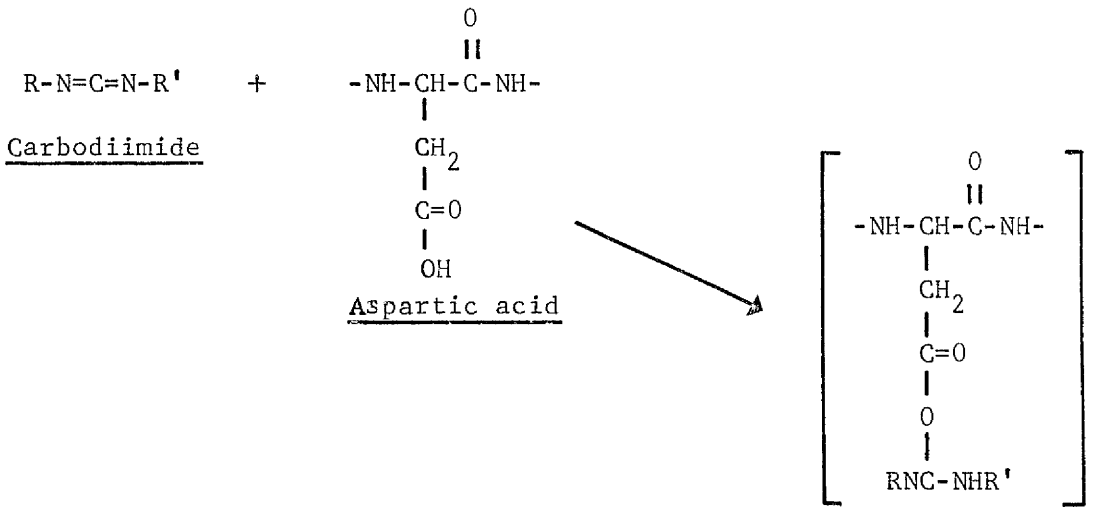
However, the products of the reaction depended on the nature of the carbodiimide, the acid, the solvent and temperature (Khorana, 1953). In general, carboxyl groups were unreactive with carbodiimide derivatives at alkaline pH (Riehm and Scherega, 1966; Hoare and Koshland, 1967; Addy et al.,1973). A further complication in the sequence was the formation of an isocyanate from the O-acylisourea (Alexandre and Rouessac, 1970), probably via the mechanism shown in [equation 18].



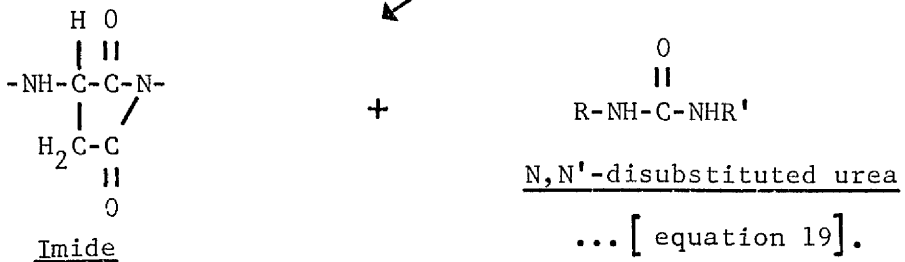
O-acylisourea — Isocyanate

... [equation 18].

Also, imide formation could result from rearrangement of the unstable O-acylisourea (Riehm and Scherega, 1966; equation 19),



Carbodiimide — Aspartic acid — O-acylisourea



Imide — N,N'-disubstituted urea

... [equation 19].

and Franzblau et al.(1963) also reported ring formation with some compounds.

Carboxyl groups could be modified using a carbodiimide and an exogenous nucleophile such as an amine. For example, glycine ethyl ester or N-(2,4-dinitrophenyl)diaminoethane were coupled to carboxyl groups in rabbit muscle phosphorylase (Ariki and Fukui, 1978). The carboxyl groups of lysozyme were modified with glycine (Hayashi et al.,1972) and sulphanilic acid (Kramer and Rupley, 1973). The action of yeast hexokinase with nitrotyrosylethylester and carbodiimide gave an inactive protein when only two carboxyl groups had been modified (Pho et al.,1974). Exposed aspartyl and glutamyl residues in proteins were converted to asparaginyl and glutaminyl residues with carbodiimide and ammonia (Lewis and Shafer, 1973, equation 20).

$$R'CH_2COOH + RNCNR \xrightarrow{NH_3} R'CH_2CONH_2$$

Aspartyl, glutamyl — Modified residue

... [equation 20].

Another modification was the reaction of yeast enolase carboxyl groups with water-soluble carbodiimides (George and Borders, 1979). The observation was made that non-inhibitors or non-substrates such as phosphate or glucose-6-phosphate protected the enzyme against inactivation; these species - in effect the phosphate moiety - were assumed to react with the carbodiimide in competition with the protein. Active-site carboxyl groups were also protected by substrates or inhibitors at saturating concentrations (Lin and Koshland, 1969; Eyl and Inagami, 1970; Riensch and Dunlap, 1980). In most cases, substrate or substrate analogues often protected the enzyme against carbodiimide modification, either by enzyme-substrate binding, thus preventing access of the carbodiimide, or direct competition from the substrate for the carbodiimide.

Examples of other modifications at essential carboxyl groups with carbodiimides are given in Table 4.

Apart from the modification of protein carboxyl groups with carbodiimide and an exogenous nucleophile, the reagents could introduce both inter- and intramolecular cross-links. For example, Lötscher et al.(1984a) found that one of the three β-subunits of E. coli ATPase F_1 complex was cross-linked to the ε-subunit by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC); and that another carbodiimide, dicyclohexylcarbodiimide (DCCD), inhibited ATPase activity of F_1 by covalent modification of a single glutamic acid residue in the β-subunit (Lötscher and Capaldi, 1984). Lötscher et al.(1984b) also found that EDAC promoted a cross-link between the C-terminal carboxyl group (Ala-79) in subunit C of the Fo portion of the enzyme and a near-neighbour phosphatidylethanolamine; this resulted in an inhibition of F_1-Fo ATPase activity which was postulated as due to conformational constraint imposed by cross-linking. Pedemonte and Kaplan (1985) found that EDAC inhibited function of the Na, K-ATPase system isolated from dog kidney, presumably by reaction of the reagent between a carboxyl group and an endogenous nucleophile (a side-chain amine). Therefore, the formation of an intramolecular cross-link resulted in the enzyme being frozen in a non-functional conformation.

In another study, modification of beef-heart cytochrome C oxidase with EDAC or 1-ethyl-3-(3-trimethylaminopropyl)carbodiimide significantly inhibited reaction of the enzyme with its substrate, cytochrome C (Millett et al., 1982). The loss in activity was accompanied by modification of one (or more) partially buried carboxyl groups on subunit II of the enzyme to form a positively charged N-acylurea which inhibited cytochrome C binding. In the presence of cytochrome C, EDAC promoted the formation of amide cross-links between lysine amino groups on cytochrome C and

Table 4. Modification of protein carboxyl groups with carbodiimides

Reference	Protein	Nucleophile	Modifying Reagent
Riehm and Scheraga (1966)	Ribonuclease	-	CMC
Hoare and Koshland (1967)	Insulin	glycine methyl ester	BDC
Armstrong and McKenzie (1967)	β-lactoglobin A	-	CMC
Carraway et al.(1969)	chymotrypsin/ chymotrypsinogen	glycine methyl ester	EDAC
Fersht and Sperling (1973)	"	glycinamide/ semicarbazide	EDAC
Frater (1971)	bovine serum albumin	glycine methyl ester	EDAC
Roufogalis and Wickson (1973)	acetyl cholinesterase	glycinamide	EDAC ; DCCD
Kaminogawa et al.(1973)	casein	glycine methyl ester	EMC
Gorodetskii et al.(1976)	Acid proteinase from Aspergillus awamori	-	DCCD
Terra et al.(1979)	Insect midgut trehalase	glycine methyl ester	EDAC
Reinsch and Dunlap (1980)	Dihydrofolate reductase	-	EDAC
Kozik (1982)	Riboflavin-binding protein	glucosamine	EDAC ; CMC
Delfino et al.(1983)	Bovine growth hormone	glycine methyl ester	EDAC
el Kebbaj et al.(1984)	β-hydroxybutyrate dehydrogenase	glycine ethyl ester	EDAC ; DCCD
Takata et al.(1985)	S-adenosylhomo-cysteinase	-	CMC
Lönnroth and Holmgren (1975)	cholera toxin	glycine methyl ester	EDAC

CMC: 1-cyclohexyl-3-(2-morpholinyl (4)-ethyl)carbodiimide-metho-p-toluene sulphonate.

BDC: 1-benzyl-(3-dimethylaminopropyl)carbodiimide.

EDAC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

DCCD: dicyclohexylcarbodiimide.

EMC: 1-ethyl-3-(3-[4-morpholinyl] propyl)carbodiimide.

their complementary carboxyl groups on cytochrome C oxidase. EDAC also promoted the formation of intermolecular amide cross-links between the carboxyl groups of cytochrome C and the lysine amino groups of chloroplastid plastocyanin (Geren et al.,1983). The salt bridge between Lysine-13 (ϵ-NH_3^+) and Leucine-129 (α-COO^-) in lysozyme was converted to an amide bond by EDAC in the presence of imidazole. Absence of imidazole under similar conditions did not give this intramolecularly cross-linked lysozyme derivative, but resulted in the formation of intermolecularly cross-linked lysozyme oligomers (Yamada et al.,1983). The study of Craik and Reithmeier (1984), on carbodiimide-inhibited phosphate transport in human erythrocytes, also showed that the limiting step for inhibition was not attack by the carbodiimide on carboxylic acid groups, but the rearrangement of the resulting O-acylisourea to N-acylurea, or to intramolecular or intermolecular attack by amino groups (nucleophiles) leading to protein cross-linking. Weare and Reichert (1979a,b) found that increasing the concentration of EDAC increased the extent of covalent cross-linking between α- and β-subunits of bovine lutropin, with a concomitant decrease in the receptor-binding activity of the conjugated protein.

When lysozyme or α-chymotrypsin were treated with the water-soluble carbodiimide 1-ethyl-3-(3-morpholinopropyl)carbodiimide, both underwent intermolecular cross-linking to form thermostable proteins (Hattori,1970). A similar stability was induced in blood haemoglobin by cross-linking with EDAC (Labrude et al.,1979).

Carbodiimides have been used in a few instances for the modification of bacterial toxins, ie: cholera toxin (Lönnroth and Holmgren, 1975; Table 4) and especially the enterotoxins of E. coli. Klipstein et al.(1981) obtained encouraging results by immunizing rats

with E. coli heat-stable enterotoxin (ST) conjugated with EDAC to porcine IgG: these animals were protected against challenge with semipurified or purified ST and viable organisms of multiple heterologous serotypes. Based on these observations, Klipstein et al. (1982) prepared a bivalent toxoid by cross-linking heat-stable (ST) and heat-labile (LT) enterotoxins with EDAC. The resultant toxoid retained less than 0.15% of the biologically-active toxigenicity of the parent ST and LT components. Rats immunized with this toxoid were significantly protected against challenge with either the LT or ST toxin, or with viable heterologous strains producing these toxins either singly or together. This indicated that conjugation of ST to LT resulted in a unique new immunogen in that ST acquired immunogenicity as a function of the reaction, LT retained most of its antigenicity and the toxicity of both components was greatly reduced. Klipstein et al.(1982b) also found that an EDAC-LT-toxoid stimulated specific serum IgG and mucosal IgA antitoxin titres in mice.

More recently, these workers conjugated synthetically-produced ST to the non-toxic B subunit of LT with EDAC. Toxicity of the ST component was reduced by greater than 600-fold. The vaccine stimulated significant serum IgG and mucosal sIgA antibodies to each component, and provided significant protection against all types of enterotoxigenic E. coli (Klipstein et al., 1983a,b,c). However, Moon et al. (1983) immunized pregnant sows with the EDAC-mediated conjugate of ST and bovine immunoglobulin G of Gianella et al. (1981), and found that piglets suckled on immunized mothers had almost identical rates for diarrhoea as piglets suckled on unimmunized sows. However, there was some suggestion of a decrease in mortality from challenge with enterotoxigenic E. coli in piglets feeding from vaccinated sows.

In a separate study, glutaraldehyde replaced EDAC in the preparation of these conjugate toxoids (Klipstein et al.,1984). However, the dose-dependent mucosal secretory IgA antitoxin responses to vaccines cross-linked by either reagent were identical. In a further study (Houghten et al.,1985), the immunodeterminant region of the LT B subunit was identified by determining the antigenicity of synthetically produced peptides corresponding to various segments of its 124 amino acid sequence. This immunodeterminant region - the 26 amino acids of B subunit, sequence 58 to 83 - was chemically linked, using EDAC, to the synthetic ST toxin (consisting of only 18 amino acids). This conjugate was completely non-toxic in several in vitro and in vivo assays, antigenic in stimulating intestinal mucosal secretory IgA antitoxin titres to both components, and immunogenic in protecting rats against challenge with enterotoxigenic E. coli (Houghten et al.,1985; Klipstein, 1985).

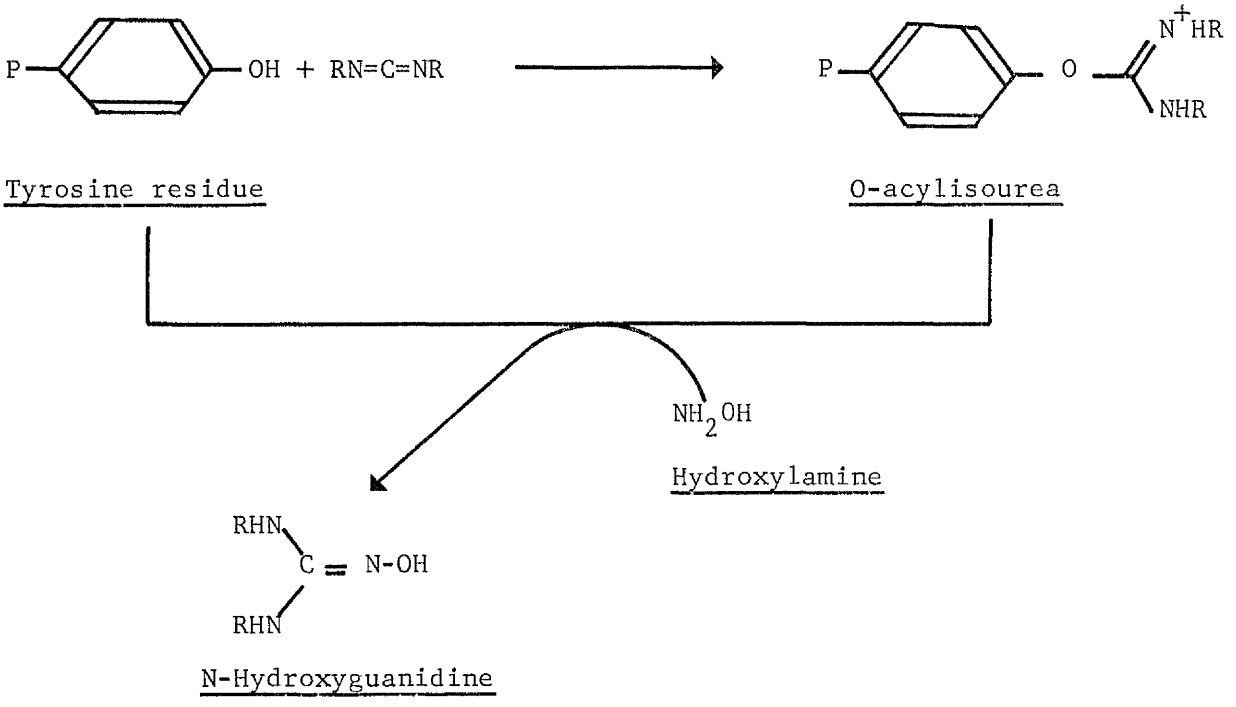
7.2 Other carbodiimide-modified protein residues

Apart from reaction with carboxyl groups and the subsequent rearrangements, carbodiimides were shown to modify tyrosine residues (Carraway and Koshland, 1968), sulphydryl residues (Carraway and Triplett, 1970), to mediate sulphation of hydroxy-amino acids, peptides and proteins (Pongor et al.,1985) and also to react with phosphate groups (Reinsch and Dunlap, 1980). α-chymotrypsin was inactivated with a water-soluble carbodiimide which reacted with the active-site serine (residue 195) to yield an O-acylisourea derivative (Banks et al.,1969).

Protein thiol groups also reacted with water-soluble carbodiimides; papain was modified at residue cysteine-25 (Perfetti et al., 1976) and the free thiol group of buckwheat α-glucosidase was partially reactive (Kanaya et al.,1979). In this latter case, introduction of a

bulky group by reaction of the sulphydryl moiety with carbodiimide was postulated to have caused a steric hindrance, or a conformational change, resulting in a reduction in enzyme activity.

The accessible tyrosine residue of yeast hexokinase was modified at pH 8.0 using EDAC, and the resulting inactivation was reversed by hydroxylamine, which presumably formed the N-hydroxyguanidine (Grouselle and Pudles, 1977; equation 21).



... [equation 21].

Also, the reaction of carbodiimides with histidine and lysine at high pH yielded substituted guanidines (Takata et al.,1985).

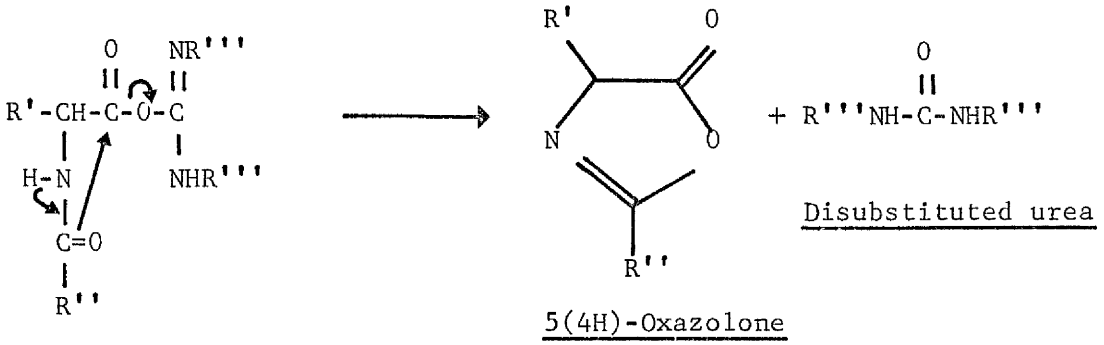
7.3 Use of carbodiimides in the preparation of immunizing conjugates

Peptide synthesis continues to utilize the carbodiimide "method", which was introduced by Sheehan and Hess (1955) with the use of DCCD to affect dehydration and peptide bond formation. The procedure of adding carbodiimide to a solution of N-protected amino acid and amino acid or peptide ester won wide acceptance because of its simplicity,

speed and compatability with water.

The carbodiimide "method" is represented by the flow sequence in Fig. 6. There appears to be essentially two competing reactions, ie: the O⟶N acyl shift to form the N-acylurea, and the reaction of the labile O-acylisourea with an amino group to give the peptide link. Alternatively, the acid anhydride, resulting from reaction of the O-acylisourea with a second carboxylate, may react with an amino group to regenerate the first carboxyl group and yield a peptide link.

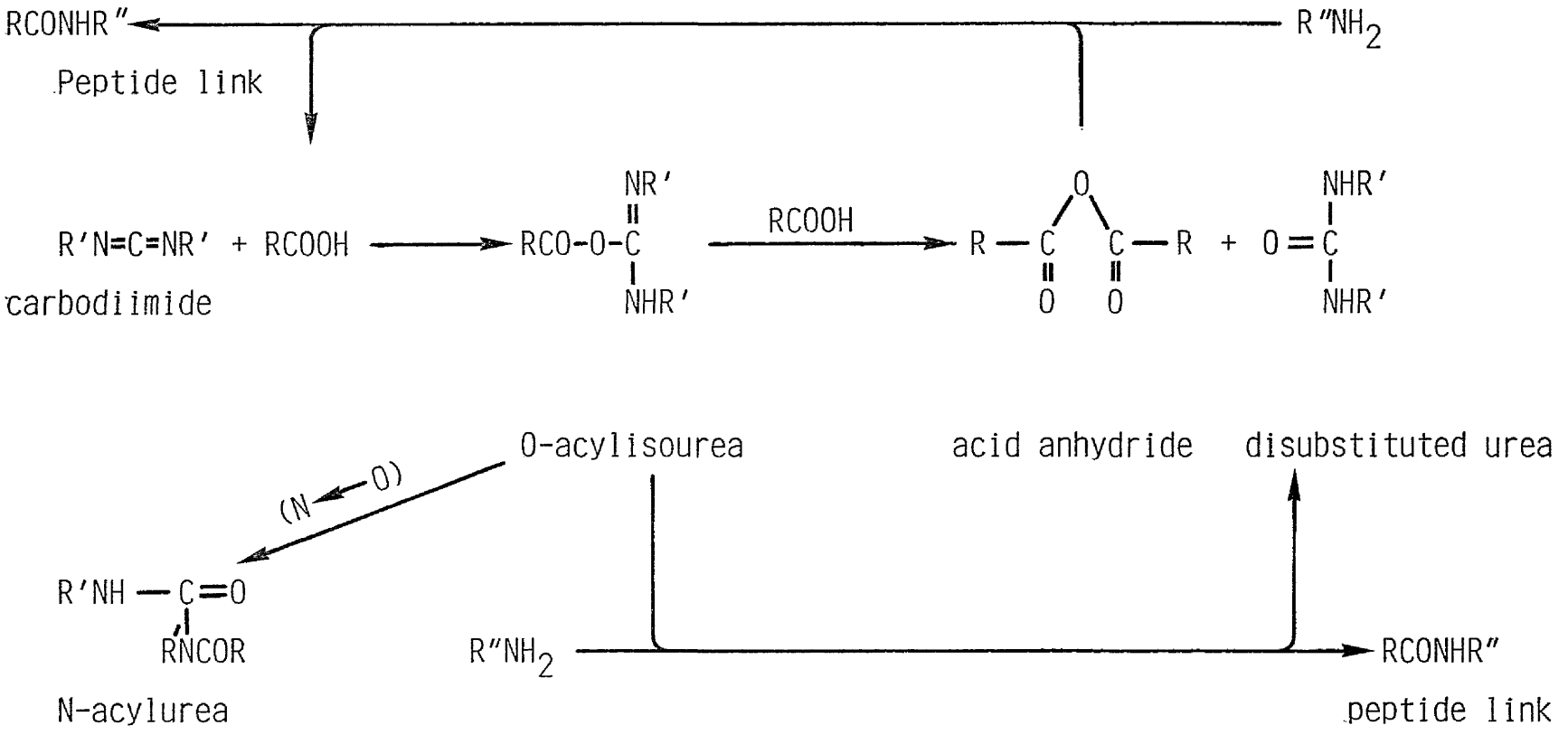
However, aminolysis can be accompanied by competing reactions from other nucleophiles proximate to the O-acylisourea, eg: intramolecular amide groups giving rise to 5(4H)-oxazolones [equation 22],

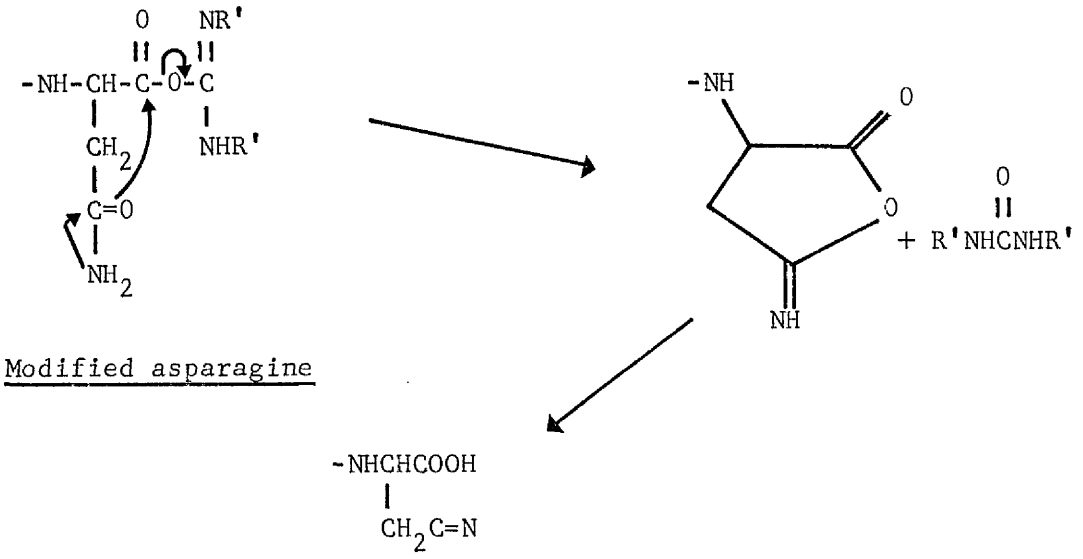


... [equation 22].

secondary carboxylates forming acid anhydrides (Fig. 6), and also activation of glutamine or asparagine carboxyl groups may cause partial dehydration to form the nitrile (Rich and Singh, 1979; equation 23).

Figure 6. CHEMISTRY OF CARBODIIMIDE MODIFICATION





... [equation 23].

Therefore, reaction conditions that favoured intermolecular nucleophilic attack on the O-acylisourea led to fewer side reactions and to cleaner products, because 5(4H)-oxazolone and N-acylurea formation were suppressed.

The N-acylureas were the main side-products at elevated temperatures, and, in order to shift the reaction towards peptide bond formation, temperatures around $0^{\circ}C$ were recommended (Bauminger and Wilchek, 1980). Alternatively, the formation of N-acylurea was significantly depressed by the use of asymmetrical carbodiimides (Ito et al., 1977). Presence of an amino group, either exogenous or endogenous during the reaction, also reduced the formation of side-products. Additives such as N-hydroxysuccinimide or 1-hydroxybenzotriazole were highly effective in suppressing side-reactions in peptide synthesis (Rich and Singh, 1979).

In the field of immunology, the main use of carbodiimides has been in the conjugation of weakly immunogenic or non-immunogenic compounds to larger carrier proteins or to synthetic antigens. The

conjugation of two compounds by this method requires the presence of an amino group and a carboxyl group. In some cases, the amino groups involved in the reaction were lysyl residues of the protein carriers (Goodfriend et al.,1964; McGuire et al.,1965; Koch et al.,1973; Bauminger et al.,1974) or lysyl and alanyl residues of synthetic polypeptide carriers (Arnon and Sela, 1969; Bauminger et al.,1969). The carboxyl groups were often contributed by the hapten: if they were absent, they were introduced into the molecule using a variety of chemical procedures (Bauminger and Wilchek, 1980).

The specificity of the antibodies produced to the hapten was affected mainly by the site of attachment of the hapten to the protein carrier. Due to the stable nature of the N-acylurea-carbodiimide-adducts of carboxylic acid residues of the carrier protein or hapten, immunization with carbodiimide-coupled hapten-carrier conjugates allowed for the production of antibody not only to both hapten and carrier, but possibly also to the carbodiimide-adducts of either (Goodfriend et al.,1964). Davis et al.(1984) showed that when EDAC was used as a coupling agent, antibody was produced to N-acylurea-EDAC-carrier, the anbtibody being directed primarily to the 3-dimethylaminopropyl end of the reagent. Therefore, the carbodiimide introduced a new antigenic determinant.

Antibodies to naturally occurring compounds were produced by attaching them to proteins that stimulated an antigenic response (Erlanger, 1980). For example, protein-heparin complexes, prepared with an EDAC coupling technique, were used to produce anti-heparin antibodies in rabbits (Gitel et al.,1985). Antisera which recognized carbodiimide-treated heparin, but not untreated heparin, were obtained: this indicated the formation of new epitopes as a result of conformational

changes due to either inter- or intramolecular cross-linking. Examples of other carbodiimide-mediated conjugations are listed in Table 5.

EDAC has been used to conjugate the synthetic adjuvant muramyl dipeptide (MDP) - the minimum adjuvant-active structure that substituted for Mycobacteria in FCA - to a synthetic polypeptide carrier. This potentiated both the immunostimulatory and pyrogenic activities of the adjuvant (Chedid et al.,1979). Reichert et al.(1980) described the EDAC-mediated synthesis of MDP-protein conjugates which stimulated an immune response to the MDP-hapten.

The alkaline-detoxified lipopolysaccharide from Pseudomonas aeruginosa was rendered antigenic by coupling to several carrier proteins including tetanus toxoid (Seid and Sadoff, 1981). Coupling of LPS was promoted by the formation of active esters with carboxyl groups of KDO residues, in the presence of EDAC and hydroxylamine; such activated products could then react with the amino groups of protein carriers (Fig. 7).

Water-soluble carbodiimides have been used to couple nucleotides directly to proteins, presumably by formation of N-P bonds as the principle type of linkage (Halloran and Parker, 1966a). The results of immunological studies indicated that nucleotide-, oligonucleotide- and DNA-protein conjugates induced the formation of antibodies with nucleotide specificity (Halloran and Parker, 1966b).

Table 5. Use of carbodiimides in the preparation of immunological conjugates

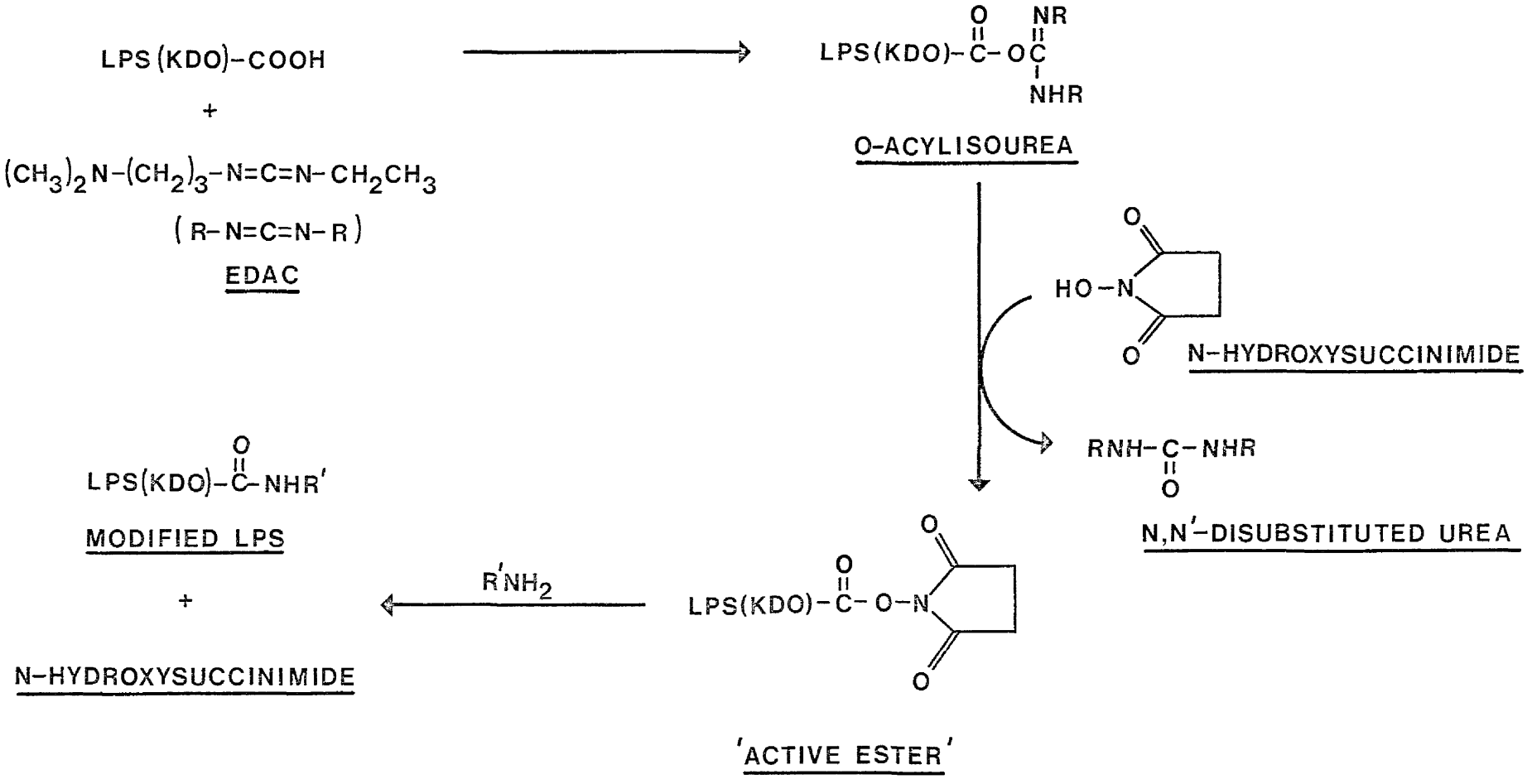
Reference	Hapten	Protein carrier	Conjugating Reagent
Goodfriend et al.(1964,1966)	Bradykinin/angiotensin	RSA	EDAC;CMC
McGuire et al.(1965)	α-MSH/ACTH	RSA	EDAC
Arnon and Sela (1969)	lysozyme fragment	synthetic polypeptide	EDAC
Steiner et al.(1969)	Succinyl cAMP	HSA	EDAC
Bauminger et al.(1969)	Genistein	Synthetic polypeptide	EDAC
Fuchs and Fuchs (1969)	Indole acetic acid/ gibberellic acid	BSA	DCCD
Levine and van Vunakis (1970)	Prostaglandins	Polylysine	EDAC
van Vunakis et al.(1971)	D-lysergic acid	Polylysine	EDAC
Lewis et al.(1972)	Gentamicin	BSA, HSA, KLH, PT*	EDAC
van Vunakis et al.(1972)	Morphine	Polylysine	EDAC
Bauminger et al.(1973)	Prostaglandin E_2	BSA	DCCD
Koch et al.(1973)	Gonadotropin-releasing hormone	BSA	EDAC
Bauminger et al.(1974)	Gonadol steroids	BSA, thyroglobulin	EDAC
Arnon et al.(1976)	Carcinoembryonic antigen (cancer tissue protein)	Polyalanine	EDAC
Kaul et al.(1976)	Cocaine metabolite	Sheep gamma globulin	EDAC
Kopp et al.(1977)	α-melanotropin	HSA	EDAC
Kohen et al.(1979)	Progesterone	BSA	not identified
Shirouzu et al.(1983)	Thyrotropin-releasing hormone	BSA	not identified
Beuvery et al.(1983)	*N. meningitidis* group C polysaccharide	Tetanus toxoid	EDAC
Clough et al.(1985)	Synthetic peptide representing the immunodominant epitope of *Plasmodium knowlesi*	Tetanus toxoid	EDAC
Petrov et al.(1985)	Isolated influenza virus antigens	Polyelectrolyte	CMC
Jacob et al.(1986)	Synthetic cholera toxin B-subunit peptide sequences	Tetanus toxoid	EDAC

BSA: Bovine serum albumin. RSA: rabbit serum albumin. HSA: horse serum albumin.

α-MSH: α-melanocyte stimulating hormone. ACTH: adrenocorticotrophic hormone.

KLH: keyhole limpet haemocyanin. *PT: porcine thyroglobulin.

Figure 7. Modification of LPS with EDAC and N-hydroxysuccinimide

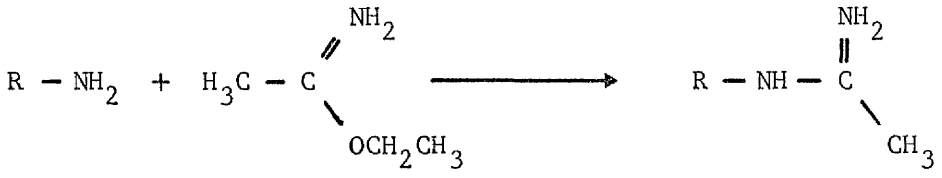


SECTION 8. TOXOIDING OF PERTUSSIS TOXIN

The component now designated as PT has been detoxified most frequently with the 'classic' reagent formaldehyde. Matsui and Kuwajima (1959) used formalin to detoxify the histamine-sensitizing factor present in culture fluid. Munoz and Hestekin (1966) found that histamine-sensitizing activity of a *B. pertussis* saline extract decreased in the presence of formalin at 37°C at a faster rate than mouse-protective activity. They postulated that since the HSF and protective activities were actually part of the same molecule, the action of formaldehyde was similar to the detoxification of other bacterial exotoxins, with the formation of immunologically-active toxoids.

The purification of PT to homogeneity led to more studies on the production and biological properties of toxoids. The properties of pertussis antigen-toxoids, prepared with formaldehyde or glutaraldehyde, will be mentioned in more detail in the discussion section of this thesis.

In the study of Nogimori *et al.*(1984a), the lysine residues of PT were modified using ethyl acetimidate [equation 24].

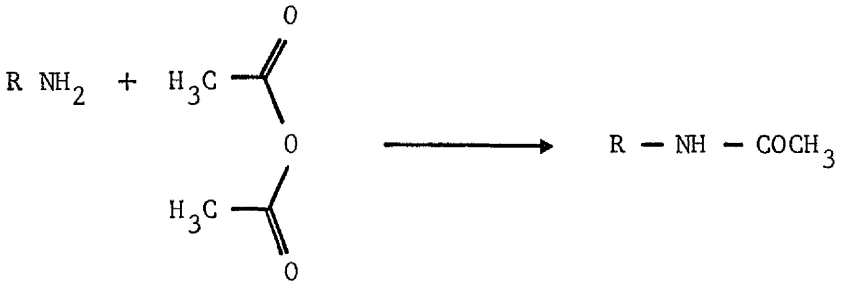


PT-lysine Ethyl acetimidate PT-derivative

... [equation 24].

The interesting outcome from this was a separation of the biological properties of the toxin into those not significantly affected by acetamidination, and those markedly suppressed by acetamidination of intrapeptide lysine residues.

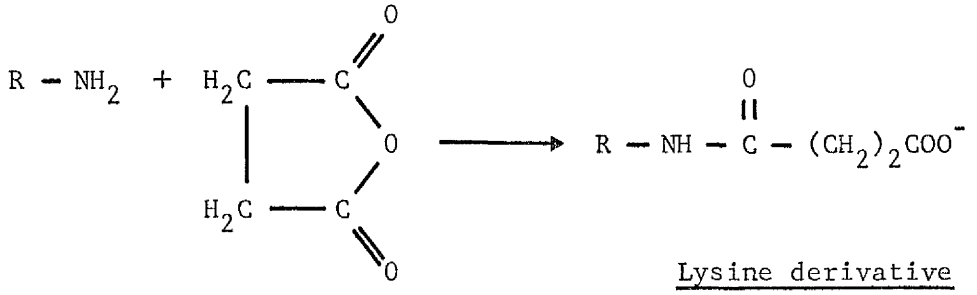
Pertussis toxin was also treated with acetic anhydride [equation 25],



Lysine residue — Acetic anhydride — Lysine derivative

... [equation 25].

maleic anhydride [equation 15], and succinic anhydride (Nogimori et al., 1984b; equation 26).



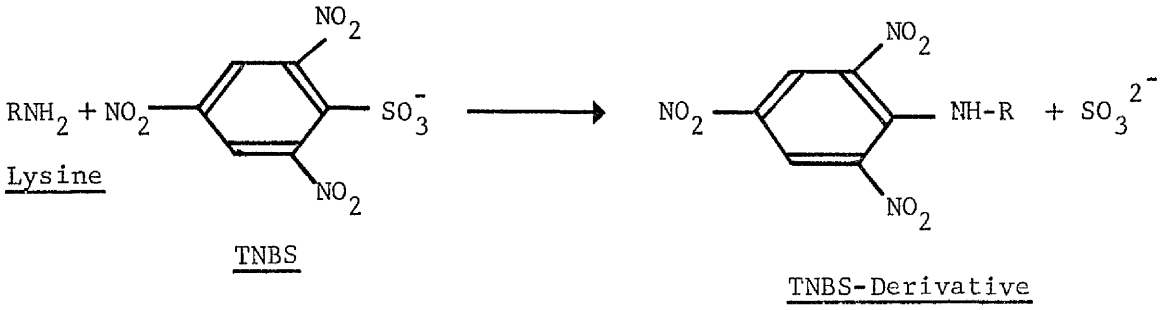
Lysine residue — Succinic anhydride — Lysine derivative

... [equation 26].

These modified proteins exhibited no biological activity in vivo because of destruction of the quaternary structure, when more than 90% of the free amino groups were acylated.

In fact, reductive methylation with formaldehyde, in the presence of a reducing agent, was preferred to acetamidination or acylation because it proceeded under milder reaction conditions. The methylated toxin exhibited the same biological properties as acetamidinated toxin (Ui et al., 1985).

Fish et al.(1984) modified PT with trinitrobenzenesulphonic acid (TNBS) - an ϵ-amine modifying reagent [equation 27] - or potassium iodide (iodination).



... [equation 27].

The mouse-protective activities of the modified proteins of Fish et al.(1984) or the acetamidinated, acylated or methylated derivatives mentioned above were not reported.

OBJECT OF RESEARCH

The primary objective of this research is to develop a toxoided pertussis antigen preparation with satisfactory immunogenicity which can be considered as a replacement for conventional whole-cell pertussis vaccine.

The aims are to

(1) culture *Bordetella pertussis* to obtain optimum yields of extracellular pertussis toxin and filamentous haemagglutinin in a suitable antigen:antigen ratio,

(2) extract these antigens using a simple procedure,

(3) determine the optimum conditions of toxoiding with a carbodiimide which render the antigen preparation non-toxic. A carbodiimide was chosen from a variety of chemical reagents, including formaldehyde and glutaraldehyde, which were initially considered for toxoiding studies.

Thereafter, the objective is to demonstrate the low toxicity, stability, antigenicity and immunogenicity of the experimental acellular vaccine in several *in vivo* and *in vitro* assays.

MATERIALS AND METHODS

SECTION 1. STRAINS, MEDIA AND CULTURE CONDITIONS

1.1 Strains

Three phase I strains and one mutant strain of *B. pertussis* were used.

B. pertussis strain 18334 was a phase I vaccine strain obtained originally from the Connaught Laboratories Ltd., Toronto, Canada.

B. pertussis strain 77/18319 was a phase I fresh isolate kindly provided by Dr. F. Fallon, Ruchill Hospital, Glasgow.

B. pertussis strain NCTC 10739 (18323) was a phase I organism, highly virulent in the ICMPT, and kindly provided by Dr. F. Sheffield of the National Institute for Biological Standards and Controls (NIBSC), Holly Hill, Hampstead, London, NW3 6RB.

The mutant organism was *B. pertussis* strain 357, which was deficient in the production of PT (Weiss *et al.*,1984), and was kindly provided by Dr. A.A. Weiss, University of Virginia School of Medicine, Charlottesville, Virginia, USA.

All the strains were kept frozen at -70°C in Casamino acids (1% w/v) containing glycerol (10% v/v), (Appendix 1).

1.2 Media

The following were used for the cultivation of *B. pertussis*.

Bordet Gengou (BG) agar. The agar base and defibrinated horse blood (final concentration, 17% v/v) were obtained from Gibco Bio-cult Diagnostics Ltd., Paisley, Scotland.

A Stainer and Scholte liquid medium modified from the original (Stainer and Scholte , 1971) and designated SS-X.

The cyclodextrin liquid (CL) medium of Imaizumi *et al.*(1983) containing methylated-β-cyclodextrin (MeβCD).

SS-X medium plus MeβCD (1.0g L^{-1}).MeβCD (heptakis (2,6-O-dimethyl)β-cyclodextrin) was a gift from Dr. Shimizu, Medical and Pharmaceuticals Division, Teijin Ltd., Chiyoda-ku Tokyo, Japan.

The compositions and preparation of these media are given in Appendix 1.

1.3 Growth of cultures from frozen suspensions

Cultures maintained at -70°C were thawed at room temperature and grown on BG agar. The plates were incubated in a humidified plastic box at 37°C for 48h. The organisms were then subcultured onto fresh BG agar and incubated for a further 48h at 37°C.

Thawed suspensions of the challenge strain, B. pertussis 18323, were plated onto BG agar and incubated at 37°C for 24h. These were then heavily inoculated onto fresh BG agar plates and incubated for a further 24h at 37°C.

1.4 Growth of batch cultures

(i) Static cultures

The solid bacterial growth from BG agar was removed with a sterile loop and transferred into 1.0L Fernbach and/or Carrell flasks, each containing 500 ml SS-X medium. These were incubated statically for 5 days at 37°C, the cultures being swirled once each day.

(ii) Shaken cultures

The solid bacterial growth from BG agar plates was transferred directly into 2.0L Erlenmeyer flasks, each containing either 1.0L of SS-X medium, with or without MeβCD, or 1.0L of the CL medium. These were incubated for 36-48h at 37°C on a rotary shaker (L.H. Engineering Co. Ltd., Bell's Hill, Stokes Poges, Bucks., England) set at 60-100 rpm.

(iii) Fermenter Growth

Growth of a B. pertussis batch culture was attempted in a Type LHE 1/1000 laboratory fermenter (L.H. Engineering Co. Ltd., Bell's Hill, Stokes Poges, Bucks., England). The fermenter vessel was filled with 15 litres of SS-X medium: this, along with the attendant tubing, air filters and inoculum flask, was autoclaved for 1h at 121°C, 103.4kPa, to ensure thorough heat penetration for complete sterilization.

An overnight shake-flask culture of B. pertussis 77/18319 in 500 ml of SS-X medium, was checked for purity by Gram staining and used to inoculate the fermenter vessel. The culture was initially stirred overnight at 450 rpm and then for only 1 min a day for the next four days. Static growth conditions were necessary for efficient production of FHa in SS-X medium. The vessel was continuously aerated by a steady stream of sterile air, maintained at 37°C, and was not subject to pH monitoring or antifoam additions. After incubation, thimerosal (0.01% (w/v) final concentration: Sigma Chemical Co., Fancy Road, Poole, Dorset) was added before removal of the culture from the fermenter vessel.

All batch cultures of B. pertussis were checked for purity by Gram staining and the bacterial cells removed by centrifugation, twice at 10,000 rpm (13,300 x g) for 30 min at 4°C. Culture fluid was stored at 4°C with thimerosal (0.01% (w/v) final concentration) acting as a preservative. The cell pellet was stored either at -20°C or processed to make a vaccine.

(iv) Culture fluid fractionation

Culture fluid was fractionated using a Hollow Fibre dialysis/ concentration system, model DC2 (Amicon Corporation, Danvers, Ma., USA), a self-contained unit with reservoir, adjustable pump, withdrawal ports,

prefilter and interchangeable Diaflo Hollow Fibre cartridges (Amicon Corporation, USA).

Culture fluid was circulated through a HIP100-20 cartridge (mol.wt cut off 100,000) and separated into a concentrated retentate and a filtrate. The filtrate was circulated through a HIP10-20 cartridge (mol.wt cut off 10,000) to give a concentrate and a filtrate. This filtrate was discarded and both retentates (10,000 mol.wt and 100,000 mol.wt) stored at -20°C with thimerosal (0.01% (w/v) final concentration).

SECTION 2. PREPARATION OF A STANDARD B. PERTUSSIS WHOLE-CELL VACCINE (WCV)

B. pertussis strain 18334 was used for the production of a standard whole-cell vaccine. From frozen stocks, cultures of the organism were grown on BG agar as previously described (p. 82) and their purity checked by Gram staining. Shaken cultures of the organism in SS-X medium were produced as described (p. 82), checked for purity and harvested. Culture was poured into 500 ml screw-cap centrifuge bottles (which had been steam-sterilized for 30 min) and the bacterial cells removed by centrifugation at 10,000 rpm (13,300 x g) for 30 min at 4°C. The cell pellet was resuspended in sterile Sorensen's phosphate buffer, pH 7.2 (Appendix 2) and the centrifugation repeated. A bacterial cell suspension was finally made in sterile Sorensen's phosphate buffer and the concentration adjusted to 100 opacity units (ou). This was done by comparison with the International Reference Preparation of Opacity from the World Health Organization International Laboratory for Biological Standards (NIBSC, Holly Hill, Hampstead, London). This standard was defined as 10 ou, and consisted of a plastic rod mounted in a 6" x $^{5}/_{8}$" test tube. The standard and vaccine suspension were matched by viewing

a printed card through both tubes, and the suspension diluted until its opacity equalled that of the opacity rod (Perkins et al.,1973). The dilution factor used to obtain matching of the bacterial vaccine suspension with the reference was calculated and multiplied by 10, to give the concentration of the undiluted suspension in ou. This was adjusted to 100 ou accordingly.

A sample of the final suspension was removed for estimation of protein content (p. 99) and the remainder heated for 30 min in a water bath at 56°C, to destroy heat-labile toxin activity. Thimerosal (0.01% (w/v) final concentration) was added as a preservative and sterility checked by plating 0.1 ml aliquots of the vaccine onto nutrient agar and BG agar plates. The vaccine was then tested for toxicity and histamine-sensitizing activity in mice as described (p. 87).

Glass ampoules (Jencons (Scientific) Ltd., Bedfordshire, England) containing vaccine (2 ml) were shell-frozen in a dry ice-acetone mixture and lyophilized in a Multi-Dry Freeze-Dryer (FTS Systems Inc., Stone Ridge, NY, USA) at -50°C under vacuum. The ampoules were heat-sealed and the vacuum checked with a 'spark' gun.

After lyophilization, the contents of several ampoules were resuspended in sterile PBS, pH 7.4, and tested for histamine-sensitizing activity and sterility. The ampoules were stored at 4°C.

SECTION 3. EXTRACTION OF PT AND FHA FROM CULTURE FLUID

A mixture of PT and FHa was prepared by dye-ligand affinity chromatography. The pH of culture fluid was adjusted to 6.0 with 2.5N HCl and Blue Sepharose CL-6B (Pharmacia, Uppsala, Sweden), 10 ml packed

gel L^{-1}, added. (Blue Sepharose CL-6B consisted of the dye Cibacron Blue F3G-A covalently attached to the agarose gel Sepharose CL-6B.) The suspension was gently stirred overnight at $4^{o}C$. The gel was collected by passing the suspension through a G1 sintered glass filter (Gallenkamp, Loughborough, Leicestershire, England) connected to a vacuum flask. It was packed into a column (3 cm x 34 cm, LKB-Produkter AB, Stockholm, Sweden) and washed with 0.05M Tris-HCl buffer, pH 8.0. After washing, total haemagglutinating activity was eluted with 0.05M Tris-HCl buffer, pH 8.0 containing 1.0M NaCl. Column fractions of 10 ml were collected with an Ultrorac Fraction Collector (LKB-Produkter AB, Stockholm, Sweden) and the elution profile monitored with a Uvicord II-UV absorptiometer (LKB-Produkter AB, Stockholm, Sweden). The protein content of column fractions was also monitored by recording the absorbance at $\lambda\ 280_{nm}$ in a Pye Unicam SP6-550 UV/VIS spectrophotometer, using 1.0 cm quartz cells.

After elution, the gel was regenerated by washing with 0.1M Tris-HCl buffer, pH 8.0 containing 0.5M NaCl, 6M urea, to remove any strongly bound material, followed by alternate washing cycles of high and low pH, ie: with 10 column volumes of 0.1M Tris-HCl buffer, pH 8.5 containing 0.5M NaCl and 10 column volumes of 0.1M sodium acetate buffer, pH 4.5 containing 0.5M NaCl. The gel was finally washed with 0.05M Tris-HCl buffer, pH 8.0 and stored at $4^{o}C$ with thimerosal (0.01% (w/v) final concentration) as preservative.

The procedures for washing, elution and regeneration of the gel were done at room temperature, with a buffer flow rate of 80 ml $cm^{-2}h^{-1}$ maintained by a peristaltic pump (LKB-Produkter AB, Stockholm, Sweden).

Column fractions containing haemagglutinating activity (p. 98) were pooled and stored at $-20^{o}C$ until needed. If necessary, the eluate

pool was concentrated by placing in dialysis tubing (Medicell International, Liverpool Rd., London) and covering the sealed tubing with Calbiochem Aquacide II-A (mol.wt 500,000; Behring Diagnostics, La Jolla, California, USA). After a 5-10 fold concentration, the Aquacide was removed from the outside of the dialysis tubing, the tubing washed thoroughly with distilled water and the contents emptied. Alternatively, the eluate pool was concentrated by pressure dialysis on a 43 mm YM30 Diaflo ultrafiltration membrane (Amicon Corporation, USA) in a stirred ultrafiltration cell, model 52 (Amicon Corporation, USA).

Samples from each eluate pool produced were removed for analysis by SDS-PAGE (p. 100), and for estimation of protein (p. 99), PT and FHa (p.96-97).

SECTION 4. ANIMALS AND ANIMAL PROCEDURES

4.1 Mice

Male mice were from a randomly-bred, closed colony originally derived from the HaM/ICR strain (Charles River (UK) Ltd., Manston Rd., Margate, Kent). They were kept in a thermostatically-controlled room at 22-23°C with a 12h light-dark cycle, and allowed free access to food and water.

4.2 Histamine-sensitizing activity (HSA)

Mice of 7-8 weeks of age were injected intraperitoneally (ip), in groups of 5, with 0.5ml volumes of graded doses of test samples in sterile PBS. Injections were administered in 2 ml plastic syringes (Becton, Dickinson and Co. Ltd., Ireland) with 26 gauge, $^{5}/_{8}$" needles (Becton, Dickinson and Co. Ltd., Ireland). Five days later, each mouse

was injected ip with 3 mg of histamine dihydrochloride (Sigma) in 0.5 ml of PBS, pH 7.4. The survivors were counted 2-4h after challenge with histamine. The standard whole-cell vaccine (WCV) was titrated in each experiment as a reference preparation.

The results of the assay were expressed as the HSD_{50} value - the dose/mouse which sensitized 50% of the mice to the lethal effect of a challenge of histamine - with 95% confidence limits, as calculated by the probit method (Wardlaw, 1985).

4.3 Leucocytosis-promoting activity (LPA)

Groups of five mice of 3-4 weeks of age were injected either intravenously (iv) via the tail vein with 0.2 ml, or ip with 0.5 ml volumes of graded doses of test samples in sterile PBS. Control mice were injected with sterile PBS only. Five days after injection, mice were anaesthetized (in a glass jar fitted with an ether-soaked pad in the lid) and bled from the retro-orbital plexus with heparinized capillary tubes (Harshaw Chemicals Ltd., Daventry, Northants.,England). The blood sample (40 µl) was immediately transferred with a Coulter pipette to plastic vials containing 20 ml Isoton II diluent (Coulter Electronics Ltd., Harpenden, Herts., England) to give a final blood concentration of 1 in 501 (vol:vol).

Immediately before counting, 6 drops of Zap-Oglobin (Coulter Electronics Ltd., England) were added to each vial to lyse the red blood cells. The leucocytes were counted in a Coulter Counter, model FN (Coulter Electronics Ltd., England) with attenuation set at 0.707, aperture at 8.0 and threshold at 14.0. To correct for coincidence losses during counting, counts above 10,000 WBC/mm^3 were adjusted with a coincidence correction chart and background counts from diluent alone were then subtracted.

The geometric mean and 95% confidence limits (Wardlaw, 1985) were calculated from the corrected leucocyte counts obtained from each group of mice injected with a test sample.

4.4 Toxicity as determined by a mouse-weight-gain test (MWGT)

Groups of 5 mice of 3-4 weeks of age and approximately the same weight, were injected ip with 0.5 ml volumes of graded doses of test samples in sterile PBS, pH 7.4. Control mice were injected with the diluent alone. Mice were weighed just before injection and at the same time each day for the next seven days, and deaths recorded.

4.5 Intracerebral mouse-protection test (ICMPT)

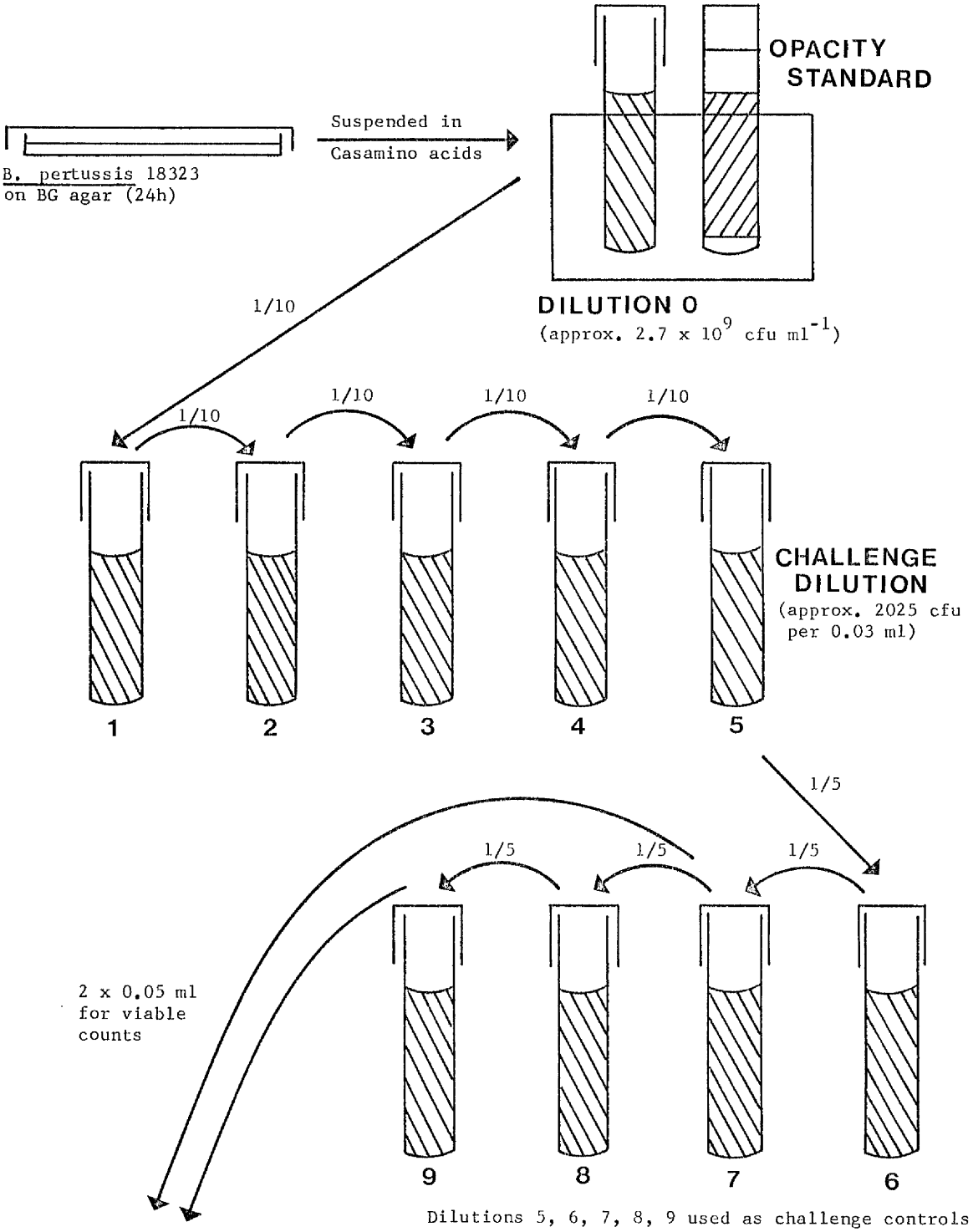
(i) Challenge suspension

The growth from 24h BG agar plates of B. pertussis 18323 was suspended in Casamino acids (Appendix 1) and matched against the International Reference Preparation of Opacity. The matched 10 ou suspension (approximately 2.7×10^9 colony forming units (cfu) ml^{-1}) was diluted as shown in Fig. 8. The challenge suspension contained approximately 6.75×10^4 cfu ml^{-1} and each mouse received 0.03 ml or approximately 2025 cfu. The viable count of each challenge suspension was determined by spreading 0.05 ml of suitable dilutions on each of two BG agar plates which were incubated at $37^{o}C$ for 3-4 days.

(ii) Intracerebral administration of challenge

Randomized groups of 10 mice of 3-4 weeks of age were immunized ip with 0.5 ml volumes of graded doses of test preparations in sterile PBS. After 14 days, the mice were anaesthetized and challenged intracerebrally with 0.03 ml of challenge suspension containing approximately 2025 cfu of B. pertussis 18323. The challenge was given at right angles to the skull, through the parietal bone and using a 1.0 ml syringe (Becton, Dickinson and Co. Ltd., Ireland) with a 26 gauge, $^3/_8$"

Figure 8. Preparation of challenge suspension of B. pertussis for mouse-protection tests



needle (Becton, Dickinson and Co. Ltd., Ireland). The virulence of the challenge culture was determined by injecting groups of unimmunized mice with 0.03 ml/mouse of dilutions 5, 6, 7, 8 or 9 respectively. Surviving mice were counted daily for fourteen days and those dying within 3 days of challenge were excluded from the results. Deaths in the latter case were considered to be due to mechanical injury to cerebral tissue.

In some cases, moribund mice were killed by cervical dislocation and samples of brain tissue plated onto BG agar in order to demonstrate that the infection was due to B. pertussis.

4.6 Intranasal infection

(i) Challenge suspension

A 10 ou suspension of B. pertussis 18323 was prepared as described (p. 89) and suitable 10-fold dilutions made in Casamino acids (Appendix 1). The challenge suspension (dilution 2) contained approximately 2.7×10^7 cfu ml^{-1}. The viable count of each challenge suspension was determined by making further 10-fold dilutions in Casamino acids and spreading 0.05 ml on each of two BG plates, and incubating at $37^{\circ}C$ until the colonies were visible.

(ii) Intranasal administration of challenge

Randomized groups of 10 mice of 3-4 weeks of age were immunized ip with 0.5 ml volumes of graded doses of test preparations in sterile PBS. After 14 days, the mice were anaesthetized with ether until unconscious and breathing deeply at a regular rate. The challenge was administered by placing two drops of 0.025 ml volume (approximately 1.4×10^6 cfu) from a dropping pipette onto the nares. The drops were usually inhaled rapidly, but if breathing was irregular the mice created nasal aerosols or swallowed the suspension.

The mice were weighed every other day after infection and any deaths recorded. Blood samples were also taken from the retro-orbital plexus during the infection for estimation of leucocytosis (p. 88).

(iii) Autopsy

Individual mice were killed by cervical dislocation and pinned out on a dissection board. Using scissors and forceps sterilized by dipping in alcohol and flaming, the thorax was opened and the lungs excised. These were homogenized in 10 ml sterile Casamino acids and dilutions from this spread as 0.1 ml on each of two BG agar plates. After incubation at 37°C for 4-5 days, the plates were checked for any bacterial growth. Any organisms on the plates were checked by Gram staining and slide agglutination with anti-*B. pertussis* antiserum.

4.7 Raising of antisera

(i) Immunization schedule

Groups of 5 or 10 mice of 3-4 weeks of age, were immunized ip with 0.5 ml volumes of test preparations in sterile PBS. The mice were kept for 21 days and on each of the first 7 days body weights were recorded as a matter of routine. On the twenty-first day post-immunization, the mice were killed and blood collected as described below.

(ii) Raising of a standard antiserum, RA

A reference antiserum for the enzyme-linked immunosorbent assays for anti-PT IgG and anti-FHa IgG antibodies, was raised against a carbodiimide-toxoided antigen preparation ((AP-17) T33) according to the immunization schedule of Evans and Perkins (1953). A group of 50 mice of 3-4 weeks of age was injected ip with the toxoid using a two-dose immunization schedule. Each immunization with 20 μg of toxoid/mouse was separated by an interval of 14 days, and mice were bled 10 days after the second dose. Sera were separated as described below and pooled.

The anti-PT IgG and anti-FHa IgG antibody responses of the pooled reference antiserum, RA, were determined by ELISA (p.97-98).

(iii) Preparation of sera

Mice were anaesthetized individually in an ether jar, then secured to a dissection board and kept under anaesthesia (with the aid of a small beaker, containing cotton wool soaked in ether, held over the animal's head). A 2 ml syringe with a 26 gauge, $^{5}/_{8}$" needle was used to withdraw blood by heart puncture through the rib cage. Alternatively, mice were anaesthetized and blood collected from the trunk after cervical dislocation followed by decapitation. The volume of blood collected by either procedure was usually 1.0 to 1.5 ml/mouse.

Individual samples were stored overnight at 4°C in plastic micro-centrifuge tubes (Treff AG, Degersheim, Switzerland) to allow clotting. Serum was separated by rapid centrifugation (Wifug Lab Centrifuges, Bradford, England) and individual samples were placed in fresh micro-centrifuge tubes and stored at -20°C.

4.8 Endotoxin assay in mice

Assay of endotoxin by the measurement of fluctuation of mouse rectal temperature was based on the method of Prashker and Wardlaw (1971) and Wardlaw et al.(1971). The preparation of pyrogen-free saline (PFS) and glassware is given in Appendix 3. B. pertussis lipopolysaccharide was obtained from List Biological Laboratories Inc., Campbell, California, USA. Mice of 3-4 weeks and 7-8 weeks of age were used: they were kept in a thermostatically-controlled room at 22-23°C under artificial light for the duration of the experiment.

Groups of 5 mice were injected ip with 0.5 ml volumes of graded doses of test samples or B. pertussis LPS as a standard preparation. Dilutions of test samples and the standard preparation were made in sterile PFS, and control mice were injected with 0.5 ml of

sterile PFS alone. Pyrogen-free, 2 ml disposable plastic syringes (Becton, Dickinson and Co. Ltd., Ireland) with 26 gauge, $^{5}/_{8}$" needles were used for the injections. Rectal temperatures were measured at 1.5h and 3h post-injection with an electric thermometer (Grant Instruments, London, England). The probe was lubricated with mineral oil, inserted into the rectum to a depth of 2 cm and temperature read after 1.0 min.

4.9 Assays for hypoglycaemia and hyperinsulinaemia

Groups of 5 mice of 7-8 weeks of age were injected ip with 0.5 ml volumes of graded doses of test preparations in sterile PBS. Control mice were injected with sterile PBS only. Five days after injection, blood was collected by the following method. The mice were individually anaesthetized in an ether jar for exactly 90 seconds, removed and allowed to recover for 30 seconds. Blood was collected from the trunk after cervical dislocation and decapitation, and sera separated as described (p. 93).

(i) Hypoglycaemia

Glucose (mMol L^{-1}) was measured enzymatically by the glucose oxidase method using a Beckman Glu-2 glucose analyzer (Beckman, Palo Alto, California, USA).

(ii) Hyperinsulinaemia

Serum immunoreactive insulin (IRI) levels were measured by the double-antibody method of Hales and Randle (1963) using antibodies and ^{125}I-labelled insulin (Amersham, UK), membrane filters (N25/N45/UP, Oxoid) and a crystalline rat insulin standard (Novo, Denmark). Briefly, the radioimmunoassay depended on the competition between insulin and ^{125}I-labelled insulin for a limited number of binding sites on an antibody raised against insulin. This insulin-specific antibody was combined with a second antibody to give an 'insulin-binding reagent'.

The proportion of the radioactive insulin bound to the 'insulin-binding reagent' varied inversely with the concentration of unlabelled insulin in the serum sample under investigation. Therefore, the ^{125}I-count was inversely proportional to the immunoreactive insulin concentration in the sample, and by constructing a standard curve of counts per minute (cpm) ^{125}I versus rat insulin concentration (ng ml^{-1}), immunoreactive insulin for test samples was calculated by interpolation onto that curve.

SECTION 5. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

5.1 Reagents

Purified PT was obtained from Professor M. Ui, Department of Physiological Chemistry, Hokkaido University, Sapporo, Japan or List Biological Laboratories Inc., California, USA. Fetuin, Tween-20 and O-phenylenediamine (O-PD) were obtained from Sigma Chemical Co., Fancy Rd., Poole, Dorset. Hydrogen peroxide (H_2O_2) and sulphuric acid (H_2SO_4) were obtained from May and Baker Ltd., Dagenham, England, and the anti-mouse IgG-horseradish peroxidase (HRP) conjugate from the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland.

Purified FHa, F_2 IgG anti-FHa capture antibody, F_3 IgG anti-FHa-HRP conjugate and mouse anti-PT monoclonal antibodies L_4, L_5, L_9 and L_{10} were kindly donated by Dr. L.A.E. Ashworth and Dr. A. Robinson, PHLS, Centre for Applied Microbiological Research, Porton Down, Salisbury, Wiltshire, England. Human haptoglobin type 2-2 was kindly supplied by Dr. J.B. Hertz, Department of Clinical Microbiology, Hvidovre Hospital, Copenhagen, Denmark. Rabbit anti-PT polyclonal antibody was a gift from Professor A.C. Wardlaw, Department of Microbiology, University of Glasgow.

5.2 Detection of PT antigen by Fetuin-ELISA

The wells of flat-bottomed, Nunc-Immunoplate 1-96F ELISA plates (A/S Nunc, Kamstrup, Roskilde, Denmark) were each coated with 200 µl of fetuin at 2.0 µg ml^{-1} in 0.05M carbonate buffer, pH 9.6. The plates were kept at 4°C overnight in a humidified box. They were washed three times with PBS, pH 7.4 containing Tween-20 (0.05% v/v) with each wash being left at room temperature for 3 min. After washing, the plates were inverted and tapped vigorously onto absorbent tissue to remove any remaining buffer from the wells. Test samples were serially diluted in PBS-Tween (PBST) and 200 µl added to the wells. As a reference, purified PT was used. The samples and reference toxin were titrated in duplicate. The plates were incubated for 1h at 37°C in a humidified box and washed and dried as described above. To each well was added mouse anti-PT monoclonal antibody L_{10} (200 µl of a 1 in 200 dilution in PBST). The plates were incubated for 1h at 37°C in a humidified box and washed. Anti-mouse IgG-HRP-enzyme conjugate (200 µl of a 1 in 3000 dilution in PBST) was added to each well and the plates incubated for 1h at 37°C and washed as described above.

The enzyme reaction was initiated by the addition to each well of 200 µl of a solution of O-phenylenediamine (34 mg ml^{-1}) and H_2O_2 (20 µl of a 20 vols. solution) in 100 ml of citrate-phosphate buffer, pH 5.0. The reaction occurred for 30 min in the dark at room temperature and was terminated by addition to each well of 50 µl of H_2SO_4 (12.5% v/v). Absorbance was measured at 492 nm in a Titertek Multiscan MC ELISA reader (Flow Laboratories, Hertfordshire, England) using substrate alone as a blank for the colour reaction. A graph of absorbance at 492 nm versus log PT (ng $well^{-1}$) was drawn, and the PT content of the test samples calculated with a parallel line assay by comparison of the absorbance

values for PT reference and test preparations.

5.3 Detection of FHa antigen by ELISA

FHa was detected by modification of the method of Dr. L.A.E. Ashworth (personal communication). The wells of flat-bottomed Nunc-Immunoplates were each coated with 200 µl of F_2 IgG anti-FHa antibody at 1.0 µg ml^{-1} in 0.05M carbonate buffer, pH 9.6. The plates were kept overnight at 4°C and washed as described above. Test samples were serially diluted in PBST and 200 µl added to the wells: purified FHa was used as a reference preparation. The plates were incubated for 2h at 37°C, washed,and 200 µl of F_3 IgG anti-FHa-HRP conjugate (0.5 µg ml^{-1} in PBST) added to each well. After incubation for 2h at 37°C and washing, the enzyme reaction was initiated and terminated as described above and absorbance measured at 492 nm.

A graph of absorbance at 492 nm versus log FHa (ng $well^{-1}$) was drawn, and the FHa content of the test samples calculated with a parallel line assay by comparison of the absorbance values for the FHa test and reference preparations.

5.4 Detection of anti-PT IgG antibodies by Fetuin-ELISA

The wells of Nunc-Immunoplates were each coated with 200 µl of fetuin (2 µg ml^{-1}) in 0.05M carbonate buffer, pH 9.6. The plates were kept overnight at 4°C, washed, and 200 µl of PT (1.0 µg ml^{-1} in PBST) was added to each well. The plates were incubated for 1h at 37°C and washed. Dilutions of test and reference sera were prepared in PBST and 200 µl volumes added to duplicate wells. The plates were incubated for 1h at 37°C, washed, and anti-mouse IgG-HRP conjugate (200 µl of a 1 in 3000 dilution in PBST) added to each well. After incubation for 1h at 37°C and washing, the enzyme reaction was initiated and terminated, and absorbance measured at 492 nm, as described above.

5.5 Detection of anti-FHa IgG antibodies by ELISA

Antibody to FHa was detected by ELISA using FHa as the solid phase. The wells of Nunc-Immunoplates were each coated with 200 µl of FHa (1.0 µg ml^{-1}) in coating buffer. The plates were kept at $4^{o}C$ overnight and were washed with PBST. Dilutions of test and reference sera were prepared in PBST and 200 µl volumes added to duplicate wells. The plates were incubated for 1h at $37^{o}C$ and washed. Anti-mouse IgG-HRP conjugate (200 µl of a 1 in 3000 dilution in PBST) was added to each well and the plates incubated for 1h at $37^{o}C$. After washing, the enzyme reaction was initiated and terminated and absorbance measured at 492 nm as described above.

The antibody activity of test sera, compared to the reference antiserum RA, was expressed in theoretical Antibody Units ml^{-1} mouse serum, AbU ml^{-1} (Appendix 9) or actual antibody titres (Appendix 10).

The composition of buffers used in these assays is given in Appendix 4.

SECTION 6. MISCELLANEOUS PROCEDURES

6.1 Haemagglutination assay

Haemagglutination assays were done in plastic round-bottomed microtitre plates (Sterilin Ltd., Sussex, England) with Titertek micro-droppers and microdiluters (Flow Laboratories, Hertfordshire, England). Horse erythrocytes were washed four times in sterile saline (0.145M NaCl) and the packed cell suspension diluted to give a 0.5% (v/v) suspension, which was stored at $4^{o}C$.

Test samples were serially diluted in saline (50 µl) in the microtitre wells and two drops (50 µl) of the horse erythrocyte suspension added to each well. The control for each sample, titrated in duplicate, was sterile saline to which horse erythrocytes were added. The plates were kept at room temperature for 4-6h or at $4^{o}C$ overnight. The haemagglutination titre was the reciprocal of the highest dilution showing complete agglutination of the erythrocytes.

6.2 Estimation of total protein

Protein was estimated by the method of Herbert, Phipps and Strange (1972), using bovine serum albumin (Sigma) as a standard. The compositions of the reagents used are shown in Appendix 5.

(i) 0.5 ml volumes of distilled water (or buffer) were pipetted into duplicate test tubes as reagent blanks;

(ii) 0.5 ml volumes of samples were dispensed into duplicate test tubes;

(iii) the bovine serum albumin protein was diluted in distilled water (or buffer) to concentrations of 500, 400, 300, 200, 100 and 50 µg ml^{-1}, and 0.5 ml volumes pipetted into duplicate tubes;

(iv) 0.5 ml of 1.0N NaOH was added to each test tube. They were placed in a boiling water bath for 5 min, removed and allowed to cool;

(v) 2.5 ml of Reagent C was added to each test tube and the mixtures incubated at room temperature for 10 min;

(vi) 0.5 ml of Reagent D was added to each tube, the contents immediately mixed and left at room temperature for at least 30 min for full colour to develop;

(vii) absorbance was measured at 750 nm in 1.0 cm plastic cuvettes in a Pye Unicam SP6-550 UV/VIS spectrophotometer. A standard curve of absorbance (750 nm) versus BSA concentration (µg ml^{-1}) was plotted and the protein concentrations determined by interpolation onto the curve.

6.3 Detection of Cibacron Blue F3G-A in Blue Sepharose eluates

A 0.01% (w/v) solution of the dye (Ciba-Geigy, Paisley, Scotland) was made in 0.05M Tris-HCl buffer, pH 8.0 containing 1.0M NaCl, and the absorption spectrum scanned in 2.0 cm silica cuvettes (Pye Unicam, Cambridge, UK) in a Pye Unicam SP8-100 UV spectrophotometer with the dilution buffer as the blank.

The solution was diluted in two-fold steps in 0.05M Tris-HCl buffer, pH 8.0 containing 1.0M NaCl and absorbance measured at λ 291 nm and λ 613 nm (ie: the absorption maxima of the dye). Eluate samples were also assayed at these wavelengths.

Standard curves of absorbance 291 nm and 613 nm versus concentration of dye ($\mu g\ ml^{-1}$) were plotted, and estimations of the content of Cibacron Blue F3G-A in Blue Sepharose eluates determined by interpolation onto the curves.

6.4 SDS-PAGE

SDS-PAGE, ie: polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulphate, was done using a discontinuous buffer system based on the methods of Ames (1974) and Laemmli (1970).

Separating and stacking gels contained 11% and 5% (w/v) acrylamide (BDH Ltd., Poole, England) respectively. Both the gels and buffers contained SDS (0.1% w/v; Sigma). The dimensions of the glass moulds were 8.0 cm x 7.0 cm x 0.3 cm; the bottom 6 cm consisted of separating gel whilst the remaining 2 cm was stacking gel. Samples for SDS-PAGE were prepared by the addition of 0.5 ml solubilizing buffer to 0.5 ml sample. The mixture was heated at 100°C for 5 min prior to electrophoresis.

Electrophoresis was done in a Uniscil slab gel electrophoresis unit (Universal Scientific Ltd., London) at a constant current of 15 mA/gel

for approximately 2-4h, or until the tracking dye reached the limit of the gel. The gels were carefully removed from the plates and immersed in fixing-staining solution for 60-90 min. Destaining was done by soaking in several changes of destaining solution.

The compositions of reagents used are given in Appendix 6.

6.5 Chemiluminescence

(i) Neutrophil isolation

Rabbit peritoneal exudate neutrophils were obtained as follows. Briefly, 500 ml of 0.85% (w/v) saline containing glycogen (0.1% (w/v) oyster glycogen; Sigma) was injected ip into a New Zealand white female rabbit. Peritoneal exudate was removed 4h later and stored at $4^{o}C$ until used, usually within 2 days of isolation. Before use, the cells were washed once in divalent cation-free HBS-EDTA (Appendix 7) and then in HBS. Contaminating erythrocytes were removed from all neutrophil preparations after the first wash by hypotonic lysis. To obtain a monodispersed population of cells, the suspension was passed through a 10 μM Nitex filter (Plastok Associates, Birkenhead, England). Cell suspensions contained greater than 95% neutrophils and cell viability was greater than 95% as judged by Trypan Blue exclusion. These suspensions were used within 2h of preparation.

(ii) Assay

The assay was done using a LKB 1251 Luminometer used in automode and controlled with an Acorn BBC'B microcomputer; both were connected via the RS232C interface. The number of neutrophils per assay tube was 1.0×10^{6} and luminol (Sigma) was used as a light-enhancing reagent at a final concentration of 1.0×10^{-5}M. The synthetic chemotactic peptide N-formyl methionyl-leucyl-phenylalanine (fMLP; Sigma) was used as the stimulus at a final concentration of 1.0×10^{-7}M.

The final assay volume per assay tube was 700 µl. Before simultaneous addition of luminol and fMLP, neutrophils were pre-incubated in the presence of the test agent(s) for 1h at 37°C with occasional mixing. After stimulation, samples were counted for 0.5 sec.

The results were represented as graphs of millivolts (mV) versus time (min) for each sample assayed in triplicate.

6.6 LAL assay for endotoxin

The *Limulus* amoebocyte lysate (LAL) test was used for the detection of endotoxin. Endotoxin-free glassware was prepared as described in Appendix 3. Pyrogen-free water and the amoebocyte lysate were obtained from Sigma, and *B. pertussis* LPS from List Biological Laboratories Inc., California, USA. All assays were done in endotoxin-free glass tubes.

Dilution series of the samples were made in pyrogen-free water. To 0.1 ml of each sample was added 0.1 ml of E-Toxate amoebocyte lysate. To reduce possible cross-contamination, lysate was added to tubes containing the least endotoxin first. The tube contents were mixed gently and incubated for 1h at 37°C undisturbed.

After incubation, the tubes were gently removed from the water bath and slowly inverted through 180° whilst observing for gelation. A positive test was the formation of a hard gel which permitted the complete inversion of the tube without disruption of the gel. All other results, eg: soft gels, turbidity, increase in viscosity and clear liquid were considered to be negative. The following controls were used:

(i) a negative control, being 0.1 ml of endotoxin-free water and 0.1 ml of E-Toxate working solution;

(ii) a series of positive controls, i.e: dilutions of the endotoxin reference preparation with E-Toxate solution.

SECTION 7. TOXOIDING OF ANTIGEN PREPARATION

The antigen preparation was dialyzed overnight at 4°C against 20 mM sodium phosphate buffer, pH 5.0 containing 0.5M NaCl. A solution of the reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.HCl (EDAC; Sigma) was made in the same buffer and added to the buffered antigen preparation so that the final concentration of protein in the mixture was 50 $\mu g\ ml^{-1}$ and of EDAC 4,000 $\mu g\ ml^{-1}$. This was incubated at 37°C for 24h without agitation. The mixture was dialyzed at 4°C for three days against daily changes of 20 mM sodium phosphate buffer, pH 7.6 containing 0.5M NaCl and thimerosal (0.01% (w/v) final concentration) as a preservative. The toxoid was stored at 4°C.

As a control, the antigen preparation was incubated at 37°C for 24h without EDAC and dialyzed.

Many toxoids were prepared by variations on this general procedure and these are mentioned in the Results section.

RESULTS

SECTION 1. EXTRACTION OF ANTIGEN PREPARATION (AP) CONTAINING PT AND FHA

1.1 Preliminary experiments: a comparison of blue and red dye-ligand media

A series of experiments was done to compare the affinity of red and blue dye-ligand media for the haemagglutinating (HA) activity in culture fluid fractions. Several affinity chromatography dye-ligand media were compared: Blue Sepharose CL-6B, Reactive Red-120 cross-linked agarose (Sigma) and a Blue Sepharose matrix prepared and supplied by Dr. R. Parton.

Mini-columns, made from the barrels of 5 ml plastic syringes (Becton Dickinson, Ireland) and plugged with glass wool to prevent leakage, were filled with 3 ml of gel and equilibrated with 0.05M Tris-HCl buffer, pH 8.0. Samples of an Amicon 100 kDal culture fluid retentate, with detectable HA activity, were passed through the columns and eluted with different buffers.

All three gels bound HA activity, as shown by the absence of detectable activity in the initial eluate fractions. HA activity was eluted from both Blue Sepharose matrices with 0.05M Tris-HCl buffer, pH 6.5 containing 0.75M $MgCl_2$, but the Reactive Red-120 agarose gel required a buffer containing at least 2.0M $MgCl_2$ before activity was detected in the collected fractions.

Blue Sepharose CL-6B contains the dye Cibacron Blue F3G-A which has an affinity for enzymes which require adenylyl-containing cofactors, including NAD. Since PT catalyzes the transfer of ADP-ribose from intracellular NAD to a membrane-bound 41 kDal protein (Tamura *et al.*, 1982), an experiment was done to test whether NAD (Sigma) could specifically elute PT from the gel matrices. After the Amicon retentate

was passed through the gel columns, these were washed with 0.05M Tris-HCl buffer, pH 8.0 containing either 10 mM NAD or 20 mM NAD. This experiment was repeated and no HA activity was eluted with either buffer.

As a result of several preliminary experiments, it was found that 0.05M Tris-HCl buffer, pH 8.0 containing 1.0M NaCl eluted HA activity from all 3 matrices. However, for routine use, Blue Sepharose CL-6B was chosen for the extraction of HA activity from culture fluid.

1.2 Characteristics of culture fluid before and after extraction with Blue Sepharose

From two experiments (Table 7; experiment 2,3), samples of culture fluid before and after extraction with Blue Sepharose were retained for analysis. There were detectable levels of PT and FHa in culture fluid before extraction as determined by haemagglutination and by antigen-ELISA (0.2-1.2 μg PT ml^{-1} and 0.1-1.0 μg FHa ml^{-1} respectively). The culture fluid after extraction contained no HA activity or PT and FHa detectable by ELISA, thus showing efficient extraction of the desired components.

Samples of culture fluid (Table 7; experiment 2,3) before and after extraction, were heated at 56°C in a water bath for 30 min. The leucocytosis-promoting activity (LPA) of the unheated and heated culture fluid samples was determined (p. 88), and the test animals were also weighed daily.

(a) LPA: undiluted culture fluid before extraction induced a highly significant leucocytosis in mice compared to mice injected with PBS as controls(Table 6). After heating, there was a slight but significant reduction in LPA.

After extraction, unheated culture fluid induced a geometric mean WBC/mm^3 count of 8,843 (95% confidence limits (CL) 7,371, 10,610).

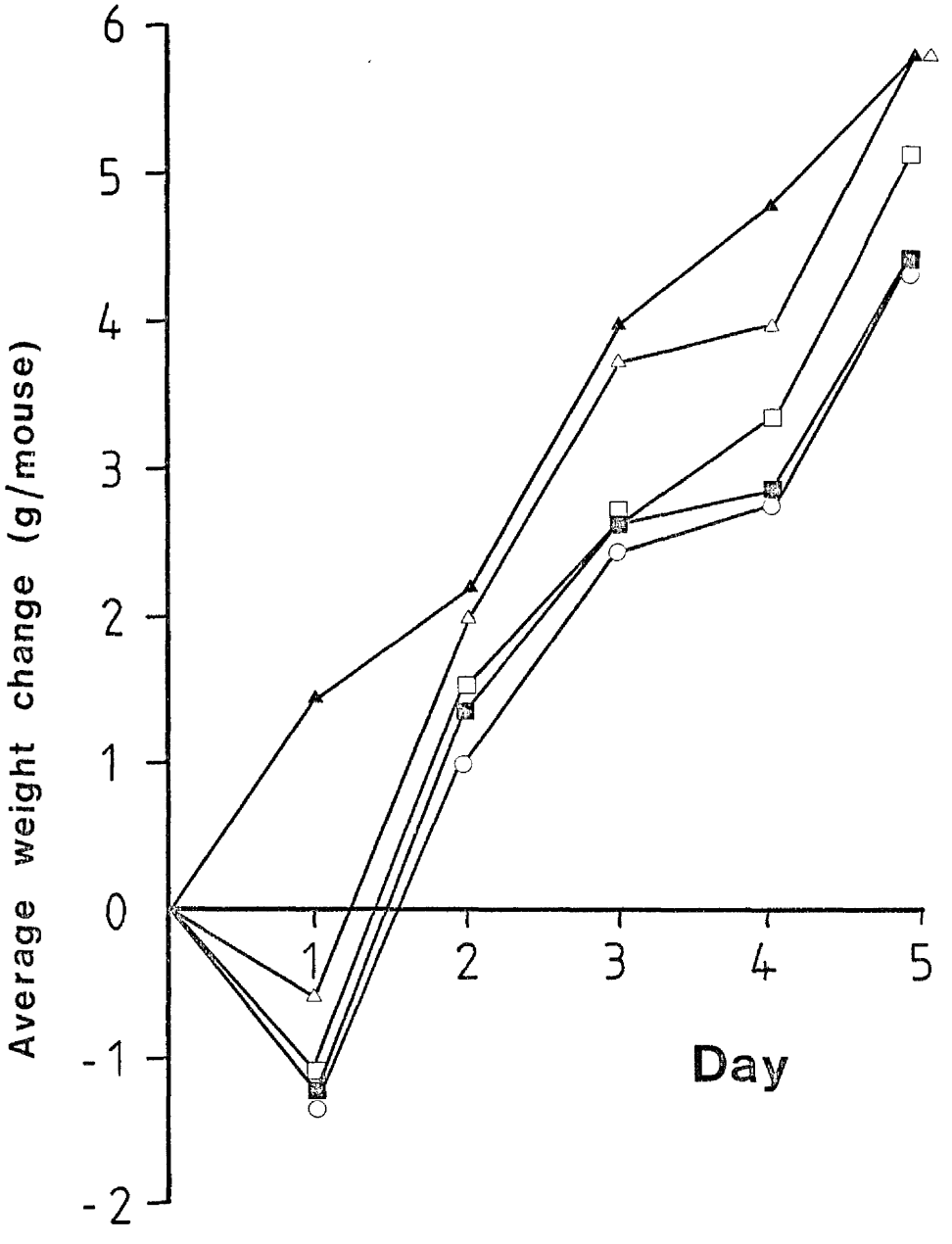
Table 6. Leucocyte response in mice to *B. pertussis* culture fluid before and after extraction with Blue Sepharose

Sample		Leucocyte counts (WBC/mm^3) Geometric mean (95% CL)	
Culture fluid before Blue Sepharose.	unheated	38,115	(32,095, 45,265)
	heated at 56° for 30 min	27,146	(23,627, 31,188)
Culture fluid after Blue Sepharose.	unheated	8,843	(7,371, 10,610)
	heated at 56° for 30 min	6,891	(5,690, 8,347)
PBS		6,700	(6,277, 7,154)

Figure 9. Mouse-weight gain test with B. pertussis culture fluid before and after extraction with Blue Sepharose

Groups of 5 mice were injected iv with PBS, or samples of unheated or heated culture fluid before and after extraction with Blue Sepharose. Mice were weighed daily for 5 days.

	Sample injected
▲——▲	PBS
■——■	Culture fluid before Blue Sepharose - unheated
△——△	" " " - heated at 56°C for 3 min
□——□	Culture fluid after Blue Sepharose - unheated
○——○	" " " - heated at 56°C for 30 min.


Average weight change (g/mouse)
6
5
4
3
2
1
0
-1
-2
1
2
3
4
5
Day

Heated culture fluid after extraction induced a geometric mean WBC/mm^3 count of 6,891 (95% CL 5,690, 8,347), which was not significantly different from the mean response in mice injected with unheated culture fluid or PBS (Table 6).

(b) Mouse-weight-gain test: there was no difference in the pattern of weight change with all four samples tested (Fig. 9). The undiluted culture fluid samples, heated and unheated, induced an initial weight loss in mice. All the mice regained normal weight by day 2, and no deaths were observed in any of the test animals.

The results for LPA and mouse-toxicity for the culture fluid samples from both experiments were similar.

1.3 Presence of Cibacron Blue F3G-A in antigen preparations (AP)

It was essential to determine if the Blue Sepharose dye, Cibacron Blue F3G-A, was present in the antigen preparations (AP). Initially, the absorption spectrum for a 0.01% (w/v) solution of the dye was scanned. The dye showed two absorption maxima at wavelengths (λ) 613 nm and 291 nm (Fig. 10a). At these two maxima, standard curves of absorbance versus concentration of dye ($\mu g\ ml^{-1}$) were constructed (Fig.10b).

Several AP samples were assayed for the dye; these were AP-12, AP-13 and AP-16 (Table 8). At λ 291 nm there was interference from protein in the estimations, but at λ 613 nm all three undiluted samples gave absorbance values less than or equal to 0.05. Therefore, there was no detectable dye, ie: less than 3.125 $\mu g\ ml^{-1}$ in the eluates.

1.4 Effect of cultural conditions on yield of antigen preparation (AP)

Table 7 summarizes the effect of cultural conditions on the yield of antigen preparation (AP) extracted from culture fluid. *B. pertussis* was grown in different media, and the antigens extracted

Figure 10a. Absorption spectrum for Cibacron Blue F3G-A

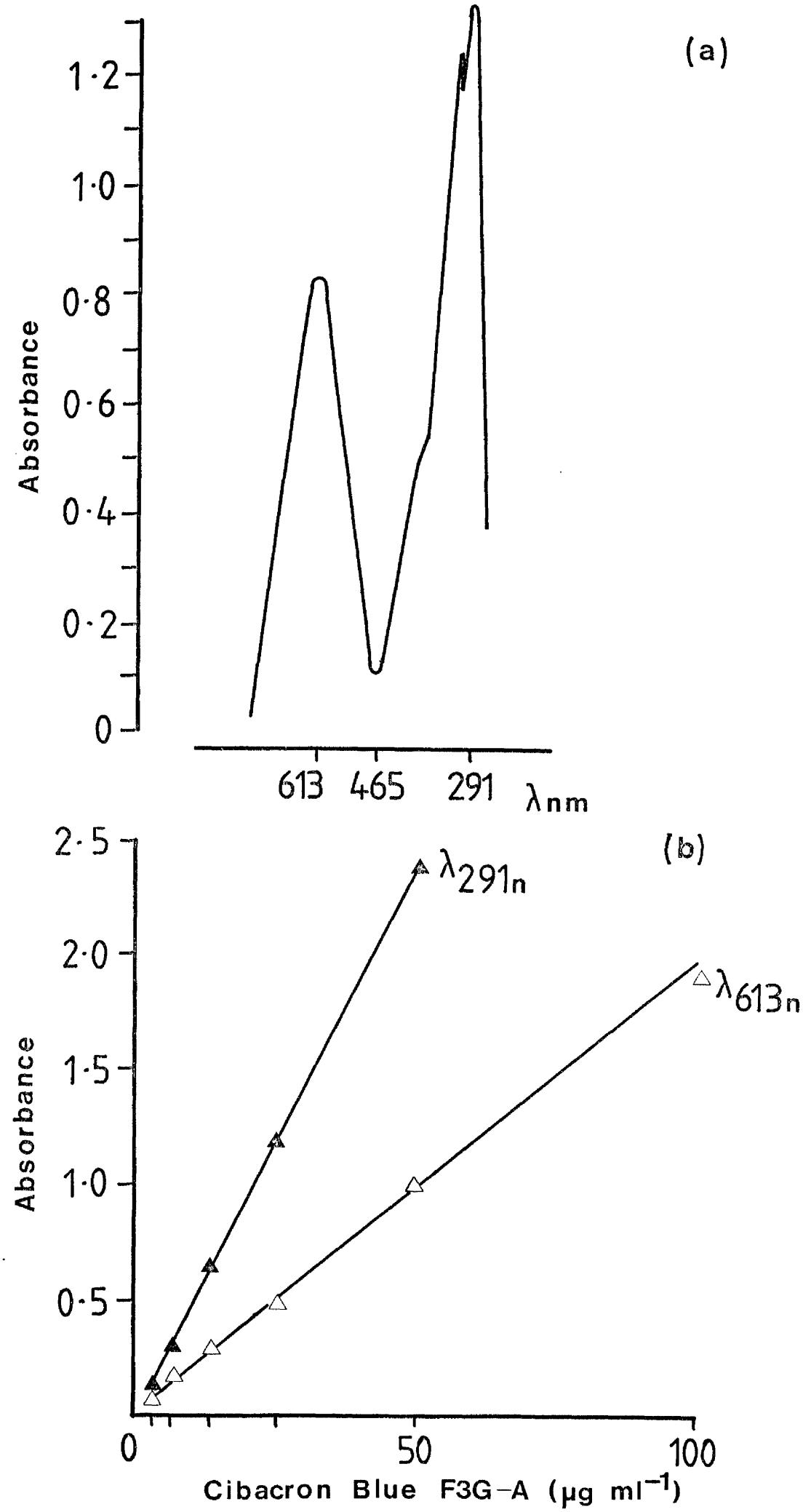
The absorption spectrum for a 0.01% (w/v) solution of the dye was scanned in 2.0 cm silica cuvettes in a Pye Unicam SP8-100 UV spectrophotometer (p. 100).

Figure 10b. Standard curves for Cibacron Blue F3G-A

Standard curves of absorbance versus dye concentration ($\mu g\ ml^{-1}$) at the two absorption maxima were constructed (p. 100).

▲——▲ Standard curve at λ 291 nm

△——△ Standard curve at λ 613 nm


(a)
Absorbance
1·2
1·0
0·8
0·6
0·4
0·2
0
613
465
291
λnm
(b)
λ291n
λ613n
Absorbance
2·5
2·0
1·5
1·0
0·5
0
50
100
Cibacron Blue F3G-A (μg ml⁻¹)

with Blue Sepharose (p. 85). Representative elution profiles of HA activity from Blue Sepharose are shown in Fig. 11 for two growth experiments (Table 7; experiment 6,12). Elution of HA activity with 0.05M Tris-HCl buffer, pH 8.0 containing 1.0M NaCl corresponded with the increase in absorbance measured at λ 280 nm. After elution, the gel was washed with 0.1M Tris-HCl buffer, pH 8.0 containing 0.5M NaCl, 6.0M urea, which resulted in a second absorbance peak in which no HA activity was detected.

The addition of cyclodextrin and aeration greatly enhanced the amount of protein extracted from culture fluid with Blue Sepharose. The yield of protein from static cultures of B. pertussis in SS-X medium varied from 0.47-1.45 mg L^{-1} of culture fluid (mean 0.79 mg L^{-1}), and 0.93 mg L^{-1} were obtained from the fermenter static culture. In one experiment (Table 7; experiment 2), B. pertussis vaccine strain 18334 was cultured in SS-X medium, static, and was shown to produce extracellular protein, PT and FHa. When cyclodextrin was added to SS-X medium and the cultures shaken for 36h, 2.35 mg and 3.22 mg protein per litre were extracted (mean 2.78 mg L^{-1}). With the CL medium shaken for 36h, 2.80-5.54 mg protein per litre were extracted (mean 4.16 mg L^{-1}), but if the organisms were cultured for 48h, only 1.22 mg and 1.45 mg per litre were extracted (mean 1.34 mg L^{-1}). By comparison of the mean yields, the CL medium produced (i) 5 times more protein per litre than SS-X medium (static, in Carrell and Fernbach flasks or the fermenter vessel) and (ii) 1.5 times more protein per litre than SS-X medium supplemented with cyclodextrin (shaken, 36h). However, if the CL medium was shaken for 48h instead of 36h, there was a three-fold reduction in the yield of protein per litre of culture fluid, which was extracted with Blue Sepharose.

Figure 11. Elution profile of haemagglutinin from Blue Sepharose

Representative elution profiles of haemagglutinin are shown for experiments in which B. pertussis strain 77/18319 was cultured in SS-X medium, static, 120h (Table 7, expt. 6) and in CL medium, shaken, 36h (Table 7, expt. 12). Absorbance (280 nm), and haemagglutinating activity (p. 98) were determined for each column fraction collected.

Expt. 12

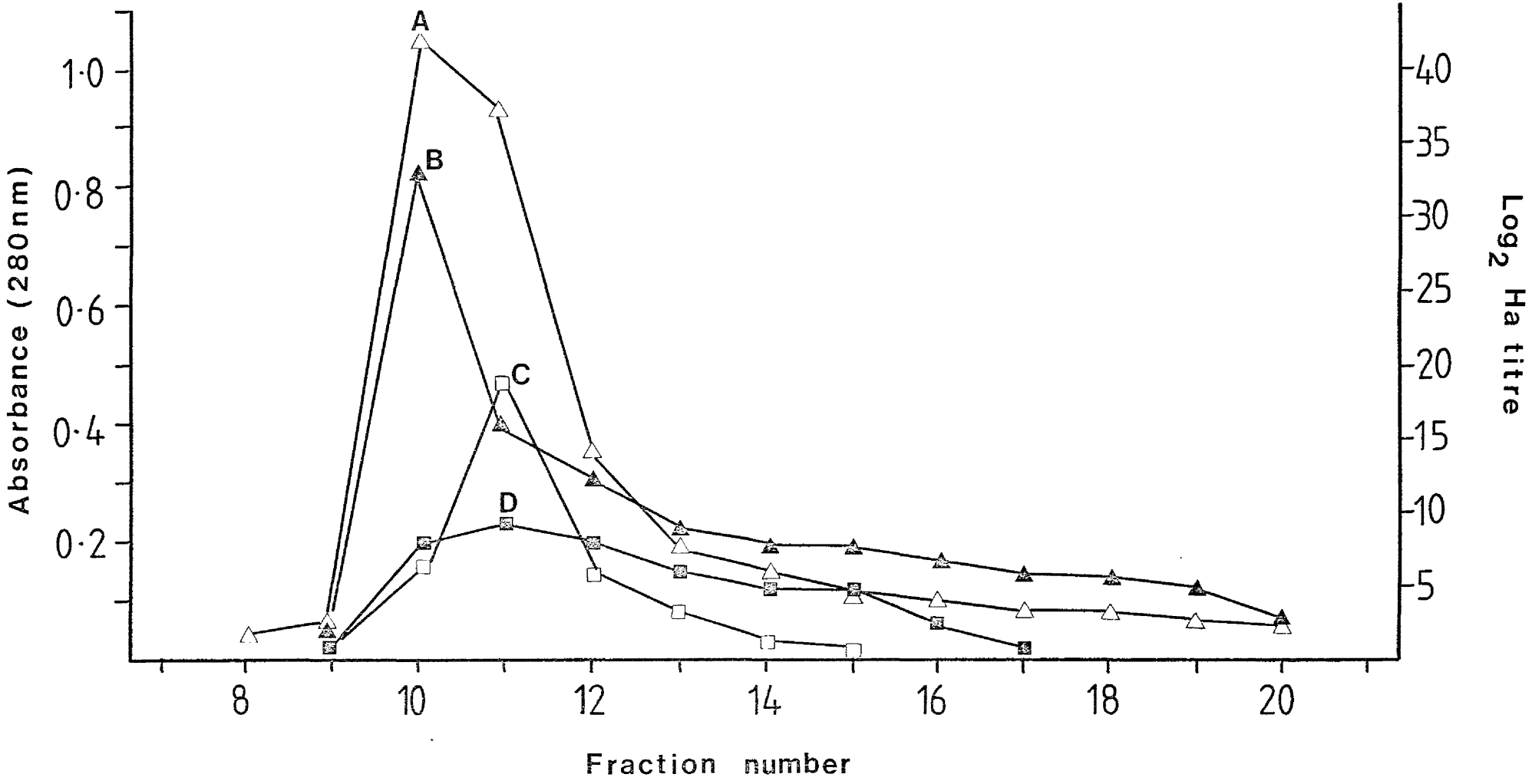
A. Absorbance at λ 280 nm for the elution of gel-bound material

B. Haemagglutinin titre ($\log_2$).

Expt. 6

C. Absorbance at λ 280 nm for the elution of gel-bound material

D. Haemagglutinin titre ($\log_2$).


A
B
C
D
1·0
0·8
0·6
0·4
0·2
Absorbance (280nm)
40
35
30
25
20
15
10
5
Log2 Ha titre
8
10
12
14
16
18
20
Fraction number

From static cultures of B. pertussis in SS-X medium, 0.12-1.18 mg PT per litre were extracted (mean 0.45 mg L^{-1}) and only 0.08 mg per litre were obtained from fermenter growth of the organism (Table 7). With SS-X medium supplemented with cyclodextrin (shaken), 2.08 mg and 2.83 mg PT per litre were extracted (mean 2.45 mg L^{-1}). The CL medium (shaken, 36h) yielded 0.36-0.91 mg PT per litre (mean 0.55 mg L^{-1}) and when shaken for 48h, 0.38 mg and 0.96 mg PT per litre (mean 0.67 mg L^{-1}). Only 0.01 mg PT per litre were extracted from the culture fluid of B. pertussis strain 357 (PT^-, FHa^+) grown in the CL medium. Therefore, the best yields of PT were obtained from SS-X medium supplemented with cyclodextrin and shaken with aeration for 36h. This medium yielded approximately,

(i) 5 times and 31 times more PT than SS-X medium, static (in Carrell and Fernbach flasks) and SS-X medium, static in a fermenter vessel, respectively,

(ii) 4-5 times more PT than the CL medium shaken for either 36 or 48 h. There was no significant difference in the yields of PT from the CL medium shaken for either 36 or 48h.

Filamentous haemagglutinin (FHa) was also produced by B. pertussis in all of these media (Table 7). From static cultures in SS-X medium, 0.03-0.91 mg FHa per litre (mean 0.25 mg L^{-1}) were extracted, and fermenter growth of the organism yielded 0.21 mg FHa per litre. With SS-X medium plus cyclodextrin, 0.94 mg and 1.0 mg FHa per litre were extracted (mean 0.97 mg L^{-1}). The CL medium, shaken for 36h, yielded 2.74-3.96 mg FHa per litre (mean 3.5½ mg L^{-1}); however, if shaken for 48h, only 0.19 mg and 0.36 mg FHa per litre (mean 0.28 mg L^{-1}) were extracted. By comparing the mean yields of FHa per litre of culture fluid, the CL medium (shaken, 36h) yielded approximately

Table 7. Effect of cultural conditions on the yield of antigen preparation (AP)

Experiment	B. pertussis strain	Cultural conditions	Culture fluid processed (litres)	Yield (mg per litre culture fluid)		
				Protein	PT by ELISA	FHa by ELISA
1	77/18319	SS-X (static, 120h)	5.0	0.47	0.12	0.11
2	18334	"	5.0	1.45	1.18	0.91
3	77/18319	"	11.5	0.64	0.61	0.03
4	"	"	13.0	0.51	0.13	0.12
5	"	"	6.0	0.90	0.30	0.23
6	"	"	12.4	0.79	0.37	0.07
7	"	SS-X(fermenter,static,120h)	12.4	0.93	0.08	0.21
8	"	SS-X + cyclodextrin (shaken,36h)	10.0	3.22	2.83	1.00
9	"	"	10.0	2.35	2.08	0.94
10	"	CL medium (shaken, 36h)	10.0	4.42	0.37	3.96
11	"	"	10.0	3.87	0.36	3.74
12	"	"	10.0	5.54	0.91	2.74
13	"	CL medium (shaken, 48h)	10.0	1.45	0.96	0.19
14	"	"	3.0	1.22	0.38	0.36
15	357	CL medium (shaken, 36h)	10.0	2.80	0.01	3.60

Table 8. Characteristics of antigen preparations (AP)

Antigen preparation (AP)	Protein ($mgml^{-1}$)	Eluate volume (ml)	Total yield (mg)	PT ($mgml^{-1}$) by ELISA	FHa ($mgml^{-1}$) by ELISA	PT and FHa as a % (w/w) of total protein	PT/FHa ratio
AP-1	0.11	22	2.35	0.03	0.03	56	1.0
AP-2	0.31	23	7.25	0.26	0.20	146	1.3
AP-3	0.18	40	7.36	0.18	0.01	103	18.0
AP-4	0.16	53	6.63	0.03	0.03	48	1.0
AP-5	0.14	40	5.40	0.05	0.03	59	1.6
AP-6	0.13	74.5	9.80	0.06	0.01	53	6.0
AP-7	0.37	31.5	11.50	0.03	0.08	31	0.4
AP-8	2.15	15	32.20	1.89	0.67	119	2.8
AP-9	0.39	60	23.50	0.35	0.16	129	2.2
AP-10	0.88	50	44.20	0.07	0.79	98	0.1
AP-11	0.78	50	38.70	0.07	0.75	106	0.1
AP-12	0.58	96	55.40	0.09	0.29	66	0.31
AP-13	0.18	82.5	14.50	0.12	0.02	80	6.0
AP-14	0.67	5.5	3.67	0.21	0.19	60	1.1
AP-15	0.50	56	28.00	0.008	0.64	129	0.003
AP-16	0.24	168	39.70	0.09	0.04	55	2.25
AP-17	0.82	-	-	0.30	0.47	94	0.64

(i) 14 times more FHa than SS-X medium, static,

(ii) 17 times more FHa than SS-X medium, static, fermenter,

(iii) 4 times more FHa than SS-X medium plus cyclodextrin, shaken.

However, there was approximately a 13-fold reduction in the yield of FHa if the CL medium was shaken for 48h instead of 36h.

Therefore, most PT was produced by organisms grown in SS-X medium supplemented with cyclodextrin (shaken, 36h), whereas most FHa was produced in the CL medium (shaken, 36h).

1.5 Characteristics of antigen preparations (AP)

During this investigation, fifteen independent antigen preparations (AP) were processed and characterized (Table 8). These preparations, AP-1 to AP-15, are the corresponding Blue Sepharose extracts from the numbered experiments shown in Table 7. The differences in eluate volumes (ml) in Table 8 were due to the degree to which the pooled fractions containing HA activity were concentrated by either Amicon ultrafiltration or Aquacide (p. 87).

The most important data are summarized in the last two columns of Table 8. For convenience, PT and FHa as a percentage of the total protein (w/w) was calculated for each AP. (However, estimation of protein by the method of Herbert *et al.* (1971) used bovine serum albumin as the standard and thus cannot strictly be correlated with the estimation of PT and FHa by ELISA, since these procedures use purified antigens as standards.) Nevertheless, from the culture fluids of *B. pertussis* grown in static SS-X medium (Table 7; experiments 1-7), four antigen preparations (AP-1, AP-4 to AP-6) contained approximately 50% (w/w) as PT and FHa: in another two (AP-2, AP-3), approximately 100% (w/w) of the total protein was accounted for as both antigens. Only 31% of protein in the

preparation extracted from the culture fluid of fermenter-grown B. pertussis (AP-7) was accounted for as PT and FHa. With antigen preparations from SS-X medium plus cyclodextrin, shaken, (AP-8, AP-9), approximately 100% (w/w) of the total protein was accounted for as both PT and FHa. The antigen preparations from CL medium, shaken for either 36 or 48h (AP-10 to AP-15), contained PT and FHa from 60-129% (w/w) of the total protein.

The last column of Table 8 summarizes the data as a PT/FHa ratio. Four preparations extracted from culture fluid of B. pertussis grown in SS-X medium, static, (AP-1, AP-2, AP-4, AP-5), had a PT/FHa ratio of approximately 1.0. In preparations AP-3 and AP-6 the ratios were approximately 18.0 and 5.0 respectively. In AP-7, the PT/FHa ratio was approximately 0.4. Antigen preparations AP-8 and AP-9 had PT/FHa ratios of approximately 2.5. Those preparations extracted from the culture fluid of CL medium shaken for 36h (AP-10 to AP-12) had PT/FHa ratios of approximately 0.1-0.3: but when cultured with shaking for 48h, the observed ratios were approximately 6 (AP-13) and 1.1 (AP-14). Antigen preparation AP-15, extracted from the culture fluid of B. pertussis strain 357 (PT^-, FHa^+) grown in CL medium (Table 7; experiment 15) had a PT/FHa ratio of approximately 0.003.

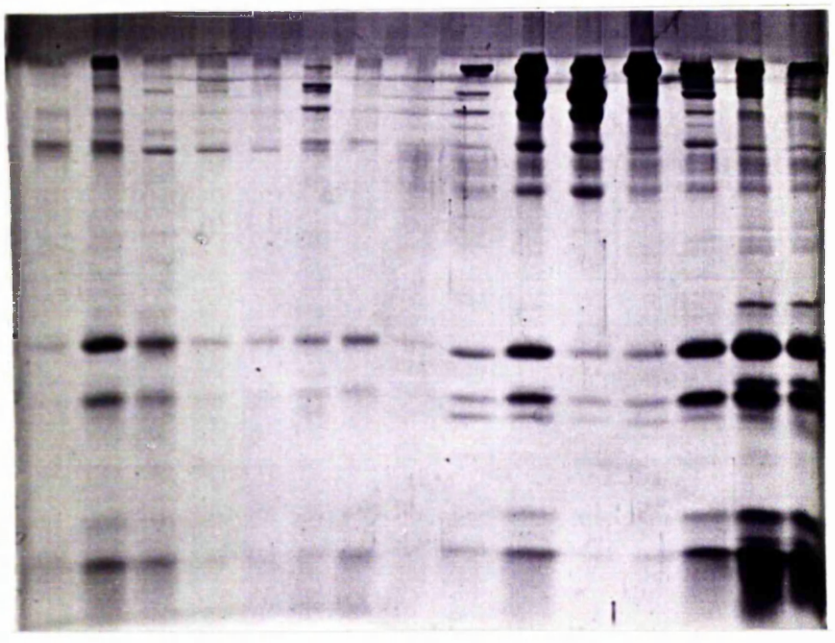
Several of the antigen preparations were analyzed by SDS-polyacrylamide gel electrophoresis (Plates 1,2). The gel profiles were similar for all of the preparations, with a series of high mol.wt bands (possibly FHa) and a collection of 5 bands of lower mol.wt, which, when run in parallel with marker proteins had mol.wts similar to those reported for PT. The differences in the intensities of the profiles were presumably due to the amount of protein in the samples. In some cases, the SDS-PAGE profiles indicated the presence of other minor proteins.

Plate 1. Analysis of antigen preparations (AP) by SDS-polyacrylamide gel electrophoresis

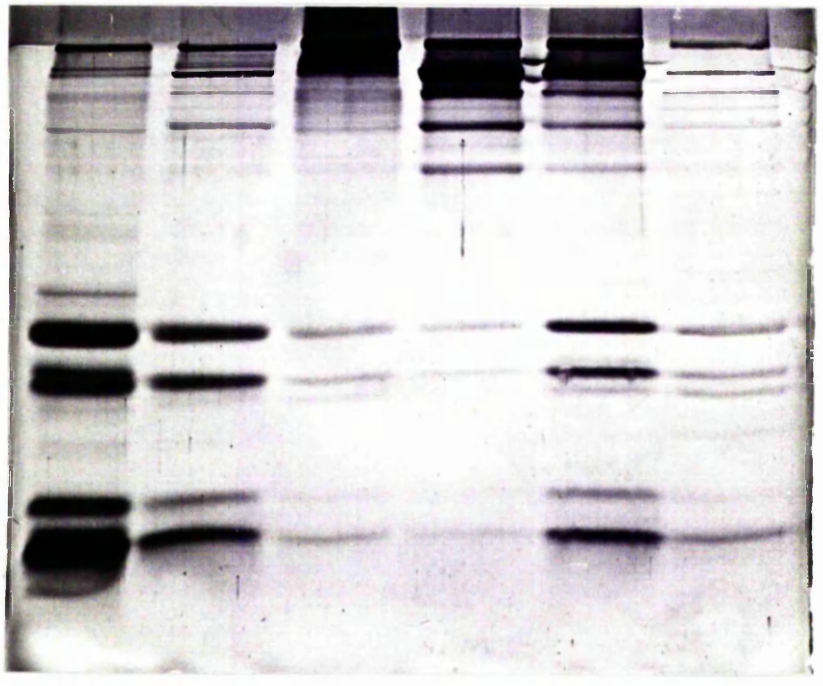
Lane	Preparation		
1	AP-1	9	AP-7
2	AP-2	10	AP-12
3	AP-3	11	AP-11
4	AP-4	12	AP-10
5	AP-5	13	AP-9
6	AP-6	14,15	AP-8
7,8	AP-13		

Plate 2. Analysis of antigen preparations (AP) by SDS-polyacrylamide gel electrophoresis

Lane	Preparation
1	AP-8
2	AP-9
3	AP-10
4	AP-11
5	AP-12
6	An antigen preparation prepared and supplied by Dr. R. Parton, but not characterized in this investigation.

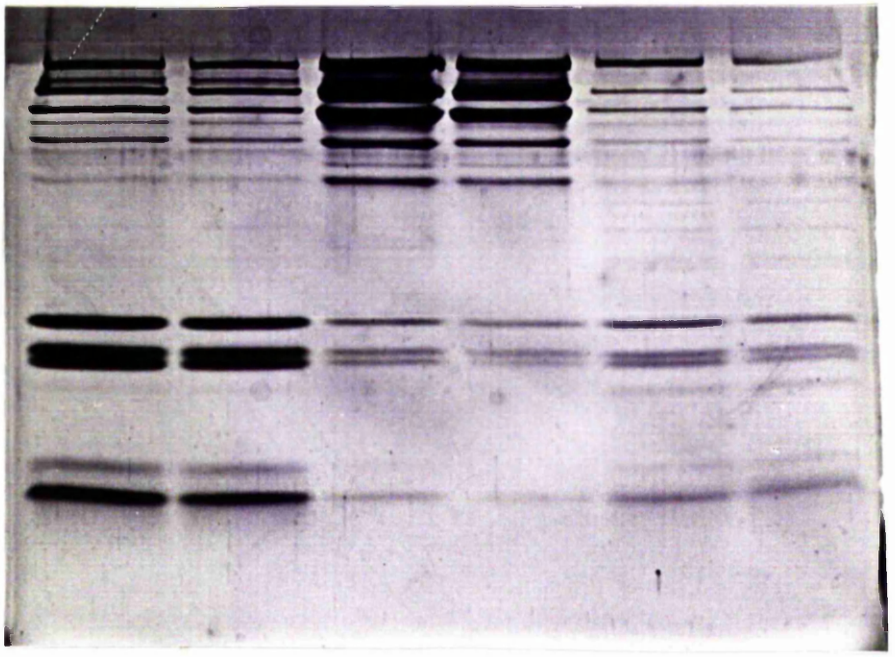


I 3 5 7 9 II I3 I5



I 2 3 4 5 6

Plate 3. Analysis of antigen preparations (AP), before and after filtration, by SDS-polyacrylamide gel electrophoresis



I 2 3 4 5 6

Lane	Preparation
1	AP-9 before filtration
2	AP-9 after "
3	AP-11 before "
4	AP-11 after "
5	An antigen preparation prepared and supplied by Dr. R. Parton - before filtration.
6	An antigen preparation prepared and supplied by Dr. R. Parton - after filtration.

Samples were passed through 0.45 μm filters (Millipore) and analyzed.

Several antigen preparations were filtered through 0.45 μM filters (Millipore (UK) Ltd., Harrow, Middlesex) and analyzed by SDS-PAGE (Plate 3). There were no apparent differences in the intensities of any of the bands in the gel profiles for each preparation before and after filtration.

For the toxoiding studies to be described, two mixtures of antigen preparations were made and characterized (Table 8). Preparation AP-16 was made by pooling samples AP-1 to AP-6. This mixture contained PT and FHa to only approximately 55% (w/w) of the total protein and had a PT/FHa ratio of approximately 2.25. Preparation AP-17 was made by mixing different volumes of samples AP-8 to AP-11; this mixture contained PT and FHa to approximately 94% (w/w) of the total protein and had a PT/FHa ratio of approximately 0.64 (Table 8).

1.6 Detection of endotoxin in antigen preparations (AP)

(a) Endotoxin assay in mice.

In preliminary experiments, male mice of 3-4 weeks of age were injected ip with graded doses of *B. pertussis* LPS (List Biological Laboratories, Inc.), AP-17 or WCV, or PBS (as a control), and rectal temperatures measured (p. 93). No change in rectal temperature was observed in mice at 1.5h or 3h post-injection with any of the test preparations, when compared to mice injected with PBS. To determine whether age of the mouse was a significant factor, the experiment was repeated in adult mice. Again, there was no detectable hypo- or hyperthermic response to any of the test preparations. Therefore, the HaM/ICR mouse strain was not sensitive for an *in vivo* assay, and endotoxin was subsequently detected with the *Limulus* assay.

(b) *Limulus* assay.

Samples AP-16, AP-17, culture fluid before and after extraction with Blue Sepharose, and the elution buffer (0.05M Tris-HCl, pH 8.0 containing 1.0M NaCl) were tested for endotoxin by the Limulus assay. The minimum detectable amount of endotoxin in the assay was 0.5 ng ml^{-1} (Table 9). No gelation of the amoebocyte lysate was observed beyond 1 in 10,000 of AP-16 or AP-17, or beyond 1 in 100,000 of culture fluid before and after Blue Sepharose. Culture fluid samples tested undiluted, at dilutions of 1 in 10 or 1 in 100, did not cause gelation, indicating the presence of an inhibitor.

Assuming that at a 1 in 10,000 dilution of AP-16 or AP-17 there was approximately 0.5 ng ml^{-1} of endotoxin, therefore, in the undiluted samples there was approximately 5 μg ml^{-1} of endotoxin. Relative to protein content, this indicated that

(i) AP-16 contained approximately 5 μg ml^{-1} endotoxin and 240 μg ml^{-1} protein, ie: 2% (w/w) endotoxin,

(ii) AP-17 contained approximately 5 μg ml^{-1} endotoxin and 500 μg ml^{-1} protein, ie: 1% (w/w) endotoxin.

Culture fluid before and after extraction with Blue Sepharose contained approximately 50 μg ml^{-1} endotoxin, but an estimation of total protein was not accurately made because the samples contained insufficient protein to be detected by the method of Herbert et al. (1971).

Table 9. Detection of endotoxin in various samples by the *Limulus* assay

Sample	Gelation at sample dilution						
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
AP-16	+	+	+	+	+	-	-
AP-17	+	+	+	+	+	-	-
Culture fluid before Blue Sepharose	-	-	+	+	+	+	-
" " after " "	-	-	-	+	+	+	-
Elution buffer, 0.05M Tris-HCl, pH 8.0, + 1M NaCl	-						
Negative control*	-						

	ng ml^{-1}		
B. pertussis LPS	1.0	0.5	0.1
	+	+	-

+, indicates gelation

-, indicates absence of gel formation

*, 0.1 ml endotoxin-free water and 0.1 ml E-Toxate working solution

The concentration of protein in undiluted AP-16 and AP-17 was 240 μg ml^{-1} and 500 μg ml^{-1} respectively.

SECTION 2. TOXOIDING STUDIES WITH 1-ETHYL-3-(3-DIMETHYLAMINOPROPYL) CARBODIIMIDE.HCL (EDAC)

EDAC was used in this investigation to detoxify antigen preparations (AP) containing predominantly PT and FHa. In this section, experiments were done to determine the optimum conditions of EDAC-treatment for detoxification, as measured by loss of histamine-sensitizing and leucocytosis-promoting activities.

2.1 HISTAMINE-SENSITIZING ACTIVITY (HSA)

(i) Preliminary experiment.

Antigen preparation AP-1 (Table 10) was treated with various amounts of EDAC and at different temperatures. The untoxoided preparation, toxoids and whole-cell vaccine (WCV) were tested for their ability to sensitize mice to the lethal effect of a histamine challenge (ie: one of the activities associated with pertussis toxin; Table 2).

Untoxoided antigen preparation AP-1 kept at $4^{o}C$ overnight, had a HSD_{50} value of approximately 0.189 μg/mouse, but 95% confidence limits (CL) could not be calculated from the data. When kept at $25^{o}C$ or $37^{o}C$ overnight, AP-1 had approximately 64% and 53% respectively of the HSA of the preparation kept at $4^{o}C$.

Toxoids (AP-1)T1, (AP-1)T2 and (AP-1)T3 each sensitized one mouse to the lethal effect of histamine challenge, whereas all the mice immunized with preparation (AP-1)T4 survived (Table 10). All these toxoids had less than or equal to 5% of the HSA of untoxoided preparation AP-1 kept at $4^{o}C$.

The whole-cell vaccine (WCV) had a HSD_{50} value of approximately 0.189 ou.ml/mouse (Table 10) and was included in the HSF assay as a control. Subsequently, each time an assay was done, a sealed ampoule

Table 10. Effect of toxoiding with different amounts of EDAC and at different temperatures on the HSA of AP

Sample code (EDAC:protein ratio and temperature of treatment)	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
	μg/mouse		
AP-1 (4°C)	2.0	5/5	
	1.0	5/5	0.189 μg
	0.5	4/5	
	0.25	3/5	
" (25°C)	2.0	5/5	
	1.0	5/5	0.294 μg
	0.5	4/5	(0.23, 0.38)
	0.25	2/5	
" (37°C)	2.0	5/5	
	1.0	4/5	0.355 μg
	0.5	3/5	(0.22, 0.57)
	0.25	1/5	
(AP-1)T1.(40:1, 25°C)	4.0	1/5	⩾ 4 μg
" T2.(80:1, 25°C)	4.0	1/5	" 4 μg
" T3.(40:1, 37°C)	4.0	1/5	" 4 μg
" T4.(80:1, 37°C)	4.0	0/5	> 4 μg
	ou.ml/mouse		
Whole-cell vaccine	0.75	5/5	
(WCV)	0.37	4/5	0.189 ou.ml
	0.19	3/5	(0.14, 0.24)
	0.09	1/5	

Toxoids were prepared by treatment of AP-1 with EDAC overnight, with the ratios of reagent to protein and at the temperatures described above.

of lyophilized vaccine was opened and the contents resuspended and tested.

(ii) Determination of EDAC:protein ratio required to remove the HSA of AP.

Antigen preparation AP-2 (Table 8) had a HSD_{50} value of approximately 0.069 μg/mouse (95% CL 0.04, 0.125; Table 11). Increasing the amount of EDAC in the toxoiding mixtures led to a progressive loss in the HSA of preparation AP-2. Toxoids (AP-2)T9 and (AP-2)T10 did not sensitize mice to the lethal effect of histamine challenge (Table 11). These toxoids, therefore, had less than 1% of the original HSA of preparation AP-2.

The data in Table 11 indicated that an EDAC to protein ratio of at least 40:1 by weight was required to completely remove the HSA of AP at the dose tested (10 μg/mouse).

(iii) Determination of time of EDAC-treatment required to remove the HSA of AP.

Experiments were done to determine the time of EDAC-treatment required to remove HSA, using either a single addition of reagent or a series of multiple additions. In both experimental designs the same preparation of AP-2 and whole-cell vaccine (WCV) were used as controls (Tables 12, 13).

Untoxoided antigen preparation AP-2 had a HSD_{50} value of approximately 0.093 μg/mouse (95% CL 0.06, 0.15; Tables 12, 13). Preparation (AP-2)T11, prepared by treatment and incubation of AP-2 with EDAC for 30 min, sensitized 4/5 mice to the lethal effect of a histamine challenge (Table 12). Complete removal of HSA was observed after treatment and incubation of AP-2 with EDAC for at least 60 min followed by exhaustive dialysis (Table 12). Toxoids (AP-2)T12-(AP-2)T16,

Table 11. Determination of EDAC:protein ratio required to remove the HSA of AP

Sample code (and EDAC:protein ratio)	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
	μg/mouse		
AP-2	1.0	5/5	0.069 μg (0.04, 0.125)
	0.33	4/5	
	0.11	3/5	
	0.03	0/5	
(AP-2)T5 (1:1)	10.0	5/5	< 10 μg
" T6 (5:1)	10.0	5/5	< 10 μg
" T7 (10:1)	10.0	3/5	≪ 10 μg
" T8 (20:1)	10.0	2/5	≫ 10 μg
" T9 (40:1)	10.0	0/5	> 10 μg
" T10 (80:1)	10.0	0/5	> 10 μg
	ou.ml/mouse		
Whole-cell vaccine (WCV)	0.75	5/5	0.259 ou.ml (0.217, 0.31)
	0.37	4/5	
	0.19	1/5	
	0.09	0/5	

Toxoids (AP-2)T5 to (AP-2)T10 were prepared by treatment of AP-2 with EDAC as described (p. 103), except that the EDAC:protein ratio was varied.

Table 12. Determination of time of EDAC-treatment required to remove the HSA of AP

Sample code (and time of EDAC-treatment, min)	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
	μg/mouse		
AP-2	0.5	5/5	0.093 μg (0.06, 0.15)
	0.25	5/5	
	0.12	3/5	
	0.06	2/5	
(AP-2)T11 (30)	10.0	4/5	< 10 μg
" T12 (60)	10.0	0/5	> 10 μg
" T13 (120)	10.0	0/5	"
" T14 (240)	10.0	0/5	"
" T15 (480)	10.0	0/5	"
" T16 (720)	10.0	0/5	"
	ou.ml/mouse		
Whole-cell vaccine (WCV)	0.75	5/5	0.216 ou.ml (0.17, 0.27)
	0.37	4/5	
	0.19	3/5	
	0.09	1/5	

Toxoids (AP-2)T11 - (AP-2)T16 were prepared by treatment of AP-2 as described (p. 103), but with an EDAC:protein ratio of 40:1 by weight. The untoxoided preparation was incubated in toxoiding buffer without EDAC for 720 min and dialyzed, as a control.

Table 13. Effect of multiple additions of EDAC during incubation time on removal of the HSA of AP

Sample code (and incubation time, min)	Dose	Mice dead/challenged	HSD_{50} (95% CL)
	μg/mouse		
AP-2	0.5	5/5	
	0.25	5/5	0.093 μg
	0.12	3/5	(0.06, 0.15)
	0.06	2/5	
(AP-2)T17 (60)	10.0	0/5	>10 μg
" T18 (120)	10.0	0/5	"
" T19 (240)	10.0	0/5	"
" T20 (360)	10.0	0/5	"
	ou.ml/mouse		
Whole-cell vaccine	0.75	5/5	
(WCV)	0.37	4/5	0.216 ou.ml
	0.19	3/5	(0.17, 0.27)
	0.09	1/5	

Toxoids (AP-2)T17 - (AP-2)T20 were prepared by treatment of AP-2 with a reagent to protein ratio of 40:1 by weight, achieved by the stepwise addition of four equal amounts of EDAC within the incubation times.

which showed no detectable HSA in mice at the dose tested (10 μg/mouse), had less than 1% of the original HSA of untoxoided preparation AP-2.

Similarly, toxoids prepared by multiple additions of EDAC during incubation did not induce histamine-sensitization in mice at the dose tested (Table 13). These preparations ((AP-2)T17-(AP-2)T20) also had less than 1% of the original HSA of AP-2.

(iv) Effect of EDAC concentration on removal of the HSA of AP.

Antigen preparation AP-16 (Table 8) was treated with various amounts of EDAC and tested for HSA in mice (Table 14). The untoxoided preparation, AP-16, had a HSD_{50} value of approximately 0.063 μg/mouse (95% CL 0.05, 0.075). Toxoids (AP-16)T23 and (AP-16)T24 had HSD_{50} values of greater than 27 μg/mouse and therefore retained less than 0.2% of the original HSA of the untoxoided preparation. However, whereas mice immunized with 27 μg/mouse of toxoid (AP-16)T24 survived histamine challenge, one mouse died in the group immunized with 27 μg/mouse of toxoid (AP-16)T23, possibly indicating the presence of a trace of HSA in this preparation.

Toxoid (AP-16)T22 had a HSD_{50} value of approximately 12.6 μg/mouse, and therefore retained approximately 0.5% of the original HSA of AP-16.

Toxoid preparation (AP-16)T21 was the most active, as judged by its ability to sensitize mice to the lethal effect of a histamine challenge, with a HSD_{50} value of approximately 1.73 μg/mouse (95% CL 1.14, 2.65; Table 14). This preparation had approximately 3.6% of the original HSA of untoxoided preparation AP-16.

This experiment indicated that treatment with EDAC at a ratio of 80:1 reagent to protein by wt was necessary to remove <u>all</u> detectable (ie: $>$ 99.8%) HSA of AP.

(v) Removal of the HSA of AP with EDAC-treatment at different reaction temperatures.

Table 14. Effect of EDAC concentration on removal of the HSA of AP

Sample code (and EDAC:protein ratio)	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
	μg/mouse		
AP-16	0.18	5/5	
	0.09	4/5	0.063 μg
	0.04	1/5	(0.05, 0.075)
	0.02	0/5	
(AP-16)T21 (10:1)	27.0	5/5	
	9.0	5/5	1.73 μg
	3.0	4/5	(1.14, 2.65)
	1.0	1/5	
" T22 (20:1)	27.0	5/5	
	9.0	2/5	12.6 μg*
	3.0	0/5	
" T23 (40:1)	27.0	1/5	
	9.0	0/5	>27 μg
	3.0	0/5	
" T24 (80:1)	27.0	0/5	
	9.0	0/5	>27 μg
	3.0	0/5	
	ou.ml/mouse		
Whole-cell vaccine (WCV)	0.75	5/5	
	0.37	3/5	0.258 ou.ml
	0.19	2/5	(0.176, 0.377)
	0.09	1/5	

*The data could not be analyzed directly by the probit method, and this HSD_{50} value was estimated by drawing a dose-response curve parallel to that of the whole-cell vaccine (included in each assay as a standard).

Toxoids were prepared by treatment of AP-16 with EDAC as described (p. 103) except that the EDAC to protein ratios were varied.

Untoxoided antigen preparation AP-16 had a HSD_{50} value of approximately 0.039 μg/mouse (95% CL 0.03, 0.054; Table 15). There was no significant reduction in the HSA of AP-16 incubated at 25°C or 37°C by comparison of the confidence limits for all three control preparations (Table 15).

Removal of the HSA of AP-16 with EDAC-treatment was not influenced by reaction temperature. All three toxoid preparations ((AP-16)T25-(AP-16)T27) did not sensitize mice to the lethal effect of a histamine challenge at the dose tested (10 μg/mouse) and therefore had less than 0.4% of the HSA of AP-16 kept at 4°C.

(vi) Effect of reaction pH on removal of the HSA of AP with EDAC-treatment.

An experiment was done to determine the effect of reaction pH on the toxoiding of antigen preparation AP-16 with EDAC. There was a marked difference in the HSA of toxoids, which was dependent on the conditions of their preparation (Table 16). Toxoids (AP-16)T29-(AP-16)T31, prepared in 20 mM sodium phosphate buffer of different pH, had no detectable HSA at the dose tested (10 μg/mouse). These toxoids had less than 0.7% of the HSA of untoxoided preparation AP-16 kept at 4°C in the dialysis buffer, pH 7.6. Therefore, the toxoiding reaction was not pH-dependent over the range of pH 5-9. However, toxoid (AP-16)T28 prepared in the citrate-phosphate buffer, pH 3.5, sensitized all 10 mice to the lethal effect of a histamine challenge (Table 16). This was probably due to the competitive effect of the free carboxyl groups of citric acid for the carbodiimide.

The untoxoided preparation kept at 4°C in the dialysis buffer, pH 7.6 (AP-16, 7.6), had a HSD_{50} value of 0.073 μg/mouse (95% CL 0.04, 0.124; Table 16). By comparison of the confidence limits, the HSA of AP-16 was fairly stable from pH 3.5-7.0. However, there was a significant reduction in the HSA of the preparation at pH 9.0 (Table 16).

Table 15. Removal of the HSA of AP with EDAC-treatment at different reaction temperatures

Sample code (and reaction temperature)	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
	μg/mouse		
AP-16 (4°C)	0.2	5/5	
	0.08	4/5	0.03 μg
	0.03	2/5	(0.03, 0.054)
	0.01	0/5	
" (25°C)	0.2	5/5	
	0.08	3/5	0.063 μg
	0.03	1/5	(0.047, 0.085)
	0.01	0/5	
" (37°C)	0.2	5/5	
	0.08	4/5	0.05 μg
	0.03	1/5	(0.04, 0.063)
	0.01	0/5	
(AP-16)T25 (4°C)	10.0	0/5	>10 μg
" T26 (25°C)	10.0	0/5	"
" T27 (37°C)	10.0	0/5	"
	ou.ml/mouse		
Whole-cell vaccine (WCV)	0.75	5/5	
	0.37	3/5	0.265 ou.ml
	0.19	2/5	(0.176, 0.40)
	0.09	0/5	

Toxoids were prepared by treatment of AP-16 with EDAC as described (p. 103) except that different reaction temperatures were used. As controls, samples of AP-16 were incubated at these temperatures without EDAC.

Table 16. Effect of reaction pH on removal of the HSA of AP with EDAC-treatment

Sample code (and reaction pH)	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
	µg/mouse		
AP-16 (7.6)	0.116	3/5	
	0.046	2/5	0.073 µg
	0.018	1/5	(0.04, 0.124)
	0.007	0/5	
" (3.5)	0.5	4/5	
	0.1	2/5	0.117 µg
	0.02	1/5	(0.06, 0.23)
" (5.0)	0.5	5/5	
	0.1	2/5	0.17 µg
	0.02	1/5	(0.07, 0.40)
" (7.0)	0.5	5/5	
	0.1	2/5	0.17 µg
	0.02	1/5	(0.07, 0.40)
" (9.0)	0.5	4/5	
	0.1	1/5	0.224 µg
	0.02	0/5	(0.15, 0.34)
(AP-16)T28 (3.5)	10.0	10/10	<10 µg
" T29 (5.0)	10.0	0/10	>10 µg
" T30 (7.0)	10.0	0/10	"
" T31 (9.0)	10.0	0/10	"
	ou.ml/mouse		
Whole-cell vaccine	0.75	5/5	
(WCV)	0.37	4/5	0.212 ou.ml
	0.19	3/5	(0.169, 0.266)
	0.09	0/5	

Toxoids were prepared by treatment of AP-16 with EDAC as described (p. 103), but in toxoiding buffers of different pH, ie: 0.1M citrate-0.2M phosphate buffer, pH 3.5 or 20 mM sodium phosphate buffers, pH 5.0-9.0. All toxoiding buffers contained 0.5M NaCl. Samples of AP-16 were also incubated at these pH without EDAC as controls. A sample was also kept at 4°C in the dialysis buffer, pH 7.6, for the duration of the experiment.

The experiment was repeated and the data obtained were similar.

(vii) Determination of HSA at 5 and 12 days after injection of various samples.

The rationale in this experiment was to determine if the toxoid preparation manifested 'late HSA' (Isawa et al., 1985).

The HSD_{50} value of the untoxoided preparation at day 5 or day 12 was less than 0.11 μg/mouse (Table 17). The toxoid (AP-16)T32 did not sensitize any of the mice to the lethal effect of a histamine challenge on day 5 or day 12 post-injection. Therefore, the preparation had less than 1% of the original HSA of the untoxoided material.

The data indicated that the toxoid manifested no 'late HSA', and the histamine-sensitizing activities of the whole-cell vaccine (WCV) or untoxoided preparation (AP-16) were not significantly different at day 5 and day 12.

(viii) Removal of the HSA of antigen preparation AP-17 with EDAC-treatment.

Toxoids were independently prepared by treatment of antigen preparation AP-17 (Table 8) with EDAC. In the first experiment, the untoxoided preparation had a HSD_{50} value of approximately 0.039 μg/mouse (Table 18; 1). Toxoid (AP-17)T33 had a HSD_{50} value of greater than 25 μg/mouse (Table 18; 1,2) and therefore had less than 0.16% of the original HSA of untoxoided AP-17.

In a preliminary titration (data not shown), 5 mice immunized with 25 μg/mouse of toxoid (AP-17)T34 died after histamine challenge. When retitrated, the preparation had a HSD_{50} of approximately 16.6 μg/mouse (95% CL 14.8, 18.6; Table 18; 1), ie: retained 0.24% of the original HSA of untoxoided AP-17. This toxoid preparation was treated with EDAC to give preparation (AP-17)T35 (Table 18; 2).

Table 17. Determination of HSA at 5 and 12 days after injection of various samples

Challenge day	Sample code	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
		µg/mouse		
5	AP-16	1.0	5/5	<0.11 µg*
		0.33	5/5	
		0.11	5/5	
	(AP-16) T32	10.0	0/10	>10 µg
		ou.ml/mouse		
	Whole-cell vaccine (WCV)	0.75	5/5	0.195 ou.ml (0.133,0.285)
		0.37	4/5	
		0.19	2/5	
		µg/mouse		
12	AP-16	1.0	5/5	<0.11 µg*
		0.33	5/5	
		0.11	5/5	
	(AP-16) T32	10.0	0/10	>10 µg
		ou.ml/mouse		
	Whole-cell vaccine (WCV)	0.75	5/5	0.265 ou.ml (0.124,0.566)
		0.37	3/5	
		0.19	2/5	

*Confidence limits not calculable from the data.

Toxoid (AP-16) T32 was prepared by treatment of AP-16 with EDAC as described (p. 103).

Table 18. Removal of the HSA of antigen preparation AP-17 with EDAC-treatment

	Sample code	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
		µg/mouse		
1.	AP-17	0.75	5/5	0.039 µg*
		0.37	4/5	
		0.19	4/5	
		0.09	3/5	
	(AP-17) T33	25.0	5/5	>25 µg
	" T34	25.0	9/10	16.6 µg
		12.5	2/10	(14.8, 18.6)
		ou.ml/mouse		
	Whole-cell vaccine (WCV)	0.75	5/5	0.174 ou.ml
		0.37	4/5	(0.13, 0.26)
		0.19	3/5	
		0.09	1/5	
		µg/mouse		
2.	AP-17	0.17	5/5	0.075 µg
		0.09	3/5	(0.06, 0.09)
		0.04	1/5	
		0.02	0/5	
	(AP-17) T33	50.0	3/5	>25 µg
		25.0	0/5	
	" T35	50.0	0/5	>50 µg
		25.0	0/5	
		12.5	0/5	
		ou.ml/mouse		
	Whole-cell vaccine (WCV)	0.75	4/5	0.263 ou.ml
		0.37	3/5	(0.18, 0.38)
		0.19	1/5	
		0.09	0/5	

*Confidence limits not calculable from the data.

Toxoids (AP-17)T33 and (AP-17)T34 were prepared as described (p. 103). Toxoid (AP-17)T35 was prepared by a second treatment of toxoid (AP-17) T34 as described (p. 103).

In the second experiment, the untoxoided preparation had a HSD_{50} value of 0.075 μg/mouse (95% CL 0.06, 0.09). Toxoid (AP-17)T35 did not sensitize mice at the highest dose tested (50 μg/mouse) and therefore had less than 0.15% of the original HSA of the untoxoided preparation. When toxoid (AP-17)T33 was retested, 3/5 mice immunized with 50 μg/mouse of the preparation died after histamine challenge (Table 18; 2). However, all 5 mice immunized with 25 μg/mouse of (AP-17)T33 survived.

2.2 LEUCOCYTOSIS-PROMOTING ACTIVITY (LPA)

Data for the leucocyte response to antigen preparation AP-16 treated with various amounts of EDAC are shown in Table 19.

Mice injected with PBS had a geometric mean WBC/mm^3 count of 7,000 (95% CL 4,000, 10,000). The lowest dose of untoxoided preparation, AP-16 (1.5 μg/mouse), induced a significant increase in leucocytosis when compared to the normal response of mice injected with PBS (Table 19). At the highest dose of AP-16 tested (12 μg/mouse), WBC/mm^3 counts of the order of 121,000 (95% CL 97,000, 151,000) were observed. The whole-cell vaccine (WCV) also induced a highly significant leucocytosis in mice (Table 19).

Toxoids (AP-16)T23 and (AP-16)T24, which had no detectable HSA (Table 14), also had no detectable LPA at the doses tested (Table 19). However, the two preparations which had HSA, ie: (AP-16)T21 and (AP-16)T22 (Table 14), also demonstrated significant LPA. Toxoid (AP-16)T22 induced a significant increase in the geometric mean WBC/mm^3 count only at a dose of 27 μg/mouse, whereas toxoid (AP-16)T21 was more active, inducing a significant increase (compared to the normal response in mice injected with PBS) at a dose of 9 μg/mouse (Table 19).

Table 19. Leucocyte response to PT-FHa preparation AP-16 treated with various amounts of EDAC

Sample code (and EDAC:protein ratio)	Dose	No. of mice tested	Leucocyte counts ($WBC/mm^3 x10^3$), Geometric mean (95% CL)
	μg/mouse		
AP-16	12.0	2	121 (97, 151)
	6.0	4	80 (69, 93)
	3.0	4	37 (32, 43)
	1.5	5	18 (14, 23)
(AP-16)T21(10:1)	27.0	5	22 (15, 31)
	9.0	5	16 (11, 24)
	3.0	5	9 (7, 12)
(AP-16)T22(20:1)	27.0	5	16 (13, 20)
	9.0	5	10 (9, 11)
	3.0	5	7 (6, 9)
(AP-16)T23(40:1)	27.0	5	9 (7, 11)
	9.0	5	6 (4, 8)
	3.0	5	6 (5, 7)
(AP-16)T24(80:1)	27.0	5	8 (7, 10)
	9.0	5	6 (5, 8)
	3.0	5	5 (4, 8)
	ou.ml/mouse		
Whole-cell vaccine (WCV)	5.0	5	38 (26, 53)
	ml/mouse		
PBS	0.5	5	7 (4, 10)

Toxoids (AP-16)T21 - (AP-16)T24 were prepared as described in Table 14.

2.3 MOUSE-WEIGHT-GAIN TEST (MWGT)

(i) Mouse-toxicity of antigen preparations AP-16 and AP-17 after EDAC-treatment.

The untoxoided preparations AP-16 and AP-17 were lethal for mice. Preparation AP-16, at a dose of 6 or 12 μg/mouse (Fig. 12a), and AP-17 at 5 μg/mouse (Fig. 13), significantly retarded average mouse-weight-gain. From the data in Fig. 12a, the lethal $dose_{50}$ (LD_{50}) of AP-16 was calculated by the probit method as 8.4 μg/mouse (95% CL 5.8, 12.1). The LD_{50} dose of untoxoided preparation AP-17 was 5 μg/mouse (Fig. 13).

The toxoids were non-lethal for mice (Fig. 12b,c; 13). However, toxoid (AP-16)T21 which had residual HSA (Table 14) and LPA (Table 19), manifested some mouse-toxicity at the highest dose tested (Fig. 12b), although with the lower doses weight-gain was normal (Fig. 12c). Mice injected with any of the other toxoids, ie: (AP-16)T22-(AP-16)T24 (Fig. 12b,c), (AP-17)T33-(AP-17)T35 (Fig. 13), gained weight normally.

Mice injected with the whole-cell vaccine (WCV) gained weight normally after an initial weight-loss (Fig. 13) or no weight-gain (Fig. 12a) on the first day.

(ii) Acute toxicity of EDAC.

An experiment was done to determine whether EDAC alone was toxic for mice (Fig. 14).

EDAC was non-lethal for mice at the doses tested (0.001-1.0mg/mouse). However, mice showed lethargy, unsteadiness and exaggerated body movements within 0.5h of injection of EDAC (0.1-1.0 mg/mouse). These reactions persisted for several hours but there was no permanent paralysis, or death. Mice injected with 1.0 mg/mouse of EDAC did not gain body-weight until day 6 (Fig. 14). Those injected with 0.1 mg/mouse

Figure 12. Toxicity of antigen preparation AP-16, treated with various amounts of EDAC, in a mouse-weight-gain test

Mice were injected ip with test samples, weighed daily for 7 days and any deaths recorded. (Leucocyte counts were done on these mice 5 days after injection and the results shown in Table 19).

(a) Toxicity of AP-16 and whole-cell vaccine (WCV)

	Sample injected	Dose/mouse	No. deaths
▲——▲	PBS	0.5 ml	0/5
■——■	Whole-cell vaccine	5.0 ou.ml	0/5
▼——▼	AP-16	12.0 µg	3/5
◺——◺	"	6.0 µg	1/5
◿——◿	"	3.0 µg	1/5
▽——▽	"	1.5 µg	0/5

Toxicity of toxoids (and EDAC:protein ratio)

(b) 27 µg/mouse	+ (c) 9 µg/mouse	
▲——▲	PBS	
○——○	Toxoid (AP-16)	T24(80:1)
◣——◣	"	" T23(40:1)
□——□	"	" T22(20:1)
◺——◺	"	" T21(10:1)

No deaths were observed in mice injected with 27 µg/mouse, 9 µg/mouse or 3 µg/mouse of any of the toxoids. The pattern of weight-gain in mice injected with 3 µg/mouse of any of the toxoids was similar to that observed with 9 µg/mouse, and therefore not shown.

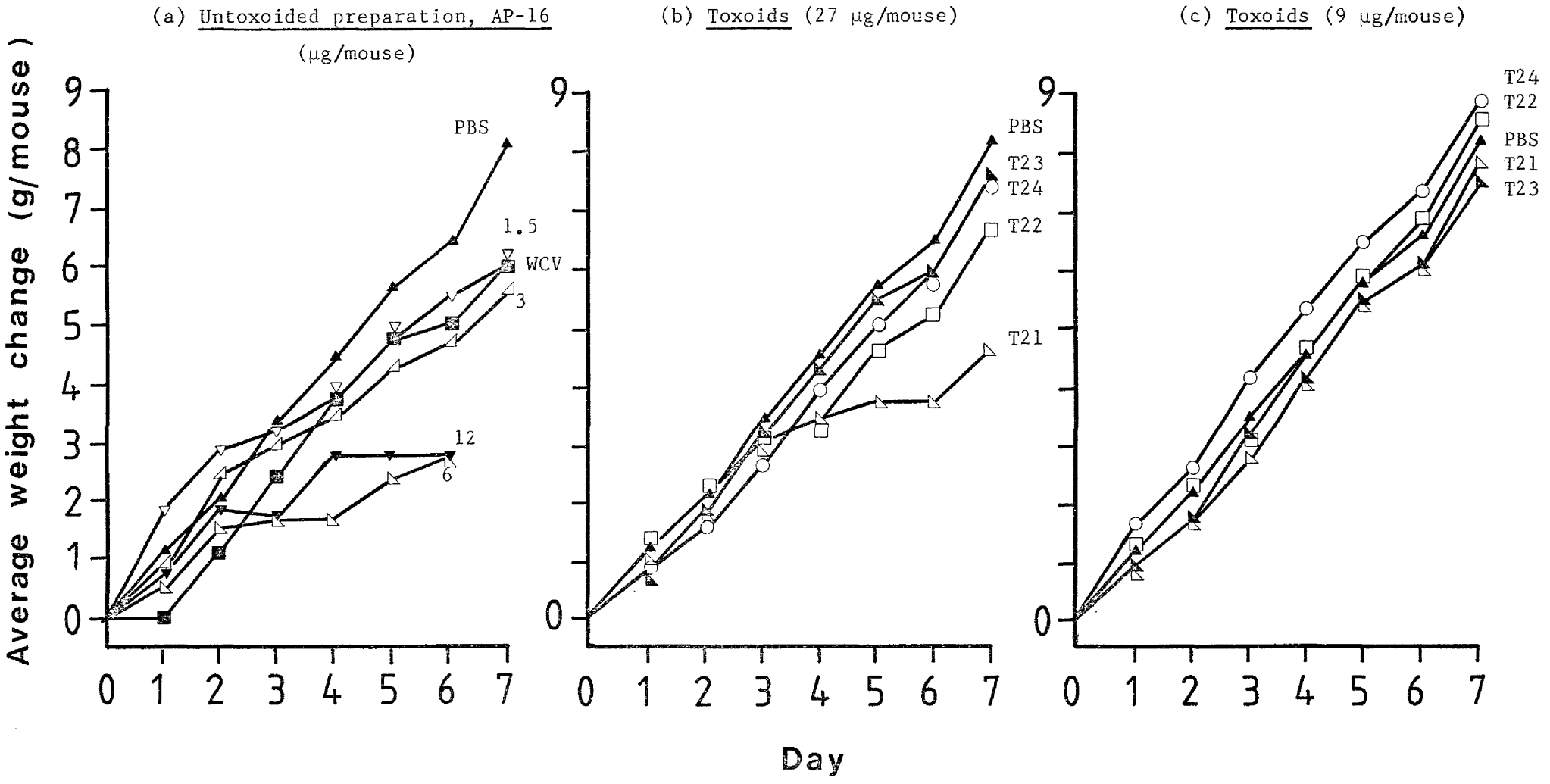

(a) Untoxoided preparation, AP-16
(μg/mouse)
(b) Toxoids (27 μg/mouse)
(c) Toxoids (9 μg/mouse)
Average weight change (g/mouse)
Day
PBS
1.5
WCV
3
12
6
PBS
T23
T24
T22
T21
T24
T22
PBS
T21
T23
0
1
2
3
4
5
6
7
8
9

Figure 13. Toxicity of untoxoided antigen preparation AP-17 and various toxoids in a mouse-weight-gain test

Groups of 10 mice were injected ip with 5 μg/mouse of untoxoided preparation AP-17 or toxoids (AP-17)T33, (AP-17)T34 or (AP-17)T35. Also, groups of 5 mice were injected with 25 μg/mouse of toxoid or whole-cell vaccine, or PBS (as controls). Mice were weighed daily for 7 days and any deaths recorded.

	Sample injected	dose/mouse
▲——▲	PBS	0.5 ml
□——□	AP-17	5 μg*
○——○	(AP-17)T33	25 μg
△——△	" T34	"
▼——▼	" T35	"
◘——◘	Whole-cell vaccine (WCV)	5 ou.ml

*5/10 mice died with this preparation within 7 days. No deaths were observed in mice injected with whole-cell vaccine or any of the toxoids. The average weight-gains of mice injected with 5 μg/mouse of any of the toxoids were comparable to those seen with the higher dose tested (25 μg/mouse).

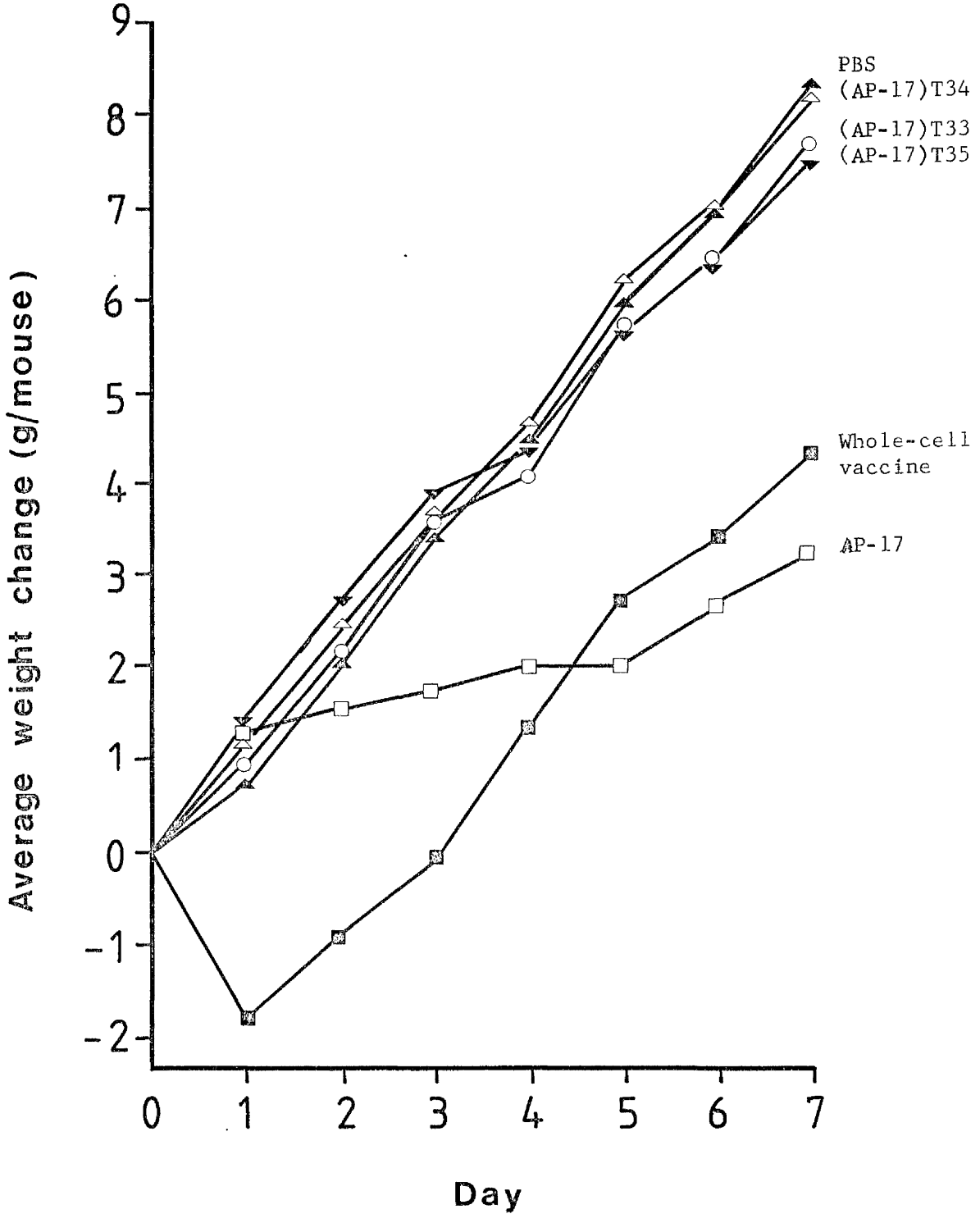

Average weight change (g/mouse)
9
8
7
6
5
4
3
2
1
0
-1
-2
0
1
2
3
4
5
6
7
Day
PBS
(AP-17)T34
(AP-17)T33
(AP-17)T35
Whole-cell vaccine
AP-17

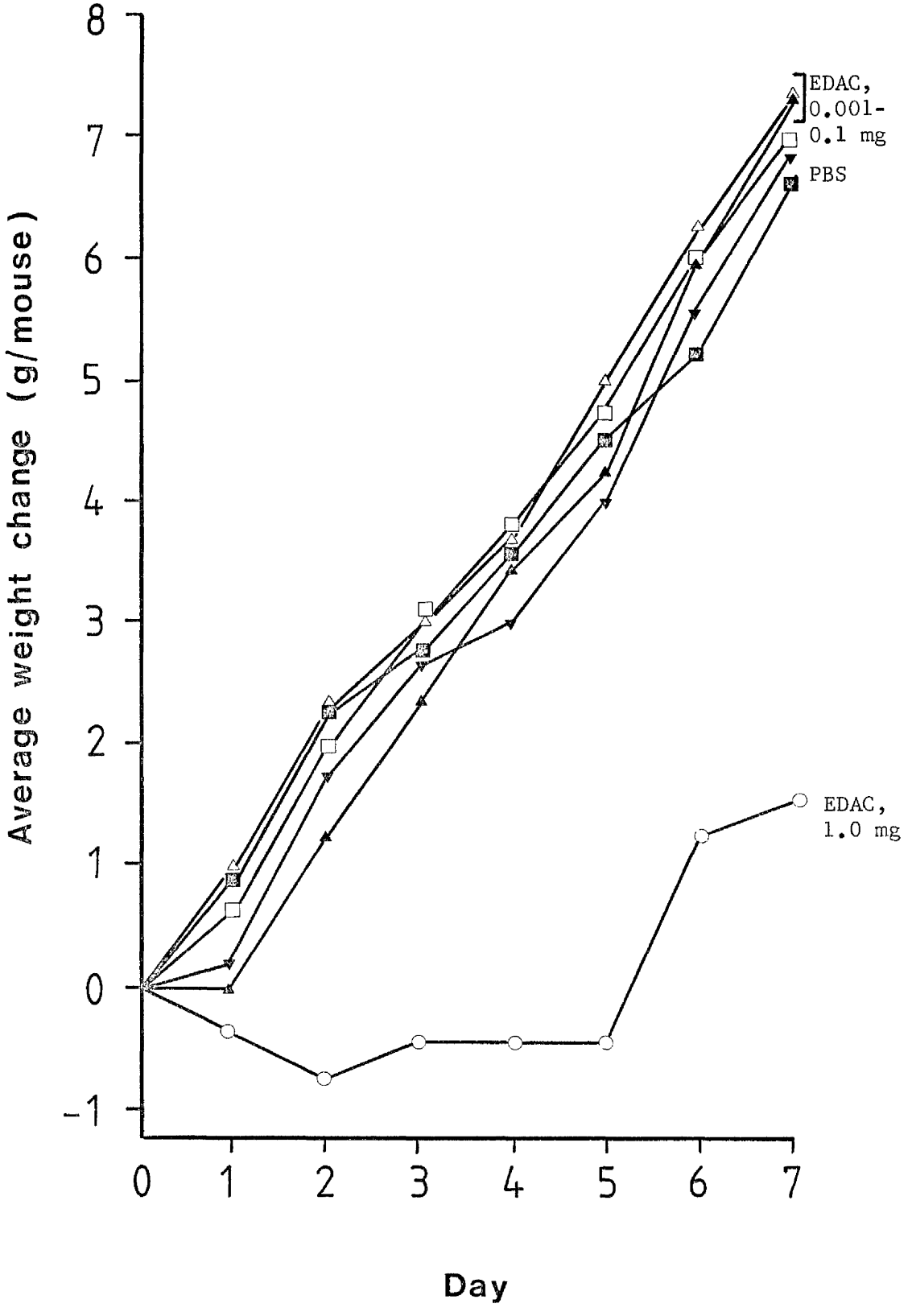
Figure 14. Acute toxicity of EDAC in a mouse-weight gain test

Groups of 3 mice of 3-4 weeks of age were injected ip with graded doses of EDAC in 20 mM sodium phosphate buffer, pH 7.6 containing 0.5M NaCl. Also, a solution of EDAC (4 mg ml^{-1}, ie: the amount present in the toxoiding mixture, p. 103) was dialyzed against 20 mM sodium phosphate buffer, pH 7.6, containing 0.5M NaCl, and mice were injected with 0.5 ml volumes of this sample. Other mice were injected with PBS or the dialysis buffer, pH 7.6, as controls.

Mice were weighed daily for 7 days and any deaths recorded.

	Sample injected	Dose/mouse
○——○	EDAC	1.0 mg
▲——▲	"	0.1 mg
△——△	"	0.01 or 0.001 mg
■——■	PBS	0.5 ml
□——□	20 mM sodium phosphate buffer, pH 7.6, + 0.5M NaCl	0.5 ml
▼——▼	Sample after dialysis	0.5 ml

No deaths were observed in any of the groups of mice injected with EDAC or the controls.


Average weight change (g/mouse)
8
7
6
5
4
3
2
1
0
-1
0
1
2
3
4
5
6
7
Day
EDAC, 0.001-0.1 mg
PBS
EDAC, 1.0 mg

of EDAC gained no body-weight on day 1 but gained weight normally thereafter. The other mice injected with 0.001-0.01 mg/mouse of EDAC or 0.5 ml/mouse of the dialyzed sample showed no signs of intoxication and gained weight normally (Fig. 14).

2.4 EFFECT OF UNTOXOIDED AND CARBODIIMIDE-TOXOIDED PREPARATIONS ON THE CHEMILUMINESCENCE OF RABBIT NEUTROPHILS IN RESPONSE TO A CHEMOTACTIC PEPTIDE

In a series of *in vitro* experiments, rabbit peritoneal neutrophils were pre-treated with various samples and stimulated with the chemotactic peptide N-formyl methionyl-leucyl-phenylalanine (fMLP) and the chemiluminescence-enhancing reagent luminol (p. 101). The following samples were assayed: (i) untoxoided preparation AP-16 (Table 8) and toxoid (AP-16)T29 (Table 16), (ii) purified FHa (Porton) and EDAC-treated purified FHa. The rationale was to develop a possible *in vitro* assay for toxoids in the knowledge that the presence of active PT inhibits the chemiluminescence response.

(i) Effect of untoxoided preparation AP-16 on the chemiluminescence response.

The normal response of rabbit neutrophils to fMLP is shown in Fig. 15 as a primary peak response followed by a secondary peak response which gradually falls towards background (0.8-1.0 mV). Rabbit neutrophils without the stimulus did not respond and gave a constant background of 0.8-1.0 mV.

The untoxoided preparation caused a dose-dependent suppression of chemiluminescence with almost complete suppression occurring with 50 ng/assay tube of AP-16 (Fig. 15). A dose of 10 ng/assay tube caused a suppression of the secondary peak and a slight reduction in the primary peak, whereas 2 ng/assay tube of AP-16 did not affect the chemiluminescence response (Fig. 15).

Figure 15. Effect of different amounts of AP on the chemiluminescence of rabbit neutrophils in response to fMLP and luminol

Rabbit peritoneal neutrophils were pre-treated with untoxoided preparation AP-16 for 1h at 37°C and stimulated with fMLP and luminol (p. 101). The response was observed for 1h after stimulation, but only the most important features occurring within 30 min are shown.

a, control : no pre-treatment

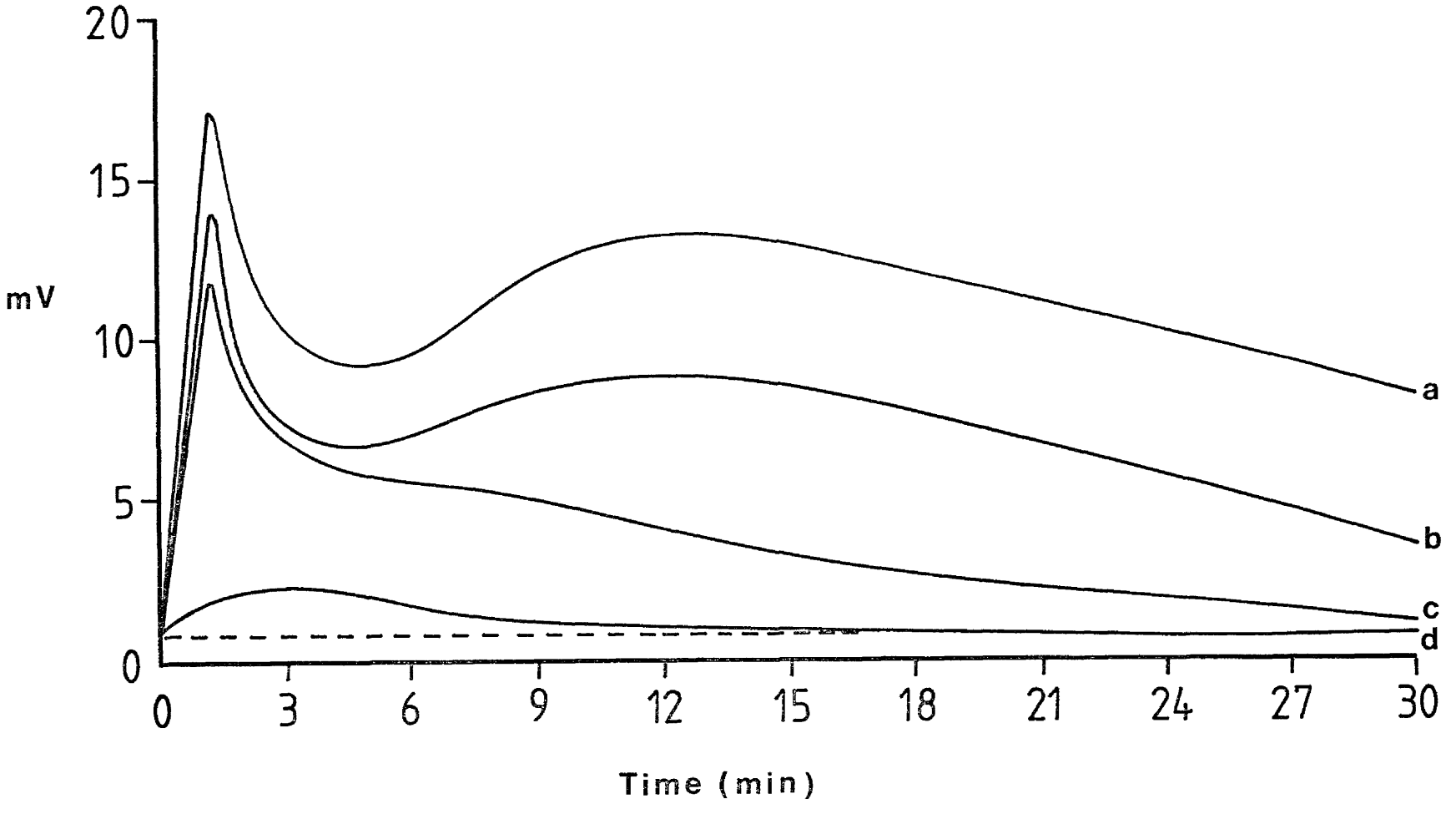
b, rabbit neutrophils pre-treated with 2 ng of AP-16

c, " " " " 10 " " "

d, " " " " 50 " " "

- - -, " " without fMLP stimulus, ie: background.

mV, the measurement of light-enhancement in millivolts.


20
15
10
5
0
mV
0
3
6
9
12
15
18
21
24
27
30
Time (min)
a
b
c
d

The experiment was repeated twice and similar results obtained.

(ii) Effect of toxoid on the chemiluminescence response.

Toxoid preparation (AP-16)T29, which had less than 0.7% of the HSA of untoxoided preparation AP-16 (Table 16), markedly enhanced the chemiluminescence of rabbit neutrophils in response to fMLP and luminol (Fig. 16). Doses of 10 μg or 1 μg/assay tube significantly enhanced the primary peak response, but no secondary peak response was observed at the maximum dose tested. A dose of 0.1 μg of (AP-16)T29 per assay tube gave a response comparable to the control, whereas 0.1 μg of the untoxoided preparation completely suppressed the response (Fig. 16).

Toxoid (AP-16)T29 was heated at 80^{o}C for 30 min in a water bath and assayed. A dose of 1.0 μg/assay tube of unheated toxoid significantly enhanced both primary and secondary peaks of the bimodal response (Fig. 17), whereas the heated toxoid manifested a response comparable to the control neutrophils without pre-treatment.

Both these experiments (Fig. 16, 17) were repeated and similar results obtained.

(iii) Effect of purified FHa and EDAC-treated FHa on the chemiluminescence response.

Since the untoxoided preparation suppressed the chemiluminescence response and the toxoid enhanced it, experiments were done to determine which component(s) of the toxoid was responsible for the enhancement. The most likely candidate was FHa.

Doses of 10 or 50 ng/assay tube of purified FHa significantly enhanced the primary and secondary peaks of the chemiluminescence response (Fig. 18), whereas a dose of 2 ng/assay tube gave a response comparable to the control. Filamentous haemagglutinin (50 ng/assay tube) did not enhance the chemiluminescence response above background in the

Figure 16. Effect of different amounts of toxoid on the chemiluminescence of rabbit neutrophils in response to fMLP and luminol

Rabbit peritoneal neutrophils were pre-treated for 1h at 37°C with graded doses of toxoid (AP-16)T29 (Table 16) or 0.1 μg of untoxoided preparation AP-16 before stimulation with fMLP and luminol. The response was observed for 1h after stimulation, but only the most important features occurring with 30 min are shown.

a, pre-treatment with (AP-16)T29, 10 μg

b, " " " 1 μg

c, control: no pre-treatment

d, pre-treatment with (AP-16)T29, 0.1 μg

- - -, pre-treatment with AP-16 (0.1 μg), or rabbit neutrophils not stimulated by fMLP.

mV, the measurement of light-enhancement in millivolts.

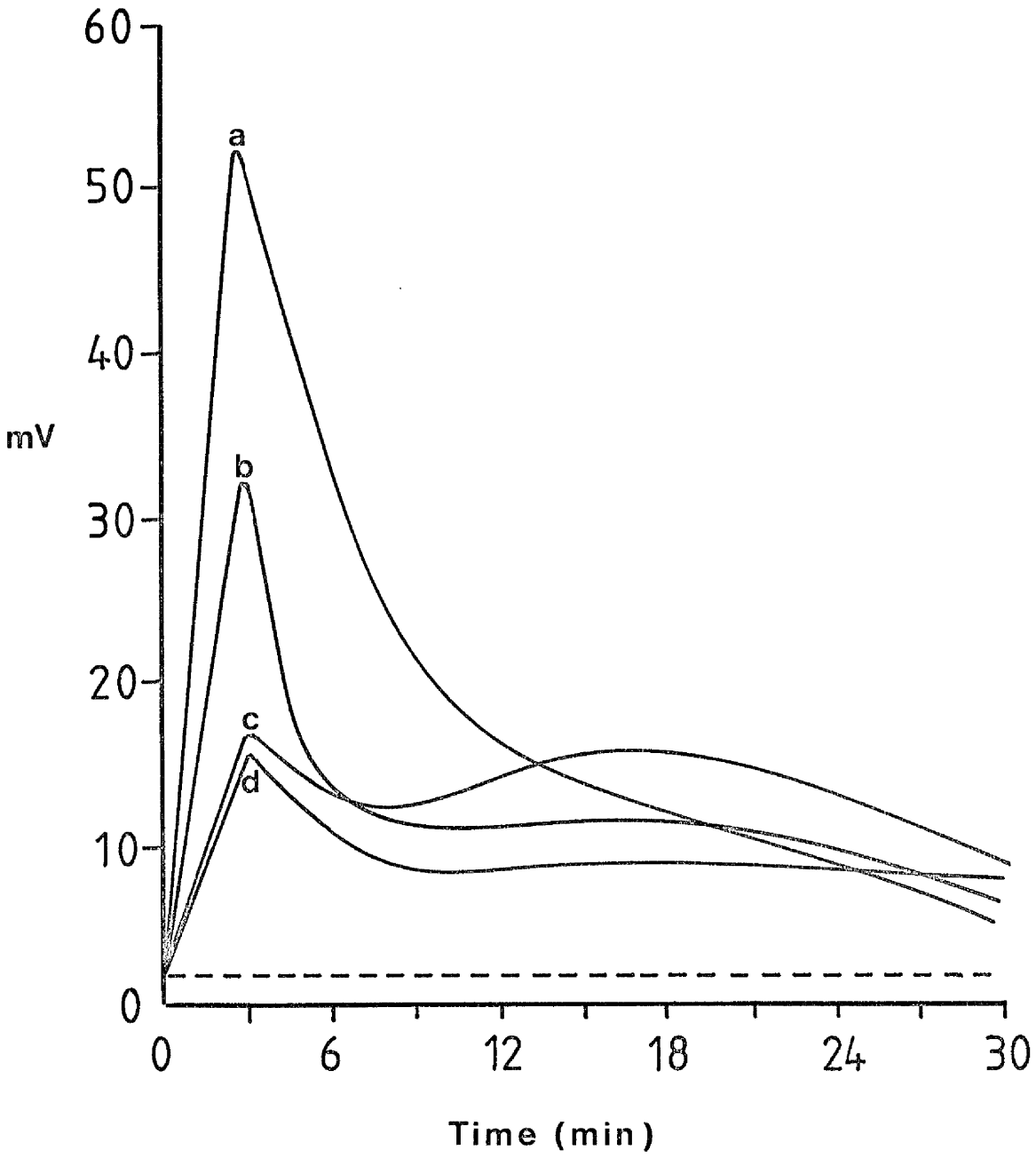

60
50
40
30
20
10
0
mV
a
b
c
d
0
6
12
18
24
30
Time (min)

Figure 17. Effect of unheated or heated toxoid on the chemiluminescence of rabbit neutrophils in response to fMLP and luminol

Rabbit peritoneal neutrophils were pre-treated for 1h at 37°C with heated or unheated toxoid and stimulated with fMLP and luminol (p. 101). The response was followed for 1h after stimulation, but only the most important features occurring within 30 min are shown.

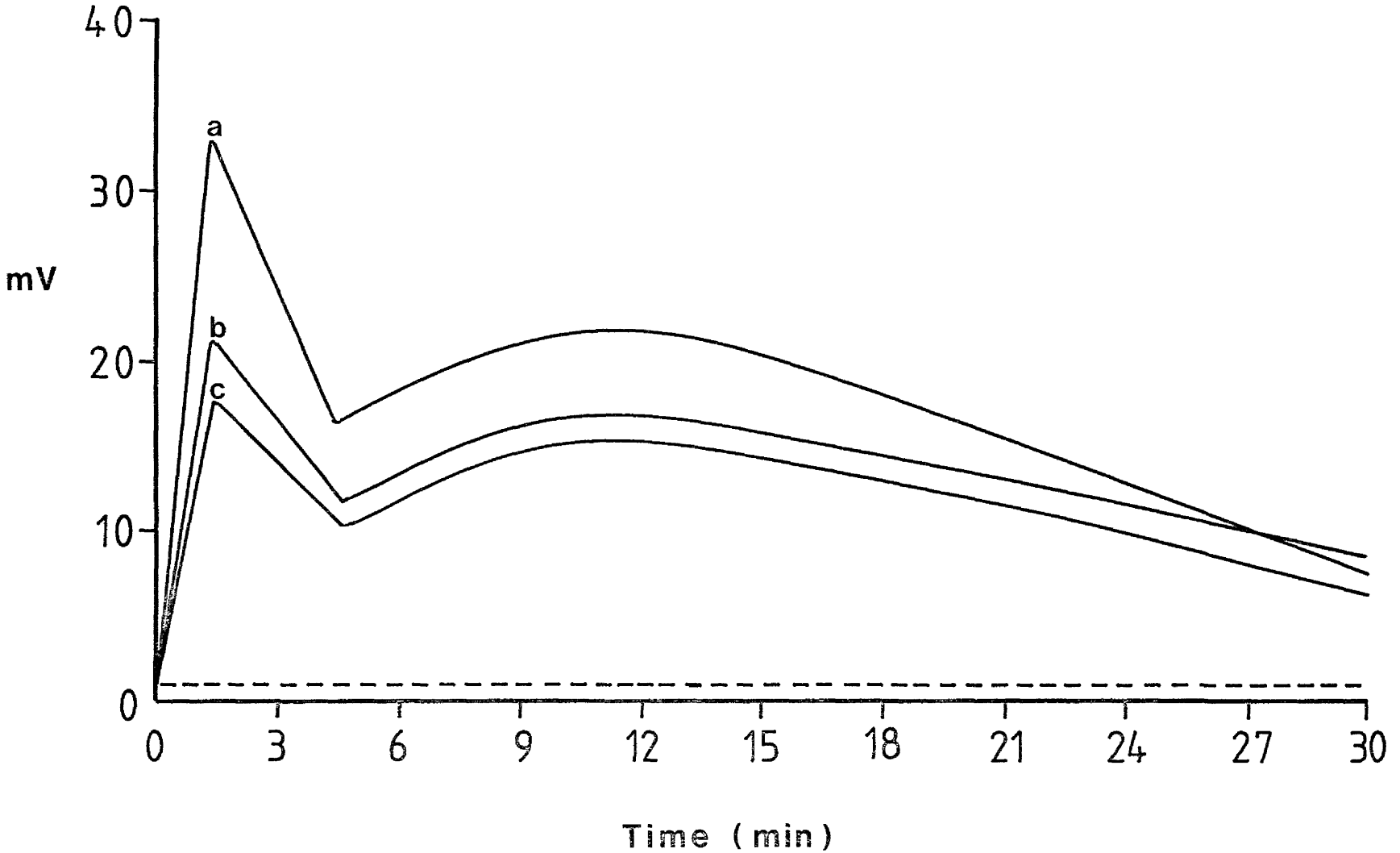
a, pre-treatment with unheated toxoid (AP-16)T29, 1.0 µg

b, " " heated " " 1.0 µg

c, control: no pre-treatment

- - -, rabbit neutrophils without fMLP stimulus.

mV, the measurement of light-enhancement in millivolts.


40
30
20
10
0
mV
a
b
c
0
3
6
9
12
15
18
21
24
27
30
Time (min)

absence of the stimulus fMLP (Fig. 18). This was also observed when the neutrophils were pre-treated with toxoid but not stimulated with fMLP.

Purified FHa was treated with EDAC and assayed. Without treatment, 0.05 μg/assay tube of FHa significantly enhanced the chemiluminescence of rabbit neutrophils, but the equivalent dose of carbodiimide-treated FHa only produced a slight enhancement compared to the control response (Fig. 19). A dose of 2.5 μg/assay tube of carbodiimide-treated FHa was required to enhance the chemiluminescence response to the level observed with 0.05 μg of unmodified FHa (Fig. 19).

Both these experiments using treated and untreated FHa preparations were repeated twice and similar results obtained.

The experimental data presented in this section is briefly summarized in Table 20.

Figure 18. Effect of different amounts of purified FHa on the chemiluminescence of rabbit neutrophils in response to fMLP and luminol

Rabbit peritoneal neutrophils were pre-treated for 1h at 37°C with graded doses of purified FHa and stimulated with fMLP and luminol (p. 101). The response was observed for 1h after stimulation, but only the most important features occurring within 30 min are shown.

a, pre-treatment with purified FHa, 50 ng

b, " " " " 10 ng

c, " " " " 2 ng

d, control: no pre-treatment

- - -, pre-treatment with FHa (50 ng) but no fMLP stimulus.

mV, the measurement of light-enhancement in millivolts.

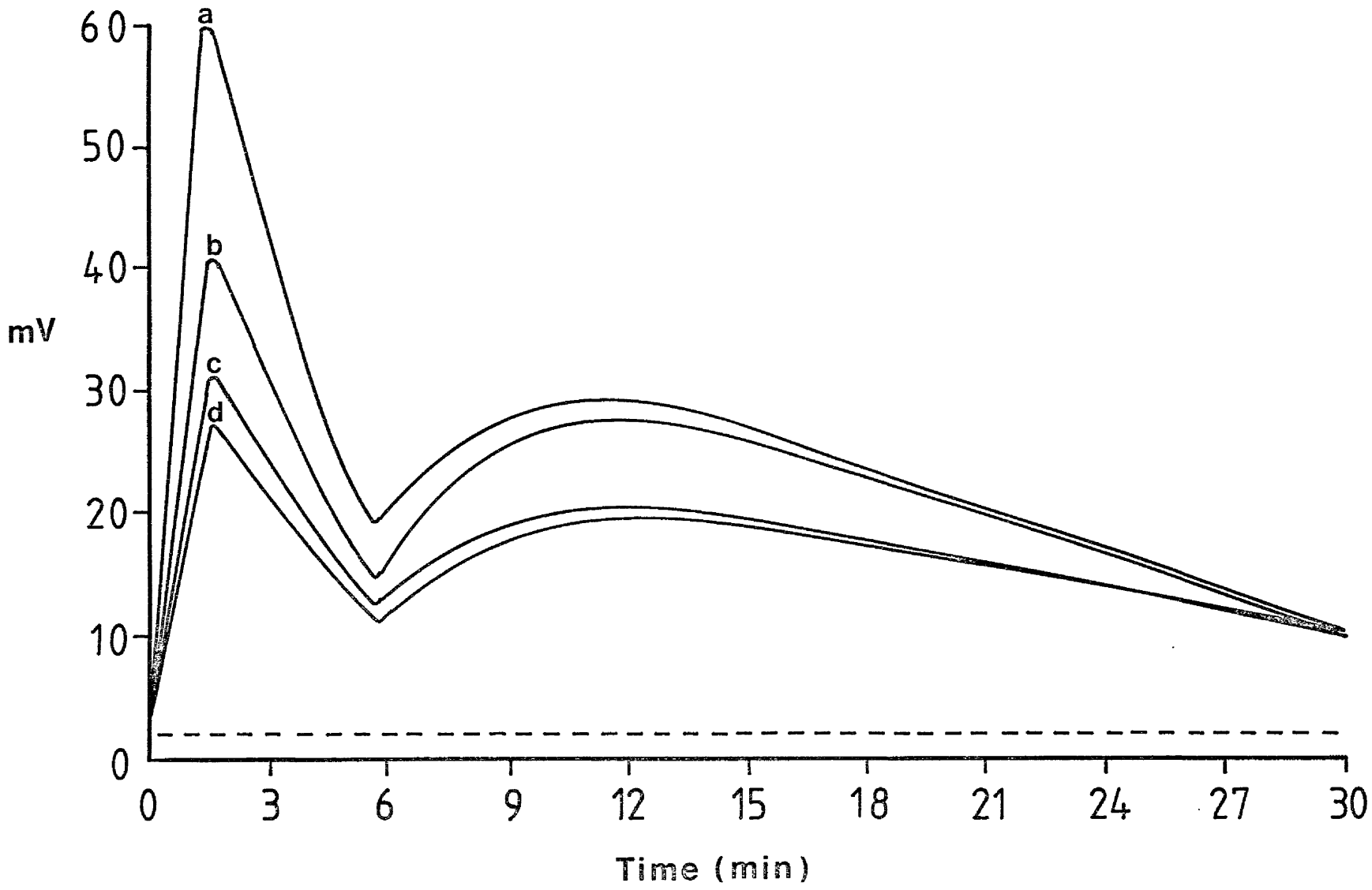

a
b
c
d
60
50
40
30
20
10
0
mV
0
3
6
9
12
15
18
21
24
27
30
Time (min)

Figure 19. Effect of carbodiimide-treated FHa on the chemiluminescence of rabbit neutrophils in response to fMLP and luminol

Purified FHa was treated with EDAC as described (p. 103) except that the preparation was dialyzed for 2 days against daily changes of HEPES buffered saline (HBS) without glucose and finally against HBS with glucose (1g L^{-1}). The control was FHa without EDAC-treatment.

Rabbit peritoneal neutrophils were pre-treated for 1h at 37°C with treated or untreated FHa and stimulated with fMLP and luminol. The response was observed for 1h after stimulation, but only the most important features occurring within 30 min are shown.

a, pre-treatment with 0.05 µg of untreated - FHa

b, " " 2.5 µg " treated - FHa

c, " " 0.5 µg " " "

d, " " 0.05 µg " " "

e, control: no pre-treatment

- - -, no fMLP stimulus to control neutrophils.

mV, the measurement of light-enhancement in millivolts.

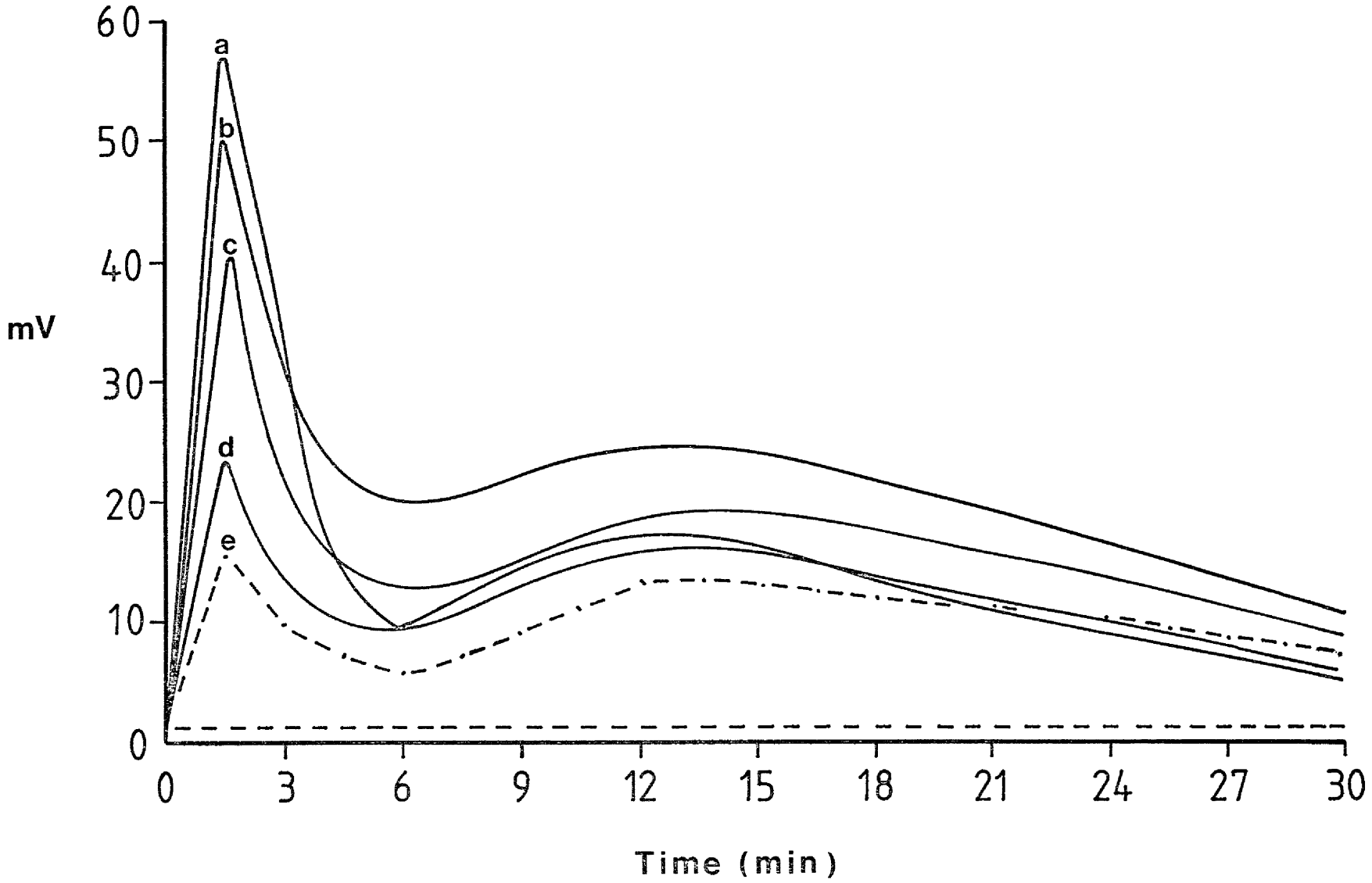

60
50
40
30
20
10
0
mV
a
b
c
d
e
0
3
6
9
12
15
18
21
24
27
30
Time (min)

Table 20. Toxoiding of antigen preparation (AP) with EDAC - a summary

Sample	Characteristics			
	HSA	LPA	LD_{50}	Effect on neutrophil chemiluminescence
Untoxoided preparation	HSD_{50}: 0.039-0.189 μg/mouse HSA present on challenge day 12	Doses as low as 1.5 μg/mouse significantly increase leucocytosis in mice	5-8.4 μg/mouse Toxic in MWGT	Inhibits response at 50-100 ng
Toxoid	>99% reduction in HSA No HSA on challenge day 12	No increase in leucocytosis at a dose of 27 μg/mouse*	> 27 μg/mouse* Non-toxic in MWGT	No inhibition of response at 10 μg*

*Highest doses tested in these assays

SECTION 3. STABILITY OF TOXOIDS

During this investigation, the stability of three independently-produced batches of toxoid was studied. The toxoids were tested during storage for histamine-sensitization, promotion of leucocytosis, hyperinsulinaemia and hypoglycaemia, toxicity and antigenicity in mice.

The stability of the following toxoids was studied: (AP-17)T33; (AP-2)T36, prepared by treatment of antigen preparation AP-2 with an EDAC to protein ratio of 40:1, with incubation for 2h at 37°C; and (AP-16)T37, prepared by treatment of antigen preparation AP-16 with an EDAC:protein ratio of 80:1 for 24h at 37°C.

3.1 HISTAMINE-SENSITIZING ACTIVITY (HSA)

In the first study, toxoid (AP-2)T36 had residual HSA when tested on day 0 (Table 21). Only 3/5 mice challenged with histamine survived, and the toxoid had approximately 0.6% of the original HSA of untoxoided preparation AP-2. In the second study, toxoid (AP-16)T37 did not sensitize any of the mice at a dose of 10 μg/mouse (Table 22), and therefore had less than 0.4% of the original HSA of untoxoided preparation AP-16.

In the third study, toxoid (AP-17)T33 was stored at -20°C for 14 days, and was therefore essentially equivalent to a preparation tested on day 0. All the mice immunized with 20 μg/mouse of this toxoid survived challenge with histamine (Table 23); this toxoid had less than 0.35% of the original HSA of untoxoided preparation AP-17.

Toxoids stored at 4°C for up to 56 days were stable. After 14 days at 4°C, the residual HSA of toxoid (AP-2)T36 was lost, since all the mice immunized with this preparation survived histamine challenge

(Table 21). Toxoid (AP-16)T37 showed no HSA after 14, 28 or 56 days at 4°C, when tested at a dose of 10 μg/mouse (Table 22). Similarly, toxoid (AP-17)T33 was stable at 4°C for up to 56 days, with no detectable HSA when tested at a dose of 20 μg/mouse (Table 23). Both these preparations still had less than 0.35-0.4% of the original HSA of their respective untoxoided preparations.

Toxoid (AP-16)T37, lyophilized and stored for 24 weeks, was also stable, with no HSA detectable at a dose of 10 μg/mouse (Table 24).

The toxoids were also stored at 37°C for up to 56 days. Under these extremely harsh conditions, all three toxoids showed partial reversion to HSA. After 14 days, toxoid (AP-2)T36 retained the HSA which was detected on day 0, but lost at 4°C (Table 21). Preparation (AP-16)T37 sensitized 9/10 mice to the lethal effect of a challenge of histamine when tested at a dose of 10 μg/mouse (Table 22) indicating reversion to toxicity.

In the preliminary studies, toxoids (AP-2)T36 (Table 21) or (AP-16)T37 (Table 22) were not stored for more than 14 days at 37°C. But, with the knowledge that partial reversion occurred with these preparations, toxoid (AP-17)T33 was kept at 37°C for up to 56 days (Table 23). When tested after 14 days, (AP-17)T33 at a dose of 10 μg/mouse, sensitized only 1/5 mice to the lethal effect of a histamine challenge. This possibly indicated some reversion to toxicity.

Toxoid (AP-17) showed reversion to detectable HSA after 28 days at 37°C (Table 23). The preparation had a HSD_{50} value of 6.68 μg/mouse (95% CL 4.63, 9.64) which was approximately a reversion to 1% of the original HSA of untoxoided preparation, AP-17. This activity was maintained at 56 days at 37°C.

Table 21. HSA of incompletely toxoided antigen preparation, (AP-2)T36, on storage

Sample code	Storage conditions	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
		µg/mouse		
AP-2	day 0	0.5	5/5	<0.06 µg*
		0.25	5/5	
		0.12	4/5	
		0.06	4/5	
(AP-2)T36	"	10.0	2/5	≥10 µg
		ou.ml/mouse		
Whole-cell vaccine (WCV)	"	0.75	5/5	0.22 ou.ml (0.1, 0.49)
		0.37	4/5	
		0.19	2/5	
		0.09	0/5	
		µg/mouse		
AP-2	14 days at 4°C	4.0	5/5	≤0.5 µg*
		2.0	5/5	
		1.0	5/5	
		0.5	3/5	
"	" 37°C	4.0	5/5	≤0.5 µg*
		2.0	5/5	
		1.0	5/5	
		0.5	3/5	
(AP-2)T36	" 4°C	10.0	0/5	>10 µg
"	" 37°C	10.0	2/5	≥10 µg
		ou.ml/mouse		
Whole-cell vaccine (WCV)	" 4°C	0.75	4/5	0.35 ou.ml (0.27, 0.46)
		0.37	3/5	
		0.19	1/5	
		0.09	0/5	

*Confidence limits not calculable from the data.

This stability study was discontinued after 14 days because of the residual toxicity of the toxoid preparation.

Table 22. HSA of toxoid preparation (AP-16)T37 on storage

Sample code	Storage conditions	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
		µg/mouse		
AP-16	day 0	1.0	5/5	0.043 µg*
		0.4	5/5	
		0.16	4/5	
		0.06	3/5	
(AP-16)T37	"	10.0	0/10	>10 µg
		ou.ml/mouse		
Whole-cell vaccine (WCV)	"	0.75	5/5	0.164 ou.ml (0.14,0.19)
		0.37	4/5	
		0.19	3/5	
		0.09	2/5	
		µg/mouse		
AP-16	14 days at 4°C	0.4	5/5	0.04 µg (0.02,0.07)
		0.16	5/5	
		0.06	3/5	
		0.03	2/5	
"	" 37°C	2.5	5/5	<0.16 µg*
		1.0	5/5	
		0.4	5/5	
		0.16	4/5	
(AP-16)T37	" 4°C	10.0	0/10	>10 µg
"	" 37°C	10.0	9/10	<10 µg
		ou.ml/mouse		
Whole-cell vaccine (WCV)	" 4°C	0.75	5/5	0.265 ou.ml (0.18,0.40)
		0.37	3/5	
		0.19	2/5	
		0.09	0/5	

* Confidence limits not calculable from the data.

Sample code	Storage conditions	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
		µg/mouse		
AP-16	28 days at 4°C	0.16	5/5	0.03 µg (0.02,0.04)
		0.06	4/5	
		0.03	2/5	
		0.01	0/5	
(AP-16)T37	"	10.0	0/10	>10 µg
		ou.ml/mouse		
Whole-cell vaccine (WCV)	"	0.75	5/5	0.268 ou.ml (0.18,0.40)
		0.37	4/5	
		0.19	3/5	
		0.09	2/5	
		µg/mouse		
AP-16	56 days at 4°C	0.16	5/5	0.03 µg (0.02,0.04)
		0.06	4/5	
		0.03	2/5	
		0.01	0/5	
(AP-16)T37	"	10.0	0/10	>10 µg
		ou.ml/mouse		
Whole-cell vaccine (WCV)	"	0.75	5/5	0.268 ou.ml (0.18,0.40)
		0.37	3/5	
		0.19	2/5	
		0.09	0/5	

Table 23. HSA of toxoid preparation (AP-17)T33 stored at various temperatures

Sample code	Storage conditions	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
		µg/mouse		
AP-17	14 days at -20°C	0.18	5/5	
		0.09	3/5	0.07 µg
		0.04	1/5	(0.06,0.09)
		0.02	0/5	
"	" 4°C	0.7	5/5	
		0.35	4/5	0.12 µg
		0.18	3/5	(0.08,0.18)
		0.09	2/5	
"	" 37°C	0.7	5/5	
		0.35	4/5	0.12 µg
		0.18	3/5	(0.08,0.18)
		0.09	2/5	
(AP-17)T33	" -20°C	20.0	0/5	>20 µg
"	" 4°C	20.0	0/5	>20 µg
"	" 37°C	10.0	1/5	>10 µg
		5.0	0/5	
		ou.ml/mouse		
Whole-cell vaccine (WCV)	" 4°C	0.75	5/5	
		0.37	4/5	0.219 ou.ml
		0.19	2/5	(0.18,0.27)
		0.09	0/5	

Sample code	Storage conditions	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
		µg/mouse		
AP-17	28 days at 37°C	0.35	5/5	
		0.18	3/5	0.147 µg
		0.09	1/5	(0.12,0.18)
		0.04	0/5	
(AP-17)T33	"	10.0	5/5	
		5.0	2/5	6.68 µg
		2.5	1/5	(4.63,9.64)
		1.25	0/5	
		ou.ml/mouse		
Whole-cell vaccine (WCV)	" 4°C	0.75	5/5	
		0.39	3/5	0.345 ou.ml
		0.19	1/5	(0.24,0.51)
		0.09	1/5	
		µg/mouse		
AP-17	56 days at 4°C	0.18	4/5	
		0.09	2/5	0.1 µg
		0.04	0/5	(0.08,0.13)
		0.02	0/5	
"	" 37°C	0.35	5/5	
		0.18	3/5	0.147 µg
		0.09	1/5	(0.12,0.18)
		0.04	1/5	
(AP-17)T33	" 4°C	20.0	0/5	>20 µg
"	" 37°C	10.0	5/5	
		5.0	2/5	6.68 µg
		2.5	1/5	(4.63,9.64)
		1.25	0/5	
		ou.ml/mouse		
Whole-cell vaccine (WCV)	" 4°C	0.75	5/5	
		0.37	2/5	0.5 ou.ml
		0.19	1/5	(0.35,0.73)
		0.09	0/5	

Table 24. HSA of lyophilized samples tested after storage for 24 weeks

Sample code	Storage conditions (24 weeks)	Dose	Mice (dead/challenged)	HSD_{50} (95% CL)
		µg/mouse		
AP-16	-20°C	0.4	5/5	0.04 µg (0.02,0.07)
		0.16	4/5	
		0.06	3/5	
		0.03	2/5	
"	Lyophilized	1.25	5/5	0.112 µg (0.08,0.17)
		0.25	4/5	
		0.05	1/5	
		0.01	0/5	
(AP-16)T37	"	10.0	0/10	>10 µg
		ou.ml/mouse		
Whole-cell vaccine (WCV)	"	0.75	3/5	0.32 ou.ml (0.25,0.40)
		0.37	3/5	
		0.19	1/5	
		0.09	0/5	

Toxoid preparation (AP-16)T37 and untoxoided preparation AP-16 were lyophilized and stored in stoppered vials in a dessicator at 4°C. After 24 weeks, the samples were resuspended in the 'reconstitution buffer' (Appendix 8) and tested.

The untoxoided preparations were also stored at 4°C or 37°C for up to 56 days as controls. There was no significant reduction in the HSA of preparations AP-16 or AP-17 at 4°C after 56 days. For example, preparation AP-16 tested on day 0 had a HSD_{50} value of approximately 0.043 μg/mouse, and after 56 days at 4°C, it was approximately 0.03 μg/mouse (95% CL 0.02, 0.04; Table 22). Similarly, AP-17 had a HSD_{50} value of approximately 0.07 μg/mouse (95% CL 0.06, 0.09) after 14 days at -20°C, and after 56 days at 4°C it was approximately 0.1 μg/mouse (95% CL 0.08, 0.13; Table 23), ie: approximately a 30% reduction in HSA.

After lyophilization and storage for 24 weeks and tested, untoxoided preparation AP-16 had a HSD_{50} value of approximately 0.112 μg/mouse (95% CL 0.08, 0.17; Table 24). This preparation retained approximately 38% of the original HSA of the preparation tested on day 0 (Table 22). Also, the untoxoided material showed no reduction in HSA after 24 weeks at -20°C (Table 24).

Untoxoided preparation AP-2 tested on day 0 had a HSD_{50} value of approximately 0.06 μg/mouse (Table 21) and after 14 days at 4°C or 37°C it was less than or equal to 0.5 μg/mouse. This possibly indicated some reduction in HSA but the data were not satisfactory and 95% confidence limits could not be calculated.

Preparation AP-16 (Table 22) showed a reduction in HSA when tested after 14 days at 37°C, but was not stored at this temperature for longer periods.

Untoxoided preparation AP-17 was still highly active even after 56 days at 37°C (Table 23), with a HSD_{50} value of approximately 0.147 μg/mouse (95% CL 0.12, 0.18). This preparation retained approximately 48% of the original HSA of untoxoided material tested after 14 days at -20°C.

There was no significant reduction in the HSA of whole-cell vaccine (WCV) after 56 days at 4°C in the stability study in Table 22. In this experiment, the HSD_{50} value of the vaccine tested on day 0 and after 56 days at 4°C was 0.164 ou.ml/mouse (95% CL 0.14, 0.19) and 0.268 ou.ml/mouse (95% CL 0.18, 0.40), respectively. Similarly, there was no reduction in the HSA of the vaccine after 14 days at 4°C in the preliminary study in Table 21. However, when the 95% confidence limits of the sample tested after 14 or 56 days at 4°C were compared (stability study, Table 23) there was a slight, but significant reduction in the HSA of the vaccine. This was an unexpected finding and might have been due to insufficient dispersion of the whole-cells before injection into animals.

3.2 LEUCOCYTOSIS-PROMOTING ACTIVITY (LPA)

In the preliminary study, toxoid preparation (AP-2)T36 which had residual HSA (Table 21), induced a significant increase in circulating leucocytes, with a geometric mean WBC/mm^3 count of 12,700 (95% CL 10,800, 15,000; Table 25). This represented approximately a 2-3 fold increase in leucocyte count when compared to mice injected with the diluent, PBS. There was approximately a 6-fold difference in the geometric mean WBC/mm^3 counts in mice injected with 0.3 μg/mouse of untoxoided preparation AP-2 or 10 μg/mouse of toxoid (Table 25). On this basis, the toxoid had much less than 3% of the original LPA of untoxoided material when tested on day 0.

In the second study, toxoid preparation (AP-16)T37 which had no detectable HSA (Table 22), also had no detectable LPA at a dose of 10 μg/mouse, when tested on day 0 (Table 26).

The toxoids stored at 4°C were stable for up to 56 days.

Toxoid (AP-2)T36 which had residual LPA when tested on day 0, did not induce a significant leucocytosis when tested after 14 days at 4°C (Table 25): the residual HSA of this preparation was also lost during this storage (see above). Toxoid preparation (AP-16)T37 was also stable at 4°C, with no detectable LPA in mice when tested after 14, 28 or 56 days (Table 26).

Toxoid (AP-16)T37 when tested after lyophilization and storage for 24 weeks (Table 27), induced a geometric mean WBC/mm^3 count of 8,500 (95% CL 6,900, 10,400). This was not significantly different from the geometric mean response of control mice injected with PBS, indicating that the toxoid was stable to lyophilization and prolonged storage.

Both toxoids (AP-2)T36 and (AP-16)T37 showed partial reversion to HSA when tested after 14 days at 37°C (see above). A similar reversion to detectable LPA was also observed. Toxoid (AP-2)T36 tested after 14 days at 37°C, induced a geometric mean WBC/mm^3 count of 33,500 (95% CL 30,300, 39,600) at a dose of 10 μg/mouse (Table 25). This was a highly significant increase in leucocytosis when compared to the geometric mean response in mice injected with PBS as controls. Similarly, toxoid (AP-16)T37 showed a partial reversion to LPA (Table 26). When tested after 14 days at 37°C, a dose of 10 μg/mouse induced a geometric mean WBC/mm^3 count of 16,900 (95% CL 14,200, 20,000), which was a slight, but significant increase in leucocytosis when compared to the control (Table 26). No samples of either toxoid were stored for more than 14 days at 37°C, and the LPA of toxoid (AP-17)T33 was not tested during storage.

Untoxoided antigen preparations AP-2 and AP-16 were also stored at 4°C or 37°C as controls. When tested on day 0, both preparations were highly active in promoting leucocytosis in mice. Untoxoided

preparation AP-2 was lethal for mice at doses of 1.25 μg/mouse or more (Fig. 20b; Table 25). At non-lethal doses highly significant leucocytosis was observed, eg: 0.3 μg/mouse of AP-2 elevated the WBC count to levels of 76,600/mm^3 (95% CL 65,300, 89,800), which was approximately a 15-fold increase in circulating leucocytes compared to the controls. With preparation AP-16, as little as 0.12 μg/mouse induced a significant increase in leucocytosis,when compared to the geometric mean response of mice injected with PBS as controls (Table 26).

When tested after 14 days at 4°C, there was a slight decrease in the LPA of untoxoided preparation AP-2. For example, when tested on day 0 a dose of 0.6 μg/mouse induced a WBC/mm^3 count of 83,900 (95% CL 71,500, 98,600), whereas after storage the geometric mean WBC/mm^3 count was 62,200 (95% CL 58,100, 66,600). Also, doses of 5.0 and 2.5 μg/mouse were not as lethal when tested after storage, although the surviving mice had leucocyte counts of over 100,000 WBC/mm^3 (Table 25). When tested after storage at 37°C for 14 days, untoxoided preparation AP-2 was non-lethal at doses of 1.25, 2.5 or 5.0 μg/mouse, but induced geometric mean WBC/mm^3 counts of 78,000 to 107,500 (Table 25).

There was no decrease in the LPA of untoxoided preparation AP-16 tested after 14, 28 or 56 days at 4°C or 14 days at 37°C (Table 26), or after lyophilization and storage for 24 weeks (Table 27).

In the preliminary study (Table 25), there was a slight reduction in the LPA of whole-cell vaccine (WCV) tested after 14 days at 4°C, when compared to the activity of the preparation tested on day 0. In the second study (Table 26), there was no decrease in LPA after 14, 28 or 56 days at 4°C.

Table 25. LPA of incompletely toxoided antigen preparation,(AP-2)T36, on storage

Sample code	Storage conditions	Dose	No. of mice tested	Leucocyte counts (WBC/mm^3, x 10^3) Geometric mean (95% CL)
		µg/mouse		
AP-2	day 0	5.0	1+	68.2
		2.5	1+	94.2
		1.25	1+	66.5
		0.6	5	83.9 (71.5, 98.6)
		0.3	5	76.6 (65.3, 89.8)
(AP-2)T36	"	10.0	5	12.7 (10.8, 15.0)
		ou.ml/mouse		
Whole-cell vaccine (WCV)	"	5.0	5	51.7 (46.0, 58.0)
		ml/mouse		
PBS*	"	0.5	5	5.1 (4.6, 5.6)
		µg/mouse		
AP-2	14 days at 4°C	5.0	3	121.0 (113.6, 129.0)
		2.5	3	110.5 (104.0, 117.0)
		1.25	5	85.6 (82.5, 88.8)
		0.6	5	62.2 (58.1, 66.6)
"	" " 37°C	5.0	5	107.5 (90.8, 127.2)
		2.5	5	107.5 (101.5, 114.0)
		1.25	5	78.0 (66.6, 91.4)
(AP-2)T36	" " 4°C	10.0	5	9.5 (7.5, 11.9)
"	" " 37°C	10.0	5	33.5 (30.3, 39.6)
		ou.ml/mouse		
Whole-cell vaccine (WCV)	" " 4°C	5.0	5	33.9 (29.9, 38.5)
		ml/mouse		
PBS*	" " "	0.5	5	9.2 (8.7, 9.5)

*PBS contained gelatin (0.1% w/v) and thimerosal (0.01% (w/v) final concentration) as a preservative. Mice were injected iv with samples and leucocyte counts done as described (p. 88).
+4/5 mice died within 5 days of injection with sample dose.

Table 26. LPA of toxoid preparation (AP-16)T37 on storage

Sample code	Storage conditions	Dose	No. of mice tested	Leucocyte counts ($WBC/mm^3, x10^3$) Geometric mean (95% CL)
		µg/mouse		
AP-16	day 0	3.0	5	78.7 (66.6 , 93.0)
		0.6	5	36.6 (31.8 , 42.1)
		0.12	5	20.4 (17.6 , 23.6)
		0.02	5	10.2 (9.3 , 11.1)
(AP-16)T37	"	10.0	10	10.7 (9.6 , 12.1)
		ou.ml/mouse		
Whole-cell vaccine (WCV)	"	5.0	5	57.4 (49.1 , 67.2)
		ml/mouse		
PBS*	"	0.5	5	9.2 (7.6 , 11.2)
		µg/mouse		
AP-16	14 days at 4°C	3.0	5	96.6 (88.3 ,105.7)
		0.6	5	37.0 (26.0 , 52.8)
		0.12	5	17.8 (13.6 , 23.3)
"	" 37°C	9.0	3+	108.3 (89.8 ,130.7)
		3.0	5	68.3 (53.7 , 87.1)
		0.6	5	24.3 (20.2 , 29.1)
(AP-16)T37	" 4°C	10.0	10	9.6 (8.0 , 11.6)
"	" 37°C	10.0	10	16.9 (14.2 , 20.0)
		ou.ml/mouse		
Whole-cell vaccine (WCV)	" 4°C	5.0	5	55.7 (45.5 , 68.3)
		ml/mouse		
PBS*	" "	0.5	5	11.0 (8.7 , 13.9)

		ug/mouse		
AP-16	28 days at 4°C	3.0	5	73.1 (58.1, 92.1)
		0.6	5	25.2 (19.9, 31.8)
		0.12	5	19.9 (14.3, 27.6)
(AP-16)T37	"	10.0	10	8.7 (7.7, 10.3)
		ou.ml/mouse		
Whole-cell vaccine (WCV)	"	5.0	5	47.1 (39.1, 56.8)
		ml/mouse		
PBS*	"	0.5	5	10.4 (9.0, 12.1)
		ug/mouse		
AP-16	56 days at 4°C	3.0	5	64.0 (56.1, 7.31)
		0.6	5	15.2 (12.6, 18.3)
		0.12	5	12.1 (10.6, 13.0)
(AP-16)T37	"	10.0	10	8.6 (7.7, 9.6)
		ou.ml/mouse		
Whole-cell vaccine (WCV)	"	5.0	5	57.2 (44.3, 73.9)
		ml/mouse		
PBS*	"	0.5	5	6.6 (5.2, 8.5)

*PBS contained gelatin (0.1% w/v) and thimerosal (0.01% (w/v) final concentration) as a preservative.
Mice were injected iv with test samples and leucocyte counts done as described (p. 88).
+2/5 mice died within 5 days of injection of sample.

Table 27. LPA of lyophilized samples tested after 24 weeks at $4^{o}C$

Sample code	Dose	No. of mice tested	Leucocyte counts (WBC/mm^3, x 10^3) Geometric mean (95% CL)
	µg/mouse		
AP-16	3.0	5	86.7 (59.1, 127.3)
	0.6	5	20.8 (15.9, 27.2)
	0.12	5	14.3 (12.8, 15.9)
	0.02	5	7.8 (6.7, 9.1)
(AP-16)T37	10.0	10	8.5 (6.9, 10.4)
	ou.ml/mouse		
Whole-cell vaccine (WCV)	5.0	5	36.1 (20.2, 63.8)
	ml/mouse		
PBS*	0.5	5	9.1 (7.2, 11.7)

*PBS contained gelatin (0.1% w/v) and thimerosol (0.01% (w/v) final concentration) as a preservative, and was freshly prepared for this experiment.

Lyophilized samples were stored at $4^{o}C$ in glass stoppered vials (in a dessicator with phosphorous pentoxide, P_2O_5). The lyophilized samples were resuspended in the 'reconstitution buffer' (Appendix 8) and diluted in PBS*. Mice were injected iv with test samples and leucocyte counts done as described (p. 88).

3.3 INDUCTION OF HYPOGLYCAEMIA AND HYPERINSULINAEMIA IN MICE

The ability of toxoid preparation (AP-17)T33, stored at 4°C or 37°C for up to 56 days, to induce hypoglycaemia and/or hyperinsulinaemia in mice was studied (Table 28). These activities are associated with the A-protomer of PT, whereas histamine-sensitization and promotion of leucocytosis are dependent on the B-oligomer (Nogimori et al., 1984a).

(i) Hypoglycaemia

The minimum detectable amount of untoxoided preparation AP-17 or whole-cell vaccine (WCV) which induced a significant hypoglycaemia in mice was 0.5 μg/mouse and 0.75 ou.ml/mouse respectively, at the p = 0.005 level (Table 28).

Toxoid (AP-17)T33 kept at -20°C for the duration of the experiment did not induce hypoglycaemia in mice. Similarly, no significant hypoglycaemia was induced in mice injected with toxoid after 14, 28 or 56 days at 4°C or 37°C (Table 28). There was indication of a slight hypoglycaemia in mice injected with toxoid stored at 37°C for 56 days, but this was not significantly different from the control (PBS), at the p = 0.005 level.

(ii) Hyperinsulinaemia

The minimum detectable amount of activity, at the p = 0.005 level in this assay, was 0.5 μg of AP-17/mouse (Table 28). The whole-cell vaccine (WCV) induced a slight hyperinsulinaemia in mice at a dose of 0.75 ou.ml/mouse, but this was not significantly different from the control (PBS).

Toxoid kept at -20°C or stored at 4°C for 56 days did not induce hyperinsulinaemia at a dose of 10 μg/mouse (Table 28). However, when tested after 14 days at 37°C, toxoid (AP-17)T33 induced a significant hyperinsulinaemia in mice when compared to the control (PBS). By con-

Table 28. Induction of hypoglycaemia and hyperinsulinaemia in mice with various preparations: measurement of serum glucose and insulin levels

Sample code	Storage conditions	Dose	Serum Glucose, mMol L^{-1} (Mean and Standard deviation)*	Serum Immunoreactive Insulin ($ngml^{-1}$) Geometric mean (95% CL)*
		µg/mouse		
AP-17	Not stored under test conditions (-20°C)	2.5	8.8 ± 1.15	33.05 (6.00 , 181.7)
		0.5	9.22 ± 0.54	11.19 (3.40 , 36.7)
		0.1	10.88 ± 0.55	5.77 (2.30 , 14.7)
		0.02	11.44 ± 0.81	2.33 (1.40 , 3.8)
(AP-17)T33	"	10.0	11.8 ± 0.80	2.04 (1.40 , 3.0)
	14 days at 4°C	"	12.2 ± 0.40	2.56 (1.24 , 5.3)
"	" " 37°C	"	11.1 ± 0.60	11.66 (3.46 , 39.3)
"	28 " "	"	11.3 ± 1.20	4.23 (1.94 , 9.2)
"	56 " 4°C	"	12.3 ± 0.44	1.63 (1.19 , 2.2)
"	" " 37°C	"	10.3 ± 1.63	7.35 (2.60 , 20.6)
		ou.ml/mouse		
Whole-cell vaccine (WCV)	Not stored under test conditions	0.75	10.1 ± 0.65	4.85 (2.15 , 10.9)
		0.37	11.7 ± 0.41	1.88 (1.30 , 2.7)
		0.19	12.7 ± 0.21	4.56 (1.95 , 10.7)
		0.09	12.3 ± 0.99	1.45 (1.14 , 1.8)
		ml/mouse		
PBS	"	0.5	11.7 ± 0.36	1.59 (1.18 , 2.1)

Samples of toxoid (AP 17)T33 were removed after 14, 28 and 56 days at 4°C or 37°C and kept at -20°C until assayed. Also from day 0 of the stability study, a sample of untoxoided preparation AP-17 and toxoid (AP-17)T33 were kept at -20°C as controls. These preparations were tested for HSA after 14 days and the data shown in Table 23.

*Compared by Student's t-Test (p = 0.005).

structing a standard curve of immunoreactive insulin, IRI (ng ml^{-1}) versus $\log_{10}$ µg AP-17/mouse, and by interpolation onto that curve, the toxoid showed approximately a 5% reversion to hyperinsulinaemia activity.

When tested after 28 or 56 days at 37°C, the toxoid still induced a slight hyperinsulinaemia in mice, but this was not significantly different from the control (PBS) at the $p = 0.005$ level (Table 28).

3.4 MOUSE-WEIGHT-GAIN TEST (MWGT)

In all three stability studies, the toxicity of carbodiimide-toxoided antigen preparations was monitored in a mouse-weight-gain test (MWGT). In the preliminary study, mice injected with toxoid (AP-2)T36, which had residual HSA (Table 21) and LPA (Table 25) when tested on day 0, gained weight normally (Fig. 20a). Similarly, mice injected with the toxoid stored at 4°C or 37°C for 14 days also gained weight normally (Fig. 21a).

Toxoid (AP-16)T37 tested on day 0 (Fig. 22a) and after storage at 4°C for 14 (Fig. 23a), 28 (Fig. 24a) or 56 days (Fig. 25a) did not affect normal mouse-weight-gain. Also, the toxoid which had detectable HSA (Table 22) and LPA (Table 26) when tested after 14 days at 37°C, was non-toxic (Fig. 23a).

Toxoid (AP-16)T37 was also lyophilized and stored at 4°C for 24 weeks. When tested in the MWGT, this preparation was also non-toxic at a dose of 10 µg/mouse (Fig. 26).

In the third study, mice injected with toxoid preparation (AP-17)T33 kept at -20°C for 14 days gained weight normally (Fig. 27). This trend was also observed with the toxoid tested after 14 (Fig. 28a) or 56 days (Fig. 28b) at 4°C. The toxoid stored at 37°C for 14 days and which induced a significant hyperinsulinaemia (Table 28), had no

adverse effect on mouse-weight-gain at a dose of 20 μg/mouse (Fig. 27). Similarly, normal mouse-weight-gain was unaffected when the toxoid was tested after 28 or 56 days at 37°C (Fig. 28a,b) when a reversion to HSA was observed (Table 23).

In summary, these toxoids were non-lethal and non-toxic for mice at the doses tested (see Figures). The slight reversion to histamine-sensitization, promotion of leucocytosis or hyperinsulinaemia activities with some of these toxoids under extremely adverse storage conditions, was also insufficient to affect normal weight-gain (see Figures).

Conversely, the untoxoided preparations were toxic and lethal for mice. When tested on day 0, preparation AP-2 was highly toxic for mice via the iv route. At all the doses tested, average weight-gain for mice was significantly retarded with numerous deaths also (Fig. 20b). From these data (Fig. 20b), a LD_{50} value for AP-2 was calculated by the probit method as 0.88 μg/mouse (95% CL 0.74, 1.06). The mouse-lethality of this preparation was reduced during storage at 4°C or 37°C for 14 days, but it was still toxic, in that normal weight-gain was significantly retarded (Fig. 21b,c).

Preparation AP-16 was not lethal when tested on day 0, and only a dose of 3 μg/mouse significantly retarded normal mouse-weight gain (Fig. 22b). However, when tested after 14 days at 37°C, mice injected with 3 μg/mouse of AP-16 gained weight normally, but a dose of 9 μg/mouse was lethal or toxic (Fig. 23b). Mice injected with 3 μg/mouse of the preparation stored at 4°C for 14 (Fig. 23c) or 28 days (Fig. 24b) did not gain weight normally, but after 56 days at 4°C this body-weight-decreasing toxicity was lost (Fig. 25b).

After lyophilization and storage for 24 weeks, preparation AP-16

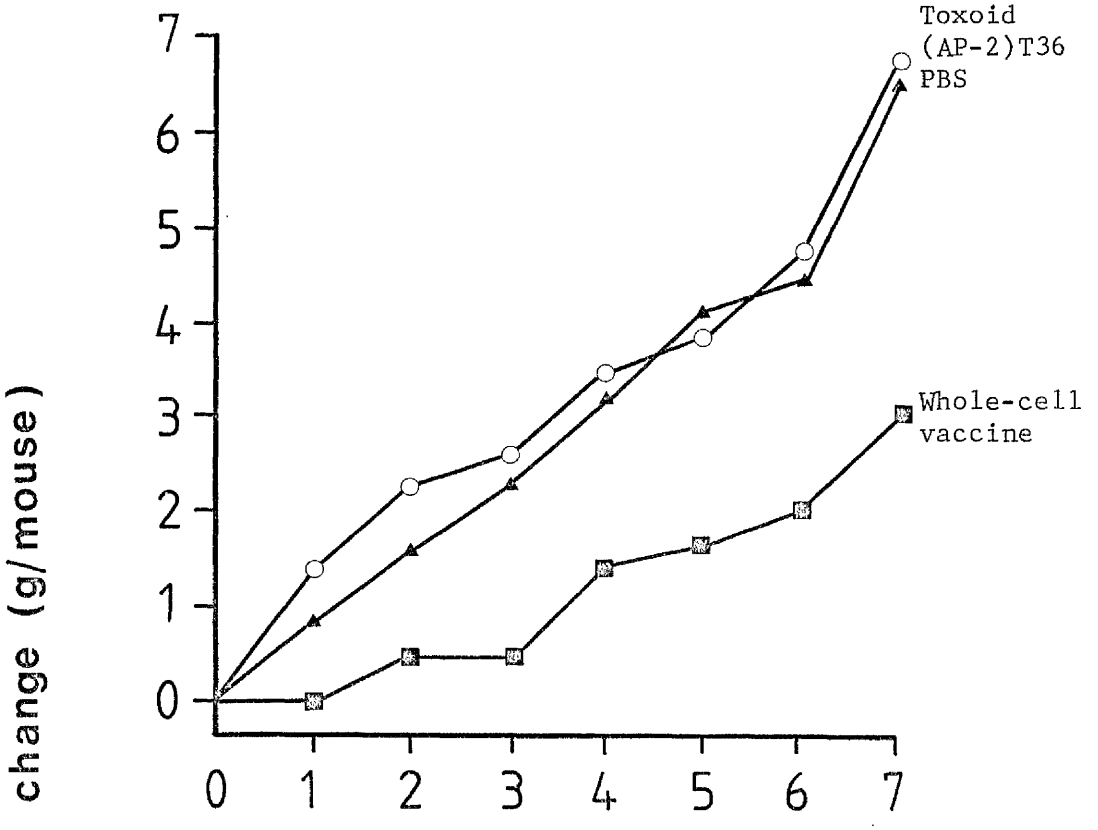
Figure 20. Mouse-weight-gain (toxicity) test for untoxoided preparation AP-2 and incompletely toxoided preparation (AP-2)T36 tested on day 0

Mice were injected iv with test samples, weighed daily for 7 days and any deaths recorded. (Leucocyte counts were also done 5 days after injection and these data shown in Table 25).

		Sample injected	Dose/mouse	Mice (dead/injected)
a.	▲——▲	PBS	0.5 ml	0/5
	○——○	Toxoid (AP-2)T36	10.0 μg	0/5
	◘——◘	Whole-cell vaccine (WCV)	5.0 ou.ml	0/5
b.	▲——▲	PBS	0.5 ml	0/5
	○——○	Untoxoided preparation, AP-2	5.0 μg	4/5
	▼——▼	"	2.5 μg	4/5
	□——□	"	1.25 μg	4/5
	▽——▽	"	0.6 μg	1/5
	◘——◘	"	0.3 μg	0/5

In Fig.(b) the average weight change (g/mouse) with AP-2 (1.25-5.0 μg/mouse) is only shown for the first three days.

(a)


Toxoid
(AP-2)T36
PBS
Whole-cell
vaccine
Average weight change (g/mouse)
0
1
2
3
4
5
6
7

(b)

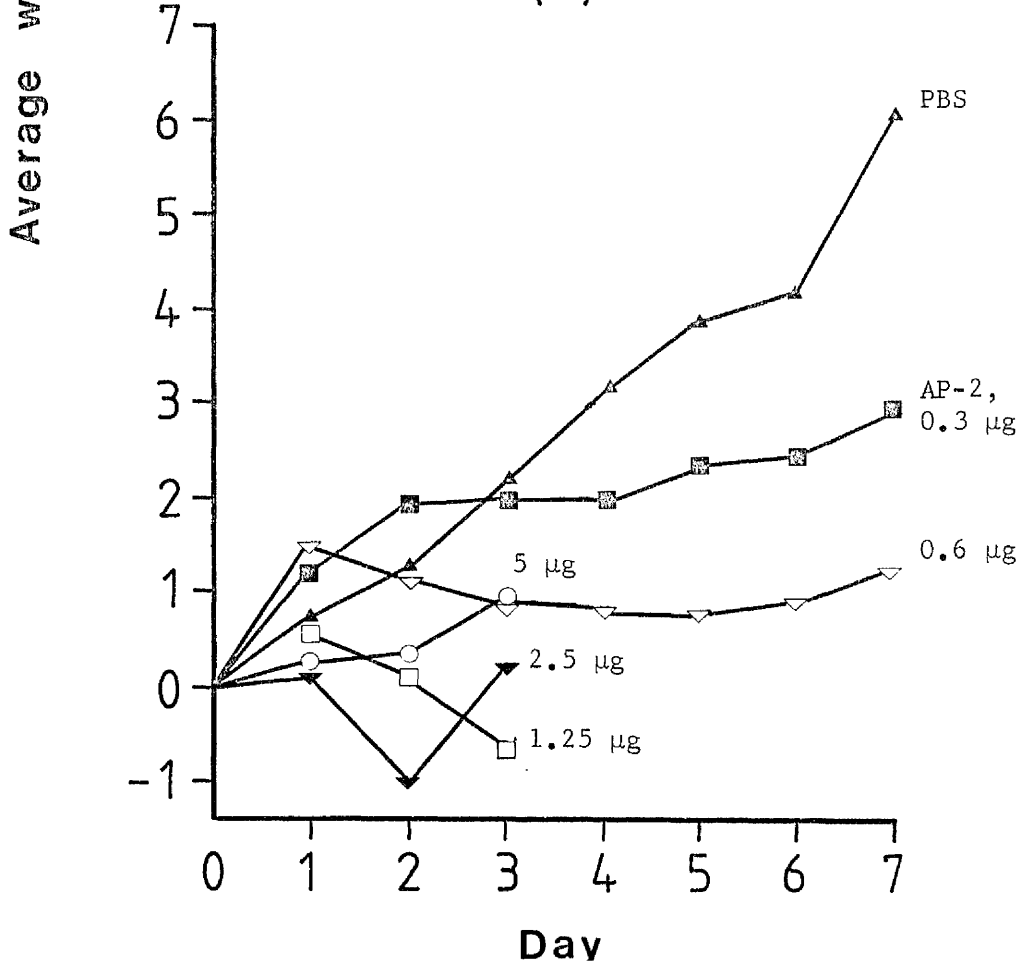

PBS
AP-2,
0.3 μg
0.6 μg
5 μg
2.5 μg
1.25 μg
-1
0
1
2
3
4
5
6
7
Day

Figure 21. Mouse-weight-gain (toxicity) test for untoxoided preparation AP-2 and toxoid (AP-2)T36 tested after storage

Mice were injected iv with samples stored for 14 days at 4°C or 37°C, weighed daily for 7 days and any deaths recorded. (Leucocyte counts were also done 5 days after injection and these data shown in Table 25).

	Sample injected	Storage conditions	Dose/ mouse	Mice (dead/ injected)
a. ▲——▲	PBS	14 days at 4°C	0.5 ml	0/5
○——○	(AP-2)T36	"	10.0 µg	0/5
△——△	"	" 37°C	"	0/5
■——■	Whole-cell vaccine (WCV)	" 4°C	5.0 ou.ml	0/5
b. ▲——▲	PBS	(as above)	(as above)	
◿——◿	AP-2	14 days at 37°C	5.0 µg	0/5
◢——◢	"	"	2.5 µg	0/5
□——□	"	"	1.25 µg	0/5
c. ▲——▲	PBS	(as above)	(as above)	
◿——◿	AP-2	14 days at 4°C	5.0 µg	2/5
◢——◢	"	"	2.5 µg	2/5
□——□	"	"	1.25 µg	0/5
▽——▽	"	"	0.6 µg	0/5

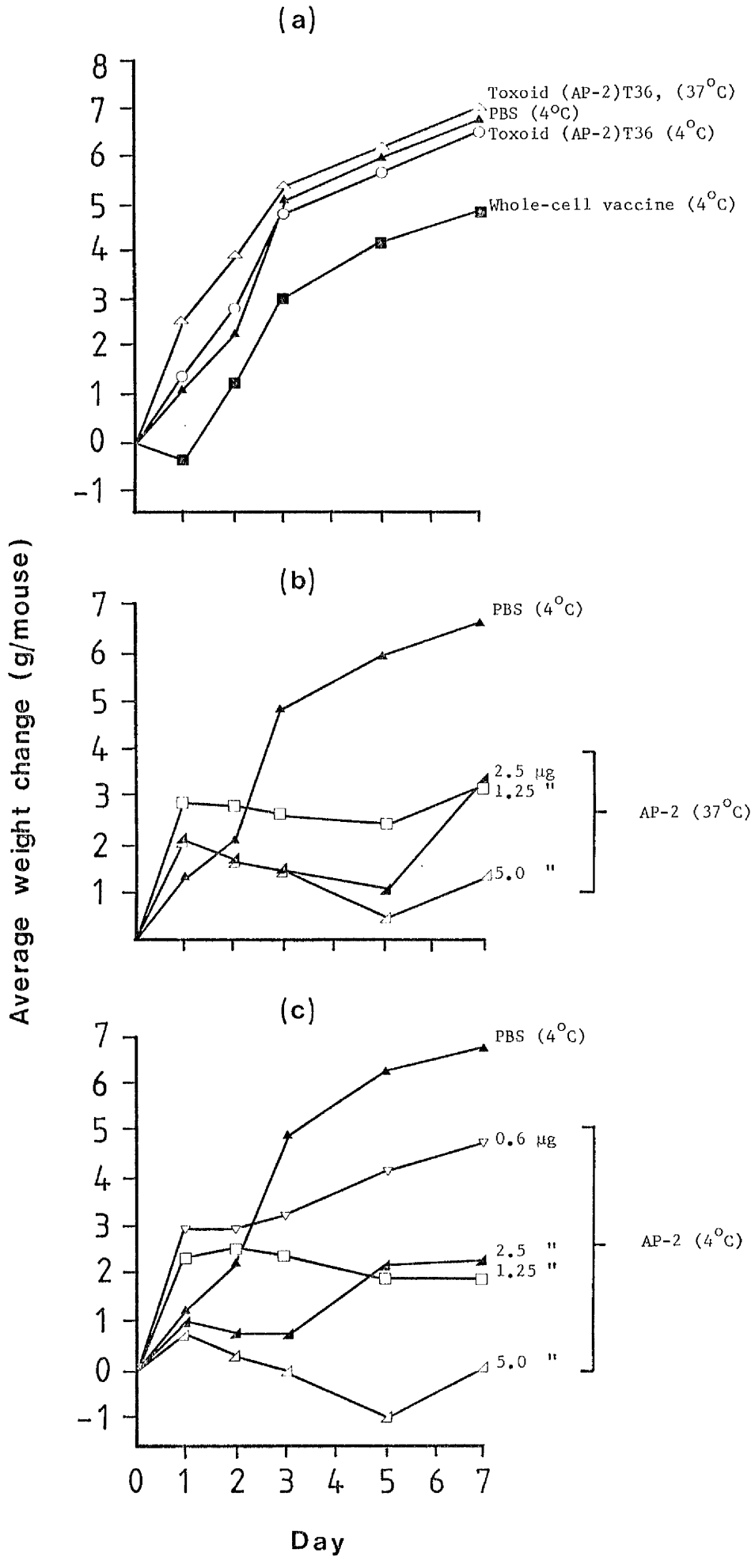

(a)
Toxoid (AP-2)T36, (37°C)
PBS (4°C)
Toxoid (AP-2)T36 (4°C)
Whole-cell vaccine (4°C)
(b)
PBS (4°C)
2.5 μg
1.25 "
5.0 "
AP-2 (37°C)
(c)
PBS (4°C)
0.6 μg
2.5 "
1.25 "
5.0 "
AP-2 (4°C)
Average weight change (g/mouse)
Day

Figure 22. Mouse-weight-gain (toxicity) test for untoxoided preparation AP-16 and toxoid (AP-16)T37 tested on day 0

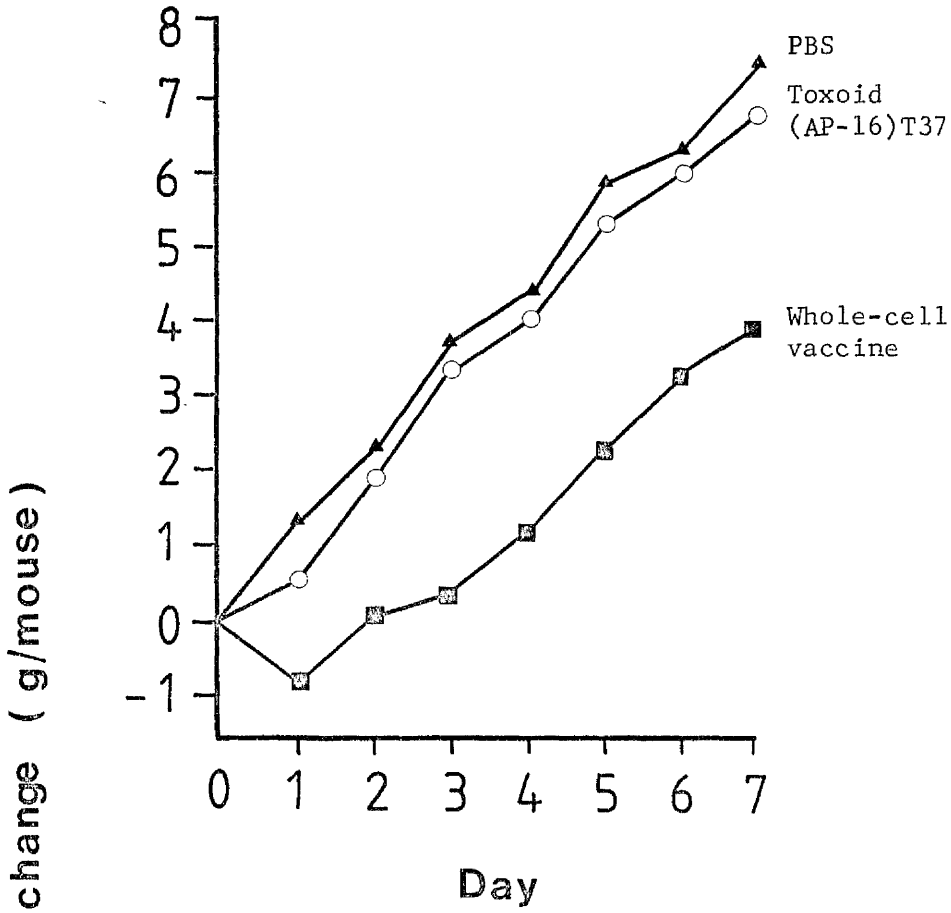
Groups of 5 mice were injected iv with graded doses of untoxoided preparation AP-16, or single doses of whole-cell vaccine or PBS; a group of 10 mice was injected with toxoid. Animals were weighed daily for 7 days, and any deaths recorded. (Leucocyte counts were also done 5 days after injection and these data shown in Table 26).

		Sample injected	Dose/mouse
a.	▲——▲	PBS	0.5 ml
	○——○	(AP-16) T37	10.0 μg
	◘——◘	Whole-cell vaccine (WCV)	5.0 ou.ml
b.	▲——▲	PBS	(as above)
	◢——◢	AP-16	3.0 μg
	◿——◿	"	0.6 μg
	□——□	"	0.12 μg
	▼——▼	"	0.02 μg

In Fig. (b) mice injected with the untoxoided preparation were not weighed after 5 days.

No deaths were observed in mice injected with the untoxoided or toxoided preparations, whole-cell vaccine or PBS.

(a)


Average weight change (g/mouse)
8
7
6
5
4
3
2
1
0
-1
0 1 2 3 4 5 6 7
Day
PBS
Toxoid
(AP-16)T37
Whole-cell
vaccine

(b)

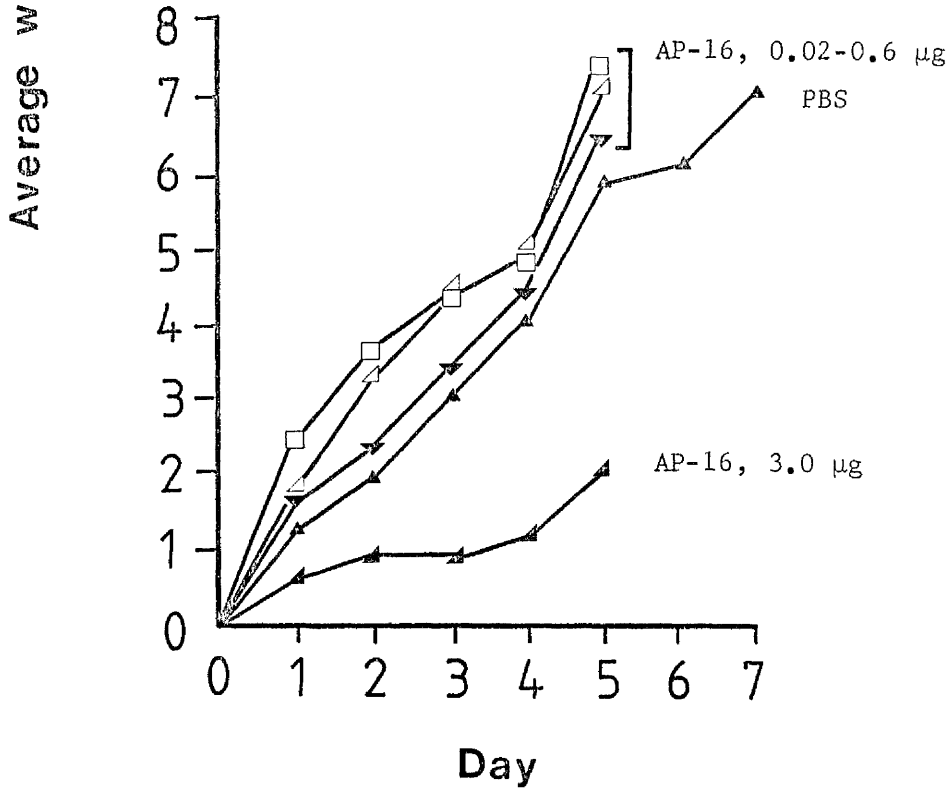

8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7
Day
AP-16, 0.02-0.6 μg
PBS
AP-16, 3.0 μg

Figure 23. Mouse-weight gain (toxicity) test for untoxoided preparation AP-16 and toxoid (AP-16)T37 stored for 14 days at $4^{o}C$ or $37^{o}C$

Groups of 5 or 10 mice were injected iv with graded doses of untoxoided preparation AP-16 or single doses of toxoid (AP-16)T37 stored at $4^{o}C$ or $37^{o}C$, or single doses of whole-cell vaccine or PBS stored at $4^{o}C$. Animals were weighed daily for 7 days and any deaths recorded. (Leucocyte counts were done 5 days post-injection and these data shown in Table 26).

	a.				b.				c.		
	Sample injected	Storage conditions	Dose/mouse		Sample injected	Storage conditions	Dose/mouse		Sample injected	Storage conditions	Dose/mouse
▲——▲	PBS	14 days at $4^{o}C$	0.5 ml	▲——▲	PBS	14 days at $4^{o}C$	0.5 ml	▲——▲	PBS	14 days at $4^{o}C$	0.5 ml
○——○	(AP-16)T37	" "	10.0 µg	◣——◣	AP-16	" $37^{o}C$	+9.0 µg	◿——◿	AP-16	" "	3.0 µg
△——△	"	" $37^{o}C$	"	◿——◿	"	" "	3.0 µg	▽——▽	"	" "	0.6 µg
■——■	Whole-cell vaccine (WCV)	" $4^{o}C$	5.0 ou.ml	▽——▽	"	" "	0.6 µg	□——□	"	" "	0.12 µg

+2/5 mice died within 7 days of injection.

No deaths were observed in any of the other mice injected with toxoids, whole-cell vaccine or untoxoided preparations.

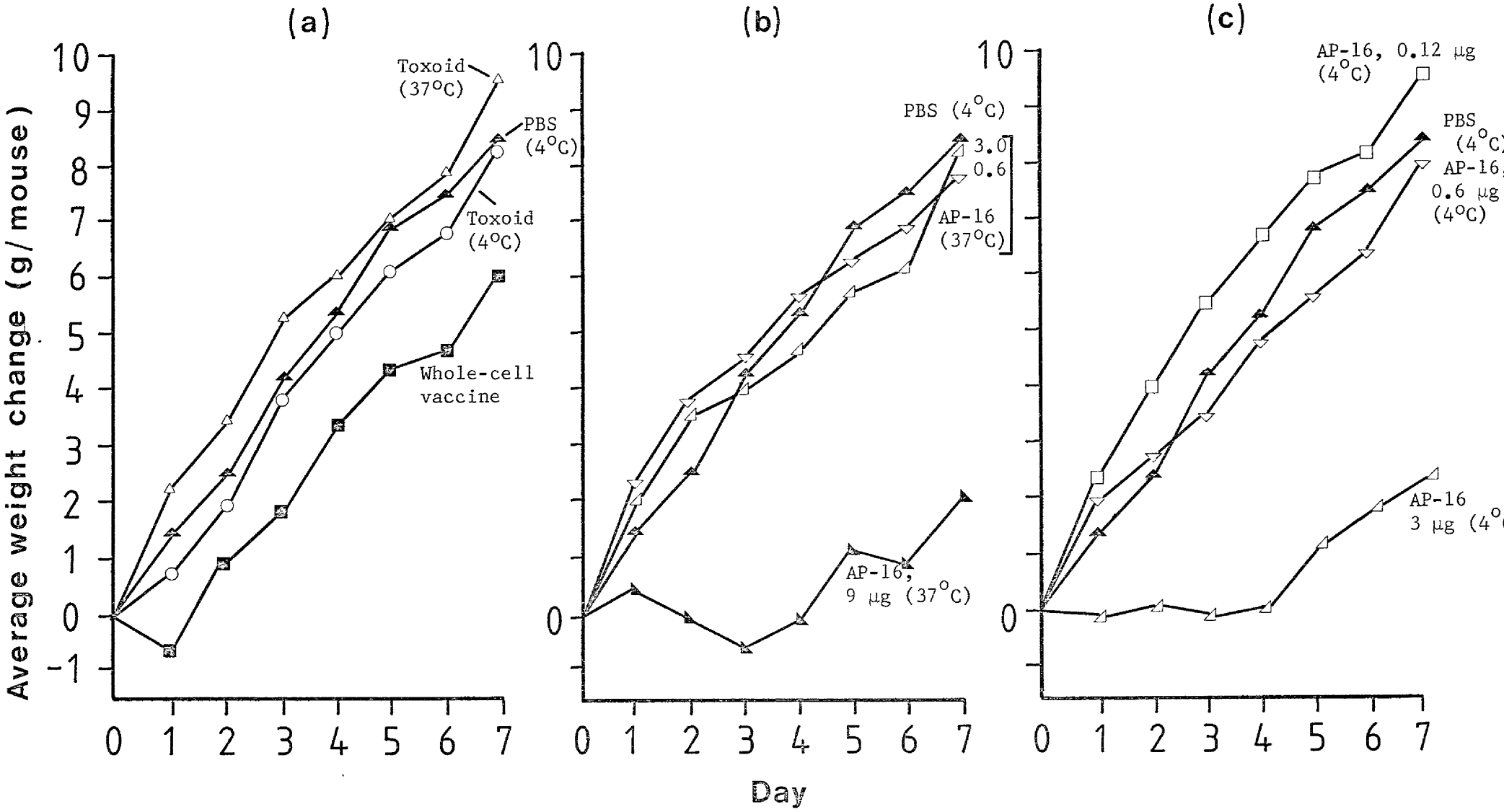

(a)
(b)
(c)
Average weight change (g/mouse)
Day
Toxoid (37°C)
PBS (4°C)
Toxoid (4°C)
Whole-cell vaccine
PBS (4°C)
3.0
0.6
AP-16 (37°C)
AP-16, 9 μg (37°C)
AP-16, 0.12 μg (4°C)
PBS (4°C)
AP-16, 0.6 μg (4°C)
AP-16 3 μg (4°

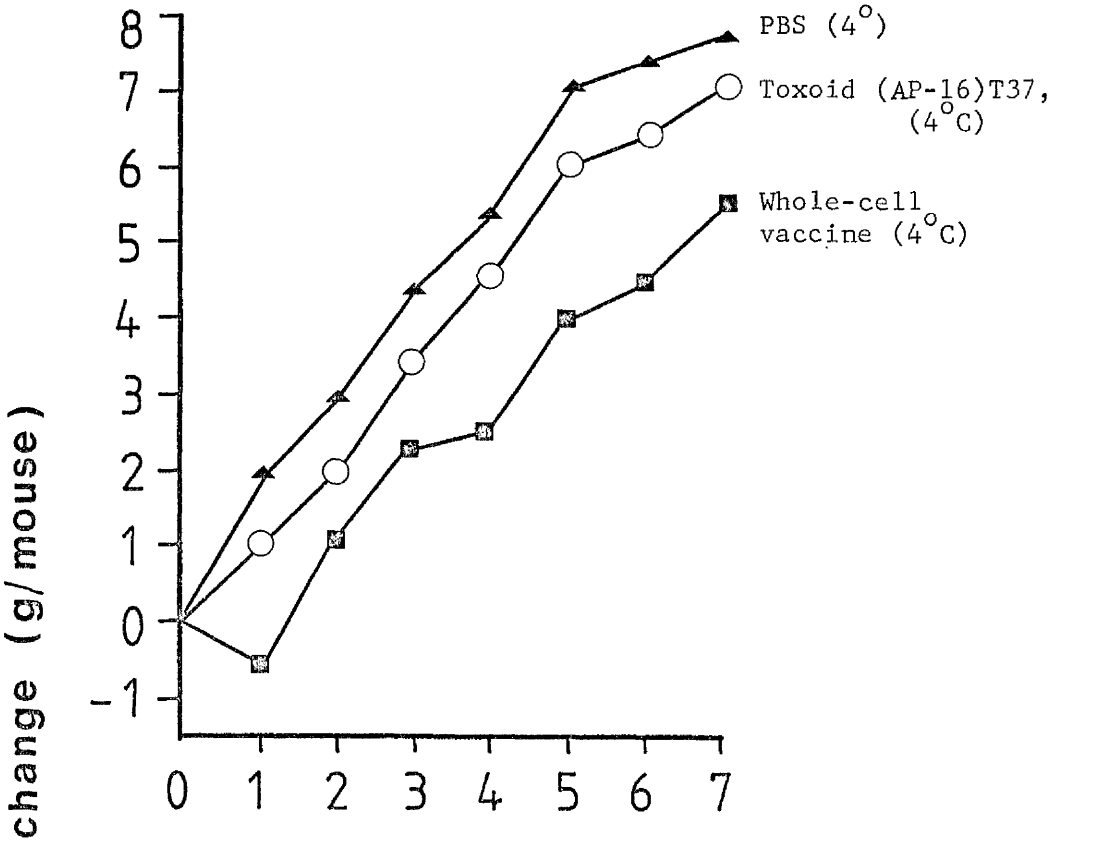
Figure 24. Mouse-weight-gain (toxicity) test for untoxoided preparation AP-16 and toxoid (AP-16)T37 stored for 28 days at 4°C

Groups of 5 or 10 mice were injected iv with graded doses of AP-16 or single doses of whole-cell vaccine, toxoid (AP-16)T37 or PBS stored at 4°C for 28 days. Animals were weighed daily for 7 days and any deaths recorded. (Leucocyte counts were done 5 days post-injection, and these data shown in Table 26).

	Sample injected	Dose/mouse
a.	▲——▲ PBS	0.5 ml
	○——○ (AP-16)T37	10.0 µg
	■——■ Whole-cell vaccine (WCV)	5.0 ou.ml
b.	▲——▲ PBS (as above)	0.5 ml
	◿——◿ AP-16	3.0 µg
	◺——◺ "	0.6 µg
	□——□ "	0.12 µg

No deaths were recorded with any of these samples tested.

(a)


PBS (4°)
Toxoid (AP-16)T37, (4°C)
Whole-cell vaccine (4°C)
Average weight change (g/mouse)
8
7
6
5
4
3
2
1
0
-1
0 1 2 3 4 5 6 7

(b)

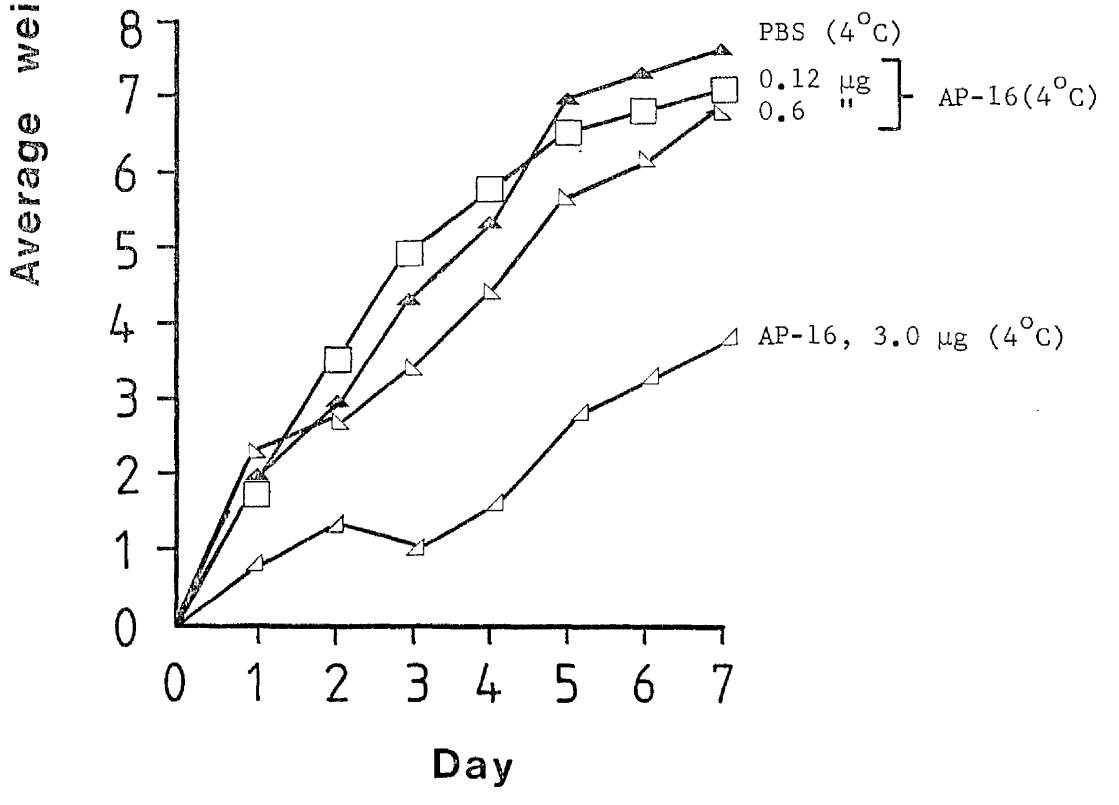

PBS (4°C)
0.12 µg
0.6 "
AP-16(4°C)
AP-16, 3.0 µg (4°C)
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7
Day

Figure 25. Mouse-weight-gain (toxicity) test for untoxoided preparation AP-16 and toxoid (AP-16)T37 stored for 56 days at 4°C

Groups of 5 or 10 mice were injected iv with graded doses of untoxoided preparation AP-16 or single doses of toxoid (AP-16)T37, whole-cell vaccine or PBS stored at 4°C for 56 days.

Animals were weighed daily for 7 days and any deaths recorded. (Leucocyte counts were also done 5 days after injection and these data shown in Table 26).

	Sample injected	Dose/mouse
a.	▲——▲ PBS	0.5 ml
	○——○ (AP-16)T37	10.0 µg
	◘——◘ Whole-cell vaccine (WCV)	5.0 ou.ml
b.	▲——▲ PBS (as above)	0.5 ml
	◿——◿ AP-16	3.0 µg
	◺——◺ "	0.6 µg
	□——□ "	0.12 µg

No deaths were recorded in any of the test groups.

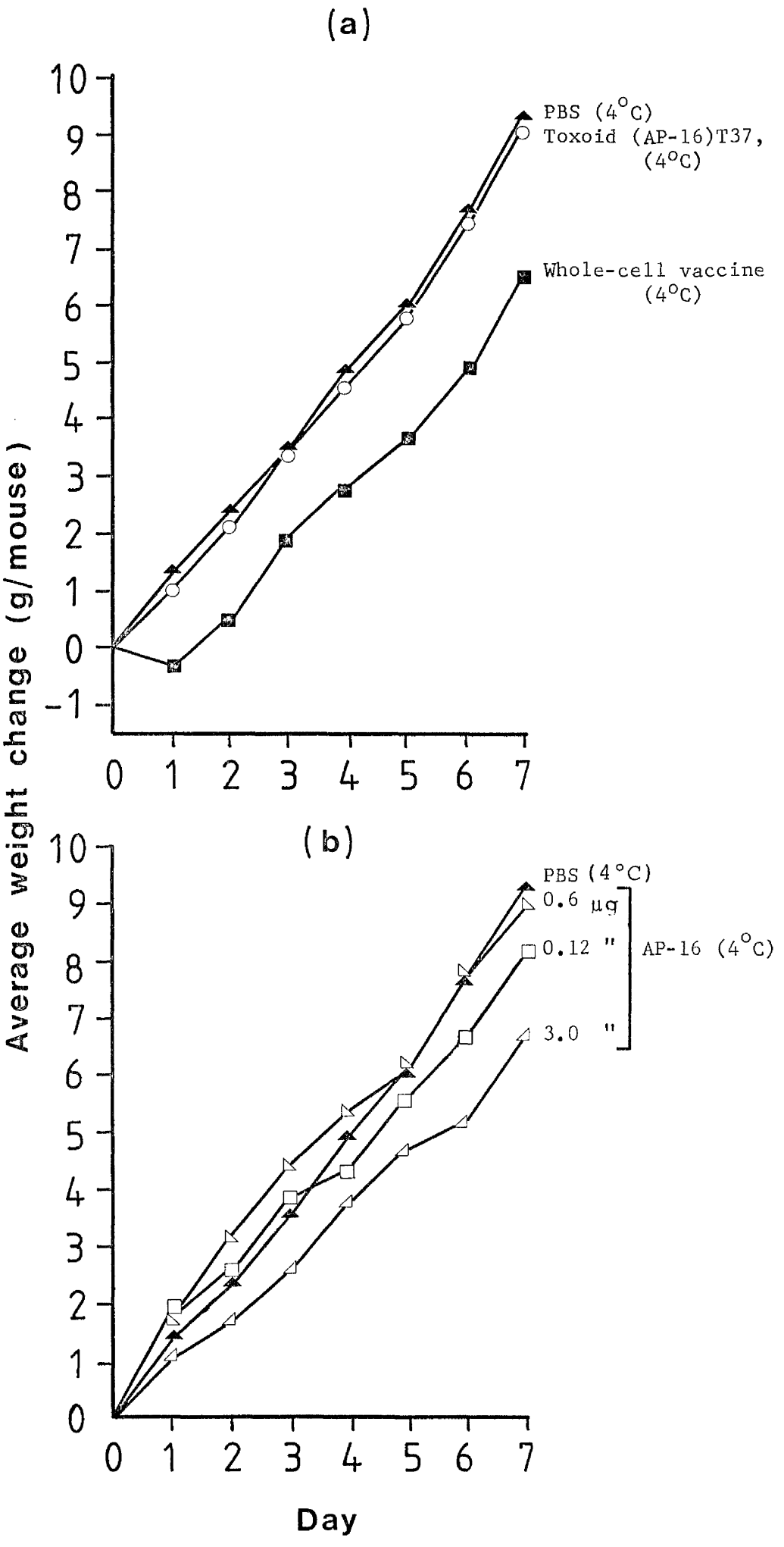

(a)
10
9
8
7
6
5
4
3
2
1
0
-1
0 1 2 3 4 5 6 7
PBS (4°C)
Toxoid (AP-16)T37, (4°C)
Whole-cell vaccine (4°C)
(b)
10
9
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7
PBS (4°C)
0.6 µg
0.12 "
3.0 "
AP-16 (4°C)
Average weight change (g/mouse)
Day

Figure 26. Mouse-weight-gain (toxicity) test for various lyophilized samples stored for 24 weeks at $4^{o}C$

Groups of 5 or 10 mice were injected iv with graded doses of untoxoided preparation AP-16 or single doses of toxoid (AP-16)T37 or whole-cell vaccine, or PBS as controls.

Animals were weighed daily for 7 days and any deaths recorded. (Leucocyte counts were also done 5 days after injection and these data shown in Table 27).

	Sample injected	Dose/mouse
▲——▲	PBS	0.5 ml
○——○	Toxoid (AP-16)T37	10.0 µg
	Untoxoided preparation,	
▽——▽	AP-16	3.0 µg
▼——▼	"	0.6 µg
◿——◿	"	0.12 µg
◺——◺	"	0.02 µg
◘——◘	Whole-cell vaccine (WCV)	5.0 ou.ml

No deaths were observed in any of the test animals.

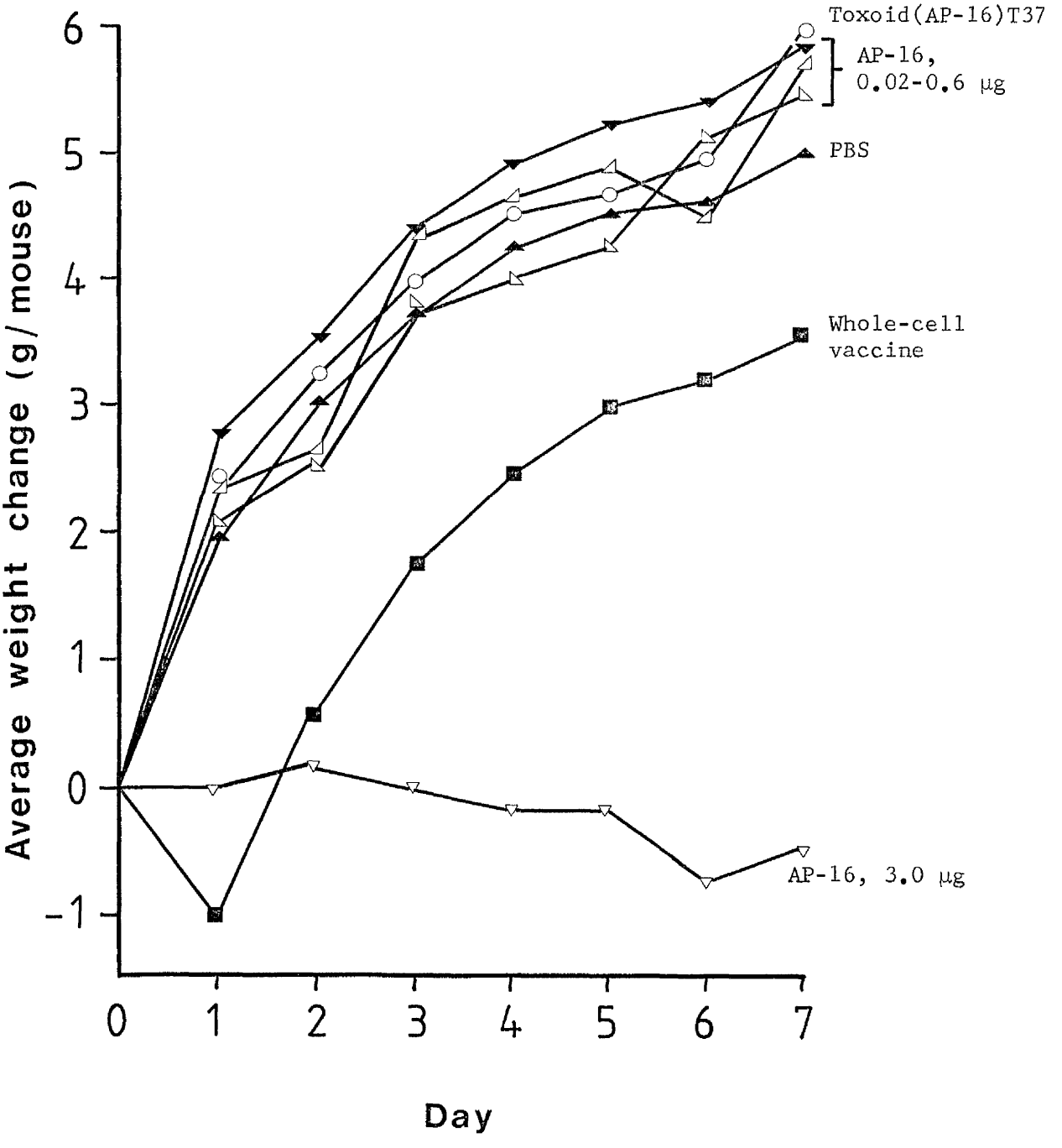

Toxoid(AP-16)T37
AP-16,
0.02-0.6 μg
PBS
Whole-cell
vaccine
AP-16, 3.0 μg
Average weight change (g/mouse)
Day
6
5
4
3
2
1
0
-1
0
1
2
3
4
5
6
7

Figure 27. Mouse-weight-gain (toxicity) test for untoxoided and toxoided preparations stored for 14 days at different temperatures

Groups of 5 mice were injected ip with untoxoided preparation AP-17 or toxoid (AP-17)T33 stored at -20°C, 4°C or 37°C for 14 days. Other mice were injected with the whole-cell vaccine (WCV), or PBS as controls. Animals were weighed daily for 7 days and any deaths recorded.

	Sample injected	Storage conditions	Dose/mouse
▲——▲	PBS	14 days at 4°C	0.5 ml
○——○	(AP-17)T33	" -20°C	20.0 µg
△——△	"	" 4°C	"
▼——▼	"	" 37°C	"
◣——◣	AP-17	" -20°C	8.0 µg*
□——□	"	" 4°C	" *
◺——◺	"	" 37°C	" *
◘——◘	Whole-cell vaccine (WCV)	" 4°C	5.0 ou.ml

*2/5 mice died in each group within 7 days of injection. No deaths were observed in any of the other groups.

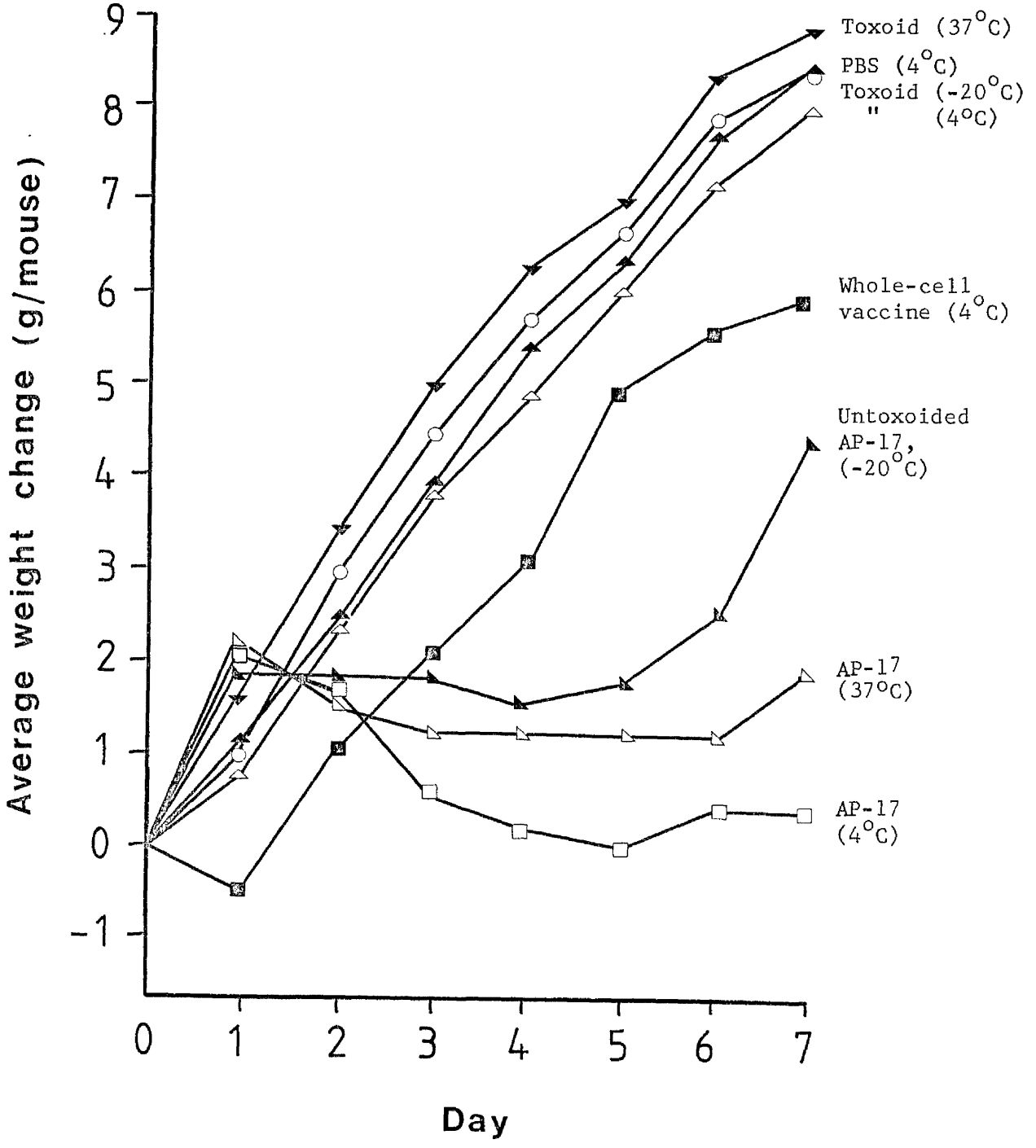
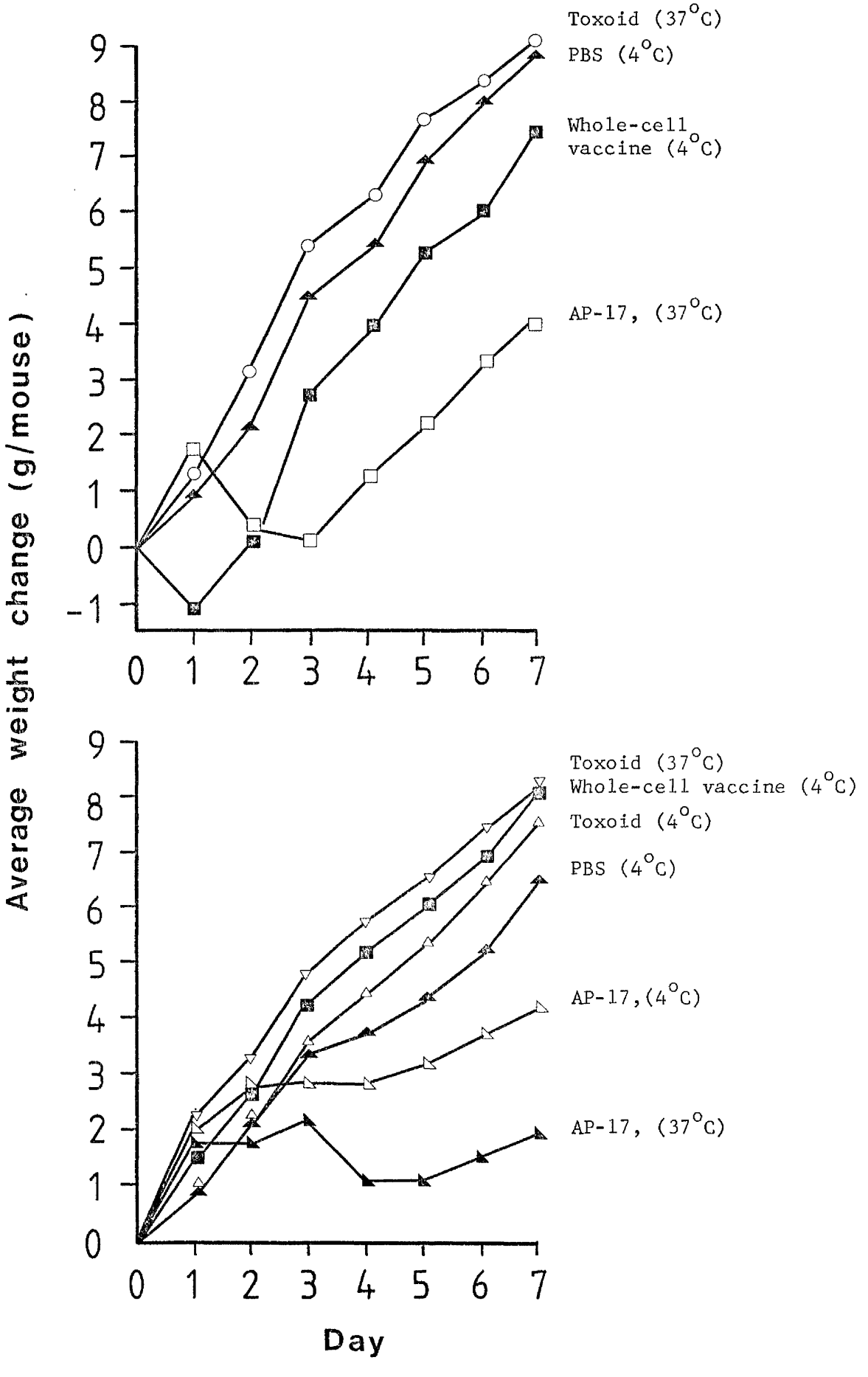

Average weight change (g/mouse)
9
8
7
6
5
4
3
2
1
0
-1
0
1
2
3
4
5
6
7
Day
Toxoid (37°C)
PBS (4°C)
Toxoid (-20°C)
" (4°C)
Whole-cell vaccine (4°C)
Untoxoided AP-17, (-20°C)
AP-17 (37°C)
AP-17 (4°C)

Figure 28. Mouse-weight-gain (toxicity) test for untoxoided and toxoided preparations stored for 28 or 56 days at 4°C or 37°C

Groups of 5 mice were injected ip with untoxoided preparation AP-17 or toxoid (AP-17)T33 stored at 4°C or 37°C for 28 or 56 days, or with whole-cell vaccine or PBS stored at 4°C for 28 or 56 days. Animals were weighed daily for 7 days and any deaths recorded.

	Sample injected	Storage conditions	Dose/mouse
a. ▲——▲	PBS	28 days at 4°C	0.5 ml
○——○	(AP-17)T33	" 37°C	20.0 µg
□——□	AP-17	" "	8.0 µg
▣——▣	Whole-cell vaccine (WCV)	" 4°C	5.0 ou.ml
b. ▲——▲	PBS	56 days at 4°C	0.5 ml
▽——▽	(AP-17)T33	" 37°C	20.0 µg
△——△	"	" 4°C	"
◣——◣	AP-17	" 37°C	8.0 µg*
◺——◺	"	" 4°C	" *
▣——▣	Whole-cell vaccine (WCV)	" "	5.0 ou.ml

*1/5 mice died in each group within 7 days of injection. No deaths were observed in the other groups.


Toxoid (37°C)
PBS (4°C)
Whole-cell vaccine (4°C)
AP-17, (37°C)
Toxoid (37°C)
Whole-cell vaccine (4°C)
Toxoid (4°C)
PBS (4°C)
AP-17,(4°C)
AP-17, (37°C)
Average weight change (g/mouse)
Day
0 1 2 3 4 5 6 7
-1 0 1 2 3 4 5 6 7 8 9

was still toxic but not lethal at a dose of 3 μg/mouse, when tested (Fig. 26).

The untoxoided preparation AP-17 stored at -20°C, 4°C or 37°C for 14 days was toxic or lethal at a dose of 8 μg/mouse (Fig. 27). When tested after 28 days at 37°C, the preparation was not lethal but normal weight-gain for mice was significantly retarded (Fig. 28a). Similarly, mice injected with AP-17 stored at 4°C or 37°C for 56 days did not gain weight normally (Fig. 28b) and by day 7, 1/5 mice from each group had also died.

In any of the experiments, mice injected with the whole-cell vaccine (WCV) showed an initial weight loss on the first day, presumably due to endotoxin, with original weight regained usually by the second day. In one experiment (Fig. 28b) this initial weight loss was not observed with the whole-cell vaccine tested after 56 days at 4°C; this may have been due to inadequate dispersion of the whole-cells before injection. In all cases, this dose of vaccine was non-lethal for mice.

The data in this section is summarized in Table 29, which compares the stability of untoxoided and carbodiimide-toxoided preparations and whole-cell vaccine during storage.

Table 29. Comparison of the storage stability of various preparations

SAMPLE	PROPERTIES
UNTOXOIDED PREPARATION	No significant reduction in HSA, LPA or mouse-toxicity on storage at 4°C for 56 days: stable at -20°C and 4°C (lyophilized) for 24 weeks. Slight reduction in biological activities at 37°C during 56 days storage.
TOXOID PREPARATION	Stable at 4°C for 56 days, and 24 weeks (lyophilized). Slight reversion on storage at 37°C: HSA detected after 14-28 days, approximately 1.0% of the original activity of untoxoided preparation. Detectable reversion to LPA after 14 days at 37°C; also reversion to approximately 5% hyperinsulinaemia activity, labile on further incubation.
WHOLE-CELL VACCINE (WCV)	No significant reduction in HSA, LPA or mouse-toxicity on storage at 4°C for 56 days.

SECTION 4. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

4.1 Development of ELISA for pertussis toxin

Preliminary experiments were done using anti-PT monoclonal antibodies L_4, L_5 and L_{10}, a rabbit anti-PT polyclonal antibody, fetuin and human haptoglobin type 2-2. In the first experiment, the routine ELISA procedure was used (p. 96), except that wells were coated with fetuin at 0.2-1.6 $\mu g\ well^{-1}$, the antigen was an Amicon 100 kDal culture fluid retentate with detectable HSA, and L_4 monoclonal antibody (10.5 ng $well^{-1}$) was used after the bound antigen. There was no difference in the ELISA dose-response curves when the concentration of fetuin was varied (data not shown), and in all subsequent experiments fetuin at 0.4 $\mu g\ well^{-1}$ was the solid phase.

The affinity of the monoclonal antibodies L_4, L_5 and L_{10} for the antigen bound to fetuin was next evaluated. The wells of Nunc ELISA-plates were coated with fetuin (0.4 $\mu g\ well^{-1}$) and the Amicon 100 kDal retentate titrated as described above. After incubation, 200 μl volumes of L_4 (0.21 $\mu g\ ml^{-1}$), L_5 (0.21 $\mu g\ ml^{-1}$) or L_{10} (1 in 200 dilution of stock)were added to duplicate wells. The routine ELISA procedure (p. 96) was followed. In this experiment, the ELISA dose-response curves for the titrated antigen were similar in all three fetuin-monoclonal antibody models (data not shown), and in all subsequent experiments L_{10} was routinely used after the fetuin-bound antigen phase.

A comparison of different solid phase coats was done using two different antigen preparations; (1) purified islet-activating protein (IAP-PT) and (2) the Amicon 100 kDal culture fluid retentate. The wells of Nunc ELISA-plates were coated with either fetuin, haptoglobin, mono-

clonal antibody L_4, L_5 or polyclonal antibody (Fig. 29). The positive end-point for each ELISA was chosen as the absorbance value (A492nm) above the background control values: with fetuin as the solid phase, background values were approximately 0.05. Using haptoglobin, monoclonal antibodies or polyclonal antibody as the solid phase, background values were approximately 0.15.

Taking into account background absorbance, the minimum detectable amount of IAP(PT) with the fetuin-L_{10}, haptoglobin-L_{10}, polyclonal antibody-L_{10} and L_5-L_{10} ELISA systems was 4.0 ng well^{-1} (Fig. 29). The L_4-L_{10} ELISA model detected 2.0 ng well^{-1} above background absorbance (Fig. 29).

When purified IAP was substituted with the crude preparation of PT, the fetuin-L_{10}, L_4-L_{10} and L_5-L_{10} ELISA systems gave similar dose-response curves (Fig. 30). However, the polyclonal-L_{10} and haptoglobin-L_{10} systems did not give the expected type of sigmoidal ELISA dose-response curve (Fig. 30).

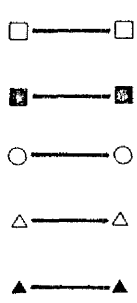
The ELISA chosen for routine detection and quantitation of PT was the fetuin-L_{10} model. Several other fractions were tested in this system, eg: culture fluid concentrates of X- and C-mode B. pertussis 18334, and of B. parapertussis. Only the X-mode B. pertussis fraction demonstrated ELISA-PT activity (A492nm $>$ 0.05).

Throughout this investigation, culture fluid samples and antigen preparations were frequently assayed for PT using the fetuin-L_{10} ELISA with purified islet-activating protein (IAP-PT) as the standard. There was a marked reduction in the binding affinity of this standard preparation with repeated freezing and thawing during storage (Fig. 31). When the preparation was first titrated, 4 ng well^{-1} of IAP was detected above background absorbance: however, in the final titration only 16 ng well^{-1}

Figure 29. Comparison of different coating layers in ELISA for purified islet-activating protein (IAP-PT)

The wells of Nunc ELISA-plates were coated in duplicate with 0.4 μg well^{-1} of fetuin, human haptoglobin, monoclonal L_4, L_5 or polyclonal antibody. The antigen preparation, IAP, was titrated in duplicate. Control wells were coated as above, but no antigen was added. Monoclonal antibody L_{10} was used after the antigen phase. The addition of conjugate, enzyme initiation and termination steps were done as described (p. 96).

Coating layer



□——□ Mouse anti-PT L_4 monoclonal IgG antibody

■——■ " " L_5 " " "

○——○ Fetuin

△——△ Haptoglobin

▲——▲ Rabbit anti-PT polyclonal IgG antibody

Absorbance values with 128 ng well^{-1} of IAP in the fetuin-L_{10} haptoglobin-L_{10}, L_4-L_{10} and L_5-L_{10} systems were beyond the detectable range of the ELISA reader (A492nm $>$ 2.0).

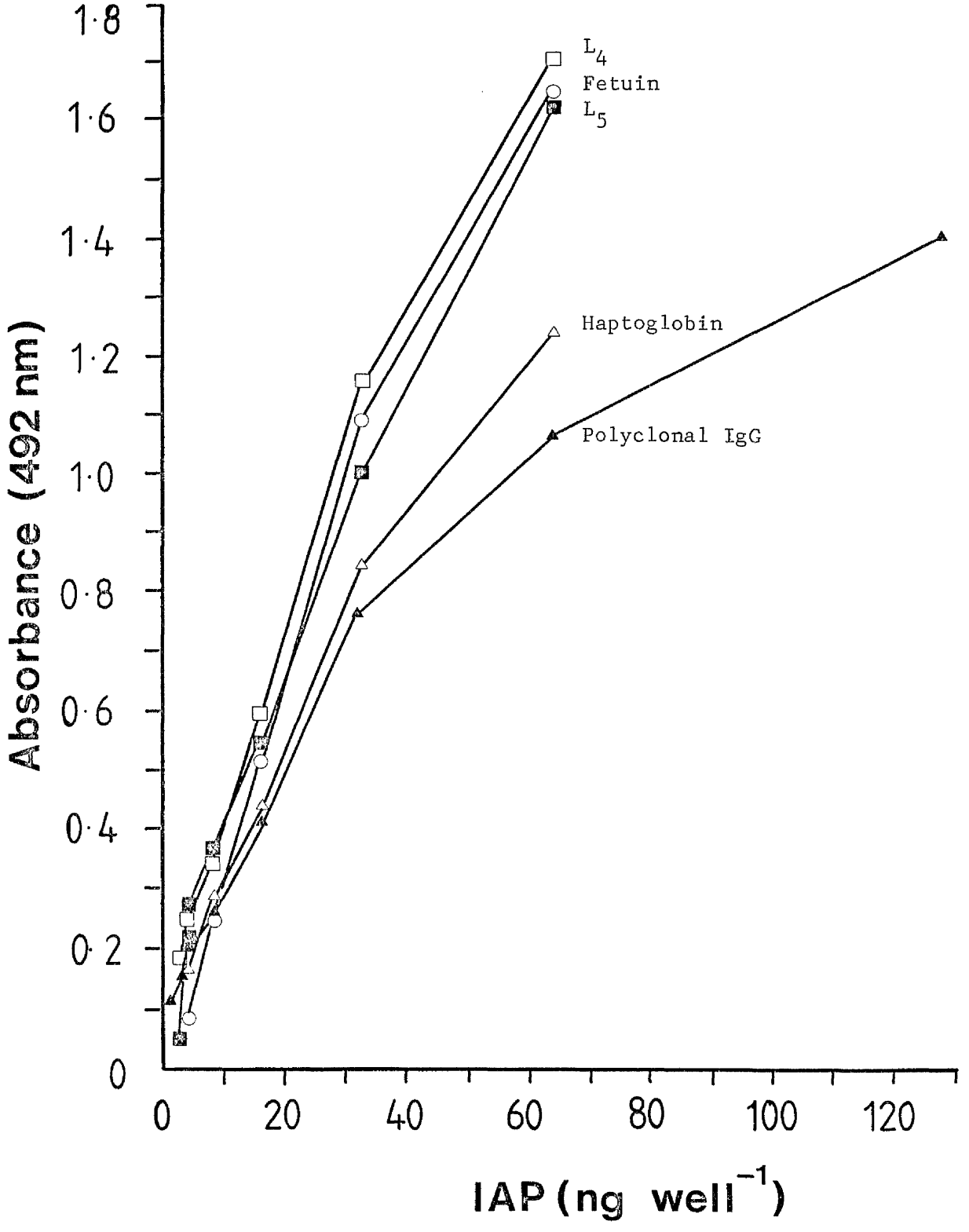
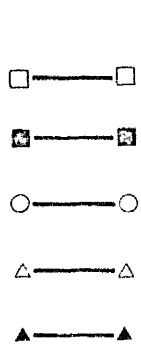

Absorbance (492 nm)
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0
20
40
60
80
100
120
IAP (ng well^{-1})
L_4
Fetuin
L_5
Haptoglobin
Polyclonal IgG

Figure 30. Comparison of different coating layers in ELISA for a crude preparation of PT

The wells of Nunc ELISA-plates were coated in duplicate with 0.4 μg well^{-1} of fetuin, haptoglobin, L_4, L_5 or polyclonal antibody. The antigen sample titrated was an Amicon 100 kDal *B. pertussis* culture fluid retentate. Monoclonal antibody L_{10} was used in each assay after the bound antigen. The addition of conjugate, enzyme initiation and termination steps were done as described (p. 96).



□——□ Mouse anti-PT L_4 monoclonal IgG antibody

▣——▣ " " L_5 " " "

○——○ Fetuin

△——△ Haptoglobin

▲——▲ Rabbit anti-PT polyclonal IgG antibody

Absorbance values with undiluted sample in the monoclonal antibody (L_4, L_5) systems were beyond the detectable range of the ELISA reader (A 492nm $>$ 2.0).

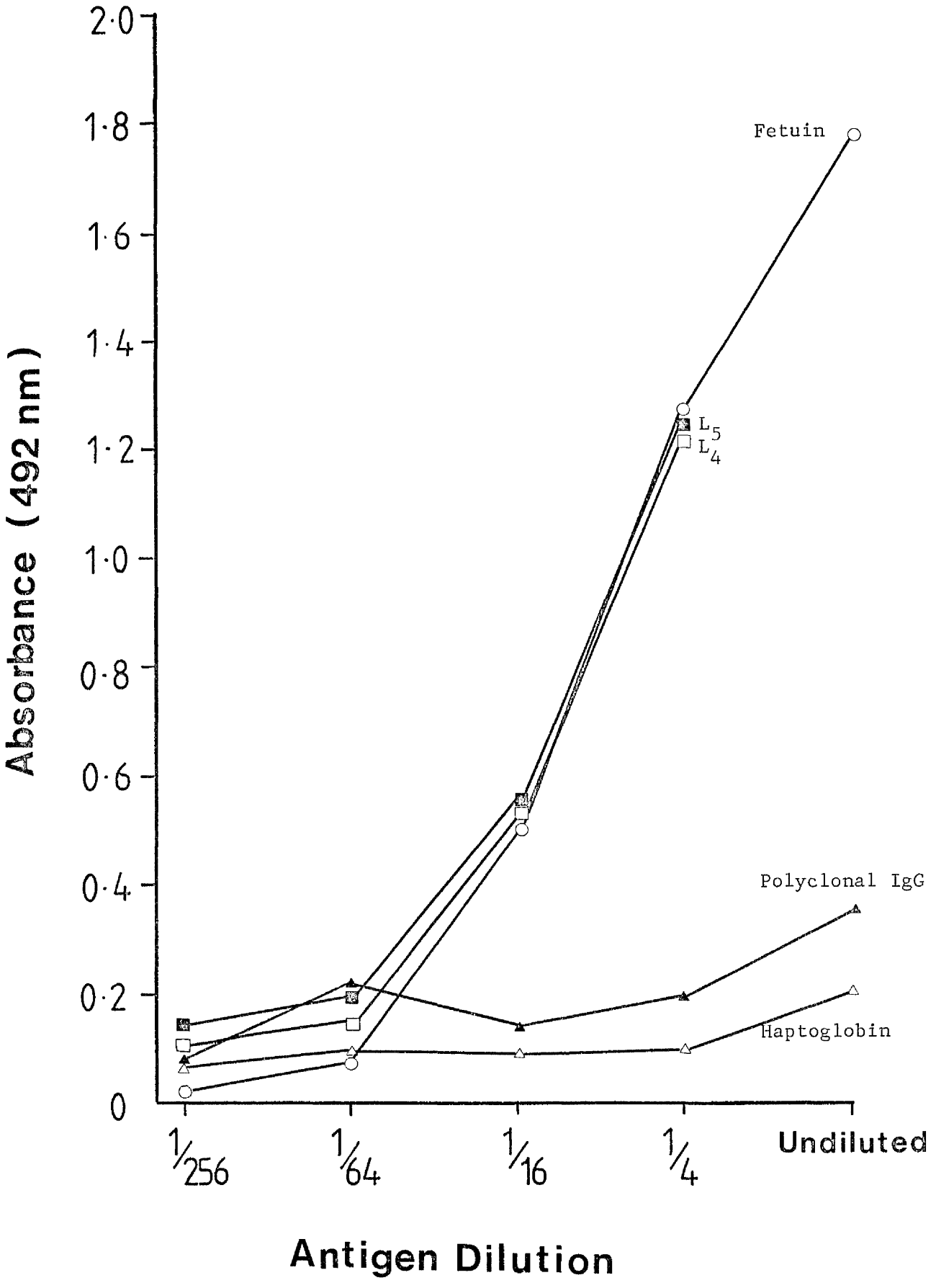

2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Absorbance (492 nm)
Fetuin
L_5
L_4
Polyclonal IgG
Haptoglobin
1/256
1/64
1/16
1/4
Undiluted
Antigen Dilution

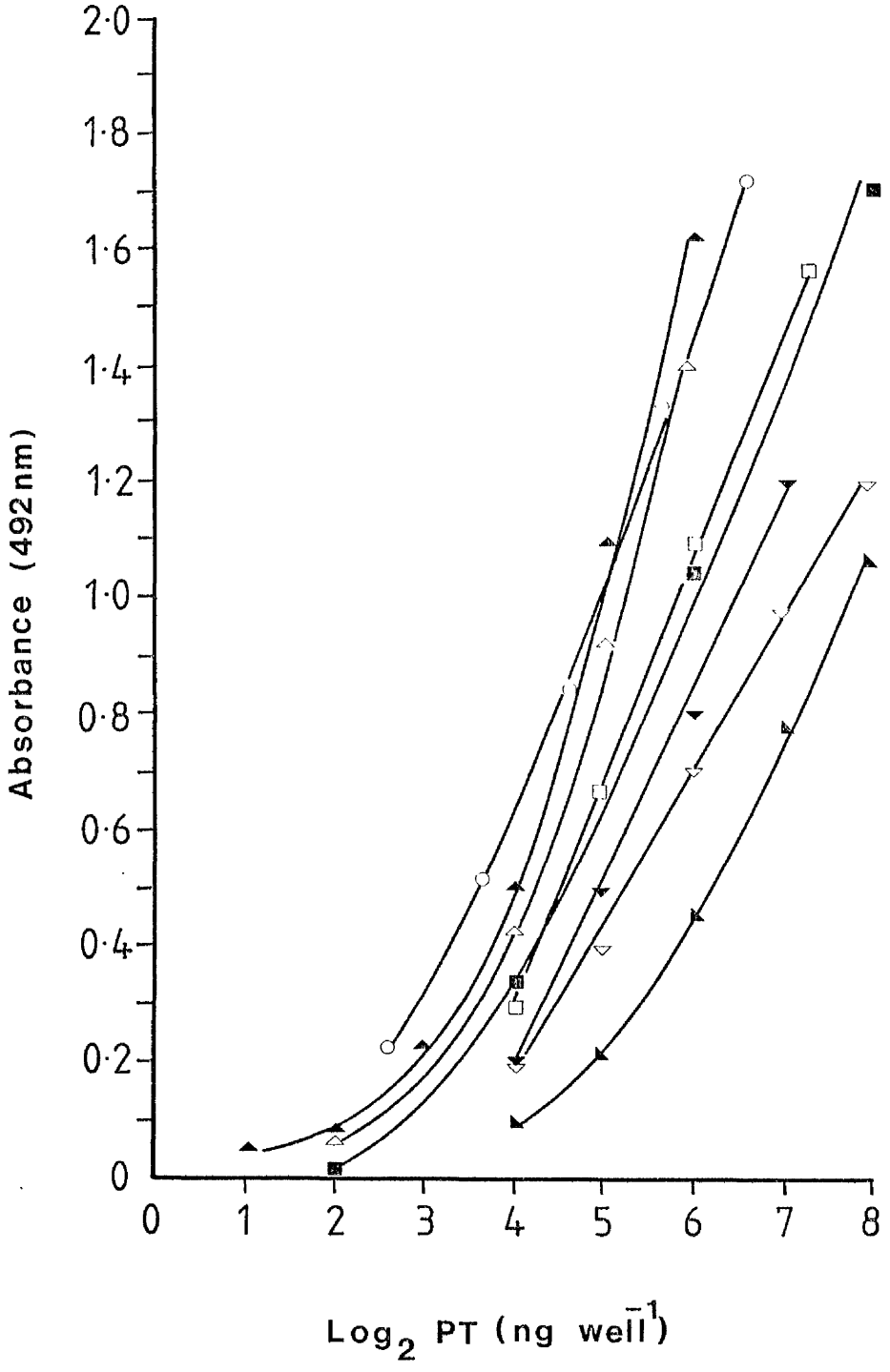
Figure 31. Binding affinity, during storage, of purified IAP(PT) in the fetuin-L_{10} ELISA

Islet-activating protein, IAP(PT), was used as a standard reference preparation for the quantitation of PT in samples. This reference was stored frozen (-20°C) between assays and titrated in the fetuin-L_{10} ELISA (p. 96).

IAP	Weeks after toxin storage
▲——▲	0
△——△	2
◧——◨	3
□——□	69
▼——▼	79
▽——▽	86
◣——◣	88
PT (List Biological Laboratories, Inc.).	
○——○	0

Commercial, purified PT (List) was titrated alongside IAP (stored for 88 weeks at -20°C with repeated freezing and thawing).

This commercial preparation of PT was used as the standard for the quantitation of PT in antigen preparations; these data are presented in column 5 of Table 8.


2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Absorbance (492 nm)
0
1
2
3
4
5
6
7
8
Log2 PT (ng well-1)

was detected. The parallelism of the dose-response curves was fairly uniform from one titration to the next, although each curve was progressively shifted to the right as the IAP preparation was repeatedly used (Fig. 31). For comparison, IAP and fresh, commercial PT were titrated at the same time in the fetuin-L_{10} ELISA. There was considerable difference in the ELISA dose-response curves with the IAP preparation, tested after repeated freezing and thawing during storage, and the fresh PT preparation (Fig. 31).

4.2 Detection of serum IgG antibodies to PT and FHa

Antibodies to PT and FHa in mouse sera were detected with the enzyme-linked immunosorbent assays (see pages 97 - 98).

Initially, experiments were done to determine if partially-purified preparations of PT and FHa could replace the purified antigens in these assays. Comparisons were made of a partially-purified preparation of PT (AP-2, Table 8) and purified PT (List Biological Laboratories, Inc.), and of FHa extracted from shaken cultures of B. pertussis 357 (AP-15, Table 8) and purified FHa (Porton). The antiserum was prepared from mice immunized with 30 μg of preparation AP-2 toxoided with EDAC (p. 103).

Initially, the PT content of AP-2 was quantitated in the fetuin-L_{10} ELISA using purified PT (List) as the standard; this value was shown in column 5 of Table 8. Serum anti-PT IgG antibodies were detected by fetuin-ELISA (p. 97), except that in one titration purified PT was replaced by the equivalent of partially-purified (AP-2) PT. This experiment (Fig. 32) indicated that commercial, purified PT could be replaced with a partially-purified PT preparation (AP-2) as the antigen phase in ELISA for detection of serum IgG antibodies to PT.

Figure 32. Comparison of partially-purified PT and purified PT as antigens in fetuin-ELISA for anti-PT IgG antibody

The wells of Nunc ELISA-plates were coated with fetuin (0.4 μg well^{-1}). The antigen was either 0.2 μg well^{-1} of purified PT (List) or the equivalent of antigen preparation AP-2 (Table 8) containing 0.2 μg of PT as quantitated by fetuin-L_{10} antigen ELISA (p. 96). The antiserum was raised against AP-2. Serum anti-PT IgG antibodies were detected as described (p. 97).

Antigen

△———△ Purified PT (List Biological Laboratories, Inc.)

▲———▲ Partially-purified preparation of PT (AP-2)

Each point represents the arithmetic mean, and the bars the standard deviation, of the absorbance values of three tests.

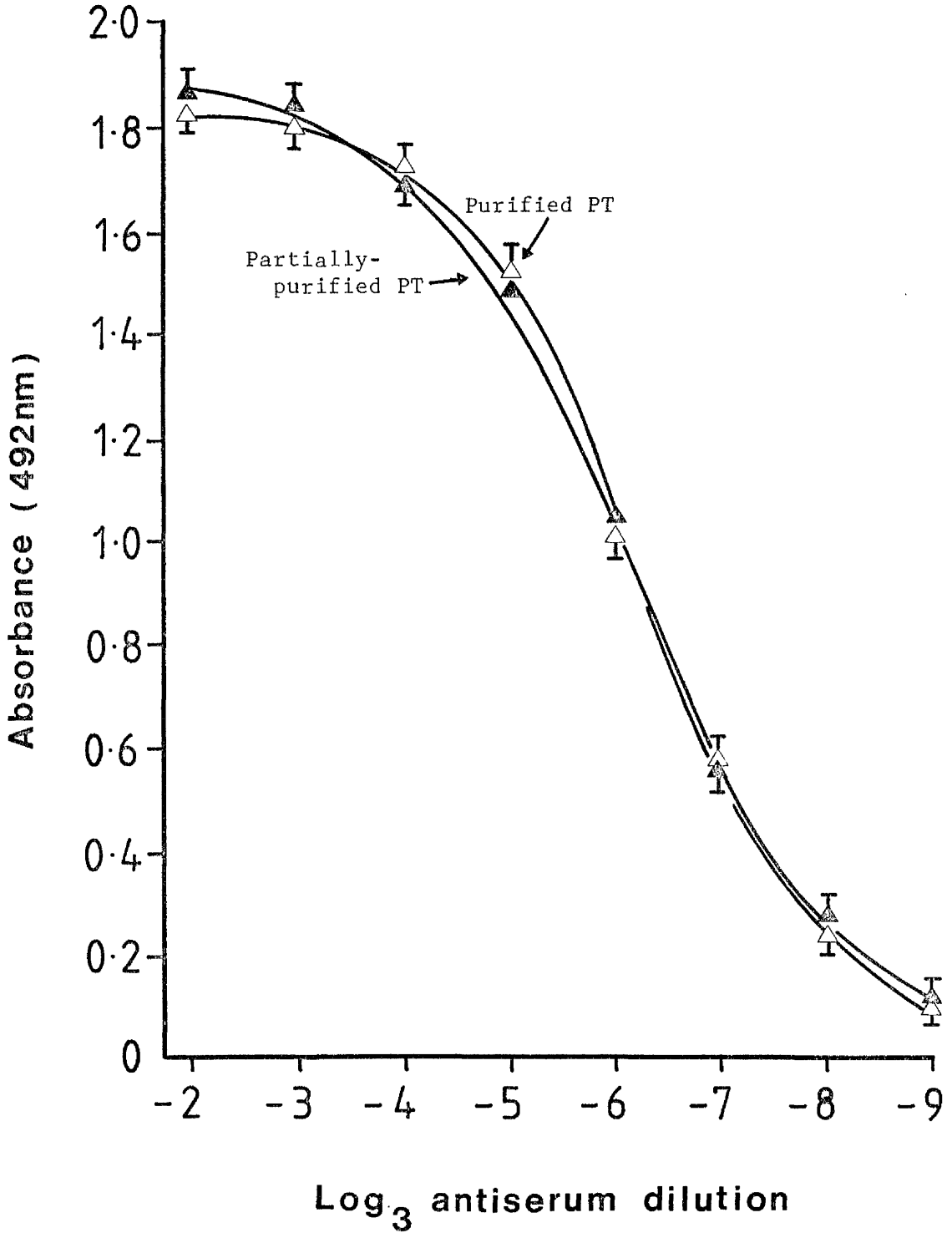

2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Absorbance (492nm)
Purified PT
Partially-purified PT
-2
-3
-4
-5
-6
-7
-8
-9
Log3 antiserum dilution

Figure 33. Comparison of purified FHa and a FHa-rich preparation (AP-15), as antigens in ELISA for anti-FHa IgG antibody

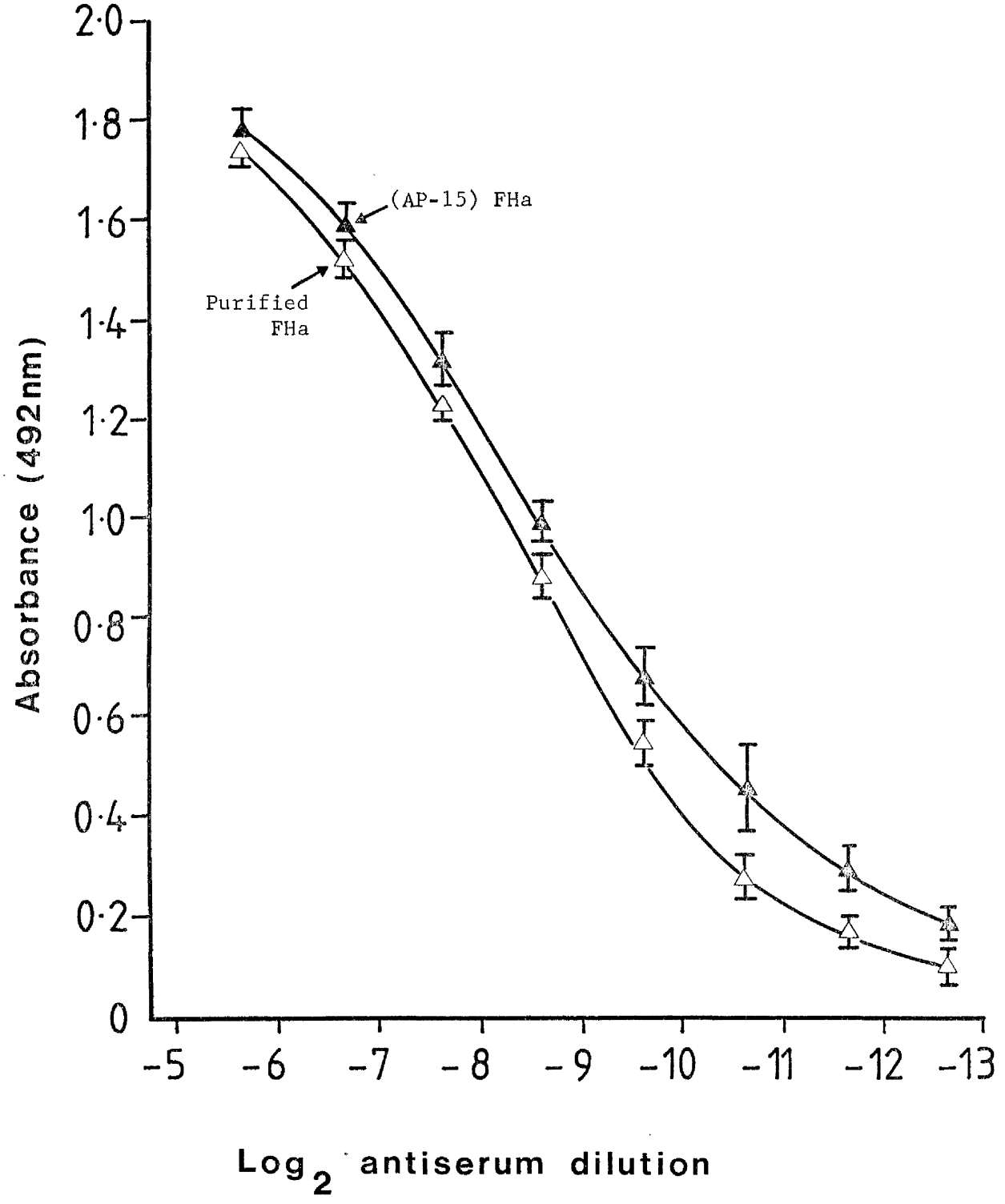
The wells of Nunc ELISA-plates were coated with either 0.2 μg $well^{-1}$ of purified FHa (Porton) or the equivalent of preparation AP-15 (Table 8) containing 0.2 μg FHa as quantitated by antigen-ELISA (p. 97). The antiserum was raised against AP-2. Serum IgG anti-FHa antibodies were detected as described (p. 98).

Antigen

△———△ Purified FHa (Porton)

▲———▲ (AP-15)-FHa (Table 8)

Each point represents the arithmetic mean, and the bars the standard deviation, of the absorbance values of three tests.


2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Absorbance (492nm)
(AP-15) FHa
Purified FHa
-5
-6
-7
-8
-9
-10
-11
-12
-13
Log2 antiserum dilution

A similar experiment was done to determine whether purified FHa (Porton) could be replaced with a FHa-rich preparation extracted from culture fluid (AP-15)。 The relative amounts of PT and FHa in this extract were quantitated by antigen-ELISA and the values obtained shown in columns 5, 6 of Table 8。 There was no difference in the antibody titration curves using purified FHa (Porton) or AP-15 (FHa) as the antigen phase in ELISA (Fig。 33)。 Consequently, this preparation of FHa (AP-15) was used as the solid antigen phase for detection of serum IgG antibodies to FHa。

Enzyme-linked immunosorbent assays for IgG antibodies to PT and FHa were done on antisera from mice immunized with antigen preparations (AP), carbodiimide-toxoided antigen preparations (EDAC-AP) or whole-cell vaccine (WCV)。 The controls were antisera from mice injected with phosphate-buffered saline。 Individual antiserum samples were assayed in duplicate and on each ELISA plate the reference antiserum, RA (p。 92), was also titrated。

Examples of the types of responses with several preparations are shown in Fig。 34, 35 and 36a,b。 The IgG antibody response to PT and FHa as components in untoxoided and toxoided preparations are shown in Fig。 34 and 35 respectively。 With untoxoided preparation AP-17, there was no detectable serum IgG antibody response to either component, since the mean absorbance values of all antisera tested were not greater than 0。1 (the background absorbance value chosen for these determinations)。 Untoxoided antigen preparations injected into mice consistently failed to stimulate anti-PT and anti-FHa IgG antibody responses (see later)。

Toxoid preparation (AP-17)T34 stimulated highly significant IgG anti-PT and anti-FHa antibody titres in mice, compared to the responses in mice immunized with untoxoided preparation AP-17 (Fig. 34,35).

The IgG antibody response to PT and FHa as components in whole-cell vaccine (WCV) are shown in Fig. 36a,b. The vaccine stimulated significant IgG anti-PT and anti-FHa antibody titres compared to mice injected with PBS.

In both ELISAs for detection of serum IgG antibody to PT and FHa, there was no apparent non-specific binding of IgG antibodies to either the antigen or the polystyrene plate, since the responses of sera from mice injected with PBS were not significantly different from background (Fig. 36a,b). Normal mouse serum was similarly unresponsive.

For convenience, such raw graphical data were converted into theoretical Antibody Units ml^{-1} serum (Appendix 9) or antibody titres (Appendix 10) by comparison with the reference antiserum preparation (RA).

Throughout this investigation, many sera were titrated in these ELISA; consequently, different plates were used and titrations made at different times. However, on each ELISA plate the reference antiserum (RA) was titrated in parallel. Therefore, a whole series of standard curves were obtained for the reference titrated in antibody-ELISAs for PT and FHa. To test for variability, the slope of each standard curve was estimated and an arithmetic mean and standard deviation calculated for each ELISA. For the anti-PT IgG antibody-ELISA the arithmetic mean slope was 0.689 with a standard deviation of 0.1, and for the anti-FHa IgG antibody-ELISA it was 0.67 with a standard deviation of 0.1. Therefore, there was little variability in the assays.

Figure 34. IgG antibody response to PT in untoxoided and toxoided antigen preparations determined by fetuin-ELISA

Mice were immunized with 5 μg/mouse of untoxoided preparation AP-17 or 25 μg/mouse of toxoid (AP-17)T34 and killed 21 days later. Individual sera were titrated in duplicate in a fetuin-ELISA for anti-PT IgG antibody (p. 97). The reference antiserum was RA (p. 92).

———— Individual mouse sera

▲———▲ Reference antiserum, RA

------- Maximum background absorbance

(Individual points for the test sera are only shown in one titration for better presentation).

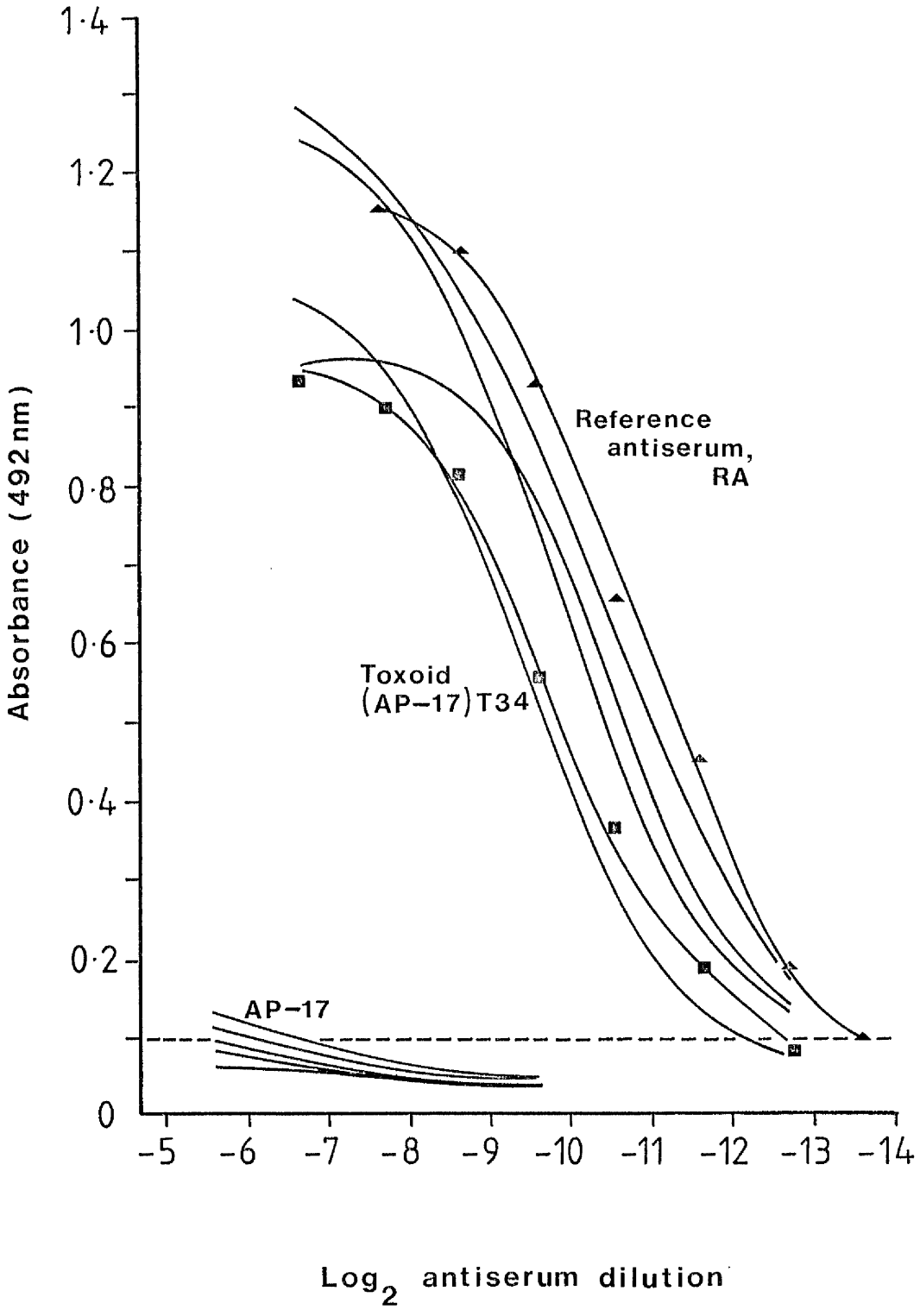

1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Absorbance (492 nm)
Reference antiserum, RA
Toxoid (AP–17)T34
AP–17
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
Log2 antiserum dilution

Figure 35. IgG antibody response to FHa in untoxoided and toxoided antigen preparations determined by ELISA

Mice were immunized with 5 μg/mouse of untoxoided preparation AP-17 or 25 μg/mouse of toxoid (AP-17)T34 and killed 21 days later. Individual sera were titrated in duplicate in ELISA for anti-FHa IgG antibody (p. 98). The reference antiserum was RA (p. 92).

——— Individual mouse sera

▲———▲ Reference antiserum, RA

- - - - - - Maximum background absorbance

(Individual points for the test sera are only shown in one titration for better presentation.)

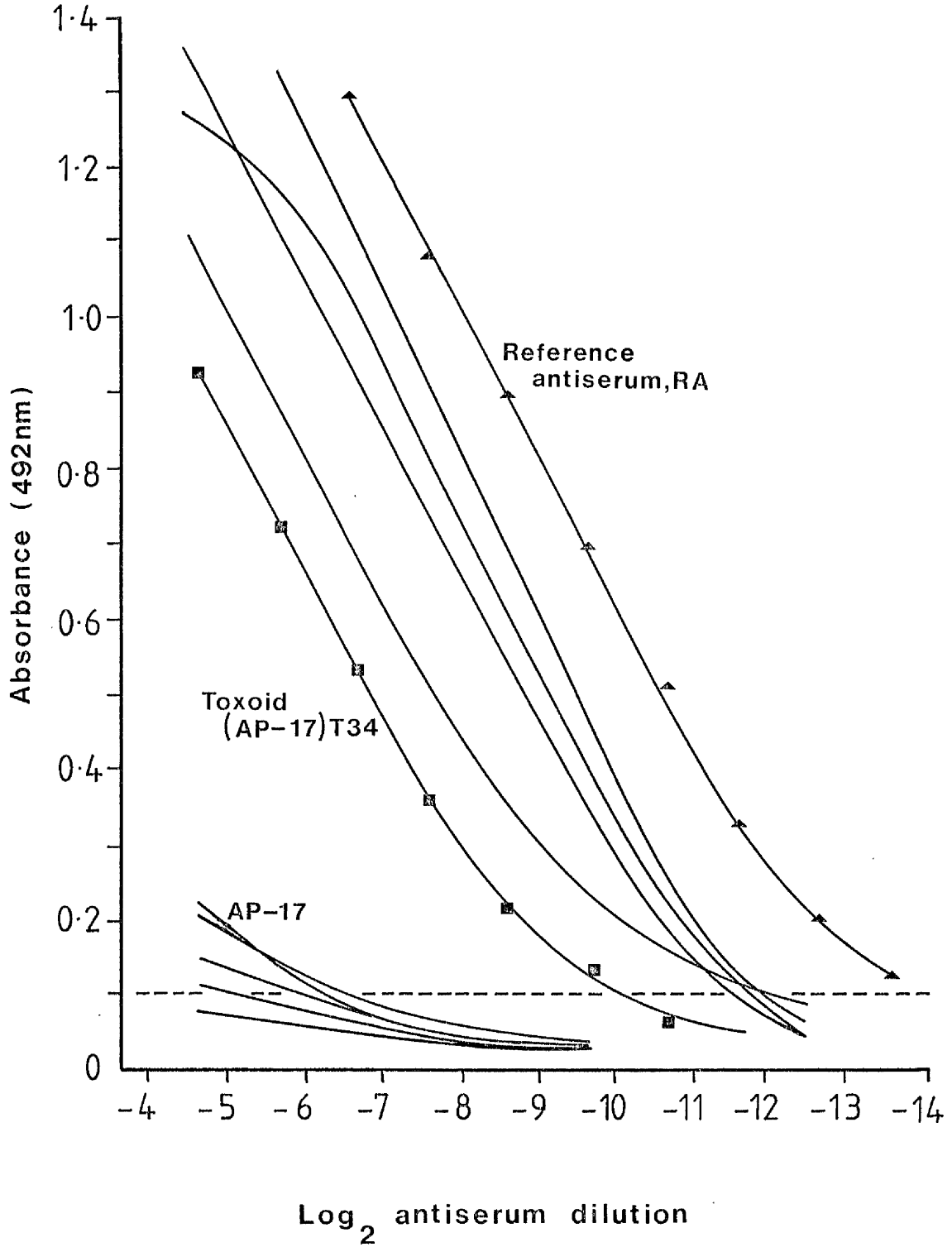

1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Absorbance (492nm)
Reference antiserum,RA
Toxoid (AP-17)T34
AP-17
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
Log2 antiserum dilution

Figure 36a. Anti-PT IgG antibody response stimulated by whole-cell vaccine (WCV), determined by fetuin-ELISA

Mice were immunized with 5 ou.ml/mouse of WCV and killed 21 days later. Individual sera were titrated in duplicate in a fetuin-ELISA for IgG anti-PT antibody (p. 97). The reference antiserum was RA (p. 92).

——— Individual mouse sera (representative data)

▲———▲ Reference antiserum, RA

- - - - - - - Maximum background absorbance

Figure 36b. Anti-FHa IgG antibody response stimulated by whole-cell vaccine (WCV), determined by ELISA

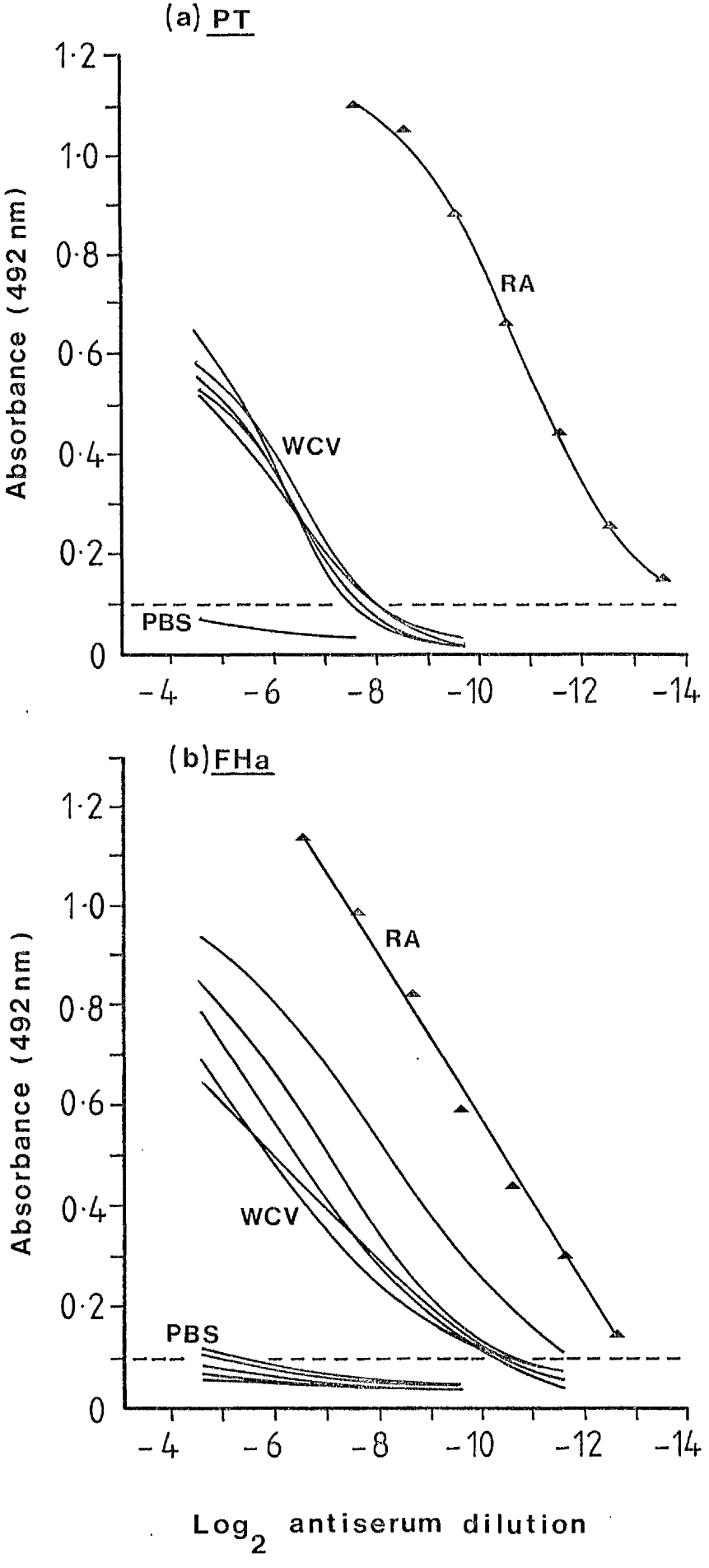
Mice were immunized as described above, and individual sera titrated in duplicate in ELISA for IgG anti-FHa antibody (p. 98).

——— Individual mouse sera (representative data)

▲———▲ Reference antiserum, RA

- - - - - - - Maximum background absorbance

(Individual titration points for each test sera are not shown, for better presentation.)


(a) PT
1·2
1·0
0·8
0·6
0·4
0·2
0
Absorbance (492 nm)
RA
WCV
PBS
-4
-6
-8
-10
-12
-14
(b) FHa
1·2
1·0
0·8
0·6
0·4
0·2
0
Absorbance (492 nm)
RA
WCV
PBS
-4
-6
-8
-10
-12
-14
Log2 antiserum dilution

4.3 Antibody-binding affinity of PT in toxoids

The binding affinity of PT in untoxoided antigen preparation AP-16 and toxoids (AP-16)T21-(AP-16)T24 was studied by ELISA, using a rabbit anti-PT polyclonal antibody and mouse anti-PT monoclonal antibodies.

In the first experiment, using the anti-PT polyclonal antibody (Fig. 37), increasing the concentration of EDAC used to toxoid AP-16 caused a significant decrease in absorbance measured at 492nm. The carbodiimide-toxoided preparations (AP-16)T21 to (AP-16)T23 showed similar decreases in absorbance (Fig. 37). Toxoid preparation (AP-16)T24 manifested a more significant decrease, but the greatest difference in absorbance from the control (untoxoided preparation AP-16), was observed with the toxoid prepared with an EDAC to protein ratio of 160:1.

Untoxoided preparation AP-16 gave similar ELISA dose-response curves with monoclonal antibodies L_5, L_9 or L_{10} (Fig. 38). However, toxoid (AP-16)T24 manifested a highly significant decrease in absorbance with all three antibodies (Fig. 38).

Figure 37. Antibody-binding affinity of PT in antigen preparation AP-16 after treatment with various amounts of EDAC

Toxoids (AP-16)T21 - (AP-16)T24 were prepared as described in Table 14. A toxoid was also prepared by treatment of AP-16 with an EDAC:protein ratio of 160:1 but was not tested in any in vivo assays.

ELISA. The wells of Nunc ELISA-plates were coated in duplicate with graded amounts of untoxoided preparation AP-16 or toxoids: the plates were kept at 4°C overnight, washed and anti-PT polyclonal antibody (0.2 μg well^{-1}) added. The plates were incubated for 2h at 37°C, washed and conjugate added as described (p. 96). After incubation for another 2h at 37°C, the enzyme initiation and termination steps were done as described and absorbance measured at 492 nm (p. 96).

	Coating layer (and treatment; EDAC:Protein ratio)
○——○	Untoxoided preparation, AP-16
■——■	Toxoid (AP-16)T21, (10:1)
□——□	" " T22, (20:1)
▲——▲	" " T23, (40:1)
△——△	" " T24, (80:1)
▼——▼	Toxoid preparation, (160:1)

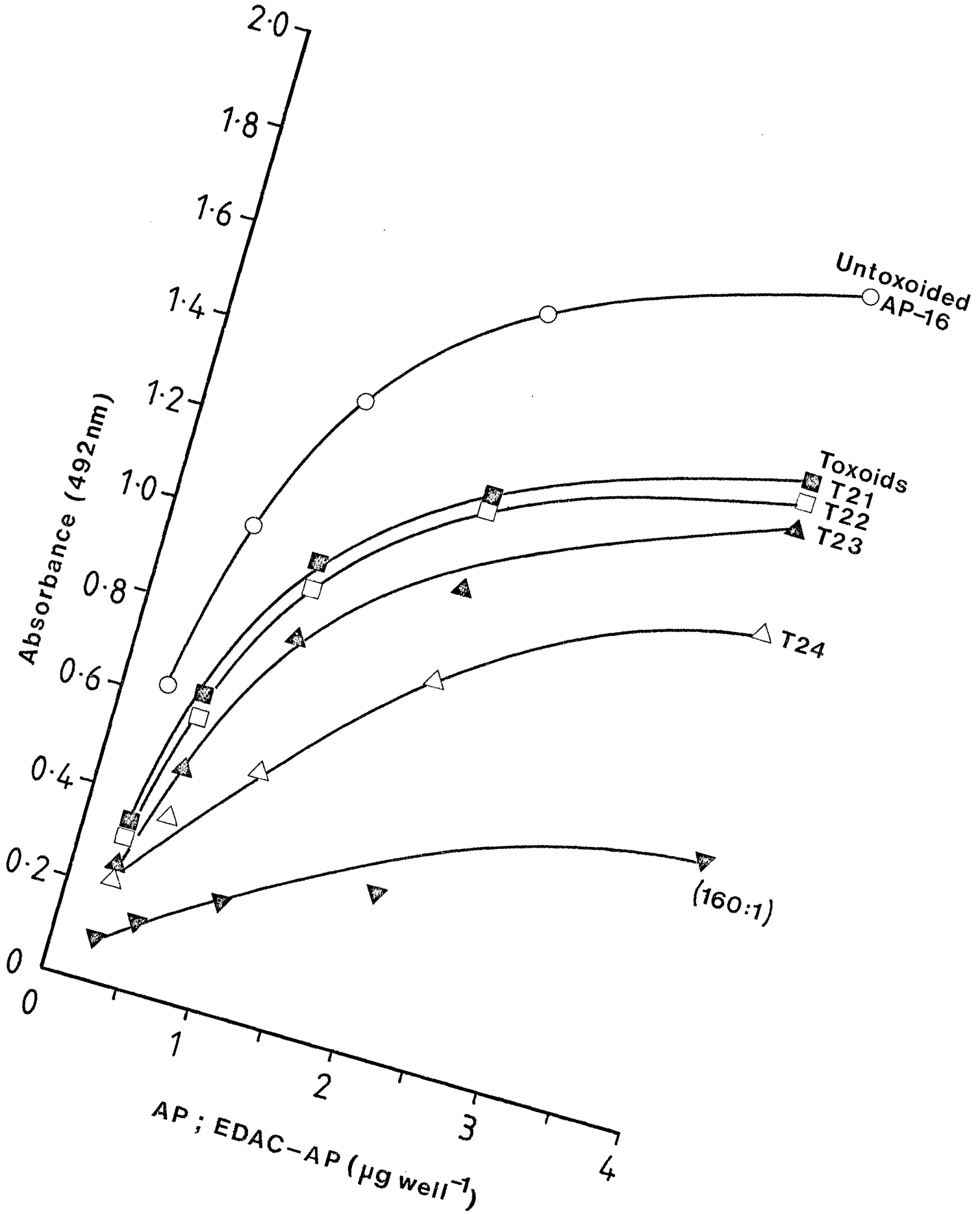
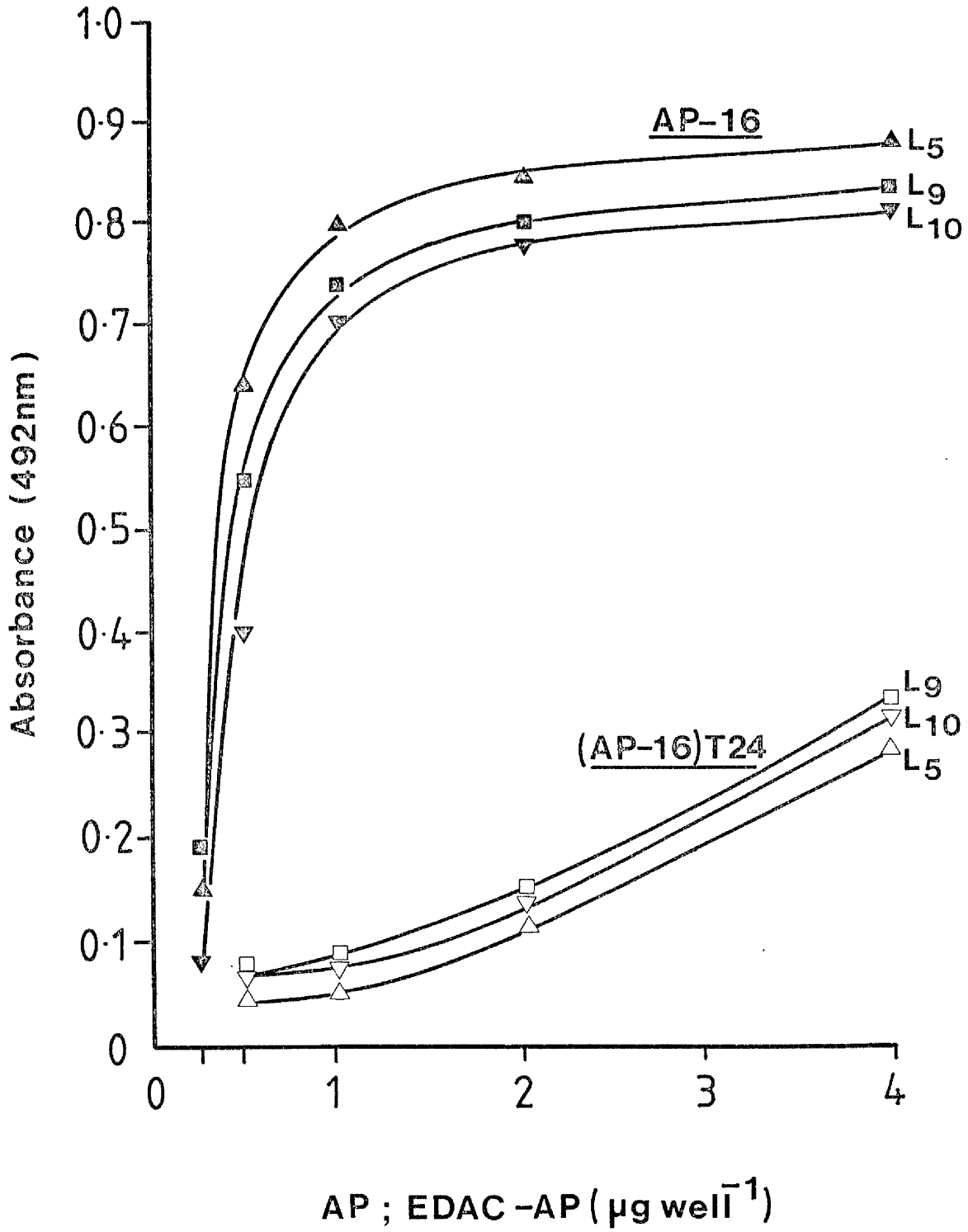

2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Absorbance (492nm)
0
1
2
3
4
AP ; EDAC–AP (μg well⁻¹)
Untoxoided AP–16
Toxoids
T21
T22
T23
T24
(160:1)

Figure 38. Antibody-binding affinity of PT in toxoid preparation (AP-16)T24 studied by ELISA

Toxoid (AP-16)T24 was prepared as described in Table 14.

ELISA. The wells of Nunc ELISA-plates were coated in duplicate with graded amounts of untoxoided preparation AP-16 or toxoid (AP-16)T24. The plates were kept at 4°C overnight and washed. Anti-PT mouse monoclonal antibody L_5, L_9 or L_{10} (200 µl of a 1 in 200 dilution of stock) was added to each well, and the plates incubated for 2h at 37°C and washed. Conjugate was added, the plates reincubated for 2h at 37°C and the enzyme initiation and termination steps done as described and absorbance measured at 492nm (p. 96).

	Coating Layer	Monoclonal Antibody
	Untoxoided preparation,	
▲——▲	AP-16	L_5
■——■	"	L_9
▼——▼	"	L_{10}
△——△	Toxoid (AP-16)T24	L_5
□——□	"	L_9
▽——▽	"	L_{10}


1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Absorbance (492nm)
AP-16
L5
L9
L10
(AP-16)T24
L9
L10
L5
0
1
2
3
4
AP ; EDAC-AP (μg well⁻¹)

SECTION 5. ANTIGENICITY OF TOXOIDS

The antigenicity of PT and FHa in untoxoided and carbodiimide-toxoided preparations injected into mice, was determined by ELISA (p. 97 - 98).

5.1 Antigenicity of PT and FHa in carbodiimide-toxoided antigen preparations

In two experiments, untoxoided preparation AP-16 (Table 30) and AP-17 (Table 31) did not stimulate significantly detectable IgG anti-PT or anti-FHa antibody responses in mice, at any of the doses tested. Higher doses of AP-16 (> 12 μg/mouse) or AP-17 (> 5 μg/mouse) were not tested for antigenicity because of the inherent toxicity of the preparations. Examples of the IgG anti-PT and anti-FHa antibody titrations in ELISA of antisera against AP-17 are shown in Fig. 34, 35. In both figures, the mean absorbance values with the titrated sera were not significantly different from background.

When equivalent doses, ie: 3 μg/mouse of untoxoided preparation AP-16 and any of the toxoids (AP-16)T21 to (AP-16)T24 were compared, there was a highly significant increase in the detectable IgG antibody response to both PT and FHa in mice immunized with the toxoids (Table 30). This was also observed when untoxoided preparation AP-17 (5 μg/mouse) and toxoids (AP-17)T33 to (AP-17)T35 (5 μg/mouse) were similarly compared (Table 31).

All toxoid preparations were highly antigenic in stimulating anti-PT IgG and anti-FHa IgG antibody responses in mice. In one experiment (Table 30), the antigenicity of toxoids prepared with various amounts of EDAC was compared, and the following conclusions drawn:

Table 30. Antigenicity of PT and FHa in preparation AP-16 treated with various amounts of EDAC

Sample code (and EDAC:protein ratio)	Dose	No. of mice tested	Anti-PT IgG by ELISA*		Anti-FHa IgG by ELISA*	
			Theoretical AbU ml^{-1} serum**	Titre ($x10^3$)***	Theoretical AbUml^{-1} serum**	Titre ($x10^3$)***
	µg/mouse					
AP-16	12	2	Not detected+	Not detected	Not detected	Not detected
	6	4	"	"	"	"
	3	4	"	"	"	"
	1.5	5	"	"	"	"
(AP-16)T21	27	5	1.1 (0.3, 5.7)	0.26 (0.02 , 3.3)	3.7 (0.3 , 5.4)	2.5 (0.6 , 10.5)
(10:1)	9	5	2.9 (1.3, 6.6)	0.85 (0.17 , 4.3)	5.9 (3.5 , 9.9)	1.5 (0.7 , 3.2)
	3	5	0.9 (0.09, 9.6)	0.07 (0.02 , 0.2)	0.8 (0.5 , 1.3)	0.03 (0.02, 2.5)
(AP-16)T22	27	5	72.5 (44.8,117.0)	12.8 (4.2 , 39.8)	44.5 (27.0, 73.6)	2.2 (0.98, 4.9)
(20:1)	9	5	36.8 (17.7, 76.7)	7.1 (1.8 , 28.0)	5.9 (3.0, 11.5)	2.9 (1.4 , 6.4)
	3	5	14.8 (5.4 , 40.3)	1.6 (0.3 , 8.2)	3.8 (2.0 , 7.4)	1.6 (0.4 , 7.3)
(AP-16)T23	27	5	20.2 (3.0 ,136.0)	3.8 (0.07 ,186.0)	4.5 (0.8 , 23.3)	3.8 (1.0 , 14.0)
(40:1)	9	5	9.0 (1.5 , 53.2)	2.9 (0.07 , 12.7)	4.2 (0.6 , 26.6)	1.5 (0.3 , 9.0)
	3	5	15.3 (4.2 , 54.6)	1.9 (0.12 , 29.0)	2.5 (0.8 , 6.9)	0.4 (0.2 , 0.9)
(AP-16)T24	27	5	23.6 (16.0, 34.6)	3.0 (1.9 , 4.8)	4.2 (2.1 , 8.5)	3.6 (1.0 , 14.3)
(80:1)	9	5	6.6 (1.5 , 28.6)	1.3 (0.3 , 5.5)	3.3 (2.4 , 4.5)	1.0 (0.8 , 4.2)
	3	5	4.2 (0.9 , 19.1)	1.6 (0.8 , 3.4)	0.8 (0.2 , 3.8)	0.3 (0.02, 3.0)
	ou.ml/mouse					
Whole-cell vaccine (WCV)	5	5	2.2 (0.66, 7.1)	0.4 (0.3 , 0.5)	1.5 (0.8 , 2.8)	0.3 (0.1 , 0.9)
	ml/mouse					
PBS	0.5	5	Not detected	Not detected	Not detected	Not detected

*Data expressed as the geometric mean (95% CL), and analysed by Student's t-test (p = 0.005).

Appendix 9. * Individual data shown in Appendix 11.

+Not detected; mean absorbance values < 0.1 (background) at antiserum dilutions tested.

Mice were injected ip with samples; leucocyte counts were done 5 days later (Table 19) and animals weighed daily for 7 days (Fig. 12).

Table 31. Antigenicity of PT and FHa in various toxoid preparations.

Sample code	Dose	No. of mice tested	Anti-PT IgG by ELISA*		Anti-FHA IgG by ELISA*	
			Theoretical AbU ml^{-1} serum**	Titre($x10^3$)***	Theoretical AbUml^{-1} serum	Titre ($x10^3$)***
	µg/mouse					
AP-17	5	5	+Not detected	Not detected	Not detected	Not detected
(AP-17)T33	25	5	30.6 (12.0 , 78.0)	3.6 (1.4 , 9.2)	18.2 (7.5 , 43.7)	2.3 (0.5 , 10.6)
	5	10	3.6 (2.0 , 6.3)	1.2 (0.3 , 5.2)	0.75(0.5 , 1.2)	0.1 (0.02 , 0.5)
(AP-17)T34	25	5	42.5 (23.0 , 78.0)	4.8 (1.7 , 13.1)	28.9 (10.0 , 83.0)	2.3 (0.5 , 9.7)
	5	10	1.8 (0.7 , 4.6)	0.2 (0.08 , 3.2)	0.8 (0.4 , 1.7)	0.2 (0.04 , 1.4)
(AP-17)T35	25	5	15.9 (5.7 , 44.5)	2.8 (1.8 , 3.7)	36.5 (6.4 ,209.0)	2.4 (0.1 , 47.9)
	5	10	6.8 (3.2 , 14.5)	1.9 (0.5 , 7.6)	2.4 (1.4 , 4.0)	1.1 (0.1 , 11.8)
	ou.ml/mouse					
Whole-cell vaccine (WCV)	5	5	1.4 (1.0 , 1.7)	0.19 ***	4.9 (3.1 , 7.7)	0.2 (0.1 , 0.4)
	ml/mouse					
PBS	0.5	5	Not detected	Not detected	Not detected	Not detected

*Data expressed as the geometric mean (95% CL), and analyzed by Student's t-test (p = 0.005).

**Appendix 9. ***Individual data in Appendix 12.

+Not detected; mean absorbance values < 0.1 (background) at antiserum dilutions tested.

Mice were injected with the test preparations as described in Fig. 13.

(i) toxoid preparations (AP-16)T22, (AP-16)T23 and (AP-16)T24 stimulated similar anti-PT IgG antibody responses in mice,

(ii) toxoid preparation (AP-16)T21, which had residual HSA (Table 14) and LPA (Table 19), and manifested some mouse-toxicity (Fig. 12b), was the least effective in stimulating an anti-PT IgG antibody response. Nevertheless, this toxoid was significantly more antigenic than the untoxoided preparation (Table 30),

(iii) by Student's t-test analysis ($p = 0.005$), there was a significant increase in the theoretical anti-FHa IgG antibody response in mice immunized with 27 μg/mouse of toxoid (AP-16)T22 compared to mice immunized with 27 μg/mouse of toxoids (AP-16)T21, (AP-16)T23 or (AP-16)T24. However, there was no significant difference in the actual anti-FHa antibody titres stimulated by any of these toxoids (Table 30).

Toxoids (AP-17)T33, (AP-17)T34 and (AP-17)T35 were similarly antigenic in mice (Table 31). At a dose of 5 μg/mouse or 25 μg/mouse, there were no significant differences in the IgG antibody responses, (theoretical or actual titres), to PT or FHa in mice immunized with any of the toxoids.

The whole-cell vaccine (WCV) was also antigenic in stimulating significantly detectable IgG anti-PT and anti-FHa antibody responses in mice (Table 30, 31). Examples of antisera titrated for IgG antibody to PT and FHa in whole-cell vaccine are shown in Fig. 36a,b.

5.2 Antigenic stability of PT and FHa in carbodiimide-toxoided antigen preparations

The antigenic stability of PT and FHa in three toxoid preparations, (AP-17)T33, (AP-2)T36 and (AP-16)T37, was studied during storage.

In the first study, toxoid (AP-2)T36 was antigenic in stimulating

Table 32. Antigenic stability of PT and FHa in incompletely toxoided preparation,(AP-2)T36, on storage

Sample code	Storage conditions	Dose	No. of mice tested	Anti-PT IgG by ELISA*		Anti-FHa IgG by ELISA*	
				Theoretical $AbUml^{-1}$ serum**	Titre***	Theoretical $AbUml^{-1}$ serum**	Titre***
		µg/mouse					
AP-2	day 0	5.0	1	Not detected+	Not detected	Not detected	Not detected
		2.5	1	"	"	"	"
		1.25	1	"	"	"	"
		0.6	5	"	"	"	"
(AP-2)T36	"	10.0	5	8.7 (5.2 , 14.8)	1,500 (570, 4,000)	11.1 (5.9 , 21.1)	3,200 (1,200 , 8,500)
		ou.ml/mouse					
Whole-cell vaccine (WCV)	"	5.0	5	0.67	111	0.2	315
		ml/mouse					
PBS	"	0.5	5	Not detected	Not detected	Not detected	Not detected
		µg/mouse					
AP-2	14 days at 4°C	5.0	3	Not detected	Not detected	Not detected	Not detected
		2.5	3	"	"	"	"
		1.25	5	"	"	"	"
	" 37°C	5.0	5	"	"	"	"
		2.5	5	"	"	"	"
		1.25	5	"	"	"	"
(AP-2)T36	" 4°C	10.0	5	12.6	2,000	10.4	2,900
	" 37°C	10.0	5	11.0	1,900	12.6	3,300
		ou.ml/mouse					
Whole-cell vaccine (WCV)	" 4°C	5.0	5	0.8	97	0.4	256
		ml/mouse					
PBS	" "	0.5	5	Not detected	Not detected	Not detected	Not detected

*Data expressed as the geometric mean (95% CL). Where confidence limits not shown, pooled sera were titrated.
**Appendix 9. ***Individual data shown in Appendix 13.
+Not detected; mean absorbance values <0.1 (background) at antiserum dilutions tested.
Mice were injected iv with test samples and leucocyte counts done (Table 25). Mice were also weighed daily for 7 days (Fig. 20, 21).

significantly detectable anti-PT IgG and anti-FHa IgG antibody responses in mice (Table 32). There was no loss in the antigenicity of PT or FHa in toxoid preparation (AP-2)T36, when tested after 14 days at $4^{o}C$ or $37^{o}C$ (Table 32).

In the second study, toxoid (AP-16)T37 tested on day 0, also stimulated highly significant anti-PT IgG and anti-FHa IgG antibody responses in mice, and was antigenically stable on storage (Table 33). By a Student's t-test ($p = 0.005$) of (i) the theoretical $\log_2$ $AbUml^{-1}$ serum values and (ii) the $\log_2$ antibody titres, there was a slight but insignificant decrease in the antigenicity of PT in toxoid (AP-16)T37 stored at $37^{o}C$ for 14 days or $4^{o}C$ for 14, 28 or 56 days (Table 33). No significant loss in the antigenicity of FHa in toxoid (AP-16)T37 after 14 days at $37^{o}C$ or 14, 28 or 56 days at $4^{o}C$, was found (Table 33).

Toxoid (AP-16)T37 was also lyophilized and stored at $4^{o}C$. After 24 weeks, the toxoid was resuspended in the 'reconstitution buffer' (Appendix 8) and tested for antigenicity. In this single experiment, only 2/10 sera from mice immunized with the toxoid had detectable anti-PT IgG antibody, and only 3/10 had detectable anti-FHa IgG antibody (Table 34).

However, a 'carbodiimide-precipitated toxoid' (AP-2)T38, which was not tested for toxicity or stability on storage, was lyophilized, resuspended and sonicated and tested for antigenicity in mice (Table 35). This preparation was highly antigenic in stimulating anti-PT IgG and anti-FHa IgG antibody responses in mice (Table 35), thus indicating that the procedure of lyophilization did not impair antigenicity.

In the third stability study, toxoid (AP-17)T33 was also antigenically stable on storage at $4^{o}C$ or $37^{o}C$ for the test period of 56 days (Table 36). The data indicated that there was

Table 33. Antigenic stability of PT and FHa in toxoid preparation (AP-16)T37 stored at 4°C or 37°C

Sample code	Storage conditions	Dose	No. of mice tested	Anti-PT IgG by ELISA*		Anti-FHa IgG by ELISA*	
				Theoretical $AbUml^{-1}$ serum**	Titre***	Theoretical $AbUml^{-1}$ serum**	Titre***
		µg/mouse					
AP-16‡	day 0	3.0	5	Not detected+	Not detected	Not detected	Not detected
(AP-16)T37	"	10.0	10	5.6 (3.3 , 9.3)	1,300(460, 3,800)	7.2 (5.1 , 10.1)	2,100(1,000, 4,500)
		ou.ml/mouse					
Whole-cell vaccine (WCV)	"	5.0	5	0.8 (0.3 , 1.8)	120(20, 750)	2.1 (0.8 , 5.8)	280(62, 1,300)
		ml/mouse					
PBS	"	0.5	5	Not detected	Not detected	Not detected	Not detected
		µg/mouse					
AP-16‡	14 days at 4°C	3.0	5	Not detected	Not detected	Not detected	Not detected
	" 37°C	9.0	3	"	"	"	"
(AP-16)T37	" 4°C	10.0	10	3.1 (1.3 , 7.4)	600(67, 5,500)	6.0 (4.0 , 9.2)	1,100(450, 2,700)
	" 37°C	10.0	10	3.8 (2.4 , 5.9)	1,300(340, 4,700)	9.2 (6.4 , 13.2)	1,100(510, 2,000)
		ou.ml/mouse					
Whole-cell vaccine (WCV)	" 4°C	5.0	5	0.7 (0.2 , 1.9)	189(9 , 4,100)	2.0 (0.8 , 5.2)	480(47, 4,900)
		ml/mouse					
PBS	" "	0.5	5	Not detected	Not detected	Not detected	Not detected

		µg/mouse					
AP-16‡	28 days at 4°C	3.0	5	Not detected	Not detected	Not detected	Not detected
(AP-16)T37	" "	10.0	10	1.6 (0.7 , 3.4)	780(160, 3,800)	3.5 (2.1 , 5.8)	810(230, 2900)
		ou.ml/mouse					
Whole-cell vaccine (WCV)	" "	5.0	5	0.4 (0.12 , 0.8)	83(13, 500)	2.2 (0.8 , 5.6)	480(100, 2,200)
		ml/mouse					
PBS	" "	0.5	5	Not detected	Not detected	Not detected	Not detected
		µg/mouse					
AP-16‡	56 days at 4°C	3.0	5	Not detected	Not detected	Not detected	Not detected
(AP-16)T37	" "	10.0	10	2.0 (0.8 , 4.8)	340(34, 3,400)	5.4 (2.9 , 10.2)	1,500(300, 7,000)
		ou.ml/mouse					
Whole-cell vaccine (WCV)	" "	5.0	5	0.4 (0.2 , 1.0)	81(17, 380)	4.0 (1.4 , 11.6)	990 (450, 2,200)
		ml/mouse					
PBS	" "	0.5	5	Not detected	Not detected	Not detected	Not detected

*Data expressed as the geometric mean (95% CL), and analyzed by Student's t-test ($p = 0.005$).

**Appendix 9. ***Individual data shown in Appendix 14.

+Not detected; mean absorbance values < 0.1 (background) at the antiserum dilutions tested.

‡Lower doses of untoxoided preparation were also tested, but IgG anti-PT or anti-FHa antibody responses were not detected.

Groups of 5 or 10 mice were injected iv with test preparations and leucocyte counts done 5 days later (Table 26).

Animals were also weighed daily for 7 days (Fig. 22-25).

Table 34. Antigenicity of pertussis antigens in lyophilized samples stored for 24 weeks at 4°C

Sample code	Dose	No. of mice tested	Anti-PT IgG by ELISA*		Anti-FHa IgG by ELISA*	
			Theoretical $AbUml^{-1}$ serum**	Titre***	Theoretical $AbUml^{-1}$ serum**	Titre***
	µg/mouse					
AP-16	3.0	5	Not detected+	Not detected	Not detected	Not detected
	0.6	5	"	"	"	"
	0.12	5	"	"	"	"
	0.02	5	"	"	"	"
(AP-16)T37	10.0	10	1.0 ; 0.5	84 , —	0.09 (0.05, 0.16)	37 (14, 98)
	ou.ml/mouse					
Whole-cell vaccine (WCV)	5.0	5	0.58 (0.4 , 0.95)	35 (1.0 , 105)	2.2 (0.96, 5.2)	970 (290, 3,200)
	ml/mouse					
PBS	0.5	5	Not detected	Not detected	Not detected	Not detected

*Data expressed as the geometric mean (95% CL).

Appendix 9. * Individual data shown in Appendix 15.

+Not detected; mean absorbance values < 0.1 (background) at antiserum dilutions tested.

Mice were injected iv with test preparations and leucocyte counts done (Table 27).

Animals were also weighed daily for 7 days (Fig. 26).

Table 35. Antigenicity of PT and FHa in a 'carbodiimide-precipitated toxoid'

Sample code	Dose	No. of mice tested	Anti-PT IgG by ELISA*		Anti-FHa IgG by ELISA*	
			Theoretical $AbUml^{-1}$ serum***	Titre***	Theoretical $AbUml^{-1}$ serum**	Titre***
	µg/mouse					
AP-2	5.0	5	+Not detected	Not detected	Not detected	Not detected
(AP-2)T38	30.0	5	18.5 (3.6 , 95.4)	7,600(900, 64,900)	8.9 (1.7, 47.9)	3,000(170, 50,400)
"	15.0	5	15.9 (5.7 , 44.6)	3,700(1,400, 9,600)	6.7 (1.5, 29.3)	3,900(630, 24,000)
	7.5	5	2.2 (1.0 , 5.2)	Not calculable***	1.2 (0.4, 3.1)	320(9, 10,700)
	ml/mouse					
PBS	0.5	5	Not detected	Not detected	Not detected	Not detected

*Data expressed as the geometric mean (95% CL).

**Appendix 9. ***Individual data are shown in Appendix 16. A geometric mean antibody titre (95% CL) for sera from mice immunized with 7.5 µg/mouse of toxoid could not be calculated from the data.

+Not detected; mean absorbance values <0.1 (background) at antiserum dilutions tested.

Toxoid (AP-2)T38 was prepared by treatment of antigen preparation AP-2 with EDAC as described (p. 103). An insoluble precipitate was formed which was dialyzed against distilled water and lyophilized. The lyophilized product was resuspended in the 'reconstitution buffer' (Appendix 8), sonicated, and tested for antigenicity. The toxoid was not tested for HSA, LPA or mouse-toxicity.

(i) no loss in the antigenicity of PT or FHa in toxoid (AP-17)T33 after 14 days at -20°C, 4°C or 37°C, 28 days at 37°C or 56 days at 4°C,

(ii) a slight but insignificant decrease in the antigenicity of PT and FHa in toxoid (AP-17)T33 after 56 days at 37°C.

In all these studies, the untoxoided preparations did not stimulate detectable anti-PT IgG or anti-FHa IgG antibody responses in mice at any of the doses tested during storage (Tables 32-36).

In these studies, there was no loss in the antigenicity of PT or FHa in whole-cell vaccine (WCV) stored at 4°C. However, in one study (Table 36), there was no detectable antibody to PT or FHa in sera from mice immunized with the vaccine stored for 56 days at 4°C. The assays for detection of these antibodies were not at fault, since the reference antiserum (RA) titrated in parallel gave normal ELISA dose-response curves. This unexpected loss in antigenicity might have been due to inadequate dispersion of the whole-cell vaccine before injection into animals.

Table 36. Antigenic stability of PT and FHa in toxoid preparation (AP-17)T33 stored at different temperatures

Sample code	Storage conditions	Dose	No. of mice tested	Anti-PT IgG by ELISA*		Anti-FHa IgG by ELISA*	
				Theoretical $AbUml^{-1}$ serum**	Titre ($x10^3$)***	Theoretical $AbUml^{-1}$ serum**	Titre ($x10^3$)***
		µg/mouse					
AP-17	14 days at -20°C	8.0	3	Not detected+	Not detected	Not detected	Not detected
"	" 4°C	8.0	3	"	"	"	"
"	" 37°C	8.0	3	"	"	"	"
(AP-17)T33	" -20°C	20.0	5	13.8 (3.8, 51.1)	3.8 (0.5, 29.8)	6.1 (3.0, 12.8)	1.1 (0.3, 4.8)
"	" 4°C	20.0	5	23.4 (11.2, 49.0)	7.3 (3.1, 17.2)	12.9 (7.0, 23.7)	1.8 (0.7, 5.1)
"	" 37°C	20.0	5	13.2 (5.8, 30.2)	2.8 (0.6, 15.5)	16.9 (5.9, 48.1)	14.7 (0.6, 35.5)
		ou.ml/mouse					
Whole-cell vaccine (WCV)	" 4°C	5.0	5	0.6 (0.5, 0.7)	0.15‡	0.9 (0.3, 2.9)	0.6 (0.06, 4.7)
		ml/mouse					
PBS	" "	0.5	5	Not detected	Not detected	Not detected	Not detected

			µg/mouse					
AP-17	28 days at 37°C		8.0	5	Not detected	Not detected	Not detected	Not detected
(AP-17)T33	"		20.0	5	23.1 (0.9, 57.9)	2.1 (0.5, 9.0)	7.2 (1.5, 35.2)	3.1 (0.4, 22.5)
			ou.ml/mouse					
Whole-cell vaccine (WCV)	"	4°C	5.0	5	0.4 (0.2, 0.9)	64‡	2.0 (0.6, 6.8)	0.7 (0.06, 8.3)
			ml/mouse					
PBS	"	"	0.5	5	Not detected	Not detected	Not detected	Not detected
			µg/mouse					
AP-17	56 days at 4°C		8.0	3	Not detected	Not detected	Not detected	Not detected
"	"	37°C	8.0	4	"	"	"	"
(AP-17)T33	"	4°C	20.0	5	18.9 (9.9, 35.8)	4.2 (1.4, 12.1)	12.4 (4.2, 37.3)	3.9 (0.5, 32.5)
"	"	37°C	20.0	5	6.7 (2.5, 17.5)	1.2 (0.3, 5.6)	1.7 (0.6, 5.0)	1.0 (0.05, 18.0)
			ou.ml/mouse					
Whole-cell vaccine (WCV)	"	4°C	5.0	5	Not detected	Not detected	Not detected	Not detected
			ml/mouse					
PBS	"	"	0.5	5	"	"	"	"

*Data expressed as the geometric mean (95% CL) and analyzed using Student's t-test ($p = 0.005$).

**Appendix 9. ***Individual data are shown in Appendix 17.

‡Other data calculated in Y/X ratios (Appendix 10; 17).

+Not detected; mean absorbance values < 0.1 (background) at antiserum dilutions tested.

Mice were injected ip with test preparations and weighed daily for 7 days (Fig. 27, 28), and killed at 21 days post-injection.

SECTION 6. IMMUNOGENICITY OF TOXOIDS

6.1 Intracerebral mouse-protection test (ICMPT)

The immunogenicity of toxoids was determined by their ability to protect mice from lethal intracerebral challenge with live *B. pertussis* 18323. The results from two identical experiments with groups of 10 mice were pooled and shown in Table 37, experiment 1. In each case the challenge dose was approximately 125-130 LD_{50}. Both the toxoid preparation and the whole-cell vaccine were highly protective with PD_{50} values as follows:

whole-cell vaccine (WCV) - 0.22 ou.ml/mouse

toxoid (AP-17)T33 - 0.8 μg/mouse.

In the next experiment, groups of 10 mice were immunized with graded doses of the untoxoided preparation, toxoids or the whole-cell vaccine (Table 37, experiment 2). Each immunized mouse was challenged with approximately 150 LD_{50}.

All three toxoids were highly protective and had similar PD_{50} values as follows:

toxoid (AP-17)T33 - 1.11 μg/mouse

" " T34 - 0.8 " "

" " T35 - 1.11 " "

The whole-cell vaccine was also protective with a PD_{50} value of 0.31 ou.ml/mouse (95% CL 0.2, 0.48).

The untoxoided preparation AP-17 was tested for mouse-protective activity, and some protection was observed in mice immunized with less than 5 μg/mouse of the preparation. All the mice immunized with this maximum dose died within 14 days after challenge. Therefore, a PD_{50}

Table 37. Immunogenicity of various preparations in the intracerebral mouse-protection test (ICMPT)

	Sample code	Dose	Mice (Survived/challenged)	PD_{50} + (95% CL)
1.		µg/mouse		
	(AP-17)T33	4.0	18/18	
		0.8	9/18	0.8 µg
		0.16	0/20	
		ou.ml/mouse		
	Whole-cell vaccine	5.0	15/18	
	(WCV)	1.0	17/20	0.22 ou.ml
		0.2	9/19	(0.17, 0.29)
		cfu/mouse		
	Controls	85	0/10	
		17	5/10	—
		3	10/10	
2.		µg/mouse		
	(AP-17)T33	4.0	8/10	
		0.8	3/10	1.11 µg
		0.16	0/10	(0.8, 1.55)
	(AP-17)T34	4.0	9/10	
		0.8	5/10	0.8 µg
		0.16	1/10	(0.56, 1.13)
	(AP-17)T35	4.0	7/9	
		0.8	3/10	1.11 µg
		0.16	1/9	(0.75, 1.64)
	AP-17	5.0	0/10	
		1.0	4/9	
		0.2	4/10	Not calculable
		0.04	2/10	
		0.008	0/10	
		ou.ml/mouse		
	Whole-cell vaccine	1.0	8/10	
	(WCV)	0.2	3/9	0.31 ou.ml
		0.04	1/10	(0.2, 0.48)
		cfu/mouse		
	Controls	96	2/10	
		19	2/10	—
		4	9/10	

+ PD_{50}; the immunizing dose/mouse which protected 50% of the mice from a lethal challenge of live, virulent B. pertussis. In experiment 1, each mouse was challenged with approximately 125-130 LD_{50}, and in experiment 2, approximately 150 LD_{50}.

value with 95% confidence limits could not be calculated from these data (Table 37, experiment 2).

6.2 Protection against intranasal infection in mice

In a preliminary experiment, mice were immunized with toxoid or whole-cell vaccine and challenged intranasally with B. pertussis 18323 after 14 days. Leucocyte counts (p. 88) and determination of the degree of mouse-lung colonization were done (p. 92).

Determination of viable counts of the challenge suspension indicated that each mouse was instilled with approximately 3×10^5 cfu of B. pertussis 18323 (Table 38). After 14 days, a slight leucocytosis was observed in mice immunized with toxoid (AP-17)T33 (4 μg/mouse) but not with the whole-cell vaccine (1.0 ou.ml/mouse). Control mice infected with the challenge suspension had a geometric mean WBC/mm^3 count of 19,900 (95% CL 12,400, 31,900). There were no deaths in any of the groups of mice including the infected controls, and normal weight-gain was unaffected (data not shown). Thus, the infection was sub-lethal.

The lungs of control mice infected with B. pertussis showed extensive colonization; approximately 820,000 cfu were recovered per mouse lungs (Table 38). Mice immunized with toxoid (AP-17)T33 at doses of 4.0, 0.8 or 0.16 μg/mouse had 0, 1,400 and 5,800 cfu/mouse lungs respectively. At the dose of whole-cell vaccine tested (1.0 ou.ml/mouse) only 1,240 cfu/mouse lungs were recovered (Table 38).

These experiments indicate that the carbodiimide-toxoided antigen preparations and the whole-cell vaccine, (i) protected mice against intracerebral challenge with B. pertussis and (ii) prevented lung colonization by B. pertussis.

The properties of the untoxoided antigen preparation, carbodiimide-toxoided antigen preparation and whole-cell vaccine are briefly summarized in Table 39.

Table 38. Protection against intranasal infection in mice

Sample code	Dose	Challenge	Leucocyte counts (WBC/mm^3) Geometric mean (95% CL)	Lung colonization	
				Organisms recovered*	Organisms recovered as a % of the control+
	µg/mouse	cfu/mouse		cfu/mouse lungs	
(AP-17)T33	4.0	2.94×10^5	15,200 (13,300, 17,300)	0	0
	0.8	"	ND	1,400	0.17
	0.16	"	ND	5,800	0.71
	ou.ml/mouse				
Whole-cell vaccine (WCV)	1.0	"	9,700 (6,700, 14,200)	1,240	0.15
	ml/mouse				
PBS	0.5	"	19,900 (12,400, 31,900)	820,000	100
"	"	No challenge	8,500 (7,100, 10,300)	0	0

ND, not determined.
*, arithmetic mean of 5 pairs of mouse lungs.
+ $\left[\frac{\text{Organisms recovered from immunized mice}}{\text{Organisms recovered from unimmunized infected control mice}}\right] \times 100$

Groups of 10 mice of 3-4 weeks of age were immunized ip with graded doses of toxoid or a single dose of whole-cell vaccine. Other mice were injected with PBS, as controls. The animals were infected intranasally 14 days later except for one control group. After 2 weeks, leucocyte counts were done (p. 88)on 5 mice per group; these mice were killed, the lungs excised and viable counts of the organism made (p. 92).

No deaths were observed in any of the test or control mice, and weight-gain was normal for the duration of the experiment.

Table 39. Comparison of the biological activities of various preparations

Sample	Characteristics		
	Pathophysiological Activities	Stability	Protective Activities
CARBODIIMIDE-TOXOIDED ANTIGEN PREPARATION	No HSA, LPA, induction of hyperinsulinaemia or hypoglycaemia in mice. Non-toxic and non-lethal for mice. Enhances neutrophil chemiluminescence *in vitro*.	Stable and antigenic at 4°C. Slight reversion at 37°C.	Highly antigenic and immunogenic: PD_{50} (ICMPT), 0.8-1.11 µg/mouse. Protects against intranasal infection and lung colonization in mice.
UNTOXOIDED ANTIGEN PREPARATION	Possesses HSA, LPA and induces hypoglycaemia and hyperinsulinaemia; toxic and lethal for mice. Inhibits neutrophil chemiluminescence *in vitro*.	Toxic, lethal and non-antigenic on storage.	Non-antigenic, when tested at doses permitted by its toxicity. Non-immunogenic in the ICMPT.
WHOLE-CELL VACCINE (WCV)	Possesses HSA, LPA and induces hypoglycaemia; toxic but non-lethal for mice at a dose of 5 ou.ml/mouse.	Antigenically stable at 4°C, but no loss in HSA, LPA or mouse-toxicity on storage.	Antigenic and immunogenic: PD_{50} (ICMPT), 0.2-0.31 ou.ml/mouse. Protects against intranasal infection and lung colonization in mice.

DISCUSSION

SECTION 1. PREPARATION AND CHARACTERIZATION OF ANTIGEN PREPARATIONS (AP)

Pertussis toxin (PT) and filamentous haemagglutinin (FHa) are currently accepted as probably the two most important antigens for inclusion in acellular pertussis vaccines (Robinson et al., 1985a). In the present study, both antigens were extracted by a single-step procedure using dye-ligand affinity chromatography. Sekura et al. (1983) used Affi-Gel Blue as the first step in the purification of PT to homogeneity. Both Affi-Gel Blue and Blue Sepharose CL-6B used in this study contain the dye Cibacron Blue as the ligand. This dye is thought to have a structure similar to NAD, the substrate in the PT-catalyzed ADP-ribosyltransferase reaction. However, in this study, elution buffers containing NAD did not remove haemagglutinating (HA) activity, and thus the dye probably does not serve as a substrate analogue to which PT binds. An important finding was that Blue Sepharose also had an affinity for FHa, or for a PT-FHa complex, which was not reported by Sekura et al. (1983) using Affi-Gel Blue.

In this investigation, culture fluid was used as the starting material for extraction. This had demonstrable HA and leucocytosis-promoting activity (LPA), and PT and FHa by ELISA. After extraction with Blue Sepharose, no PT or FHa were detected by ELISA and HA and LPA were absent. Therefore, extraction of both antigens was successfully achieved, and with simplicity, using dye-ligand affinity chromatography.

There was much less than 3.0 $\mu g\ ml^{-1}$ of Cibacron Blue F3G-A in the antigen preparations as determined by a simple spectrophotometric analysis. Trace amounts of this dye may be undesirable in the experimental acellular pertussis vaccines described in this study. Also,

if infants are to be immunized with these vaccines it is essential that possible toxic contaminants such as dyes are absent.

Since PT and FHa are currently accepted as essential acellular vaccine components (Manclark and Cowell, 1984; Pittman, 1984; Robinson et al., 1985a) procedures for increasing their yields in culture are desirable. In this investigation, the addition of methylated-β-cyclodextrin to media greatly enhanced the amount of total protein, PT and FHa extracted with Blue Sepharose.

The cyclodextrin liquid (CL) medium yielded the greatest amount of total protein per litre when B. pertussis was cultured with aeration at 37°C for 36h; however, when cultured for 48h there was a significant reduction in the yield.

The best yields of PT were obtained from B. pertussis cultured in SS-X medium supplemented with cyclodextrin and shaken for 36h at 37°C. Production of PT was also enhanced in the CL medium, although the mean yield was only slightly greater than that obtained by growth of B. pertussis in SS-X (static).

In this study, only approximately 0.01 mg of PT per litre were extracted from the culture fluid of B. pertussis 357 grown in the CL medium. By comparison, approximately 0.36-0.91 mg of PT per litre were produced by wild-type, phase I B. pertussis 77/18319 in the same medium. This transposon-induced mutant strain, B. pertussis 357, was originally isolated as PT-deficient and was shown to be avirulent in mice by the intranasal route of infection (Weiss et al., 1984). In this investigation, the organism released a large amount of FHa but little PT into the medium, thus confirming the characterization of this strain, ie: PT^-, FHa^+ by Weiss et al. (1984).

Imaizumi et al. (1983) made no mention of the production of FHa by B. pertussis in the shaken CL medium. In this study, the best yields of FHa were obtained from B. pertussis grown in the CL medium, shaken for 36h at 37°C. However, when cultured for 48h there was a significant reduction in the yield of this antigen similar to the reduction in total protein mentioned above. Therefore, incubation time may be a critical factor in determining the maximum yield of FHa from shaken cultures. Increasing the incubation time did not significantly reduce the mean yield of PT per litre obtained in the CL medium, possibly indicating that cyclodextrin stabilizes PT more effectively than it does FHa. Whereas previously, FHa could only be produced in sufficient quantities in static SS-X medium (Arai and Munoz, 1979), the culture of B. pertussis in cyclodextrin-supplemented media (CL and SS-X in this study) should be carefully monitored to determine when maximum FHa is produced. Suzuki et al. (1985) also reported maximum production of FHa by B. pertussis strain Tohama in the CL medium after 36h, but no decrease in the amount of FHa obtained after 48h of culture.

Approximately 100% (w/w) of the total protein extracted from culture fluid of B. pertussis grown in cyclodextrin-supplemented SS-X medium, was accounted for as PT and FHa. Similarly, approximately 69-129% (w/w) of the total protein extracted from culture fluid of organisms grown in the CL medium was PT and FHa. However, in the majority of the experiments in which B. pertussis was cultured in SS-X (static), only approximately 50% of the total protein extracted was accounted for as both antigens. It should be noted that in all of these growth experiments the initial inoculum was not a standardized bacterial suspension, and this in part may explain some of the differences observed.

Growth of B. pertussis in SS-X (static) in a fermenter vessel was the least effective method for obtaining high yields of both antigens. Only 31% (w/w) of the total protein extracted was accounted for as PT and FHa, the rest possibly coming from cell lysis. This experiment was only done once, and culture of B. pertussis in cyclodextrin-supplemented media in a fermenter vessel should not be ruled out as a means of improving antigen yields.

Cultural conditions also influenced the ratio of PT to FHa in the antigen preparations. In the production of acellular pertussis vaccines, the desirable PT to FHa antigen ratio could be achieved by mixing different volumes of these preparations. Therefore, it is possible to produce vaccines of uniform antigen composition without needing to purify the antigens separately and combine them after toxoiding. Nevertheless, the question still remains as to the amount and relative proportions of these (and other) antigens for inclusion in human acellular vaccines.

To summarize, the use of different cultural conditions followed by dye-ligand affinity chromatography to achieve a particular ratio for PT and FHa, deserves serious consideration in the development of acellular pertussis vaccines.

Analysis of the antigen preparations described in this study by SDS-PAGE, revealed the presence of distinct high molecular weight bands which appeared to be FHa. A semi-purified haemagglutinin fraction prepared by a different method (sodium acetate extraction of B. pertussis whole-cells) had a similar profile of high molecular weight bands (R. Parton, personal communication). FHa purified from liquid culture

supernates was found to be heterogeneous when examined by SDS-PAGE, and these various fragments probably arose by degradation of a polypeptide of higher molecular weight, 220k (Irons et al., 1983).

There were also five distinct and characteristic bands of lower molecular weight, which, when run alongside molecular weight standard protein markers, had similar molecular weights to those reported in the literature for PT (data not shown). Also, when antigen preparations were analyzed by SDS-PAGE alongside a purified, commercial PT preparation, the band profiles in the lower molecular weight region were identical (data not shown).

Minor proteins were detected in most of the PT and FHa antigen preparations by SDS-PAGE. It may be that these would be useful antigens in a commercial acellular pertussis vaccine and as such would require further characterization. B. pertussis produces and releases some extracytoplasmic adenylate cyclase during exponential growth (Hewlett et al., 1977) and it is possible that this enzyme was extracted with PT and FHa by Blue Sepharose. Recently, Novotny et al. (1985) prepared an adenylate cyclase extract from B. pertussis which was about 95% pure, and found that it protected mice from intracerebral challenge with B. pertussis 18323. Trace contaminating proteins were observed in this extract by SDS-PAGE, but PT was absent since hyperimmunization of rabbits did not stimulate detectable anti-toxin antibodies. Therefore, since adenylate cyclase may be useful in an effective vaccine formulation, it would be of interest to ascertain whether the antigen preparations described in this study contained the enzyme.

Other proteins present in culture fluid may include tracheal cytotoxin (Goldman et al., 1982), outer membrane proteins and agglutinogens. There is evidence that fimbriae, certain outer membrane proteins and FHa

protect mice against intracerebral challenge with B. pertussis, when given with low levels of native, non-protective PT (Robinson and Irons, 1983). Native PT also had a pronounced synergistic effect on the protective activities of various antigens against intranasal infection in mice (Robinson et al., 1985b). In this investigation, the agglutinogen profile and other minor proteins in the antigen preparations were not characterized. Nevertheless, the potency of acellular vaccines for use in humans may be improved by the inclusion of these various antigens.

The presence of some or all of these proteins may explain in part the discrepancies where the total protein in the antigen preparations was not accounted for as PT and FHa.

Endotoxin was detected in antigen preparations AP-16 and AP-17 by the Limulus amoebocyte lysate (LAL) assay. There was approximately 5 μg of endotoxin ml^{-1} in AP-16 and 8 μg of endotoxin ml^{-1} in AP-17, where the protein concentrations were 240 μg ml^{-1} and 820 μg ml^{-1} respectively.

The disadvantages of the Limulus assay are that it is semi-quantitative, and gelation of the lysate can be inhibited by unknown components in the antigen preparations or culture fluid. The assay of endotoxin by measurement of temperature responses in mice (Prashker and Wardlaw, 1971; Wardlaw et al., 1971) was unsuitable in this investigation, because the HaM/ICR mouse-strain was unresponsive to purified B. pertussis endotoxin. An alternative method for detection and quantitation of endotoxin involves resolution of test and reference endotoxin samples by SDS-PAGE with silver-staining. The stained gels are subjected to densitometric scanning and an estimate of endotoxin content in test samples can be made (Y. Perera, personal communication). This procedure may be useful in preference to the mouse and rabbit pyrogenicity tests

and the Limulus assay for detection and quantitation of contaminating endotoxin in acellular pertussis vaccines.

Endotoxin is generally regarded as an undesirable constituent of pertussis vaccine (Manclark and Cowell, 1984). However, recent research indicates that endotoxin could play a role in acellular vaccine formulations. Ashworth et al. (1982b) found the LPS of B. pertussis to be antigenic, and antibodies to LPS were produced in animals and humans after vaccination with whole-cell pertussis vaccines (Ashworth et al., 1983). Winsnes et al. (1985) reported that children immunized with adsorbed DTP vaccine had detectable IgA, IgM and IgG antibodies to LPS, and that two individuals protected against clinical infection with B. pertussis had high concentrations of IgG anti-LPS antibodies. Sultzer et al. (1985) reported that proteins usually complexed to endotoxin had adjuvant properties in enhancing the immunogenicity of cholera toxoid in mice. Consequently, in the toxoided PT and FHa preparations which had demonstrably low toxicity in this investigation, traces of endotoxin could possibly have functioned as an adjuvant to enhance immunogenicity.

It is possible that the LPS-endotoxin present in the acellular preparations described in this study was chemically modified by treatment with EDAC. The KDO residues in the LPS core contain free carboxyl groups which could be accessible to carbodiimide-modification at low pH (Seid and Sadoff, 1981; Fig. 7). Consequently, LPS in an immunogenic and non-toxic form may be necessary in order to prepare a highly effective acellular pertussis vaccine.

Endotoxins could possibly be removed from acellular vaccines by passage through a polymyxin-Sepharose gel matrix (Bannatyne et al., 1986), or their in vivo pyrogenicity reduced by administration of the

antibiotic itself (Larter et al., 1984). However, with the antigen preparations described herein, these procedures may be unnecessary for the reasons mentioned above.

The acellular vaccine manufactured at the PHLS, CAMR, Porton Down, Salisbury, Wiltshire, also contains traces of endotoxin (Robinson et al., unpublished observations). By the Limulus assay this vaccine contains 30-60 ng of endotoxin ml^{-1}, and approximately 60 μg of protein ml^{-1}. Therefore, a single human dose (30 μg of protein) would contain approximately 15-30 ng of endotoxin, ie: 0.05-0.1% endotoxin. By comparison, a single human dose of the antigen preparations in this study would contain approximately 300-600 ng of endotoxin, ie: 1-2% endotoxin. However, in the preparation, toxoiding and storage of these antigen preparations no special precautions such as pyrogen-free equipment were used.

An important finding in this investigation was that the antigen preparations could be filter-sterilized without an apparent loss of protein; this indicated that there was no gross aggregation in these preparations, and that the samples were amenable to sterilization, which would be an essential step when preparing a vaccine for use in humans.

SECTION 2. TOXOIDING STUDIES WITH 1-ETHYL-3-(3-DIMETHYLAMINOPROPYL) CARBODIIMIDE.HCl (EDAC)

Classically, formaldehyde and glutaraldehyde have been used to detoxify bacterial exotoxins, including pertussis toxin. For reasons which will be explained below, in this study the PT and FHa antigen preparation was toxoided with a water-soluble carbodiimide, EDAC.

The antigen preparations (AP) prepared in this study had many diverse biological activities including induction of histamine-sensitization, promotion of leucocytosis, hypoglycaemia and hyperinsulinaemia, and lethality in mice. Experiments were done to determine the optimum conditions of toxoiding for elimination of both the A-protomer and B-oligomer activities of PT in the antigen preparation. With this preparation, the minimum amount of EDAC required to remove completely HSA was a ratio of reagent to protein of at least 40:1 by weight. In this study, reaction of EDAC with AP at a ratio of 40:1 at 37^{o}C, pH 5.0 for 60 min, followed by exhaustive dialysis at 4^{o}C, was sufficient to remove > 99% of the original HSA. However, for routine toxoiding a ratio of EDAC to protein of 80:1 was chosen to allow for a greater margin of safety. At this ratio of EDAC to protein, toxoids were successfully produced by reaction at various temperatures and over a pH range of 5.0-9.0.

The toxoiding conditions most suitable for elimination of these activities were reaction of EDAC with protein at a ratio of 80:1, pH 5.0 at 37^{o}C for 24h. These toxoids were greatly reduced in HSA and LPA, activities dependent on the B-oligomer of PT (Nogimori *et al.*, 1984a). The toxicity of the A-protomer was also eliminated by carbodiimide-treatment, as judged by the inability of toxoids to induce hyperinsulinaemia in mice.

These toxoids were non-lethal and non-toxic for mice. There was evidence of some toxicity for mice injected with a toxoid prepared with an EDAC to protein ratio of 10:1. This toxicity was observed 4-5 days after injection and was probably due to residual active toxin (which was detected in this toxoid as HSA and LPA).

Other toxins modified with EDAC were cholera toxin (Lonnröth and Holmgren, 1975) and the heat-labile (LT) enterotoxin of E. coli (Klipstein et al., 1982b). Lonnröth and Holmgren (1975) treated cholera toxin with EDAC (10 mM in 0.15M NaCl buffer, pH 4.3) for 1h at 23°C. There was no reduction in the toxicity of treated cholera toxin in the rabbit intradermal assay, nor in the capacity to bind to G_{M1} ganglioside (the membrane binding site, monosialosyl-N-tetra-glycosylceramide, in target cells) in vitro. Klipstein et al. (1982b) treated the LT enterotoxin of E. coli with EDAC at reagent to toxin ratios from 10 to 75 by weight, for 18h at 4°C. At the maximum amount of toxoiding reagent used, the LT-toxoid had less than 1% residual toxicity in the in vitro YI adrenal cell assay. Apart from these two reports, no other bacterial exotoxins have been toxoided with this reagent until the studies described in this thesis.

As mentioned above, PT has been toxoided with formaldehyde (Sato et al., 1974; Sato and Sato, 1984) and glutaraldehyde (Munoz et al., 1981a,b; Munoz and Arai, 1982; Cowell et al., 1982; Robinson and Irons, 1983; Oda et al., 1984; Watanabe, 1984), but in only a few instances were biological activities other than mouse-protection investigated. Sato et al. (1974) found that the HSA, LPA and HA activities of PT (not intended for inclusion in an acellular vaccine) decreased gradually with formaldehyde-L-lysine treatment. After treatment

for 11 days, all 3 biological activities were $<0.1\%$ of the untoxoided preparation. Sato and Sato (1984) treated PT with formaldehyde in the absence of L-lysine and reported that the toxoid showed neither LPA at 10 μg/mouse nor haptoglobin-binding activity by ELISA at 1.0 μg: the minimum detectable amount of active PT by the former and latter assays was 10 ng and 1.0 ng respectively (Sato et al., 1983b).

The toxicity of pertussigen was also reduced by treatment with glutaraldehyde-L-lysine (Munoz et al., 1981a,b; Munoz and Arai, 1982). For example, HSA and HA activities of the toxoid were less than 25% and 4% respectively of the untoxoided material, and doses of 10 μg of glutaraldehyde-toxoid/mouse were non-lethal. By comparison, the carbodiimide-toxoided antigen preparations described in this study had $<0.5\%$ of the original HSA of untoxoided material and were non-lethal at much higher doses than those tested above. The native LPF(PT) of Robinson and Irons (1983) was toxic for mice at about 2 μg/mouse, but after toxoiding with glutaraldehyde-L-lysine, 10 μg/mouse was non-toxic but produced a marginal increase in leucocytosis. No LPA was observed in mice injected with doses of up to 27 μg/mouse of the toxoids prepared with EDAC in the present investigation.

Apart from these studies and observations, no thorough investigations on the toxoiding of purified PT with formaldehyde or glutaraldehyde have been reported. This is a serious deficiency in view of the fact that current acellular pertussis vaccines contain antigens treated with these reagents.

Recently, a curious HSA was shown by the formalin-toxoided Japanese acellular pertussis vaccine (Isawa et al., 1985). It is generally accepted that HSA in mice injected with whole-cell vaccine or

purified PT, increases to reach a plateau after 4-5 days and then gradually decreases for the next 3-4 weeks (Wardlaw and Parton, 1983b). However, it was found that HSA of the Japanese acellular vaccine was absent on day 4 but present on day 12 after injection into mice. Isawa et al. (1985) postulated that either,

(i) when bulk materials were toxoided with formaldehyde a portion of the entity responsible for HSA might have escaped detoxification by forming an aggregate. In vivo, this aggregate might gradually dissolve and release active toxin.

(ii) the toxoided acellular vaccine might reactivate in vivo.

Both these possibilities could lead to serious pathophysiological side reactions in immunized infants and cause renewed criticism of pertussis vaccines.

(iii) formaldehyde might modulate the existing state of the entity responsible for early (day 4-5) HSA so as to delay the time course of histamine-sensitization, or

(iv) different moieties might be responsible for these activities.

In this investigation, when the antigen preparation was toxoided with EDAC there was no detectable HSA on day 5 or day 12 after injection into mice. The fact that this toxoid preparation did not manifest HSA on day 12 might indicate that,

(i) there was no in vivo reversion,

(ii) if different moieties were responsible for the early (day 4-5) or late (day 12) HSA, both were inactivated with EDAC-treatment,

(iii) EDAC did not modulate the existing state of the entity responsible for early (day 4-5) HSA so as to delay the time course of histamine-sensitization. In this last respect, it would be interesting to monitor HSA and other biological activities beyond 12 days.

Without doubt, the formalin-toxoided Japanese acellular vaccine manifests a late (day 12) HSA in mice which may have a profound significance for human vaccination. This activity may have to be taken into account as a laboratory test for toxicity of pertussis vaccine, and the results in this study indicate that EDAC may be a more suitable toxoiding agent than formaldehyde.

Pertussis toxin has been chemically modified with trinitrobenzenesulphonic acid, TNBS (Fish et al., 1984), ethyl acetimidate (Nogimori et al., 1984a), acetic, maleic and succinic anhydrides (Nogimori et al., 1984b) and formaldehyde in the presence of sodium borohydride, $NaBH_4$ (Ui et al., 1985). All these reagents react primarily with the free ϵ-amino groups of protein lysine residues. However, the A-protomer (S1 subunit, Fig. 2) of PT lacks lysyl residues (Nicosia et al., 1986). Consequently, treatment of PT with these reagents could leave A-protomer activity intact. These authors observed that following treatment of PT with glutaraldehyde, subunits S2, S3, S4 and S5 (B-oligomer, Fig. 2) were cross-linked to form aggregates of high molecular weight, while the S1 subunit (A-protomer) retained its original size (Nicosia et al., 1986).

Nogimori et al. (1984a) showed that acetamidination of islet-activating protein, IAP(PT), modified the biological activities of the B-oligomer, eg: leucocytosis-promotion and histamine-sensitization, whereas the A-protomer remained biologically-active in its ability to catalyze ADP-ribosylation and potentiate insulin secretion in vivo. Reductive methylation (Ui et al., 1985) produced the same results. In the studies described in this thesis, carbodiimide-treatment abolished the biological activities associated with the A- and B-subunits of PT in the antigen preparations. However, Nogimori et al. (1984b) observed that

acylation abolished all biological activities *in vivo* but by destruction of the quaternary structure of the protein.

Because of the problems encountered with the reagents described above, carbodiimides may be preferred toxoiding reagents since they react primarily with the carboxyl groups of glutamyl and aspartyl residues as well as with the C-terminal carboxyl, when low pH is used (Hoare and Koshland, 1967). As a result, a sequence of complex chemical reactions could occur leading ultimately to peptide bond formation. Also, other amino acid residues might be modified, eg: tyrosine(Carraway and Koshland, 1968), cysteine (Carraway and Triplett, 1970), histidine (Takata *et al.*, 1985), serine (Banks *et al.*, 1969) and asparagine (Rich and Singh, 1979).

Analysis of the amino acid sequence of subunit S1 of PT (Nicosia *et al.*, 1986; Locht and Keith, 1986) showed that lysine residues were absent, but that carbodiimide-reactive residues were present. Without an exact amino acid analysis of protein before and after carbodiimide-treatment, the exact nature of the modified residues remains uncertain. However, modification of tyrosine can be reversed at pH 8.0 using hydroxylamine to indicate one possible reaction (Grouselle and Pudles, 1977).

Biological activity may be abolished by conformational constraints induced by carbodiimide-modification and cross-linking. In this study, indirect evidence of successful carbodiimide-mediated antigen conjugation was sometimes provided by the formation of precipitates. When slight precipitates or a colloidol suspension were formed, the preparations were gently inverted to ensure uniform distribution before injection into animals. These precipitates were probably caused by changes in the

solubility of the antigens when cross-linked by the carbodiimide reaction or by the formation of N-acylurea adducts of the proteins (Goodfriend et al., 1964). In this study, macromolecular cross-linking of the antigens was apparent from the inability of a toxoid preparation, (AP-17)T33, to run in SDS-PAGE gels, even after boiling at 100°C for 5 min in solubilizing buffer (containing 2% (w/v) SDS and 5% (v/v) β-mercapto-ethanol).

The introduction of inter- and/or intramolecular cross-links might perturb the spatial organization of the molecule (Gill et al., 1968), and render binding sites of the B-oligomer and enzymatic sites of the A-protomer biologically redundant. Also, the formation of N-acylurea adducts of proteins (Timkovich, 1977; Davis et al., 1984) might cause a steric hindrance or a conformational change and concomitantly the reduction of enzyme activity. However, it has to be stressed that the complex reactions of formaldehyde (French and Edsall, 1945) or glutar-aldehyde (Cheung and Nimni, 1982a) with amino acids and proteins might also abolish biological activities by perturbation of the normal conformational structure of PT.

To summarize, the studies described in this thesis indicate that EDAC is an excellent toxoiding reagent for pertussis toxin in the antigen preparations.

SECTION 3. POSSIBLE NEW ASSAYS FOR TOXOIDS

The advent of acellular pertussis vaccines is an opportune time to reconsider the toxicity testing of isolated pertussis antigens. Also, the recent legislation limiting the use of animals in medical research would support the continued search for alternative procedures. A small study was undertaken jointly with Mr. F. Craig to determine the effect of untoxoided and carbodiimide-toxoided preparations on the chemiluminescence response of rabbit peritoneal neutrophils to a chemotactic stimulus N-formylmethionyl-leucyl-phenylalanine (fMLP) and a light-enhancing reagent luminol.

When polymorphonuclear leucocytes (PMNL) and soluble or particulate matter interact, the cells produce chemiluminescence (light-emission). The generation of chemiluminescence-reactive molecules, eg: superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen and hydroxyl radicals as a result of respiratory burst activation in PMNL, is essential for host defence against microorganisms (Briheim et al., 1984). Chemiluminescence of PMNL is a phenomenon related to this respiratory burst activation and is a bimodal response (Bender and Van Epps, 1983). Chemotactic factors such as fMLP, which are important in attracting neutrophils to inflammatory sites, also stimulate oxidative metabolism resulting in increased chemiluminescence and release of superoxide anions. In the bimodal response, the primary peak is a result of extracellular reactions and the secondary peak a result of intracellular reactions responsible for the oxidation of luminol and enhanced chemiluminescence.

The study indicated that an untoxoided, partially purified PT preparation (AP-16) totally inhibited the chemiluminescence response of rabbit neutrophils to fMLP and luminol, at a dose of 50-100 ng/assay tube.

In a parallel study published in 1985, Becker et al. showed that a dose of 200 ng ml^{-1} of purified, crystalline PT inhibited neutrophil chemotaxis induced by fMLP, possibly by ADP-ribosylation of a membrane-bound GTP-binding regulatory protein which mediated the inhibition or activation of a target protein(s) required in neutrophil activation. In the present study, toxoid (AP-16)T29, with $>$ 99% reduction in HSA, enhanced the chemiluminescence response of rabbit neutrophils to fMLP and luminol. This indicated the absence of biological activity associated with the A-protomer, the active ADP-ribosyltransferase moiety of PT.

The antigen preparations (AP) contain predominantly PT and FHa. Consequently, purified FHa was tested to see how it modulated the chemiluminescence response of rabbit neutrophils. Filamentous haemagglutinin markedly enhanced chemiluminescence, although modification with EDAC caused a significant reduction in the response when comparable doses (50 ng/assay tube) of modified and unmodified FHa were assayed. This reduction might be associated with the introduction of carbodiimide-mediated cross-links (inter- and/or intramolecular), and/or the formation of carbodiimide-adducts of FHa, which in some way prevent interaction with neutrophils. Similarly, toxoided PT in the antigen preparation did not inhibit neutrophil chemiluminescence, possibly by inability of the toxin B-oligomer to interact with neutrophil membrane-receptors, thus preventing entry of the enzymatic A-protomer. Nevertheless, the toxoid ((AP-16)T29) contained sufficient carbodiimide-modified FHa to significantly enhance the chemiluminescence response.

Other factors cannot be dismissed, since B. pertussis LPS also enhances the chemiluminescence response. A dose of 1.0 μg of LPS/assay tube produced an enhancement comparable to that expressed with FHa

(50 ng/assay tube; Fig. 18), whereas 0.1 μg of LPS/assay tube did not enhance the response above the control (F. Craig, personal communication). Clearly, FHa is a better enhancer of the chemiluminescence response than LPS. In the toxoid preparation (AP-16)T29, tested at 10 μg/assay tube, there would be approximately 0.2 μg of endotoxin, an amount insufficient to cause the enhancement of chemiluminescence observed (F. Craig, personal communication).

The usual enhancement of chemiluminescence observed with purified FHa was abolished when the protein was heated at 80°C for 30 min. However, B. pertussis LPS was unaffected by this heat-treatment and still enhanced the response (F. Craig, personal communication). When toxoid (AP-16)T29 was similarly heated, the enhancement was also abolished. This indicates that the trace of LPS in the toxoid preparation did not play a role in enhancement of the chemiluminescence response of rabbit neutrophils to fMLP and luminol.

It was noticed in this assay that there was variation in the responsiveness of different batches of neutrophils, eg: in the size of the primary response peak and sometimes in the absence of the secondary peak if the primary response was very large. This variation might be overcome by comparison with a standard toxoid preparation run in parallel each time an assay was done.

The most important conclusions from this study are that, (i) PT in the antigen preparation suppresses chemiluminescence of rabbit neutrophils even in the presence of enhancers such as FHa and LPS, (ii) the toxoid preparation,devoid of HSA, enhances the chemiluminescence of rabbit neutrophils. Carbodiimide-treated PT presumably does not inhibit neutrophil chemotaxis nor the chemiluminescence-enhancing properties of other proteins in the toxoid.

Because of its simplicity and rapid generation of results, this *in vitro* assay of PT could be used to screen toxoids for residual toxicity. These toxoids could be evaluated in parallel with a standard toxoid preparation, and it would be interesting to compare the many emerging commercial acellular pertussis vaccines with the carbodiimide-toxoided preparations described in this study.

Another possible *in vitro* assay for toxoids was examined in this investigation, using the enzyme-linked immunosorbent assay (ELISA). In the study of Klipstein *et al.* (1982b), there was a progressive reduction in the antibody-binding affinity of *E. coli* heat-labile (LT) enterotoxin after treatment with increasing amounts of EDAC. The antibody-binding affinity of toxoids was assessed in an ELISA by comparing the dose-response curves of untreated LT or the toxoids, as the coating layers, against monospecific hyperimmune antiserum to LT. This reduction correlated with the compromised effectiveness of toxoided LT to evoke an antitoxin response and provide protection in immunized animals.

Similarly, in the present investigation, the antibody-binding affinity of toxoided preparations was assessed by comparing the dose-response curves of untoxoided AP or the toxoids, as coating layers, against either monospecific rabbit anti-PT polyclonal IgG antibody or mouse anti-PT monoclonal IgG antibodies. In these experiments, there was a progressive reduction in either the antibody-binding affinity of PT in AP when treated with increasing amounts of EDAC, or in the binding affinity of toxoids for the polystyrene ELISA plates. Klipstein *et al.* (1982b) did not report whether their data represented a genuine reduction in antibody-binding affinity, or binding affinity of the toxoids for polystyrene. If the former possibility was the case, then

a preliminary screening of toxoids for reduced antibody-binding affinity - which must be correlated with a concomitant reduction in toxicity - could be used to expedite the evaluation of toxoiding reagents and conditions. A major advantage would be to obviate the need to examine all toxoid preparations with the more cumbersome and time-consuming animal studies.

SECTION 4. STABILITY OF TOXOIDS

Product stability is an ultimate requirement for the preparation of commercial vaccines. There is ample evidence that some bacterial exotoxins revert both *in vitro* and *in vivo*. This was shown with diphtheria toxin (Wadsworth *et al.*, 1937; Stainer, 1967; Akama *et al.*, 1971a), tetanus toxin (Akama *et al.*, 1971b), *Pseudomonas aeruginosa* exotoxin A (Cryz *et al.*, 1981), cholera toxin (Northrup and Chisari, 1972; Rappaport *et al.*, 1974) and pertussis toxin (Sato *et al.*, 1974).

In this investigation, independent stability studies were done on three toxoid preparations. In these studies, the toxoids were stable at $4^{o}C$ for the duration of the test (56 days), as judged by their inability to induce histamine-sensitization, promote leucocytosis or hyperinsulinaemia or affect weight-gain in mice. A toxoid preparation was also lyophilized and stored at $4^{o}C$ for 24 weeks, and tested. This preparation was also non-toxic for mice.

However, when stored at $37^{o}C$, the toxoids showed partial reversion. Toxoid (AP-16)T37 showed slight reversion to HSA and LPA when tested after 14 days at $37^{o}C$. Reversion was also detected with toxoid (AP-17)T33 when tested after 28 days at $37^{o}C$; 1.0% of the original HSA of untoxoided material was detected. There was also approximately a 5% reversion to hyperinsulinaemia activity of the A-protomer of PT in toxoid (AP-17)T33 when tested after 14 days at $37^{o}C$, but this was labile on further incubation.

However, these toxoids which showed partial reversion to both A- and B-subunit activities of PT in this study, were non-lethal for mice and did not affect normal weight-gain.

In the literature, toxoids prepared with formaldehyde were stable at 4°C but showed *in vitro* reversion during storage at elevated temperatures. The formalin-L-lysine treated PT of Sato *et al.* (1974), which was not used in any vaccine formulation, regained lethal toxicity (HSA, LPA) upon removal of formalin by dialysis at 37°C. This reversion is especially interesting because evidence with other bacterial exotoxins (Linggood *et al.*, 1963; Scheibel and Christensen, 1965; Stainer, 1968; Cryz *et al.*, 1981) suggests that the addition of L-lysine to the reaction mixtures substantially improves toxoid stability. The pertussis component vaccine of Sato *et al.* (1984) consisted of a crude haemagglutinin extract which was toxoided with formaldehyde. However, no mention was made of the use of L-lysine in the toxoiding reaction. Therefore, it would be surprizing if this or any other acellular pertussis vaccine proved to be totally stable after treatment of the antigens with formaldehyde, and the possibility of *in vivo* reversion cannot be discounted. When toxoiding with carbodiimide, the addition of exogenous amino acids such as L-lysine would probably not improve toxoid stability and may have undesirable consequences for antigenicity.

The studies described herein do not indicate exactly when toxoid reversion occurs at elevated temperature. To determine this, samples should be assayed daily during storage for reversion of biological activities such as HSA, LPA and induction of hyperinsulinaemia in mice. Also, the exact nature of the reversible reaction remains unclear. From the chemistry of carbodiimide-modification of proteins, two major reactions can occur, ie: formation of a peptide bond or intramolecular rearrangement to a stable N-acylurea. The formation of N-acylurea is markedly suppressed by the use of asymmetrical carbodiimides which include EDAC (Ito *et al.*, 1977), and peptide bond formation is probably the major

reaction product for reasons described earlier. Reversion might be associated with the thermal instability of modified amino acids such as tyrosine, etc., but no evidence is presented to support this. The pH of the storage buffer might also be important, and experiments should be done to determine if toxoids are stable at 37°C in buffers of other pH values.

An interesting feature of these studies was the stability of HSA and LPA of PT in the untoxoided preparations at 4°C or 37°C. There was remarkably little reduction in these biological activities, although mouse-lethality was often reduced on storage at 37°C. The whole-cell vaccine (WCV) showed no reduction in HSA or LPA at 4°C in these stability studies. In fact, lyophilized whole-cell vaccine, kept at 4°C in sealed ampoules, showed no significant reduction in HSA during the 3 year period of independent research described in this thesis.

SECTION 5. ENZYME-LINKED IMMUNOSORBENT ASSAY

Irons and MacLennan (1979a,b) showed a specific interaction between PT and the side-chain groups of haptoglobin that contained sialic acid residues, and used haptoglobin bound to a Sepharose 4B matrix in affinity chromatography purification of PT. Since other sialic acid-containing proteins will bind PT, the more readily available fetuin replaced haptoglobin in purification studies (Askelöf et al., 1982; Sekura et al., 1983).

Sato et al. (1983b) used haptoglobin as the solid (ie: polystyrene-bound) phase in an ELISA for PT. In the studies described herein, fetuin was used as the solid phase in experiments to develop an ELISA for detection and quantitation of PT-antigen.

5.1 Quantitation of antigen

The end results of the quantitative enzyme immunoassay of antigen concentration, determined by a standard curve method, are expressed in absolute terms, eg: $\mu g\ ml^{-1}$ antigen. In this study, ELISA systems were developed using either fetuin, haptoglobin, rabbit anti-PT IgG polyclonal antibody or mouse anti-PT IgG monoclonal antibodies as the solid phase to provide specificity for the PT-antigen. Extra specificity was provided in these assays by the use of a monoclonal antibody, L_{10}, for antigen bound to the coating layer. These ELISA systems detected as little as 2-4 ng of PT $well^{-1}$; in the haptoglobin-ELISA of Sato et al. (1983b) the minimum detectable amount of PT was reported as 1.0 ng $well^{-1}$.

The fetuin-L_{10} monoclonal antibody system was the most acceptable of all these ELISA since background absorbance was negligible, whereas the haptoglobin-, polyclonal-, L_4- and L_5-L_{10} systems gave high

backgrounds. It is possible that specific binding of anti-mouse IgG-HRP conjugate to the monoclonal antibodies L_4 and L_5 as coating layers, was responsible for the high background absorbance in these assays. However, the high background observed with haptoglobin or polyclonal antibody as the solid phase might have resulted from some non-specific interaction with the conjugate.

Both the haptoglobin-L_{10} and polyclonal-L_{10} systems were unsuitable for assay of PT in crude material. Non-specific binding of other components in the crude preparation to the coating layer, or a hindrance of specific pertussis toxin binding by the total concentration of these components, are among the various possibilities to explain this finding.

The fetuin-L_{10} ELISA was reproducible and specific for PT. The advantages of this ELISA over the *in vivo* assays of PT activity (eg: HSA, LPA and induction of hyperinsulinaemia) for the quantitative determination of PT, are the rapidity, sensitivity, specificity and cost of the procedure. Also, recent legislation limiting experimental animal research would support the use of this alternative procedure. Although the haemagglutination reaction can be used as an *in vitro* assay for PT, it has low specificity and sensitivity, and the presence of FHa in the majority of assay samples in this study is a major problem.

Although ELISA does not measure a true biological activity, Sato *et al.* (1983b) reported a highly significant correlation between PT determinations obtained by haptoglobin-ELISA and the leucocytosis assay in mice. The detectable amounts of PT in several *in vivo* assays are summarized in Table 2 (this thesis) and the review of Wardlaw and Parton (1983b). For example, the HSD_{50} value of PT was in the order of 0.5-60 ng/mouse, and doses of 20-200 ng/mouse induced significant leucocytosis

in mice (Table 2). In this study, the fetuin-ELISA could detect as little as 4 ng of PT well^{-1}, and it would be interesting to compare directly the sensitivity of this assay with the *in vivo* determinations of PT activity.

5.2 Quantitation of antibody

A major problem in the assay of serum IgG antibody to PT and FHa by ELISA was the availability of an inexpensive supply of both purified antigens. This was alleviated by the use of semi-purified antigen preparations, and, in the determination of anti-PT IgG antibody, with fetuin as the specific coating layer. Initially, these semi-purified preparations were standardized against purified antigens for use in ELISA. It was found that the purified antigens in antibody-ELISAs were successfully replaced with these standardized preparations, without significant alterations in the antibody titration curves.

In contrast to antigen quantitation, the measurement of antibody by ELISA is much more difficult. Since ELISA measures the combined effects of antibody concentration and antibody affinity, the standard curve method has not been used to produce results in absolute units of specific antibody concentration, eg: μg IgG ml^{-1} serum. Instead, a variety of alternative approaches have been used to estimate the relative 'antibody activity' rather than absolute antibody concentration. De Savigny and Voller (1980) listed a variety of methods and concluded that none of them satisfied all the essential criteria of an ideal assay, ie: to be quantitative, reproducible, efficient and economical, and free of introduced assumptions such as parallelism of dose-response curves.

In the pertussis literature, many methods have been used to report 'antibody activity' of test sera. These included reporting end-

point titres (Granström et al., 1982a) and absorbance values at a single serum dilution (Granström et al., 1982b). Burstyn et al. (1983) and Oda et al. (1984, 1985) compared the linear portions of plots of absorbance versus log serum dilutions for test and reference sera. Relative antibody titres were calculated by determining the antilog difference between the X-intercepts (log serum dilutions) of the linear portions of test and reference sera and multiplying this by 100, the assigned unitage of the reference antiserum. However, in this calculation the linear portion of each curve was redrawn by using a common slope, ie: parallelism of dose-response curves was assumed. Therefore, no single method for reporting 'antibody activity' has been universally adopted, and those mentioned above are not totally satisfactory.

In this investigation, many pooled and individual sera were titrated for IgG anti-PT and anti-FHa antibody responses by ELISA. The data were presented as either theoretical Antibody Units ml^{-1} serum (Appendix 9) or actual antibody titres (Appendix 10). In neither calculation was any parallelism of the ELISA antibody titration curves of test and reference sera assumed. The ELISA titration curves of some test sera were significantly non-parallel to the reference antiserum titration curve and were therefore assigned Y/X values rather than valid antibody titres (Appendix 10). The major advantage in presenting data as actual antibody titres in this investigation, is that the combined effects of antibody affinity (absorbance 492nm) and antibody concentration (log titre) in test sera are evaluated. To validate the data even further a reference antiserum against a commercial acellular vaccine,as an international ELISA reference antiserum,could be made available. Such a comparison would give a crucial indication of the relative antigenicities of the experimental acellular vaccines prepared in this and other studies.

SECTION 6. ANTIGENICITY AND IMMUNOGENICITY OF TOXOIDS

In this thesis, the distinction of Finklestein (1984) between antigenicity, ie: the capacity to stimulate an immune response, and immunogenicity, ie: resulting in resistance or immunity, is used. All untoxoided preparations in this study failed to stimulate significantly detectable anti-PT IgG or anti-FHa IgG antibody responses in mice; normal mouse serum or sera from mice injected with PBS were similarly unresponsive. The lack of stimulation by untoxoided preparations might be due to the fact that the dose of antigen required to initiate a good immune response was lethal. Alternatively, Asakawa (1969) observed that LPF (PT) suppressed the formation of circulating antibody in mice and Suzuki et al. (1978) postulated that active PT might directly suppress the phagocytosis or antigen processing by macrophages. Therefore, the antigen preparations containing active PT described in this investigation might be immunosuppressive.

When equivalent doses of untoxoided and carbodiimide-toxoided preparations were tested for antigenicity, the toxoid stimulated significantly greater anti-PT IgG and anti-FHa IgG antibody responses in mice. There was also no difference in the antigenicities of independently produced batches of toxoid.

There was no loss in the antigenicity of toxoids stored at $4^{o}C$. Also, toxoids which showed slight reversion during storage at $37^{o}C$ were antigenically stable. Rappaport et al. (1974) reported that glutaraldehyde-treated cholera toxin was not nearly as effective an antigen as a formalin-toxoid, and this was probably related to the observation that the formalin-toxoid reactivated in vivo and in vitro, whereas the glutaraldehyde-toxoid did not. In this study, there was no evidence to

suggest that the presence of active toxin in the carbodiimide-toxoided preparations enhanced antigenicity.

When toxoid was lyophilized and stored at $4^{o}C$ for 24 weeks and tested, both the PT and FHa components were less antigenic in mice. This lost antigenicity could possibly be restored with immunostimulatory adjuvants. Nevertheless, the experiment merits repetition since a 'carbodiimide-precipitated toxoid', lyophilized and resuspended by sonication, was still highly antigenic in mice.

Carbodiimide-modification and cross-linking has pronounced effects on antigenicity. In the carbodiimide-toxoided PT and FHa antigen preparations, the possibility exists of new epitope formation. This may occur by conformational changes which either expose new antigenic determinants or stabilize existing antigenic sites on the molecules. Carbodiimide-mediated cross-links markedly alter the specificity of antibodies stimulated, and sites involving cross-links are more potent determinants than those on linear portions of the polypeptide chain (Gill et al., 1968). Alternatively, the possibility exists of a carbodiimide-adduct being the new epitope (Goodfriend et al., 1964; Davis et al., 1984). In all these cases, antibody might be produced to altered protein and this antibody might not react with native protein (Erlanger, 1980). Obviously, this was not solely the case with the carbodiimide-toxoided antigen preparations in this study, since antigenicity was enhanced, with a concomitant reduction in toxicity.

Toxoiding with formaldehyde and glutaraldehyde also affects the antigenicities of bacterial exotoxins. Rittenberg et al. (1976) and Cryz et al. (1980) demonstrated that formaldehyde-treatment partially destroyed or altered the antigenic determinants of both A and B domains

of diphtheria toxin. Similarly, formaldehyde-treatment produced minor alterations in the structure of Pseudomonas aeruginosa exotoxin A but antigenicity was not compromised (Pollack and Prestcott, 1982). More importantly, studies by Warren et al. (1975a,b) showed that formaldehyde-polymerized staphylococcal enterotoxoids were antigenic whereas monomeric enterotoxoids were not. Similarly, glutaraldehyde-polymerized Clostridium perfringens α-toxin was more antigenic than the monomeric toxoid (Petrov et al., 1977). In the present study, the polymeric nature of the carbodiimide-toxoided antigen preparation, a result of extensive intermolecular cross-linking, may also be important in the stimulation of anti-PT IgG and anti-FHa IgG antibody responses in animals. It may be that polymeric toxoids are more efficiently processed by macrophages or provide a more efficient presentation of antigenic sites for attachment with, or activation of, immunocompetent cells (Warren et al., 1975b).

In addition to enhanced antigenicity, an important finding in this study was the associated immunogenicity of the toxoids.

The untoxoided PT and FHa preparation described in this study showed evidence of partial protection in the intracerebral mouse-protection test (ICMPT) at doses non-toxic for mice. Munoz et al. (1981a) demonstrated that a pertussis toxin preparation, extracted from B. pertussis cells with buffer containing Triton X-100, was protective in the ICMPT with a PD_{50} value of 1.4 μg/mouse. However, this extract was well tolerated by mice at a dose of 10 μg, and more toxic crystalline preparations were not protective (Munoz et al., 1981a,b; Munoz and Arai, 1982). Irons and MacLennan (1979b) and Robinson and Irons (1983) also found that purified native LPF(PT) was non-protective in the ICMPT, when

tested at doses permitted by its toxicity. The failure of purified or crude preparations of PT to provide complete protection might be a consequence of its purity or its toxic inhibition of antibody response (Pittman, 1980).

Protection against intracerebral challenge in mice was demonstrated by the carbodiimide-toxoided antigen preparations, which had PD_{50} values from 0.8-1.11 μg/mouse. These values are as low as have ever been reported for B. pertussis antigen(s) toxoided with formaldehyde or glutaraldehyde (Table 2). For example, the glutaraldehyde-toxoided crystalline PT of Munoz et al. (1981a,b) had a PD_{50} value of 1.7 μg/mouse. Sato and Sato (1984) reported that PT toxoided with formaldehyde had a PD_{50} value of 0.93 μg/mouse. However, mouse-protective activity of this toxoid was enhanced if the immunizing mixture contained formaldehyde-treated FHa (Sato and Sato, 1984). Similarly, the presence of carbodiimide-treated FHa may contribute to the excellent protective activity of the toxoids in this study.

Robinson and Irons (1983) found that low levels of native, purified LPF(PT) enhanced the protective activities, as judged by the ICMPT, of several B. pertussis antigens, including FHa. However, glutaraldehyde-treated LPF did not exhibit a synergistic effect with these antigens. Also, Robinson and Irons (1983) did not report whether the protective activity of the glutaraldehyde-toxoid could be enhanced by the presence of a low level of native toxin. It is possible that the mouse-protective activity of the carbodiimide-toxoided PT-FHa preparation in the ICMPT might be due to a trace amount of native PT, acting synergistically to enhance the protective activities of other antigens. However, since all three toxoids had similar PD_{50} values, there was no evidence to suggest

that the presence of some residual HSA (PT) enhanced mouse-protective activity.

Pertussis toxin may not be the only component active in the ICMPT, for Novotny et al. (1985) demonstrated mouse-protection with a preparation containing adenylate cyclase. Therefore, the presence of minor protein components in the carbodiimide-toxoided PT-FHa preparations might play an important role in protecting mice against intracerebral challenge.

In a preliminary experiment, the carbodiimide-toxoided PT-FHa preparation also protected mice against sublethal intranasal infection and lung colonization with B. pertussis. In this experiment, the lungs of control mice showed extensive and persistent colonization with B. pertussis (approximately 820,000 cfu/mouse). Mice immunized with the toxoid at doses of 4.0, 0.8 or 0.16 μg/mouse had 0, 1,400 and 5,800 cfu/mouse respectively, indicating extensive clearance of the infecting organism.

Concerning the role of antibody in protection against pertussis, the following assumptions were made (Pittman, 1980; Sato et al., 1984): FHa antibody inhibits attachment of the organism to the ciliated epithelium and PT antibody neutralizes toxin produced by the infecting organism. Passive protection tests with antisera to PT protected mice from intracerebral infection, whereas sera lacking anti-PT antibodies but containing high concentrations of anti-FHa antibodies did not (Munoz et al., 1981a). In the literature, the exact nature of the antibodies protective against infection is unclear since the evidence is conflicting. In the studies of Sato et al. (1981, 1982) and Sato and Sato (1984), passive immunization with serum anti-FHa or anti-PT antibody protected

mice against aerosol challenge. However, Oda et al. (1984) showed that passive immunization with serum anti-PT, but not anti-FHa, antibody protected mice from aerosol challenge.

Ashworth et al. (1982b) suggested that the ability of FHa to reduce lung colonization in rabbits after intranasal infection with B. pertussis was due to production of secretory IgA (sIgA) to the antigen. Oda et al. (1985) showed that the enriched sIgA or IgG antibody fractions from human colostrum, with relatively high titres of anti-PT, -FHa and -agglutinogen antibodies, protected mice against respiratory challenge with B. pertussis. However, fractions containing anti-FHa sIgA or IgG antibodies, but low levels of anti-PT or anti-agglutinogen antibodies, gave little if any protection.

Passive immunization tests could be done to see whether carbodiimide-toxoided antigen preparations protect mice against intracerebral and aerosol challenge with B. pertussis. Also, antisera could be titrated for anti-agglutinogen antibodies which may play a role in protection.

The protective antigens in the carbodiimide-toxoided preparations are most likely to be PT and FHa. Since B. pertussis is not an invasive organism, protection against infection at the mucosal surface may be mediated mainly by sIgA antibody to B. pertussis adhesins, or other antigens including agglutinogens. Both PT and FHa appear to be critical for adhesion (Tuomanen et al., 1985). In this study, the mouse-protection data indicate that the toxoids stimulate

(i) protective antibody levels, presumably of sIgA, at the mucosal surface,

(ii) anti-PT and anti-FHa serum antibodies essential for protection against the infection and disease symptoms.

SECTION 7. PERTUSSIS ACELLULAR VACCINES OF THE FUTURE

Whooping cough (pertussis) is still a severe, highly contagious respiratory disease especially for young infants. Pertussis whole-cell vaccine has been an effective vaccine but also the subject of considerable debate with regard to safety (Wardlaw and Parton, 1983a). There is a need for acellular pertussis vaccines of low toxicity which, ideally, will prevent colonization and protect against the disease symptoms. Vaccine design can rely increasingly on knowledge of the roles of individual bacterial components in the pathogenesis of pertussis, and at present, vaccines containing detoxified PT plus one or more purified adhesins are envisaged (Robinson et al., 1985a).

Criteria for assessing a new pertussis vaccine should be related to the disadvantages and imperfections of current whole-cell vaccines (Weihl et al., 1963). New acellular vaccines should (1) be non-toxic, thereby ensuring the absence of local and systemic reactions, severe neurological reactions or permanent sequelae in immunized children, (2) stimulate a more predictable antibody response and a higher incidence of adequate antibody response in very young infants, and (3) give a higher incidence of clinical protection.

Considering that an acellular pertussis vaccine was made as long ago as the early 1950s (Pillemer et al., 1954) and shown to be effective in children, it is surprising that whole-cell preparations are still in use today. Much research has been directed towards the identification of protective antigens for inclusion in acellular vaccines. An acellular pertussis vaccine composed of mainly two extracellular proteins of B. pertussis, pertussis toxin and filamentous haemagglutinin, has been used in Japan since 1981 (Sato et al., 1984). This vaccine was

manufactured as follows: culture supernate of B. pertussis was treated with ammonium sulphate and HA activity extracted from the precipitate with high salt. This crude extract was fractionated by sucrose density gradient centrifugation to obtain a HA preparation which was then treated with formaldehyde. In the present study, HA activity was removed from culture supernate using dye-ligand affinity chromatography. Clearly, this method is much more simple and cost-effective to use for the preparation of pertussis antigens for inclusion in acellular vaccines.

Production lots of the Japanese component vaccine were not only protective in the ICMPT, but also less than one-tenth as toxic as whole-cell vaccine as judged by leucocytosis-promotion, histamine-sensitization and endotoxicity assays in mice (Sato et al., 1984). The vaccine appeared to be safe and less reactogenic than whole-cell preparations when administered to children starting at 12-24 months of age. These limited field trials also showed that PT and FHa were antigenic. However, only the epidemiological results of mass immunization will reveal whether this vaccine is effective against pertussis in children and completely safe.

Similar vaccines are being prepared elsewhere, but information in the literature is not extensive. Garcia-Sanz et al. (1985) prepared an acellular extract from culture supernate whose principle component was PT. Although simple to prepare and protective in the ICMPT, the extract was toxic and residual HSA and LPA were not removed by toxoiding. In a computer search of the World Patents Index done in April, 1986, there was no vaccine formulation which matched the carbodiimide-toxoided preparations described in this study. In the few patents appearing in this search where B. pertussis whole-cells or pertussis antigens for

inclusion in vaccines were toxoided, the reagent used was invariably formaldehyde.

The first generation of acellular pertussis vaccine is exemplified by the preparations described above (Manclark and Cowell, 1984). Second-generation pertussis vaccines would be more uniform in antigen composition, but the fundamental requirement would still be for a suitable method to modify PT so that it no longer possessed its pathophysiological activities but retained protective activity. Therefore, acellular vaccines containing such toxoids would be safer and more potent. The antigen composition of the experimental acellular vaccine prepared in this study is fundamentally similar to the Japanese component vaccine (Sato et al., 1984). However, in a departure from toxoiding with formaldehyde or glutaraldehyde, use was made of a carbodiimide, a member of a group of compounds which have been extensively used in the preparation of other immunizing conjugates (Bauminger and Wilchek, 1980). It would be interesting to compare the carbodiimide-toxoided vaccine alongside emerging commercial acellular pertussis vaccines manufactured in this country and abroad.

Third-generation acellular pertussis vaccines would be a natural progression from the preceding formulations (Manclark and Cowell, 1984). They may be characterized by the addition of other antigens, such as surface antigens involved in the attachment and infection of the organism, and toxins (suitably toxoided) that have been found to be important in vaccine prophylaxis. Nevertheless, such vaccine formulations may be too complex, for the pathology of the disease is primarily an exotoxinosis (Pittman, 1984). Identification of PT as the reactive and protective antigen in pertussis, with new information about its pathophysiological mode of action and detoxification, gives high hope for a

toxoid vaccine - possibly combined with one or two other components of the bacillus - that would be effective against the disease. Therefore, it would be important to evaluate a suitably toxoided PT preparation in a series of limited clinical field trials.

As an improvement to third-generation vaccines, molecular subunits and chemically-synthesized and/or genetically-engineered antigens would replace components extracted from B. pertussis. With knowledge of the nucleotide and amino acid sequence of PT (Nicosia et al., 1986; Locht and Keith, 1986), the important epitopes may soon be identified. This raises the possibility of preparing synthetic peptides representing limited but antigenically important portions of the relevant protective antigens. The antigenicity and immunogenicity of such peptide sequences (haptens) would be enhanced by efficient coupling to carrier proteins. The 'carbodiimide method' is the most popular technique for coupling haptens to carriers, because the reaction is carried out under mild conditions and is relatively fast (Bauminger and Wilchek, 1980). In peptide synthesis and coupling reactions, competing reaction pathways to the formation of peptide bonds can also be efficiently suppressed (Rich and Singh, 1979).

The major advantage of carbodiimides as coupling agents is the production of 'zero-length' amide cross-links, without introducing 'unnatural and antigenically potent chemical bridges' which are undesirable in conjugate vaccines. The products of protein modification and conjugation with formaldehyde or glutaraldehyde are complex, uncertain and not reproducible, with respect to the size and number of polymers obtained and to the extent of intramolecular cross-linking. Examples of successful carbodiimide-mediated conjugate vaccines include meningococcal

capsular polysaccharide-tetanus toxoid conjugates (Beuvery et al., 1983) and E. coli enterotoxin conjugates (Klipstein et al., 1982a; 1983a,b,c; 1984; Houghten et al., 1985).

It is unlikely that any peptide vaccine will be useful in the prevention of human disease unless given in conjunction with an immunostimulatory adjuvant (Dorner and McDonel, 1985). The use of immunopotentiating conjugates, with a backbone of carrier immunopotentiator and protective antigen(s), can be envisaged in the near future (Stewart-Tull, 1985). In fact, carbodiimide-conjugated adjuvant-antigen complexes have been prepared using muramyl dipeptide (Mozes et al., 1980) or non-toxic synthetic polyelectrolyte, a polymer synthesized from copolymerization of acrylic acid and N-vinylpyrrolidone (Petrov et al., 1985), as immunostimulatory agents.

A potentially exciting vaccine formulation would contain the synthetic antigenic determinants of pertussis, diphtheria and tetanus toxins coupled to a non-toxic carrier immunopotentiator.

A possible alternative to these postulated synthetic antigens is the prospect of genetically-engineering pertussis components for inclusion in acellular vaccines. For example, the cloned and sequenced PT gene will facilitate development of safer pertussis vaccines, since it would be possible to modify the toxin 'active sites' (of ADP-ribosylation in the A-protomer, and target cell-binding in the B-oligomer) by site-directed mutagenesis of the B. pertussis genome. These modifications would abolish the pathophysiological activities of the toxin without impairing antigenicity or immunogenicity ('in vivo toxoids').

In conclusion, a carbodiimide-toxoided antigen preparation containing predominantly PT and FHa was an excellent experimental acellular pertussis vaccine. In this study, the toxoid was (i) easily produced, (ii) stable for long periods at $4^{o}C$, (iii) greatly reduced in levels of A-protomer and B-oligomer biological activities associated with PT, (iv) non-toxic, highly antigenic and highly immunogenic in mice. The use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.HCl (EDAC) offers an excellent alternative to current toxoiding reagents, and in the future development of synthetic peptide and/or conjugate vaccines it may prove indispensable.

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APPENDICES

Appendix 1.

1% Casamino acids solution.

Casamino acids (Difco Technical)	10.0 g
$MgCl_2 . 6H_2O$	0.1 g
$CaCl_2$	0.015 g
NaCl	5.0 g

The ingredients were dissolved in 980 ml distilled water and pH adjusted to 7.1 with 1N NaOH. Aliquots were dispensed and autoclaved at 121°C, 103.4 kPa for 15 min. For stock cultures, glycerol (10% v/v) was added before autoclaving.

Bordet Gengou (BG) agar plates.

The BG agar base (9 g) was dissolved in 250 ml distilled water containing glycerol (4% v/v), autoclaved at 121°C, 103.4 kPa for 15 min and cooled to 45°C. Defibrinated horse blood (50 ml) was added aseptically to give a final concentration of 17% (v/v), mixed gently with the agar and poured into plastic petri dishes. These were allowed to set, stored at 4°C and used within 2 weeks of manufacture.

Nutrient agar plates.

Oxoid nutrient agar (28 g) was dissolved in 1.0 litre of distilled water and sterilized by autoclaving at 121°C, 103.4 kPa for 15 min. The agar was poured into plastic petri dishes, set and stored at 4°C.

SS-X liquid medium.

1.	L-glutamate (monosodium salt; BDH)	10.72 g
2.	L-proline (Sigma)	0.24 g
3.	NaCl	2.5 g
4.	KH_2PO_4	0.5 g
5.	KCl	0.2 g

6.	$MgCl_2.6H_2O$	0.1 g
7.	$CaCl_2$	0.02 g
8.	Tris(hydroxymethyl-methylamine)	1.52 g

(Koch Light Laboratory, Colnbrook, Bucks., England)

9.	L-cysteine (BDH)	0.04 g
10.	$FeSO_4.7H_2O$	0.01 g
11.	Ascorbic acid (BDH)	0.02 g
12.	Nicotinic acid (BDH)	0.004 g
13.	Glutathione (Sigma)	0.1 g

Ingredients 1-8 were dissolved in 800 ml of distilled water and pH adjusted to 7.6 with 2.5N HCl. The volume was made up to 990 ml and autoclaved at 121°C, 102.4 kPa for 15 min. To obtain cyclodextrin-modified SS-X, 1.0 g of MeβCD was added to ingredients 1-8 before autoclaving.

Immediately before use, ingredients 9-13 were dissolved in 10 ml distilled water and filter-sterilized with a millipore filter of 0.45 μM pore size (Millipore (UK) Ltd., Harrow, Middlesex). This was added aseptically to the bulk medium.

Cyclodextrin liquid (CL) medium (Imaizumi et al., 1983).

Ingredients 1-8		As for SS-X liquid medium
9.	L-cysteine	0.04 g
10.	$FeSO_4.7H_2O$	0.01 g
11.	Ascorbic acid	0.4 g
12.	Nicotinic acid	0.004 g
13.	Glutathione	0.15 g
14.	Casamino acids	10.0 g
15.	MeβCD	1.0 g

Ingredients 1-8, 14 and 15 were dissolved in 800 ml distilled water, pH adjusted to 7.6 with 2.5N HCl and the volume made up to 990 ml with distilled water. This was autoclaved as described above. Ingredients 9-13 were dissolved in distilled water, filter-sterilized and added to the bulk medium as described above.

Appendix 2.

Sorensen's phosphate buffer, pH 7.2

Stock solutions:

A. 1/15M monopotassium phosphate — 9.08 g KH_2PO_4 L^{-1}

B. 1/15M disodium phosphate — 11.88 g $Na_2HPO_4.2H_2O$ L^{-1}

x ml A + (100-x) ml B.

For a buffer at pH 7.2, 285 ml of solution A and 715 ml of solution B were mixed.

Appendix 3.

Preparation of pyrogen-free glassware and saline.

Pyrogen-free glassware was prepared as follows:

(i) glassware was soaked overnight in a 1% (w/v) solution of the detergent E-Toxa-Clean (Sigma),

(ii) it was rinsed 8-10 times in warm, running tap water and five times in distilled water before baking in an oven at 180°C for 4h.

Pyrogen-free saline was prepared by dissolving baked NaCl in freshly drawn distilled water to give a 0.85% (w/v) solution, which was autoclaved at 121°C, 103.4 kPa for 15 min.

Appendix 4.

ELISA methodology.

Coating buffer, 0.05M carbonate buffer, pH 9.6.

$Na_2 CO_3$	1.59 g
$Na HCO_3$	2.93 g

Both ingredients were dissolved in 1.0 litre of distilled water and used within 2 weeks of manufacture.

Washing/incubation buffer, PBST, pH 7.4.

NaCl	8.0 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g
KCl	0.2 g

Ingredients were dissolved in 1.0 litre of distilled water to which was added Tween-20 (polyoxyethylene sorbitan mono-laurate, 0.05% (v/v) final concentration).

0.15M citrate-phosphate buffer, pH 5.0

Solution A: 0.1M citric acid (21.01 g L^{-1})

Solution B: 0.2M disodium phosphate (35.6 g L^{-1}).

x ml A + (100-x)ml B.

For a 0.15M buffer, pH 5.0, 49 ml of solution A was mixed with 51 ml of solution B. The substrate buffer was filtered through Whatman no. I (Whatman Ltd., Maidstone, Kent, England) before use.

Appendix 5.

Reagents for protein estimation.

Reagent A: Na_2CO_3, 5% (w/v) in distilled water.

Reagent B: $CuSO_4.5H_2O$, 1% (w/v) in distilled water.

Sodium potassium tartrate, 2% (w/v) in distilled water.

Equal volumes were mixed before use.

Reagent C: to 50 ml of Reagent A was added 2 ml of Reagent B. This was prepared and used immediately.

Reagent D: Folin and Ciocalteu Phenol Reagent (BDH), used as a solution of 50% (v/v) in distilled water.

Appendix 6.

SDS-PAGE solutions.

1. Acrylamide 30.0 g

   N,N'-methylenebisacrylamide 0.8 g

The reagents were dissolved in 100 ml distilled water, filtered and stored at 4°C.

2. 1M Tris-HCl buffer, pH 8.8.

   2M Tris 50 ml

   1N HCl 16.2 ml

   distilled water 33.8 ml

3. 0.5M Tris-HCl buffer, pH 6.8.

   1M Tris 50 ml

   1N HCl 45 ml

   distilled water 5 ml

Buffer was stored frozen.

4. Tris-glycine buffer, pH 8.3 (Running buffer).

   Glycine 72.06 g

   Tris 15.14 g

   SDS 5.0 g

Reagents were dissolved in 5.0 litres of distilled water and stored at 4°C.

5. Ammonium persulphate: 0.8% (w/v) in distilled water.

This was made fresh each time and discarded after use.

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

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

8. Solubilizing buffer.

Tris	0.024 g
EDTA	0.058 g
SDS (20% w/v)	20.0 ml
β-mercaptoethanol	10.0 ml
Glycerol	20.0 ml
Bromophenol blue	4.0 ml

The pH was adjusted to 8.0 with 1N HCl and the volume made up to 100 ml with distilled water.

9. Fixing-staining solution.

Coomassie blue R250	1.25 g
Methanol, 50% (v/v)	454.0 ml
Glacial acetic acid	46.0 ml

10. Destaining solution.

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

Preparation of gel plates.

These were prepared with glass plates separated with spacers and sealed on three sides with adhesive tape. The corners were sealed by dipping in molten paraffin wax.

Preparation of separating gel.

Acrylamide	36.7 ml
1M Tris-HCl, pH 8.8	37.5 ml

SDS (20% w/v)	0.5 ml
TEMED	25.0 µl
(N ,N ,N',N'-tetramethyl-ethylenediamine)	
Ammonium persulphate	10.0 ml
Distilled water	15.3 ml

The solutions were mixed thoroughly without aeration. The gel plates were filled to three-quarters of their volume with this solution. Before polymerization, the meniscus was removed from the gel surface by carefully overlaying with ethanol (5% v/v).

Preparation of stacking gel.

Acrylamide	3.4 ml
0.5M Tris-HCl, pH 6.8	5.0 ml
SDS (20% w/v)	0.1 ml
TEMED	10.0 µl
Ammonium persulphate	2.5 ml
Distilled water	9.0 ml
	20.0 ml

The stock solutions were mixed thoroughly without aeration. The ethanol overlay was removed and the separating gel blotted dry. The gel plate was then filled with stacking gel solution to 3-4 mm from the top. Sample 'combs' were suspended in the solution and any remaining spaces filled with stacking gel solution. The gel polymerized and the combs were removed under running buffer.

Electrophoresis

A lower electrode vessel was filled with running buffer. After removal of the sealing tape from their bases, the gel plates were inserted into the top electrode vessel with a liberal amount of grease

around the gaskets to prevent leakage. The upper electrode vessel was replaced and filled with buffer. Samples were carefully pipetted in volumes of 50-100 µl into the wells.

The electrode vessel was connected to a power supply and run at a constant current of 15 mA/gel. When the tracking dye reached the end of the gel, power was switched off and the plates removed.

Appendix 7.

HEPES buffered saline (HBS)

NaCl	8.0 g
KCl	0.4 g
$MgCl_2 \cdot 6H_2O$	0.2 g
$CaCl_2$	0.14 g
Glucose	1.0 g
HEPES (hydroxyethylpiperazine sulphonic acid, Sigma)	2.388 g

The chemicals were dissolved in 900 ml of distilled water and pH adjusted to 7.4 with 1N NaOH. The volume was made up to 1.0 litre with distilled water and autoclaved at 121°C, 103.4 kPa for 15 min. The divalent cation-free HBS-EDTA was prepared by the omission of Ca- and Mg-containing salts and the addition of 0.292 g of monosodium EDTA.

Appendix 8.

Reconstitution buffer, pH 3.5

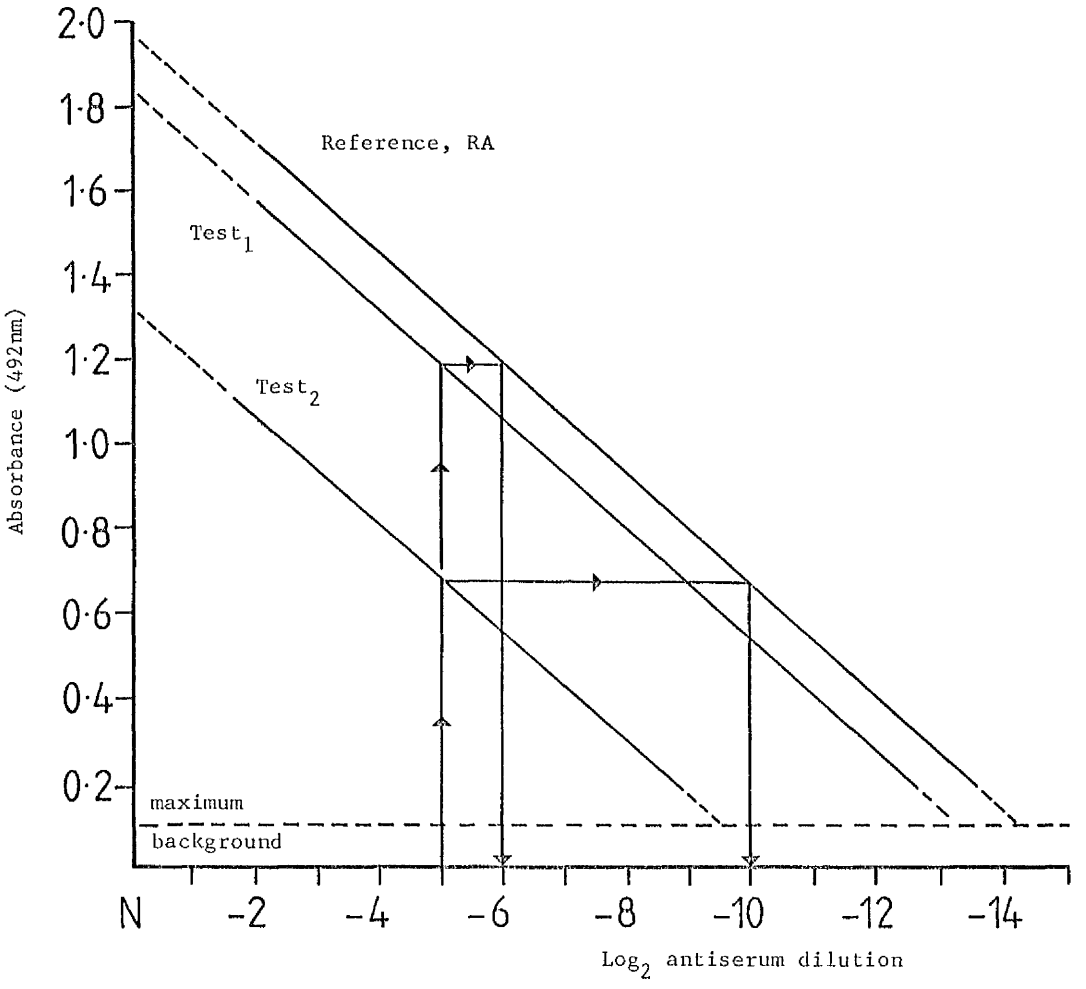
DL-α-alanine	6.78 g
Formic acid (HCOOH)	0.7 g

Chemicals were dissolved in 1.0 litre of dissolved water and the buffer stored at 4°C.

Lyophilized samples were resuspended in this low pH buffer and immediately diluted with an equal volume of 0.05M Tris-HCl buffer, pH 8.0 containing 1.0M NaCl.

Appendix 9.

Calculation of theoretical Antibody Units ml^{-1} serum (AbU ml^{-1})



Volume of serum sample assayed (µl)	200	50	12.5	3	0.8	0.2	0.05	0.01

By assigning an arbitrary unitage of 100 AbU ml^{-1} to the reference antiserum, RA, therefore;

AbU ml^{-1}	100	25	6.25	1.5	0.4	0.1	0.025	0.005

By comparing linear portions of curves and interpolating from test to reference antiserum, RA, as shown above, $Test_1$ at a dilution of $^1/_{32}$ (log_2 -5) contains 1.5 AbU ml^{-1} serum, ie: 48 AbU ml^{-1} neat mouse serum

$Test_2$, at the same dilution contains 0.1 AbU ml^{-1} serum, ie:

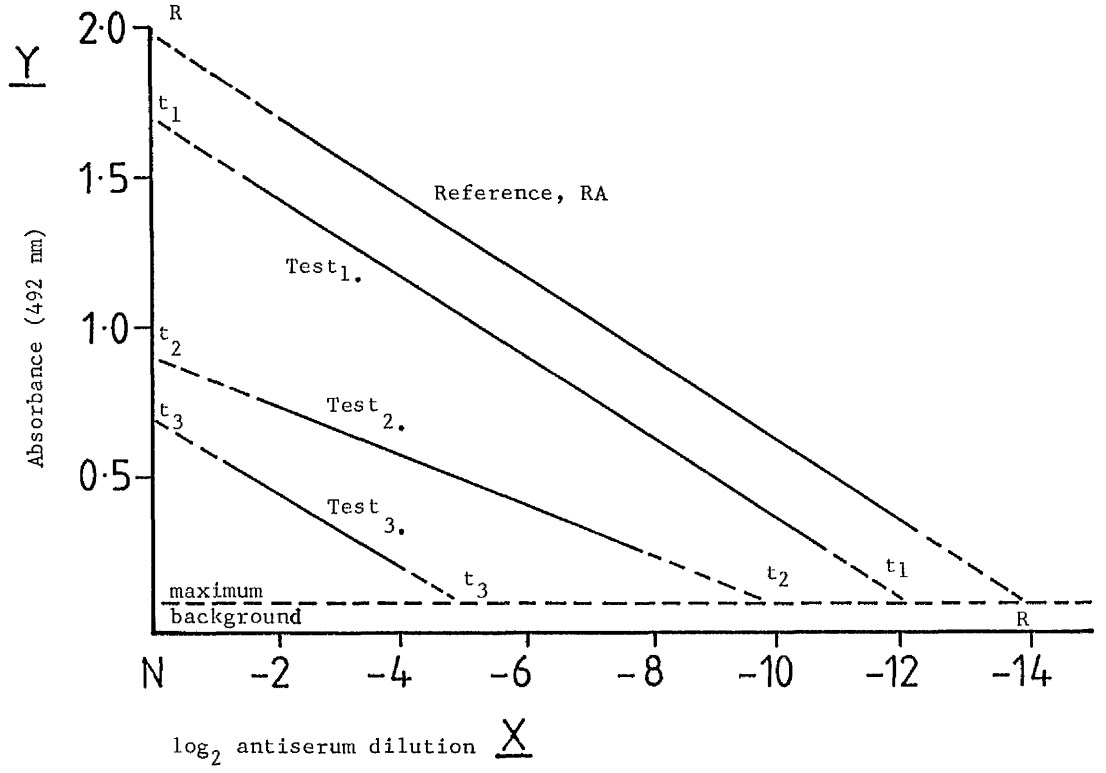
3.2 AbU ml^{-1} neat mouse serum.

If the linear portion of the test titration curve was significantly non-parallel to the reference, then 3 or more interpolations from test to reference were made and an average taken.

In tables, results were expressed as the geometric mean and 95% confidence limits.

Appendix 10.

Calculation of actual antibody titres



The linear portions of curves are compared by calculating test/reference ratios for absorbance (Y axis) and $\log_2$ antiserum dilution (X axis) as follows.

	Y	X	
If ratio of test/reference is	>0.5	>0.5	Then high titre reading is valid
" " "	>0.5	<0.5	" low " " "
" " "	<0.5	>0.5	Titre is invalid, state Y/X ratio
" " "	<0.5	<0.5	Then low titre reading is valid

In the above example,

		Y	X	
t_1/R	=	0.85	0.86	High titre valid
t_2/R	=	0.45	0.71	Titre invalid (Y/X value)
t_3/R	=	0.35	0.36	Low titre valid

The results shown in tables are the geometric mean and 95% confidence limits; data for individual sera (including Y/X values) are shown in the relevant appendices to follow.

Antibody titre is the 'reciprocal antilog of the ($\log_2$) antiserum dilution'.

Appendix 11. ELISA data, as actual antibody titres, for Table 30

Anti-PT IgG antibody

Sample code (dose/mouse)		Y (test/reference)	X (test/reference)	$\log_2$ titre or (Y/X)
(AP-16)T21	(27 µg)	0.47	0.5	-6.8
		0.59	0.61	-8.4
		0.61	0.64	-8.8
		-	-	ND*
		-	-	ND
"	(9 µg)	0.66	0.74	-10.3
		0.5	0.71	-9.9
		0.55	0.66	-9.0
		0.45	0.62	(0.73)
		0.36	0.62	(0.58)
"	(3 µg)	0.54	0.61	-6.7
		0.33	0.57	(0.58)
		0.32	0.44	-6.0
		0.23	0.43	-5.8
		-	-	ND
(AP-16)T22	(27 µg)	1.3	0.96	-13.6
		0.89	1.0	-14.5
		0.95	0.94	-13.3
		0.90	0.94	-13.2
		0.44	0.77	(0.57)
"	(9 µg)	0.99	0.99	-14.1
		0.92	0.92	-13.0
		0.85	0.88	-12.5
		0.85	0.97	-12.4
		0.81	0.85	-12.0
"	(3 µg)	1.0	0.87	-11.9
		0.8	0.82	-11.3
		0.94	0.74	-10.1
		0.7	0.77	-10.6
		0.6	0.69	-9.5
(AP-16)T23	(27 µg)	1.0	1.10	-13.9
		0.89	0.92	-12.3
		0.88	0.75	-12.1
		0.94	0.59	-13.0
		0.58	0.5	-8.0
"	(9 µg)	1.0	0.89	-11.9
		0.92	0.89	-11.8
		0.53	0.74	-10.8
		0.36	0.72	(0.5)
		0.23	0.68	(0.34)
"	(3 µg)	1.3	0.88	-11.7
		0.8	0.85	-11.3
		0.7	0.78	-10.4
		0.7	0.77	-10.2
		0.43	0.74	(0.58)
(AP-16)T24	(27 µg)	1.0	0.87	-11.9
		0.9	0.85	-11.7
		0.76	0.85	-11.6
		0.75	0.84	-11.5
		0.76	0.81	-11.2
"	(9 µg)	0.76	0.85	-11.0
		0.67	0.7	-9.9
		0.53	0.72	-11.0
		0.47	0.77	(0.61)
		0.43	0.75	(0.57)
"	(3 µg)	0.6	0.63	-10.6
		0.61	0.62	-10.4
		0.68	0.73	-11.0
		0.49	0.64	(0.77)
		0.45	0.56	(0.80)
WCV (5 ou.ml)		0.72	0.66	-8.6
		0.7	0.65	-8.5
		0.3	0.63	(0.48)
		0.2	0.58	(0.34)
		-	-	ND

Anti-FHa IgG antibody

Sample (dose/mouse)	Y (test/reference)	X (test/reference)	$\log_2$ titre or (Y/X)
(AP-16)T21 (27 µg)	0.79	0.78	-11.7
	0.77	0.77	-11.5
	0.79	0.71	-10.6
	0.27	0.54	(0.5)
	-	-	ND
" (9 µg)	0.78	0.74	-11.0
	0.84	0.72	-10.8
	0.74	0.69	-10.3
	0.70	0.68	-10.2
	-	-	ND
" (3 µg)	0.6	0.6	-9.1
	0.62	0.5	-7.6
	0.48	0.55	(0.87)
	0.47	0.54	(0.87)
	0.51	0.48	-7.4
(AP-16)T22 (27 µg)	1.0	0.92	-11.6
	0.9	0.9	-11.3
	0.97	0.86	-10.8
	0.94	0.85	-10.7
	-	-	ND
" (9 µg)	0.74	0.8	-12.2
	0.69	0.76	-11.6
	0.69	0.76	-11.6
	0.64	0.74	-11.3
	0.56	0.68	-11.0
" (3 µg)	0.75	0.76	-11.6
	0.71	0.75	-11.4
	0.66	0.71	-10.8
	0.64	0.66	-10.0
	0.63	0.63	-9.6
(AP-16)T23 (27 µg)	0.94	0.83	-12.2
	0.84	0.86	-12.6
	0.85	0.8	-11.8
	0.64	0.75	-11.0
	0.40	0.57	(0.7)
" (9 µg)	0.72	0.76	-10.8
	0.66	0.73	-10.4
	0.47	0.77	(0.61)
	0.16	0.54	(0.30)
	-	-	ND
" (3 µg)	0.76	0.69	-9.3
	0.6	0.64	-8.6
	0.57	0.6	-8.5
	0.5	0.6	-8.3
	0.48	0.55	(0.87)
(AP-16)T24 (27 µg)	0.6	0.86	-12.7
	0.58	0.81	-12
	0.56	0.8	-11.8
	0.57	0.74	-11
	0.45	0.66	(0.68)
" (9 µg)	0.62	0.75	-11.4
	0.62	0.72	-11
	0.53	0.72	-11
	0.59	0.71	-10.8
	0.53	0.66	-10.1
" (3 µg)	0.68	0.77	-9.5
	0.64	0.59	-8.8
	0.47	0.47	-7.1
	0.46	0.46	-6.9
	0.39	0.52	(0.75)
WCV (5 ou.ml)	1.27	0.56	-8.3
	1.2	0.55	-8.2
	0.88	0.62	-9.3
	1.1	0.64	-8.0
	0.64	0.5	-7.5

*ND, not detected

Sample code (dose/mouse)	Serum Anti-PT IgG antibody Y	X	$\log_2$ titre or (Y/X)	Serum Anti-FHa IgG antibody Y	X	$\log_2$ titre or (Y/X)
(AP-17)T33 (5 µg)	0.64	0.83	-11.6	0.65	0.61	-8.3
	0.6	0.8	-11.2	0.61	0.55	-7.48
	0.67	0.7	-9.8	0.61	0.55	-7.48
	0.67	0.69	-9.6	0.5	0.54	-7.24
	0.57	0.73	-10.2	0.42	0.45	-6.1
	0.52	0.74	-10.4	0.42	0.44	-5.9
	0.46	0.76	(0.61)	0.41	0.44	-5.9
	0.57	0.64	-8.9	0.4	0.43	-5.8
	0.4	0.61	(0.66)	0.34	0.39	-5.3
	0.36	0.54	(0.67)	-	-	ND*
" (25 µg)	1.0	0.95	-12.5	1.0	0.85	-11.5
	1.0	0.93	-12.3	0.89	0.9	-12.2
	0.85	0.86	-11.3	0.56	0.84	-11.4
	0.54	0.88	-11.6	0.53	0.79	-10.8
	0.53	0.86	-11.4	0.61	0.72	0.98
(AP-17)T34 (5 µg)	0.56	0.62	-8.6	0.64	0.67	-9.8
	0.53	0.66	-8.3	0.6	0.61	-8.9
	0.59	0.61	-8.7	0.83	0.57	-8.3
	0.43	0.43	-6.1	0.57	0.59	-8.6
	0.15	0.33	-4.8	0.57	0.58	-8.4
	0.46	0.81	(0.57)	0.76	0.53	-7.7
	0.42	0.69	(0.61)	0.73	0.54	-7.9
	0.33	0.65	(0.51)	0.29	0.48	-7.0
	0.33	0.64	(0.51)	0.26	0.45	-6.5
	0.33	0.56	(0.59)	0.25	0.40	-5.8
" (25 µg)	1.0	0.96	-13	1.1	0.91	-11.7
	1.1	0.89	-12	0.92	0.92	-11.8
	0.86	0.92	-12.4	0.9	0.91	-11.6
	0.72	0.91	-12.3	0.73	0.84	-10.8
	0.89	0.84	-11.4	0.55	0.77	-9.8
(AP-17)T35 (5 µg)	0.82	0.84	-11.9	0.75	0.82	-11.2
	0.74	0.83	-11.7	0.62	0.7	-9.6
	0.77	0.82	-11.6	0.61	0.69	-9.4
	0.72	0.81	-11.4	0.47	0.7	(0.67)
	0.72	0.8	-11.3	0.46	0.69	(0.67)
	0.76	0.75	-10.6	0.47	0.61	(0.77)
	0.7	0.74	-10.4	0.45	0.59	(0.76)
	0.68	0.7	-9.9	0.45	0.58	(0.78)
	0.63	0.65	-9.2	0.32	0.58	(0.55)
	0.4	0.52	(0.77)	0.28	0.61	(0.46)
" (25 µg)	0.96	0.84	-11.3	1.0	0.99	-12.5
	0.85	0.86	-11.7	0.97	0.98	-12.3
	0.82	0.84	-11.4	0.98	0.94	-11.8
	0.77	0.81	-10.9	1.0	0.89	-11.2
	0.41	0.59	(0.69)	0.63	0.65	-8.2
WCV (5 ou.ml)	0.59	0.57	-7.6	0.8	0.6	-7.7
	0.59	0.57	-7.6	1.0	0.62	-7.9
	0.49	0.59	(0.83)	1.0	0.58	-7.4
	0.42	0.62	(0.68)	1.0	0.62	-8.0
	0.42	0.62	(0.68)	1.0	0.625	-8.0

*ND, not detected

Appendix 13. ELISA data, as actual antibody titres, for Table 32

Sample code (dose/mouse)	Storage conditions	Anti-PT IgG antibody Y (test/reference)	Anti-PT IgG antibody X (test/reference)	Anti-PT IgG antibody $\log_2$ titre or (Y/X)	Anti-FHa IgG antibody Y (test/reference)	Anti-FHa IgG antibody X (test/reference)	Anti-FHa IgG antibody $\log_2$ titre or (Y/X)
(AP-2)T36 (10 µg)	day 0	0.83	0.81	-11.2	0.96	0.83	-12.5
		0.6	0.8	-11.1	1.0	0.77	-11.6
		0.8	0.73	-10.2	0.83	0.79	-11.8
		0.62	0.75	-10.4	0.9	0.75	-11.3
		0.53	0.71	-9.9	0.8	0.73	-11.0
" *	14 days at 4°C	0.79	0.76	-11.0	0.78	0.78	-11.5
" *	" 37°C	0.78	0.75	-10.9	0.79	0.81	-11.7
WCV (5 ou.ml) *	day 0	0.49	0.43	-6.8	0.42	0.43	-8.3
" *	14 days at 4°C	0.46	0.47	-6.6	0.4	0.45	-8.0

*Pooled sera of 5 samples were tested

Appendix 14. ELISA data, as actual antibody titres, for Table 33

Sample code (dose/mouse)	Storage conditions	Serum Anti-PT IgG antibody Y	X	log_2 titre or (Y/X)	Serum Anti-FHa IgG antibody Y	X	Log_2 titre or (Y/X)
(AP-16)T37 (10 µg)	day 0	0.83	0.83	-11.6	0.77	0.78	-11.35
		0.74	0.79	-11	0.76	0.79	-11.5
		0.77	0.74	-10.4	0.64	0.79	-11.4
		0.57	0.74	-10.4	0.55	0.79	-11.5
		0.58	0.74	-10.3	0.52	0.8	-11.6
		0.54	0.71	-10	0.56	0.76	-11.0
		0.52	0.7	-9.8	0.54	0.75	-10.8
		0.49	0.66	(0.74)	0.53	0.74	-10.75
		0.48	0.66	(0.73)	0.52	0.73	-10.55
		0.50	0.67	-9.4	0.55	0.69	-10.05
"	14 days at 4°C	1.0	0.84	-11.7	0.82	0.81	-11.3
		0.89	0.74	-10.3	0.75	0.74	-10.4
		0.75	0.66	-9.3	0.77	0.71	-9.9
		0.76	0.64	-9.0	0.67	0.74	-10.4
		0.75	0.64	-8.9	0.72	0.69	-9.6
		0.68	0.56	-7.8	0.62	0.73	-10.2
		0.57	0.55	-7.7	0.61	0.74	-10.4
		0.44	0.59	(0.75)	0.66	0.68	-9.5
		0.43	0.59	(0.73)	0.53	0.67	-9.4
		0.3	0.66	(0.45)	-	-	ND*
"	14 days at 37°C	0.65	0.83	-11.6	0.86	0.80	-11.4
		0.6	0.75	-10.6	0.84	0.76	-11.3
		0.58	0.75	-10.6	0.78	0.83	-11.9
		0.76	0.66	-9.3	0.76	0.82	-11.8
		0.53	0.73	-10.2	0.76	0.82	-11.8
		0.48	0.69	(0.7)	0.67	0.76	-11
		0.56	0.65	-9.2	0.66	0.75	-10.8
		0.46	0.6	(0.77)	0.63	0.76	-10.9
		0.43	0.58	(0.74)	0.61	0.72	-10.4
		0.58	0.75	-10.6	-	-	ND
"	28 days at 4°C	0.82	0.74	-10.6	0.83	0.74	-10.8
		0.73	0.69	-9.9	0.81	0.74	-10.7
		0.64	0.64	-9.2	0.81	0.73	-10.6
		0.6	0.6	-8.7	0.80	0.65	-9.4
		0.43	0.6	(0.72)	0.76	0.66	-9.6
		0.42	0.59	(0.71)	0.76	0.64	-9.3

"	"	0.46	0.54	(0.85)	0.61	0.58	-8.4
		0.38	0.55	(0.69)	0.46	0.61	(0.75)
		0.23	0.57	(0.40)	0.77	0.63	-9.2
		-	-	ND	0.76	0.62	-9.0
"	56 days at 4°C	0.72	0.74	-10.0	0.85	0.8	-11.9
		0.71	0.71	-9.7	0.81	0.8	-12.0
		0.71	0.7	-9.55	0.8	0.71	-10.7
		0.65	0.68	-9.3	0.79	0.71	-10.7
		0.57	0.59	-8.05	0.78	0.7	-10.5
		0.51	0.56	-7.65	0.76	0.69	-10.4
		0.50	0.55	-7.45	0.73	0.69	-10.3
		0.48	0.54	(0.89)	0.70	0.64	-9.6
		0.17	0.41	-5.6	0.50	0.57	-8.6
		-	-	ND	-	-	ND
WCV (5 ou.ml)	day 0	0.58	0.54	-7.6	0.84	0.61	-8.8
		0.43	0.59	(0.74)	0.82	0.59	-8.5
		0.42	0.57	(0.73)	0.75	0.625	-9.0
		0.54	0.5	-7.0	0.82	0.51	-7.3
		0.22	0.44	-6.1	0.53	0.49	-7.1
"	14 days at 4°C	0.5	0.63	-9.0	0.93	0.74	-10.5
		0.49	0.56	(0.88)	0.72	0.6	-8.5
		0.22	0.50	-7.1	0.56	0.64	-9.1
		0.27	0.46	-6.6	0.61	0.54	-7.6
		-	-	ND	0.4	0.59	(0.68)
"	28 days at 4°C	0.52	0.54	-7.8	0.79	0.69	-10.2
		0.43	0.59	(0.73)	0.95	0.63	-9.2
		0.45	0.42	-6.1	0.62	0.61	-8.9
		0.39	0.41	-5.9	0.62	0.56	-8.2
		0.31	0.39	-5.7	0.53	0.54	-8.0
"	56 days at 4°C	0.48	0.57	(0.84)	0.77	0.85	-9.6
		0.42	0.53	(0.79)	0.77	0.65	-9.6
		0.41	0.47	-6.8	0.61	0.7	-10.4
		0.4	0.46	-6.6	0.55	0.69	-10.2
		0.19	0.39	-5.6	0.41	0.69	(0.59)

*ND, not detected

Appendix 15. ELISA data, as actual antibody titres, for Table 34

	Serum anti-PT IgG antibody			Serum anti-FHa IgG antibody		
Sample code (dose/mouse)	Y	X	$\log_2$ titre or (Y/X)	Y	X	$\log_2$ titre or (Y/X
(AP-16)T37 (10 μg)	0.53	0.47	-6.4	0.22	0.4	-5.7
	0.24	0.53	(0.45)	0.17	0.34	-5.0
				0.14	0.34	-5.0
	< Rest not detected >			< Rest not detected >		
WCV (5 ou.ml)	0.25	0.53	(0.47)	0.68	0.7	-10.3
	0.24	0.53	(0.45)	0.65	0.7	-10.3
	0.24	0.53	(0.45)	0.63	0.68	-10.1
	0.22	0.44	-5.25	0.51	0.61	-9.0
	0.19	0.39	-5.0	0.48	0.57	(0.84)

Appendix 16. ELISA data, as actual antibody titres, for Table 35

Sample code (dose/mouse)	Serum anti-PT IgG antibody			Serum anti-FHa IgG antibody		
	Y	X	$\log_2$ titre or (Y/X)	Y	X	$\log_2$ titre or (Y/
(AP-2)T38 (30 µg)	0.88	0.95	-13.9	0.89	0.88	-13.4
	0.87	0.94	-13.7	0.84	0.79	-12.0
	0.81	0.86	-12.5	0.69	0.70	-10.6
	0.99	0.79	-11.5	0.66	0.66	-10.1
	0.31	0.69	(0.45)	0.30	0.70	(0.43)
" (15 µg)	1.0	0.90	-12.6	0.82	0.86	-12.8
	0.97	0.83	-11.6	0.79	0.84	-12.4
	0.84	0.83	-11.6	0.76	0.80	-11.9
	0.59	0.83	-11.6	0.64	0.72	-10.6
	0.35	0.76	(0.46)	0.34	0.65	(0.52)
" (7.5 µg)	0.40	0.78	(0.51)	0.55	0.58	-9.0
	0.35	0.72	(0.49)	0.57	0.62	-9.2
	0.33	0.69	(0.48)	0.48	0.55	(0.87)
	0.30	0.66	(0.45)	0.34	0.57	(0.60)
	-	-	ND*	0.25	0.45	-6.6

*ND, not detected

Appendix 17. ELISA data, as actual antibody titres, for Table 36

Sample code (dose/mouse)	Storage conditions	Serum anti-PT IgG antibody Y	X	$\log_2$ titre or (Y/X)	Serum anti-FHa IgG antibody Y	X	$\log_2$ titre or (Y/X)
(AP-17)T33 (20 µg)	14 days at -20°C	1.1	0.94	-13.75	0.79	0.81	-11.4
		0.76	0.83	-12.05	0.74	0.76	-10.6
		0.74	0.82	-11.95	0.69	0.7	-9.8
		0.67	0.75	-10.9	0.67	0.69	-9.6
		0.66	0.74	-10.75	0.67	0.67	-9.4
"	" 4°C	0.81	0.94	-13.5	0.85	0.82	-11.4
		0.7	0.88	-12.6	0.83	0.81	-11.2
		0.66	0.88	-12.7	0.76	0.76	-10.5
		0.64	0.87	-12.55	0.76	0.74	-10.3
		-	-	ND*	-	-	ND
"	" 37°C	0.84	0.88	-12.35	0.94	0.92	-13.6
		0.83	0.86	-12	0.84	0.88	-13
		0.76	0.86	-12	0.91	0.82	-12.2
		0.59	0.76	-10.7	0.77	0.78	-11.6
		0.57	0.74	-10.3	0.74	0.72	-10.6
"	28 days at 37°C	1.0	0.94	-12.3	0.85	0.88	-13
		0.83	0.88	-11.5	0.72	0.81	-11.9
		0.78	0.83	-10.8	0.56	0.79	-11.6
		0.73	0.79	-10.3	0.46	0.8	(0.58)
		0.60	0.80	-10.4	0.62	0.66	-9.8
"	56 days at 4°C	0.85	0.93	-12.9	0.89	0.88	-13
		0.93	0.88	-12.3	0.8	0.87	-12.8
		0.77	0.86	-12	0.79	0.84	-12.4
		0.69	0.83	-11.6	0.75	0.79	-11.6
		0.59	0.81	-11.3	0.62	0.68	-10
"	" 37°C	0.79	0.84	-11.3	0.7	0.74	-11.2
		0.65	0.77	-10.3	0.69	0.73	-11
		0.60	0.74	-9.9	0.69	0.73	-10.9
		0.51	0.70	-9.4	0.64	0.63	-9.4
		0.32	0.64	(0.49)	0.48	0.48	-7.2
WCV (5 ou.ml)	14 days at 4°C	0.36	0.52	(0.69)	0.74	0.75	-11
		0.35	0.51	(0.69)	0.66	0.74	-9.4
		0.34	0.51	(0.66)	0.6	0.63	-8.7
		0.31	0.49	-7.2	0.53	0.6	-8.8
		-	-	ND	0.44	0.46	-7.6
"	28 days at 4°C	0.31	0.58	(0.53)	0.77	0.75	-11.3
		0.28	0.51	(0.55)	0.65	0.66	-9.9
		0.34	0.42	-6.0	0.63	0.65	-9.8
		0.16	0.51	(0.31)	0.58	0.6	-9
		-	-	ND	0.49	0.49	-7.4
"	56 days at 4°C	< no detectable response in any sera >			< no detectable response in any sera >		

*ND, not detected

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1615 BIOLOGICAL ACTIVITIES OF A TOXOIDED CULTURE FLUID EXTRACT FROM BORDETELLA PERTUSSIS. M. Christodoulides, R. Parton, D.E.S. Stewart-Tull, Department of Microbiology, University of Glasgow.

There is a great deal of current interest in the development of acellular pertussis vaccines. Whole-cell vaccines have been described as compromises that accept a certain level of toxicity in order to achieve an acceptable level of potency (Manclark and Cowell, 1984). Recently, the Japanese made a significant step forward in the preparation of acellular vaccines with greatly reduced toxicity (Sato et al., 1984).

Attempts have been made in this study to detoxify further, a mixture of extracellular products of B. pertussis containing predominantly pertussis toxin (PT) and filamentous haemagglutinin (FHA). B. pertussis was grown statically in Stainer and Scholte medium or in a medium containing β-methyl cyclodextrin (Imiazumi et al., 1983) in which large amounts of FHA and PT were released into the culture supernate. These were recovered by dye-ligand chromatography for toxoiding studies. Treatment with the cross-linking reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl yielded a product which - 1) did not affect normal weight gain in mice, 2) had <1% of the original PT activity as measured by histamine sensitization, 3) did not induce significant leukocytosis, but ..4) was both highly antigenic and immunogenic compared to the untoxoided material.

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