Effects of Toxoiding Agents on Protective Antigens of *Bordetella pertussis* and on Other Proteins

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DEDICATION

To my mother and the memory of my father

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SUMMARY

In the preparation of acellular pertussis vaccines (usually containing pertussis toxin (PT), filamentous haemagglutinin (FHA) and agglutinogens (AGGs), inactivation of the toxic PT component is necessary.

This investigation stems from the work of Christodoulides *et al.*, (*Vaccine* , **5**, 199-207, 1987), who observed that toxoiding of a mixed antigen preparation from *B. pertussis* consisting of PT and FHA in a 1:1 ratio with a commercial conjugation reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), resulted in an enhanced immunogenic response to these antigens. The objectives of the present study were to determine if this effect could be confirmed and whether the enhanced response was still elicited after immunization of PT or FHA treated *separately* with EDAC. Also, by using other antigens namely ovalbumin (OA) and lysozyme (LZ), the possible restriction of this toxoiding/adjuvanting effect to the antigens of *B. pertussis* was examined. As controls, the antibody responses after immunization with formaldehyde and glutaraldehyde treatments were also explored.

The work started with two model antigens, ovalbumin and lysozyme, to test the specificity of the adjuvanting effect of EDAC. These proteins were treated with EDAC under conditions described for toxin inactivation and at several other concentrations. On SDS polyacrylamide gels, an extra band (thought to be a dimer) was observed for LZ but not with OA. Immunization of mice followed by ELISA on the sera gave anti-OA and anti-LZ titres. Two independent experiments showed that whilst EDAC treatment resulted in enhanced anti-LZ titres, it actually depressed anti-OA titres. Another experiment where EDAC concentration was varied during OA treatment showed this repressive response was apparent only when OA was treated with 11 and 21 mM EDAC. Treatment with 55, 110 and 220mM EDAC did not decrease nor increase the antibody response after immunization.

Using loss of bacteriolytic activity to monitor the extent of modification, LZ was treated with EDAC, formaldehyde and glutaraldehyde. These agents caused a

respective loss in activity of 30 to 40% for EDAC and >99.9 for formaldehyde and glutaraldehyde. With all three agents, gel profiles showed higher MW bands indicating polymerization. ELISA results on antisera from experiments comparing antibody responses obtained by injecting treated preparations showed that formaldehyde and glutaraldehyde had no adjuvanting effects; however two out of the three LZ preparations treated with 21 mM EDAC gave a significant enhanced antibody response. Treatment with 800 mM EDAC depressed the response.

From *B. pertussis* transposon-mutant strains, preparations of PT and FHA free from each other were made. Some PT preparations were tested for their ability to cause the clustering of Chinese hamster ovary cells. These preparations were toxoided and post-fixed with the letters E, F or G for treatment with EDAC, formaldehyde and glutaraldehyde respectively.

The haemagglutinating activity of FHA was used as an indicator of the extent of modification of this protein. Formaldehyde and glutaraldehyde treatment destroyed all detectable activity but the effect of EDAC on HA activity (between 51 to 75% loss in HA) was less. Gel profiles showed high molecular weight bands after glutaraldehyde and formaldehyde and a similar but with a slightly lesser degree of polymerization after EDAC treatment. Immunization experiments with preparations of FHA also gave variable results. In one experiment, all treated FHA samples induced lower responses compared with FHA alone. However, in a repeat experiment with independently treated samples, FHA-E showed a similar response but FHA-F and FHA-G elicited significantly higher responses compared with FHA alone.

For experiments with PT, histamine-sensitizing activity was used to assess inactivation of the toxin. PT-F preparations were invariably ≥99.9 % inactivated. Inactivation levels for PT-G and PT-E were 66 and 96% respectively. Anti-PT serum titres were best after immunisation with PT-E. The PT-G and PT-F showed equivocal responses to untreated PT. Four PT/FHA preparations assessed by SDS-PAGE and ELISA methods for their relative concentrations, were toxoided with the three agents.

As with PT only, formaldehyde consistently inactivated the PT component in the mixtures to the limits of detection (≥99.9%). Immunisation with these preparations for anti-PT showed that PT/FHA-E preparations responded best followed by PT/FHA-G. PT/FHA-F responded poorly with titres sometimes falling below the untreated control preparation. For anti-FHA, formaldehyde treatment depressed the antibody response significantly, whilst EDAC or glutaraldehyde treatment did not appreciably affect responses.

In conclusion, the enhancing effect of EDAC does not seem to be specific to PT and FHA mixtures alone. It is variable with the antigen used and in some cases can actually depress the response. With respect to the antigens of *B. pertussis* adjuvanting by EDAC was obtained with PT and a PT/FHA mixture. Immunisation with FHA-E alone did not elicit an increased response. Conditions for the treatment with EDAC would have to be investigated further to ensure reliable inactivation of PT. However, with respect to antibody response, EDAC-treated PT was superior to formaldehyde or glutaraldehyde PT.

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LIST OF ABBREVIATIONS

A : amp(s)
ACPV : acellular pertussis vaccine
ACT : adenylate-cyclase toxin
AGG : agglutinogens
a.m. : arithmetic mean
arg : arginine
BCA : bicinchoninic acid
BCA-WR : bicinchoninic acid working reagent
BG : Bordet Gengou
BSA : bovine serum albumin
° C : degrees Celsius
CBD : carbodiimide
CHO : chinese hamster ovary cells
95% CL : 95% confidence limits
cm : centimeter
CT : cholera toxin
DCCD : dicyclohexylcarbodiimide
DT : diphtheria toxin
DTP : diphtheria tetanus pertussis vaccine
DW : distilled water
EDAC : 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA : enzyme-linked immunosorbent assay
FHA : filamentous haemagglutinin
g : gram
glu : glutamate
g.m. : geometric mean
h : hour(s)
HA : haemagglutinating activity
HLT : heat-labile toxin
HLY : haemolysin
HRP : horseradish peroxidase
HSA : histamine-sensitising activity
HSD_{50} : dose which sensitises 50% of pertussis-injected mice to histamine
HSF : histamine-sensitizing factor
IAP : islets-activating protein
i.c. : intra-cerebral
ICMPT : intra-cerebral mouse protection test

i.d. : intra-duodenal
i.n. : intra-nasal
i.p. : intra-peritoneal
IU : international units
i.v. : intra-venous
KDa : kilodaltons
L : litre
LD_{50} : dose which kills 50% of mice injected
Lf/ml : flocculating units per milliliter
LPF : lymphocytosis-promoting factor
LPF-HA : lymphocytosis-promoting factor haemagglutinin
LPS : lipolysaccharide
LZ : lysozyme
M : molar
mA : milliamp(s)
mg : milligram
ml : millilitre
mm : millimetre
mM : millimolar
MPA : mouse protective antigen
MRC : Medical Research Council
MW : molecular weight
MWGT : mouse weight gain test
N : normality
ng : nanogram
NAD : nicotinamide adenine dinucleotide
nm : nanometer
OA : ovalbumin
OMP : outer membrane protein
P.69 : pertactin (69 KDa OMP)
PD_{50} : dose which protects 50% of mice
PBS : phosphate buffered saline
PN/ml : protein nitrogen per milliliter
PT: pertussis toxin
rpm : revolutions per minute
RT : room temperature
SD : standard deviation
TBS : tris-buffered saline
TCT : tracheal cytotoxin

Tn : transposon
TT : tetanus toxin
TTS : tris,tween saline
v/v : volume per volume
w/v : weight per volume
WCPV : whole-cell pertussis vaccine
WHO : World Health Organization
µg : microgram
µl : microlitre
µm : micrometre

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INTRODUCTION

BORDETELLA PERTUSSIS AND WHOOPING COUGH

The Organism

Discovery and classification

The respiratory tract pathogen that causes whooping cough was first cultured by Bordet and Gengou (1906) from secretions coughed up by a five-year-old child which were also examined microscopically. As well as noting large numbers of leucocytes, the presence of "small ovoid bacteria resembling micrococci" was observed. These bacteria were identified as Gram negative and their pleomorphic nature was also recorded.

The whooping cough bacillus was reclassified from the *Haemophilus* genus into the new genus of *Bordetella* as *Bordetella pertussis* (Moreno-Lopez, 1952). Other members assigned to the bordetellae are *B. parapertussis*, which causes a similar but milder form of whooping cough; *B. bronchiseptica*, responsible for broncho-pneumonia in pigs, dogs and other mammals and *B. avium*, the causative agent of turkey coryza and bordetellosis in other species of birds. The pathogenic mechanisms and the virulence factors produced by these organisms were reviewed by Wardlaw (1988) and Parton (1996). Since the present work started, two other bordetellae have been recognized, *B. hinzii* isolated from the respiratory tracts of poultry (Vandamme *et al.*, 1995) and *B. holmesii* isolated from blood cultures of patients (Weyant *et al.*, 1995).

Growth

B. pertussis was first grown by Bordet and Gengou (1906) on an agar medium containing glycerinated potato extract and blood which is still used today and known by their names (BG medium). Subsequently, a wide variety of liquid and solid media were developed to support growth of *B. pertussis* (reviewed by Stainer, 1988). The growth

of this organism is easily inhibited by peptone, sulphur, peroxide and fatty-acids; however, the toxic effects can be overidden by adding blood, charcoal or starch to the medium.

A particularly notable development was the defined liquid medium of Stainer and Scholte (1970) containing glutamate as the main carbon and energy source. This medium was further developed by Imaizumi *et al.* (1983), who added ß-methylated cyclodextrin. This additive not only enhanced growth of *B. pertussis* but also increased yields of pertussis toxin (PT) and allowed growth from small inocula.

In 1931, Leslie and Gardner found by repeated subculture from a single colony that *B. pertussis* underwent phase variation. Phase I and II produced smooth colony forms that were toxic to guinea-pigs, whereas phases III and IV had rough colony morphologies and were avirulent. Many decades later, it was found that this alteration coincided with loss of several virulence factors as discussed below (Coote and Brownlie, 1988).

A similar process leading to the loss of virulence components was identified by Lacey (1960). Certain environmental conditions induce growth in X-mode or C-mode. Fully virulent X-mode organisms arose from growth at 37 °C in the presence of NaCl. C-mode organisms were produced by growth at lower temperatures (28 °C) or by high concentrations of $MgSO_4$ or nicotinic acid. With the advent of genetic techniques, Weiss and Falkow (1984), reported the expression of these virulence factors was controlled by the *vir* gene now known as *bvg* for Bordetella virulence gene (Arico *et al.*, 1989). Phase I, II or X-mode organisms result when the *bvg* gene is functional. When *bvg* is switched off through environmental changes or mutation, the organisms became avirulent. These authors suggest that this phenomenon occurs either to facilitate transmission or to allow avirulent organisms to exist in the host in an inoffensive carrier state.

The Disease

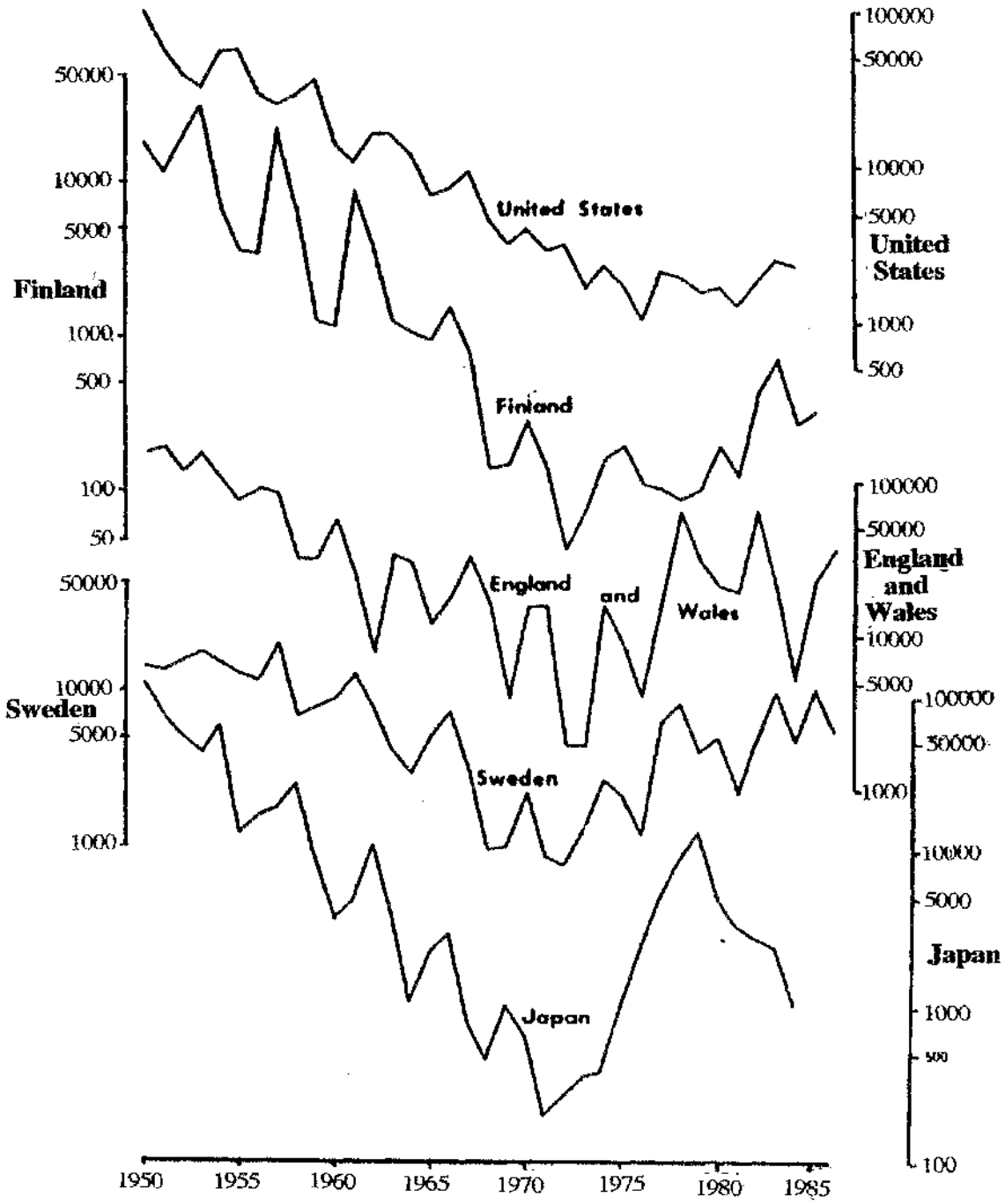
Occurrence

The earliest report of a whooping cough epidemic was in Paris in 1578 and reported by Guillame de Baillou (cited by Mortimer, 1988). In more recent times, the disease has occured worldwide in 3-4 year epidemics, with 60 million cases reported annually (Muller *et al.*, 1986). Figure 1 presents epidemiological data on the incidence of pertussis in England and Wales, Finland, Japan, Sweden and the United States. As many as 0.5 to 1.0 million deaths per annum are attributable to whooping cough, with the highest incidence found in developing countries. Reviews on pertussis epidemiology include those of Muller *et al.* (1986); Cherry *et al.* (1988) and Hodder and Mortimer (1992).

Stocks (1933) noted that most cases of pertussis in the period 1919 to 1931 occured in children between birth and 8 years old with the incidence and the mortality rates being higher in girls. Later, Gordon and Hood (1951) examined cases of whooping cough in Massachusetts from 1918 to 1949. Again, they found that 47 to 55% of cases were in children ≤ 5 years of age. Socio-economic conditions such as low education of parents and crowding were correlated strongly with mortality rates. Attack rates for pertussis occuring in children of White, Negroid, Chinese or Indian extraction were similar, therefore race was not an important factor in susceptibility to the disease. It is accepted that control of whooping cough has been largely achieved through a combination of vaccination and improved socio-economic conditions (Cherry *et al.*, 1988).

Mink *et al.* (1992) isolated *B. pertussis* from 26% of university students who exhibited a persistent cough of 6 or more days duration. Farizo *et al.* (1992) also reported a large increase of pertussis in adolescents and adults. In a study conducted by Nelson (1978), 12 out of 14 infants were found to have contracted the disease from an

Figure 1: Annual pertussis notifications between 1950 and 1986 for the United states, Finland, England and Wales, Sweden and Japan. The pattern is one of epidemics with a cycle of three to four years in each country despite major changes in pertussis incidence. (Reproduced with permission from the *Reviews of Infectious Diseases* , University of Chicago Press; Thomas, 1989)



infected adult. Such occurrences suggest that these older groups may provide a reservoir of infection for susceptible infants and children.

Transmission of *B. pertussis* occurs through direct respiratory contact with organisms shed from an infected person through coughing. In 1968, Linnemann *et al.* attempted to identify the carrier state by isolation of *B. pertussis* from nasopharyngeal swabs from children in an epidemic year. These swabs were plated on BG plates containing penicillin or analyzed for the presence of the organism by fluorescent antibody staining. However, from a total of 1102 asymptomatic children only 5 were positive, suggesting that the carrier state was rare if it exists at all.

Clinical aspects

Typically, a case of whooping cough exhibits four phases: the incubation, catarrhal, paroxysmal and convalescent. Details of the disease symptoms have been described in the reviews by Mortimer, (1988); Walker, (1988) and Cherry, (1988).

Infection with *B. pertussis* begins with the incubation phase which is of between 7 and 14 days duration. The catarrhal phase (7-10 days) follows. In these early stages, the infected individual exhibits symptoms similar to the common cold with rhinorrhea, sneezing and a mild cough. Sometimes, a slight fever occurs but usually is not perpetuated throughout the disease. Treatment with antibiotics at this stage are of the most benefit in reducing the length and the severity of the disease (Linnemann *et al.*, 1975). Also, at this time, *B. pertussis* can be isolated at a greater frequency from nasopharyngeal cultures. The patient is most infectious at this stage.

After this period, the mild cough becomes more severe and the individual enters into the paroxysmal stage which can last for many weeks. Paroxysmal coughing is described as a series of short violent coughs without inspiration which ends in a final cough clearing the air passage of mucus and often accompanied by vomiting. The inspiration of air against a narrowed glottis produces the characterisitic 'whoop'. Paroxyms can be induced by feeding, crying, examination of the pharynx or excitement

and seem to occur more frequently at night. As the paroxysms become milder and less frequent, the patient begins to recover slowly but with a persistent cough. This is the convalescent phase and may last from 1-6 months. Other respiratory infections cause the recurrence of paroxysms. During this phase, the patient is not infectious and the organism cannot be isolated.

Complications

The force of the expiratory cough during paroxysms sometimes causes minor complications in infants and children (Mortimor, 1988). Oedema of the face and ulceration of the tongue caused by its protrusion during paroxysms has been noted. Venous congestion induces subconjuctival haemorrhages. Fever is not usually associated with pertussis, however, its occurrence is usually indicative of secondary bacterial infection. Another feature of secondary infection is otitis media, caused by *Haemophilus influenzae*, *S. pneumoniae*, *S. pyogenes*, or *Staphylococcus aureus*.

Respiratory problems and encephalitic complications have more serious consequences. The obstruction of blood flow by raised intrathoracic and intra-abdominal pressures may lead to cyanosis with subsequent impairment of normal respiratory function. Anoxia is associated with convulsions and brain damage. Persistent seizures or a loss of consciousness (particularly in infants) have been noted.

Diagnosis

An important clinical sign in pertussis is lymphocytosis, noted as early as 1897 by Frolich (cited Wardlaw and Parton, 1983) and later by Bordet and Gengou (1906). Confirmation of *B. pertussis* requires its isolation from a nasopharyngeal swab plated on BG or other media. Such medium may be supplemented with penicillin or other antibiotics to reduce the growth of contaminants. However, even under favourable conditions, the isolation rate may not exceed 60% (Kwantes *et al.*, 1983). Serological evidence of infection may be obtained by ELISA. For example, Viljanen *et al.* (1982),

developed an ELISA test for anti-pertussis IgM and IgA. *B. pertussis* has also been effectively detected from nasopharyngeal swabs by molecular biological techniques such as the polymerase chain reaction (Lichtinghagen and Glaubitz, 1995).

VIRULENCE FACTORS AND PATHOGENESIS

Survey of Virulence Factors

B. pertussis produces a variety of virulence factors which are summarized in Table 1. Numerous reviews have been published detailing the purification, structure, activities and possible functions of the virulence factors produced by this organism. These components will be discussed briefly with particular emphasis on filamentous haemagglutinin (FHA) and pertussis toxin (PT). Further information may be obtained from the reviews by Munoz and Bergman (1977); Wardlaw and Parton (1983); Pittman (1984); Weiss and Hewlett (1986); Wardlaw (1988) and Wardlaw and Parton (1988).

Infection occurs through inhalation of the organisms followed by attachment to the ciliated epithelium in the pulmonary airways (Figure 2). Thereafter the organisms multiply and release a variety of components which act as aggressins and toxins. The role of most virulence factors in the pathogenesis of the disease is not clearly defined, although in some cases a function has been attributed.

Adhesins

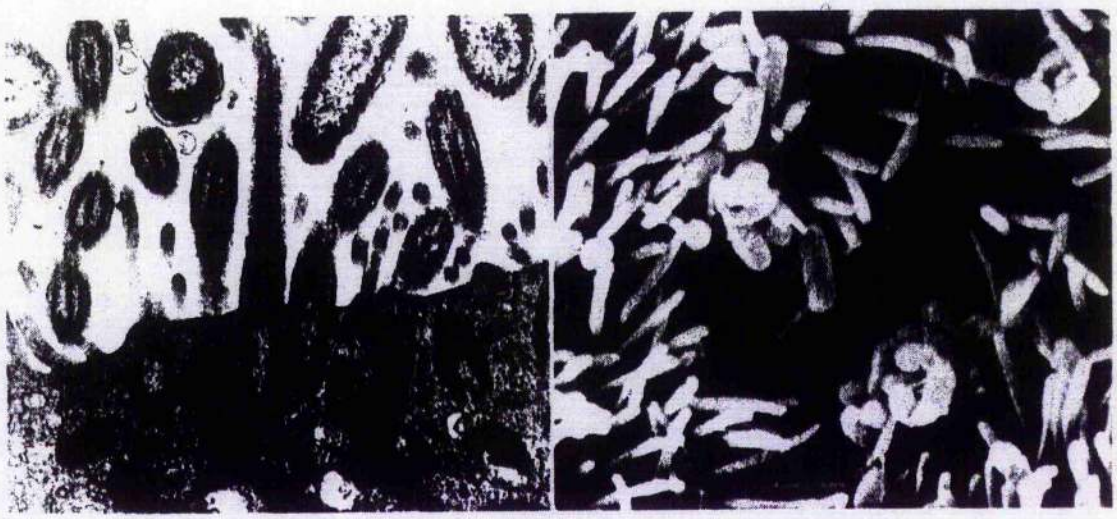
The virulence factors believed to be involved in the colonization of *B. pertussis* to the respiratory epithelium are: pertactin (Leininger *et al.*, 1990), the agglutinogens (AGG), filamentous haemagglutinin (FHA) and pertussis toxin (PT), (Weiss and Hewlett, 1986).

Table 1 : Virulence Factors of *B.pertussis* .

Virulence Factor	Role in pathogenesis	Reference to initial discovery
Toxins:		
Heat-labile toxin	local tissue damage	Bordet and Gengou (1909)
Pertussis toxin (PT) identified for histamine-sensitizingactivity	bacterial attachment, systemic toxicity	Parfentjev and Goodline (1948)
Lipopolysaccharide (LPS)	systemic toxicity	MacLennan (1960)
HLY-ACT Haemolysin (HLY) Adenylate cyclase (ACT)	 local damage evades host defences	 Lautrop (1960) Wolff and Cook (1973)
Tracheal cytotoxin (TCT)	local damage	Goldman *et al.*, (1982)
Envelope proteins:		
Agglutinogens	bacterial attachment?	Leslie and Gardner, (1931)
Filamentous haemagglutinin (FHA)	bacterial attachment	Arai and Sato, (1976)
Kawai haemagglutinin	bacterial attachment?	Kawai *et al.*, (1982)
Pertactin (69-KDa non-fimbrial agglutinogen)	bacterial attachment?	Brennan *et al.*, (1988)
Brk A	bacterial adhesion?	Fernandez and Weiss (1994)
Tracheal colonization factor	bacterial adhesion?	Finn and Stevens (1995)

Based on the reviews of Weiss and Hewlett, (1986) and Wardlaw and Parton, (1988).

Figure 2: Left: Scanning electron micrograph showing *B.pertussis* infiltration of the ciliary layer. Right: Transmission electron micrograph of a ciliated epithelial cell infected with *B.pertussis* for 24 h. Note the close apposition between the bacterium and host cell membrane. Magnification is x16,500 (Reproduced with permission from Muse *et al.*, 1978)



The agglutinogens were recognized by Andersen (1953) and Eldering (1957). Of the 14 AGG's described, six of these were unique to *B. pertussis*. AGG 1 is common to all *B. pertussis* strains. AGG 1, 2 and 3 are major antigens and AGG 4, 5 and 6 are minor antigens. Three of these surface proteins were later identified as fimbriae. These were AGG 2 by Carter and Preston (1984), AGG 3 by Ashworth *et al.* (1985), and AGG 6 by Cowell *et al.* (1987). An alternative scheme for serotyping *B. pertussis* strains was proposed by Robinson *et al.* (1989), where AGG 2 was referred to as fim 2 (F 2) and AGG 3 as fim 3 (F 3). The AGG 6 identified as fimbriae by Cowell *et al.* (1987), was thought to be identical to AGG 3 and therefore also termed fim 3.

The role of agglutinogens in adhesion is unclear. Some studies have shown that fimbriated *B. pertussis* can adhere to non-ciliated cell-lines and that anti-fimbriae monoclonal antibodies blocked their adherence (Sato *et al.*, 1979; Gorringe *et al.*, 1985; Redhead *et al.*, 1985). However, Urisu *et al.* (1985), reported that a transposon Tn5 mutant deficient in fim 2 still adhered well to WiDr cells indicating that this protein was not important in adhesion. Robinson *et al.*, 1990, confirmed that mutant strains deficient in fim 2 and fim 3 still adhered well to mammalian cells but the same study also showed that antisera raised against these antigens could prevent bacterial adherence. Thus, a minor role has been implicated for fimbriae in the process of attachment.

The first evidence for the protectivity of agglutinogens came from the results of the MRC trials (MRC, 1959). Protective efficacies of whole cell vaccines in children correlated well with agglutinin content. Studies by Robinson *et al.*, (1985) and Zhang *et al.* (1985) demonstrated protection in animal models of infection following immunization with purified fimbriae.

Novotny *et al.* (1985), isolated an adenylate cyclase (AC) related protein which could protect mice against atrophic rhinitis. Later, Brennan *et al.* (1988) identified this protein of 69 KDa as present on the surface of all virulent *B. pertussis* strains.

Leininger *et al.* (1990) initiated studies into the function of this protein and found monoclonal antibody against it could block the adherence of *B. pertussis* to CHO cells by 33%. This protein was named 'pertactin' and was thought to be important for mediating cell-attachment. This protein was therefore another candidate for inclusion in new acellular vaccine preparations.

Studies by Shahin *et al.* (1990) and Capiau *et al.* (1990), analyzed the capacity of the 69 KDa OMP to protect mice from respiratory challenge with virulent *B. pertussis*. The former workers showed that glutaraldehyde-treated pertactin could still induce 92% survival of challenged neonatal mice. Passive administration of an anti-pertactin monoclonal induced 90% survival. Furthermore, these workers demonstrated the immunogenicity of pertactin in humans. The sera from four humans vaccinated with the Takeda type Japanese acellular vaccine showed the presence of anti-69 KDa IgG. Capiau *et al.* (1990) protected mice by immunization with pertactin upon *i.n.* challenge (particularly potent when combined with FHA and PT) but not after *i.c.* challenge.

The production of P.69 has been achieved in high yields by Romanos *et al.* (1991) through use of the industrial yeast *Pichia pastoris* as an expression vector. These workers observed that the addition of native or recombinant P.69 enhanced the *i.c.* protective activity of pertussis toxoid. Roberts *et al.* (1992) showed immunization with recombinant pertactin was just as effective as the native protein in clearing organisms from the upper respiratory tract and inhibition of growth in the lungs.

Filamentous haemagglutinin (FHA) and PT were respectively identified as adhesins by Sato *et al.* (1981a) and Tuomanen and Weiss (1985). In the former case, inhibition of *B. pertussis* attachment to mammalian cells could occur by preincubation with anti-FHA. Tuomanen and Weiss (1985) carried out adhesion assays with transposon mutant strains deficient in the production of PT or FHA. These strains did not adhere, therefore these workers concluded that these proteins were important for adhesion. Redhead (1985) also showed that anti-sera against X-mode organisms, agglutinogens 1, 2 and 3 or purified FHA could block binding. Further evidence for the

role of FHA in attachment was provided by Urisu *et al.* (1985). These workers demonstrated inhibition of binding with anti-FHA preincubated organisms to WiDr cells. Also, mutant strains deficient in the production of FHA adhered less well than the parent strain.

When the organism is secured to the ciliated epithelium, it begins to multiply and accquires nutritional factors for growth. Redhead (1987) showed that *B. pertussis* unlike other bacteria did not procure iron with siderophores. The analysis of outer-membrane protein preparations suggested that *B. pertussis* obtained iron directly from host iron-binding proteins.

Aggressins and toxins

As the organism multiplies, a range of toxins are produced which enable the evasion of host defences, damage to surrounding tissue in the respiratory tract and the manifestation of systemic disease. Heat-labile toxin (HLT); pertussis toxin (PT); lipopolysaccharide (LPS); tracheal toxin (TCT) and adenylate-cyclase/haemolysin toxin (ACT-HLY), are proposed to be involved (Table 1).

To survive, the organism must evade the host defences. Two toxins of *B. pertussis* ACT and PT are shown to inhibit the function of immune effector cells. Confer and Eaton (1982) demonstrated the inhibition of chemotaxis, phagocytosis, superoxide generation and microbial killing by polymorphonuclear leucocytes through exposure to ACT. The contribution of ACT and HLY to the disease process was evaluated by Weiss *et al.* (1986), where a Tn5 mutant deficient in the production of both ACT and HLY was unable to cause a lethal infection of infant mice even after very high doses. PT was shown to inhibit macrophage migration *in-vitro* (Meade *et al.*, 1984a) and in a further study, *in-vivo* (Meade *et al.*, 1984b). The oxidative response of neutrophils was also inhibited (Bokoch *et al.*, 1984).

Local tissue pathology may occur through the concerted effects of TCT, HLT and ACT-HLY. Goldman *et al.* (1982) first identified a fragment of peptidoglycan as

TCT and showed that exposure of a partially purified fraction containing this component to organ cultures of hamster tracheal rings, inhibited ciliary activity. A cytopathology similar to that seen with *B. pertussis* infection was also exhibited. The intracellularly located HLT (Cowell *et al.*, 1979) caused oedematous lesions in rabbits and haemorrhagic necrotic lesions in guinea-pigs and mice (Munoz, 1971; and Cowell *et al.*, 1979). The pathogenic role of HLT was to damage tissues of the respiratory tract and thought to occur through vasoconstriction and rapid cytopathic changes in the peripheral blood vessels of vascular smooth muscle from guinea-pig and suckling mouse skin (Nakase and Endoh, 1988)

In the review by Pittman (1984) PT and LPS were the factors responsible for the manifestation of systemic disease. The most notable effect of PT is the enhanced leukocytosis commonly associated with pertussis cases (Morse and Morse, 1976). Other biological effects such as hyperinsulinaemia and hypoglycaemia can be reproduced in laboratory animals.

PT appears to be the major protective antigen and its protective activity will be discussed in the section below. Aside from PT, the protective activity of the other *B. pertussis* toxins is not known. Guiso *et al.* (1989) reported passive or active immunization with anti-ACT or ACT respectively protected mice against lethal respiratory challenge with *B. pertussis* or *B. parapertussis*. LPS was found to be non-protective by Robinson *et al.*, (1985). Protection studies for TCT and HLT have not been done. Their role as protective antigens remains to be determined.

Pertussis Toxin

Isolation and purification

In 1948, Parfentjev and Goodline observed that mice injected with whole-cell pertussis vaccine became more susceptible to histamine. In order to produce a less reactogenic

product, research was conducted into the segregation of the many activities of the vaccine (reviewed by Munoz and Bergman, 1977).

Since PT has many biological activities, a number of preparations possessing at least one of the activities now known to be caused by this molecule were purified and given various names. The procedure for preparing saline extracts from acetone-dried cells was reviewed in detail by Munoz (1963). Although many of the cells antigens could be separated by starch-block electrophoresis, the mouse protective antigen activity (MPA) could not be separated from the histamine-sensitizing activity.

Morse and Bray (1969) detected leukocytosis and lymphocytosis activity in late cultures of *B. pertussis* . They extracted this activity through a caesium chloride density gradient centrifugation technique. Subsequently, Sato and Arai (1972), purified the lymphocytosis promoting factor (LPF) from solid cultures noting its insolubility in low salt buffers. Further work by these workers (Arai and Sato, 1976) purified LPF and reported their preparation to have two distinct types of haemagglutination activity which were separable by molecular sieving through a 6% agarose column. These preparations were termed LPF-HA and fimbrial- haemagglutinin (FHA).

Irons and Maclennan (1979b) identified haptoglobin as having receptor affinities for LPF-HA. They purified this preparation (Irons and Maclennan, 1979a) with human haptoglobin linked to Sepharose-4B as an affinity chromatography medium. Arai and Munoz (1981) constructed another affinity medium which consisted of purified 'antipertussigen' antibodies free of anti-FHA antibodies conjugated to Sepharose-4B. Sato *et al.* (1983), separated LPF from HA by the particulate material hydroxylapatite which bound FHA but allowed LPF to pass through. The purification of PT utilizing a two step purification procedure with two different affinity media was done by Sekura *et al.*, (1983). PT was firstly adsorbed onto Affi-gel blue (which contained an NAD substrate analogue) and eluted with a buffer containing 1.0 M NaCl then passed through a fetuin-sepharose column made according to Askelof *et al.*,

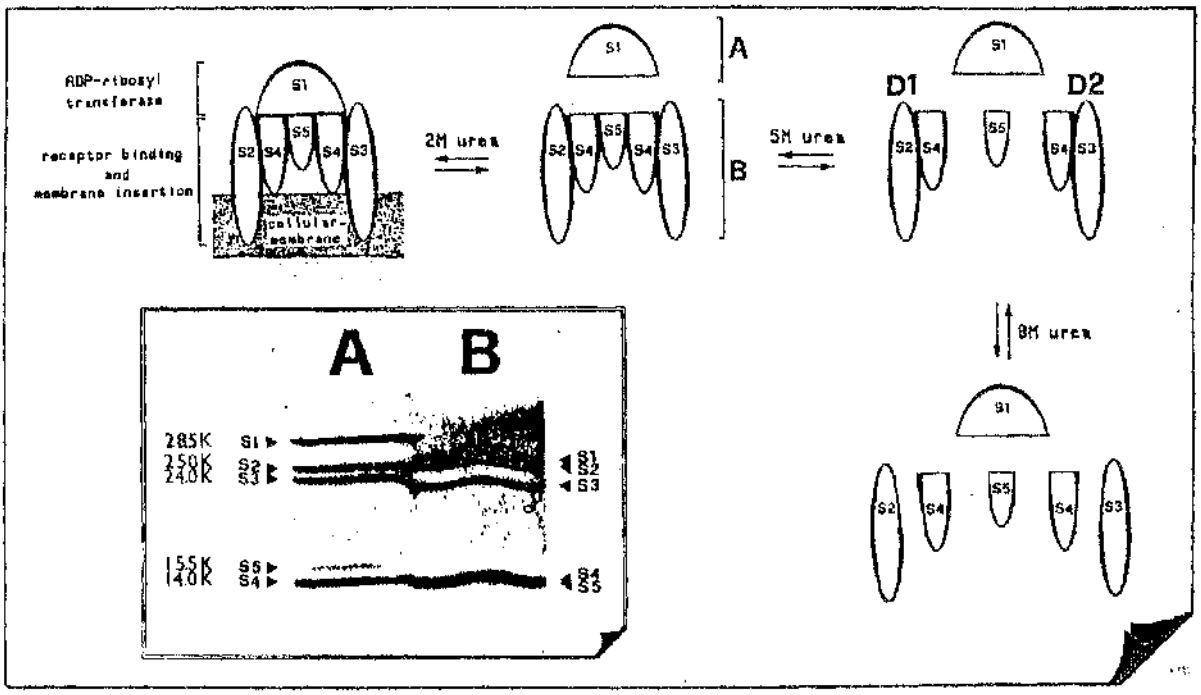
(1982). This method yielded a preparation which was pure and retained haemagglutinating and lymphocytosis-promoting activity.

Yajima *et al.* (1978), purified a protein from *B. pertussis* culture supernates which increased the secretion of insulin by Beta cells of the pancreas. This preparation was termed islets-activating protein (IAP). Pittman (1979) suggested that these many preparations MPA, LPF, LPF-HA, HSF and IAP were activities of the same substance and suggested the name of pertussis toxin.

Structure

Morphology of the LPF-HA preparation was investigated by Arai and Sato (1976), using electron microscopy and negative staining techniques. Molecules of 'PT' showed up as spherical structures of approximately 6nm diameter. Tamura *et al.* (1982), showed that PT possessed the A-B subunit structure similar to some other bacterial toxins. Figure 3 shows the assembly of the subunits and their breakdown with increasing concentrations of urea. An SDS-PAGE profile is also shown in the insert. Tamura *et al.* (1982) determined that the PT molecule was comprised of six subunits, five of which were dissimilar by SDS-PAGE and densitometry analysis . These five subunits were termed S1, S2, S3, S4 and S5 and were found in the relative ratios of 1:1:1:2:1. The S1 subunit could be isolated by urea treatment under mild dissociating conditions followed by haptoglobin-Sepharose chromatography. The B-oligomer of PT was composed of two dimers, S2-S4 and S3-S4 connected by the S5 subunit. The assembly of the toxin was investigated by Montecucco *et al.* (1986) by studying the interaction of the toxin molecule with membrane-lipids The S1 subunit or A-protomer was positioned on top of the five radially distributed subunits constituting the B-oligomer. The genes coding for pertussis toxin were cloned and their nucleotide sequences elucidated by Nicosia *et al.*, (1986) and Locht and Keith, (1986).

Figure 3: Structure and properties of pertussis toxin. The insert shows the sodium do decyl sulphate polyacrylamide gel electrophoresis of purified pertussis toxin under reducing (A) or non-reducing conditions (B). Reproduced with permission from Rappuoli and Silvestri (1987).



Biological activities

The many activities of pertussis toxin are summarized in Table 2. A number of these activities were reported initially as biological activities of whole-cell pertussis vaccine before PT was recognized.

Lymphocytosis-promoting activity In 1897, Frolich (cited by Wardlaw and Parton, 1983), was the first to report an increase in the number of white blood cells from whooping cough patients. Munoz and Bergman (1977) reported levels of up to 175,000 cells/mm^3 compared with normal levels of between 7,000 to 11,000. The studies of Morse and Riester (1967a and 1967b) showed that there was a migration of circulating cells in mice injected with pertussis vaccine. The increased number of lymphocytes arose from a transfer of cells from the spleen, thymus, and lymph nodes rather than a multiplication of cells. Later, Morse and Morse (1976), reported that the majority of lymphocytes were T-cells with a smaller but more notable rise in B-cells. Monocytes and polymorphonucleocytes were also increased.

Isles activation: PT has a marked effect on the metabolic functions of the infected host. Blood-glucose levels were reported as being lower than normal in infants with pertussis by Regan and Tolstoouhov, 1936. Hyperinsulinaemia and changes in blood-glucose levels were also reported to occur in mice and rats injected with pertussis vaccine by Gulbenkian *et al.* (1968). Yajima *et al.* (1978), showed that highly purified preparations of islets-activating protein also exhibited similar changes in rats. The increase in insulin levels occurred by a block in the inhibitory effect of the alpha-adrenoceptors in pertussis infected animals resulting in continuous stimulation by beta-adrenoceptors of insulin release by pancreatic islet Beta-cells (Katada and Ui, 1981).

Mouse protective actvity : Kendrick *et al.* (1947) devised a method for assaying the mouse protective antigen content of whole-cell pertussis vaccines. Purification of the MPA from HSF actvity was not obtained (Munoz and Bergman, 1977). However, Pillemer *et al.* (1954) purified the stromata protective antigen which was later shown to contain MPA with most workers concluding that the MPA was identical to the HSF and

Table 2: Biological activities of pertussis toxin.

Activity	First described by:
Leukocytosis promotion	Frolich (1897, cited by Wardlaw and Parton, 1983)
Islets activation	Regan and Tolstoouhov (1936)
Mouse protective antigen (MPA)	Kendrick *et al.*,(1947)
Histamine sensitization	Parfentjev and Goodline (1948)
Adjuvant activity	Levine and Pieroni (1966)
Haemagglutination	Keogh *et al.*, (1947)
Mitogen	Kong and Morse (1977a)
ADP-ribosyltransferase	Katada and Ui (1982a)

an activity of the same protein (Joo and Putszai, 1960; Levine and Pieroni, 1966; Irons and Maclennan, 1979a; Munoz *et al.*, 1981a).

Immunization with inactivated PT has been shown by several workers to protect mice against intracerebral (*i.c.*) challenge (Munoz *et al.* 1981a; Sato *et al.*, 1981a; Robinson and Irons 1983; Oda *et al.*, 1984). Passively administered goat anti-PT or mouse anti-PT monoclonal also protected mice after aerosol challenge (Oda *et al.*, 1984). Munoz *et al.* (1981a) and later Gupta *et al.* (1990), found that immunization of mice with sub-lethal doses of native PT alone did not protect mice against *i.c.* challenge. The latter workers reported however that mice immunized with FHA as well as a small amount of native PT protected 60% of mice. Burstyn *et al.* (1983) reported an IgG and IgM response to PT after vaccination of children. Similarly, Thomas *et al.* (1989), also detected an IgG response to PT in children after vaccination and the disease.

Histamine-sensitizing activity : Pertussis-vaccine injected mice were shown to become sensitized to a subsequent injection of histamine (Parfentjev and Goodline, 1948). Maitland *et al.* (1955), termed this factor the histamine-sensitzing factor (HSF). Munoz and Bergman (1968) found that mice and rats were highly resistant to histamine whereas guinea-pigs and rabbits were highly susceptible to the lethal effects of histamine. Thus, injection of HSF would show a greater sensitizing effect in an animal normally resistant to histamine therefore mice are routinely used. Female mice were more sensitive than male mice to histamine after pertussis vaccine injection (Maitland *et al.*, 1955). Bergman and Munoz (1964) reported older mice (>7 weeks) were more responsive to histamine after HSF than younger mice (3 to 7 weeks old). The intra-peritoneal (*i.p.*) route of injection of HSF or pertussis vaccine is commonly used in the assay however, administration via the intra-venous route (*i.v.*) appeared to elicit the best responses (Munoz and Bergman, 1966).

Adjuvant activity : Pertussis toxin has a number of adjuvant effects however, it was not until 1966 when Levine and Pieroni showed that this activity was attributed

specifically to PT and not all as due to LPS. Pertussis vaccine containing PT could enhance antibody titres to antigens injected simultaneously (Munoz and Bergman, 1977). Munoz (1964) noted that antitoxin to diphtheria and tetanus toxoids was raised when injected with pertussis vaccine. Also, Munoz *et al.* (1981a), observed that *i.v.* injection of PT increased the IgG1 and IgE response to mice immunized with OA. Increase of IgE was first noted by Mota (1958), described then as 'mast-cell sensitizing antibody'.

The autoimmunity condition hyperacute experimental allergic encephalomyelitis was induced or accelerated in rats by whole-cell pertussis vaccine (Levine *et al.*, 1966). Other types of hypersensitivity reactions (immediate and delayed type) are also affected by pertussis toxin (reviewed by Munoz, 1988).

Haemagglutinating activity : The haemagglutinating activity although first described by Keogh *et al.* (1947) in *B. pertussis* culture supernates was not attributed to PT until 1976, when Arai and Sato purified the LPF-HA and found it to haemagglutinate goose erythrocytes.

Mitogenic activity : Kong and Morse (1977a, 1977b) found purified PT preparations had a mitogenic effect on T-lymphocytes which was only slightly lower in potency than the lectin concanavalin A. This activity was a function of the B-oligomer (Nogimori *et al.*, 1984a).

ADP-ribosyltransferase activity : The ADP-ribosylating activity of the S1 subunit of PT was identified by Katada and Ui (1982a, 1982b). These workers found that PT catalyzed the ADP-ribosylation of GTP-binding regulatory proteins (G-proteins) of eukaryotic cells. PT mediates the transfer of the ADP-ribose moiety of NAD to the G-protein substrate. Casey and Gillman (1988) demonstrated that PT ADP-ribosylated a family of GTP proteins including Gi, Go and Gt. These proteins are involved in signal transfer to effector molecules within the cell. Gi (identified as the MW 41,000 protein substrate of Katada and Ui, 1982a, 1982b), regulates adenylate cyclase activity. Thus,

when Gi is ADP-ribosylated by PT, an increase in cAMP levels is observed since the adenylate cyclase is no longer inhibited.

The results of PT causing interference of these cell-signalling systems are numerous, e.g. *in vitro* effects of PT activity are: increased release of insulin by pancreatic rat islet Beta cells (Katada and Ui, 1980); enhanced cAMP production due to adenylate cyclase activity in rat C6 glioma cells (Katada and Ui, 1982b) and clustered growth of chinese hamster ovary cells (Hewlett *et al.*, 1983).

Pertussis toxin has many diverse biological activities both *in vitro* and *in vivo*. Since the discovery of its A-B structure by Tamura *et al.* (1982), its mode of action was investigated by Tamura *et al.* (1983) who proposed that the B-oligomer mediated the binding of the holotoxin to the eukaryotic cell membrane and translocated the S1 subunit across into the cytoplasm of the cell. Nogimori *et al.* (1984a) acetaminidated the epsilon groups of lysine residues on the IAP molecule and then assayed the *in-vitro* and *in-vivo* biological actvities of PT. Comparison of IAP and its acetaminidated derivative revealed that certain activities (ADP-ribosylation of membrane proteins, stimulation of insulin release and haemagglutination) were not affected by chemical modification whereas activities such as the mitogenic activity, lymphocytosis promotion, histamine-sensitization and adjuvant activity were. These workers concluded that the activities not affected by lysine modification were due to the A-protomer which entered the cell through action of the B-oligomer. Neither of these functions was altered by lysine modification. However, activities associated with the B-oligomer which were affected, indicated that the lysine modification interfered with correct binding of the B-oligomer to the cell membrane.

Filamentous Haemagglutinin

Isolation and purification

A haemagglutinin (HA) activity for chicken erythrocytes was found in cells from young cultures or in the supernate of older cultures of *B. pertussis* (Keogh *et al.*, 1947). HA was extracted by Masry (1952), by incubating cells in 2M NaCl or by methanol precipitation. The latter preparation yielded purer preparations which were toxic. However, it was later found that HA activity was not related to toxic activity (Munoz and Bergman, 1977). These workers stated in their review that these early preparations were crude and contaminated with many other components of the *B. pertussis* cell.

In 1976, Arai and Sato purified filamentous haemagglutinin (FHA) although at that time it was termed fimbrial haemagglutinin. These workers found two distinct peaks with HA activity upon separation through gel filtration. One peak had a high HA activity whereas the second peak had low HA activity but possessed high lymphocytosis-promoting and histamine-sensitizing activities. Irons and Maclennan (1979b) investigated the receptor specificity of FHA and determined that it bound to cholesterol. An affinity chromatography medium was prepared and FHA was subsequently purified. Static growth of *B. pertussis* was superior to shaken cultures for the production of FHA (Arai and Munoz, 1979). Askelof *et al.* (1982) described the purification by preparing sodium acetate extracts according to Masry (1952) followed by chromatography on Sephacryl S-300 gel.

FHA purified by Arai and Sato (1976) and Arai and Munoz (1981) were contaminated with PT, since FHA made by their methods could sensitize mice to histamine (Munoz *et al.*, 1981a). Subsequently, Sato *et al.* (1983) purified FHA which contained <0.002% active PT as assayed by histamine-sensitization.

FHA is highly susceptible to breakdown (Arai and Munoz, 1979). The former workers noted that all HA activity was lost upon shaking a solution of FHA at 37 °C overnight. Sato *et al.* (1983) reported FHA could be stored at -70 °C for at least one

year without any loss in HA. Also, FHA is not specifically produced only by *B. pertussis* but also by *B. parapertussis* and *B. bronchiseptica.* (Blom *et al.*, 1983).

Structure and Biological activity

Electron microscopy studies on FHA by Arai and Sato (1976), revealed that the protein appeared as filamentous structures of approximately 2 nm width x 40 nm length. Further studies on FHA morphology by Blom *et al.* (1983) showed FHA as a mixture of small thin filaments, 3 nm wide and 40 to 100 nm long and long fimbriae (3 nm wide and up to several nm long). These fimbriae also showed a tendency to form bundles. The highest HA titres however, corresponded to an increased number of shorter filaments.

The approximate MW of FHA was judged by SDS-PAGE was 126,000 (Arai and Sato, 1976). Irons and Maclennan (1979b) estimated the MW of the FHA bands at 127 KDa and 95 KDa by the SDS-PAGE. In a further study, Irons *et al.* (1983) analyzed the heterogeneity of FHA by western blotting with monoclonal antibodies. Bands of 220 , 127, 95, 76 and 58 KDa were obtained. These were assumed to be degradation products of FHA and all derived from the 220 KDa band. Domenighini *et al.* (1990) also examined the SDS-PAGE profile of FHA. FHA was initially seen as one band of 220 KDa, but after storage, it was accompanied by breakdown products of 140, 125 98, 75 and 58 KDa bands with a concurrent loss in the intensity of the 220 KDa band.

The gene encoding FHA has been sequenced (Domenighini *et al.*, 1990) and found to code for a basic protein (pK, 9.65). Alanine and glycine are the two most commonly occurring amino-acids making up 27% of the total residues. An arginine-glycine-aspartic acid (RGD) sequence occurred twice in the primary structure. Relman *et al.* (1989), provided evidence that this sequence was involved in FHA-receptor binding.

The haemagglutinating activity of FHA has already been described. It haemagglutinates sheep, chicken and goose cells (Aral and Sato, 1976; Irons and Maclennan, 1979b). FHA has also been described as an important adhesin and binds to a number of cell types including rabbit-ciliated cells (Tuomanen*et al.*, 1985) WiDr cells -an epithelial-like cell line from a human intestinal carcinoma (Urisu *et al.*, 1986) and chinese hamster ovary cells (Relman *et al.*, 1989).

Protective activity in the mouse

Tuomanen and Weiss (1985) showed FHA played an important role in adhesion. A transposon mutant strain deficient in the production of FHA did not adhere well to human ciliated epithelial cells but this could be overcome by the addition of exogenous FHA.

The inclusion of FHA in acellular vaccine preparations could theoretically provide antibodies which block binding of the organism to the cells in the respiratory tract thereby preventing infection. A number of studies therefore were undertaken to evaluate the protective potency of FHA. Protection of mice immunized with FHA and challenged by the aerosol infection model was obtained by Oda *et al.* (1984). However, FHA alone was not protective when mice were challenged *i.c.* (Munoz *et al.*, 1981b; Sato and Sato, 1984; Oda *et al.*, 1984) unless containing pertussis toxoid (Munoz *et al.*, 1981b; Sato and Sato, 1984). Passive immunization studies in mice receiving murine anti-FHA and goat IgG to FHA were protective against aerosol challenge as reported by Sato *et al.* (1981a) and Sato and Sato (1984). Conversely, Oda *et al.* (1984) found passive administration of murine monoclonal antibody or affinity-purified goat anti-FHA gave little protection from disease after respiratory challenge. Ashworth *et al.* (1982) showed rabbits immunized with FHA cleared *B. pertussis* more effectively from the nasopharynx relative to control animals.

The protective activity of FHA was investigated further by Kimura *et al.* (1990) using the mouse respiratory infection model. These workers reported that immunization

with FHA resulted in reduced numbers of bacteria recovered from the lungs and caused less colonisation of the trachea. Passive immunization with goat and rat anti-FHA resulted in a marked reduction in colonization of lungs and trachea after challenge.

The serological response to FHA in humans after vaccination and the disease was examined by Burstyn *et al.* (1983) and Thomas *et al.* (1989). Both studies reported high IgG titres to FHA in both convalescents and vaccinees. IgA was found only in the former group. Since these high IgA titres were found in convalescents, Shahin *et al.* (1992) proposed long-lived immunity might be obtained through mucosal immunization. These workers immunized BALB/c mice *i.n.* and intra-duodenally (*i.d.*) and analysed antibody isotypes in sera, nasal washes and bronchoalveolar lavage fliud. IgA and IgG was detected in lung lavage fluid of both *i.d.* and *i.n.* immunized mice. Increased numbers of FHA specific B lymphocytes were also noted in the lungs. More recently, Roberts *et al.* (1993) demonstrated a mucosal memory response to FHA in immunized mice that were boosted nine months later.

IMMUNITY TO PERTUSSIS

Naturally Accquired Immunity

Linneman (1979) reported that immunity to pertussis after the disease was of a longer-lasting nature compared with that induced by vaccination. Following infection, IgA, IgG and IgM antibodies were detected against *B. pertussis* by ELISA in sera from suspected cases of whooping cough (Viljanen *et al.*, 1982). Comparatively, Ruuskanen *et al.* (1980) reported that IgM and IgG responses were very low in sera from DTP vaccinees. An anti-pertussis IgA response was also found in nasopharyngeal secretions after natural infection but not after parenteral vaccination by Goodman *et al.* (1981).

Infection produces higher levels of IgG, IgA and IgM isotypes compared with

vaccination (Nagel *et al.*, 1985). The antibody response to *B. pertussis* was followed by Finger and Wirsing von Koenig (1985). These workers found that an antibody response was stimulated one to two weeks after the beginning of the clinical symptoms of the disease. IgM and IgA appeared first followed by IgG with a subsequent decrease in IgM and IgA titres for most cases within two to five months.

Antibody responses against the various virulence factors of *B. pertussis* were examined in a number of studies. The most important antibody for long-term protection from the disease is anti-PT. Viljanen *et al.* (1985) reported the production of an IgM, IgA and IgG response to PT during infection. The anti-PT IgG titre increased progessively with the paroxysmal cough whilst the IgM titre declined (Wisnes *et al.*, 1985).

An anti-FHA response was not detected by Nagel *et al.* (1985) in sera from suspected pertussis cases although Winsnes *et al.* (1985) found both anti-FHA IgG and IgM in convalescent and in vacinee's sera. Granstrom *et al.* (1982b) measured anti-FHA responses in human sera during natural infection. The data showed a significant rise in IgG, IgM and IgA levels. Antibodies to LPS were thought to be of little protective value (Pittman, 1984). The heat-labile toxin (HLT) induced low titres of antibody if they could be detected at all (Evans and Maitland, 1939).

Whole-Cell Pertussis Vaccines

Preparation and testing of whole-cell pertussis vaccines.

According to WHO specifications (1979), pertussis vaccines are prepared usually from a mixture of several strains of *B. pertussis* to ensure the presence of agglutinogens types 1, 2 and 3. In the review of Wardlaw and Parton (1983), the preparation and testing of WCPV is described in detail.

Well characterized strains of *B. pertussis* are grown in bulk culture either on solid or in liqiud media under conditions which vary considerably between

manufacturers. The suspension is killed by treatment with formaldehyde, thiomersal, long-term storage at 2-8 °C, or by heating at 56 °C for 30 min. Thiomersal (as preservative) and mineral adjuvants aluminium hydroxide, aluminium phosphate or calcium phosphate (if required in the final vaccine) are added at this stage. After undergoing rigorous tests for pH, sterility, toxicity, potency and stability, the product is aliquoted into sterile vials.

Lapin (1943) reviewed the preparation and protective efficacy of early pertussis vaccines. These mixtures contained *B. pertussis* with other upper respiratory tract flora (e.g. staphylococci and streptococci); and precipitated with alum. These vaccine strains were grown on Bordet-Gengou medium containing horse, human or sheep blood. Methods of killing included heating or chemical treatment with formalin, phenol or thiomersal (Pittman *et al.*, 1952). When Leslie and Gardner (1931) discovered that *B. pertussis* underwent phase changes, subsequent pertussis vaccines were purposely prepared from virulent phase I strains. Since the report by Hewlett *et al.* (1977) that formalin-treated WCPV contains active PT and LPS, other detoxifying agents have been tested. Relyveld (1973) first treated the organisms with glutaraldehyde as an alternative inactivating agent to yield vaccine preparations. Later, Gupta *et al* (1987a) reinvestigated the effects of different inactivating agents on potency, toxicity and stability of whole-cell pertussis vaccine. Formaldehyde and glutaraldehyde inactivation showed similar potencies to heat inactivation, although heat-inactivated vaccines were less effective in the mouse weight-gain test. Glutaraldehyde was a better agent for reducing the histamine-sensitizing activity of PT (Gupta *et al.*, 1987b, Gupta *et al.*, 1988). Under optimal conditions of inactivation (0.05% glutaraldehyde at room temperature for 10 min) produced a vaccine which was as potent but less toxic than if heat-inactivated.

Safety testing of vaccines utilise the mouse weight gain test (MWGT). Toxicity in this test is indicative of the presence of active LPS, PT or HLT. Groups of mice are injected *i.p.* with the test preparation with at least half a human dose and then weighed

daily. Loss of weight after 24 h is due to LPS. Slow weight gain or later death is attributed to bioactive PT.

A laboratory test designed to assess the protective capacity or potency of vaccine preparations was developed by Kendrick *et al.* (1947) and termed the *intra--cerebral* mouse protection test (ICMPT). Essentially, graded doses of test and standard vaccines are injected *i.p.* into groups of mice. After 14-17 days the animals are challenged by intracerebral (*i.c.*) injection with 0.03 ml of *B. pertussis* W18-323 (a highly virulent strain following *i.c.* injection). The number of survivors are counted after 14 days and the potency of the test vaccine is estimated relative to a reference preparation. This reference is the Second International Standard for Pertussis Vaccine and each ampoule of freeze-dried material contains 46 International Units (IU) of protective potency. Test vaccines must have at least 4 IU lower limit 2 IU per human dose immunized.

Protective Efficacy

Protection afforded by immunisation with some early pertussis vaccines was demonstrated by clinical field trials in children from the 1920's and onwards.

Madsen (1933) used a formaldehyde-killed suspension of *B. pertussis* to vaccinate a total of 3,926 children (combined figures from two trials) with 1,073 children in the unvaccinated group. The percentage fatalities were less (only 0.15%) in the vaccinated group compared with unvaccinated children (2.4%). Sauer (1937) immunised 1,122 children. Only six of 128 children subsequently exposed to pertussis developed the disease. In the study of Kendrick and Eldering (1939) 4,212 children were vaccinated. After home exposure, 89.4% of unvaccinated children contracted whooping cough compared with 34.9% of vaccinated individuals.

Not all clinical trials reported successful results however. McFarlan *et al.* (1945) tested two vaccines produced by the Wellcome laboratories in Britain. Children from day nurseries, welfare clinics in Oxford and from residential nurseries were

immunised Based on clinical diagnosis, no beneficial effect of vaccination against whooping cough was apparent.

To ascertain definitively whether pertussis vaccines were effective in protecting children from the disease, the MRC conducted a number of trials during the 1950's. In the first of these, (MRC, 1951), plain or alum precipitated vaccines manufactured either in the U.K. or U.S.A. were administered in three injections to 3,801 children. A similar number of children (3757) were included in the control group. Upon home exposure, 87.3% of unvaccinated children compared with only 18.2% of vaccinated children became infected. Figures for exposure outside the home were 38.0% unvaccinated and 8.3% vaccinated. The infection was also noted to be milder in vaccinated children. Immunised children (44.3%) had a cough of shorter duration (<6 weeks) compared with 14.4% in the unvaccinated group. The vaccines varied considerably in their protective effect.

Trials to compare the efficacy of 14 pertussis vaccine preparations in 28,799 immunised children were completed in 1956 (MRC, 1956). Nine of the test vaccines used in the first trial were poor whereas the five in the second trial gave better protection. The third series of MRC trials, (MRC, 1959) reported protective capacity of a number of test vaccines which had been assessed by two laboratory tests - ICMPT and agglutinin-production. One of the aims of these clinical trials was to establish which laboratory test gave the best correlation with protective efficacy in children. Comparisons were made between vaccines prepared from fluid medium and solid medium and of pertussis vaccine alone or mixed with diphtheria toxoid. An antigenic fraction of *B. pertussis* prepared by Pillemer *et al.* (1954) consisting of a sonic disintegrate treated with autoclaved human red cell stromata was also included. It was shown that the two vaccines prepared from solid and liquid medium did not differ significantly in their protective capacity, however, the third prepared from liquid medium was more effective. Protection rates afforded with pertussis vaccine administered alone, or as a mixture with diphtheria toxoid, were similar. Although the

Pillemer vaccine was more effective than WCPV, it also induced more adverse reactions. Both ICMPT and agglutinin-production tests correlated well with protective efficacy with the exception of the Pillemer vaccine. It was highly potent in protection of mice by the ICMPT but produced a poor agglutinin-response (Evans and Perkins, 1955). Thus, the ICMPT was selected as the laboratory test for assessing the protective potency of pertussis vaccines. The potency of these vaccines were compared to the first British Standard Vaccine.

In a report by the Public Health Laboratory Service (PHLS, 1973), the efficacy of whooping cough vaccines was thought to have decreased. Perkins (1969) amended the potency of the British Reference vaccine from 2.1 IU to 4 IU. Altered serotypes of infective pertussis strains to explain the drop in effectiveness were also suggested. Prevailing serotypes were of 1, 2 or 1, 2, 3 but by 1963-1964 during a pertussis epidemic, they changed to predominantly 1,3 types. This led to the WHO recommendation (WHO, 1979) to include strains of all three types in future pertussis vaccine preparations.

Since as evidenced in the MRC field trials, pertussis vaccines provided good protection, it was considered unethical to withhold vaccination from susceptible children. As a result indirect methods of evaluating the efficacy of pertussis vaccines by careful surveillance and documentation of pertussis cases were developed. In a recent study by Onorato *et al.* (1992), a vaccine efficacy rate of 96% was estimated for home exposure infection in preschool children. Thus pertussis vaccines today offer good protection from the disease.

Toxicity

It has long been demonstrated that pertussis vaccine shows toxicity. In the early study of Madsen (1933) two children were reported to have died within hours of their second pertussis vaccination. Reactions to pertussis vaccines are classified by Mortimer (1988) as mild, worrisome or severe. Local reactions include swelling, redness and pain at the

site of injection. Systemic reactions are fever, vomiting, irritability and anorexia. These minor or mild adverse reactions occur 12-24 h after immunisation and are normally of a transient nature. Reactions of a more worrisome sort are excessive sleepiness, persistent crying, an uncommon shock-like state termed an hypotonic-hyporesponsive episode and fever-related seizures. The most serious reaction to pertussis vaccination is acute encephalopathy, however, since this reaction occurs so rarely it has been difficult to attribute it without reservation to WCPV.

In 1974 two deaths were reported in Japan following pertussis vaccination. Further vaccination of children in this country was discontinued. In Sweden, an article by Strom (1960) suggested as many as 1 in 6000 children were left brain-damaged after immunisation. However this was corrected to 1 in 50,000 after recalculation of the data. Kulenkampff *et al.* (1974) published the results of a study where 36 children exhibiting neurological illness had been followed over a period of 11 years after recieving DTP. The results showed a clustering of neurological complications in the first 24 h after immunisation and because of this, it was concluded that DPT vaccine had a 'causal' rather than a 'coincidental' effect and vaccination of ill children should be withheld. Adverse media coverage of this article claiming that pertussis vaccine was unsafe, resulted in reduced vaccine acceptance rates in Great Britain.

Since the vaccine was thought to cause neurological complications, the need for mass immunisation against whooping cough was further questioned by Bassili and Stewart (1976). They reported a strong correlation between socioeconomic conditions and attack rates, whereas attack rates and vaccine-acceptance rates correlated weakly. The authors suggested the decline of the incidence of pertussis was not vaccine related and found from an outbreak of pertussis in Glasgow, a third of the notified cases were in fully-immunised individuals. In 1977, Stewart estimated the incidence of vaccine-associated encephalopathy to occur at a frequency of 1 in 10,000 to 1 in 60,000 children. He stated the risk of developing neurological complications after vaccination surpassed the injury caused by the disease. However, Grist (1977) recommended

continuing with vaccination arguing that the decline in whooping cough was due to a combination of improved socioeconomic factors and vaccine use. He estimated the rate of developing neurological complications at 1 in 135,000 children. Pollard (1980) also favoured continuing with vaccination. He found a direct relation between the increase in notification rates of pertussis and the decrease in vaccine acceptance in England and Wales.

The reactogenicity of WCPV was assessed by Barkin and Pichichero (1979). DTP vaccine was administered to 1232 children. A postal questionnaire was completed by parents and on this basis, 72% were found to have local reactions. Cody *et al.* (1981) reported reactions occurring within 48 h of vaccination in 784 DT and 15,752 DTP immunizations. Mild adverse reactions occurred significantly more frequently in DTP compared with DT-immunized individuals. Later, Blumberg *et al.* (1993), carried out a detailed study of sixty children experiencing severe reactions within 48 h of DTP immunisation. They attributed persistent crying to painful local reactions and vaccine endotoxin as the cause of fever. Lymphocyte counts, insulin and glucose levels in these children were investigated and acute serum samples examined for active PT. No active PT was found in immunised chidren therefore there was no evidence that PT played a role in severe vaccine reactions.

To settle the question of whether pertussis vaccine was responsible for acute neurological illness in vaccinees and to provide an estimate of the frequency of its occurrence, the National Childhood Encephalopathy Study was set up. During 3 years, 1,167 children with certain neurological illnesses from hospitals in Scotland, England and Wales were notified in a case-control design method with two matched controls for each case. The risk estimate was 1 in 330,000 doses with a 95% confidence interval of 1 in 50,000 to 1 in 18,000,000. This study concluded that DTP was responsible for acute neurological illness in some children. However, despite many epidemiological surveys, the link between pertussis vaccination and *permanent* brain damage could not be proved.

A number of reviews surveying the information on adverse reactions to pertussis vaccines have been published. These include Miller *et al.* (1982); Wardlaw and Parton (1983); Mortimer (1988); Wentz and Marcuse (1991); Robinson (1981); Fine and Chen (1992); Hodder and Mortimer (1992).

Acellular Pertussis Vaccines (ACPV)

Development of acellular pertussis vaccines was undertaken with the purpose of producing a less reactogenic more efficacious product which conferred longer lasting immunity. Fears over the safety of WCPV led to greatly reduced vaccine acceptance rates causing a subsequent rise in the number of pertussis cases in unprotected individuals (Pollard, 1980).

A number of experimental vaccine preparations have been constructed and are composed of protective antigens identified from animal models of infection. These acellular preparations differ in their composition and in the method of detoxification.

Origins

Early "component" whooping cough vaccine preparations include the stromata protective antigen of Pillemer *et al.* (1954) and the "Tri-Solgen" vaccine of Weihl *et al.* (1963). These vaccine preparations did not consist of purified defined antigens but the protective antigen component was obtained from supernates of sonicated cells or through removal by chemical extraction from culture supernates. Both vaccines were tested by the ICMPT. Further information on earlier vaccine preparations made from cell extracts can be obtained from the review by Wardlaw (1992).

In 1972, Sato and Arai were successful in purifying the lymphocytosis-promoting factor from the culture supernate of *B. pertussis* strain Tohama. These cells had been grown on solid medium. This preparation had leukocytosis-promoting,

histamine-sensitizing and haemagglutinating activities (Sato *et al.*, 1973) and was toxic but had protective activity. After a mild formalin treatment (0.2% formalin at 25 °C for 7 to 11 days) this preparation could be made atoxic and passed testing by both the active and passive ICMPT assays. This discovery was an important finding in the formulation of acellular pertussis vaccines (ACPV). Arai and Sato (1976) discovered that this LPF preparation consisted of two types of haemagglutinin, LPF-HA and FHA which were co-purified by their extraction procedures. Then, PT was identified as a major protective antigen both in active and passive *i.c.* and *i.n.* challenge mouse models (Munoz *et al.*, 1981a; Sato *et al.*, 1981a; Robinson and Irons, 1983 and Oda *et al.*, 1984). FHA was not protective by *i.c.* challenge but was after *i.n.* challenge (Munoz *et al.*, 1981b; Sato and Sato, 1984; Oda *et al.*, 1984; Sato *et al.*, 1981b) therefore it also was included.

By 1981, a pertussis component vaccine containing formalin-detoxified PT and FHA was being used for mass immunization in Japan (Sato *et al.*, 1984). This vaccine was a preparation of FHA and PT. Culture supernates obtained from scraping the growth off solid medium were the starting material for the purification of these antigens. Extraction was by ammonium precipitation, and LPS was removed from this crude extract by ultracentrifugation followed by detoxification with formalin. The final vaccine mixture contained : FHA (5 to 10μg protein nitrogen/ml (PN/ml)); PT (0.5 to 5.0μg PN/ml); diphtheria toxoid (30 to 40 flocculating units (Lf/ml); tetanus toxoid (3 to 8 Lf/ml); formalin ≤0.01%; merthiolate ≤0.01% and aluminium hydroxide ≤0.2 mg/ml as adjuvant.

In Japan, there are six different vaccine manufacturers of acellular vaccines, producing basically two types of preparations. The Biken type (B-type) which contains inactivated PT and FHA in approx equal amounts and the Takeda type (T-type) which is comprised mostly of FHA, PT and agglutinogen 2 (Noble *et al.*, 1987). Figure 4 shows the composition of the various ACPV preparations available in Japan. Preparations of ACPV's that vary in antigenic composition and the method of PT

detoxification were formulated in America, England, France and Italy (Table 3) were evaluated by clinical trials.

Iwasi *et al.* (1985) reported that formaldehyde-detoxified PT could revert *in vivo* by showing mice were sensitized to histamine when challenged on day 12 instead of the normal third day. Also, formaldehyde reacts primarily with the epsilon-amino group of lysine residues and when Nicosia *et al.* (1986) cloned and sequenced the PT gene it was found that the immunodominant S1 subunit did not contain any lysine residues for reaction with the detoxification agent.

At the Sclavo research centre in Italy, the PT molecule was genetically detoxified in order to produce a less toxic immunogenic vaccine (reviewed by Rappuoli *et al.*, 1991). Pizza *et al.* (1988), identified the amino-acids important for the ribosyltransferase activity of the S1 subunit in the PT molecule. Thereafter, using DNA recombination techniques, the mutated genes were inserted into the chromosomes of *B. pertussis*. The resulting mutant producing a defective S1 subunit and even though this protein was 10 to 1000 times less toxic than wild type, it was still too toxic for use in vaccines (Pizza *et al.*, 1989). Double mutants containing amino-acid substitutions were constructed and the mutant PT molecules tested for toxicity, ADP-ribosyl-transferase activity, antibody recognition and T-cell recognition. A mutant PT molecule obtained by substituting Arg^{9} with lysine and Glu^{129} with glycine was stabilized with formaldehyde (Nencioni *et al.*, 1991) and tested in adult volunteers (Podda *et al.*, 1991). Two vaccines were constructed. A monocomponent vaccine containing 15 μg of the mutant PT-9K/129G and the other containing 7.5 μg of this double mutant PT, 10 μg of FHA and 10 μg of the 69 KDa protein. Vaccinated individuals showed good antibody responses to each antigen with only mild local reactions obtained both in the vaccinees and placebo. The results of recent phase III clinical trials are shown in the discussion section.

Figure 4 : Composition of Japanese acellular pertussis vaccines.
(Reproduced with permission from Kimura 1988)

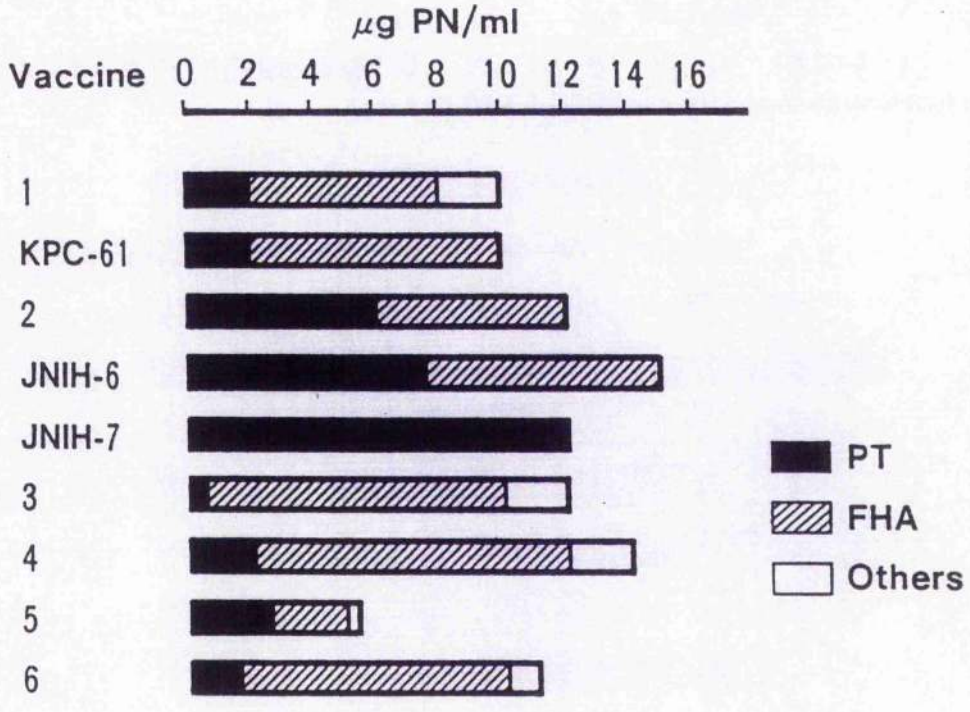


Table 3 : Formulations of acellular pertussis vaccines

Antigen composition	Method of PT inactivation	Manufacturer	Reference
PT, FHA, pili	formaldehyde	Centre for Applied Microbiological Research	Rutter *et al.*,1987
PT ,	hydrogen peroxide	National Institute of Child Health and Human Development	Sekura *et al.*, 1988
PT,FHA (1:1)	glutaraldehyde	Institute Merieux	Edwards *et al.*, 1989
PT PT, FHA, 69 KDa	genetic detoxification	Sclavo Research Centre	Podda *et al.*,1990

Based on Rappuoli *et al.*, 1991

Laboratory characterization

The *i.c.* mouse protection test of Kendrick *et al.* (1947) is recommended by WHO guidelines (WHO, 1979) for potency testing of WCPV. Acellular preparations are also evaluated by this same method (Sato *et al.*, 1984). Tiru *et al.* (1990) tested ten candidate acellular vaccine preparations with the ED_{50} for the Japanese monocomponent vaccine (JNIH-7) as reference. Four were comparable to JNIH-7, two preparations were one third as effective and four were of very low potency. This test has been suggested also as a way of following the consistency of preparations during production.

In the past, Preston and Stanbridge (1976), have questioned the relevance of infecting the brain of mice to test preparations made to prevent respiratory infection in children. Respiratory infection models may be more relevent to the testing of ACPV. In this case, vaccine potency is judged by its ability to protect colonization of the lungs after *i.n.* or aerosol challenge. This test was described by Sato *et al.* (1980). It requires large numbers of mice and *i.n.* infection can lead to variable results (Robinson and Funnel, 1992).

Other tests for monitoring the toxicity of ACPV preparations are the MWGT for LPS content and CHO cell clustering for active PT (Hewlett *et al.*, 1983). Japanese acellular vaccines are much reduced in endotoxin content and biological activities of PT compared with WCPV (Sato *et al.*, 1984).

Protective efficacy

The acellular vaccine produced by Pillemer *et al.* (1954) underwent testing in children in the MRC clinical trial reported in 1959 (MRC, 1959). Although the product protected against whooping cough, it was too toxic for further use. The 'Trisolgen' vaccine produced by Weihl *et al.* (1963) was licensed for use in the United States. It produced less local reactivity and showed less pyrogenicity than WCPV; however, the product did not come into general use.

In Japan, the death of two infants in 1974, 24 h after recieving DTP, caused much public concern (Sato *et al.*, 1984). The DTP vaccine was reassesssed, resulting in the ages of administration increasing from 3 months to 2 years in an effort to avoid adverse events. Small scale field trials were conducted with newly formulated Japanese acellular vaccines (described above) to test for side-effects and protective efficacy. From 1978 to 1981, 5,000 children aged under 48 months were tested in total (Sato *et al.*, 1984). with both ACPV and WCPV being administered. Attack rates after household exposure to pertussis were 82.8% in unvaccinated children, 14.3% in WCPV recipients and 11.1% in ACPV recipients. Adverse reactions were considerably lower for ACPV compared with WCPV. Since 1981, acellular vaccines have been used for routine immunizations in Japan in two year old children

In 1985, a group of public health service scientists travelled to Japan to monitor the development, production and use of these vaccines. Noble *et al.* (1987) reported since the introduction of ACPV's the incidence of pertussis was showing a downward trend in the country. These authors summarized the results of three Japanese studies with the Takeda-type vaccine administered under a four-dose shedule. When the results of the three studies were combined, efficacy was 87%.

Vaccination with WCPV was discontinued in Sweden in 1979, due to poor protection and fears about its safety (Fine and Clarkson, 1987). Two ACPVs formulated in Japan were investigated in a phase II clincal trial. One contained 7.5 µg protein nitrogen/ml each of PT and FHA (JNIH-6) and the other (especially formulated for the trial) was a monocomponent vaccine containing 12.0 µg protein nitrogen/ml of formalin-detoxified pertussis toxin. A double-blind randomized field trial was designed, involving a total of 3801 children of 5-11 months age. The placebo (vaccine solvent) was given to 954 children, 1419 received JNIH-6, 1428 received JNIH-7. Point estimates of vaccine efficacy against culture-confirmed pertussis with cough was 69% and 54% for JNIH-6 and JNIH-7 respectively. These workers also reported that antibodies to PT and FHA were similar in vaccinated children whether or not they

subsequently had pertussis. Thus, protection against whooping cough was not correlated with antibody levels. Fewer adverse reactions were reported in comparison with reported rates for WCPV. During the 17 to 19 months of follow up, five children died. Three children given JNIH-6 and the other two children were given JNIH-7. Causes of deaths were: *Haemophilus influenzae* type B meningitis, heroin intoxication with concomitant pneumonia, *Neisseria meningitidis* group B septicaemia, suspected pneumococcal septicaemia and a brain tumour. The results of the follow-up study (Storsaeter and Olin, 1992) indicated that the two-component vaccine containing pertussis toxoid and filamentous haemagglutinin provided better long-term protection against pertussis than monocomponent pertussis toxoid vaccine.

TREATMENT OF PROTEINS WITH TOXOIDING REAGENTS

Toxoiding of Bacterial Toxins

General

Many agents exist which have been used for the toxoiding of bacterial toxins, none however, appear to have been used as extensively as formaldehyde and glutaraldehyde. This section therefore focuses on these two agents with some information on carbodiimides which are of particular interest in this project.

The conversion of bacterial toxins to non-toxic products by detoxifying agents has been known since the late 19th century. Much of the early work was on the detoxification of diphtheria (DT) and tetanus toxins (TT). Behring and Kitasato,1890, (cited Parish, 1958) used iodine trichloride to reduce the toxicity of TT before the immunization of rabbits. Then subsequently in 1898, Salowski described toxoiding with formalin (cited by Parish, 1958). During the 1920's, the detoxification of DT and TT with formaldehyde was further investigated to prepare toxoids for human

immunization (Glenny *et al.*, 1923; Ramon, 1923). Widespread immunization with the first toxoids led to greatly decreased incidences of diphtheria and tetanus.

Glutaraldehyde was used as a toxoiding agent by Relyveld, 1978, although previously, it had been used widely for sterilization and disinfection purposes. In their review, Relyveld and Ben-Efraim (1983) reported that glutaraldehyde detoxification resulted in a more stable product than formaldehyde-detoxified proteins.

A large body of information exists for the wide variety of conditions employed in the treatment of bacterial toxins. Conditions differ for each toxin to be treated. Important variables of the toxoiding treatment are: length of exposure to the reagent, temperature, pH, concentrations of toxin and toxoiding agent and, with formaldehyde and glutaraldehyde, the addition of certain amino-acids usually lysine.

Effect of pH : French and Edsall (1945) reported the importance of pH during chemical reactions with formaldehyde. Detoxification of bacterial toxins is generally done at neutral or alkaline pH with both formaldehyde and glutaraldehyde. The effect of pH on the rate of inactivation and the effect on antigenicity was studied with diphtheria toxin (Eaton, 1937), shiga toxin (Dubos and Geiger, 1946), staphyloccocal enterotoxin B (Warren *et al.*, 1973), *Staphylococcus aureus* enterotoxins A and C1 (Warren *et al.*, 1975) and cholera toxin (Rapapport et al., 1974). These workers found that highly alkaline pH reduced toxicity to the greatest extent however, this was also accompanied by the greatest loss of antigenicity. Warren *et al.* (1973), and in a later study (Warren *et al.* 1975) analysed formaldehyde-treated staphyloccocal enterotoxins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) reporting that treatment at acidic or neutral pH caused the formation of high MW polymers which could not penetrate the gel, indicating intermolecular cross-linking; whereas, treatment at alkaline pH 9.5 caused the formation of a heterogeneous group of monomeric derivatives indicating intra-molecular cross-linking. During the toxoiding reaction, Rappaport *et al.* (1974) and Pollack and Prescott (1982) observed no major fluctuations of pH in the reaction mixture.

Effect of concentration : The ratio of formaldehyde or glutaraldehyde to protein concentrations are critical to the end products obtained. Too high a concentration of the toxoiding agent caused damage to the toxin and a loss or complete alteration of antigenicity (Eaton , 1937; Wadsworth *et al.*, 1938; Rappaport *et al.*, 1974; Relyveld *et al.*, 1978). Murphy *et al.* (1967) showed that treatment of TT with formaldehyde resulted in a shift from a mixture of monomers, dimers and polymers to the formation of large polymers. An increase in polymerization also occurred with increasing protein concentration when the formaldehyde was kept constant. Rappaport *et al.* (1974) reported that a high ratio of cholera toxin to glutaraldehyde gave insoluble products.

Dawson and Mauritzen (1969) examined the incorporation of radioactive formaldehyde during modification of TT. The rate of binding of formaldehyde increased with increasing concentrations resulting in an immediate loss of lethality (80%) of the toxin. Following this, slower detoxification was noted with binding of the agent becoming more gradual. In the classic study by Pittman *et al.* (1952), the effects of different agents on the detoxification of *B. pertussis* cells for vaccine production were evaluated. The subjection of cells to physical factors (heat, UV radiation) or chemical agents (phenol, merthiolate or formalin) were examined. Detoxification was monitored by the newly developed *i.c.* mouse protection test (ICMPT) for the evaluation of the protective potency of vaccines (Kendrick *et al.*, 1947). Heating to 56 ^{o}C for 30 min and then preservation of the bacteria with merthiolate yielded the best preparation due to stabilised potency. Formalin treatment reduced the toxicity but also caused some loss in potency. Clumping of bacteria was also noted. Kendrick *et al.* (1955) however, observed no loss of potency upon formaldehyde treatment.

Effect of temperature and time : Higher temperatures of 30-39 ^{o}C appear to reduce toxicity rapidly, whereas treatment at room temperature resulted in slower detoxification. However, antigenicity was better retained at lower temperatures. For example, this was shown by Wadsworth *et al.* (1938) with DT and formalin, and much later by Pollack and Prestcott (1982) for detoxification of *Pseudomonas aeruginosa*

exotoxin A with formaldehyde. The conversion of poorly immunogenic heat-labile toxin to an immunogenic toxoid by formaldehyde treatment in rabbits was reported by Livey and Wardlaw (1984). HLT underwent a rapid loss of toxicity at 37 °C and a slower loss at 4 °C.

The incubation period of preparations detoxified with glutaraldehyde were much shorter than with formaldehyde and varied according to the type of toxin used. Relyveld (1973), inactivated *B. pertussis* with glutaraldehyde at 0.0131 M the bacteria being completely killed after only one min exposure. Relyveld (1978), detoxified TT for 10 min whereas DT required 3 h before it became atoxic under the same conditions. Dubos and Geiger (1946), found Shiga toxin very difficult to detoxify even after several months incubation with formaldehyde. Light did not appear to be an important factor in the detoxification reaction since their's was the only study where the reaction was stated to have been carried out in the dark (Pollack and Prestcott, 1982).

Stability : Toxins inactivated by formaldehyde were noted to show a reversal to toxicity upon removal of the toxoiding agent by dialysis or after storage. The instability of formaldehyde-treated bacterial toxins was reported by Wadsworth *et al.*, 1938; Linggood *et al.* (1963) and Relyveld *et al.* (1978) for DT; by Relyveld *et al.* (1978) for TT; *in vivo* reversion of cholera toxin by Northrup and Chisari (1972); PT (Sato *et al.*, 1974); and Pseudomonas exotoxin A (Cryz *et al.*, 1981). Also, Relyveld *et al.* (1978), reported that detoxification of DT and TT with glutaraldehyde, instead of formaldehyde, resulted in a stable product which showed no reversal upon storage, yet retained antigenicity.

Hewlett *et al.* (1977), reported that *B. pertussis* whole-cell vaccines contained pertussis toxin (PT) and lipopolysaccharide (LPS) which had not been inactivated by the formaldehyde treatment used to kill the cells. Work by Gupta *et al.* (1987a), Gupta *et al.* (1987b), Iiada and Horiuchi (1987); and Gupta *et al.* (1988) investigated glutaraldehyde as a detoxifying agent for *B. pertussis* cells which would result in a

more stable, completely inactivated product. Iida and Horiuchi (1987) found treatment with 10 mM glutaraldehyde at 37 °C for 30 min was sufficient to produce a marked drop in toxicity with virtually no loss in potency. Arya *et al.* (1989) however, urged caution when using glutaraldehyde as a detoxifying agent for this purpose since impurities in the glutaraldehyde solution were noted to give different reactions with amino-acids. Furthermore, they reported that detoxification of PT occurred but glutaraldehyde did not detoxify the LPS component.

Bunney (1931), added amino-acids, peptones or proteins to the detoxification mixture, stating that they were essential to prevent damage to the toxoid during the long periods of incubation required. Eaton (1937) suggested the addition of peptones may have had a protective effect on the antigenic properties of the toxin. Linggood *et al.* (1963) reported that lysine stabilised formaldehyde-treated proteins and helped retention of antigenicity. Relyveld (1978), found that addition of lysine neutralised excess glutaraldehyde and stopped its further interaction with DT and TT.

Immunogenicity : Glutaraldehyde-toxoided preparations were reported to elicit higher antibody titres than formaldehyde toxoids (Relyveld, 1978; Rappaport *et al.*, 1974). Relyveld (1978) observed DT alone was more immunogenic than a formalin toxoid of DT adsorbed to $Al(OH)_3$. In contrast, Pavlovskis *et al.* (1981) reported that a glutaraldehyde toxoid of *P. aeruginosa* exotoxin A was not as immunogenic as the formaldehyde toxoid. The immunogenicity of this toxin after treatment with formaldehyde was low anyway according to Cryz *et al.* (1981) and Cryz and Iglewski (1983). Porro *et al.* (1980) found diphtheria toxoid and a non-toxic mutant of DT (crm197) had equivalent immunogenicity after formalin treatment. Enhanced immunogenicity was not always due to polymerization of the toxin molecule. Relyveld (1978) noted the dimeric fraction of glutaraldehyde-treated DT was a better immunogen than the monomeric or the polymeric fraction. Stearne and Birkbeck (1980) found the non-immunogenic delta haemolysin of *S. aureus* to become highly immunogenic after formaldehyde treatment but no polymerization was observed.

The detoxification of bacterial toxins with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) has been reported only in the detoxification of CT by Lonnroth and Holmgren (1975) and for *Escherichia coli* heat-labile enterotoxin by Klipstein *et al.* (1982), aside from the studies of Christodoulides *et al.* (1987, 1989) with PT. For CT, there was no loss in toxicity or compromized antigenicity with EDAC treatment whereas, in comparison, glutaraldehyde-treatment decreased toxicity and affected antibody-binding capacity. *E. coli* enterotoxin exhibited rapid loss of activity and retained antigenicity. A mixture of PT and FHA not only lost toxicity, but also showed enhancement of immunogenicity after toxoiding with EDAC (Christodoulides *et al.* , 1987).

Pertussis toxin

Matsui and Kuwajima (1959), reported treatment of HSF with formalin led to complete detoxification, as monitored by loss of histamine-sensitizing activity. Mice immunized with this toxoid were protected from sensitization with *B. pertussis* culture supernate. In 1966, Munoz and Hestekin detoxified soluble extracts of *B. pertussis* containing histamine- sensitizing and protective activity. These workers reported protective activity as more resistant to inactivation by formaldehyde than the HSF activity, and suggested that formaldehyde treatment altered the molecule in such a way as to destroy HSF activity but retain protective activity. Sato *et al.* (1974) and later, Sato *et al.* (1984) and Sato and Sato (1984), reported detoxification of PT with formaldehyde for the production of acellular vaccines. Sato *et al.* (1974) carried out Ouchterlony gel diffusion of toxoided PT and found a line of complete identity to native PT. Sato and Sato (1984) showed that formaldehyde-treated pertussis toxin had lost lymphocytosis-promoting activity in the mouse at 10 μg/mouse. Antigenicity was not impaired, as shown by the ability of the pertussis toxoid to bind to its receptor haptoglobin in ELISA.

Glutaraldehyde treatment of PT was used by Munoz *et al.* (1981a), Munoz *et al.* (1981b), Robinson and Hawkins (1983), Robinson and Irons (1983) and Oda *et al.* (1984), for investigating the protective activity of inactivated PT. Munoz *et al.* (1981a, 1981b) found the histamine-sensitizing and haemagglutinating activities of treated PT were reduced markedly (25% and 4% of the original values respectively). Also, glutaraldehyde-treated pertussigen did not lose its antigenicity, as it actively precipitated with antibody. Toxicity was much reduced since 10 μg/mouse was atoxic from the previously toxic 2 μg/mouse of native PT .

Detoxification of PT has also been carried out with hydrogen peroxide (Sekura *et al.*, 1986), with EDAC (Christodoulides *et al.*, 1987), and tetranitromethane (Winberry *et al.*, 1988). Trinitrobenzenesulphonic acid (TNBS), potassium iodide and chlorane were used by Fish *et al.* (1984). While elucidating mechanisms of PT action, a number of agents which modified lysine residues were used by Nogimori *et al.* (1984a, 1984b). Genetic modification of two amino acids (Arg^{9} and Glu^{129}) resulted in an enzymatically-inactivated PT molecule (Pizza *et al.*, 1989 and Nencioni *et al.*, 1990).

Information on the alteration of protein molecules by formaldehyde, glutaraldehyde and carbodiimides must begin with a discussion on the chemical groups of proteins which are susceptible to attack by these agents. A short account of their interactions with proteins follows.

Reactions of Proteins with Formaldehyde

The reactions of formaldehyde with proteins are numerous and complex as reviewed by French and Edsall, 1945; Fraenkel-Conrat and Olcott, 1948a, 1948b; Fraenkel-Conrat and Mecham, 1949. Formaldehyde (HCHO) is commercially available as formalin, a solution which contains 37% (w/v) formaldehyde and small amounts of methanol, in water. The methanol is added as a stabilizer to inhibit the formation of

polymers. In aqueous solutions more than 99.9% of formaldehyde exists in its hydrated form as $CH_2(OH)_2$. Formaldehyde adds to compounds containing an active hydrogen atom resulting in the formation of hydroxymethyl compounds (Equation 1).

Equation 1 :

$$R\text{-}H + CH_2O \rightleftharpoons R\text{-}CH_2(OH)$$

If the hydrated form of formaldehyde is present, then the hydroxymethyl compound is produced by a condensation reaction, with the liberation of water (Equation 2)

Equation 2 :

$$R\text{-}H + CH_2(OH)_2 \rightleftharpoons R\text{-}CH_2(OH) + H_2O$$

hydroxymethyl compound

The amino groups of proteins react rapidly and reversibly with two moles of formaldehyde in neutral or alkaline solutions to give methylol amines (Equation 3).

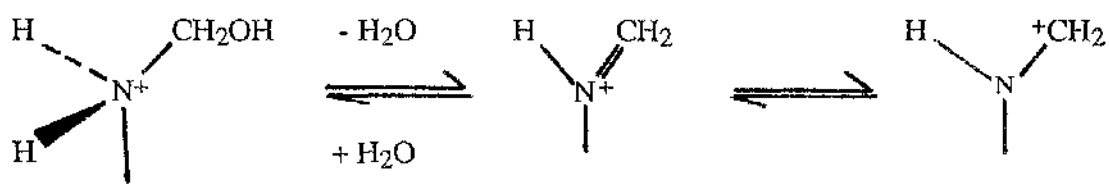
Equation 3 :

$$R\text{-}NH_2 + HCHO \rightleftharpoons R\text{-}NHCH_2OH + HCHO \rightleftharpoons R\text{-}N(CH_2(OH)_2$$

methylol amines

Formaldehyde reacts with adjacent amino-acid side chains through electrophilic immonium cations (Equation 4) such as tyrosine, tryptophan, histidine, asparagine and cysteine residues (French and Edsall, 1945) resulting in additional rings being formed.

Equation 4 :



When steric relationships are favourable, formaldehyde can react with two groups to form methylene bridges (Equation 5)

Equation 5 :

$$R\text{-}CH_2(OH) + H\text{-}R' \rightleftharpoons R\text{-}CH_2\text{-}R' + H_2O$$

Numerous groups found in amino-acids, peptides and proteins are capable of undergoing addition and condensation reactions with formaldehyde. The epsilon amino group of lysine residues have a much stronger tendency to associate with formaldehyde. This rapid primary reaction of formaldehyde with lysine residues results in the formation of Schiff bases (Equation 6) which can then undergo methylene bridge formation with a variety of groups.

Equation 6 :

$$R\text{-}NH_2 + HCHO \rightleftharpoons R\text{-}NHCH_2OH + H^+ \rightleftharpoons R\text{-}NH^+{=}\ CH_2 + H_2O$$

lysine Schiff base

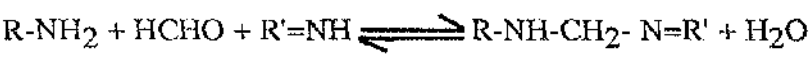
Figure 5 shows the reaction of formaldehyde-mediated methylene bridge formation with some of the functional groups found in amino-acids. The reaction of thiols is much more rapid than its reaction with amines, since the sulfhydryl group is a better nucleophile. Only one molecule of formaldehyde, however, can react with one sulfhydryl.

Fraenkel-Conrat and Mecham (1949), showed by means of molecular weight analysis and osmotic pressure measurements that methylene bridges were indeed being formed between two or more protein molecules. Thus, bond formation can take place intra-molecularly, forming ring structures, or intermolecularly with the formation of molecular aggregates. Polyoxymethylene chains and bridges could also result when more formaldehyde molecules entered the reaction.

During the detoxification of bacterial toxins the cross-linking reaction led to polymerization of toxin molecules, and was regarded unfavourably, as reversal to

Figure 5 : Modification of amino-acids by formaldehyde

The imino group e.g. arginine

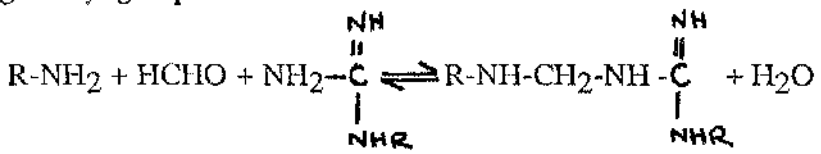


$$R\text{-}NH_2 + HCHO + R'\text{=}NH \rightleftharpoons R\text{-}NH\text{-}CH_2\text{-}\ N\text{=}R' + H_2O$$

The amide group e.g. asparagine, glutamine

$$R\text{-}NH_2 + HCHO + NH_2COR' \rightleftharpoons R\text{-}NH\text{-}CH_2\text{-}\ NHCOR' + H_2O$$

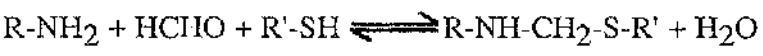
The guanidyl group



The hydroxyl group e.g. serine

$$R\text{-}NH_2 + HCHO + R'\text{-}OH \rightleftharpoons R\text{-}NH\text{-}CH_2\text{-}O\text{-}R' + H_2O$$

The sulfhydryl group e.g. cysteine



$$R\text{-}NH_2 + HCHO + R'\text{-}SH \rightleftharpoons R\text{-}NH\text{-}CH_2\text{-}S\text{-}R' + H_2O$$

toxicity could occur (Murphy, 1967). Low concentrations of formaldehyde gave incomplete toxoiding, therefore Linggood *et al.* (1963) examined the effects of the addition of various amino-acids. They found the best preparation was obtained by the addition of a small amount of lysine. They suggested instead of taking the reaction to the point of methylene bridge formation between toxin molecules, the addition of lysine disrupted polymerization by the production of highly stable antigenic side chains. (Equation 7).

Equation 7 :

$$\text{Toxin-H} + \text{HCHO} + \text{H}_2\text{N.CH.(COOH).(CH}_2)_4\text{.NH}_2 \rightleftharpoons \text{Toxin.CH}_2\text{NH.CH.(COOH).(CH}_2)_4\text{.NH}_2 + \text{H}_2\text{O}$$

toxin-lysine side chains

Formaldehyde seems to have a stabilising effect on proteins. As far back as 1896, Blum (cited French and Edsall, 1945) noticed that treatment of egg-albumin with formaldehyde rendered the egg-albumin resistant to coagulation. Collagen and casein also became more resistant to the action of trypsin and other proteolytic enzymes. Habeeb (1969) showed increased resistance of formalinized BSA to unfolding with urea, indicating the formation of intra-molecular bonds. Formaldehyde-mediated modification of proteins was found to be dependent on a number of important factors such as temperature, pH and the concentration of the agent utilised during the reaction. The chemical nature of the protein itself contributes to the reaction products obtained.

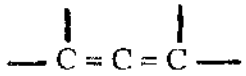
Reactions of Proteins with Glutaraldehyde

Glutaraldehyde is a bifunctional aldehyde. Its chemical formula is $CHO(CH_2)_3CHO$. The mechanism of cross-link formation is analogous to formaldehyde. Richard and Knowles (1968) suggested that the reactive species present in aqueous solutions of glutaraldehyde was a condensation polymer. However, Korn *et al.* (1972) repeated the analysis of glutaraldehyde in water and found solutions to consist of free glutaraldehyde, the cyclic hemiacetal of its hydrate, and oligomers of this. These workers also reported that glutaraldehyde reacted with the epsilon amino groups of lysine residues in the ratio of four moles of glutaraldehyde per mole lysine. Cheung and Nimni (1982) elucidated that glutaraldehyde underwent a number of condensation polymerization reactions with the epsilon groups of lysine residues to form, like formaldehyde, Schiff-based derived products.

Habeeb and Hiramoto (1968) investigated the reaction of glutaraldehyde with amino-acids and peptides. They found modification of the N-terminal group of some peptides, the sulfhydryl group of cysteine residues and partial reactivity with the phenolic and the imidazole of tyrosine and histidine residues. Habeeb (1969) studied glutaraldehyde-treated bovine serum albumin (BSA). This protein was found to have undergone more extensive modification of free amino groups with the formation of molecular cross-links. The conformation of the molecule was altered, as shown by increased reduction of disulphide linkages compared to native BSA. Antigenicity of the molecule was also affected. New antigenic determinants created by glutaraldehyde treatment of the protein were apparent. Treatment with glutaraldehyde gave a product which was irreversibly modified and resistant to breakdown with urea, semicarbazide, wide ranges of pH and ionic strength and temperature (Richard and Knowles, 1968).

Reactions of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC)

Carbodiimides are one of the several classes of unsaturated compounds belonging to the heterocumulenes family. This family contains compounds based on an allene structure and are characterized as such because of the presence of twinned double bonds.



The general formula of carbodiimides is denoted by RN = C = NR' where R and R' may be either aliphatic or aromatic groups. A commonly used carbodiimide is the water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and its formula is shown below.

$$CH_3CH_2\text{-}N{=}C{=}N\text{-}CH_2CH_2\ CH_2\ N(CH_3)_2$$

Carbodiimide chemistry has been reviewed in detail by Khorana (1953), Kurzer and Douraghi-Zadeh (1967) and Williams and Ibrahim (1981).

Being unsaturated compounds, carbodiimides undergo addition reactions readily. Primarily addition of a carbodiimide to a protein results in carboxyl group modification (Figure 6). Reactivity of carboxyl groups increases with increasing acidity (Williams and Ibrahim, 1981). The reaction begins with the attachment of a proton to the carbodiimide. This is followed by attack of the carboxyl group to form the O-acylisourea. This can rearrange to form the N-acylurea. Alternatively, attachment of a second molecule results in cation formation. The subsequent attack of an acid anion forms a N,N'- disubstituted urea and the acid anhydride. Undesirable side reactions can also take place via the rearrangement of the O-acylisourea, being quite apart from the major side product of the N-acylurea. These include imide formation (Riehm and

Scherega, 1966), formation of acid anhydride cross-links between adjacent carboxyl groups and addition of the carbodiimide on the protein carboxyl group as a substituted amide with isocyanate formation (Williams and Ibrahim, 1981). Addition of an exogenous nucleophile has been used to suppress the rate of side reactions during the analysis of carboxyl group content of enzymes (Carraway and Koshland, 1972).

Reactions of carbodiimides may occur with a number of functional groups. Modification of the phenolic groups of tyrosine (Carraway and Koshland, 1968); sulfhydryl groups of cysteine residues, (Carraway and Triplett, 1970); hydroxyl groups of serine residues (Banks, Blossey and Shafer, 1969) and imidazole groups (Williams and Ibrahim, 1981) have been shown. Carraway and Triplett (1970), found the rate of modification of sulfhydryl groups by ethyldimethylcarbodiimide and carboxyl groups to be equivalent, whereas tyrosine modification was slower. Regeneration of tyrosine residues was possible using hydroxylamine (Carraway and Koshland, 1968); however regeneration of sulfhydryl groups was not obtained under conditions which would preserve protein conformation (Carraway and Triplett, 1970). Carraway and Koshland (1972), reported protein amino groups were not reactive at the lower pH values used in cardodiimide-nucleophile reactions.

Modification with 1-ethyl-3-(3-morpholinopropyl)carbodiimide was reported to give inter-molecularly linked thermostable lysozyme and alpha-chymotrypsin (Hattori, 1970). Pedemonte and Kaplan (1985) found intramolecular bond formation occurred through reaction with a carboxyl group and a side chain amine (acting as an endogenous nucleophile), to inhibit the activity of Na^+-K^+ATP-ase from dog kidney.

In 1955, Sheehan and Hess discovered peptide bond formation could be affected by condensation reactions with dicyclohexylcarbodiimide (DCCD). This procedure involved the addition of the carbodiimide to a solution of N-protected amino-acids or peptides. The mechanism of peptide bond formation with carbodiimide is shown in Figure 7. After protonation of the carbodiimide, reaction with a carboxylate anion yields the O-acylisourea. This can rearrange to form the N-acylurea, react with

an amino-acid ester to form peptide linkage or react with another carboxylate anion to produce urea and acid anhydride. The latter participates in further peptide formation. In the review by Rich and Singh, (1979), the formation of side products during peptide synthesis were described. Racemization of amino-acid esters resulted in 5(4H)-oxazolone production, activation of glutamine or asparaginyl carboxyl groups led to nitrile formation and the N-acylurea gave decreased yields. Again, addition of nucleophiles reduced the rate of side reactions. Nucleotide synthesis has also been reported but will not be described here (see reviews for further information).

Applications of carbodiimides

Most studies using carbodiimides to modify proteins, have used an added nucleophile to trap the activated carboxyl groups and to suppress the formation of side reactions. The use of a nucleophile with a denaturant allows quantitative evaluation of the amount of carboxyl groups in a protein. The number of free carboxyl groups may be determined by the difference in the amount of nucleophile present in the reacted protein and the unreacted protein. A common nucleophile is glycine (reviewed by Carraway and Koshland, 1971). This method was applied to a number of proteins including ribonuclease, by Riehm and Scherega (1966) and to lysozyme (Carraway and Koshland, 1972).

'Buried' carboxyl groups may also be determined using a modification of the above procedure. Reaction of the carbodiimide and the nucleophile without the addition of a denaturent results in incorporation of the nucleophile. After amino-acid analysis, the denaturent is added with radioactive nucleophile and more carbodiimide. In this way, any buried residues appearing after unfolding and exposure, are radioactively labelled. This method was applied to alpha-chymotrypsin with the identification of two buried carboxyl groups (Abeto *et al.*, 1969).

Studies of carboxyl group modification and correlated loss of enzyme activity, have also been made in attempts to assess the importance of carboxyls in binding and

Figure 6 : Protein carboxyl group modification by carbodiimide.

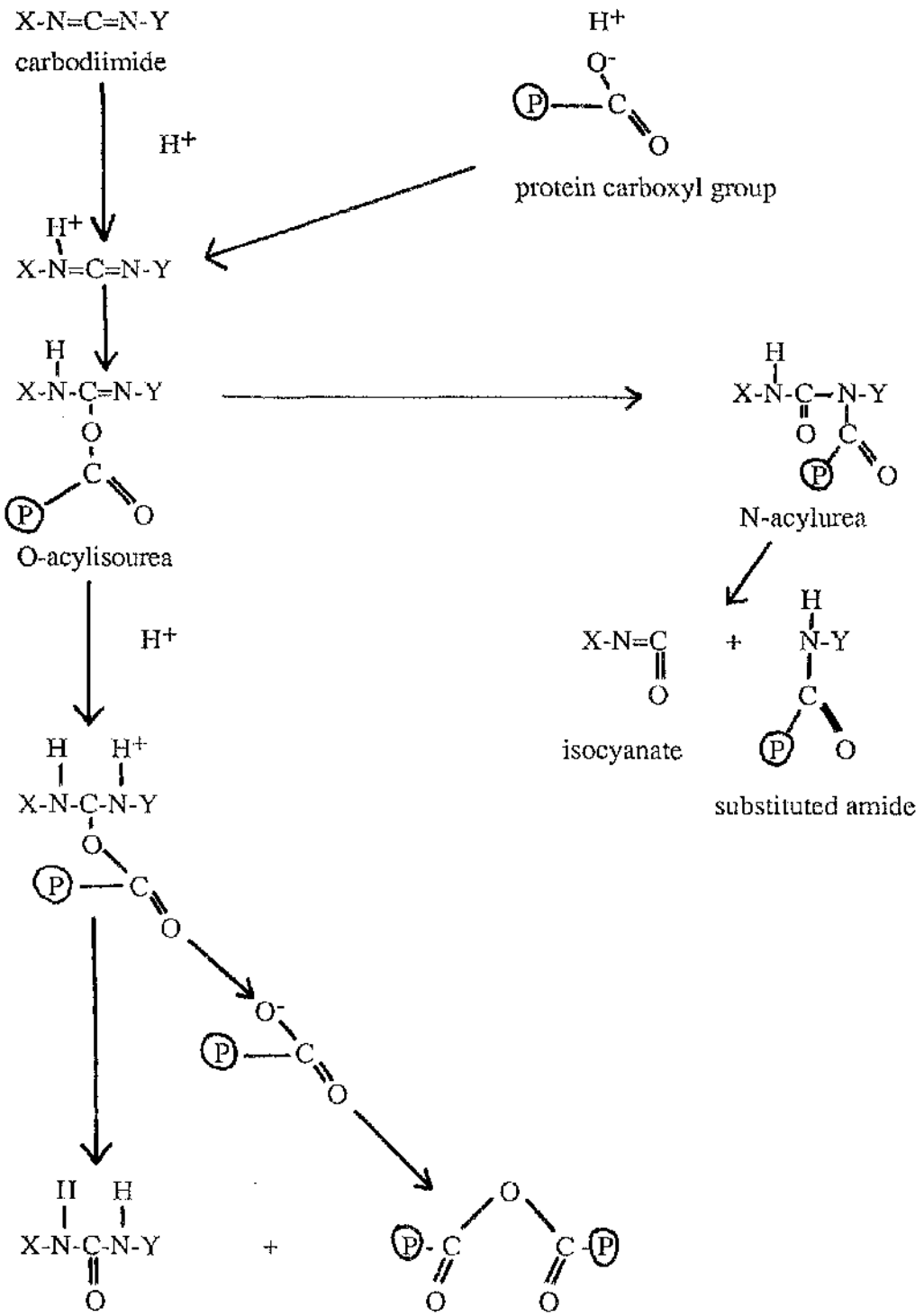
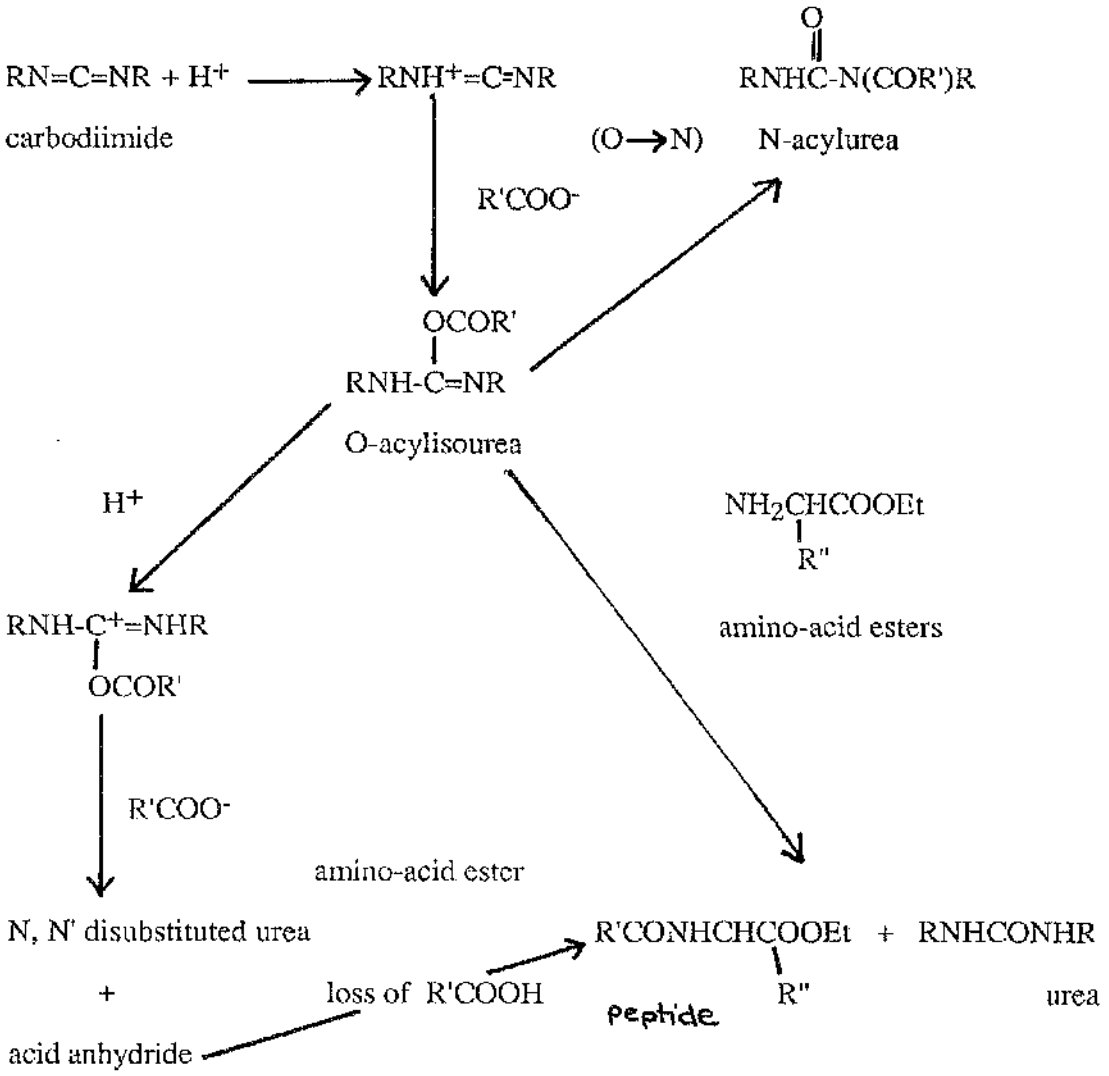


Figure 7 : Mechanism of carbodiimide-mediated peptide bond formation (from Kurzer and Douraghi-Zadeh, 1967)



catalytic groups (Toner-Webb *et al.*, 1987; Yoshida *et al.*, 1982; Parsonage *et al.*, 1988).

In the review by Bauminger and Wilchek (1980), carbodiimides were stated to be the agents of choice for the preparation of immunizing conjugates. Weakly immunogenic or non-immunogenic compounds were linked to larger carrier proteins to enhance their antibody-evoking potential. The conjugation required two groups - an amino group and a carboxyl group. Carboxyl groups were, in the majority of cases, contributed by the hapten, whereas the amino groups were supplied from the lysyl or alanyl residues of the protein carrier. Various proteins ranging from hormones to peptide fragments have been conjugated by this method (Arnon and Sela, 1969; Koch *et al.*, 1973). Table 5 however lists a selection of references which used EDAC in the preparation of conjugates for bacterial vaccines.

Table 5 : Conjugates prepared for bacterial vaccines with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC)

Hapten	Protein carrier	Reference
E. coli heat-stable enterotoxin	porcine IgG	Klipstein *et al.*, 1981
Neisseria meningitidis group C polysaccharide	tetanus toxoid	Beuvery *et al.*, 1983
E. coli ST enterotoxin	non-toxic native B-subunit of labile-toxin	Klipstein *et al.*, 1985
Synthetic cholera toxin B-subunit peptide squences	tetanus toxoid	Jacob *et al.*, 1986
Synthetic shiga toxin B-chain peptide sequences	tetanus toxoid or a synthetic carrier	Harrari *et al.*, 1988
E. coli polysaccharide	cholera toxin or *P. aeruginosa toxin A*	Cryz *et al.*, 1990

OBJECT OF RESEARCH

This study stems from the report of Christodoulides *et al.* (1987), who investigated the toxoiding of antigens of *B. pertussis* to produce an acellular pertussis vaccine. These investigations found that a mixture of pertussis toxin and filamentous haemagglutinin was not only detoxified by treatment with a water-soluble carbodiimide, EDAC, but also that this preparation had enhanced immunogenicity. The purpose of the present study was:

1. to confirm or refute the adjuvanting effect of EDAC on *B. pertussis* antigens, both as PT:FHA mixtures and with the two antigens purified independently;
2. to investigate the specificity of this EDAC-adjuvant effect by treatment of two model antigens, ovalbumin and lysozyme, with EDAC;
3. to compare EDAC-treated antigens with other toxoiding agents, namely formaldehyde and glutaraldehyde for immunogenicity.

MATERIALS AND METHODS

Bacterial Culture

Strains

The four strains of *B. pertussis* used in this study were from the departmental culture collection. Strains 353 and 357 were obtained from Dr. A.A. Weiss, University of Virginia School of Medicine, Charlottesville, Virginia, USA, and were transposon mutants lacking respectively FHA and PT. Strain 77 /18319 was a phase I isolate supplied by Dr. R. Fallon, Ruchill Hospital, Glasgow. Strain 18323 was a phase I organism highly virulent in the ICMPT and provided by Dr. F. Sheffield of the National Institute for Biological Standards and Control (NIBSC), Holly Hill, Hampstead, London.

The strains were stored either as freeze-dried cultures in vacuum-sealed glass ampoules kept at room temperature, or at -70 °C in 1% (w/v) casamino acids and glycerol (Appendix 1).

Culture media

Stock cultures : With strain 77/18319, stock cultures were started from freeze-dried or frozen material by plating on to Bordet-Gengou agar (BG) prepared as described in Appendix 1. With strain 353 and 357, kanamycin (50 µg/ ml) from Sigma Chemical Co., Fancy Rd., Poole, Dorset, was added to the medium. This was to maintain the mutant status of these strains.

Batch cultures : A modification of the CL liquid medium of Imaizumi *et. al.* (1983) was used for large-scale culture of all three strains of *B. pertussis*. This medium contained heptakis (2,6-0-dimethyl)-ß-cyclodextrin (MeßCD) which was obtained from Teijin Ltd., Chiyoda-ku Tokyo, Japan. The formula of this medium is given in Appendix 1.

Growth from stock cultures

Frozen cultures were thawed at RT for 10-15 min; freeze-dried cultures were resuspended in a 1% (w/v) solution of casamino acids (Appendix 1) and kept at RT for 20 min before plating. One or two loopfuls of suspension were streaked on four BG plates and incubated in a humidified box for 48 h at 37 °C so as to yield single colonies.

Confirmation of culture purity was obtained by visual inspection and Gram staining. Loopfuls of the growth from these plates were used to restreak fresh BG plates and again incubated for a further 48 h at 37 °C in a humidified box to provide the inoculum for batch cultures.

Growth of batch cultures

Large loopfuls of growth from BG plates were used to inoculate 1 L portions of CL medium in 2 L dimpled Ehrlenmeyer flasks. Cultures were incubated for 48 h at 37 °C and aerated by shaking at 150 rpm on a rotary shaker (L.H Engineering Co. Ltd., Stoke Poges, Bucks., England).

At the end of the incubation, culture purity was checked by Gram staining, after which the bacterial cells were removed by centrifugation in a Sorvall RC-5B centrifuge at 9000 rpm for 30 min at 4 °C. The supernate was collected and centrifuged again for further clarification prior to the extraction of PT or FHA.

Preparation of sonicated *B. pertussis*

For some immunization experiments, *B. pertussis* strain 18323 was prepared as a sonicated suspension of heat-killed bacteria.

Extraction of PT and FHA

A modification of the method of Sekura *et al.* (1983) was used to extract PT, FHA and a mixture of the two proteins from the culture supernates of appropriate *B. pertussis* strains, viz 353 for PT, 357 for FHA and 77 /18319 for the mixed-antigen preparation respectively.

Culture supernates were adjusted to pH 6.0 at RT with 2.5 M HCl before the addition of thiomersal to 0.01% (w/v) final concentration, as preservative, and 1 ml/L 5 mM phenylmethyl-sulphonylfluoride (Sigma) as a protease inhibitor. To every one L of culture, 10 ml Blue Sepharose CL-6B gel slurry (Pharmacia LKB, Uppsala, Sweden) was then added to absorb the PT and/or FHA. The mixture was stirred overnight at 4 °C for 24 h, or for 48 h if stirring had inadvertently been interrupted. The following steps were carried out at RT. The mixture was poured into a G1 sintered glass funnel (Gallenkamp, Loughborough, Leicestershire) to retain the gel. The filtrate was discarded. Without delay, the gel was washed with 0.05 M Tris-HCl, pH 8.0, and transferred to a 2.6 x 35 cm chromatography column (LKB 2137, Pharmacia LKB, Uppsala, Sweden). The gel was allowed to settle and the packed column was washed for 2.5 h with 0.05 M Tris-HCl, pH 8.0, applied with a peristaltic pump, to remove unbound material. Elution of the bound protein was done with 0.05 M Tris-HCl, pH 8.0 containing 1.0 M NaCl and 10 ml fractions were collected. The fractions were monitored for absorbancy at 280 nm. Fractions with absorbancy ≥0.2 were pooled, dialysed against PBS, pH 7.3 overnight at 4 °C, filter-sterilized through a 0.45 µm membrane (Gelman Sciences) and stored frozen at -20 °C until required.

To regenerate the gel at the end of each run, it was washed with 0.1 M Tris-HCl, pH 8.0 containing 0.5 M NaCl and 6 M urea to remove any strongly bound protein. After this, the gel was washed first with 0.1 M Tris-HCl, pH 8.5 containing 0.5 M NaCl, and next with 0.1 M sodium acetate, pH 4.5 containing 0.5 M NaCl. This alternate washing with low and high pH buffers was repeated twice more before a final

wash with 0.05 M Tris-HCl, pH 8.0 containing 0.01% w/v thiomersal was applied. Finally, the gel was removed and stored at 4 °C for reuse. Appendix 2 details the preparation of the buffers used in this procedure.

Toxoiding of Antigens

Treatment with EDAC

Essentially, the method of Christodoulides *et al.* (1987), was followed for the use of 1-ethyl-3,3-dimethylaminopropyl carbodiimide (EDAC, MW 191.7) to toxoid PT and to treat the non-toxic proteins FHA, ovalbumin (OA; Sigma) and lysozyme (LZ; Sigma). EDAC solutions were freshly made, typically at 42 mM so that after addition of an equal volume of the protein solution, a final concentration of 21 mM was obtained.

Treatment with EDAC was done at 37 °C with the protein and the EDAC dissolved in 20 mM sodium phosphate buffer, pH 5.0 with or without 0.5 M NaCl. PT and PT:FHA mixtures were dialysed at 4 °C overnight against the buffer (containing NaCl), whereas with FHA alone, the NaCl was omitted. For toxoiding PT or PT:FHA mixtures, 0.5 M NaCl was included in the EDAC stock solution, whereas with FHA alone it was omitted. For treatment of OA and LZ with EDAC, the crystalline proteins were dissolved directly in the phosphate buffer without NaCl, as was the EDAC.

With PT or PT/FHA the toxoiding mixtures contained 50 µg/ml protein and EDAC at 21 mM. With FHA there was 250 µg/ml total protein and an EDAC concentration ranging from 2.3 to 50 mM. For the treatment of LZ and OA, much higher concentrations of protein (4 mg/ml) were used in conjunction with 11 to 800 mM EDAC. Mixtures of protein and EDAC were incubated at 37 °C for 24 h without stirring and were then dialysed for three days at 4 °C against daily changes of PBS, pH 7.3 containing thiomersal at 0.01% (w/v) final concentration.

Treatment with formaldehyde

The two bacterial proteins and LZ, but not OA, were treated with formaldehyde by a combination and modification of the methods of Linggood *et al.* (1963), Porro *et al.* (1980) and Sato and Sato (1984). In particular, the usage of lysine was according to Linggood *et al.* (1963), the formaldehyde concentrations were taken from Porro *et al.* (1980), while the treatment times and temperatures were as reported by Sato *et al.* (1984). To avoid uncertainty, the concentrations are expressed both as mM *formaldehyde* and as % (v/v) *formalin* which is a 37% (w/v) solution of formaldehyde (Sigma).

Toxoiding of PT or of PT:FHA mixtures at 50 μg/ml final protein concentration was done in 20 mM sodium phosphate containing 0.5 M NaCl, pH 7.4. Freshly diluted formalin at 0.8% (v/v) was added to give a final concentration of 0.2% (v/v) formalin, equivalent to 25 mM formaldehyde. L-lysine (Sigma) at 20 mM was also included in the mixture unless otherwise stated. The mixture was incubated at 37 °C for one week, after which it was dialysed against PBS, pH 7.3 containing 0.01% (w/v) thiomersal (final concentration) for one week, with daily changes of buffer, to remove the formaldehyde and the lysine. The final product was stored at 4 °C.

The toxoiding of FHA was done with a range of formaldehyde concentrations of between 3.1 to 25 mM (with or without lysine at the corresponding concentration). The final concentration of protein in the reaction mixture was 250 μg/ml. The treatment was for 24 or 48 h at 4 °C, 22 °C or 37 °C in buffer without NaCl. Dialysis of the toxoided sample and subsequent storage was as for PT and PT:FHA mixtures.

Several different concentrations of both formaldehyde and lysine were used to treat LZ (final concentration 4 mg/ml) in 20 mM sodium phosphate, pH 7.4. Incubation was for 1 week at 37 °C followed by dialysis for three days with daily changes of buffer.

Treatment with glutaraldehyde

The two bacterial proteins and LZ, but not OA, were treated with glutaraldehyde under the conditions described by Relyveld (1978) and Munoz *et al.* (1981).

PT and PT/FHA mixtures at 50 μg/ml were dialysed overnight at 4 °C against 20 mM sodium phosphate buffer, pH 7.4 containing 0.5 M NaCl. Glutaraldehyde (Fisons) was diluted from 25% (w/v) as purchased to 2.5% (v/v) which was then added in appropriate amounts so as to give a concentration of 0.025% (v/v) or 2.5 mM glutaraldehyde when mixed with protein. Incubation was for 2 h at 37 °C, after which lysine was added to 10 mM with further incubation for 15 min to terminate the reaction. This mixture was dialysed against PBS, pH 7.3 containing 0.01% (w/v) thiomersal for three days, with daily changes of buffer, to remove the glutaraldehyde and the lysine. The final product was stored at 4 °C.

FHA (250 μg/ml) was treated exactly as above except that the buffer used throughout the procedure did not contain 0.5M NaCl and the time of incubation varied from 15-120 min at 37 °C with or without the addition of lysine at 10 mM.

LZ was treated in a similar manner except that the final concentration of protein was 4 mg/ml and the glutaraldehyde varied over the range 0.006 to 0.025% (v/v). This range corresponds to 0.6-2.5 mM. The treatment time was 2 h. The effect of lysine was explored by adding it in four-fold molar excess over that of glutaraldehyde. At the end of the 37 °C treatment, the mixture was placed in visking tubing and dialysed against PBS, pH 7.3 containing 0.01% thimerosal for three days, with daily changes of buffer. The final product was stored at 4 °C.

In Vivo Procedures

Mice

Male and female mice obtained as CD-1 (caesarean-derived animals) of the Ham/ICR strain, Charles River (UK) Ltd., Manston Rd., Margate, Kent, were used. They were maintained as a randomly-bred colony and were provided with food and water *ad lib*.

Immunogenicity of proteins

In a typical experiment, 60 to 100 mice of ages ranging from 6 to 8 weeks (exceptionally, up to 16 weeks) and of both sexes, were arranged in cages containing 12 to 16 animals of the same sex. The allocation of mice to cages was done in a randomized block fashion, each cage being taken as a block, with one recipient for each dose of each antigen. By having 5 such cages, each dose of each antigen yielded five replicate sera. Appendix 3 shows the detailed layout of a typical experiment in which 4 preparations were each tested at 3 doses, therefore requiring 12 mice per cage.

Injections were administered intra-peritoneally (*i.p.*) as a single shot of 0.5 ml of antigen preparation. To do this, for example with three antigens each at four doses, a set of twelve 2.5 ml syringes was loaded with the injection fluids and one 0.5 ml injection given from each syringe to the animals captured without preselection. The individual animals in each cage were identified by a system of positional markings with indelible dyes on the fur.

A period of three weeks was allowed for the expression of a primary immune response before the animals were killed and blood collected. This was done by transferring the mice to a closed chamber containing carbon dioxide for asphyxiation. The dead animals were pinned out on a board and blood obtained by cardiac puncture with a fresh one ml syringe and a 25 gauge 5/8 inch needle for each animal. The blood was allowed to clot either at room temparature for 3-4 h, or overnight at 4 ^{o}C, before being spun for 30 min at 3000 rpm and 4 ^{o}C in a MSE Mistral 6L centrifuge with a

swing-out rotor. Serum was collected with a pasteur pipette, taking care not to disturb the pelleted red cells, and stored frozen at -20 ^{o}C.

Histamine-sensitizing activity (HSA)

Procedures very similar to the above as regards allocation of animals to cages were used for assaying the HSA in prepartions of *B. pertussis*. Thus, groups of two or four mice (>6 weeks old) were injected *i.p.* with 0.5 ml graded doses of the test samples in a randomized block layout. After a period of five days, each mouse was challenged *i.p.* with 3 mg histamine dihydrochloride dissolved in PBS. The mice were observed for 3 h at which time the number of deaths were recorded. The 50%-histamine-sensitizing dose (HSD_{50}) was determined approximately by graphical interpolation of the dose of sensitizing material which gave 50% mortality after histamine challenge. This was expressed as the number of μg protein per mouse.

Toxicity of EDAC-treated lysozyme (LZ-E)

In a single experiment, 15 female 6-week old mice were each injected *i.p.* with 10 mg doses in 0.5 ml of LZ before and after EDAC treatment. The animals were observed closely for changes in their health and were weighed initially and then daily for three days after which they were killed by CO_2 asphyxiation.

Enzyme-Linked Immunosorbent Assay (ELISA)

Assay of antibodies

Anti-ovalbumin : For ELISA, flat-bottomed 96-well microtitre plates (Nunc Maxisorp F, Gibco BRL, Life Technologies Ltd., Renfrew Road, Paisley, Scotland) were coated with 350 μl/well of 100 μg/ml of OA. The OA was made up in coating buffer, pH 9.6 (Appendix 4). After keeping the plate for 1.5 h at RT in a humidified box, it was washed in washing buffer (PBS, pH 7.4 containing 0.05% v/v Tween 20 and 0.01%

w/v thimerosal) and allowing 3 min per wash. It was then blotted dry and used without delay.

BSA (Sigma), at 50 μg/ml (350 μl/well) in incubation buffer (PBS, pH 7.4 with 0.05% v/v Tween 20) was added to each well to block sites on the plastic not already occupied by OA. Blocking was done at RT for 5 min before further washing and blotting three times. Ten-fold dilutions of the test sera were made in incubation buffer and 300 μl added to each well. The antisera were titrated in duplicate. Incubation was for two h at RT whereupon the wells were again washed and blotted.

A 1:1000 dilution of sheep anti-mouse IgG-HRP (SAPU, Law Hospital, Carluke, Lanarkshire, Scotland) was added (250 μl per well) and incubated in a damp box overnight (to avoid the wells drying out) at 4 °C. Next day the plate was washed and blotted as before and developed with 200 μl of O-phenylene diamine (34 mg/ml) as substrate (Appendix 2). Development was done in the dark for 30 min at RT after which the reaction was stopped by adding 50 μl 12.5% (v/v) H_2SO_4 per well. The absorbancy of the fluid in each well was measured spectophotometrically at $A=492_{nm}$ using an Anthos reader 2001, (Anthos Labtech instruments, Austria).

Anti-lysozyme : The procedure for quantitating anti-LZ was similar to the above, except that the wells were coated with 2 μg/ml of LZ and the incubation time during the development stage was reduced to 15 min before the reaction was stopped and read spectophotometrically.

Anti-pertussis toxin : The general procedure was the same as above except that the wells were coated with 100 μl of a 1 μg/ml solution of fetuin (from fetal calf serum, Sigma) in coating buffer and incubated overnight at 4 °C in a humidified box. The contents of the wells were dumped out and the plate was washed three times with washing buffer. The wells were then blocked with 2% (w/v) BSA in incubating buffer for 1 h at 37 °C. The plates were washed again before adding 100 μl 10 μg/ ml PT in incubation buffer. The plate was incubated again for 1 h at 37 °C and washed. Three-fold dilutions (100 μl) of each test serum was added to the wells for another 1 h

incubation at 37 °C followed by 3X washing. A 1: 5000 dilution (100 µl) of sheep anti-mouse IgG (SAPU, Carluke, Lanarkshire) was added and incubated again for 1 h at 37 °C. Plates were washed, developed for 30 min with O-phenylene diamine and the reaction terminated with 12.5% v/v H_2SO_4 and read as before.

Anti-filamentous haemagglutinin : Purified FHA at 1.0 µg/ml in coating buffer, (FHA: courtesy of Dr. A. Robinson, PHLS, Centre for Applied Microbiological Research, Porton Down, Salisbury, Wiltshire, England) was used for the coating phase and incubated overnight at 4 °C in a humidified box. The volume of reagent added to the wells at each step throughout the test was 100 µl except 200 µl for the substrate and 50 µl for the H_2SO_4, and all incubations were performed for 1 h at 37 °C. The plate was washed three times then blocked by incubation with 2% BSA. After washing, three-fold dilutions of the test serum were added to the wells. Polyclonal FHA-HRP conjugate termed F_3 (PHLS, Centre for Applied Microbiological Research, Porton Down, Salisbury, Wiltshire, England) was added and incubated. The plates were given a final three washes before the addition of substrate and incubated in the dark for 30 min at 37 °C in a humidified box when the reaction was terminated.

Assay of bacterial antigens

Pertussis toxin : Wells of the same type of microtitre plates were coated with 200 µl of 20 µg/ml fetuin in carbonate buffer, pH 9.6. The plate was incubated overnight at 4 °C in a humidified box, then washed three times the next day and blocked with 250 µl per well of 10% (v/v) foetal calf serum and incubated for 1 h at 37 °C. A PT standard preparation (kindly provided by Dr. R. Parton of this department) and test PT samples were adjusted to 1 µg/ml protein and five-fold dilutions made. Each well was loaded with 200 µl of sample and incubated for 1 h at 37 °C. After washing, 200 µl of a 1 in 800 dilution of anti-PT monoclonal antibody (L_{10} PHLS, Centre for Applied Microbiological Research, Porton Down, Salisbury, Wiltshire) was added and incubated for 1 h at 37 °C. Plates were washed and 200 µl of a 1: 3000 dilution of

sheep anti-mouse IgG (SAPU) was added per well and again incubated for 1 h at 37 °C. The plates were given their final three washes, developed for 30 min, and finished in the usual way.

Filamentous haemagglutinin : Anti-FHA capture Ab (F_2 in ascites fluid, PHLS, Centre for Applied Microbiological Research, Porton Down, Salisbury, Wiltshire) was diluted 1:16,000 in coating buffer and 200 µl used to coat each well. The plate was incubated overnight at 4 °C in a humidified box. After plates had been washed, they were blocked with 10% (v/v) foetal calf serum (Gibco) in incubating buffer at 250 µl per well, for 1 h at 37 °C. FHA standard (CAMR) and test FHA antigen samples were adjusted to 1 µg/ml protein from which 5-fold serial dilutions were made. Test samples were titrated in duplicate and 200 µl added per well. The test plate was incubated for 1 h at 37 °C, washed and incubated for a further 2 h at 37 °C with conjugate. To each well 200 µl of F_3 IgG anti-FHA HRP (0.5 µg/ml, CAMR) were added. The plate was washed and developed as before.

Other In Vitro Procedures

Protein estimation

Protein content was measured either according to the method of Lowry *et al.* (1951) using the Folin-phenol reagent or by the Micro-BCA protocol of Smith *et al.* (1985) with bicinchoninic acid. BSA (Sigma) was used as the standard protein in both assays.

Lowry method : A series of dilutions of standard and test samples was prepared in duplicate in distilled water (DW). The BSA standard solutions ranged from 0-500 µg/ml. Each test sample (0.5 ml) was mixed with 0.5ml of 1N NaOH and placed in a boiling water bath for 5 min. Immediately before use, a solution consisting of 0.5% (w/v) $CuSO_4.5H_2O$ (1 ml), 1% (w/v) sodium potassium tartarate (1 ml) and 5%

sodium carbonate (50 ml) was prepared and 2.5 ml added to each sample. These were held at RT for 10 min then mixed with 0.5 ml of 1N Folin-Ciocalteou phenol reagent (Sigma). Test tubes were incubated for a further 30 min at RT to allow full colour development. A_{750nm} was measured in a SP6-550 UV/VIS spectophotometer (Pye Unicam) in 1 cm light path disposable plastic cuvettes. The machine was blanked against the 0 µg/ml standard.

Smith method : Dilutions of standard and test samples were prepared in DW. Standard solutions ranged from 0-50 µg/ml. These samples were mixed with an equal volume (0.5ml) of BCA-working reagent (BCA-WR) prepared as follows: Reagent A (8% $Na_2CO_3.H_2O$, 1.6% (w/v) NaOH, 1.6% (w/v) Na_2tartarate adjusted to pH 11.25 with solid $NaHCO_3$) was mixed (in equal volumes) with reagent C (0.4g of BCA.Na_2 in a plastic universal dissolved in 10 ml of DW with 0.4 ml of 4% (w/v) $CuSO_4.5H_2O$ added immediately before use). Samples were incubated in a water bath for 60 min at 60 °C and read in a UV/VIS spectophotometer at A_{562nm}. The machine was blanked against the 0 µg/ml standard.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The method of Laemmli (1970) was followed. Two glass plates (15.8 cm x 19 cm) were separated with 1.5 mm thick plastic spacers and sealed with yellow tape. To ensure the system was leakproof, the sealed glass plates were baked at 80-100 °C for 2-3 h. Separating gel, stacking gel and running buffer were prepared as described in Appendix 5. Lower gel solution was pipetted into the sealed plates to a level of approximately 11 cm, avoiding air bubbles. The solution was overlaid with ethanol so that the surface of the gel set evenly. The gel was left to set for 30 min at RT. Before pouring the upper gel (stacking gel) solution, the ethanol was removed and the surface of the gel washed with DW and a teflon comb inserted. This stacking gel solution was allowed another 30 min setting time at RT. The sealing tape was removed from the bottom of the gel to allow contact with the electrophoresis buffer. The wells were

washed with DW and the plate was inserted into a gel tank with the upper and lower reservoirs filled with running buffer. Wells were loaded with solubilised samples (which contained bromophenol blue as tracking dye, Appendix 5) with a Hamilton syringe. The samples were run through the stacking gel and into the separating gel at 18 mA then the current was turned up to 30 mA. The gel was run for 3-4 h.

Western blotting

Gels were run as above and were blotted according to the method of Towbin *et al.* (1984). The gel was overlaid with nitrocellulose membrane (mesh size of 0.4 μm, Amersham), Whatman filter paper (3mm) was laid on both sides of the gel and finally two Scotchbrite pads (Bio-Rad laboratories) were laid on either side. The sandwich was held together with plastic binders. This cassette was inserted into a transblot appparatus and filled with transfer buffer (Appendix 5). The protein was allowed to transfer overnight at 0.08 A at RT. The nitrocellulose was then removed and developed as below.

Staining of gels and blots

Proteins contained within polyacrylamide gels were stained either with Coomassie brilliant blue R250 (BDH, McQuilkin & Co., Laboratory furnishers, 21 Polmadie Avenue, Glasgow) or with silver nitrate (Sigma).

Coomassie blue staining : The gel was carefully removed from the glass plates and immersed in a fixing/staining solution of Coomassie blue R250 stain (Appendix 5) with gentle shaking overnight. The gel was destained the following day with a mixture of diluted methanol and acetic acid (Appendix 5). Destaining was carried out at RT for two days, with three changes of destain on the first day and one change on the second. Finally, the gel was rinsed thoroughly in DW and preserved by heat-sealing in plastic. The detection limit of this method was 0.3-1 μg protein per band.

Silver staining : Since this staining procedure is very sensitive to contamination (detection limit of 2-5 ng protein/band) the gel was handled with thoroughly-rinsed plastic gloves. All incubations were carried out for 30 min at RT with gentle shaking unless otherwise stated. First, the gel was prefixed in a mixture containing 30% (v/v) methanol and 10% (v/v) acetic acid in DW. This solution was poured off and, without rinsing, 10% (v/v) glutaraldehyde was added. Following this step, the gel was washed for 10 min in DW five to six times before soaking in a large volume overnight. The gel was washed again for 15 min in DW and incubated in a 5 μg/ml solution of DL-dithiothreitol (Sigma). Without rinsing, 0.1% (w/v) silver nitrate (Sigma) was added. The gel was rinsed twice in DW and twice more with 100 ml volumes of developer (3% sodium carbonate with 50 μl of 37% w/v formaldehyde). The gel was left to soak in a measured 100 ml of developer, this volume being neutralisable by the subsequently added 5 ml of 2.3 M citric acid which was poured in when bands were visible. Finally, the gel was washed in DW several times then with 0.03% (w/v) sodium carbonate before being preserved by heat sealing in a plastic sleeve.

Development of blots : Nitrocellulose was removed from the transblot apparatus and unbound sites were blocked with 2% BSA in TTS (20 mM Tris, 1% Tween 20 in saline) and incubated with gentle shaking. Anti-PT monoclonal L_{10} or polyclonal anti-FHA F_2 was added at a 1 in 1000 dilution and incubated for 1.5 h. Anti-serum was removed by five washes with TTS over 30 min. Sheep anti-mouse IgG-HRP (SAPU) was diluted to 1 in 1000 and incubated for a further 1.5 h. The blot was washed twice with TBS (20 mM Tris in saline) before the addition of the developer : 60 mg 4-chloro-1-naphthol (Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire) in 10 ml of methanol. TBS (90ml) and hydrogen peroxide (100 μl) were added just before use. When the blot had developed satisfactorily, it was rinsed several times in DW, air dried and stored at RT in the dark (wrapped in aluminium foil).

Haemagglutination assay

Test samples containing FHA were diluted in two-fold steps in PBS in polystyrene round-bottomed 96-well microtitre plates (Nunc-Immunoplate, Gibco-BRL) leaving 50 µl in each well. A 2% (v/v) suspension of washed horse red cells was added (50 µl per well) and after gentle mixing the contents were allowed to settle either at RT for 3-4 h or overnight at 4 °C. The HA titre was defined as the reciprocal dilution of the last well to show complete agglutination. Horse red cells in PBS and a known FHA standard were included as negative and positive controls on each plate.

Chinese hamster ovary cell-clustering assay

CHO cells (courtesy of Dr. E.L. Hewlett, University of Virginia School of Medicine, Charlottesville, Virginia, U.S.A.) were treated with 5 ml of trypsin/EDTA solution in Puck's saline (Gibco BRL) for 1 min. The solution was poured off and the cells were allowed to detach. After 5 min, 10 ml of growth medium (Ham's F12 containing 10% (v/v) foetal calf serum , Gibco BRL) was added to stop the activity of trypsin. The cells were transferred to a sterile universal container, centrifuged at 1000-1500g for 10 min and resuspended in Ham's F12 + 1% (v/v) foetal calf serum by gentle aspiration with a long-form pasteur pipette to break up clumps. The cell suspension was counted in a Neubauer counting chamber and used for replating or for the assay.

The wells of sterile microtitre plates (Nunclon, Gibco BRL) were seeded with approximately 10,000 CHO cells in a volume of 200 µl. The plate was incubated in a 5% CO_2 atmosphere at 37 °C for 20 h to allow attachment and stabilization. Serial 10-fold dilutions were made of test and standard PT samples in sterilized 100 mM Na_2HPO_4 + 0.5 M NaCl, pH 7.0. Each dilution was assayed in duplicate with 25 µl of sample added per well. Cells were exposed to toxin for 24 to 48 h and examined for the clustering response by light microscopy after staining. Preparation of Coomassie blue stain for staining CHO cells is given in Appendix 5. Cells were fixed with 4% (v/v)

formalin in PBS for 15 min (200 µl per well) then stained with Coomassie blue R250 (50 µl per well) for 15 min, rinsed extensively with DW and air dried.

Lysozyme Activity

A modified procedure was followed from the manufacturer product information leaflet for LZ (Sigma). A lyophilised preparation of *Micrococcus lysodeikticus* cells (Sigma) was resuspended in PBS and allowed to equilibriate until the suspension was stable at $A_{450\ nm}$= 1.0, and 2.5 ml of the suspension was delivered into 1 cm light path cuvettes. Dilutions of standard LZ and treated LZ preparations were made and tested in duplicate. To each cuvette 0.1 ml of test sample was added and thoroughly mixed using nescofilm (Alpha laboratories, Hampshire) to seal the opening. The A_{450nm} was monitored at this point and 30 min later. The % activity of LZ or treated LZ was determined from the drop in absorbance relative to a control preparation consisting of cells and 0.1 ml of buffer. A dose-response curve of *M. luteus* lysis by lysozyme is shown in Figure 8.

Statistical Procedures

Test for normality

As a preliminary step in the statistical analysis of antibody titres it was necessary to determine whether they were distributed normally, lognormally, or followed some other distribution. For this purpose rankit analysis Wardlaw (1985, p. 69-78) was adopted as a convenient procedure.

Briefly, ELISA titres from a group of typically five mice were arranged in rank order starting with the highest, and two graphs were plotted, one of rankit value against arithmetic titre and the other of rankit value against $\log_{10}$ titre. The rankit values were obtained from the rankit table. Independently, the mean and SD of both the arithmetic titres and the $\log_{10}$ titres were calculated and used to provide the theoretical straight lines for the two rankit plots (Figure 9). The goodness-of-fit of the theoretical and

experimental rankit lines was then analysed by the ordinate deviations of the individual points. With each antigen preparation, such as a particular batch of EDAC-treated LZ, the rankit deviations were tabulated according to rank number and the mean, SD and 95% CL calculated for each rank number. Summary graphs were then plotted of rankit deviation versus rank number and the decision about the underlying distribution made from the goodness-of-fit of the rankit deviations with the horizontal line at zero.

Further statistical analysis

With or without logarithmic transformation, groups of serum titres were subjected to analysis of variance to determine primary effects and interactions of the relevant experimental variables. They were also analysed as 4-point and 6-point assays for quantitative comparisons of immunogenicity and t-tests for comparison of group means. These procedures were all obtained from Wardlaw (1985).

Figure 8: Dose-response curve of *Micrococcus luteus* lysis by lysozyme. The reaction was carried out at RT and the A_{450nm} of the bacterial suspension was measured 30 min after the lysozyme was added.

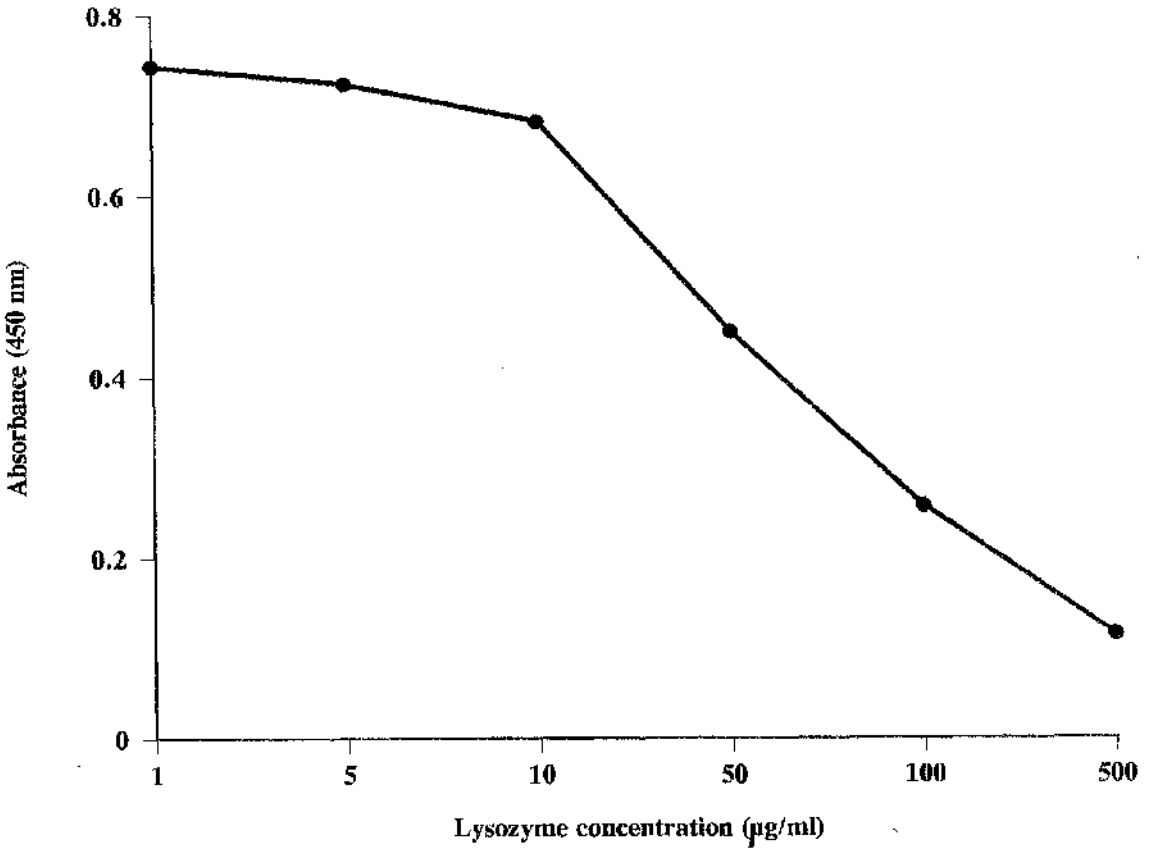
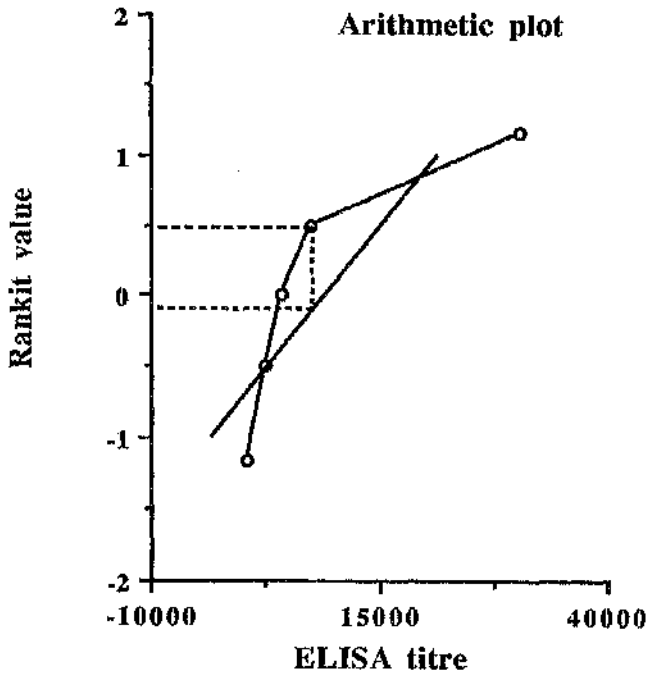
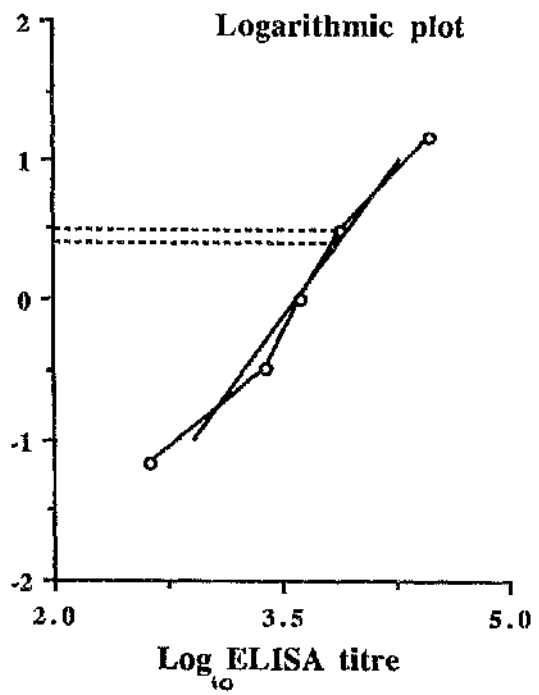


Figure 9: Example of rankit plots to determine normality or lognormality of serum titres. These 5 sera were raised against 1 mg of LZ-E. The vertical dotted line represents the ordinate deviation of the fourth rank number from the theoretical line.





RESULTS

ELISA TITRES OF ANTISERA

Before describing the effects of toxoiding agents on model antigens and on the antigens of *B. pertussis*, the procedures used for the derivation and analyses of antiserum ELISA titres are presented first.

Determination of ELISA Titres

Antibody production in mice was investigated by a single-shot immunization protocol with the various antigens, using groups of five animals at each dose level except once when groups of four were taken. A uniform time interval of 21 days was allowed for the expression of the primary response before the animals were bled and the sera obtained. The individual sera were then titrated by ELISA for antibodies specific for the relevant antigen(s). ELISA titres were obtained by plotting the $A_{492\ nm}$ against serum dilution on semi-logarithmic graph paper. Figure 10 shows an example of a plot of absorbance at 492 nm against $\log_{10}$ [reciprocal antiserum dilution]. Endpoints were obtained from the intersection of the $A_{492\ nm} = 0.5$ line with the curve, followed by interpolation down to the x-axis at $A_{492\ nm} = 0$. With low-titre sera, endpoints were obtained by extrapolation up to the line at $A_{492\ nm} = 0.5$. Where even this was not possible, an arbritary titre of one was recorded. Although read off a logarithmic scale, titres were initially recorded as their arithmetic values.

Statistical Analysis of Antiserum Titres

Rankit plots for analysis of the underlying distribution

For statistical analysis of ELISA titres it was first necessary to determine whether the data followed a normal or log-normal distribution pattern. This was done by use of rankit plots, a sample of which is given in Fig. 11 which presents selected results from

Figure 10: Derivation of an ELISA titre. Sample data illustrating interpolation of the dose-response curve with the $A_{492\ nm} = 0.5$. The resulting $\log_{10}$ titre is approximately 3.9, giving an arithmetic titre of 7900.

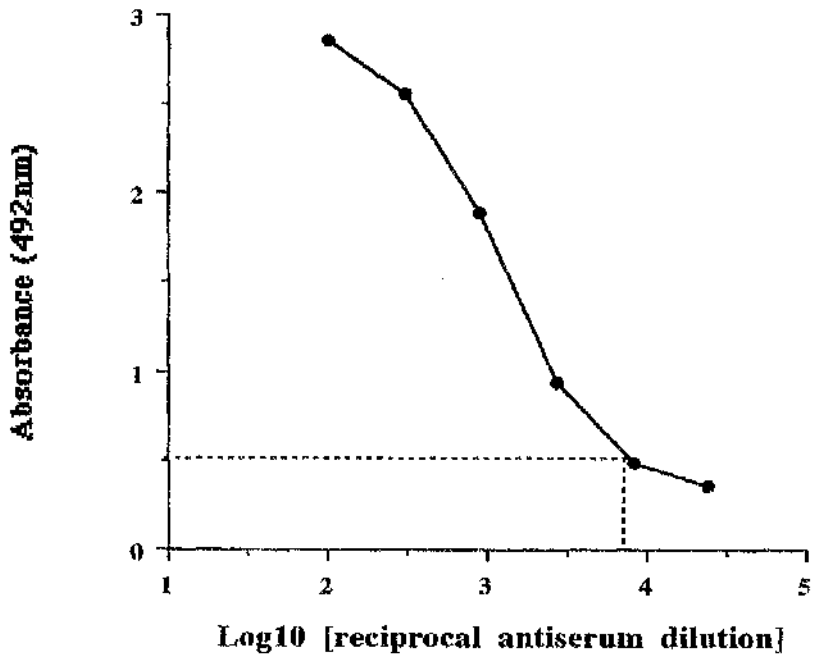
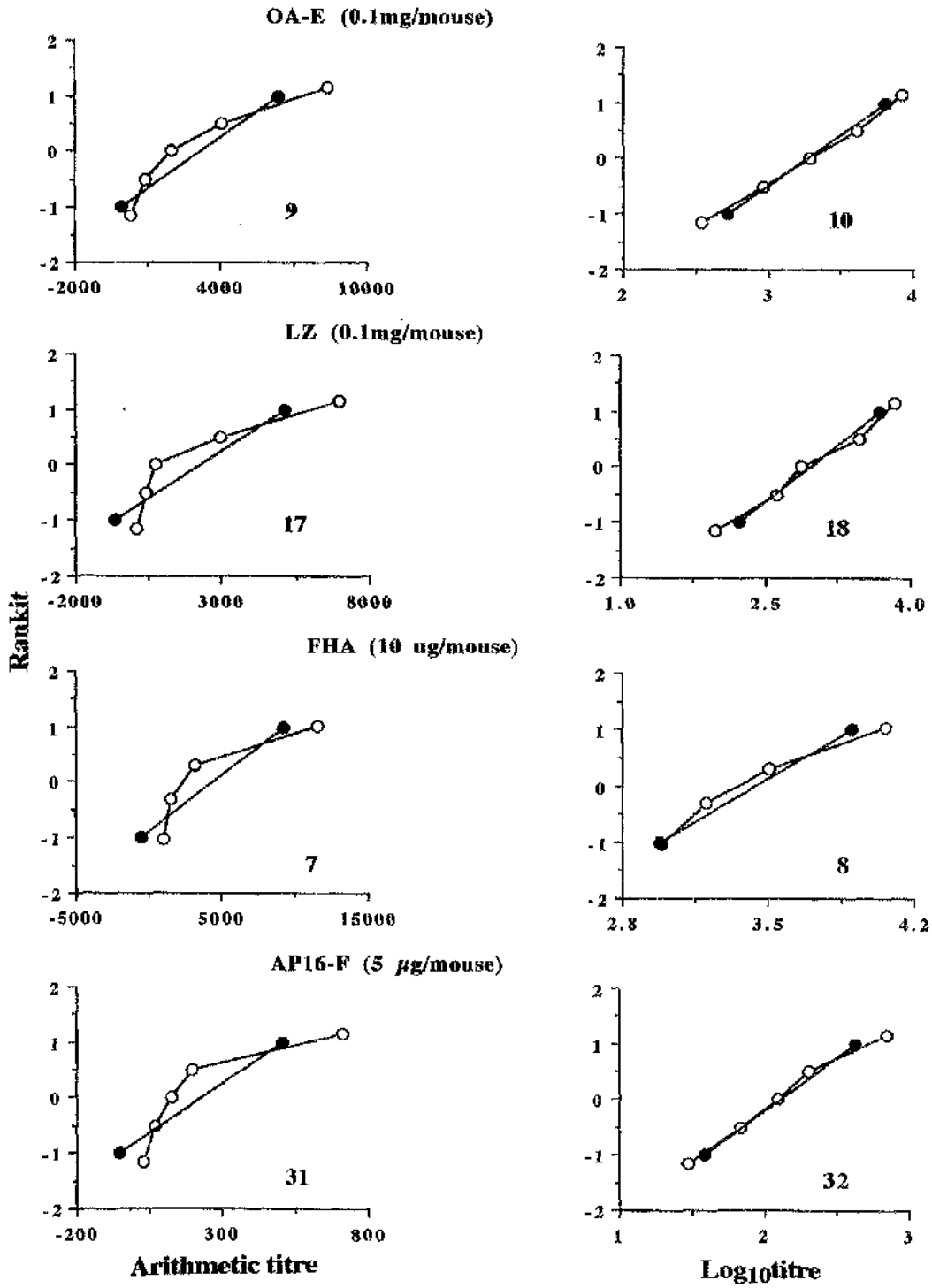


Figure 11: Rankit plots after immunization with OA-E, LZ, FHA and AP16-F. The left column shows plots with arithmetically expressed titres, the right after logarithmic transformation.



experiments with various doses of four different antigens, namely EDAC-treated OA (OA-E), lysozyme (LZ), FHA and the anti-PT titres of formaldehyde-treated AP16 (AP16-F) which was a mixture of PT and FHA. With the sera from each antigen, two different rankit plots were made, viz with the arithmetic values of the ELISA titres (left column diagrams) and the logarithmic values of the same titres (right column diagrams). In both types of diagram the observed titres are plotted with the open circles, while the straight line joining the closed circles represent the theoretical rankit line based on the mean and standard deviation (SD) of the group.

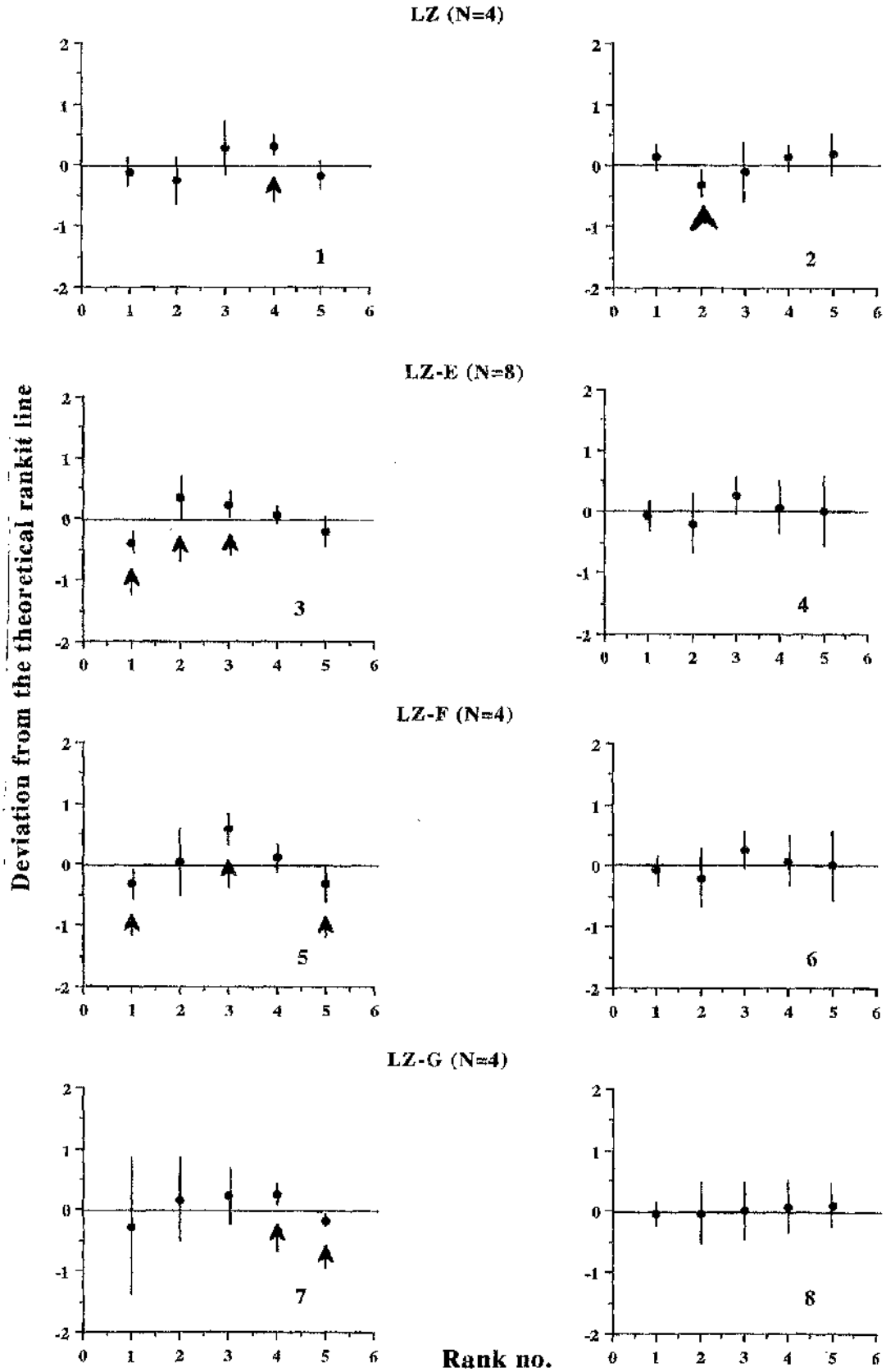
Visual inspection of the diagrams shows that all those in the left hand column exhibit the characteristic inverted L-shape of logarithmically-distributed data which have been presented on an arithmetic rankit plot, whereas those in the right hand column show a much better fit of theoretical and experimental lines indicative of an underlying log-normal distribution, or approximation thereto.

The sample of eight rankit plots in Fig. 11 were selected from the accumulated 278 rankit plots and are the results from the 57 groups of mice used in the whole study. Inspection of the totality of these results indicated that not all groups of sera benefited from logarithmic transformation of the ELISA titres in order to achieve normalization. It was therefore necessary to summarize the rankit plots in further diagrams which would highlight the differences between the experimental and the theoretical rankit lines to allow the appropriate decision on transformation.

Further analysis of rankit plots.

A sample of summarized rankit diagrams is provided in Fig. 12 for antisera raised against particular doses of four different antigens. In each diagram the deviations of the ranked points are presented with their 95% confidence limits. Wherever one of the confidence limits is outside the theoretical line an arrow has been inserted to highlight this feature. Thus it will be noted that in Fig. 12a there are 9 such arrows in the column

Figure 12a: Rankit-plot confidence-limit analysis of anti-LZ titres from experiment C. The mean vertical deviation from the theoretical line with 95% CL is plotted against rank no. for LZ treated with EDAC, formaldehyde and glutaraldehyde. Arithmetic plots are the left column diagrams, logarithmic plots are the right column diagrams. Arrows indicate deviations that are significant at the P= 5% level.



of arithmetic rankit plots of antisera against variously treated samples of LZ, but only one in the column of logarithmic plots. It therefore appeared advantageous for the ELISA titres of sera raised against LZ and its derivatives to be transformed into logarithms prior to statistical analysis.

With sera raised against OA and its derivatives, Fig. 12b shows that as with LZ, it was beneficial to make a logarithmic transformation of the titres although there are only four deviation arrows in the five diagrams of the anti-OA arithmetic plots. The results with the remainder of the anti-OA and anti-LZ sera are given in Appendix 6.

With antisera against the two *B. pertussis* antigens, PT and its derivatives (Fig.12c) and FHA and its derivatives (Fig. 12d), the logarithmic transformation had less normalizing effect than it did with anti-LZ and anti-OA and their derivatives. Nevertheless, in the interests of consistency it was decided to use logarithmically transformed titres for the further analysis of anti-PT and anti-FHA. The results with the remainder of the anti-PT and anti-FHA sera are also given in Appendix 6.

Figure 12b: Rankit-plot confidence-limit analysis of anti-OA titres from experiment C. The mean vertical deviation from the theoretical line, with 95% CL is plotted against rank no. for OA and OA-E treated to various extents. Arithmetic plots are the left column diagrams, logarithmic plots are the right column diagrams. Arrows indicate deviations that are significant at the P= 5% level.

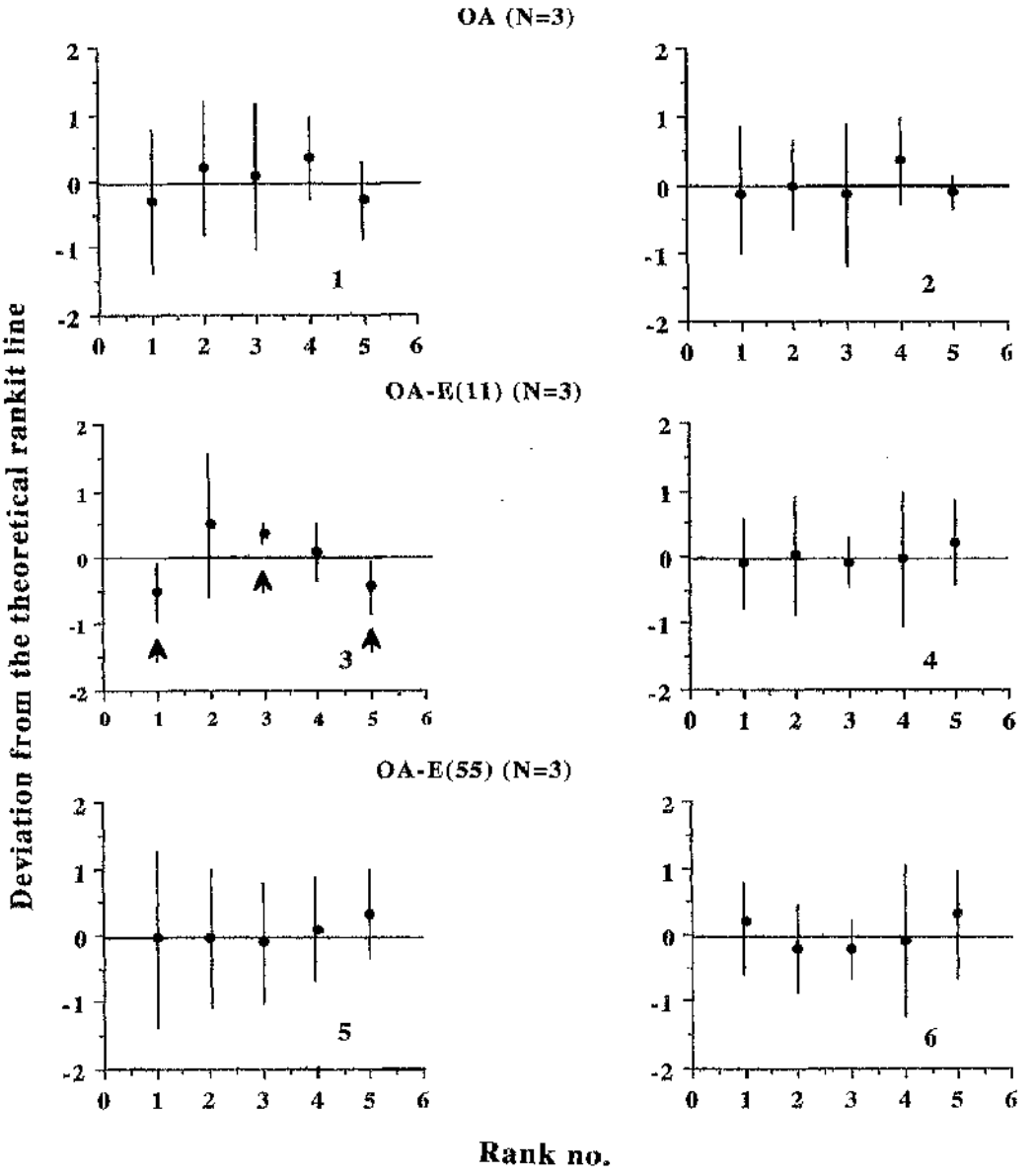


Figure 12b continued

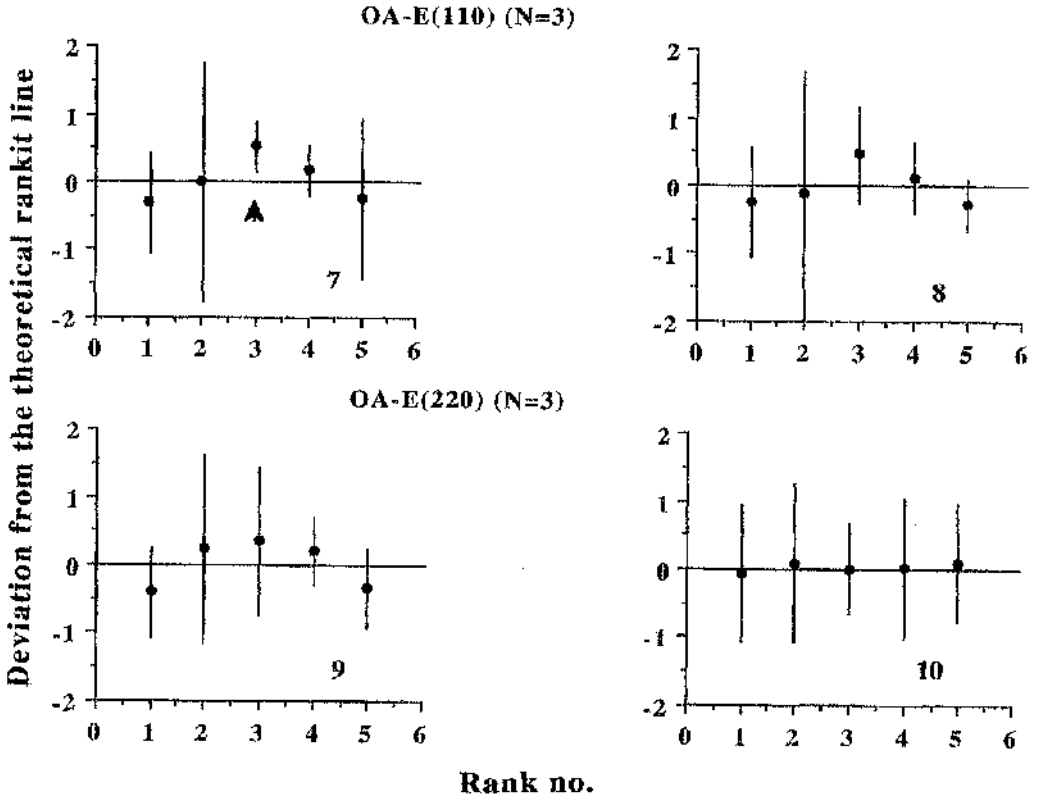


Figure 12c: Rankit-plot confidence-limit analysis of anti-PT titres after immunization with PT, PT-E, PT-F and PT-G. The mean vertical deviation from the theoretical line, with 95% CL is plotted against rank no. for each preparation. Arrows indicate deviations that are significant at the P= 5% level.

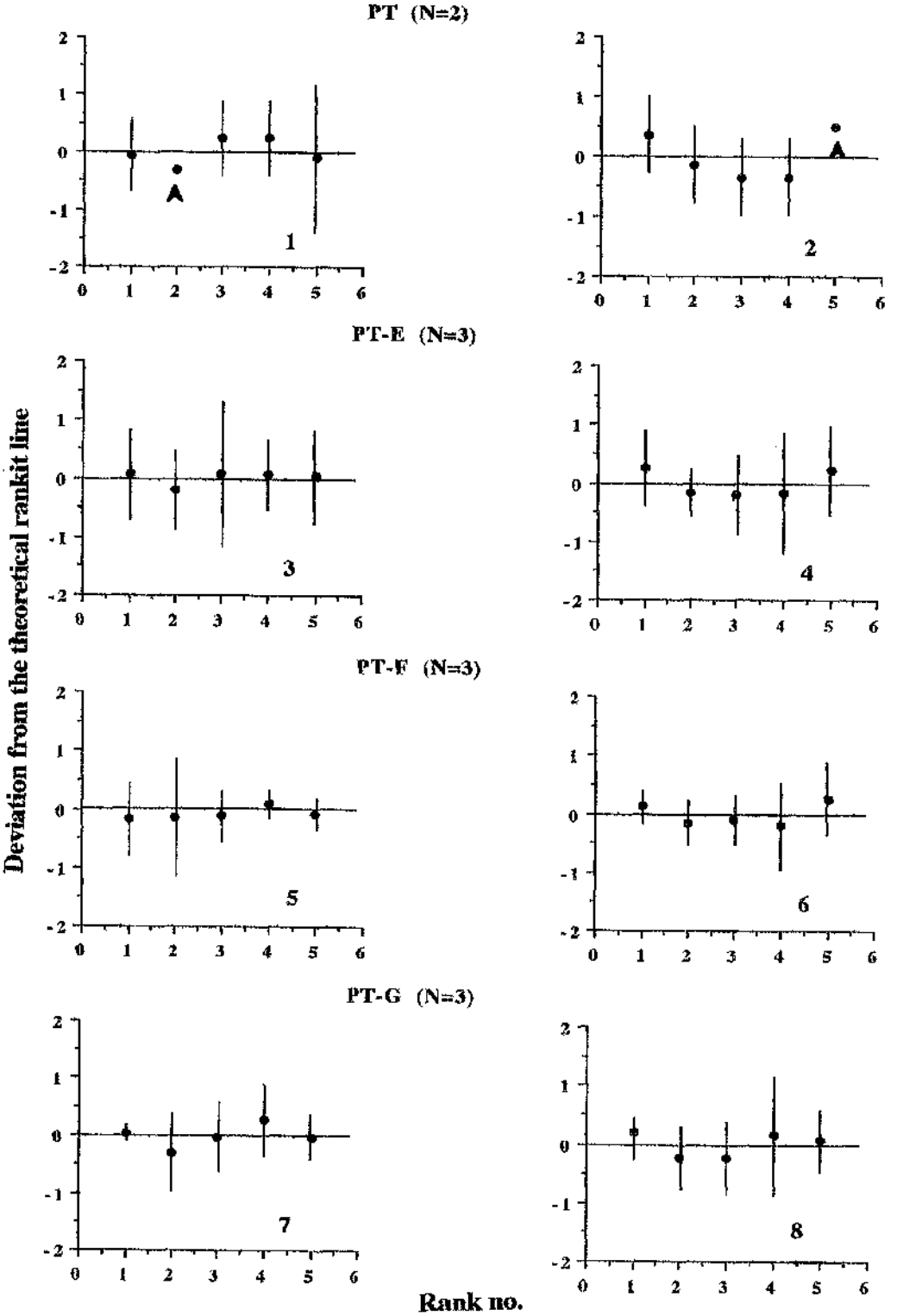
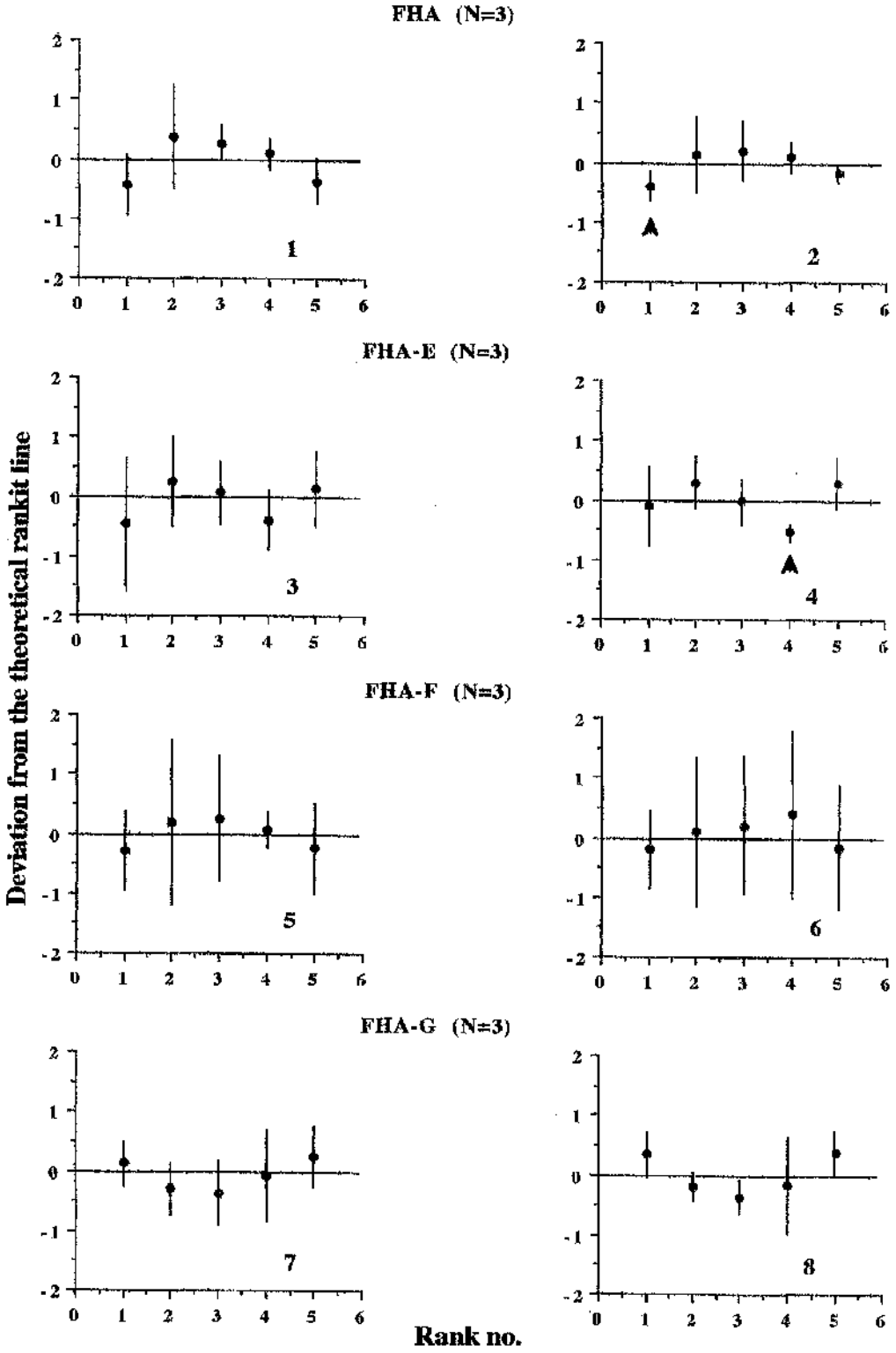


Figure 12d: Rankit-plot confidence-limit analysis of anti-FHA titres after immunization with FHA, FHA-E, FHA-F and FHA-G. The mean vertical deviation from the theoretical line, with 95% CL is plotted against rank no. for each preparation. Arrows indicate deviations that are significant at the P= 5% level.



EFFECT OF TOXOIDING AGENTS ON LYSOZYME AND OVALBUMIN

Before studying the effect of toxoiding agents on the immunogenicity of the antigens of *B. pertussis*, it was considered necessary to explore the effects of these agents on other antigens taken as models. In these studies two such antigens, ovalbumin (OA) and lysozyme (LZ), were treated with toxoiding agents. Most of the effort was eventually focused on LZ because the course of toxoiding could be monitored by loss of enzymic activity. Thus LZ was treated with EDAC, formaldehyde and glutaraldehyde, whereas OA as a non-enzymically active protein was treated with EDAC only.

Additional reasons for choosing LZ were: high NH_2-group content and the likelihood of greater modification by formaldehyde and glutaraldehyde, rather than EDAC; and high immunogenicity, which raised the question of whether toxoiding could have any significant enhancing effect on antibody production.

OA was chosen because it is a weak immunogen and is in standard use for adjuvant studies (Stewart-Tull, 1989). These features together with the high carboxyl-group content made it suitable for treatment with EDAC which combines principally with such groups (and also to a lesser extent with the hydroxyls of serine and tyrosine).

Experiments With Ovalbumin

The concentration of OA taken for treatment with EDAC was chosen so that a top immunizing dose of 1 mg per mouse would be contained in a volume of 0.5 ml for convenience of *i.p.* injection. The protein, initially at 8 mg/ml was treated with various concentrations of EDAC so as to provide molar ratios with reactive amino-acids ranging from approximately 1:1 to 20:1. According to Haurowitz (1963), the molecule of OA contains 84 carboxylic groups in glutamate and aspartate residues, 36 hydroxyl groups in serine residues, 9 phenolic groups in tyrosine residues and 5 sulfhydryl groups

associated with cysteine/cystine. In calculating the EDAC : protein ratios, it was assumed that all of these groups would be reactive. OA at 4 mg protein per ml corresponds to 11.832 mM of all the above groups taken together. Thus EDAC at 11, 21, 55, 110 and 220 mM with OA at 4 mg/ml was calculated to provide ratios of EDAC to total reactive groups of 1:1, 1:2, 1: 5, 1: 10 and 1: 20. The preparations from these were labelled respectively OA-E(11), OA-E(21), OA-E(55), OA-E(110) and OA-E(220).

It may be noted that the treatment with 21 mM EDAC was the same as used by Christodoulides *et al.* (1987) for toxoiding PT except that the latter was at 50 μg/ml.

Effect of EDAC on SDS-PAGE profiles

The above EDAC-treated samples were heated at a 100°C in solubilizing buffer and then run on a 12.5% SDS-polyacrylamide gel, with the results shown in Fig.13. The major band in each lane is due to OA at 45 KDa or modified OA at an apparently lower value of about 40 KDa. There appeared to be both bands in lane 7, while in lane 3, the 40 KDa band was much thickened. The proportion of the faster-migrating material increased with the severity of EDAC treatment. At the highest ratio of EDAC to protein (lane 3), there was also evidence of polymeric material at the top of the lane. It was clear that EDAC had had a significant effect on the electrophoretic mobility of OA.

Anti-OA ELISA

In setting up the ELISA for measuring mouse anti-OA IgG, it was first necessary to determine the appropriate concentration of OA for coating the wells in the microtitre plate. Fig. 14 shows the effect of a 125-fold range of OA coating concentrations on the A_{492} values given by 10-fold dilutions of a pool of the 40 anti-OA sera from the experiment described below. From this figure, a coating concentration of 100 μg/ml was chosen as giving a satisfactory result, i.e the serum endpoint titres increased about

Figure 13: Effect of EDAC on the SDS-PAGE profile of ovalbumin. OA and various EDAC-treated OA preparations were run on a 12.5% polyacrylamide gel. Each lane was loaded with 10 µg of protein. From left to right the lanes contained the following:

Lane	Sample
1	Sigma standards MW-SDS-70 kit[a]
2	OA
3	OA-E(220)
4	OA-E(110)
5	OA-E(55)
6	OA-E(11)
7	OA-E(21)
8	Sigma standards MW-SDS-200[b]

[a] Sigma standards MWSDS-70 kit contained a mixture of the following proteins : lysozyme (14.3 kDa), beta-lactoglobulin (18.4 kDa), trypsinogen (24 kDa), pepsin (34.7 kDa), ovalbumin (45 kDa) and bovine plasma albumin (66 kDa).

[b] Sigma standards MW-SDS-200 kit contained a mixture of the following proteins : carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine plasma albumin (66 kDa), phosphorylase B (97.4 kDa), beta-galactosidase (116 kDa) and myosin (205 kDa).

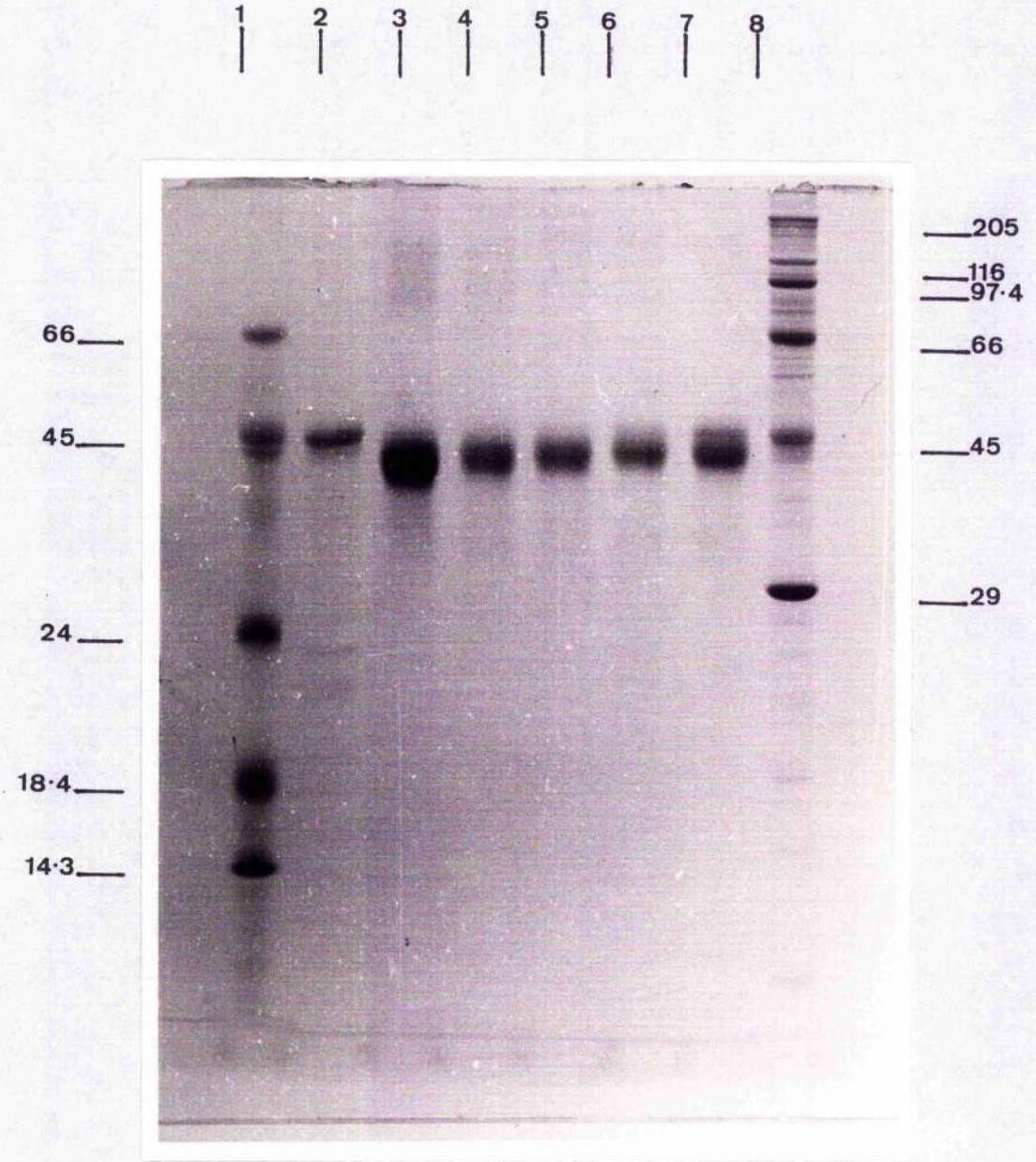
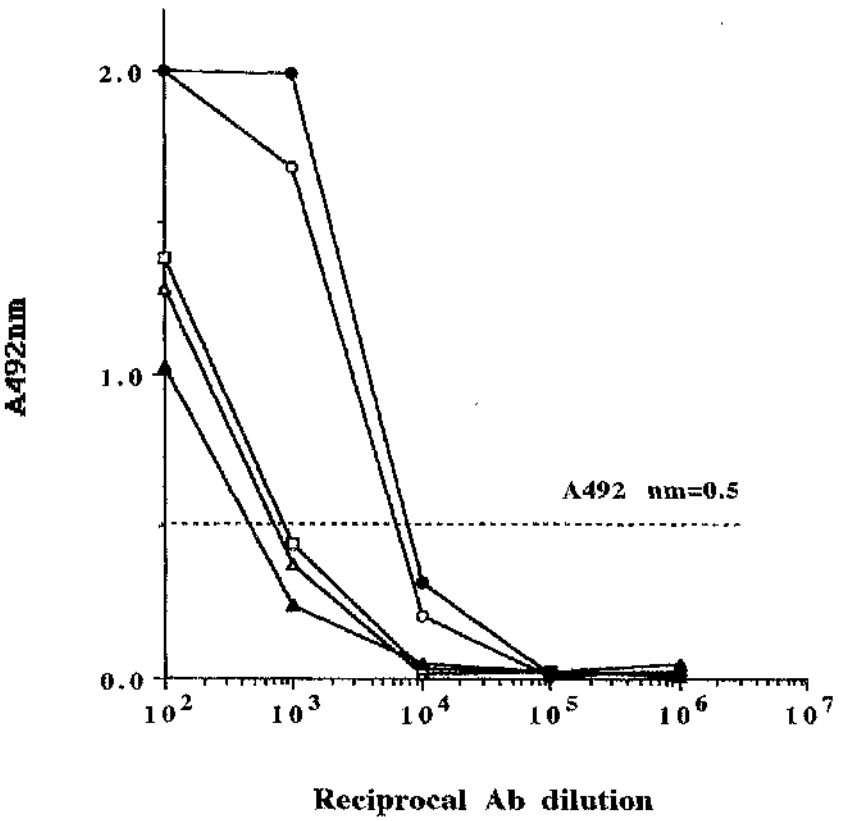

1
2
3
4
5
6
7
8
66
45
24
18·4
14·3
205
116
97·4
66
45
29

Figure 14: Effect of OA coating concentration on the $A_{492\ nm}$ in the ELISA on mouse anti-OA. The wells of microtitre plates were coated with OA at 2 µg/ml (⌓), 10 µg/ml (□), 50 µg/ml (▲), 100 µg/ml (○) and 250 µg/ml (●).



9-fold when the coating concentration was increased from 10 to 100 μg/ml but were little improved when 250 μg/ml was used.

Effect of EDAC and $Al(OH)_3$ on immunogenicity

The object of these experiments was to determine whether EDAC treatment of OA had an enhancing, depressing or no effect on antibody production. $Al(OH)_3$ was included as a further component for possible adjuvanting. OA-E treated with EDAC at 21mM only was used in this experiment. Both OA and OA-E(21) were tested as soluble antigens and after mixing with an equal weight of $Al(OH)_3$. In each of two independent experiments, experiment A and experiment B, (with separately prepared batches of OA-E(21) termed OA-E(21,1) and OA-E(21,2) respectively) groups of 5 mice were given either 0.1 or 1.0 mg doses of these preparations *i.p.* and bled 21 days later. Table 6 presents the arithmetic titres of the sera of all 80 mice while Fig.15 summarize the data as bar charts of the group geometric means (g.m.) with 95% CL. Visual inspection of this figure suggests that (a) the two experiments gave qualitatively similar results, (b) at the 0.1 mg dose, EDAC treatment *reduced* the immunogenicity of OA, and (c) $Al(OH)_3$ had an adjuvant effect but only in some instances.

In order to investigate further the influence of each variable on anti-OA production, multifactorial analysis of variance was performed (Table 7). The two independent experiments showed that EDAC had a highly significant (**) repressive effect on anti-OA formation, while $Al(OH)_3$ had a significant adjuvanting effect; however, dose was influential in experiment B but not in A. As regards the interaction terms, the two experiments differed: experiment B showed no significant interactions between the variables, whereas in experiment A there was significant interaction of doses x EDAC and $Al(OH)_3$ x EDAC. The highly significant doses x EDAC term in experiment A showed that in these mice, EDAC reversed the negative slope of response on dose and also significantly repressed the adjuvanticity of $Al(OH)_3$.

Table 6: Effect of EDAC on the immunogenicity of OA in mice; also the effect of immunizing the animals at 0.1 and 1.0 mg, with and without $Al(OH)_3$ added to the treated or untreated OA. The ELISA titres are from two experiments A and B with independently made preparations of EDAC-OA (OA-E(21,1) and OA-E(21,2)[a]. Geometric mean titres are shown in bold.

Anti-OA ELISA titre of individual mice and group **g.m.** after immunization with (mg):								
	OA				OA-E(21)			
	plain		$Al(OH)_3$		plain		$Al(OH)_3$	
	0.1	1.0	0.1	1.0	0.1	1.0	0.1	1.0
Expt A	5000	1825	7200	38000	8350	11300	875	19800
	4050	9965	9950	6500	1975	2875	1775	6700
	3100	12250	24500	2000	935	4700	1225	8075
	5300	2000	38000	18250	345	3850	430	7350
	5400	3050	32000	2000	4100	1850	1000	4600
	4475	**2653**	**18442**	**7099**	**1854**	**4050**	**961**	**8162**
Expt B	5500	6950	9500	23500	385	7600	915	6600
	8500	1700	5350	9700	1800	70	775	8100
	2775	5600	4050	36000	3500	6650	1230	7550
	1275	9200	570	2850	50	4050	2250	7100
	5800	5700	7900	20000	55	4450	3000	7800
	3946	**5107**	**3923**	**13615**	**367**	**2019**	**1425**	**7413**

[a] Mice were immunized with a single-shot dose of antigen and bled 21 days later. $Al(OH)_3$ was used at equal weights of antigen, i.e. 0.1 or 1.0 mg/mouse for the 0.1 or 1.0 mg/mouse dose .

Figure 15: Effect of EDAC and $Al(OH)_3$ on the log_{10} anti-OA ELISA titres from mice. Each bar represents the geometric mean titre from a group of five mice and the vertical line the upper 95% CL. OA (□), OA-E (21,1) and OA-E (21,2) for experiment A and B respectively (▨), OA with $Al(OH)_3$ (⊡), OA-E(21,1) and OA-E (21,2) with $Al(OH)_3$ (▤).

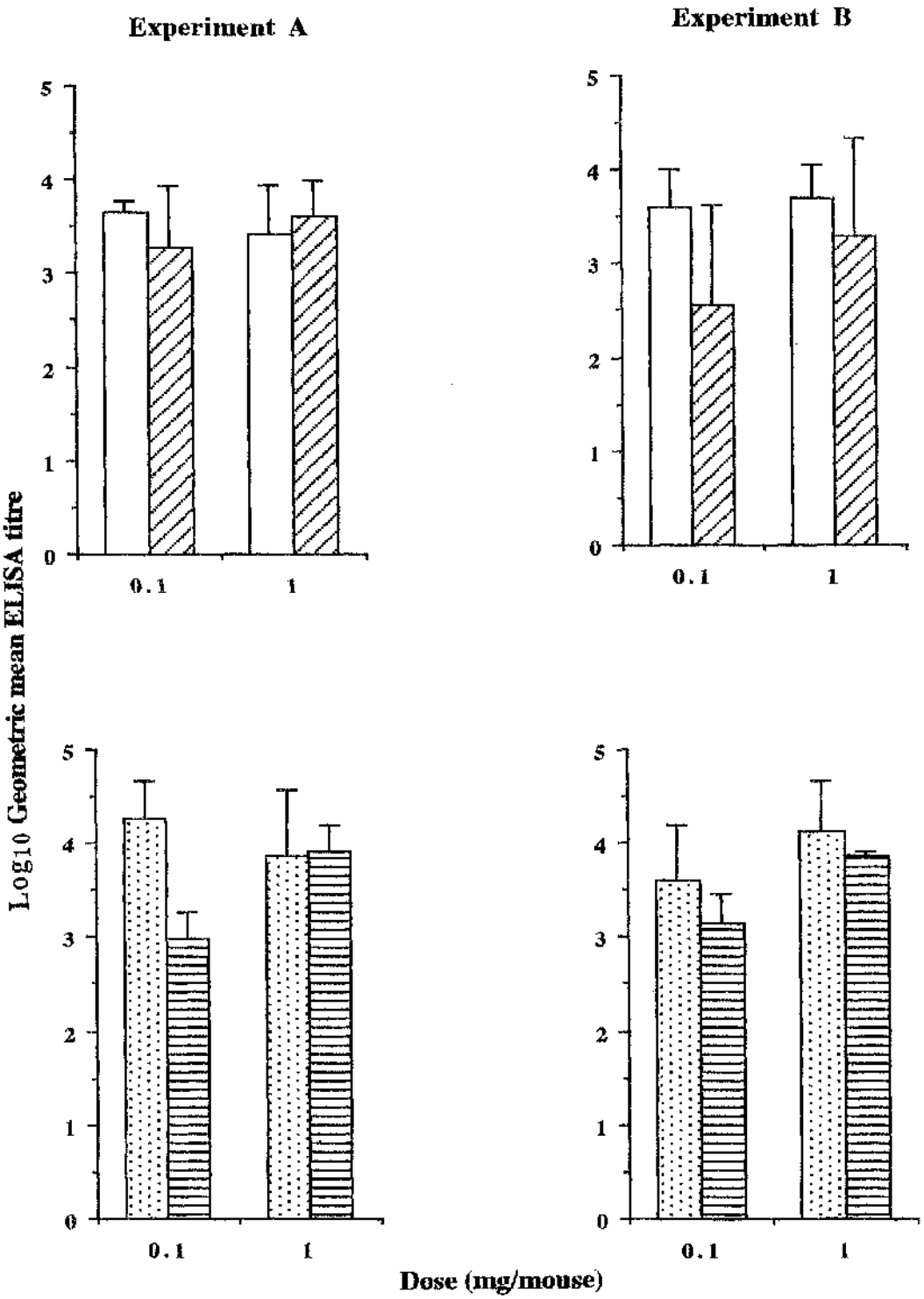


Table 7: Analysis of variance on the effect of dose, EDAC and $Al(OH)_3$ on the production of anti-OA in mice from two independent experiments A and B. Multifactorial analysis was performed on the $\log_{10}$ anti-OA titres.

Source of variation	Mean square		F value	
	Expt A	Expt B	Expt A	Expt B
Doses	0.246	2.780	1.76	11.04**
$Al(OH)_3$	0.704	1.554	5.03*	6.17*
EDAC	1.264	2.858	9.03**	11.34**
Doses x $Al(OH)_3$	0.101	0.102	0.72	0.41
Doses x EDAC	2.281	0.404	16.3**	1.61
$Al(OH)_3$ x EDAC	0.655	0.334	4.68*	1.32
Doses x $Al(OH)_3$ x EDAC	0.378	0.128	2.70	0.51
Residual	0.140	0.252	-	-

Tabulated F value for 1 and 28 degrees of freedom at P = 5% is 4.20 (*) and at the P = 1% is 7.64(**).

In a follow-up study, experiment C, OA was treated with EDAC at the four concentrations listed above. Mice were immunised with 0.01, 0.1 or 1.0 mg/mouse of OA, OA-E(11), OA-E(55), OA-E(110) and OA-E(220) with bleeding on day 21. Initially, the sera were titrated from a starting dilution of 1:100. However, the anti-OA responses were very low in some sera in that there was no detectable antibody in 19 out of the 75. These sera were therefore retested from a 1:10 starting dilution. ELISA titres and group geometric means are given in Table 8 and the results are summarized as bar charts with 95% CL in Fig. 16. The main conclusions were that (a) the lowest dose, 0.01 mg, of each preparation stimulated lower titres of antibody than the two higher doses and (b) EDAC treatment had an adjuvanting effect, with the 0.01 mg dose, and a repressive effect with the two higher doses.

In an attempt to gain further insights into the results, the titres were analyzed as for a 6-point parallel-line assay which is depicted diagramatically in Fig. 17. The output of the analysis of variance (Table 9) shows a highly significant effect of dose (the slope term), but complex effects of the other factors. Only OA-E(11) significantly reduced antibody response when compared with OA alone. In Fig. 16 the diagrams 1 and 2, for OA-E(11) and OA-E(55) each compared with OA, show lack of parallelism between the 2 dose-response lines. Diagrams 2 and 3 show the "simple kinking" which is supported by significance for "quadratic curvature" while "difference of quadratics" is illustrated by the curves in diagrams 1 and 2.

Table 8 : Effect of EDAC on the immunogenicity of OA in mice (experiment C); EDAC-treated OA preparations OA-E(11), OA-E(55), OA-E(110), OA-E(220) were injected in groups of five mice and anti-OA ELISA titres were obtained.

Sample	Anti-OA ELISA titres (and group **g.m.**) after immunisation with the following doses of antigen preparation (mg/mouse) 0.01	0.1	1.0
OA	88	3050	3375
	51	1200	2950
	34	920	2700
	17	675	860
	16	505	820
	33	**1028**	**1803**
0A-E(11)	450	415	4350
	285	94	630
	108	72	480
	45	41	195
	20	1[a]	108
	105	**41**	**488**
OA-E(55)	700	860	965
	650	315	950
	615	215	770
	455	120	620
	170	24	565
	405	**176**	**757**
OA-E(110)	845	6200	3900
	790	840	2900
	43	800	1250
	41	705	870
	40	435	715
	137	**1050**	**1545**
OA-E(220)	1100	4700	2125
	105	3600	1400
	85	440	1350
	57	330	710
	23	12	670
	105	**494**	**1138**

[a] An arbritary value of 1 was assigned to this serum for purposes of statistical analysis since anti-OA levels were similar to normal serum.

Figure 16: Effect of EDAC on the immunogenicity of OA. Each bar represents the geometric mean anti-OA ELISA titre from a group of five mice and the vertical lines the 95% CL. Four EDAC-treated OA preparations were used. OA (□), OA-E(11) (▤), OA-E(55) (▥), OA-E(110) (▨), OA-E(220) (▤).

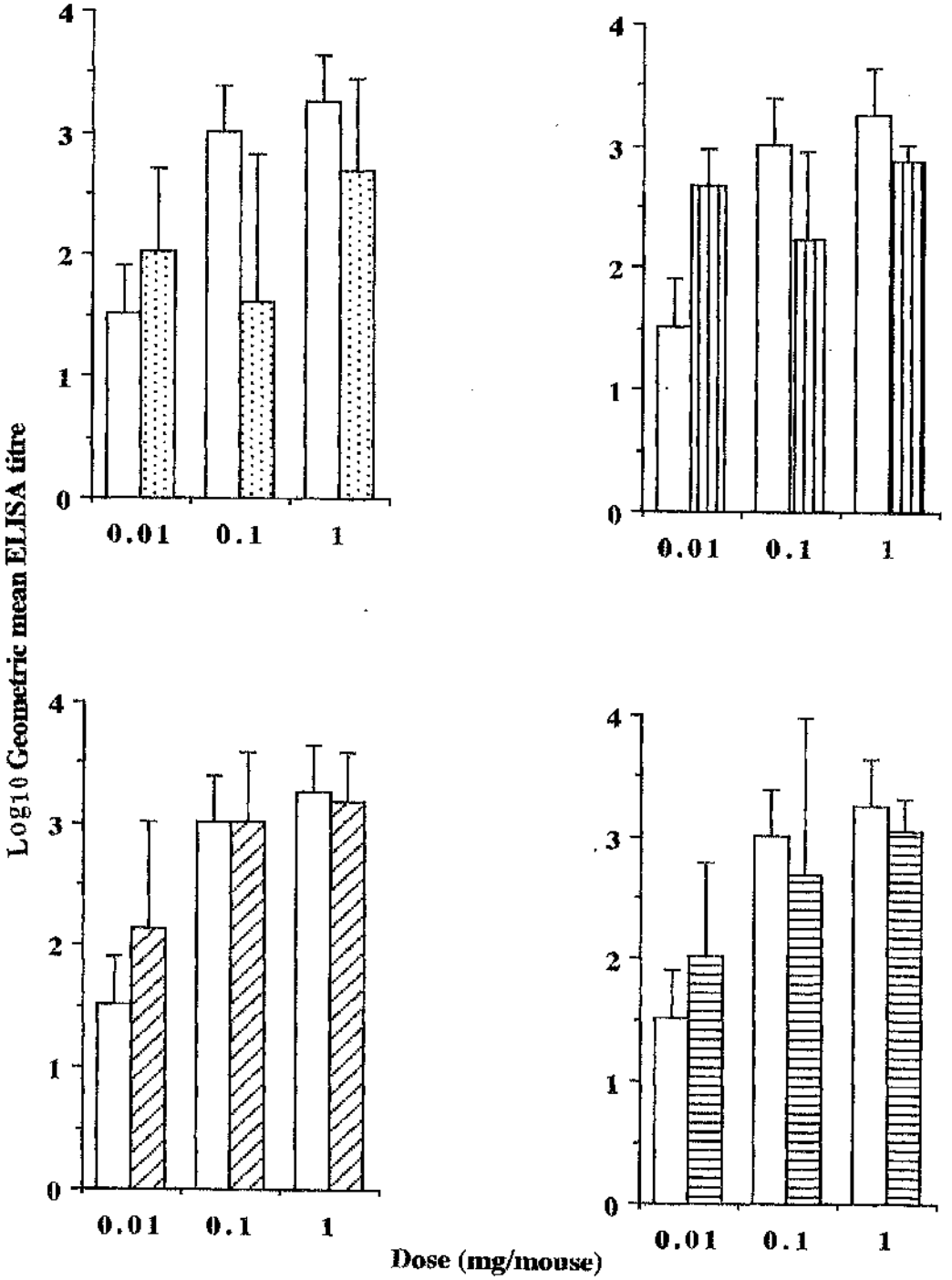


Figure 17: Antibody responses of mice to immunization with EDAC-treated OA. The four diagrams are plots of $\log_{10}$ geometric mean titres of untreated OA (o) and EDAC-treated OA (●) preparations. Diagrams 1, 2, 3, 4 denote responses obtained after immunization with OA treated with EDAC at 11, 55, 110 and 220 mM respectively.

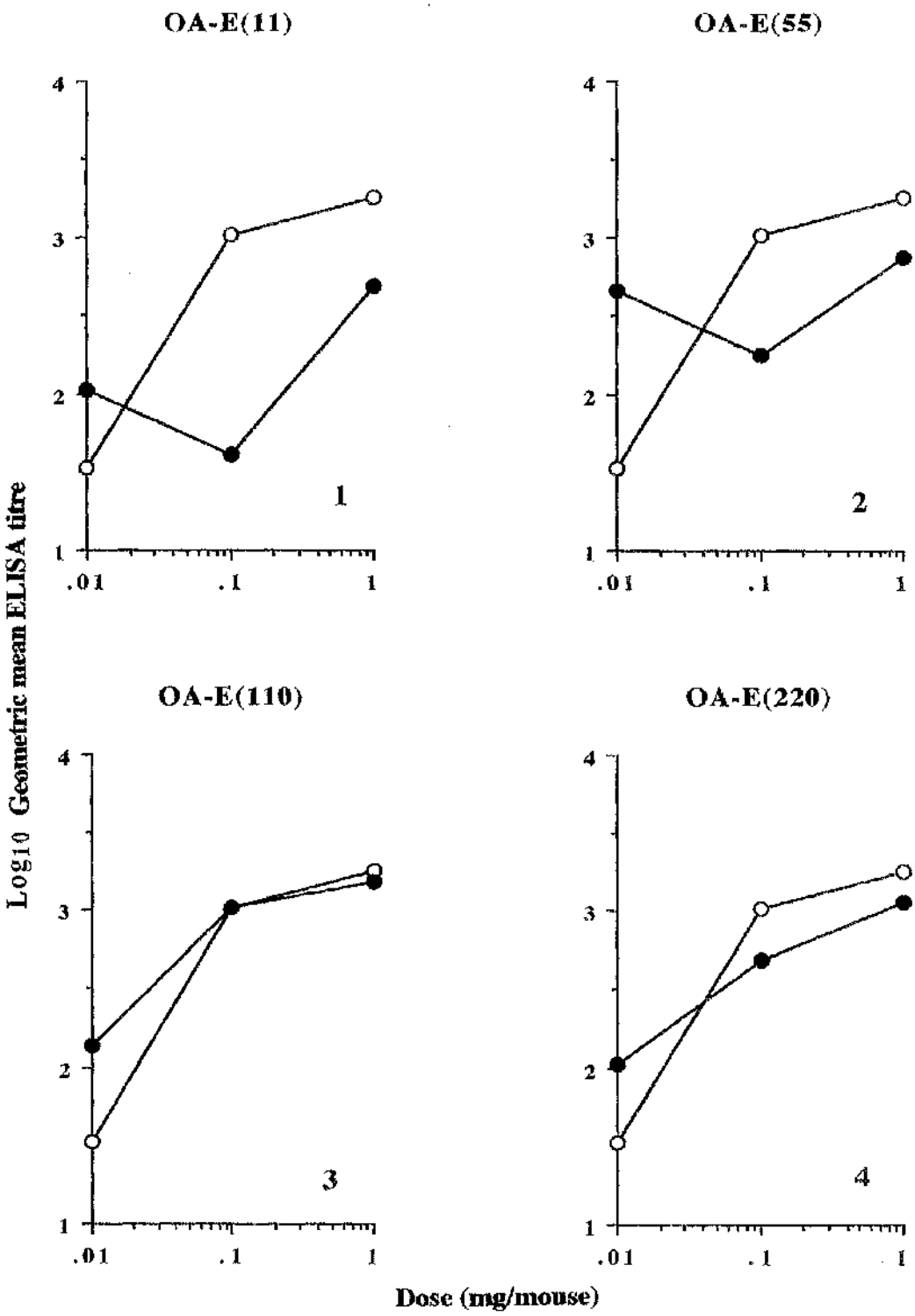


Table 9: Analysis of variance on the effect of EDAC on the production of anti-OA in mice (experiment C). Summary statistics are presented from 6-point parallel line assays.

	F-values			
Source of variation	OA-E(11)	OA-E(55)	OA-E(110)	OA-E(220)
Slope	20.97**	41.12**	53.40**	31.85**
Preparations	4.48*	0.00	1.40	0.01
Parallelism	4.41*	25.16**	3.20	2.03
Quadratic curvature	0.03	10.57**	8.80**	3.53
Difference of quadratics	8.96**	19.21**	0.60	1.21

Tabulated F value for 1 and 24 degrees of freedom at P = 5% is 4.26 (*) and at the P = 1% is 7.82 (**).

Experiments With Lysozyme

Effect of EDAC, formaldehyde and glutaraldehyde

Different considerations were adopted for planning the treatment of LZ with EDAC from those used with OA. In particular, it was decided to use the conditions of treatment with EDAC, formaldehyde and glutaraldehyde which had been applied by other workers to the detoxification of bacterial protein toxins. Thus, LZ at 4 mg/ml (final concentration) in intial experiments was treated with 21 mM EDAC at pH 5.0 for 24 h at 37°C as used by Christodoulides *et al.* (1987) for detoxification of PT which was at 50 μg/ml. Subsequently, 200, 400 and 800 mM EDAC were also used.

With formaldehyde, LZ (4 mg/ml) was treated for 168 h with 25 mM formaldehyde as used by Porro *et al.* (1980) for diphtheria toxin at 4.36 mg protein/ml except that the treatment was performed at 37 °C instead of 22 °C; in a later experiment, 12.5 and 6.25 mM formaldehyde were also employed. Similar treatments were done in the presence of lysine at concentrations equal to 80% of the molarities of formaldehyde.

With glutaraldehyde, LZ (4 mg/ml) was treated for 2 h with 2.5 mM of the reagent used by Munoz *et al.* (1981a) for the detoxification of PT (final protein concentration not stated) except that the treatment was performed at 37 °C instead of 22 °C. Lower concentrations of 0.625 and 0.312 mM were also employed. All mixtures at the end of 2 h were treated with lysine at levels equivalent to 4 times that of the glutaraldehyde so as to neutralize it before dialysis.

Enzyme activity : The effects of the above chemical treatments on the enzymic activity of LZ was monitored by bacterial lysis tests with *Micrococcus lysodeikticus* (Table 10). It is apparent that all three toxoiding agents caused loss of enzymic activity, with formaldehyde and glutaraldehyde treatments abolishing it completely, i.e. > 99% loss of enzyme activity.

Table 10: Effect of EDAC, formaldehyde and glutaraldehyde on LZ enzyme activity: EDAC, formaldehyde and glutaraldehyde treatments were for 24, 168 and 2 h at 37°C respectively. Loss of activity was followed by the lysis of *M. luteus* cells spectrophotometrically at $A_{495\ nm}$

Treated preparation	% loss in activity		
	Expt 1	Expt 2	Mean
LZ-E(21,1)	33.3	nt	-
LZ-E(21,2)	34.4	43.7	39.1
LZ-E(21,3)	37.0	nt	-
LZ-E(200)	49.0	nt	-
LZ-E(400)	38.0	nt	-
LZ-E(800)	43.0	nt	-
LZ-F(25)	>99.9	nt	-
LZ-F(12.5)	>99.9	nt	-
LZ-F(6.25)	>99.9	>99.9	>99.9
LZ-F(25) Lys(20)	>99.9	>99.9	>99.9
LZ-F(12.5) Lys(10)	>99.9	>99.9	>99.9
LZ-F(6.25) Lys(5)	>99.9	>99.9	>99.9
LZ-G(2.5)	>99.9	nt	-
LZ-G(1.25)	>99.9	nt	-
LZ-G(0.625)	>99.9	nt	-
LZ-G(2.5) Lys(10)	>99.9	nt	-
LZ-G(1.25) Lys(5)	>99.9	nt	-
LZ-G(0.625) Lys(2.5)	>99.9	nt	-
LZ-G(2.5) Lys(10)[a]	>99.9	nt	-
LZ-G(1.25) Lys(5)[a]	>99.9	nt	-
LZ-G(0.625) Lys(2.5)[a]	>99.9	nt	-

[a]Lysine was added to the mixture at the beginning of the reaction and at the end in separate preparations which were incubated for a further 15 min before dialysis.
nt = not tested

With both formaldehyde and glutaraldehyde this result was independent of whether or not lysine was present. EDAC differed from the two aldehydes in leaving between 33 and 49% of the original activity intact, and with very little regular effect with EDAC concentrations over the range 21 to 800 mM.

SDS-PAGE profile Fig. 18 shows SDS-PAGE profiles for LZ and the four LZ-E preparations, and indicates that the EDAC had produced a reduction in mobility of the main 14.3 KDa band due to LZ itself, together with dimers in the 24 to 29 KDa range. Increasing the EDAC concentration from 21 to 800 mM caused progressive changes in SDS-PAGE profile. Thus the lowest level of EDAC produced a small reduction in mobility together with a very prominent dimer band. As the concentration of EDAC was raised, the mobility of the main component decreased and at 800 mM EDAC also appeared to be attenuated. Meanwhile the intensity of the dimer bands decreased and appeared to have disappeared after treatment with the highest level of EDAC.

The effect of formaldehyde and glutaraldehyde treatments on the SDS-PAGE profile of LZ is shown in Fig. 19. Treatment with either formaldehyde, glutaraldehyde or EDAC (21 mM) gave consistently a dimer band (between 24-29 KDa). Moreover the intensity of this band corresponded to the concentration of the reagent. Thus the dimer band is only barely discernible with the 6.25 mM formaldehyde and is very pronounced with formaldehyde at 25 mM; the addition of lysine appeared to have little modifying effect. In the region above the dimer bands, there was evidence of higher polymers but exact molecular weights could not be assigned.

With glutaraldehyde (Fig. 19), the dimer bands were well developed as with formaldehyde, and at the highest concentration of agent there was a strong band just below the stacking gel (lanes 11 and 17) corresponding to a high degree of polymerization. In these particular lanes, the extra protein at the top of the gel was accompanied by weaker staining of the monomeric LZ band. Addition of lysine to the

Figure 18: Effect of EDAC on the SDS-PAGE profile of lysozyme: each lane was loaded with 10 µl of the preprations described below and run on a 12.5% polyacrylamide gel. From left to right the lanes contain the following:

Lane	Sample
1	SDS-70 MW standards[a]
2	LZ
3	LZ-E(21,3)
4	LZ-E(200)
5	LZ-E(400)
6	LZ-E(800)
7	SDS-200 MW standards[a]

[a] See foootnotes to Figure: 13 (p 94)

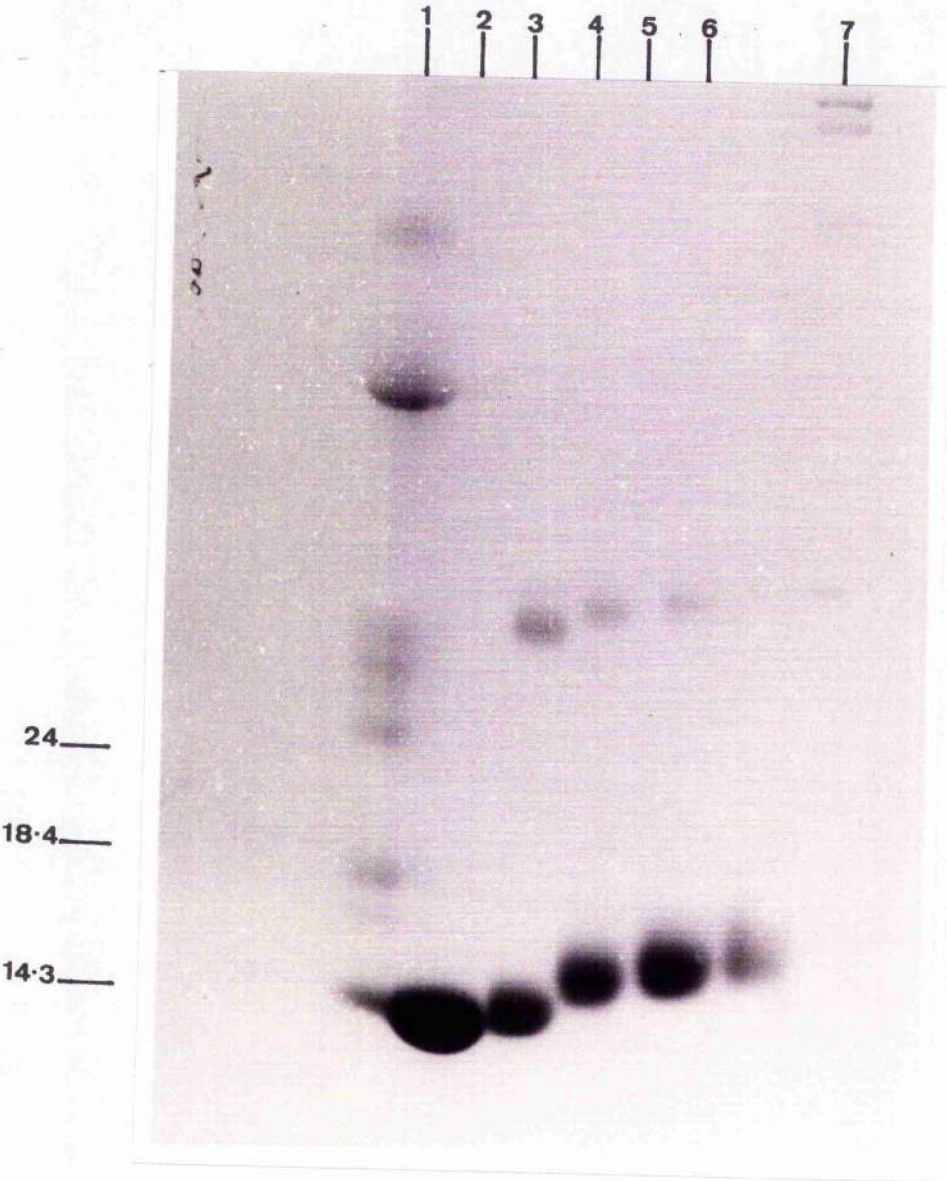
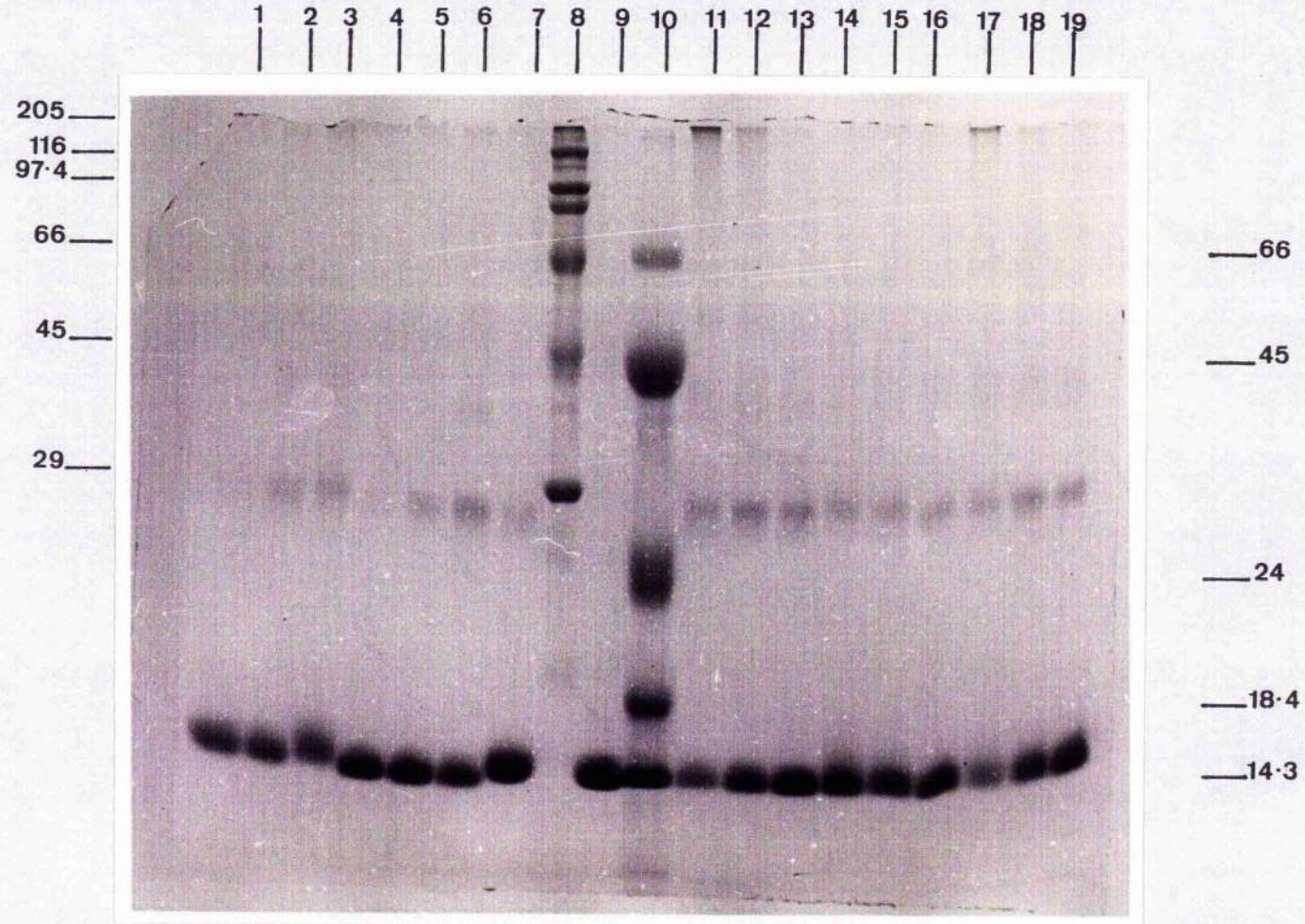

1
2
3
4
5
6
7
24
18·4
14·3

Figure 19: Effect of EDAC, formaldehyde and glutaraldehyde on the SDS-PAGE profile of LZ; each sample was loaded into the lanes of a 12.5% polyacrylamide gel as described below:

Lane	Sample
1	LZ-F(6.25)
2	LZ-F(12.5)
3	LZ-F(25)
4	LZ-F(6.25) Lys(5)
5	LZ-F(12.5) Lys(10)
6	LZ-F(25) Lys(20)
7	LZ-E(21,3)
8	SDS-6H MW standards
9	LZ
10	SDS-7 MW standards
11	LZ-G(2.5)
12	LZ-G(1.25)
13	LZ-G(0.625)
14	LZ-G(2.5) Lys(10)
15	LZ-G(1.25) Lys(5)
16	LZ-G(0.625) Lys(2.5)
17	LZ-G(2.5) Lys(10)[a]
18	LZ-G(1.25) Lys(5)[a]
19	LZ-G(0.625) Lys(2.5)[a]

[a] After glutaraldehyde treatment, lysine was added and the reaction mixture was incubated for a further 15 min before dialysis.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
205
116
97·4
66
45
29
66
45
24
18·4
14·3

system at the beginning of the treatment with glutaraldehyde allowed formation of dimers but appeared to discourage the development of higher polymers.

There appeared to be little effect of formaldehyde or glutaraldehyde on the mobility of the monomeric band and the apparently decreased mobility at the edges is attributed to uneven current during electrophoresis.

Immunogenicity

Effect of EDAC: In an initial experiment on the effect of EDAC on LZ immunogenicity, the enzyme was treated with EDAC at 21 mM. The dose-response of mice to immunization with LZ and LZ-E(21,3) was examined in groups of 5 animals which were injected *i.p* with 0.001, 0.01, 0.1, 1.0 and 10 mg/mouse of the two preparations. Anti-LZ quantitation of the sera by ELISA was done at a starting dilution of 1:100 and the data tabulated (Table 11) as the arithmetic titres, with g.m. values. Those sera with titres <100 were found by extending and extrapolating the line to the A_{492} = 0.5 level. It will be noted that LZ-E was toxic at the top dose in that 4 out of the 5 mice died, the deaths occurring between 24 and 48 h after inoculation. Inspection of Table 12, which summarizes the statistical analysis of these titres, showed that EDAC at 21 mM had no major effect on the immunogenicity of LZ as shown by the non-significant *preparations* term. However, the single surviving animal after the 10 mg dose of LZ-E had a higher titre than any of the five animals with unmodified LZ at 10 mg.

To investigate the toxicity of EDAC-treated LZ, the same preparation as used above was retested with and without further extensive dialysis (2 x 3 days), in case EDAC itself was responsible for the toxicity. Three groups of five mice were injected with 10 mg of LZ or of the two preparations of LZ-E(21,3). The mice were weighed initially and then observed closely for condition and were reweighed (Table 13). Most of the mice showed a small loss in weight after 24 h while at 48 h there were two deaths in each of the two groups receiving LZ-E. Moreover, the surviving animals in

Table 11 : Effect of dose on the immunogenicity of LZ and LZ-E in mice (preliminary experiment). Mice were immunized with five ten-fold spaced dilutions over the range 0.001 to 10 mg/mouse.

Anti-LZ ELISA titre (with group **g.m.**) after immunization (mg/mouse) of;									
LZ					LZ-E(21,3)				
0.001	0.01	0.1	1.0	10.0	0.001	0.01	0.1	1.0	10.0
144	80	4050	6300	6250	100	2010	200	8300	died
115	100	1575	2450	2400	1	118	3425	1350	died
128	448	448	690	3800	125	65	238	1375	10625
11	21	90	1550	1625	18	50	100	7300	died
12	370	370	680	2150	8	100	20	1675	died
49	**123**	**625**	**1622**	**2883**	**18**	**151**	**201**	**4517**	-

Table 12: Analysis of variance ; the effect of dose on the immunogenicity of LZ and LZ-E (preliminary experiment). Summary statistics from the 6 point parallel-line assay are given below.

Source of variation	Mean square	F-value
Slope	7.192	20.63**
Preparations	0.021	0.06
Parallelism	0.031	0.09
Quadratic curvature	0.225	0.64
Difference of quadratics	0.725	0.64
Residual	0.349	-

Tabulated F value for 1 and 24 degrees of freedom at P = 5% is 4.26 (*) and at the P = 1% is 7.82 (**).

Table 13: Toxicity of LZ-E ; groups of five mice were injected with LZ or LZ-E(21,3) at a fixed dose of 10 mg/mouse. Animals were weighed daily and their health observed.

Preparation	Weight of mouse in g (and weight change from day 0) on day :		
	0	1	2
LZ	25	26 (-1)	25 (0)
	25	25 (0)	24 (-1)
	23	24 (+1)	23 (0)
	30	29 (-1)	29 (-1)
	24	24 (0)	24 (0)
		(Av = + 0.2)	(Av = -0.4)
LZ-E(21,3)	26	25 (-1)*	26 (0)**
	23	22 (-1)*	died
	27	26 (-1)*	28 (+1)**
	25	24 (-1)*	died
	27	25 (-2)*	25 (-2)**
		(Av = -1.2)	(Av = -0.3)
LZ-E(21,3) twice dialysed	26	24 (-2)*	died
	26	24 (-2)*	24 (-2)**
	26	24 (-2)*	25 (-1)**
	22	22 (0)*	died
	26	25 (-1)*	27 (+1)**
		(Av = -1.4)	(Av = -0.4)

* sick ; ** moribund, experiment terminated.

these groups were all moribund and had to be put down, whereas the animals given untreated LZ were active and healthy. It seemed clear therefore that EDAC treatment definitely made LZ toxic for mice although a relatively high level (10 mg) had to be used to demonstrate it.

Effect of EDAC and Al(OH)$_3$: As reported above, Al(OH)$_3$ had a significant adjuvant effect with OA and it was therefore of interest to determine whether there would be a similar effect on LZ and LZ-E. For this latter purpose, LZ-E prepared with EDAC at 21 mM was used. The two preparations were mixed with equal weights of Al(OH)$_3$ and inoculated *i.p* into groups of 5 mice at doses of either 0.1 or 1.0 mg per animal in two separate experiments involving a total of 80 mice. Anti-LZ titres were obtained by ELISA on sera taken at 21 days after inoculation. Table 14 presents the individual titres and the g.m. for each group. Fig. 20 is a bar chart plot of these data with 95% CL and shows that (a) both EDAC and Al(OH)$_3$ had an appreciable adjuvant effect and (b) the two doses gave similar high titres.

These two conclusions were confirmed by analysis of variance (Table 15) in which only Al(OH)$_3$ and EDAC were consistently significant variables ($P<1\%$, $P<5\%$, respectively) and the doses term was not significant. None of the interaction terms was significant except doses x Al(OH)$_3$ in one experiment.

Effect of EDAC, formaldehyde and glutaraldehyde : In order to compare the effect of the three different toxoiding agents on the immunogenicity of LZ, experiment C was conducted where some of the same preparations examined above in SDS-PAGE and for enzymic activity were injected into mice. Four preparations of LZ-E , two of LZ-F, and two of LZ-G were compared with untreated LZ at doses of 0.1 and 1 mg in groups of five mice. The particular formaldehyde and glutaraldehyde samples chosen were selected because they showed high levels of inactivation of enzyme activity compared

Table 14 : Effect of EDAC on the immunogenicity of LZ in mice (experiment A and B); also the effect of immunizing the animals with and without $Al(OH)_3$ added to the treated or untreated LZ. The ELISA titres are from two experiments A and B with independently made preparations of EDAC-LZ[a] , LZ-E(21,1) and LZ-E(21,2) respectively..

Anti-LZ ELISA titre of individual mice and group **g.m.** after immunization with (mg) :

	LZ				LZ-E(21)			
	plain		$Al(OH)_3$		plain		$Al(OH)_3$	
	0.1	1.0	0.1	1.0	0.1	1.0	0.1	1.0
Expt A	2500	2371[b]	285	1900	5100	6350	1750	5200
	1950	1100	480	1500	1400	4400	3500	5950
	625	4400	490	11500	2500	6050	2025	8750
	4400	2150	738	3400	1800	2700	1900	950
	1750	1700	325	4300	3400	9000	315	5300
	1879	**2119**	**438**	**3437**	**2557**	**1493**	**5282**	**4236**
Expt B	738	275	7750	18000	13000	430	6400	33000
	430	628	11000	14750	1200	4200	10000	7750
	7000	26500	180	17000	3350	30500	57500	19000
	3000	425	300	14750	3900	2450	20000	43000
	95	2950	29000	32500	9500	7550	4900	47000
	913	**1418**	**2660**	**18493**	**4544**	**3996**	**12924**	**25026**

[a] Mice were immunized with a single-shot dose of antigen and bled 21 days later. $Al(OH)_3$ was used at equal weights of antigen, i.e. 0.1 or 1.0 mg/mouse for the 0.1 or 1.0 mg/mouse dose [b] The missing titre was calculated as outlined in Wardlaw, (1985)

Figure 20: Effect of EDAC and $Al(OH)_3$ on the log_{10} anti-LZ ELISA titres from mice; each bar represents the geometric mean titre from a group of five mice and the vertical line the upper 95% CL. LZ (□), LZ-E (21,1) and LZ-E (21,2) for experiment A and B respectively (▨), LZ with $Al(OH)_3$ (⊡), LZ-E(21,1) and LZ-E (21,2) with $Al(OH)_3$ (▤).

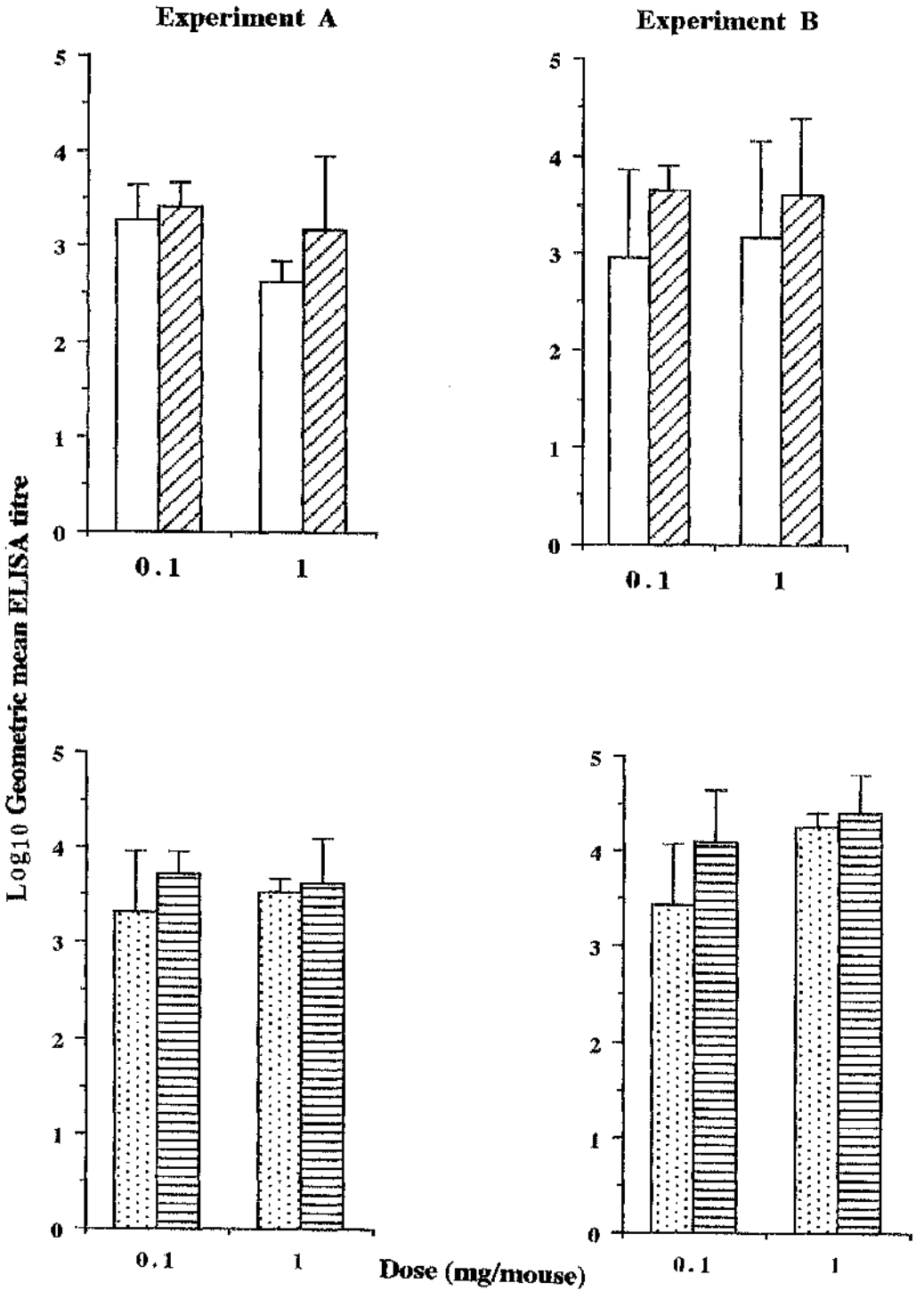


Table 15: Analysis of variance on the effect of dose, EDAC and $Al(OH)_3$ on the production of anti-LZ in mice from two independent experiments A and B. Multifactorial analysis was performed on the $\log_{10}$ anti-LZ titres.

Source of variation	Mean square		F value	
	Expt A	Expt B	Expt A	Expt B
Doses	0.354	0.100	3.93	2.50
$Al(OH)_3$	1.838	5.008	20.42**	12.51**
EDAC	0.832	2.413	9.24**	6.03*
Doses x $Al(OH)_3$	0.602	0.617	6.68*	1.54
Doses x EDAC	0.006	0.402	0.06	1.00
$Al(OH)_3$ x EDAC	0.020	0.068	0.20	0.17
Doses x $Al(OH)_3$ x EDAC	0.311	0.059	3.45	0.15
Residual	0.090	0.400	-	-

Tabulated F value for 1 and 28 degrees of freedom at P = 5% is 4.20 (*) and at the P = 1% is 7.64(**).

with the 30 to 40% inactivation produced by EDAC. Double the number of mice, i.e. two independent groups but with the same LZ preparation, were used for the immunizations with LZ alone. This was done because the sera from these groups were destined to serve as comparators for the sera raised against the various treated LZ preparations. Despite using the same preparation in this way, there were appreciable differences in the medians and the g.m.s from the two plain-LZ groups (Table 16, rows 1 and 2 at 0.1 mg/mouse and rows 11 and 12 at 1.0 mg/mouse). Figure 21 summarizes the results as bar charts with 95% CL for all the groups except LZ-B where the results were suspect.

Statistical analysis of the data was done by assembling them as a series of 4-point parallel-line assays in which each of the treated LZ preparations was compared in turn with both LZ-A and LZ-B. Table 17 shows that substantially different results were obtained depending upon which of the two plain LZ results was taken as standard. Thus with LZ-A, none of the chemical treatments had any significant effect on immunogenicity since the " preparation" terms in the analysis of variance were non-significant. In contrast when LZ-B was taken as standard, all of the chemical treatments had an adjuvanting effect. The table also shows that the chemical treatments had very little effect on the "slope" and "parallelism" terms in the analysis of variance.

The overall conclusion from this experiment was that the chemical treatments, some of which caused more than 99% inactivation of enzymic activity, certainly did not diminish the immunogenicity of LZ but it is difficult to say exactly by how much they may have enhanced it.

Table 16: Effect of EDAC, formaldehyde and glutaraldehyde on the immunogenicity of LZ in mice (experiment C); groups of five mice were immunized with 0.1 and 1.0 mg/mouse of untreated LZ and certain preparations of EDAC-treated LZ, formaldehyde-treated LZ and glutaraldehyde-treated LZ.

Preparation and dose	Anti-LZ ELISA titre (and group **g.m.**)					
0.1 mg/mouse						
LZ-A	2200	5650	158	510	4100	**1327**
LZ-B	102	50	410	10	640	**106**
LZ-E(21,3)	2000	525	910	845	790	**1449**
LZ-E(200)	3050	500	300	950	435	**717**
LZ-E(400)	1250	190	565	338	510	**471**
LZ-E(800)	670	68	545	24	1275	**238**
LZ-F(6.25)	1700	153	2975	920	740	**880**
LZ-F(6.25)Lys(5)	7850	330	5600	820	245	**1239**
LZ-G(2.5)	4350	3650	1400	2750	910	**2234**
LZ-G(2.5)Lys(10)	1650	3125	1150	1950	850	**1580**
1.0 mg/mouse						
LZ-A	230	1400	1115	80	830	**793**
LZ-B	575	130	230	160	590	**269**
LZ-E(21,3)	2525	560	3100	6300	2025	**2235**
LZ-E(200)	890	270	375	325	7050	**730**
LZ-E(400)	3600	2800	285	130	780	**781**
LZ-E(800)	2525	790	225	5600	1900	**1367**
LZ-F(6.25)	655	1150	760	8150	2350	**1614**
LZ-F(6.25)Lys(5)	1145	850	455	8900	6100	**1888**
LZ-G(2.5)	2950	445	540	815	4200	**1193**
LZ-G(2.5)Lys(10)	2180	1720	1975	490	6350	**1874**

Figure 21: Effect of EDAC, formaldehyde and glutaraldehyde on the immunogenicity of LZ in mice (experiment C); each bar represents the geometric mean titre from a group of five mice and the vertical line the upper 95% CL. LZ-E(21,3) (▩), LZ-E(200) (▦), LZ-E(400) (▩), LZ-E(800) (▤), LZ-F(6.25) (◰), LZ-F(6.25)Lys(5) (▤), LZ-G(2.5) (◧) and LZ-G(2.5)Lys(10) (◪). LZ (□)

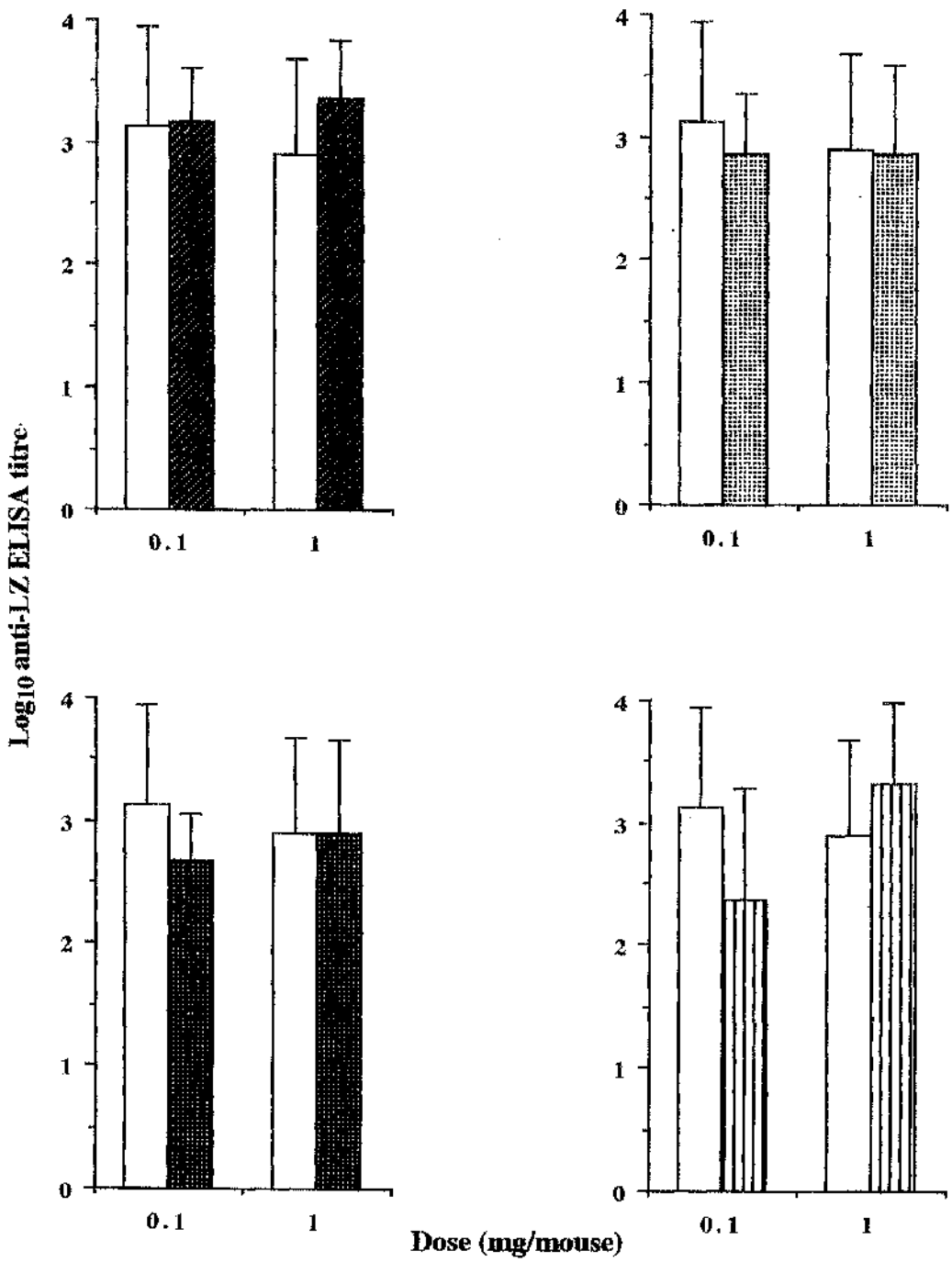


Figure 21 continued

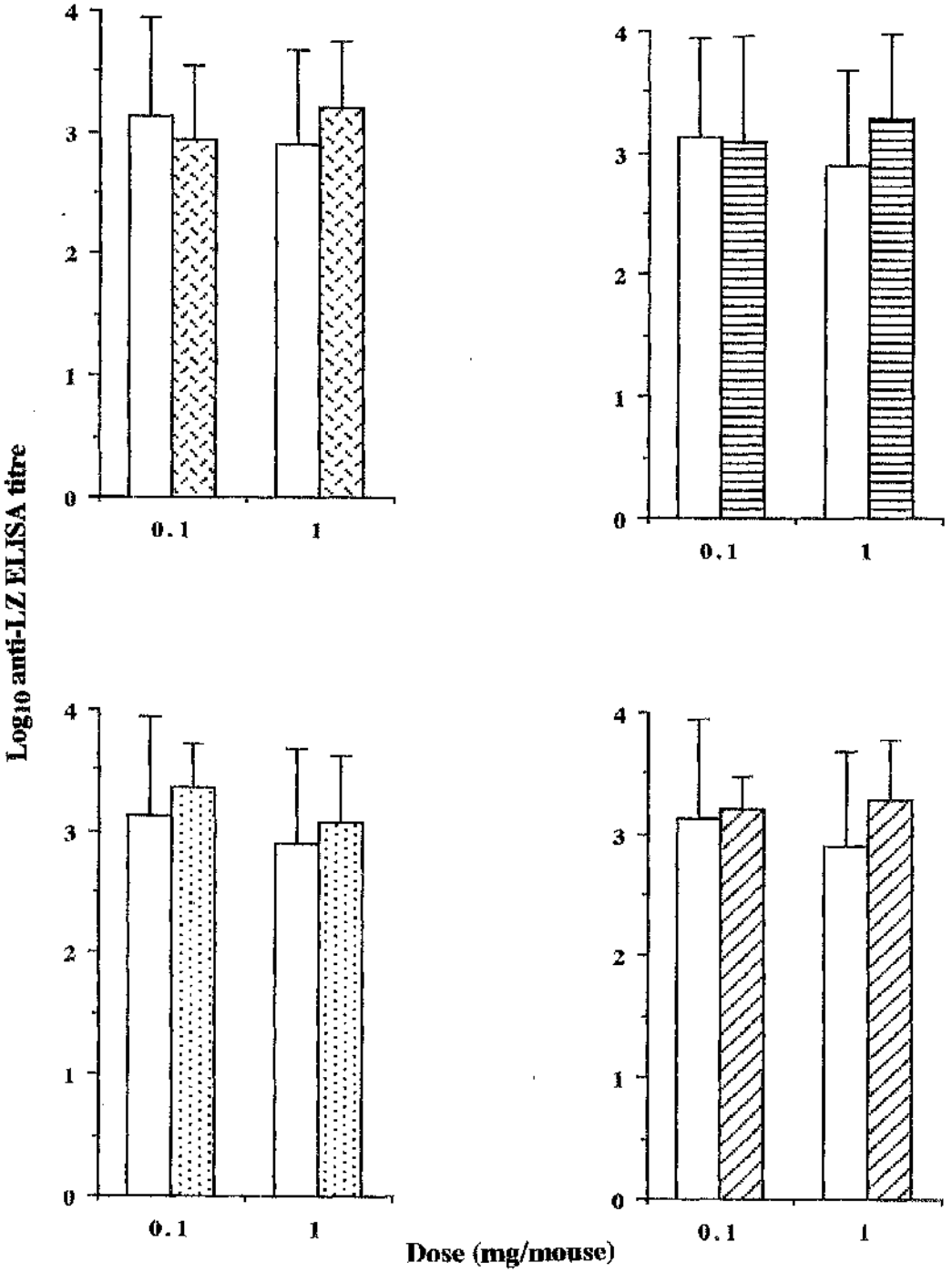


Table 17: Analysis of variance; effect of EDAC, formaldehyde and glutaraldehyde on the immunogenicity of LZ (experiment C). For each preparation , replicate ELISA titres from groups of five mice were analysed against two untreated LZ samples, LZ-A and LZ-B by the 6 point parallel-line assay. F-values and the level of significance is given below.

	Source of variation :					
Sample	Preparation		Slope		Parallelism	
	LZ-A	LZ-B	LZ-A	LZ-B	LZ-A	LZ-B
LZ-E(21,3)	0.31	24.97**	0.31	4.77*	2.34	0.00
LZ-E(200)	0.03	9.78**	0.90	1.13	0.97	1.05
LZ-E(400)	0.31	7.77*	0.30	2.61	2.56	0.25
LZ-E(800)	0.27	4.89*	0.33	6.20*	4.87*	0.53
LZ-F(6.25)	0.44	13.90**	0.12	2.27	1.77	0.12
LZ-F(6.25)Lys(5)	0.86	12.22**	0.18	1.22	1.05	0.19
LZ-G((2.5)	2.14	24.70**	2.81	0.13	0.17	3.06
LZ-G(2.5)Lys(10)	3.22	30.80**	0.99	1.85	1.93	0.90

Tabulated F value for 1 and 12 degrees of freedom at P = 5% is 4.75 (*) and at the P = 1% is 9.33 (**).

EFFECT OF TOXOIDING AGENTS ON *B. PERTUSSIS* ANTIGENS

Extraction and Characterization of PT and FHA

Pertussis toxin (PT), FHA and PT:FHA mixed antigen preparations were extracted respectively from *B. pertussis* 353, *B. pertussis* 357 and *B. pertussis* 77/18319. Strains 353 and 357 were Weiss Tn5 mutants of *B. pertussis* 338 (Weiss *et al.*, 1983). These strains were selected as they allowed the purification of PT and FHA preparations free from each other.

An affinity-chromatography method using Blue-Sepharose CL-6B which contains the dye Cibacron blue (a structural analogue of NAD) was chosen as it had affinity for both PT and FHA (Sekura *et al.*, 1983). Thus extraction of both antigens was possible with the same batch of chromatography medium regenerated between separate runs.

Fractions were monitored for absorption at 280 nm, pooled, dialysed then tested for protein content. Table 18 lists the protein yields obtained from twelve FHA extractions, five PT extractions and four PT:FHA mixed-antigen preparations. Protein yields were usually higher after extraction of FHA from *B. pertussis* 357 (a.m.= 2.33 mg/L) than PT (a.m.= 1.26 mg/L) or PT:FHA (a.m.= 1.05 mg/L) preparations.

Samples were run on SDS-containing polyacrylamide gels to confirm the presence of bands corresponding to the known MWs for PT and FHA. Figure 22 shows the SDS-PAGE profiles of FHA-containing extracts from culture supernates of *B. pertussis* 357 obtained after staining of the gel with Coomassie blue. Intense bands corresponding in MW to the 205 KDa myosin marker are visible and are consistent with the 200 KDa of undegraded FHA. The ladderlike patterns below the high MW bands appear to be degradation products of FHA. As seen from Figure 22, FHA-1 had fewer contaminating bands than FHA-6 and FHA-7. Contamination with bands in the

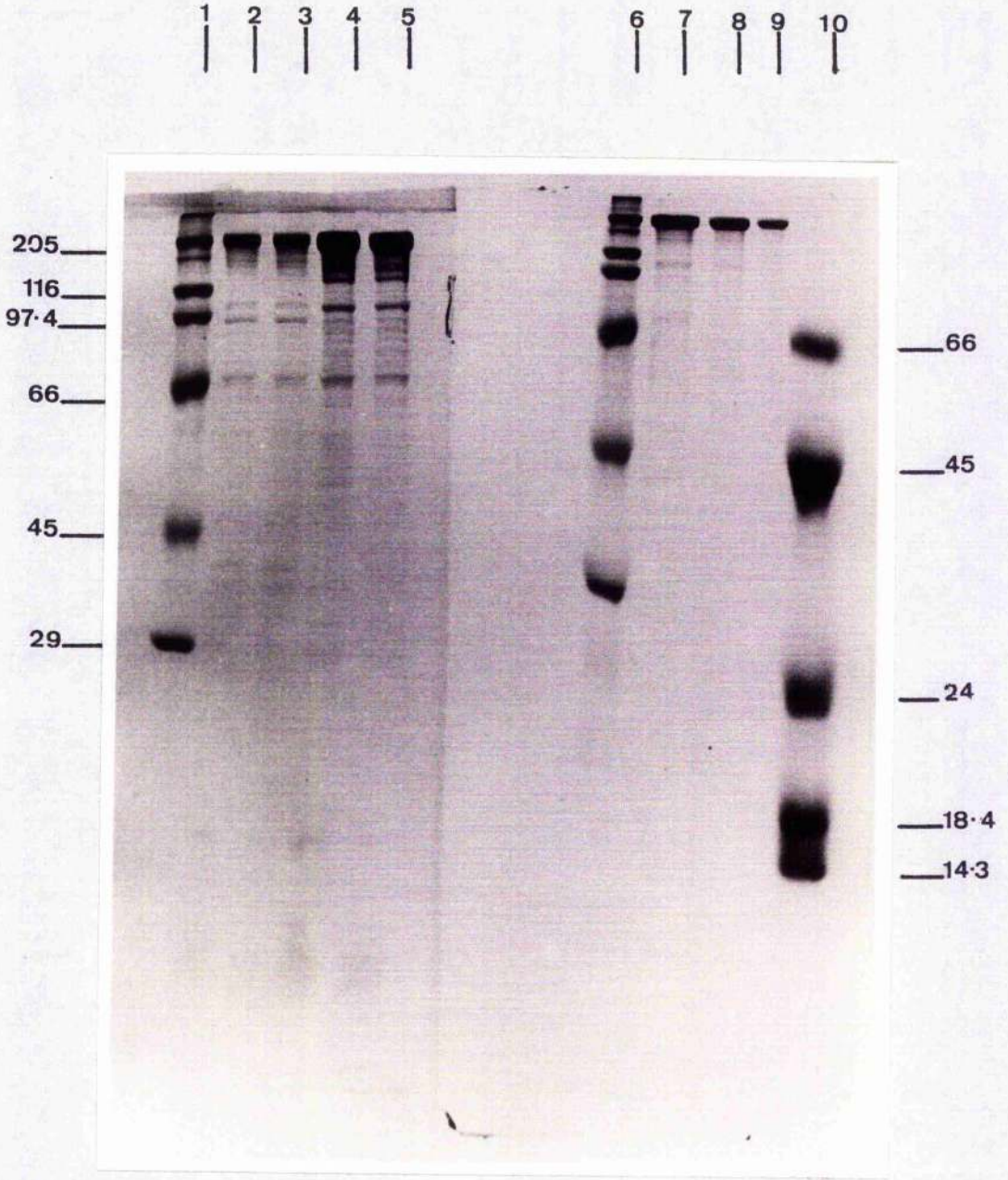
Table 18 : Protein yields from extracts of culture supernates of *B. pertussis* prepared by gel-affinity chromatography; arithmetic means are given in bold with SEM in parentheses.

Batch no.	Strain	Volume of culture (L)	Protein yield (mg/L)
FHA-1	B.p. 357	6	3.7
FHA-2	''	4	1.3
FHA-3	''	4	1.8
FHA-6	''	6	1.7
FHA-7	''	6	1.4
FHA-8	''	6	2.1
FHA-9	''	10	3.4
FHA-10	''	10	3.0
FHA-11	''	10	3.3
FHA-13	''	10	3.3
FHA-14	''	9	0.8
FHA-15	''	9	2.2
			2.33
			(0.28)
PT-1	B.p. 353	6	1.4
PT-2	''	6	1.8
PT-3	''	6	1.0
PT-4	''	9	0.6
PT-6	''	10	1.5
			1.26
			(0.21)
PT:FHA-1	B.p. 77/18319	6	1.3
PT:FHA-2	''	8	0.9
PT:FHA-3	''	10	1.0
PT:FHA-4	''	10	1.0
			1.05
			(0.09)

Figure 22 : SDS-PAGE profile of FHA preparations extracted from *B. pertussis* ; lanes were loaded with 20 µl of sample onto a 12.5% polyacrylamide gel. Each lane contains the following :

Lane	Sample
1	Sigma marker MW-SDS-70[a]
2	FHA-6
3	FHA-6
4	FHA-7
5	FHA-7
6	Sigma marker MW-SDS-200[a]
7	FHA-1
8	FHA-1
9	FHA-1
10	Sigma markers MW-SDS-70[a]

[a] See footnote to Figure 13 (p 94)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97·4
66
45
29
66
45
24
18·4
14·3

region of 14 to 28 KDa, corresponding to subunits of PT, was undetectable.

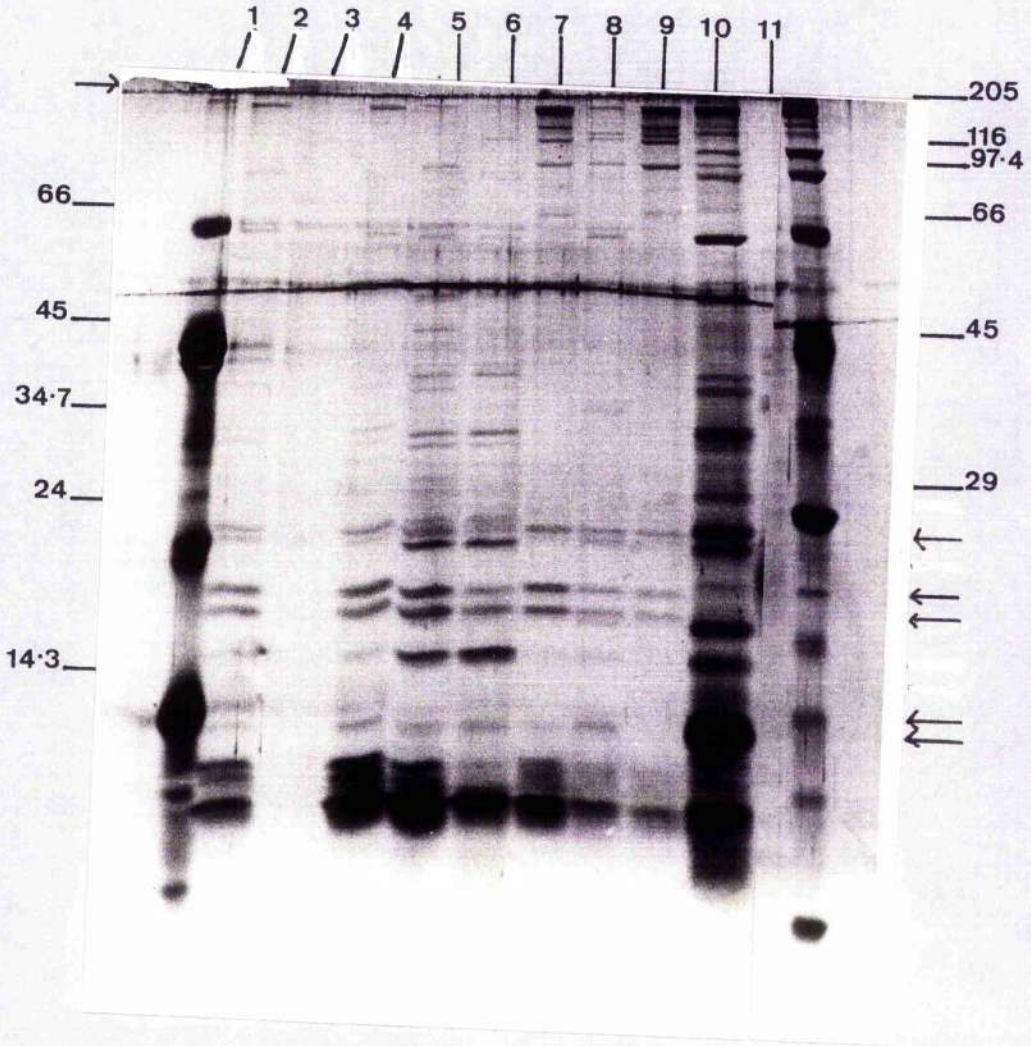
Figure 23 shows SDS-PAGE profiles of PT and PT:FHA mixed antigen preparations after silver staining of the gel. A number of independently-purified preparations of PT and PT:FHA were obtained and run on the gel for purposes of comparison. The bands thought to correspond best with reported sizes for the subunits of PT and for FHA are indicated by arrows on the figure. Lanes 2, 4 and 6 contain PT preparations extracted from *B. pertussis* 353. Independently extracted preparations of PT can be seen in lanes 5 and 6. Similarly, lanes 7 and 8 contained PT:FHA-4 and an antigen mixture prepared by the same procedure independently; for comparison, lanes 9 and 10 show independently-purified mixtures of PT and FHA. It is clear from the picture that the preparations contain many other unidentified contaminating proteins. A faint band corresponding to the 205 KDa marker was noted in lanes 2 and 4 even though the strain from which the preparation was extracted had a transposon insertion mutation disallowing the production of FHA. As adenylate cyclase (ACT) also is 200-210KDa, this band could perhaps have been ACT. These preparations were not further purified and samples PT:FHA-4, PT-6 and AP16 were used for toxoiding and immunization of animals.

Western blotting was used to confirm that the extracts did contain PT and FHA. Probing of the protein bound to nitrocellulose with an anti-PT monoclonal antibody directed against the S_1 subunit (L_{10}) and an anti-FHA polyclonal antibody conjugated to horse radish peroxidase (F_3- HRP) were used. This process would also indicate whether the PT and FHA extracts from mutant strains were free from contamination by each other. Figure 24 shows the SDS-PAGE profile of certain PT, FHA and PT:FHA preparations of an acrylamide gel stained with Coomassie blue after transfer of the proteins onto nitrocellulose. The gel shows prominent bands in lanes 2, 3 and 4 corresponding to the S_1, S_2 and S_3 subunits of PT. These bands are also faintly

Figure 23 : SDS-PAGE profiles of PT and mixed antigen preparations of PT and FHA. The 12.5% polyacrylamide gel was stained with silver. Each lane contains the following :

Lane	Sample	µg/lane
1	Sigma marker MW-SDS-70[a]	5
2	PT-5	2
3	PT-5(a fetuin sepharose extract)	0.5
4	PT-6	2
5	PT (independently purified reference)	2
6	PT(independently purified from *B.pertussis* 353)	2
7	PT:FHA-4	2
8	AP16(independently purified PT:FHA sample)	1
9	PT:FHA(independently purified PT:FHA sample)	2
10	PT:FHA(purified from fermentor grown organisms)	2
11	Sigma marker MW-SDS-200[a]	5

[a] See footnote to Figure 13 (p.94)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97·4
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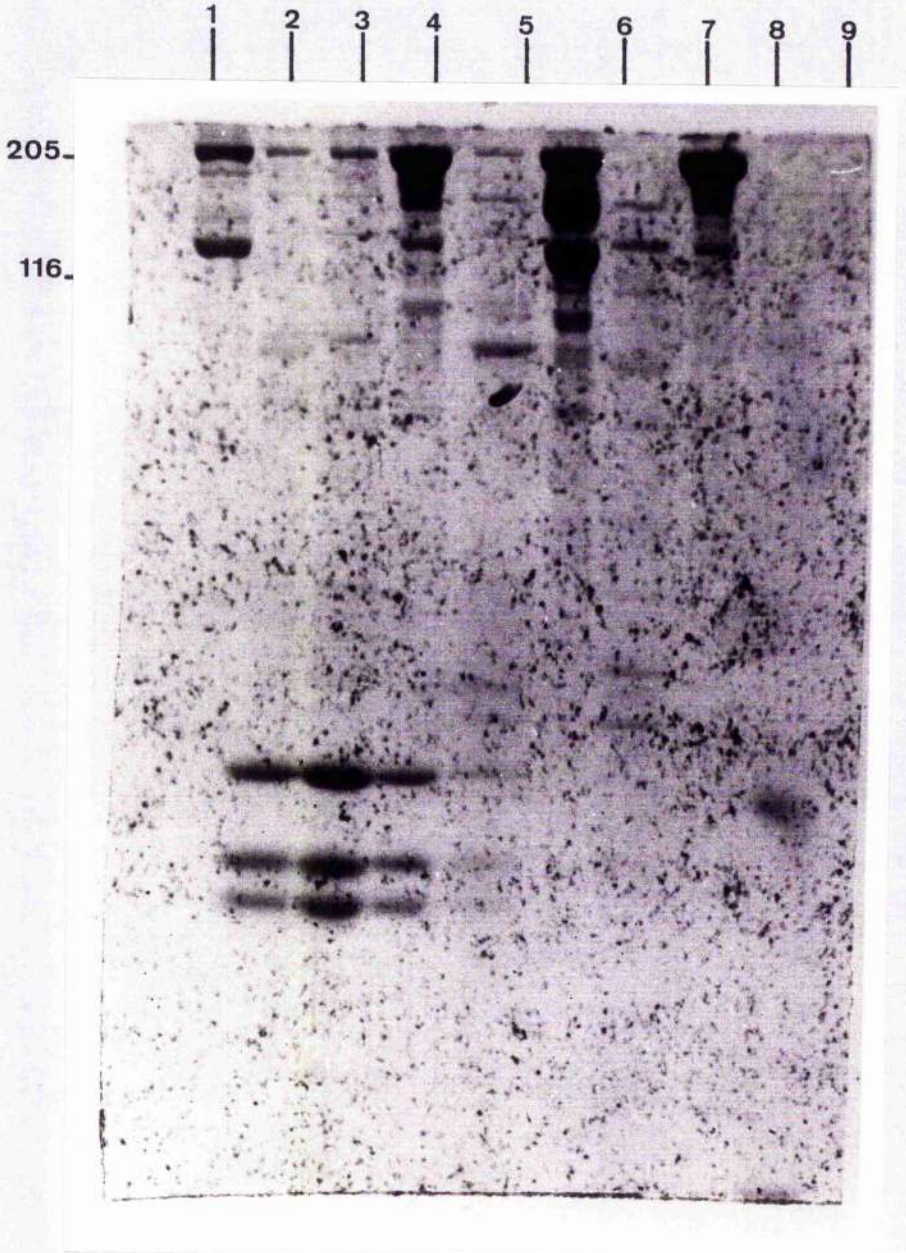
45
29
66
45
34·7
24
14·3

Figure 24 : SDS-PAGE profile of PT, FHA and PT:FHA mixed antigen preparations purified from *B.pertussis* 353, 357 and 77/18319 respectively. Each lane was loaded onto a 12.5% polyacrylamide gel with 20 μg of sample as following :

Lane	Sample
1	Sigma marker MW-SDS-200[a]
2	PT-6
3	PT-5
4	PT:FHA-3
5	PT:FHA-2
6	FHA-15
7	FHA-14
8	CAM-R FHA
9	Sigma marker MW-SDS-70[a]

[a] See footnote to Figure 13 (p 94)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present in lane 5. There were fewer contaminating bands seen on this gel for PT-5 and PT-6 preparations probably due to their transfer onto nitrocellulose. Heavily stained bands of 205 KDa are also clearly visible which may be FHA in lanes 4, 6 as they correspond to the purified FHA (CAMR) run in lane 8. This gel was run in duplicate to allow samples to be probed with both anti-PT or anti-FHA specific antibody. Each lane was loaded with 20 μg of protein. Antigens were transferred at 80 mA in a tank containing transfer buffer. Blots were developed with chloronaphthol and shown in Figure 25. Figure 25a presents the blot probed with anti-PT L_{10} and Figure 25b the blot probed with polyclonal anti-FHA. It was noticed that both the gel and blots had background mottling after staining. By comparing Figure 25a and Figure 25b, PT was present in preparations PT-6, PT-5, PT:FHA-2 and PT:FHA-3. Using this method, no PT was detected in FHA-14, FHA-15 or the CAMR FHA. From 25b, FHA was found in PT:FHA-3, FHA-15 and the reference CAMR FHA. Surprisingly, no FHA was detected in FHA-14 which was extracted from *B. pertussis* 357. Neither was any detected in PT-5, PT-6 and PT:FHA 2. Nevertheless, there was no cross contamination of PT or FHA extracts from the mutant strains as far as could be determined by this method.

PT-6, PT:FHA-3 and PT:FHA-4 were tested for histamine-sensitizing activity which reflects the potency of PT present in these preparations. These preparations were inoculated into groups of two mice > 6 weeks old at a range of dilutions and challenged five days later with three mg/mouse of histamine. Mouse deaths were recorded and the HSD_{50} was estimated. The HSD_{50} for both PT-6 and PT:FHA-4 was 250 ng, and 1000 ng for PT:FHA-3.

A pooled batch of PT preparations 1, 2 and 3 and PT-6 was tested for its ability to induce clustered growth of chinese hamster ovary (CHO) cells. Figure 26 shows photographs of control CHO cells and cells treated with PT. Cells exhibited the morphology typical after exposure to PT, with clumped growth (Figure 26b) rather than the spreading pattern of control cells treated with buffer alone (Figure 26a).

Figure 25: Western blots of PT, FHA and PT:FHA antigen preparations. The gel shown in figure 24 was blotted onto nitrocellulose and the proteins transferred. Blot (a) was probed with a 1 in 1000 dilution of anti-PT monoclonal L_{10} and blot (b) with a 1 in 1000 dilution of anti-FHA polyclonal F_3 conjugated to HRP.

Blot (a)		Blot(b)	
Lane	Sample	Lane	Sample
1	PT-6	1	PT:FHA-3
2	PT-5	2	FHA-15
3	PT:FHA-3	3	CAM-R FHA
4	PT:FHA-2		

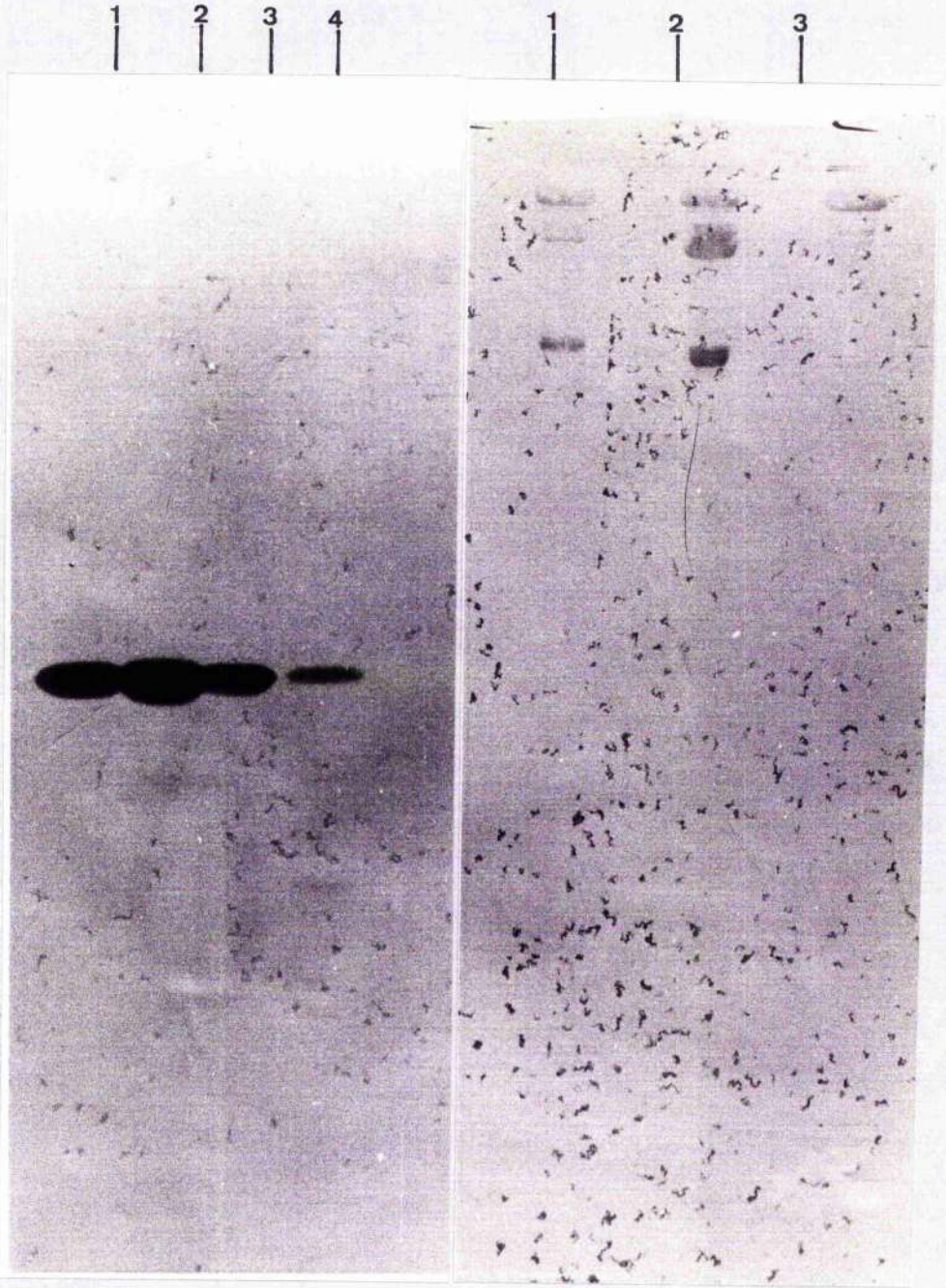
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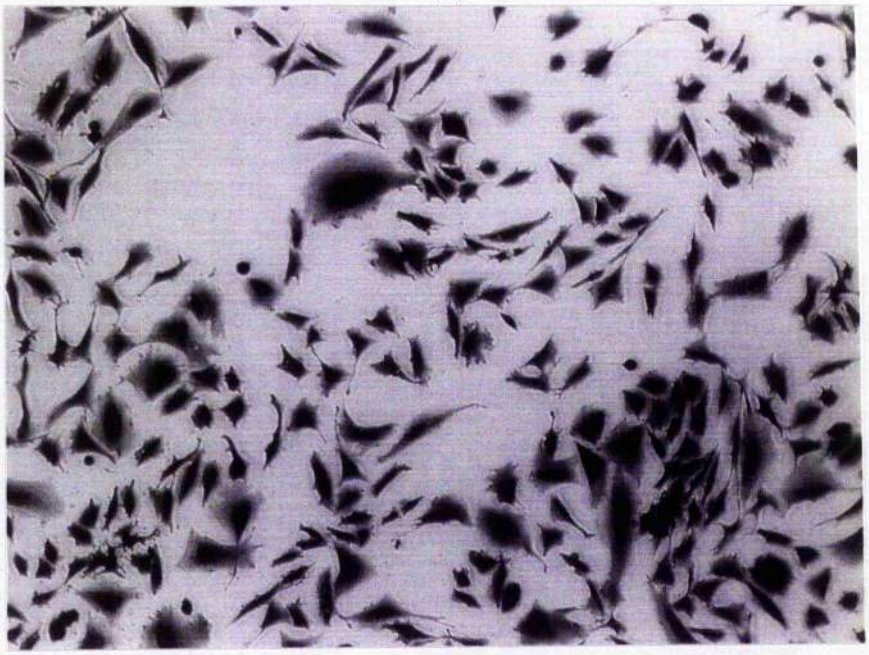
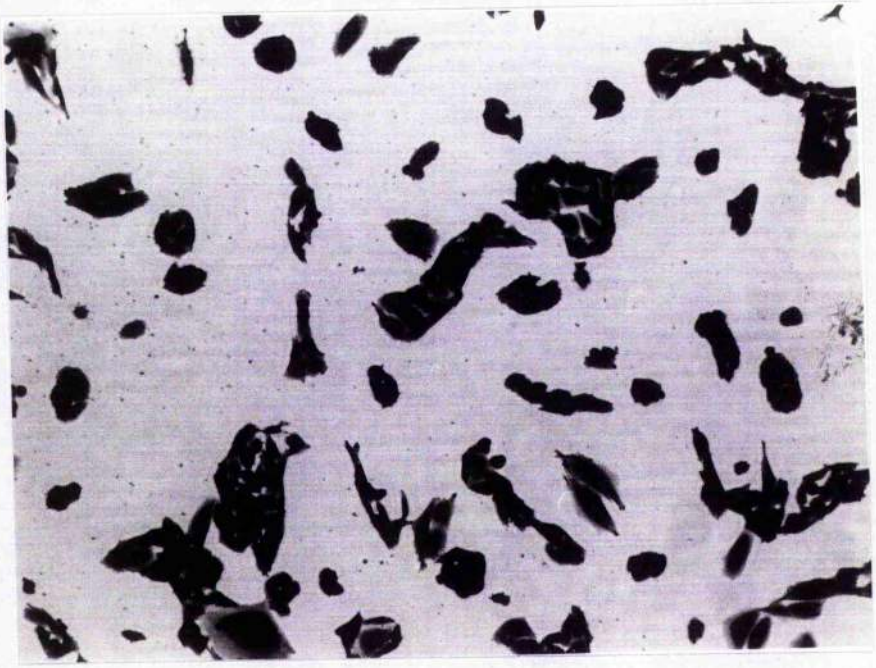
Figure 26 : Effect of PT on the morphology of Chinese Hamster Ovary (CHO) cells. Cells were exposed to (a)100 mM Na_2HPO_4 + 0.5M NaCl, pH 7.0 or (b) 12.5 ng a pooled PT preparation of PT-1, PT-2 and PT-3. Magnification x100.

(a)



(b)



PT:FHA preparations used for toxoiding and subsequent immunisation of mice were assayed for the quantity of each antigen present in the mixture by ELISA. Using a sandwich ELISA method with fetuin as the coating phase, PT was probed with an anti-PT monoclonal antibody L_{10}. Similarly, F_2 IgG anti-FHA capture antibody was the coating phase in the sandwich ELISA technique used for the detection of FHA. Standard PT was supplied (from Dr. R. Parton) while the FHA standard came from CAMR. All samples were adjusted to 1 μg/ml, with three fold dilutions of each preparation being made in PBS, pH 7.4 + 0.01% Tween 20. A plot of volume (ml) added per well of the 1 μg/ ml solution against A 492 was made. The volume of sample required to reach an A 492 = 1.2 was noted and used to calculate the amount of PT or FHA present in these preparations relative to each standard. The following PT : FHA ratios were calculated for each sample. PT:FHA-2, 4:1, PT:FHA-3, 2:3 and PT:FHA-4, 1:5. It was difficult to judge whether these results agreed with SDS-PAGE gel profiles PT:FHA-2 (Figure 24, lane 5), PT:FHA-3 (Figure 24, lane 4) and PT:FHA-4 (Figure 23, lane 7). PT:FHA-3 and PT:FHA-4 were used for toxoiding and immunization.

Experiments with FHA

In vitro effects of EDAC, formaldehyde and glutaraldehyde

Effect on HA activity : Despite FHA being non-toxic, the treatment with toxoiding agents was done under the same conditions which were also employed for the detoxification of PT, except that with formaldehyde the time was reduced from one week to 48 h. A summary of the standard treatment conditions with the three agents is presented in Table 19. However, independently of these standard conditions, the effect of varying the concentration, temperature and time was also investigated to provide additional information.

Table 19 : Summary table of standard treatment conditions for exposure of FHA to toxoiding agents.

Toxoiding agent	Concentration of FHA (µg/ml)	Concentration of agent (mM)	Temperature (°C)	Time (h)	Lysine (mM)
Formaldehyde	250	25	37	48	20
Glutaraldehyde	250	2.5	37	2	10
EDAC[a]	250	21	37	24	N/A

[a] Toxoiding conditions used by Christodoulides *et al.* (1987) for the detoxification of PT.

Loss of the HA activity of FHA was used to indicate the extent to which this protein had been modified by the various toxoiding treatments. In the case of treatments with formaldehyde and glutaraldehyde, the effect of adding lysine to the reaction mixtures was also investigated.

With formaldehyde, a modification of published conditions for inactivation of diphtheria toxin (Porro *et al.*, 1980) and pertussis toxin (Sato *et al.*, 1984) was used both as the basis for the standard conditions and for the ancillary investigation. In the context of the latter, FHA-13 was adjusted to 250 μg/ml with PBS, and treated for 48 h at 37 °C with four concentrations of formaldehyde, 3.12, 6.25, 12.5 and 25 mM. In each of these mixtures a final lysine concentration of 20 mM was included. After the treatments, the samples were dialysed against daily changes of 2 L PBS, pH 7.4, for one week at 4 °C.

The treated preparations were assayed for HA activity with a 2% suspension of horse RBCs, and the percent HA loss determined by comparison with untreated FHA. This latter was a solution of FHA-13, diluted to 250 μg/ml in PBS and stored at -20 °C to minimize degradation. The untreated FHA-13 had an HA titre of 14 on a $\log_2$ scale (Table 20). The lowest concentration of formaldehyde tested, 3.12 mM, was sufficient to reduce the HA activity by 87.5%. Thereafter, increasing the concentration to 6.25 mM and higher caused loss of all detectable activity i.e > 99.9%.

In a separate experiment the formaldehyde was kept constant at 25 mM for treatment of another batch of FHA (FHA-3) which was used at 250 μg/ ml at three different temperatures: 4 °C, RT (22 °C) and 37 °C. Samples were removed at 24 and 48 h, dialysed and assayed for HA activity as before. Untreated FHA-3 had a titre of 128 from which the percentage inactivation results shown in Table 21 were calculated. It was found that approx. 50% loss of HA activity occurred without formalin, i.e. as a result of 48 h exposure at 4, 22 and 37 °C, followed by dialysis. With formaldehyde the % loss in HA increased upon increasing the time from 24 to 48 h.

Table 20 : Effect of formaldehyde concentration on the loss of HA activity of FHA. FHA-13 (250 µg/ml) was treated with four concentrations of formaldehyde in PBS, pH 7.4, after which lysine was added as a neutralising agent at 20 mM, for 48 h at 37 °C [a].

Formaldehyde (mM)	Formaldehyde % w/v	% loss in HA activity
3.12	0.009	87.5
6.25	0.019	≥99.9
12.5	0.037	≥99.9
25.0	0.074	≥99.9

[a] FHA-13 stored at -20° C, was used as a standard on each HA test plate. No drop in HA activity was observed after incubating FHA-13 alone or with lysine (20mM) for 48 h at 37 °C.

Table 21 : Effect of temperature during formaldehyde treatment on the HA activity of FHA. FHA-3 at 250 μg/ml was treated with formaldehyde for 24 h or 48 h at 37°C with a fixed concentration of 25 mM formaldehyde in PBS, pH 7.4, with or without the addition of lysine (20mM).

FHA-3 treatment	Temperature (°C)	% loss in HA activity 24 h	48 h
control[a]	22	0	0
FHA	4	0	50
FHA	22	0	50
FHA	37	0	50
Formaldehyde FHA(FHA-F)	4	88	88
FHA-F	22	94	97
FHA-F	37	94	>99
Formaldehyde FHA with lysine(FHA-F, Lys)	4	75	75
FHA-F, Lys	22	75	>99
FHA-F,Lys	37	94	>99

[a] FHA-3 was stored at -20°C prior to use as a standard on each HA plate.

Adding lysine to the system appeared to cause less loss of activity than with formaldehyde alone, especially during the first 24 h (Table 21). However at 48 h, the difference was only appreciable with the sample treated at 4 °C.

For the glutaraldehyde treatments, the conditions chosen both for standard and exploratory treatments were modifications of those used by Relyveld (1978) with a variety of antigens, and by Munoz *et al.* (1981a) with pertussis toxin. For the exploratory treatments of FHA, the following procedure was applied: glutaraldehyde at 2.5 mM in PBS, with 10 mM lysine, at 37°C, with samples being removed at 15, 30 , 60 and 120 min. They were then immediately put to dialyse at 4 °C against 2 L PBS for three days with daily changes of buffer. The results in Table 22 show that there was complete, or almost complete inactivation of HA, and with little significant effect of adding the lysine. Compared with formaldehyde, glutaraldehyde caused much more rapid HA inactivation. For example, within 15 min, only 2-3 % of HA activity remained. As controls, incubating FHA-7 alone with or without lysine for 2 h at 37 °C showed no detectable loss of HA activity.

The treatment of FHA with EDAC was applied according to Christodoulides *et al.* (1987) for pertussis toxin, except that FHA was used at 250 µg/ml protein instead of 50 µg/ml of PT. As with the other two agents, exploratory treatments were investigated independently of the standard treatment. For the exploratory treatments, the percentage loss in HA activity after treating FHA-7 and FHA-13 with EDAC at various concentrations are shown in Table 23a and 23b respectively. All these treatments were done in 20mM phosphate buffer, pH 5.0 for 24 h at 37 °C. With FHA-7 (Table 23a), the treatments with 0, 2.4, 12.5, 25 and 50 mM EDAC were sampled at 1, 3, 6 and 24 h and dialysed for 3 days against daily changes of 2 L PBS. As is apparent from Table 23a, no loss of HA activity was observed in either the 0 mM EDAC control preparation, or in any of the reaction mixtures containing EDAC, until 24 h. Increasing

Table 22 : Effect of time during glutaraldehyde treatment on the HA activity of FHA. FHA-7 (250 mg/ml) was treated with glutaraldehyde

Table 22 : Effect of time during glutaraldehyde treatment on the HA activity of FHA. FHA-7 (250 mg/ml) was treated with glutaraldehyde at a fixed concentration of 2.5 mM glutaraldehyde in PBS, pH 7.4 with or without the addition of lysine (10 mM) at 37 °C.

Time (min)	% loss of HA activity	
	FHA-G	FHA-G + lysine
15	97	98
30	98	99
60	>99	>99
120	>99	>99

FHA-7 was used as a standard on each plate. No drop in HA activity was observed after incubating FHA-7 alone or with lysine (10mM) at 37°C.

Table 23a: Effect of EDAC on the HA activity of FHA; FHA-7 (250 µg/ml) was treated with EDAC at 4 levels in 0.02M phosphate buffer, pH 5.0 for 1, 3, 6 and 24 h at 37 °C.

EDAC (mM)	% loss in HA activity at 1	3	6	24 h
0	0	0	0	0
2.4	0	0	0	51
12.5	0	0	0	51
25.0	0	0	0	51
50.0	0	0	0	75

the concentration of EDAC approximately 10 fold, from 2.4 to 25.0 mM appeared to cause no additional reduction in activity from 51% although with the 50 mM EDAC, inactivation reached 75%. In a second experiment with a different batch of FHA and different EDAC concentrations, the results were generally similar (Table 23b) i.e. within one two-fold dilution in the HA assays. The maximum percent inactivation of HA in this second preparation, FHA-13, was 75% as before.

The FHA preparations used in the immunization experiments were treated according to the conditions summarized in Table 19. The results of these standard treatments are presented in Table 24 where three independently-extracted batches of FHA were exposed to the three toxoiding agents. Generally, formaldehyde and glutaraldehyde treatment resulted in the vast majority of HA activity consistently being lost, with only a residual level of 1% activity remaining. For EDAC however, the inactivation was much less complete, lying in the range of 50-75%. With control preparations containing no toxoiding agents there was little drop in activity after incubation for 24 and 48 h at 37 ^{o}C. Only a 50% loss in activity was noted for FHA-8 after the 48 h period.

Effect on SDS-PAGE profiles : A representative selection of the FHA preparations from above was analyzed by SDS-PAGE, with the results shown in Figs. 27. With untreated FHA-13 (stored at -20 ^{o}C), there was an intense band (lane 12) corresponding to the 205 KDa marker in lane 1. The ladder-like patterns below this band are presumably degradation products. There seemed to be no difference in the profiles of FHA which had been incubated at 37 ^{o}C either for 24 h or 48 h in 20 mM phosphate, pH 5.0 (lane 8) or PBS, pH 7.4 (lane 2).

Treatment with formaldehyde produced major changes in gel profiles. In particular, there were (a) bands corresponding to MW >205 KDa suggesting that polymerization had occurred; and (b) losses of the ladder pattern in the region of 98 to 205 KDa. The faint pattern in lane 6 may reflect an error in loading the gel.

Table 23b: Effect of EDAC on the HA activity of FHA; FHA-13 (250 μg/ml) was treated with EDAC at various concentrations in 0.02 M phosphate buffer, pH 5.0 for 24 h at 37 °C.

EDAC (mM)	% loss in HA activity
0	0
2.3	75
7.0	75
21.0	75

Table 24: Summary of three experiments on the effect of EDAC, formaldehyde and glutaraldehyde on the HA activity of FHA. Treatment conditions were as described in the text. No loss in HA activity was observed with FHA alone when incubated for 2, 24 and 48 h at 37 °C. Only FHA-8 showed a 50% loss in activity after 48 h at 37 °C.

Preparation[a]	% loss in HA activity
FHA 8-F	>99
FHA 9-F	>99
FHA 13-F	>99
FHA 8-G	99
FHA 9-G	99
FHA 13-G	>99
FHA 8-E	75
FHA 9-E	75
FHA 13-E	50

[a] The letters F, G and E refer to the treatments with formaldehyde, glutaraldehyde and EDAC respectively.

Figure 27 : SDS-PAGE profile of FHA and the effect of EDAC and formaldehyde treatments; Each lane of the 7.5% polyacrylamide gel was loaded with 6 μg protein. Formaldehyde and EDAC treatments of FHA-13 were done as detailed below.

Lane no.	Preparation	Concentration of toxoiding agent (mM)	Conditions of treatment
1	MW-SDS-200 kit[a]	-	-
2	FHA	0	48 h in PBS and 20 mM lysine
3	FHA-F	25	"
4	FHA-F	12.5	"
5	FHA-F	6.25	"
6	FHA-F	3.12	"
7	FHA	0	in PBS, kept frozen until use
8	FHA	0	24 h in 20 mM phosphate, pH 5.0
9	FHA-E	2.3	"
10	FHA-E	7.0	"
11	FHA-E	21.0	"
12	FHA	0	in 20 mM phosphate, kept frozen until use
13	MW-SDS-70 kit[a]	-	-

[a] See footnote to Figure 13 (p94)

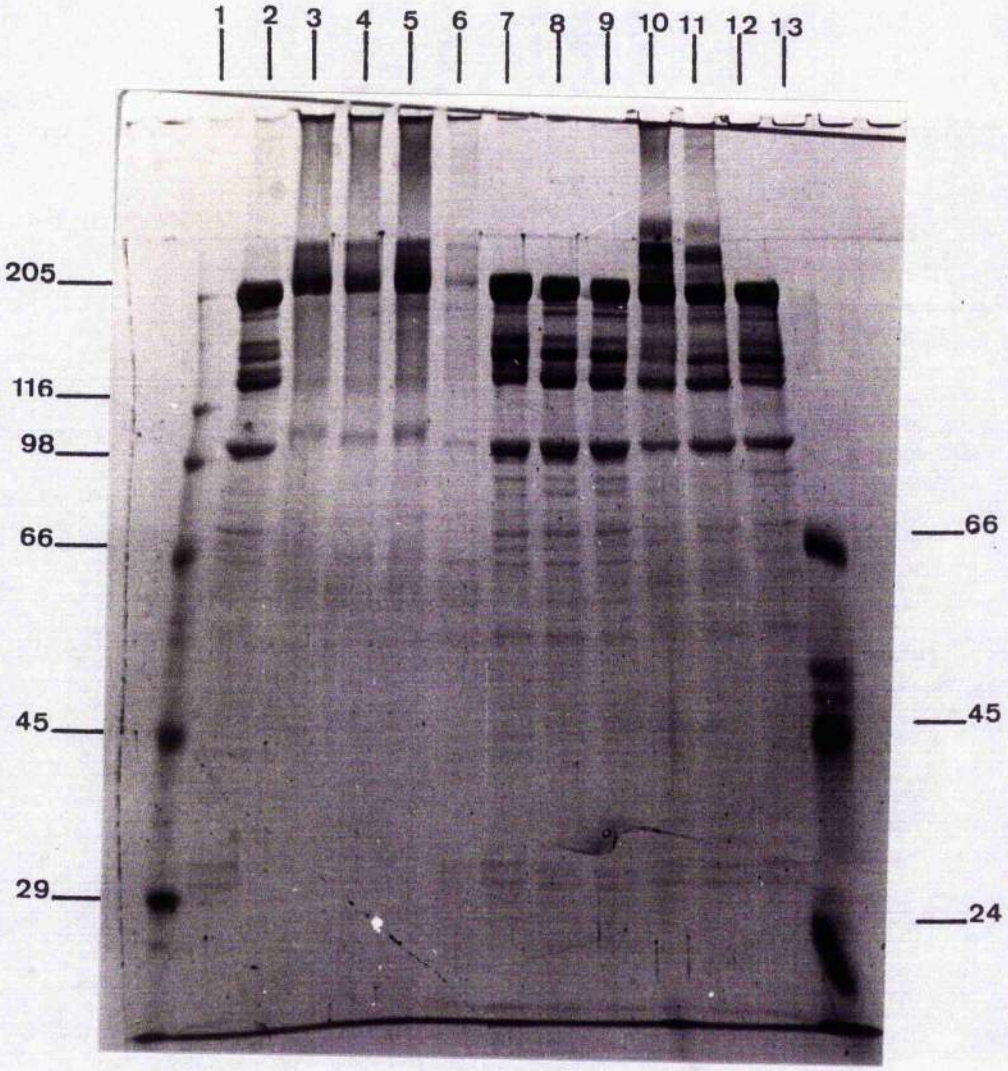
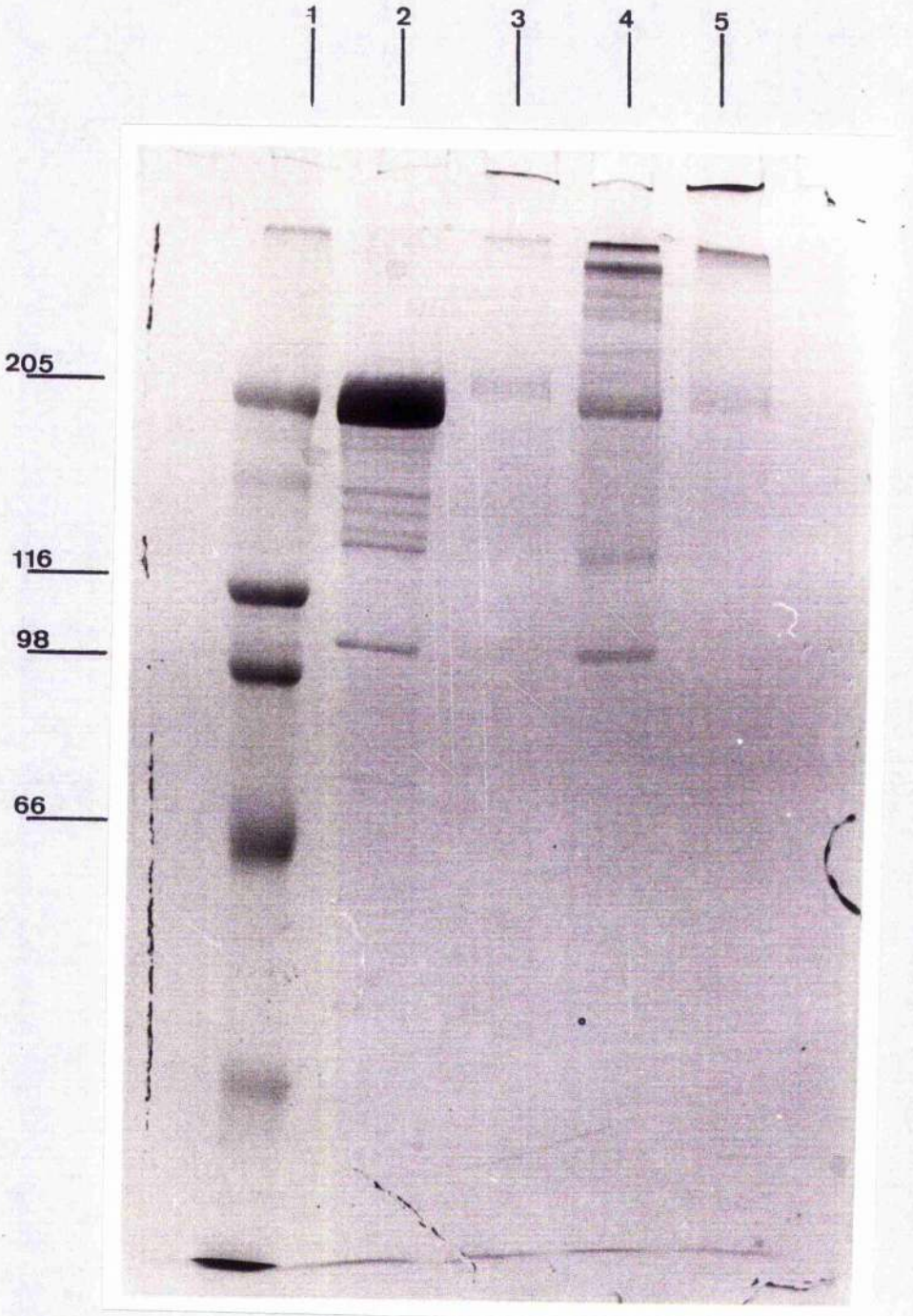

1
2
3
4
5
6
7
8
9
10
11
12
13
205
116
98
66
45
29
66
45
24

Figure 28 : SDS-PAGE profile of FHA, and of EDAC, formaldehyde and glutaraldehyde-treated FHA. Each of the toxoiding treatments was done according to the standard condition, with batch FHA9. Lanes of a 7.5% polyacrylamide gel were loaded with 12.5 µg treated or untreated FHA as below:

Lane	Sample
1	MW-SDS-200 kit[a]
2	FHA9
3	FHA9-G
4	FHA9-E
5	FHA9-F

[a] See footnote to Figure 13 (p94)


1
2
3
4
5
205
116
98
66

With EDAC, treatment at the two higher concentrations (lanes 10 and 11) there were strong bands corresponding to MW >205 KDa, again suggesting that polymerization had occurred; however the lowest concentration of EDAC (lane 9) seemed to have had little effect. Compared with formaldehyde, EDAC produced less alteration in the ladder pattern.

The effect of glutaraldehyde treatment, compared with formaldehyde and EDAC, is shown in Fig. 28 which also includes an untreated control. The glutaraldehyde preparation (FHA-G, lane 3) gave a pattern very similar to that of the formaldehyde treated FHA (FHA-F, lane 5) in showing that neither sample had moved into the stacking gel indicating a marked degree of polymerization. Correspondingly, there was very little material resembling unaltered FHA. The EDAC sample (FHA-E, lane 4) was much less altered although still extensively polymerized.

Effect on immunogenicity

To compare the effect of the three different toxoiding agents on the immunogenicity of FHA, some of the preparations made with the batches FHA9 and FHA13 (examined above in SDS-PAGE and for HA activity) were injected into mice.

In the first of the two experiments, with the batch FHA9, the untreated preparation was injected *i.p.* into male mice of six weeks age and on average weighing 25 g. Doses were at 0.01, 0.1, 1.0, 10.0 and 100 µg per mouse in groups of four mice for each dose. The treated preparations, namely formaldehyde-treated FHA9 (FHA9-F), glutaraldehyde-treated FHA9 (FHA9-G), which both were inactivated for HA activity by ≥ 99%, and EDAC-treated FHA9 (FHA9-E), inactivated to only 75%, were injected at 0.1, 1.0 or 10 µg per mouse only. Sera were collected in the usual way with mice being bled 21 days after immunization. The titres and their geometric means for each group of sera are shown in Table 25.

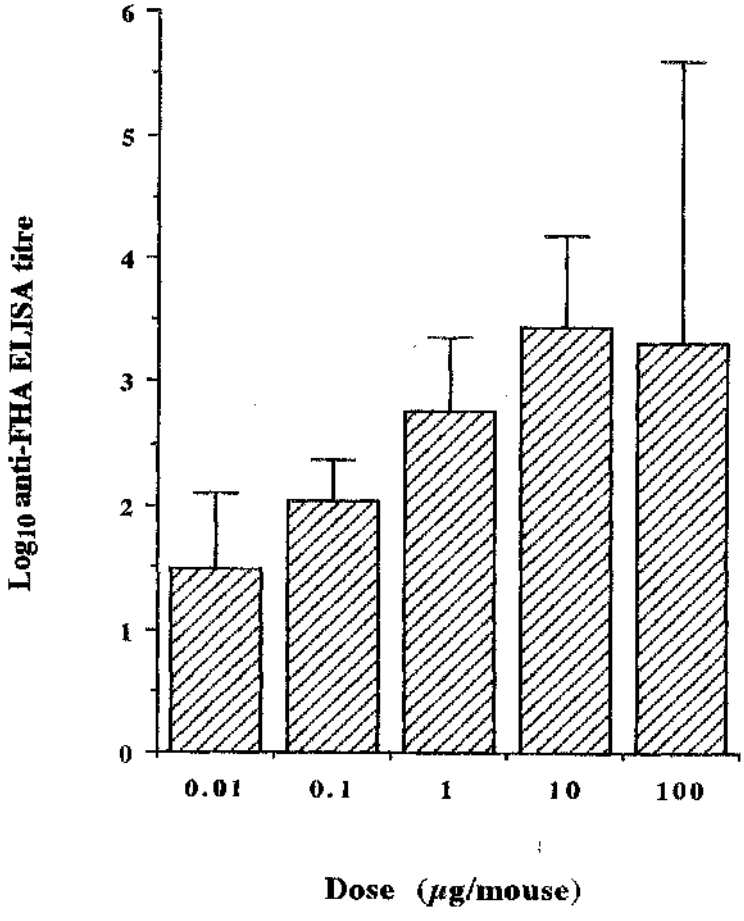
A dose-response curve of the log anti-FHA titres after immunization with the various doses of untreated FHA9 is presented in Fig. 29. A low response was obtained

Table 25: Effect of EDAC, formaldehyde and glutaraldehyde on the immunogenicity of FHA (Experiment A). Serum ELISA titres of individual mice from experiment A are shown with group geometric means in bold. Mice were immunized with various doses of untreated FHA (batch 9) or treated FHA preparations.

	ELISA titre (and **g.m.**) after immunization with dose (μg/mouse) of :				
Sample	0.01	0.1	1.0	10.0	100.0
FHA	23	80	510	1600	10800
	44	76	450	3250	8150
	80	135	235	11500	15350
	10	195	1925	970	15
	30	**113**	**568**	**2760**	**2121**
FHA-E	nt	29	21	2100	nt
	nt	20	10	5500	nt
	nt	10	150	5650	nt
	nt	20	410	680	nt
		19	**60**	**2581**	
FHA-F	nt	50	120	660	nt
	nt	97	305	860	nt
	nt	10	480	3225	nt
	nt	60	145	300	nt
		41	**225**	**861**	
FHA-G	nt	10	25	850	nt
	nt	20	20	1400	nt
	nt	100	10	910	nt
	nt	140	10	3250	nt
		41	**15**	**1370**	

nt = not tested

Figure 29: Antibody dose-responses of mice to FHA. FHA (batch 9) was immunized into groups of four mice at 0.01, 0.1, 1.0, 10.0 and 100 μg/mouse. Each bar is representative of the g.m. of each group with its upper 95% CL.



at 0.01 μg per mouse as evidenced by a g.m. of 30. However, the number of positively responding sera began to increase at 0.1 μg/ mouse (g.m. 113). At 1, 10 and 100 μg/ mouse, mice responded increasingly strongly with anti-FHA titres reaching up to 15,350 at the top dose. Exceptionally, one serum from the group injected with the top dose responded poorly (15). Maximum titres were obtained within the range of 10-100 μg/ mouse.

The $\log_{10}$ serum titres obtained after immunization with the treated preparations are summarized in Fig. 30. The main conclusions from this experiment were that (a) anti-FHA titres increased with dose for all preparations except glutaraldehyde-treated FHA, and (b) responses generally appeared lower for all the three treated preparations relative to the untreated FHA. This latter observation was statistically confirmed upon analysis of the data by 6-point parallel-line assays (Table 26). Highly significant differences in responses were found relative to the untreated group (Table 26a) and one preparation significantly different with comparison of the treated groups to each other (Table 26b).

With the other batch of FHA (FHA13) as the source of the preparations for immunization, a similar repeat experiment was carried out. Formaldehyde-treated FHA13 (FHA13-F), glutaraldehyde-treated FHA (FHA13-G) and EDAC-treated FHA13 (FHA13-E) were inactivated of HA activity by >99%, >99% and 50% respectively. These samples were injected into groups of five mice *i.p.* with 0.1, 1.0 or 10 μg/ mouse and sera collected as before. Anti-FHA arithmetic titres from this experiment are given together with geometric means in Table 27 and summarized as bar charts in Fig. 31. The figure shows that (a) generally, increased doses stimulated increased titres and (b) formaldehyde and glutaraldehyde-treated FHA stimulated higher titres than the untreated or the EDAC-treated FHA. Analysis by 6-point parallel-line assays (Table 28a and b) showed FHA13-G and FHA13-F elicited significantly better responses than the other two preparations, as indicated by the significant *preparations* terms. Untreated FHA13 and FHA13-E were very similar to each other.

Figure 30: Immunogenicity of FHA after treatment with EDAC, formaldehyde and glutaraldehyde (experiment A). FHA (batch 9) was immunized into groups of four mice at 0.1, 1.0 and 10 μg/mouse. Each bar represents the g.m. serum ELISA titre, with the upper 95% CL.

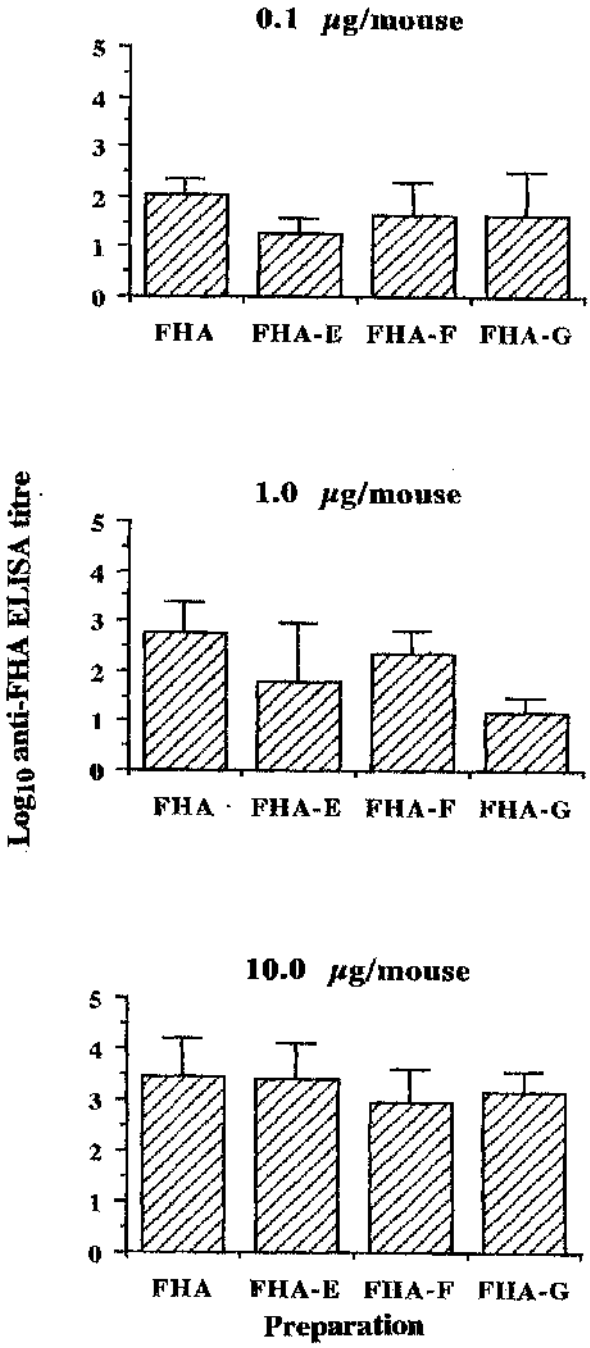


Table 26a: Analysis of variance of the effect of EDAC, formaldehyde and glutaraldehyde on the production of anti-FHA in mice in experiment A, with the untreated preparation of FHA used as a comparator. Summary statistics are presented from 6-point parallel line assays

Source of variation	F-values for toxoided FHA preparations with untreated FHA as the comparator		
	EDAC	Formaldehyde	Glutaraldehyde
Slope	63.46**	52.06**	62.47**
Preparations	10.85**	8.53**	26.46**
Parallelism	2.90	0.04	0.13
Quadratic curvature	2.07	0.06	13.92
Difference of quadratics	2.19	0.04	14.29**

Tabulated F value for 1 and 18 degrees of freedom at P = 5% is 4.41(*) and at the P = 1% is 8.29 (**).

Table 26b: Analysis of variance on the effect of EDAC, formaldehyde, and glutaraldehyde on the production of anti-FHA in mice for experiment A using the treated preparations as comparators. Summary statistics are presented from 6-point parallel line assays

Toxoided preparations taken for comparison	F-values and source of variation				
	Slope	Prep[a]	Parall[a]	Quadratic curvature	Difference of quadratics
FHA-F and FHA-E	58.57**	0.65	3.33	1.53	2.64
FHA-F and FHA-G	56.17**	4.44*	0.29	11.69**	15.06**
FHA-E and FHA-G	67.37**	0.95	1.93	20.67**	2.72

Tabulated F value for 1 and 18 degrees of freedom at P = 5% is 4.41(*) and at the P = 1% is 8.29 (**).

[a] Prep and Parall denote the 'preparations' and 'parallelism' terms in the analysis respectively.

Table 27 : Effect of EDAC, formaldehyde and glutaraldehyde on the immunogenicity of FHA (experiment B). Serum ELISA titres of individual mice from experiment B are shown with group geometric means in bold.

	ELISA titre (and g.m.) after immunization with (μg/mouse) of :		
Sample	0.1	1.0	10.0
FHA	98	120	1650
	49	90	1600
	285	405	2235
	60	125	1770
	94	205	2000
	95	**162**	**1836**
FHA-E	150	76	1960
	130	210	1500
	200	190	2600
	50	190	1900
	140	530	1900
	122	**198**	**1942**
FHA-F	500	228	4800
	180	360	2175
	590	220	4950
	165	210	7200
	200	200	3600
	281	**238**	**4221**
FHA-G	218	770	4850
	225	580	5350
	100	515	5300
	24	800	4200
	190	700	2500
	118	**664**	**4285**

Figure 31: Immunogenicity of FHA after treatment with EDAC, formaldehyde and glutaraldehyde (experiment B). FHA (batch 13) was immunized into groups of five mice at 0.1, 1.0 and 10.0 µg/mouse. Each bar represents the g.m. serum ELISA titre with the upper 95% CL.

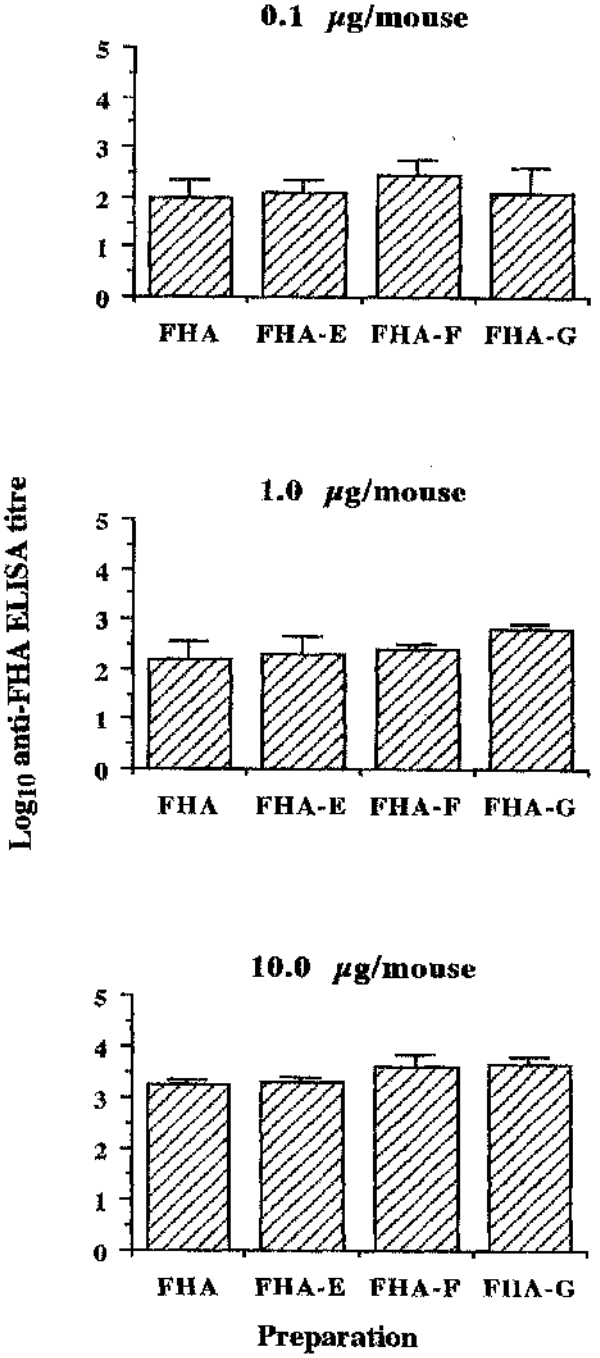


Table 28a: Analysis of variance on the effect of EDAC, formaldehyde and glutaraldehyde on the production of anti-FHA in mice for experiment B, with the untreated preparation of FHA used as a comparator. Summary statistics are presented from 6-point parallel line assays

Source of variation	F-values for toxoided FHA preparations with untreated FHA as comparison		
	EDAC	Formaldehyde	Glutaraldehyde
Slope	151.67**	164.87**	171.90**
Preparations	0.80	18.07**	16.24**
Parallelism	0.18	0.33	1.61
Quadratic curvature	20.98**	41.63**	5.46*
Difference of quadratics	0.01	2.28	4.12

Tabulated F value for 1 and 24 degrees of freedom at P = 5% is 4.26(*) and at the P = 1% is 7.82 (**).

Table 28b: Analysis of variance on the effect of EDAC, formaldehyde and glutaraldehyde on the production of anti-FHA in mice for experiment A, using the treated preparations as comparators. Summary statistics are presented from 6-point parallel line assays

Toxoided preparations taken for comparison	F-values and source of variation				
	Slope	Prep[a]	Parall[a]	Quadratic curvature	Difference of quadratics
FHA-F and FHA-E	160.59**	11.45**	0.02	41.93**	2.77
FHA-F and FHA-G	180.27**	0.10	3.58	15.27**	12.83**
FHA-E and FHA-G	167.39**	10.60**	2.86	5.14*	3.83

Tabulated F value for 1 and 24 degrees of freedom at P = 5% is 4.26(*) and at the P = 1% is 7.82 (**).

[a] Prep and Parall denote the 'preparations' and 'parallelism' terms in the analysis respectively.

The overall conclusions from these experiments were that (a) the immunogenicity of the treated preparations varied with the batch of FHA used and (b) formaldehyde and glutaraldehyde FHA samples (which were more than 99% inactivated of HA activity) from the second experiment, were slightly more immunogenic and (c) EDAC treatment of FHA did not appear to enhance immunogenicity.

Experiments with Pertussis Toxin

In vitro effects of EDAC, formaldehyde and glutaraldehyde

Effect on histamine-sensitizing activity : A pooled preparation was made from the first three batches of PT which had been processed by Blue Sepharose gel affinity chromatography, from the mutant *B. pertussis* strain (B.p. 353) deficient in FHA production. Toxoiding was done under the standard conditions given in Table 29.

The toxoiding treatment with EDAC was done exactly as described by Christodoulides *et al.* (1987) for detoxification of PT. This was with 21 mM EDAC at pH 5.0 for 24 h at 37 °C, followed by dialysis against daily changes of 2L PBS, for three days.

For formaldehyde treatment, as with FHA, the standard conditions were based on the inactivation of pertussis toxin (Sato *et al.*, 1984). The PT preparation was adjusted to 50 μg/ml protein with 20 mM phosphate, pH 7.4 containing 0.5 M NaCl. Formaldehyde at 25 mM and lysine at 20 mM (final concentrations) were added, and the mixture was incubated for 1 week at 37 °C. After the treatment, the sample was dialysed against daily changes of 2L PBS for one week.

With glutaraldehyde, PT was treated for 2 h with 2.5 mM of the reagent as used by Munoz *et al.* (1981a) for the detoxification of this toxin, except that the treatment was performed at 37 °C instead of 22 °C.

Table 29: Summary table of standard treatment conditions for exposure of PT at 50 μg/ml to toxoiding agents at 37 °C.

Toxoiding agent	Concentration of agent (mM)	Time (h)	Lysine (mM)
EDAC	21	24	N/A
Formaldehyde	25	168	20
Glutaraldehyde	2.5	2	10

The extent of inactivation after the toxoiding treatment was assessed by the loss of histamine-sensitizing activity. Groups of four mice (> 6 weeks of age and of mixed sex) were immunized *i.p.* with untreated PT and the three toxoided PT preparations each at five doses (0.06, 0.19, 0.56, 1.7 and 5.0 μg/mouse). The same PT preparation which was also incubated at 2, 24 and 168 h at 37 °C to determine the background loss of activity was tested. Mice were challenged with histamine at 3 mg/mouse on the fifth day after injection. The number of deaths after challenge were recorded and the HSD_{50} estimated (Table 30).

Formaldehyde and EDAC treatment of PT resulted in the greatest loss of HSA, with 97% and 96% inactivation respectively. PT exposed to glutaraldehyde however, showed only a 66% reduction. Incubation of the untreated control preparation at 37 °C showed that half of the HSA activity was lost after 2 h incubation. Incubation for the longer periods of 24 h and 1 week resulted in a successive decrease in activity.

The toxoiding treatment with the three agents was repeated under the standard conditions described above in order to make preparations for investigating immunogenicity. A PT extract (batch-6) was used for this purpose and termed PT-6. PT-6 alone was incubated for 2 h, 24 h and 1 week to assess the residual drop in activity due to incubation without any toxoiding agent.

As before, mice were immunized *i.p.* with these preparations to determine the extent of modification. The doses chosen were selected to indicate inactivation to levels of 99%, 90% and 50%. Only two mice per dose were injected. Table 31 showed that formaldehyde-treated PT (PT6-F) appeared to be ≥99% inactivated. The EDAC-treated (PT6-E) and glutaraldehyde-treated PT (PT6-G) were inactivated only to 50 to 90%. The toxoiding process was therefore repeated with these same samples after redialysis and reestimation of protein content. These preparations were termed PT6-EE and PT6-GG to emphasize the double treatment with the respective toxoiding agents. The HSA of the retreated PT toxoids was determined but, even after this double treatment the HSA was not completely inactivated (Table 31). Thus PT6-GG from the double

Table 30: Effect of EDAC, formaldehyde and glutaraldehyde on the histamine-sensitizing activity of PT. PT (pooled from extractions 1, 2 and 3) and the three toxoids were injected into groups of 4 mice and challenged with histamine (3 mg/mouse) five days later. The number of deaths was recorded and the HSD_{50} determined. The upper half of the table shows HSD_{50} for toxoided preparations whereas the lower half shows the progressive reduction in HSF activity of PT with time.

Sample	Dose (ug/mouse)					HSD_{50}	% inactivation
	5.0	1.7	0.56	0.19	0.06	(μg)	
	No. of deaths / 4 mice immunised						
PT	4	4	4	3	0	0.13	-
PT-E	3	1	0	0	0	3.35	96
PT-G	3	4	4	0	0	0.38	66
PT-F	2	0	0	0	0	5.00	97
PT	nt	4	4	2	0	0.19	-
PT (2 h)	nt	4	3	0	0	0.38	50
PT (24 h)	nt	4	0	0	1	1.14	83
PT (1 week)	nt	2	1	0	0	1.7	89

Table 31: Effect of EDAC, formaldehyde and glutaraldehyde on the histamine-sensitizing activity (HSA) of PT6. PT6 was treated with EDAC, formaldehyde and glutaraldehyde and the toxoids were injected into groups of two mice which were challenged with histamine (3 mg/ mouse) 5 days later. The number of deaths were noted and the HSD_{50} determined.

Sample	Immunisation with dose to test at the inactivation level (%) of :			
	99	90	50	
	No. of deaths out of 2 immunized after histamine challenge			% inactivation
PT6	2	2	2	0
PT6-G	2	2	0	> 50 < 90
PT6-E	2	1	0	> 50 <90
PT6-F	0	0	0	≥ 99
PT6-GG[a]	2	2	0	> 50 < 90
PT-EE[a]	1	0	0	> 90 < 99

[a] Due to incomplete inactivation , PT was retreated with EDAC and glutaraldehyde as above then retested for HSA activity.

glutaraldehyde treatment still retained 50 to 90% HSA. However, double treatment with EDAC, increased the inactivation from 50 to 90% to 90 to 99%.

Effect on immunogenicity

To compare the effect of the three toxoiding agents on the immunogenicity of PT, EDAC, formaldehyde and glutaraldehyde toxoids prepared above i.e. PT6-E (50 to 90% inactivated), PT6-F (≥99% inactivated) and PT6-G (50 to 90% inactivated) were injected into mice even though some of the preparations were not completely inactivated. Untreated PT6 was included as a reference preparation. Due to the inherent toxicity of PT, untreated PT6 was injected *i.p.* at doses of only 1 or 5 μg/mouse whereas the toxoided preparations were given at doses of 1, 5 and 25 μg/mouse. A heat-killed sonicate of *B. pertussis* 18323 (18323 HKS) whole cells was included for purposes of comparison. This preparation was injected at 0.5 ml containing the equivalent of 5 opacity units of bacteria and regarded as approx. one-quarter of one human dose. Mice were bled on day 21 and the sera were analyzed for anti-PT by ELISA.

Arithmetic anti-PT serum ELISA titres and geometric means are given in Table 32 and the $\log_{10}$ serum titres presented in Figure 32. From this experiment the conclusions were that anti-PT titres increased with dose for all toxoided preparations but not for the untreated PT6, and secondly, that responses appeared higher for all the three treated preparations relative to PT. However, this was not confirmed by statistical analysis. Four-point parallel-line assays with the untoxoided PT as a comparator (Table 33) were conducted. With untreated PT as the reference (Table 33), the *preparations* term for the EDAC and glutaraldehyde treatments was significant, indicating an adjuvant effect, but the formaldehyde treatment was not. Also, the response induced by 1 and 5 μg/mouse of PT and its toxoids were similar as demonstrated by the non-significant *slope* terms. Finally, the assays did not show any deviation from *parallelism*. Next, EDAC-treated PT was compared with the other two toxoids by one-

Table 32: Effect of EDAC, formaldehyde and glutaraldehyde on the immunogenicity of PT. Serum ELISA titres of individual mice are shown with group geometric means in bold. Mice were immunized with various doses of untreated PT (batch 6) or treated PT preparations.

	Anti-PT ELISA titre (and g.m.) after immunization with :			
Sample	1	5.0 (μg/mouse)	25.0	5 o.u. (0.5 ml/mouse)
PT6	420	350	nt	-
	540	60	nt	-
	1	150	nt	-
	150	1	nt	-
	40	300	nt	-
	67	**62**	nt	-
PT6-E	240	6700	9200	-
	1300	7000	1500	-
	210	2000	6000	-
	2000	1200	9000	-
	550	55	9000	-
	591	**1440**	**5824**	-
PT6-F	450	250	900	-
	450	400	150	-
	120	500	3500	-
	30	10	4900	-
	190	1080	370	-
	169	**222**	**969**	-
PT6-G	110	1400	1400	-
	300	640	1550	-
	400	880	8000	-
	120	200	3500	-
	310	1500	7800	-
	218	**749**	**3428**	-
18323 HKS[a]	-	-	-	45
	-	-	-	820
	-	-	-	1
	-	-	-	450
	-	-	-	700
	-	-	-	**103**

[a] Strain 18323 heat-killed (56 °C) and sonicated.

nt = not tested

Figure 32: Immunogenicity of PT after treatment with EDAC, formaldehyde and glutaraldehyde. PT (batch 6) was injected into groups of five mice at 1.0 or 5.0 µg/mouse. Toxoided preparations were also immunized at 25.0 µg/mouse. Each bar represents the g.m. serum ELISA titre with the upper 95% CL.

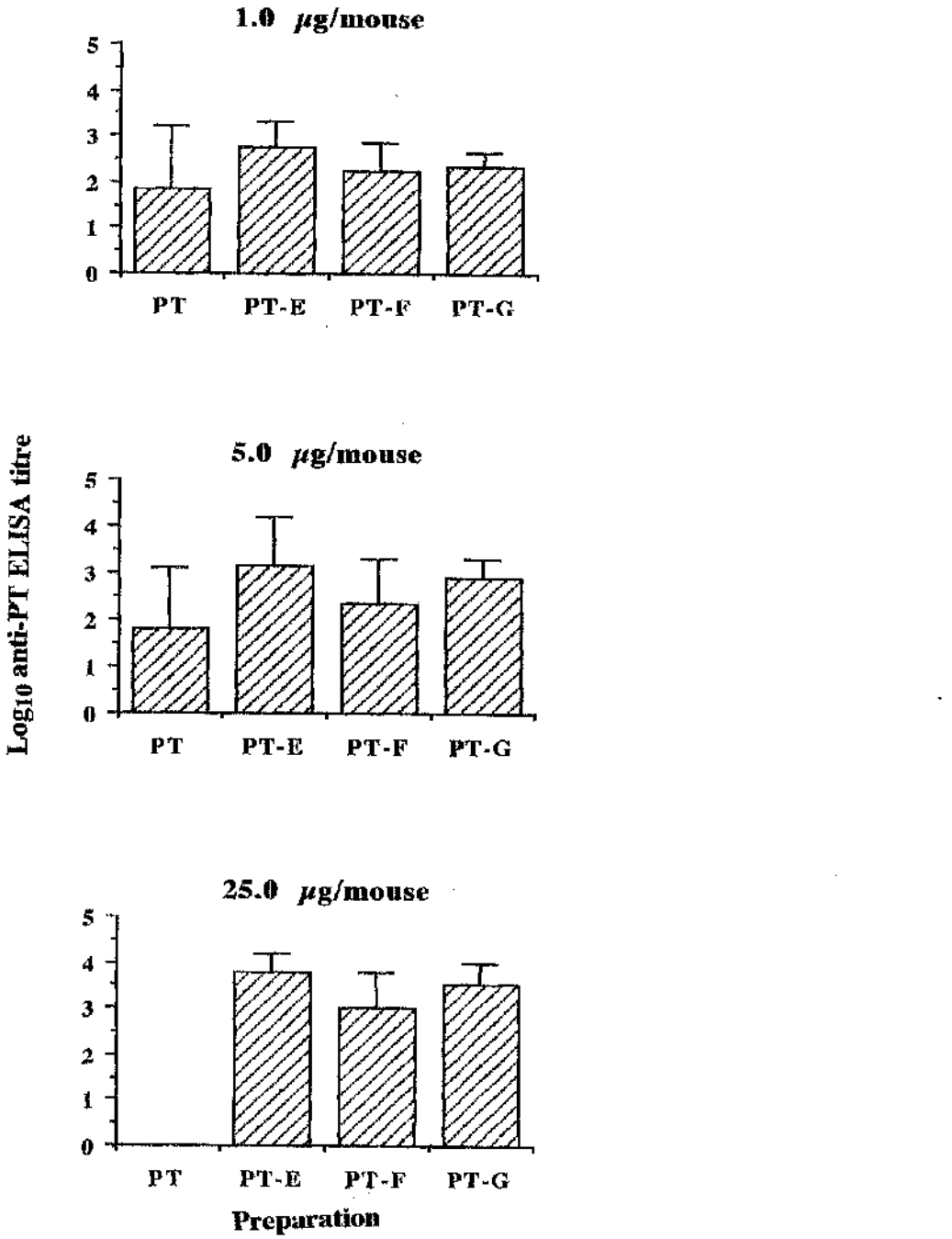


Table 33a: Analysis of variance of the effect of EDAC, formaldehyde and glutaraldehyde on the production of anti-PT in mice, with the untreated preparation of PT (batch 6) as a comparator. Summary statistics are presented from 4-point parallel line assays.

Source of variation	F-values for toxoided PT preparations with untreated PT as comparison		
	EDAC	Formaldehyde	Glutaraldehyde
Slope	0.19	0.01	0.50
Preparations	8.15*	1.42	5.00*
Parallelism	0.27	0.04	0.64

Tabulated F value for 1 and 16 degrees of freedom at P = 5% is 4.49*) and at the P = 1% is 8.53 (**).

Table 33b: Analysis of variance of the effect of EDAC, formaldehyde and glutaraldehyde on the production of anti-PT in mice with the treated preparations as comparators. Summary statistics are presented from 6-point parallel line assays.

Toxoided preparations	F-values and source of variation				
	Slope	Preparations	Parallelism	Quadratic curvature	Difference of quadratics
PT-F and PT-E	9.97**	9.86**	0.18	0.60	0.10
PT-F and PT-G	18.00**	4.42*	0.91	0.66	0.25
PT-E and PT-G	26.31**	3.28	0.23	0.22	0.02

Tabulated F value for 1 and 24 degrees of freedom at P = 5% is 4.26(*) and at the P = 1% is 7.82 (**).

tail t-tests for each dose (Table 34). This analysis revealed that (a) EDAC-treated PT was more immunogenic than formaldehyde-treated PT at 25 μg/mouse and (b) PT-E had similar immunogenicity to PT-G. Thus it was concluded that PT-E and PT-G induced the best responses followed by PT-F.

Experiments with PT and FHA Mixtures

In vitro effects of EDAC, formaldehyde and glutaraldehyde

Preparations of PT and FHA were copurified from *B. pertussis* 77/18319 by Blue Sepharose gel-affinity chromatography. Two PT/FHA mixtures extracted by this method were designated PF3 and PF4 and used for toxoiding in the following experiments. The third mixture was an independently extracted preparation by the same procedure and termed AP16, for antigen preparation 16. This was toxoided for comparative purposes. The PT:FHA ratio as determined by ELISA for AP16 was 1:2.25.

The relative amounts of PT and FHA in PF3 and PF4 were determined by ELISA and SDS-PAGE. However, the results were not consistent as judged by the two tests. PF3 had a PT:FHA ratio of 2:3 by ELISA analysis and indeed the gel profile (Figure 24, lane 4) appeared to show an excess of FHA. PF4 appeared to show equivalent amounts of PT and FHA by SDS-PAGE (Figure 23, lane 7) but this result was not confirmed upon ELISA data analysis which indicated that the PT:FHA ratio was 1:5. The toxoiding procedure was the same as described for the inactivation of PT with EDAC, formaldehyde and glutaraldehyde where the conditions are summarized in Table 29.

Effect on histamine-sensitizing activity: Toxoided AP16, PF3 and PF4 were investigated for histamine-sensitizing activity to assess the extent of modification of PT in the antigen mixtures. To begin with, the potency of the PT in the untreated antigen

Table 34: Demonstration of the superior immunogenicity of EDAC-treated PT over other PT toxoids by one-tail t-tests.

Pertussis toxoid made with	Statistic	Data from one-tail t-tests of superior immunogenicity at mouse dose (μg)		
		1	**5**	**25**
EDAC	g.m.	591	1440	5824
Formaldehyde	g.m.	169	222	969
	t	-1.865	-1.556	-2.403
	P	NS	NS	*
Glutaraldehyde	g.m.	218	749	3428
	t	-1.919	-0.680	-1.033
	P	NS	NS	NS

t = students t-test stastistic

P = probability of significance for t statistic at 8 D.f.

mixtures was assessed by injecting two mice per group with 1, 5 or 25 μg/mouse. All mice challenged on day five with 3 mg of histamine died. The endpoint HSD_{50} was therefore below 1 μg/mouse for each preparation. In order to define the HSD_{50} further, groups of two mice were immunized with 4, 16, 63, 250 and 1000 ng/mouse. The results from this second experiment showed that AP16 and PF3 had HSD_{50} of approx. 1.0 μg/ mouse while PF4 had a HSD_{50} value of approx. 0.25 μg/ mouse.

The effect of EDAC, formaldehyde and glutaraldehyde treatment on HSA is shown in Table 35. Formaldehyde treatment of all three mixtures destroyed ≥ 98% activity in AP16-F and PF3-F and PF4-F showed ≥99% inactivation. Toxoiding the mixtures with glutaraldehyde and EDAC caused less inactivation of the HSA. Thus, all glutaraldehyde-treated preparations showed <50% inactivation. AP16-E and PF3-E had >50 but <90% inactivation levels. PF4-E had >90 <99% inactivation.

Although it was subsequently realised that the glutaraldehyde and EDAC preparations were not fully inactivated for HSA, mice had already been simultaneously immunized for the raising of antisera. The process with glutaraldehyde and EDAC was therefore repeated as before on these same samples in an effort to increase the level of inactivation (Table 35). AP16-GG (double toxoiding with glutaraldehyde) and PF3-GG were ≥98% inactivated from the previous level of <50%. PF4-GG was ≥99% inactivated. Similarly, with the EDAC retoxoiding, AP16-EE and PF3-EE showed increased inactivation levels of >90<98% inactivation from >50<90%; however PF4-EE remained the same.

Effect on immunogenicity

PT and FHA antigen mixtures AP16, PF3 and PF4 were toxoided with formaldehyde, glutaraldehyde and EDAC with HSA activities as detailed above. For each toxoided preparation, 5 mice were immunized with 5 and 25 μg/mouse. Due to toxicity, the doses of untreated PT was restricted to 5 μg/mouse. As before, the experiment was terminated on day 21, and sera obtained. Anti-PT and anti-FHA were assayed by

Table 35: Effect of EDAC, formaldehyde and glutaraldehyde on histamine-sensitizing activity of PT and FHA antigen mixtures. HSD_{50} for untreated AP16 and PF3 was approx. 1.0 µg/ mouse and 0.25 µg/ mouse for PF4.

Sample	No. of deaths/ 2 injected Dose (µg/ mouse)			approx HSD_{50}	approx % inactivation
	2.0	10.0	50.0		
AP16-F	0	0	0	> 50.0	≥ 98
AP16-G	2	2	2	≥ 1.0 < 2.0	< 50
AP16-E	0	0	2	≥ 2.0 < 10	> 50 < 90
AP16-GG[a]	0	0	0	> 50.0	≥ 98
AP16-EE [a]	nt	0	2	> 10 < 50	> 90 < 98
PF3-F	0	0	0	> 50.0	≥ 98
PF3-G	2	2	2	≥ 1.0 < 2.0	< 50
PF3-E	0	2	2	> 2.0 < 10	> 50 < 90
PF3-GG[a]	0	0	0	> 50.0	≥ 98
PF3-EE [a]	nt	0	2	> 10 < 50	> 90 < 98

Sample	No. of deaths/ 2 injected Dose (µg/ mouse)			approx HSD_{50}	approx % inactivation
	0.5	2.5	25.0		
PF4-F	0	0	0	> 25.0	≥ 99
PF4-G	1	2	2	0.5	< 50
PF4-E	0	0	2	> 2.5 < 25	> 90 < 99
PF4-EE [a]	nt	0	1	25.0	≥ 90 < 99
PF4-GG[a]	0	0	0	> 25.0	≥ 99

[a] Due to insufficient inactivation, these preparations were subjected to retoxoiding with G or EDAC as before, and HSF activity determined.

ELISA and are listed in Tables 36 and 37, respectively, with group geometric means.

With respect to anti-FHA titres, bar charts (Figure 33) allowed the following conclusions to be made (a) that the anti-FHA response increased with increased dose, (b) there appeared to be differences between preparations i.e. AP16 titres were generally lower than PF3 and PF4 and (c) anti-FHA titres were the highest after immunization with EDAC-treated preparations. Statistical analysis was commenced by 1-way analysis of variance to ascertain whether there were differences between the three preparations (AP16, PF3 and PF4) after each toxoiding treatment for each dose (Table 38). These tests were performed primarily to determine whether there were significant differences in the antibody response between the untreated and treated PT:FHA preparations. The results indicated that the data were heterogeneous for anti-FHA titres with the untreated, EDAC-treated and glutaraldehyde-treated PT:FHA samples immunized at 5 μg/mouse. Statistical analysis therefore was continued by considering each PT:FHA preparation individually. The untreated PT:FHA preparation was used as the reference in t-tests for comparing the toxoided samples at the 5μg dose for each mixture. Table 39 presents a summary of the results. An adjuvant effect was noted for the EDAC and formaldehyde-treated AP16. However, formaldehyde-treated PF3 and PF4 showed significantly lower responses. The other EDAC-treated and glutaraldehyde-treated samples were not significant. In order to judge whether there was a difference between the toxoided preparations, 4-point parallel line assays were performed on the titres generated by immunization with 5 and 25 μg/mouse (Table 40). Formaldehyde-treated PF3 and PF4 were significantly lower than EDAC-treated PF3 and PF4. There were no differences between the three toxoiding treatments with the AP16 preparation despite the adjuvanting effects obtained at the 5 μg/mouse dose. The main conclusion from this experiment was that EDAC-treatment of PT:FHA gave the highest anti-FHA titres compared with the other toxoiding treatments, although a consistent adjuvant effect against the untreated preparation could not be demonstrated.

Table 36: Effect of EDAC, formaldehyde and glutaraldehyde on the anti-FHA response after immunization with PT:FHA antigen mixtures. Groups of five mice were immunized with 5 and 25 μg/mouse of each toxoided PT/FHA mixture, while the untreated mixture was used at 5 μg/mouse only. Anti-FHA ELISA titres are shown with group g.m. in bold.

	Toxoiding treatment and dose (μg/mouse)						
	Anti-FHA ELISA titre						
Preparation	Untreated	EDAC		Formaldehyde		Glutaraldehyde	
	5	5	25	5	25	5	25
AP16	1	90	2850	50	675	75	375
	30	135	2075	50	600	1	300
	25	100	2475	50	900	60	3000
	1	330	330	100	2250	30	1350
	15	1	1500	150	550	300	4000
	7	**53**	**1487**	**72**	**852**	**33**	**1127**
PF3	1000	280	2520	50	1200	2000	2250
	500	1800	1200	300	6000	630	2500
	375	300	5000	1000	530	1000	8000
	700	1800	6300	150	380	550	900
	850	1050	6000	250	4500	700	6400
	645	**789**	**3568**	**224**	**1455**	**865**	**3038**
PF4	800	435	5130	110	1500	300	90
	550	450	3200	90	600	1500	2600
	1500	345	2000	300	4900	1050	1500
	325	1000	2000	250	3000	110	3050
	460	900	7100	845	1450	745	2370
	629	**571**	**3417**	**229**	**1805**	**522**	**1205**

Table 37 : Effect of EDAC, formaldehyde and glutaraldehyde on the anti-PT response after immunization with PT:FHA antigen mixtures. Groups of five mice were immunized with 5 and 25 μg/mouse of each toxoided PT:FHA mixture, while the untreated mixture was used at 5 μg/mouse only. Anti-PT ELISA titres are shown with group g.m. in bold.

	Toxoiding treatment and dose (μg/mouse)						
	Anti-PT ELISA titre						
Preparation	Untreated	EDAC		Formaldehyde		Glutaraldehyde	
	5	5	25	5	25	5	25
AP16	690	950	5350	710	450	95	570
	355	200	2300	200	2450	430	2580
	180	1700	4500	125	3200	1800	90
	150	750	1300	30	4000	980	5600
	95	2000	6450	70	500	145	5500
	229	**865**	**3415**	**130**	**1458**	**402**	**1324**
PF3	110	760	1000	670	520	940	6600
	540	60	7000	450	170	350	2400
	200	950	5280	240	1850	1550	1700
	90	440	4000	220	45	800	1040
	600	1200	6500	85	5000	1	2700
	230	**470**	**3950**	**267**	**516**	**210**	**2376**
PF4	1200	550	1800	590	600	400	600
	360	810	2300	230	4150	2000	8500
	210	1100	6700	15	3200	1225	3530
	800	2000	7500	2150	2330	1000	7200
	760	300	8100	215	100	28	4350
	560	**783**	**4420**	**248**	**1131**	**487**	**3550**

Figure 33: Anti-FHA titres with PT:FHA antigen mixtures after treatment with EDAC, formaldehyde and glutaraldehyde. Treated preparations were immunized at 5.0 (□) and 25.0 (▨) μg/mouse into groups of five mice. Each bar represents the g.m. anti-FHA ELISA titre with the upper 95% CL.

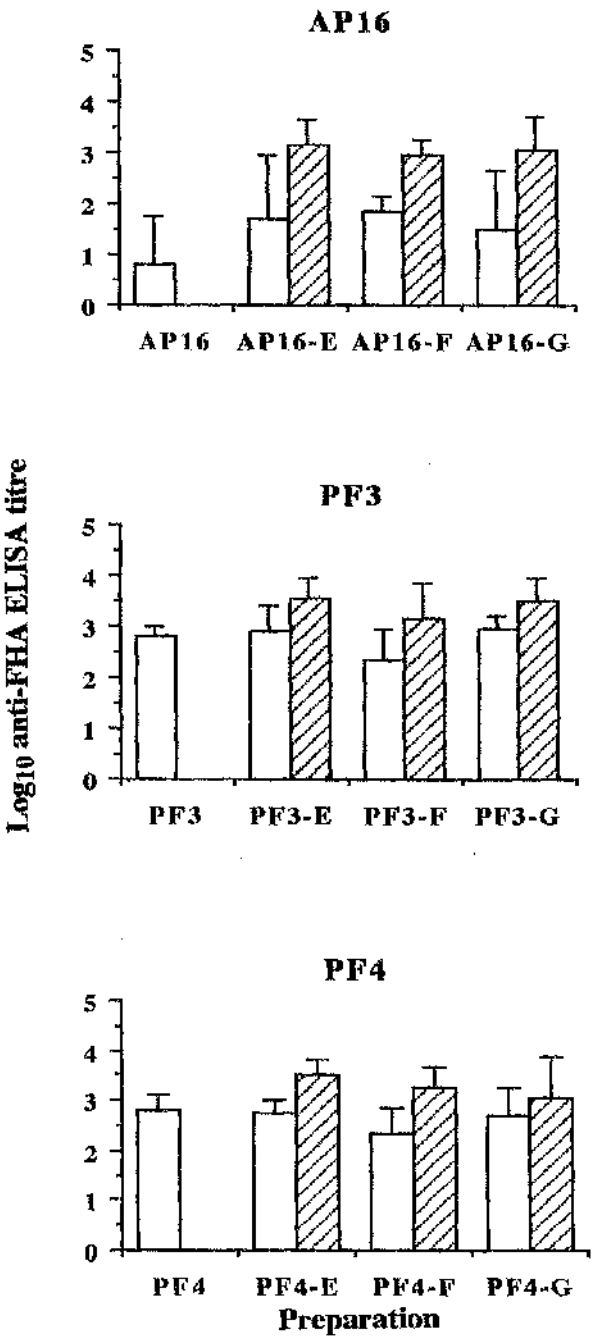


Table 38: One-way analysis of variance to determine the heterogeneity of the anti-FHA titres after immunization with EDAC, formaldehyde and glutaraldehyde-treated mixtures of PT:FHA.

Group totals and F-values of $\log_{10}$ anti-FHA ELISA titres at 5 or 25 μg/mouse

	(PT:FHA)	(PT:FHA)-E		(PT:FHA)-F		(PT:FHA)-G	
Preparation	5	5	25	5	25	5	25
AP16	4.051	8.603	15.861	9.273	14.653	7.607	15.260
PF3	14.407	14.455	17.762	11.751	15.814	14.685	17.413
PF4	13.994	13.783	17.668	11.797	16.282	13.587	15.404
	15.21**	**5.22***	**2.30**	**2.95**	**0.89**	**7.80****	**1.06**

Tabulated F value for 2 and 12 degrees of freedom at P = 5% is 3.89(*) and at the P = 1% is 6.93 (**).

Table 39: t-test analysis of anti-FHA ELISA titres to determine whether toxoiding with EDAC, formaldehyde and glutaraldehyde affects the immunogenicity of FHA in PT:FHA mixtures. The two-tailed t-tests were done on the group means and SD from the 5 µg/mouse dose only.

Untreated PT:FHA (g.m.)	Treated PT:FHA (g.m.)	t-value	Significance
AP16 (7)	AP16-E (53)	2.324	*
AP16 (7)	AP16-F (72)	4.234	**
AP16 (7)	AP16-G (33)	1.891	NS
PF3 (645)	PF3-E (789)	0.642	NS
PF3 (645)	PF3-F (224)	-2.885	*
PF3 (645)	PF3-G (865)	1.422	NS
PF4 (629)	PF4-E (571)	-0.408	NS
PF4 (629)	PF4-F (229)	-2.996	*
PF4 (629)	PF4-G (522)	-0.491	NS

N=10 and D.f.=8

Table 40: Analysis of variance to determine whether EDAC treatment of PT:FHA results in superior immunogenicity of the FHA component over formaldehyde and glutaraldehyde toxoiding. Four-point parallel line assays were performed on the anti-FHA ELISA titre group totals from the 5 and 25 μg/mouse doses.

PT:FHA preparation	F-values and source of variation		
	Slope	Preparation	Parallelism
AP16-E and AP16-F	25.97**	0.05	0.57
AP16-E and AP16-G	19.89**	0.23	0.01
PF3-E and PF3-F	14.10**	5.62*	0.15
PF3-E and PF3-G	16.05**	0.01	0.15
PF4-E and PF4-F	37.46**	6.08*	0.19
PF4-E and PF4-G	8.99**	1.67	1.18

Tabulated F value for 1 and 16 degrees of freedom at P = 5% is 4.49(*) and at the P = 1% is 8.53 (**).

A similar analysis of the anti-PT ELISA titres from the same set of sera was made. Bar charts of group g.m with 95% CL (Figure 34) showed that (a) anti-PT response increased with dose, (b) that all three PT:FHA preparations generated similar titres and (c) that two EDAC-treated PT:FHA mixtures showed the highest responses. These conclusions were confirmed by statistical analysis. Firstly, as with anti-FHA titres, the data was subjected to 1-way analysis of variance to show up differences between the three PT:FHA preparations. Table 41 shows that the data were homogeneous and that there were no significant differences between the three preparations after each toxoiding treatment for each dose. Therefore, different procedures were adopted for the statistical analysis of anti-PT titres compared with anti-FHA titres. This was done by grouping all three preparations collectively as one PT:FHA sample for each toxoiding treatment at each dose. Two-tailed t-tests on the 5 μg/mouse dose (Table 42) with the untreated preparation as comparator showed that EDAC-treatment had a significant adjuvant effect but formaldehyde and glutaraldehyde-treatment did not. Results from the 4-point parallel-line assays (Table 43) all had a highly significant slope and there was no deviation from parallelism. A clear adjuvanting effect was demonstrated for anti-PT titres after EDAC treatment relative to formaldehyde toxoiding but not when compared with glutaraldehyde. Thus, EDAC induced the highest anti-PT responses followed by glutaraldehyde. Formaldehyde treatment appeared to have no adjuvant effect.

Figure 34: Anti-PT titres with PT:FHA antigen mixtures after treatment with EDAC, formaldehyde and glutaraldehyde. Treated preparations were immunized at 5.0 (□)and 25.0 (▨)µg/mouse into groups of five mice Each bar represents the G.m. anti-PT ELISA titre with the upper 95% CL.

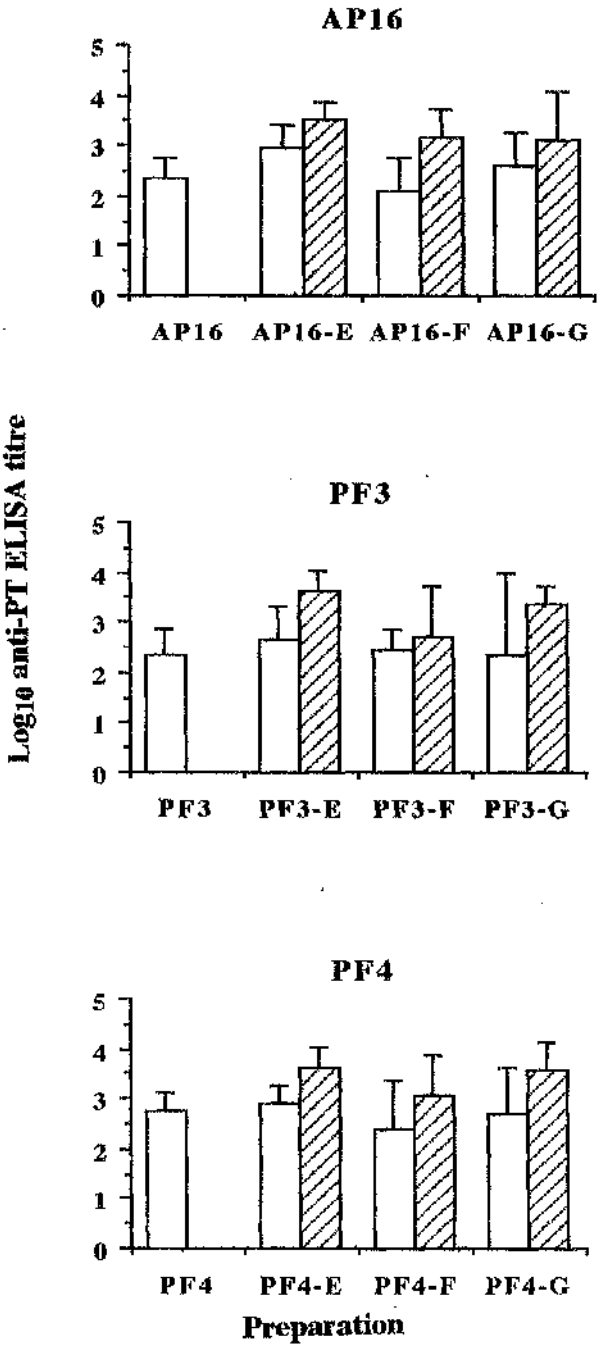


Table 41: One-way analysis of variance to determine the heterogeneity or homogeneity of anti-PT titres after immunization with EDAC, formaldehyde and glutaraldehyde-treated mixtures of PT:FHA.

Group totals and **F-values** of $\log_{10}$ anti-FHA ELISA titres at 5 or 25 µg/mouse

	(PT:FHA)	(PT:FHA)-E		(PT:FHA)-F		(PT:FHA)-G	
Preparation	5	5	25	5	25	5	25
AP16	11.798	14.686	17.667	10.571	15.819	13.019	15.610
PF3	11.806	13.360	17.983	12.130	13.565	11.610	16.879
PF4	13.741	14.468	18.227	11.973	15.268	13.468	17.751
	2.14	**0.57**	**0.31**	**0.44**	**0.55**	**0.22**	**0.78**

Tabulated F value for 2 and 12 degrees of freedom at P = 5% is 3.89(*) and at the P = 1% is 6.93 (**).

Table 42: t-test analysis of anti-PT ELISA titres to determine whether toxoiding with EDAC, formaldehyde and glutaraldehyde affects the immunogenicity of PT in PT:FHA mixtures. The two-tailed t-tests were done on the group means and SD from the 5 μg/mouse dose only.

Untreated PT:FHA (g.m.)	Treated PT:FHA (g.m.)	t-value	Significance
PT:FHA (309)	(PT:FHA)-E (682)	3.434	**
PT:FHA (309)	(PT:FHA)-F (205)	-1.461	NS
PT:FHA (309)	(PT:FHA)-G (347)	0.290	NS

N=30 and D.f.= 28

Table 43: Analysis of variance to determine whether EDAC treatment of PT:FHA results in superior immunogenicity of the PT component over formaldehyde and glutaraldehyde toxoiding. Four-point parallel line assays were performed on the anti-PT ELISA titre group totals from the 5 and 25 µg/mouse doses.

PT:FHA preparation	F-values and source of variation		
	Slope	Preparation	Parallelism
(PT:FHA)-E and (PT:FHA)-F	30.93**	19.78**	0.13
(PT:FHA)-E and (PT:FHA)-G	28.39**	3.33	0.01
(PT:FHA)-F and (PT:FHA)-G	18.41**	3.06	0.18

Tabulated F value for 1 and 56 degrees of freedom at P = 5% is 4.00-4.08(*) and at the P = 1% is 7.08-7.31 (**).

DISCUSSION

ORIGINS OF THE WORK

The impetus for the work described in this thesis was the report of Christodoulides *et al.* (1987), that pertussis toxin (PT) was not only detoxified by the carbodiimide, EDAC, but also had its immunogenicity enhanced. The immunogenicity of FHA was also enhanced when the EDAC treatment was applied to a mixture of the two antigens. In the ten years since this report there has been neither confirmation nor refutation of this finding, although the paper has been cited (Nencioni *et al.*, 1990). Meanwhile, preparations of PT and combinations of PT with other protective antigens including FHA, pertactin and the agglutinogens have been detoxified with formaldehyde (Sato *et al.*, 1984), glutaraldehyde (Quentin-Millet *et al.*, 1988), H_2O_2 (Sekura *et al.*, 1988) and tetranitromethane (Winberry *et al.*, 1988). Also, PT has been modified by genetic manipulation to produce non-toxic recombinant PT (Nencioni *et* al., 1990). These experimental vaccine preparations have been subjected to clinical trials in Sweden and Italy and are summarized in Table 44. The acellular vaccines were found to be generally more efficacious than the Connaught whole-cell preparation and showed less reactogenicity.

A detailed search of the early literature on toxoiding has uncovered very few reports where toxoiding enhanced the immunogenicity of the treated protein compared to the native molecule. With diphtheria and tetanus toxoids it is not feasible, for toxicity reasons, to compare the immunogenicity of the native protein with its non-toxic derivative. With non-toxic proteins, such as OA and BSA, the chemical changes produced by toxoiding agents have been well documented (Habeeb and Hiramoto, 1968; Habeeb, 1969) but no comparative immunogenicity studies were reported. Of particular interest therefore is the study of Nencioni *et al.* (1991) on the treatment of genetically-detoxified PT with formaldehyde. Here the formaldehyde treatment as normally applied to biologically-active PT had neither a beneficial nor a detrimental

Table 44: Efficacy results from phase III clinical trials of acellular pertussis vaccines.

Location	Type of study	Manufacturer	Method of antigen inactivation	Composition	Vaccine efficacy (%)	Reference
Gothenberg	randomised, double-blind placebo-controlled	N. American	H_2O_2 (PT)	PT	71	Trollfors *et al.* (1995)
Stockholm	randomised double-blind DT-controlled	SKB	Glutaraldehyde and formaldehyde (PT) Formaldehyde (FHA)	PT, FHA	59	Gustafsson *et al.* (1996)
		Connaught	Glutaraldehyde (PT)	PT, FHA, 69 kD, Fim 2, Fim 3	85	
		Connaught		whole-cell	48	
Italy	randomised double-blind DT-controlled	SKB	Glutaraldehyde and formaldehyde (PT)	PT, FHA, 69 kD	84	Greco *et al.* (1996)
		Biocine	genetic detoxification (PT)	rPT, FHA, 69 kD	84	
		Connaught		whole-cell	36	

effect on immunogenicity although the specificity of the antibodies changed with the severity of formaldehyde treatment. However, the storage stability of the preparation was improved by a mild formaldehyde treatment without affecting antibody specificity.

In view of the above, the decision was taken at the start of this work to make parallel comparisons of the effects of treatment with formaldehyde, glutaraldehyde and EDAC both on PT and FHA and also on a model antigens lysozyme (LZ) and ovalbumin (OA), the latter being treated with EDAC only. However, before discussing the results so obtained, it was necessary to define appropriate methods for the statistical processing of mouse serum ELISA titres and this is presented next.

SERUM ELISA TITRES AND THEIR POPULATION DISTRIBUTION

Determination of End-Points

ELISA titres throughout the study were calculated from the intersection of the A_{492nm} = 0.5 on the ordinate with the titration curve followed by interpolation down to the abscissa. The reciprocal of the $\log_{10}$ antiserum dilution was converted to its arithmetic value. It was necessary to calculate an ELISA titre for each antiserum since the chosen statistical tests depended on a full set of five replicate values (four in one experiment). In certain cases therefore, where the serum titre was less than the starting dilution, an attempt was made to obtain a titre by extending the titration curve until it reached the A_{492nm} = 0.5 intersection point. Sometimes, even this was not possible therefore that serum was assigned an abitrary titre of 1. Since a good positive reference serum was not available, this method of obtaining serum titres was used throughout this investigation.

A brief survey of the current literature revealed a number of methods for the calculation of ELISA titres. These included: comparing the linear portions from plots of absorbance versus log serum dilutions for test and reference sera by the parallel-line

assay (Huang *et al* , 1996); expressing titres as an absolute value in μg/ml by extrapolation from a standard curve of a reference serum pool (Samore and Siber, 1996); expressing ELISA titres as the reciprocal of the highest dilution of antiserum above background controls (Roberts *et al.*, 1995; Dertzbaugh and West, 1996; Fernandez *et al* , 1996); or simply as the reciprocal serum dilution giving an O.D. of 0.5 (Ott *et al* , 1995) or 0.3 (O'Dempsey *et al* , 1996).

Generally, the ELISA procedures for the quantification of anti-LZ, anti-PT and anti-FHA performed satisfactorily. However, the ELISA methodology for quantitating anti-OA required further development. It was found that an OA concentration of 100 μg/ml was required to give complete saturation of binding sites.To obtain maximal binding of specific antibody an excess of antigen had to be present in the coating phase so that the equilibrium point could be reached where most of the binding sites on the plastic are occupied (Engvall and Perlman, 1972). Kemeny (1992) reported that too high a concentration of protein during the coating phase in an ELISA resulted in protein to protein interactions leading to dissociation of 'bound' protein during the assay. Typically, a protein concentration of 1 to 10 μg/ml resulted in maximal binding of the protein to the plastic without dissociation. Also, all ELISA assays were performed with the native untreated antigens used in the coating phase. ELISA tests with the toxoided antigens for the coating phase may have revealed the formation of new epitopes of the antigens after toxoiding.

Population Distributions

In this investigation, immunogenicity experiments were designed so that each preparation was tested at certain doses in groups of five mice yielding a set of five replicate values that were required for the statistical analysis. The question arose on whether to analyse the data by parametric or non-parametric statistical tests and also whether to represent the average value for each group as the arithmetic or geometric

mean (which is the appropriate measure of central tendency for a group of lognormally distributed data). A survey of the pertussis literature revealed that there seems to be no universally adopted method for the expression of mean antibody titres. For example, Ashworth *et al* (1982), Burstyn *et al.* (1983), Sato *et al.* (1984), Thomas *et al.* (1989) and Redhead *et al.* (1993) summarized ELISA titres as arithmetic means, whereas Robinson *et al.* (1989), Christodoulides *et al.* (1989), Podda *et al.* (1990), Podda *et al.* (1991), and Nencioni *et al.* (1991) used the geometric mean.

Thus before choosing suitable statistical tests for the analysis of ELISA titres, the distribution of the population was investigated by rankit plots (Appendix 6). It was difficult to judge from the 278 rankit plots whether the data followed a normal distribution or that the data were normalizable by conversion to $\log_{10}$ titres. By summarizing the rankit plots with each immunogenicity experiment for each of the four antigens, it was decided to continue with statistical tests used for normally distributed data by first converting to logarithms.Weir (1967) stated that ELISA titre readings usually have a skewed distribution. In fact, anti-OA titres from mouse sera were cited as an example of normalizable data by transformation to logarithms. Thus, all statistical analysis was done on the $\log_{10}$ of the serum ELISA titres.

Reid (1968) reported that antibody titres in animals show asymmetry of the normal distribution curve or skewing. Logarithmic transformation of this type of data results in the curve being converted to the bell shape typical of a normal distribution. The data are thus said to follow a lognormal distribution as they are normalizable by conversion to logarithms. Such converted data are then suited for the statistical tests which are appropriate for sampling from a normal distribution (Colquhoun, 1971). A number of recent studies on vaccines stated that logarithmic transformation of antibody titres was carried out before statistical analysis because of skewing (Huang *et al.*, 1996; Mulholland *et al.*, 1996; Fernandez *et al.*, 1996; O'Dempsey *et al.*, 1996; Miller *et al.*, 1997).

CHARACTERISTICS OF 'TOXOIDED' PROTEINS

Changes in SDS-PAGE Profiles

The proteins LZ and FHA were examined by SDS-PAGE to investigate changes brought about by chemical treatment with EDAC, formaldehyde and glutaraldehyde. Ovalbumin was similarly examined after treatment with EDAC only. The mobility of the 14.3 KDa monomeric LZ band decreased with the severity of EDAC treatment. Conversely, modification of OA with EDAC appeared to result in a slight increase in mobility suggesting the protein had become more electronegative. Since EDAC predominantly modifies negatively charged carboxyl groups on glutamate and aspartate residues (Hoare and Koshland, 1967) and free sulfhydryl groups (Carraway and Triplett, 1970) when reactions are carried out at acidic pH , the increased positivity of EDAC-treated LZ observed in SDS-PAGE is probably not surprising. Also, the presence of dimers suggest cross-links introduced by chemical treatment which may restrict the binding of sodium-dodecyl sulphate (SDS) and concomitantly lower the electrophoretic mobility (Weber and Osborne, 1985). The slightly faster mobility of EDAC-treated ovalbumin is either a concentration effect or due to a large decrease in SDS binding which sometimes decreases the frictional drag because of fewer SDS molecules, thereby increasing the mobility.

Habeeb (1969) and Pass and Marcus (1970) observed that formaldehyde-treatment of BSA and rabbit serum albumin respectively resulted in the proteins becoming more electronegative in polyacrylamide disc gel electrophoresis and attributed this to the modification of positively charged amino groups. However, modification of LZ by formaldehyde and glutaraldehyde in this study did not show any changes in the mobility of the monomeric LZ band. Rather, these agents caused the formation of dimers and polymers. Formaldehyde and glutaraldehyde are widely accepted cross-linking agents and the chemical mechanisms of these effects have been documented by

Fraenkel-Conrat and Olcott (1948a and 1948b) for formaldehyde, and Hardy *et al.* (1979) for glutaraldehyde. Studies by Habeeb (1969) with BSA and OA-BSA conjugates, Onica *et al.* (1978) with rabbit serum albumin and HayGlass and Strejan (1983) with ovalbumin reported polymerization of these antigens after glutaraldehyde treatment and changes in mobility such that treated proteins cross-linked to such an extent that they were unable to enter the gel. In a study by Hopwood *et* al. (1988), where the effect of microwaves, heating and formaldehyde were examined on a number of proteins, LZ-F preparations were dimerized extensively and also formed polymers.

The major effect of treating FHA with the three chemical agents also resulted in the formation of cross-linked polymeric material although, the polymerization effect was less after EDAC-treatment compared with formaldehyde and glutaraldehyde. Polymerization after formaldehyde and glutaraldehyde treatment of bacterial toxins as assessed by SDS-PAGE has been reported by Murphy (1967), Warren *et al.* (1973), Bazaral *et al.* (1973), Saletti and Ricci (1974), Warren *et al.* (1975), Cryz *et al.*(1981), Porro *et al.* (1980) and Pollack and Prescott (1982). In a recent report, the treatment of FHA with formaldehyde was reported by Di Tommaso *et* al. (1994). These workers also observed the presence of high MW material >200 KDa which only barely entered the gel. Thus, the generation of multimeric products after chemical treatment of LZ, OA and FHA with toxoiding substances appears common.

Effects on Structure and Biological Activities

In order to assess the effect of chemical treatment, lysozyme was tested for loss of lytic acitivity for *M. lysodeikticus* cells; FHA was assayed for changes in haemagglutinating (HA) activity; and pertussis toxin was tested for alteration in histamine-sensitizing activity (HSA). An attempt has been made to explain changes in biological activities in terms of the chemical modification of reactive residues by each toxoiding reagent. In

order to do so, the number of potentially reactive residues that may react with each toxoiding agent per molecule of LZ , OA, FHA and PT is shown in Table 45.

Lysozyme (LZ)

EDAC treatment of lysozyme (LZ) resulted in between 33 to 43% loss of activity whereas formaldehyde and glutaraldehyde treatment abolished activity to >99%. Relatively low levels of inactivation were obtained with EDAC even when the reagent was used at 3, 30, 60 and 120 times molar excesses of the number of potentially reactive residues of LZ (i.e. eight aspartate , two glutamate, three tyrosine and ten serine residues). The amino-acid sequence of hen egg-white LZ is shown in Fig. 35 with residues that are part of the active site highlighted in bold print.

Studies by Blake *et* al. (1965), Blake *et* al. (1967) and Phillips (1967) on the crystallographic structure of LZ in the late 1960s deduced that all carboxyl groups appeared to be on the surface of the molecule and hypothesised that 4 out of the 10 (Asp-52, Asp-66, Asp-101 and Glu-35) were involved in the catalytic action or were important in substrate binding. Dickerson and Gels (1969) stated that charged polar residues appear on the surface and the great majority of non-polar hydrophobic groups are buried in the interior. These studies imply that the amino-acids which are reactive with EDAC are probably accessible to the reagent. However, in practice, the treatment with carbodiimides is more complex and factors such as pH, addition of a nucleophile, addition of a denaturent, and the conformation of the protein, all govern the reactivity. The modification of LZ by carbodiimides has been discussed by Hoare and Koshland (1966), Lin and Koshland (1969), Lin (1970), Hayashi *et* al, (1972), Kramer and Rupley (1973), Timkovich (1977) and Yamada *et* al., (1983).

Lin and Koshland (1969) and Lin (1970) showed that modification of the carboxyl group of Asp-52 by two different carbodiimides in the presence of a nucleophile abolished enzyme activity. Later, Kramer and Rupley (1973) found that the 'exposed' carboxyl group of Asp-101 was modified most readily whereas the 'buried'

Table 45: Potentially reactive residues of lysozyme, ovalbumin, filamentous haemagglutinin and pertussis toxin with formaldehyde, glutaraldehyde and EDAC.

Amino-acid	Reactivity with[a]	No. of residues/ molecule of[c]			
		LZ	OA	FHA	PT
Total	-	129	385	3591	972
Lysine	F, G	6	20	173	24
Tyrosine	E, F, G	3	10	39	49
Tryptophan	F	6	3	14	9
Histidine	E, F, G	1	7	62	21
Asparagine	F	13	17	47	26
Cysteine	E, F, G	8[b]	6[b]	3[b]	2[b]
Aspartate	E	8	14	203	34
Glutamate	E	2	33	153	41
Serine	E	10	38	250	70

[a] E, F and G denote the toxoiding reagents EDAC, formaldehyde and glutaraldehyde respectively.

[b] cysteine residues in LZ were present as 4 disulphide bonds. The presence of free sulfhydryl groups in OA, PT and FHA was not determined.

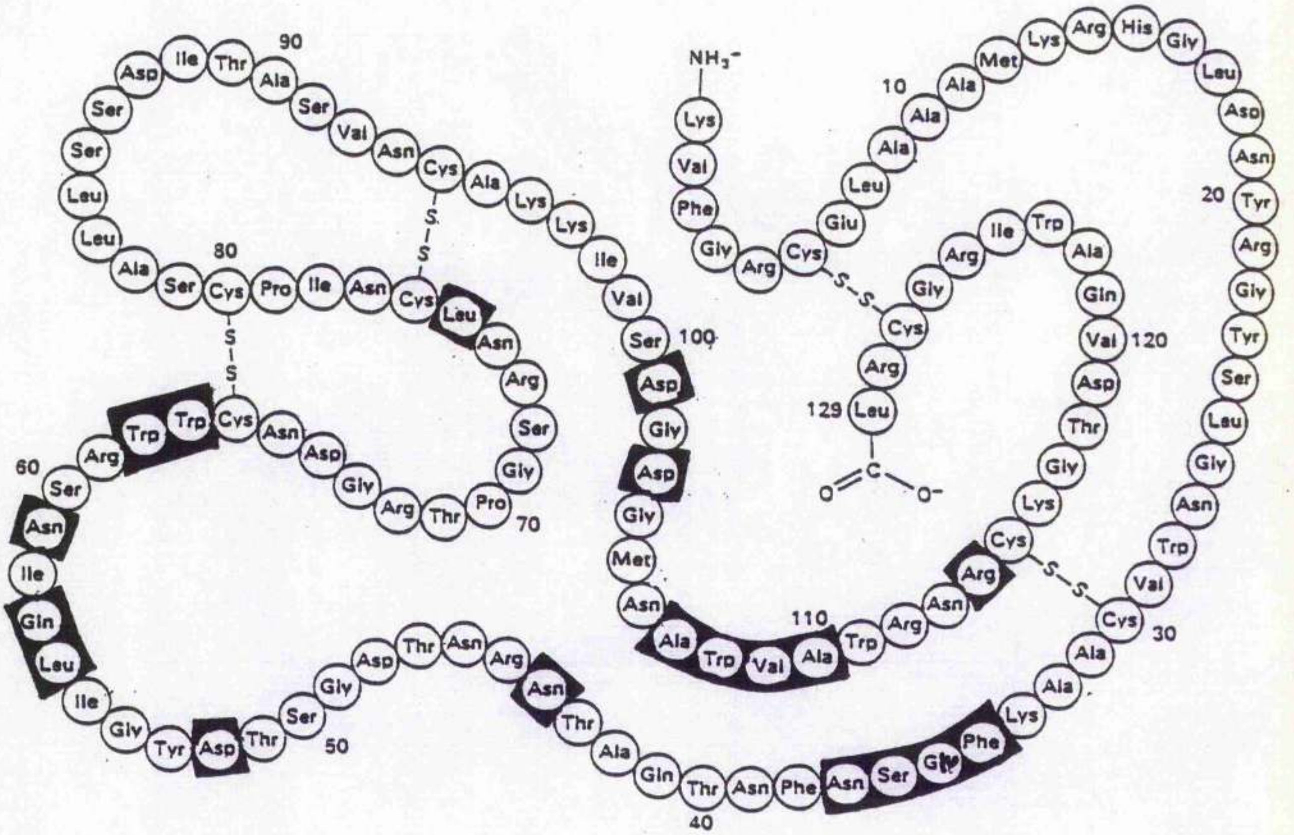
[c] The amino-acid sequences were obtained from Canfield (1965) for LZ, McReynolds *et al.* (1978) for OA, Domenighini *et al.* (1990) for FHA and Locht and Keith (1986) for PT.

carboxyl of Asp-66 and also Glu-7, Asp-18 were least reactive. The carboxyl of Glu-35 which is also largely 'buried' was again readily modified however, the rate of this reaction was dependent on the concentration of the nucleophile. These workers found that three additions of EDAC at 0.05 M to LZ at 10 mg/ml, pH 4.9-5.0 for 6 h without the addition of a nucleophile produced no significant effect on lytic activity.

The modification of LZ in this study was done without a nucleophile and also for 24 h compared with the 6 h used in the above studies. Despite these differences, some modification of the carboxyl groups in the active site probably did take place to cause the 33-43% loss in activity. The longer period of the EDAC reaction may have increased the rate of side reactions with resultant changes in protein conformation leading to reduced efficiency of substrate binding. Kramer and Rupley (1973) found the side reactions N-acylurea formation, inter- or intramolecular cross-linking and modification of tyrosine residues to be unimportant in their study. However, these reactions may have occured as evidenced by the formation of dimers and polymers of LZ which occur when activated carboxyls react with other protein groups as nucleophiles and form covalent cross-links as reported by Timkovich (1977).

Formaldehyde and glutaraldehyde predominantly react with the epsilon-amino groups of lysine (lys) residues (Fraenkel-Conrat *et* al., 1945). Since all 6 lys residues are on the surface of LZ they should be readily available for modification. There are no lysine residues involved in the binding of substrate in the active site, although Lys-33 is situated at the very bottom of the cleft. Imoto *et al* (1972) reviewed a number of studies which utilised various agents that modified the amino groups of LZ. Certain chemical reagents like acetic anhydride (Fraenkel-Conrat, 1950) which modified all 6 lys residues could completely inactivate the enzyme, whereas others, such as O-methylisourea did not affect enzyme activity; neither did the introduction of cross-links by phenyl-2-4 disulphonyl chloride (Moore and Day, 1968). This work has shown that modification of LZ with formaldehyde and glutaraldehyde, but not EDAC, can abolish activity.

Figure 35: The amino-acid sequence of hen's egg-white lysozyme. Residues that are part of the active site are highlighted in bold. Based on Canfield and Liu (1965) and Phillips (1966).



After an extensive literature search in BIDS, virtually no references were found for the inactivation of LZ with formaldehyde and glutaraldehyde. However, in an earlier study by Fraenkel-Conrat (1950), treatment with formaldehyde at neutral or acidic pH caused a 90% loss in activity in the presence of amines (e.g. alanine) and cross-linking between the amino groups and blocking of amide, guanidyl and aromatic groups was thought to occur.

Filamentous haemagglutinin (FHA)

Aside from the studies of Christodoulides *et al.* (1987, 1989), even after an extensive review of the pertussis literature, there have been no other reports on the treatment of a mixture of PT and FHA with EDAC. These authors used histamine-sensitising activity in mice as the marker for the detoxification of PT by EDAC. However, the effect of this agent on the haemagglutinating activity (HA) of FHA was not examined. While FHA in itself is not toxic (Irons and MacLennan, 1979b, Munoz *et al.*, 1981b, Sato and Sato, 1984), a number of investigations have routinely 'detoxified' the antigen preparation to ensure complete inactivation of trace amounts of PT left by the extraction and purification processes for vaccine manufacture (Sato *et al* , 1984, Tan *et al* , 1991, Ruuskanen *et al* , 1991). In none of these references was there any mention of inactivation of the HA by formaldehyde or glutaraldehyde treatment.

In the present study, the following parameters were investigated to provide more information on the effect of toxoiding agents on the HA activity of FHA: formaldehyde concentration, time and temperature of formaldehyde treatment, addition of lysine, time of glutaraldehyde treatment, EDAC concentration and time of exposure to EDAC. A formaldehyde concentration of 6.25 mM was sufficient to abolish all detectable HA activity. However, it was found necessary to use a temperature of 37 ^{o}C with exposure for 48 h. Since formaldehyde can potentially react with lysine, tyrosine, tryptophan, histidine, asparagine and cysteine residues (French and Edsall, 1945), the effect of this agent on FHA is difficult to interpret.

The reaction with the other aldehyde -glutaraldehyde was much faster in that all detectable activity was lost by 60 min either with or without the addition of lysine. The faster reaction rate with glutaraldehyde has also been reported in the review by Relyveld (1977) for the detoxification of whole-cell pertussis vaccine, tetanus toxin, diphtheria toxin and other microbial antigens. This agent modifies lysine residues, the formation of methylene bridges causing cross-links. These effects may impose conformational constraints and thus, destroy the HA. Also, Habeeb and Hiramoto (1968) found that glutaraldehyde reacted with alpha-amino groups of amino-acids, the N-terminal amino groups of some peptides, the sulphydryl group of cysteine, and were partially reactive with tyrosine and histidine residues. Again, as with formaldehyde, due to the complexity of the chemical reactions, it is difficult to predict the effect of the reagent on FHA.

Recently, Makhov *et al.* (1994) reported the structure of FHA based on electron microscopy, circular dichroism spectroscopy and computational analysis of its amino-acid sequence. FHA has a globular head with a 35nm shaft which tapers slightly from the head end. With the toxoiding agents, both inter-molecular cross-links and intra-molecular cross-links could have occurred between FHA molecules, causing the formation of large aggregates which may have resulted in the concealment of binding sites for RBCs. In the report by Tan *et al.* (1991), detoxification of FHA was achieved with formaldehyde instead of glutaraldehyde because the former reagent produced less FHA precipitation.

With EDAC, despite increasing the concentration of the reagent to 50 mM, the maximum percent inactivation of FHA was only 75%, and this was achieved in 24 h. Thus, under the conditions used in this study, there appears to be only a certain number of residues that are available for reaction with the reagent. Perhaps, cross-linking and macromolecular aggregation of EDAC-treated FHA caused the drop in HA. Modification of reactive residues may also disrupt the tertiary structure of the protein causing interference with receptor binding.

Pertussis toxin (PT)

The first acellular pertussis vaccine was produced in 1981 by Sato *et al.* (1984) and was a mixture of PT and FHA detoxified with formaldehyde. Similar preparations were tested in large-scale clinical trials in Sweden (Ad Hoc group for the study of pertussis vaccines, 1988), but the PT component was found to show some reversal to toxicity (Storsaeter *et al.*, 1990). This prompted investigations into alternative methods of detoxification.

Numerous acellular vaccine preparations have been formulated with the PT component detoxified with a variety of chemical detoxification agents of which formaldehyde has been most commonly used. Thus, formaldehyde treatment has been applied to crude extracts from *B. pertussis* culture supernates (Matsui and Kuwajima, 1959, Munoz and Hestekin, 1966) and to purified PT preparations (Sato *et al.*, 1974, Sato *et al.*, 1984, Rutter *et al.*, 1987). This present study found that formaldehyde treatment based on the method of Sato *et al.* (1984) of purified PT preparations and PT:FHA antigen mixtures removed 97 to ≥99% of the original HSA. Therefore, formaldehyde is effective in reducing the HSA of PT. In the early studies of Matsui and Kuwajima (1959) and Munoz and Hestekin (1966), 0.5% formaldehyde treatment for 7 days at 37 °C of culture supernates and saline extracts containing active PT was sufficient to destroy HSA completely. Sato *et al.* (1974), also reduced HSA to 0.1% of the original level before toxoiding by four days of formaldehyde treatment. Toxoiding conditions were generally similar to those used in this work. There were three to four additions of 0.2% formalin at pH 7.0 with 0.05 M lysine included in the reaction mixture. However, these workers found a reversal to toxicity, in that 3.3% of the original HSA was regained after storage at 37 °C.

Detoxification of PT alone with glutaraldehyde gave variable results. The inactivation of HSA was between 50 and 90%. In the present work, <50% inactivation were obtained for two of the three PT:FHA mixtures. Due to this very incomplete inactivation, the samples were retoxoided and then retested for HSA. Retoxoiding

resulted in sucessful detoxification of PT in the PT:FHA mixtures with ≤1-2% residual HSA remaining. However, with PT alone, levels remained at 50-90% inactivation. This is in disagreement with the results of Ruuskanen *et al.* (1991) and Garcia-Sanz *et al.* (1992) who obtained 100 to 1000 fold, and 98.9%, reductions in HSA respectively. The former report used a combination of glutaraldehyde and formaldehyde treatment. PT was treated with glutaraldehyde for 2 h then incubated with formalin and amino-acids for 7 days at 40 °C. Further details of the toxoiding procedure were not provided. Garcia-Sanz *et al.* (1992), used a 6-fold higher glutaraldehyde concentration and incubated for 4 rather than the 2 h used in our experiments. Interestingly, in an earlier report in which the toxoiding conditions were not specified, Garcia-Sanz *et al.* (1985), reported unsuccesful detoxification of their PT acellular vaccine preparation with glutaraldehyde, formaldehyde and heat.

In my experiments, conditions for glutaraldehyde treatment were based on the detoxification procedure of Munoz *et al.* (1981b). These authors specified that the final HSA was <25% of the original value (the dose that sensitised 50% of mice tested was >16 ng/mouse) after detoxification of a crystalline preparation of PT. Perhaps, these conditions need to be made more severe as in Garcia-Sanz *et al.* (1992) in order to obtain satisfactory destruction of HSA. In fact, the double toxoiding of the PT:FHA mixtures resulted in a sucessful detoxification of PT by glutaraldehyde.

Detoxification studies with PT and EDAC gave disappointing results despite following the toxoiding procedure exactly as described by Christodoulides *et al.* (1987). EDAC at 21 mM, which is equivalent to a weight-weight agent-protein ratio of 80:1 for 24 h at 37 °C, pH 5.0 was reported to remove >99% of the original HSA of a mixture of PT:FHA. Under these toxoiding conditions, in my experiments, however, the inactivation of HSA was generally between 50-90% except once when a PT preparation was 96% inactivated. As with glutaraldehyde, PT preparations were retoxoided with EDAC and retested for HSA. This time the residual HSA activity was

<1-2%. Possibly, this discrepancy was due to an error in the concentration of EDAC, or its deterioration upon storage.

Cloning of the gene encoding PT by Nicosia *et al.* (1986) and Locht and Keith (1986) revealed the absence of lysine residues in the S1 subunit of the toxin molecule. Therefore, since glutaraldehyde and formaldehyde react with lysine residues, A-protomer activity could be left intact. Nicosia *et al* , (1986), noted that treatment of PT with glutaraldehyde led to cross-linking of the S2, S3, S4 and the S5 subunits (which make up the B oligomer), with the formation of multimeric aggregates, whilst the S1 subunit retained its original size. Potentially reactive residues of PT with EDAC, formaldehyde and glutaraldehyde are listed in Table 44. Formaldehyde and glutaraldehyde can, in theory, react with a number of amino-acids additional to lysine; therefore modification of A-protomer activity could occur. Also, these agents introduce cross-linking which may result in conformational changes, accounting for the drop in activity. Since the only activity monitored during the detoxification of preparation in this study was HSA which is a function of the oligomeric B subunit (Nogimori *et al.* 1984a, 1984b), it is difficult to ascertain how much the A-protomer was affected by these reagents.

Barbieri and Cortina (1988) mapped the catalytic region of the S1 subunit which possessed ADP-ribosyltransferase activity and found substitution of Asp-11, Arg-13, and Trp-26 decreased enzyme activity to below detectable levels. Also, Glu-139 substitution reduced activity by 15%. Since EDAC reacts with carboxyl groups and formaldehyde with tryptophan, it is possible that A-promoter activity may be modified. In fact, Christodoulides *et al.* (1987) observed that the EDAC-treated PT:FHA mixtures did not significantly increase serum insulin levels, which is one of the biological activities of the A-promoter. Also, side reactions leading to the formation of N-acylurea adducts by reaction with EDAC (Timkovich, 1977) may also cause reduction in activity.

In conclusion, the complexity of the chemical reactions upon treatment of the four antigens examined in this study makes evaluation of the reaction products difficult. A fuller investigation into the other biological activities of PT and a complete chemical analyses of the four treated antigens is required to understand the effects of these toxoiding agents on these proteins.

IMMUNOGENICITY OF 'TOXOIDED' PROTEINS

The effect of chemical treatment on the immunogenicity of the four antigens ovalbumin (OA), lysozyme (LZ), filamentous haemagglutinin (FHA) and pertussis toxin (PT) was investigated in mice. The central questions addressed were, whether EDAC treatment caused an enhanced antibody response, whether this phenomenon occurred after EDAC treatment of selected model proteins and bacterial proteins, and whether immunogenicity obtained after immunization with the EDAC-treated antigen was superior to the conventional toxoiding agents formaldehyde and glutaraldehyde.

In the induction of an antibody response towards a foreign antigen, recognition by B-cells, T-cells and antigen presenting cells (APC) is involved (Unanue, 1984). B-cells can recognize some bacterial or parasitic antigens without any intermediary by membrane associated surface immunoglobulin (Ig). Antigens which stimulate this type of response are termed T-independent (T-ind) antigens. B-cells are capable of recognizing free or soluble exogenous antigens. T-cells require presentation of a peptide sequence of the antigen associated with certain proteins called major histocompatibility complex (MHC) molecules ultimately found on the surface of the APC. Antigens presented in this manner to T-cells are T-dependent (T-dep). T-cells possess the ability to recognize infected cells which contain foreign organisms and other antigens. Triggering of B-cells can be brought about by T-helper cells (T_H) which leads to the stimulation of a particular B-cell clone or clones and ultimately results with secretion of antibody directed towards T-cell epitopes.

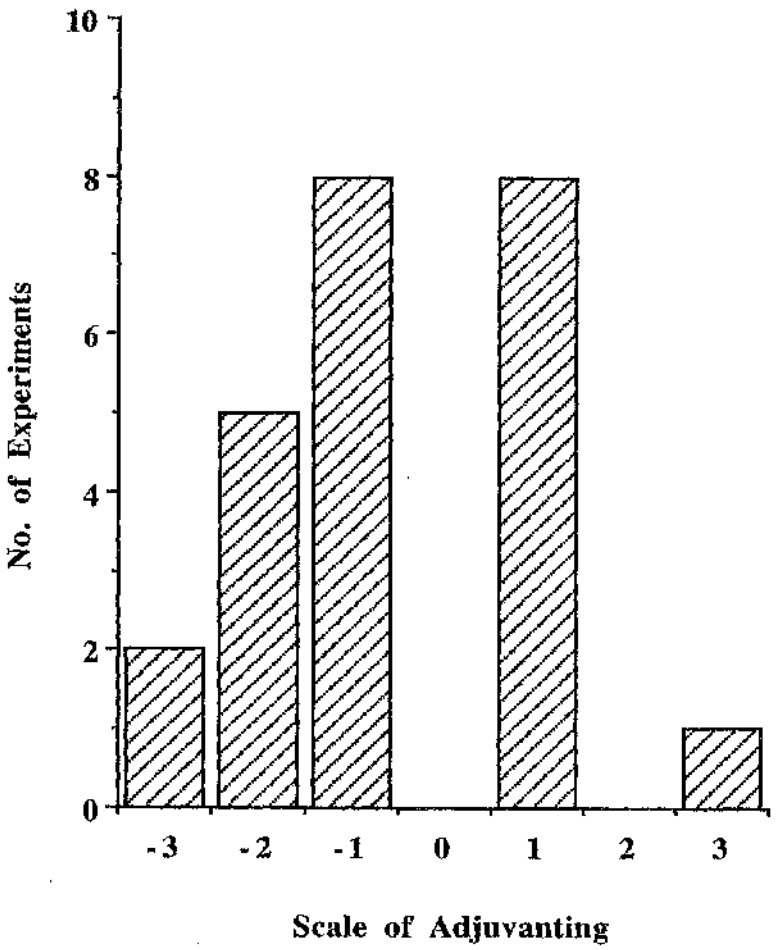
The antibody responses induced by untreated and 'toxoided' antigens are discussed separately for the four proteins used in this study. To simplify the interpretation of the effects the chemical treatments had on the immunogenicities of these antigens, summary diagrams were prepared by comparing geometric means (g.m.) ELISA titres for each antigen at each dose before and after chemical treatments by two-tailed t-tests. Each t-test was termed an experiment. A highly significant t-value ($P \leq 1\%$) was scored as a +3 or -3 for an adjuvanting or repressive effect respectively. Similarly, a score of +2 or -2 was applied for a significant t value ($P>1\leq5\%$). When differences between the two g.m.'s were not significant, a score of +1 or -1 was given depending on whether the mean of the treated antigen was higher or lower than the mean of the untreated preparation. A value of 0 was applied when the two means were identical. These scores were plotted on the x-axis as the scale of adjuvanting.

Ovalbumin

The effect of EDAC treatment of OA on antibody responses is summarized in Figure 36. An overall repressive effect is apparent with 15 'experiments' yielding negative values (7 of which were statistically significant) compared with 9 positive values (1 being statistically significant). A dose of 0.1 mg/mouse best shows this repressive effect whilst immunization with 0.01 or 1.0 mg/mouse did not always give lower g.m. titres relative to the untreated control. Statistical analysis of variance also showed that EDAC treatment of OA could reverse the adjuvanting effect of $Al(OH)_3$

$Al(OH)_3$ is a commonly used adjuvant and licenced for use in humans. In these experiments, treatment with EDAC could nullify the adjuvanting effects of $Al(OH)_3$. The OA preparations injected into mice with $Al(OH)_3$ in a formulation which should have made it more resistant to dispersal. As such, it acts as a depot for prolonged antigenic stimulation and should have perpetuated the immune response.

Figure 36: A summary of the effect of EDAC on the immunogenicity of ovalbumin



However, in this study, addition of $Al(OH)_3$ to OA-E preparations did not result in increased responses.

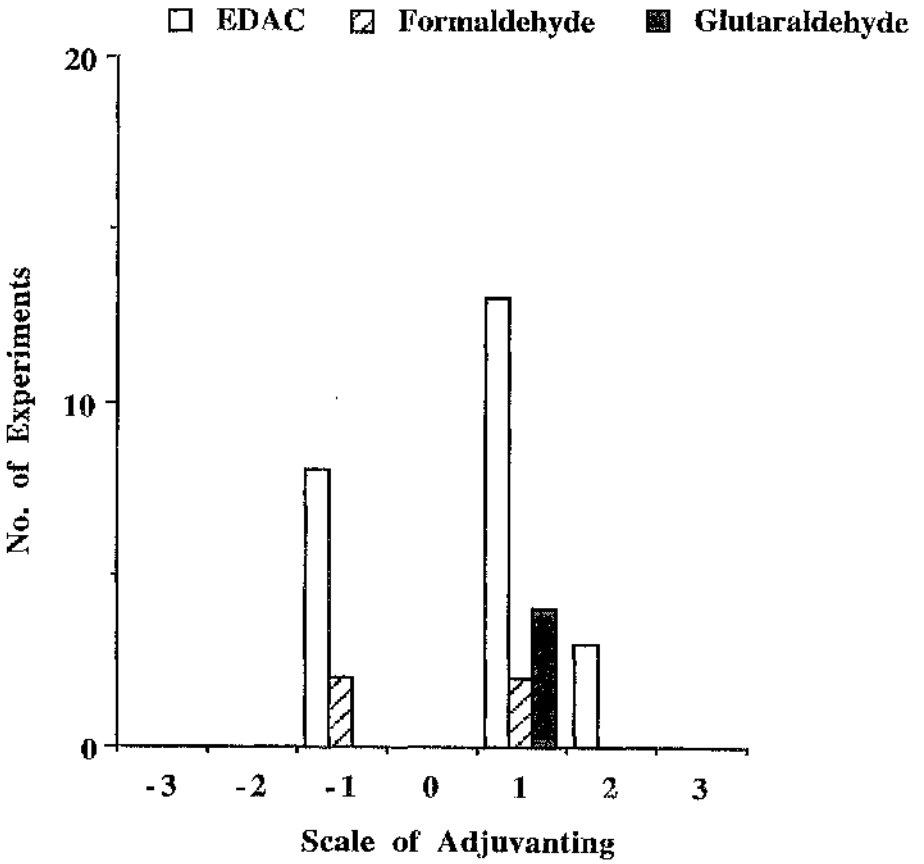
OA is a relatively small globular protein therefore it is endocytosed or pinocytosed by APC. This is followed by proteolytic processing to yield 'sequential' T-cell epitopes (Shimonkevitz *et* al., 1983). These authors indicated that preparations of denatured ovalbumin, which were largely aggregated, did not stimulate T-cell hybridoma tumour lines and suggested that this was due to insufficient exposure of critical sites. Allen (1987) reported that there was an absolute reqiurement for OA to be proteolytically cleaved before presentation could occur. Cleavage exposes epitopes which may be hidden by the tertiary conformation of the protein. In this study, the formation of polymers (visualized as faint high molecular weight bands by SDS-PAGE) by the EDAC treatment may have hindered processing of the protein, reducing the effective presentation of T-cell epitopes and therefore lower antibody titres. Vidard *et* al. (1992), generated 9 peptides which were antigenic T-cell epitopes which could be obtained from OA by digestion with different pools of proteases. Chemical modification by alkylation, denaturation and reduction of OA could reduce stimulation of certain T-cell hybridomas suggesting that these treatments were affecting OA processing. However, a large proportion of the treated protein remains in the monomeric form. Diment (1990) reported that cathepsin D, an aspartyl protease, had an important role in the processing of OA for antigen presentation. Treatment with OA primarily modifies aspartate and glutamate residues therefore perhaps the action of this enzyme is obstructed because of altered target residues. Formation of altered epitopes after EDAC treatment has been observed by Davis *et* al. (1984). Perhaps use of native OA as the coating agent in the ELISA was not appropriate for the detection of antibody against OA-E which may have altered epitopes. Thus, an ELISA developed with OA-E in the coating phase might detect antibodies to epitopes not present on the native molecule.

Lysozyme

Antibody responses towards LZ and LZ treated with EDAC, formaldehyde and glutaraldehyde were quantified from mouse sera as described previously. Summary diagrams of the results from t-tests comparing the untreated with the chemically modified preparations were constructed (Figure 37). It can be seen that with EDAC (Figure 37), 16 'experiments' had a positive adjuvanting effect, of which only 3 were statistically significant, whereas 8 statistically insignificant experiments had negative values. With formaldehyde and glutaraldehyde, no major effects on adjuvanticity could be demonstrated. This may have been due to the small number of 'experiments' from which no definite conclusions could be made. Nevertheless, a demonstrable adjuvanting effect was obtained after treatment with EDAC (Figure 37). The analysis of variance also revealed statistically significant adjuvanting effects with EDAC from two separate experiments but none with either formaldehyde and glutaraldehyde. This adjuvanting effect of EDAC was obtained only after immunization of LZ-E treated with EDAC at 21 mM compared with untreated LZ.

Lysozyme is a T-dependent antigen (Harding *et* al. 1988), therefore B-cells must interact with T-cells in the induction of an antibody response. B-cells which can also act as APCs use surface immunoglobulin (Ig) to take up and concentrate specific antigen which is then processed and presented in association with MHC class II molecules to T-cells. Lanzavecchia (1985) estimated that B-cells accumulate specific antigen up to 10^4-fold more efficiently then other antigen-presenting cells. Enhanced binding of LZ-E to membrane Ig on B-cells may result in enhanced uptake and processing. B-cells may preferentially present LZ-E to T-cells which may lead to the stimulation and proliferation of the respective B-cell clones (Chesnut and Grey, 1981 and Lanzavecchia, 1985) resulting in the observed increase in immunogenicity. EDAC treatment of LZ may slightly alter the tertiary conformation of the protein exposing

Figure 37: A summary of the effect of EDAC, formaldehyde and glutaraldehyde on the immunogenicity of lysozyme.



binding sites or make binding sites more readily accesible to membrane bound or free Ig.

Polymerization or aggregation of foreign antigen can lead to increased uptake by macrophages (Harding *et* al., 1988). EDAC treatment of LZ led to polymerization particularly dimerization but to a lesser extent than treatment with formaldehyde and glutaraldehyde. The increase in antibody response towards preparations of LZ-E could be due to the antigen being more readily "seen" and accessible to the APC system. Since increased immunogenicity did not result with formaldehyde and glutaraldehyde, excessive polymerization of the antigen may make it more resistant to proteolytic breakdown during antigen processing thereby reducing effective presentation. B-cell activation through cross-linking of B-cell antigen receptors can occur; however, excessive B-cell receptor cross-linking by antigen might decrease B-cell triggering. The LZ-F and LZ-G preparations may not have enhanced immunogenicity, but neither did they reduce it. Perhaps there is limited proteolytic degradation of these preparations, with enough stimulation to generate responses similar to the untreated LZ, but not to enhance the responses.

Filamentous Haemagglutinin

Filamentous haemagglutinin (FHA) has been shown to protect mice against respiratory challenge with *B. pertussis* (Sato *et al.* 1981b, Sato *et al.* 1982, Oda *et al.* 1984 and Sato and Sato 1984). Since these observations have been made, FHA is considered to be one of the protective antigens of this organism. Most of the acellular vaccine preparations have FHA as a component (Sato *et al.* 1984, Rutter *et al.* 1987, Podda *et al.* 1991 and Edwards, 1993).

FHA is essentially non-toxic and important in bacterial adhesion (Relman *et al.* 1989). The rationale for its inclusion therefore was for the production of antibodies which would block attachment of the organism to the respiratory mucosa and thereby

reduce the risk of infection (Edwards, 1993). Indeed, Swedish field trials with two Japanese acellular vaccine preparations showed that the two component PT:FHA preparation present in the ratio of 1:1 (JNIH-6) was more protective in infants (vaccine efficacy 69%) than the monocomponent PT (JNIH-7) for which vaccine efficacy was 54% (Ad Hoc group for the study of pertussis vaccines, 1988).

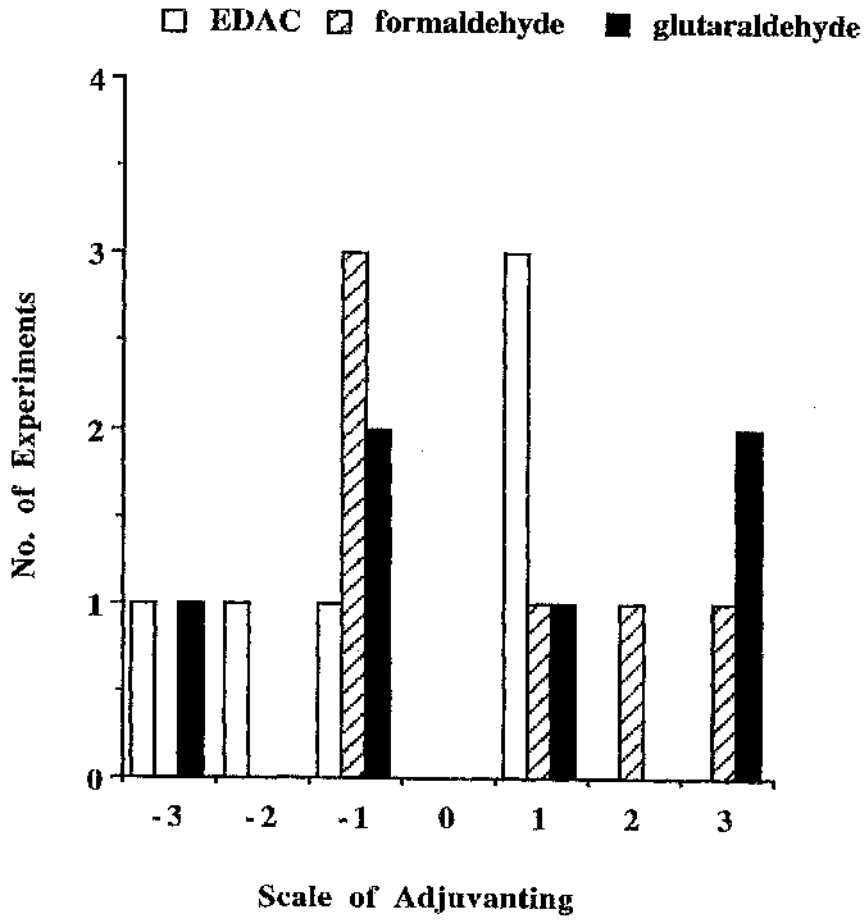
FHA was subjected to a mild chemical treatment with toxoiding agents to inactivate trace amounts of contaminating toxic material such as PT present after the extraction and purification procedures or to stabilise the protein before incorporation into a vaccine preparation (Sato *et al.* 1984, Podda *et al.* 1991).

In my work, FHA was extracted from a Tn5 mutant *B. pertussis* strain BP357 (Weiss *et* al, 1985) so that the preparation would be free of PT. Since PT is known to enhance antibody responses to antigens that are injected with it, it was important to ensure that the FHA preparation was free from the toxin in order to compare the effect of chemical toxoiding agents on the immunogenicity of FHA. The effect of injecting FHA with a quantified amount of PT after toxoiding the mixture was examined in separate experiments.

The summary diagrams depicted in Figure 38 show the positive or negative effects on immunogenicity of chemically-treated preparations of FHA. Overall, EDAC treatment had a negative adjuvanting effect, with three negative experiments (two of which were statistically significant) as opposed to three non-significantly positive experiments. Formaldehyde-treatment of FHA seemed to have a positive adjuvanting effect on immunogenicity as shown by three positive (2 significant) compared with three insignificant negative experiments. Glutaraldehyde treatment had similar effects with two out of three significant positive experiments and one out of three significant negative experiments.

A number of relatively recent publications detail the production of acellular pertussis vaccines in which FHA was treated with formaldehyde or a combination of formaldehyde and glutaraldehyde (Watanabe *et al.*, 1991; Ruuskanen *et al.*, 1991; Tan

Figure 38: A summary of the effect of EDAC, formaldehyde and glutaraldehyde treatment on the immunogenicity of filamentous haemagglutinin (FHA).



et al.,1991; Podda *et al.*, 1991). In none of these studies was a direct comparison of the treated preparation made with native FHA. Furthermore, since the FHA was made for vaccine purposes, all the preparations were injected as mixtures with PT and/or 69 KDa. It is therefore difficult to assess from the published literature exactly how the immunogenicity of FHA was affected by toxoiding. Certainly, the immunogenicity after chemical treatment as regards protective activity was not impaired since treated FHA gave satisfactory ELISA titres and aided protection from *i.c.* challenge of mice against virulent *B. pertussis* (Tan *et al.* 1991). These workers also reported that immunization with mixtures of treated-FHA and pertussis toxoid resulted in higher FHA titres than immunization with a preparation of WCPV. In all of the aforementioned publications, there were satisfactory increases in ELISA titres towards the treated FHA in the various ACPV preparations probably as a result of immunisation with the known adjuvants PT and aluminium hydroxide or aluminium phosphate.

The slight enhanced anti-FHA responses towards formaldehyde and glutaraldehyde-treated FHA observed in this study, may be a result of polymerization of the protein and therefore continuous stimulation of the immune response or increased accessibility to APCs. Indeed, as observed by SDS-PAGE, FHA-F and FHA-G preparations were polymerized to a greater extent than FHA-E.

In 1991, Di Tommaso *et al.* found that proteolytic fragments of FHA stimulated certain T-cell clones which were mainly from the carboxy terminal and amino terminal regions of the protein. In a later report (Di Tommaso *et al.* 1994), T-cell responses were investigated towards formaldehyde-treated *B. pertussis* antigens, FHA being one of them. They reported that formaldehyde-treatment of the protein resulted in the inhibition of certain T-cell clones and suggested a number of causes for this inhibition. Firstly, that formaldehyde-treated FHA was either more resistant to antigen processing with interference in proteolytic breakdown into peptides; or that there was reduced peptide binding to MHC molecules; or lastly, that there was impaired recognition by T-cell receptors of the peptide-MHC complex. The slight negative effect

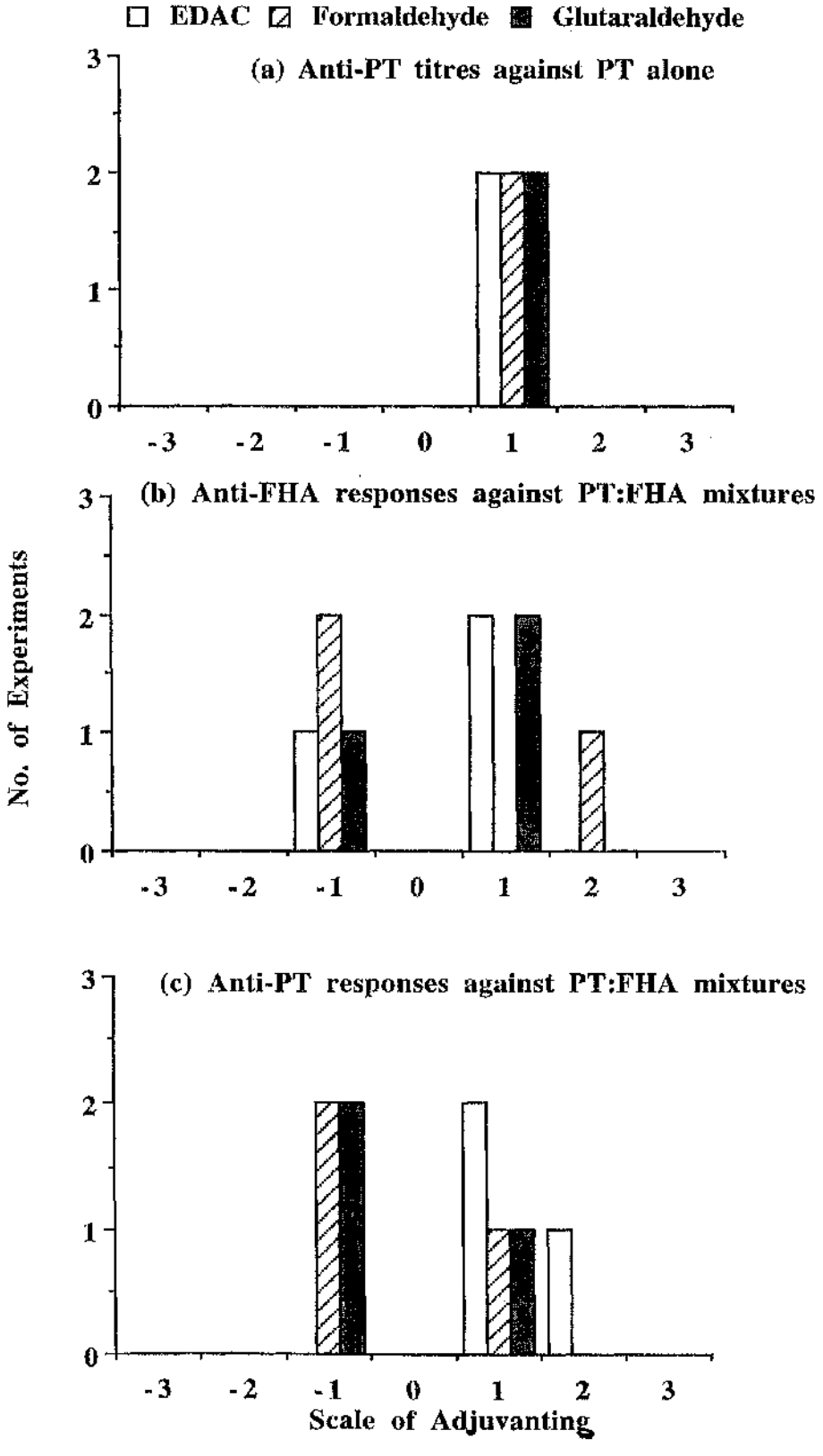
of EDAC on the immunogenicity of FHA could be due to any of the above reasons. However, the conflicting results obtained with FHA-F and FHA-G preparations suggests that treatment with the two aldehydes modified the FHA in a manner different to EDAC. Perhaps treatment with the two aldehydes which results in cross-linking of the molecules changes the status of FHA as a T-dependent antigen to a T-independent antigen due to the increased aggregation of the treated FHA.

Pertussis Toxin and Mixed Antigen Preparations of Pertussis Toxin and Filamentous Haemagglutinin

Fears over the safety of WCPV preparations due to a high concentration of endotoxin and the possibility of active PT led to the development of acellular vaccines (Griffiths, 1988). Although high levels of anti-PT and anti-FHA were found in the serum of vaccinated individuals, the vaccines were not deemed satisfactory due to some reversion of the activity of the formaldehyde-detoxified PT component (Storsaeter *et al.*, 1988). Following this observation, a number of workers attempted alternative chemical detoxification procedures with formaldehyde (Sato *et al.*1984, Watanabe *et al.*, 1991), glutaraldehyde (Quentin-Millet *et al.*, 1988), hydrogen peroxide (Sekura, 1988), tetranitromethane (Winberry *et al.*, 1988) and EDAC (Christodoulides *et al.*, 1987) as some alternatives. The latter authors observed that EDAC-treatment of PT not only reduced toxicity to below detectable levels but actually enhanced immunogenicity. This observation formed the basis of the present investigation.

The immunization of mice with chemically-detoxified PT only, or detoxified in conjuction with FHA, was examined. Summary diagrams of the effects of the three toxoiding agents on the immunogenicity of the preparations are shown in Figure 39. Figure 39a revealed that immunization with PT-E, PT-F or PT-G without FHA showed no significant adjuvanting effects in comparison with untreated PT (by t-tests carried out with ELISA titres from sera raised against 1 or 5 μg/mouse dose of PT or its

Figure 39: A summary of the effect of EDAC, formaldehyde and glutaraldehyde on the immunogenicity of pertussis toxin (PT) alone and antigen mixtures of PT and FHA.



toxoids). Statistical analysis by 4-point parallel line assays however, indicated that the PT-E and PT-G preparations were just significantly adjuvanted at the P= 5% level. When the three toxoids were compared relative to each other by one-tailed t-tests for each dose at 1, 5 and 25 µg/mouse, formaldehyde detoxified PT induced significantly lower responses when compared with EDAC-treated PT at the 25µg dose. Thus, it can be concluded that detoxification of PT alone with EDAC and glutaraldehyde *does* result in the production of a higher response relative to the native toxin but this adjuvanting only just achieves statistical significance. An important point is that the PT-E and PT-G preparations used to immunize the mice were incompletely detoxified (only between 50 to 90%). The PT-F preparation was more acceptably inactivated with HSA ≥99% inactivated. The incomplete detoxification of PT-E and PT-G were only subsequently realised as mice had already been immunized. Also, the incomplete detoxification of PT-E and PT-G preparations was unexpected since the detoxification conditions chosen from the existing literature were standard methods. Three separate preparations of PT:FHA present in the ratio's of 1:2.25 for AP16, 2:3 for PF3 and 1:5 for PF4 were tested for HSA and immunogenicity after treatment with the three toxoiding agents. Whilst formaldehyde treatment inactivated HSA by between 98 to 99%, the glutaraldehyde treatment was unsuccesful in reducing toxicity (<50% HSA inactivation). Treatment with EDAC was marginally more effective in reducing the activity of AP16 and PF3 (between 50 to 90% inactivation) and PF4 (90 to 90% inactivation). The concomitant effects on the immunogenicity were also examined and the results are summarized for anti-FHA and anti-PT titres in Figures 39b and 39c respectively. There were no major enhancing effects found with anti-FHA responses although formaldehyde-treated PF3 and PF4 were significantly lower than the respective untreated preparations. Significantly higher anti-FHA responses were obtained with AP16 treated with formaldehyde and EDAC however, the immunogenicity of the untreated preparation itself was very low. Since this preparation had been extracted previously and stored frozen for a number of years before use, it

was thought that the FHA had probably degraded, but toxoiding which results in aggregation may have restored immunogenicity. Due to the low immunogenicity of the native AP16, results from the analysis were interpreted with caution. Anti-PT responses were similar for all three toxoids therefore they were analysed collectively. A definite adjuvanting effect was noted for EDAC, whereas formaldehyde and glutaraldehyde treatments did not significantly enhance or reduce immunogenicity relative to the untreated preparations.

The inherent toxicity of bacterial toxins have made parallel comparisons of the immunogenicity of the untreated toxin and the detoxified counterpart impractical. In the few cases where the toxin could be compared with the corresponding toxoid it was found that the effect of toxoiding agents varied with the toxin being treated. Enhancement of immunogenicity was found after formaldehyde treatment of staphylococcal ∂-haemolysin (Stearne and Birkbeck, 1980), staphylococcal enterotoxin B (Warren *et al.*, 1973) and *B. pertussis* heat-labile toxin (Livey and Wardlaw, 1984). However, these toxins were poorly immunogenic before detoxification. Polymerization of the toxin molecules were suggested to be the cause of the enhanced responses. Also, the presence of free molecules of circulating toxoid (which had less efficient binding to host cell receptors as a result of the formaldehyde treatment) may have resulted in continued stimulation of the immune response. Conflictingly, formaldehyde treatment of cholera toxin (Holmgren *et al.*, 1972) and *Pseudomonas aeruginosa* exotoxin A (Pavlovskis *et al.* 1981; Cryz *et al.*, 1981; Cryz *et al.*, 1983) were reported to reduce immunogenicity. This was attributed to storage and handling of the materials used for immunization, the age of immunized animals, different aggregation states of the immunogen and an altered antigenic status of the toxoided molecules.

Results from this study also indicated that immunisation with PT-F alone repressed immunogenicity relative to the native toxin. However, the anti-PT response was not affected when mixtures of PT and FHA detoxified with formaldehyde were injected. The anti-FHA response was significantly lower for two out of the three

formaldehyde-detoxified mixtures of PT and FHA. Formaldehyde toxoiding of PT was routinely used in the manufacture of Japanese ACPV preparations. Sato *et al.* (1984) detoxified a mixture of PT and FHA with formaldehyde and stated that their preparation was one-tenth as toxic as conventional WCPV but just as effective in the production of anti-FHA and anti-PT in children. Similarly, Watanabe *et al.* (1991) also detoxified antigen mixtures of PT and FHA with formaldehyde in the production of Kitasato ACPV preparations and found anti-PT and anti-FHA responses in mice to be comparable with WCPV. In a report by Sato and Sato (1988), formaldehyde-detoxified PT:FHA mixtures induced comparable anti-PT responses to WCPV in mice but the anti-FHA responses were higher. In my study, PT:FHA mixtures treated with the three toxoiding agents were also compared to a heat-killed sonicated suspension of *B. pertussis* cells although only one-quarter of the human dose (5 OU) was injected into each mouse. Anti-PT and anti-FHA responses were lower than with the purifed detoxified antigens. This was probably a result of the lower quantity of antigen present in the sonicated preparation.

It is difficult to assess the effect of formaldehyde on the immunogenicity of PT and PT:FHA mixtures from these studies since firstly, no direct comparison was made with the native toxin and secondly being ACPV preparations, the detoxified PT was usually injected with other antigens apart from FHA. Comparisons were usually made with WCPV preparations and equivalent immunogenicity was reported. In 1989, Pizza *et al.*, constructed a non-toxic mutant of PT using molecular genetic techniques and termed it PT-9K/129G. This mutant protein contained an Arg^{9} to Lys and a Glu^{129} to Gly substitution in the S1 subunit which made it devoid of any activity. This genetically engineered protein was not only devoid of toxicity, but also did not revert to its active form, which is a problem with formaldehyde-detoxified PT (Sato and Sato, 1988). Nencioni *et al.* (1990), showed that PT-9K/129G did not change its biological and immunological properties; therefore it was possible to explore the effect of chemical treatment on immunogenicity, on a molecule which was practically identical to the

native toxin without the associated toxicity. In a later report, Nencioni *et al.* (1991), showed that after formaldehyde treatment of PT-9K/129G the total amount of antibodies was not affected. Nor did the specificity and quality of the antibodies change with the severity of the formaldehyde treatment. However, they found that the formaldehyde treatment affected the natural B-cell epitopes present on the molecule and postulated that the immune system may produce antibodies directed against non-protective epitopes, or lower affinity antibodies for protective epitopes. It is possible therefore that the lower responses obtained upon injection of my PT-F or (PT:FHA)-F may be due to altered antigenicity of the resulting toxoids. Also, aggregation or alterations in the conformation of the toxin after the formaldehyde treatment may mask immunodominant epitopes. In the review by Rappuoli *et al.* (1994), treatment of PT with formaldehyde was reported to eliminate some B-cell epitopes. Di Tommaso *et al.* (1994) reported that formaldehyde treatment of *B. pertussis* antigens impaired proteolytic processing, consquently with fewer T-cell epitopes being generated. Thus, the reduced immunogenicity of formaldehyde-detoxified antigens can be explained by these observations.

Glutaraldehyde-treatment of PT and PT:FHA mixtures did not have as deleterious effects on the immunogenicity as formaldehyde treatment. In view of the fact that glutaraldehyde modifies proteins in a similar manner to formaldehyde but with bifunctional reactivity, the antigenicity of the molecules would be expected to be modified accordingly. However, unlike formaldehyde, the glutaraldehyde treatment used in these experiments did not inactivate the HSA of PT effectively. As mentioned previously, the HSA of PT-G was inactivated only to levels of between 10 to 50% of the original. The PT:FHA mixtures treated with glutaraldehyde were only <50% inactivated. Thus, the PT molecule appears to have been minimally affected by the glutaraldehyde treatment as regards toxicity, suggesting that antigenic epitopes were probably also unchanged.

Detoxification of PT with glutaraldehyde in my experiments was done according to the conditions specified by Munoz *et al.* (1981a, 1981b). These workers reported inactivation of HSA activity >75%. I found that a double toxoiding treatment with glutaraldehyde was required before HSA could be inactivated to >98%. Unfortunately however, the immunogenicity of these retoxoided preparations was not determined. In a report by Garcia-Sainz *et al.* (1985), a *B. pertussis* cell supernate which contained PT amongst other antigens was detoxified by a more severe glutaraldehyde treatment. The antibody response towards the toxoided preparation was investigated by Western blotting (Garcia-Sainz *et al.*, 1992) and the response was mainly directed towards the A-protomer. These authors suggested that the A-protomer was associated with or cross-linked to other subunits making the A-protomer a better immunogen in the toxoided preparation compared with the untreated preparation which contained the isolated subunits. Since the HSA is a property associated with the B-oligomer (Nogimori *et al.*, 1984a, 1984b) it can be concluded that the glutaraldehyde treatment applied in this study did not affect the B-oligomer.

Ruuskanen *et al.* (1991), detoxified PT with glutaraldehyde followed by a longer incubation with formalin. Their PT toxoid was devoid of HSA at 25 µg/mouse and a good correlation was found between anti-PT measured by ELISA and neutralizing antibody measured by the CHO assay, suggesting the toxoid was still antigenic. In the study by Tan *et al.* (1991), glutaraldehyde treatment was done in a buffer containing glycerol as an anti-aggregating agent. Immunogenicity of the preparation was similar to the WCPV control. Again, no direct comparison was made with the untreated preparations. Results from these studies did not reveal any major differences between the toxin and toxoid with or without the presence of FHA. This may have due to the little change in the PT molecules as evidenced by the lack of HSA inactivation. Relyveld (1977) compared the immunogenicity of diphtheria and tetanus toxoids detoxified with formaldehyde and glutaraldehyde. Both glutaraldehyde-treated toxins had superior immunogenicity over formaldehyde toxoids. The same observation

was made in this study with PT but again, due to the incomplete inactivation of HSA it was difficult to say how much the PT was modified and therefore, the superior immunogenicity of glutaraldehyde-treated *B. pertussis* antigens is questionable.

The observation of the enhancing effect on the immunogenicity by EDAC treatment of a mixture of PT and FHA made by Christodoulides *et al.* (1987) was further investigated. A clear adjuvanting effect was demonstrated after EDAC-treatment of PT injected either alone or with FHA but not for FHA alone or as a mixture with PT. These workers reported that a much higher ELISA reading (A_{492}) was obtained with sera raised against 5 μg of toxoid compared with the same dose of toxin (per mouse). They also reported that a dose of 5 μg was not sufficient to induce a detectable anti-PT or anti-FHA response. Since their antigen mixtures had been adjusted to PT:FHA ratios of approx. 1:1, it is assumed that the PT content of a typical 5 μg dose would be 2.5 μg. The content of PT in 5 μg of samples used in this study (assuming that the total protein in the preparations were just PT or FHA), would be 1.5, 2 and 0.83 μg in AP16, PF3 and PF4 respectively. Similarly, FHA content would be approx. 3.5, 3 and 4.15 μg. The differences in the better responses could not be satisfactorily explained since the two immunization schedules, and mouse strains were identical.

The use of EDAC as a toxoiding agent has been reported by Lonnroth and Holmgren (1975) for cholera toxin and Klipstein *et al.* (1982) for *E. coli* heat-labile enterotoxin. EDAC was not succesful in reducing the toxicity, and in neither case was an enhanced response reported. On the contrary, Klipstein *et al.* (1982) found that treatment with EDAC actually reduced both antigenicity and immunogenicity. Thus, to my knowledge the only instances of an enhanced response from EDAC treatment of a protein antigen was by Christodoulides *et al.* (1987) and partially confirmed in my studies. These latter demonstrate that treatment of PT does result in enhanced antibody responses. However, what proportion of these antibodies were neutralizing and thus protective was not investigated. Stimulation of protective antibodies was demonstrated by *i.c.* and *i.n.* challenge of immunized mice by Christodoulides *et al.* (1987). Thus, it

can be concluded that EDAC-treated PT does produce protective antibody. Again however, as with glutaraldehyde, the toxicity of PT was not effectively reduced by treatment with EDAC at the concentrations used and therefore, the quantity of antibody generated towards modified epitopes would also be reduced. The report by Nencioni *et al.* (1991) stated that the total amount of antibodies did not change after injection with formaldehyde-detoxified PT-9K/129G. Further investigation into the specificity and protective capacity of these antibodies generated by EDAC-treated PT is required.

The enhanced response towards PT may reflect a change in antigen clearance so that the antigen remains localised in the lymph nodes providing a constant source of stimulation, coupled with increased resistance to antigen degradation. Cross-linking of PT molecules may change the size and conformation of the protein so that immunodominant epitopes become more accessible and more effectively presented. Antigen processing of the treated protein may yield sequential T-cell epitopes which stimulate a greater population of T-lymphocytes. An altered conformation may present non-protective epitopes which are more antigenic. EDAC-treatment may alternatively increase susceptibility of the molecules to APC proteases with generation of an increased number of T-cell epitopes. Thus, the interaction of the immune system with toxin and toxoid is complicated, and further experiments would be required to study these facets.

IMPLICATIONS OF THIS STUDY FOR FUTURE ACELLULAR VACCINES

As noted above (p. 187) various formulations of ACPV provided protection against pertussis and some of them were more effective than the Connaught WCPV. The most efficacious ACPVs appeared to be the 5-component Connaught product, the 3-

component Smith-Kline Beecham vaccine, and the three-component Biocine vaccine which contained the components in the quantities shown below.

Connaught product:	Glutaraldehyde-inactivated PT (10 μg)
(5-component)	FHA (5 μg)
	Fimbriae 2 and 3 combined (5 μg)
	Pertactin (3 μg)
Smith-Kline Beecham product:	Formalin and glutaraldehyde inactivated PT (25 μg)
(3-component)	FHA (25 μg)
	Pertactin (8 μg)
Biocine product:	Genetically-inactivated PT (5 μg)
(3-component)	FHA (2.5 μg)
	Pertactin (2.5 μg)

The clinical trial conducted in Italy by Greco *et al.* (1996), revealed that 5 μg genetically-inactivated PT stimulated greater anti-PT IgG both in ELISA and by CHO-cell clustering assay for neutralizing antibody than 25 μg chemically-detoxified PT, even though both preparations were of similar protective efficacy. According to Rappuoli *et al.* (1995), the superior immunogenicity of the genetically-detoxified pertussis toxoid was due to the structural integrity of the molecule with retention of T and B-cell epitopes. The induction of a satisfactory neutralizing antibody response with only one fifth of the dose normally required to elicit protective antibody with chemically-detoxified PT also resulted in fewer adverse reactions.

Marsili *et al.* (1992) investigated the suitability of a strain of pertussis producing the genetically detoxified PT molecule for production of a WCPV preparation. They found that seological responses towards pertussis antigens did not differ from conventional WCPV. However, responses towards the diphtheria and tetanus toxoids

were reduced, suggesting that the genetically-inactivated pertussis toxoid had not retained its adjuvanting activity. A mild chemical treatment with formaldehyde was applied to stabilize genetically-inactivated PT (Nencioni *et al.*, 1990) and preparations of FHA and pertactin (Podda *et al.*, 1991). The use of EDAC instead of formaldehyde to stabilize the genetically detoxified PT should therefore be investigated. EDAC treatment might result in further stimulation of protective antibody towards the PT itself and also to other antigens injected with it. The serological responses towards the other pertussis vaccine components, namely pertactin and fimbriae could be evaluated after a mild EDAC treatment and also whether their shelf life might benefit from the treatment. The immune responses towards EDAC-modified PT and LZ could be studied further to analyze how changes in structural conformation could lead to the stimulation of enhancement of antibody production.

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APPENDICES

Appendix 1

1% (w/v) 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 of DW and the pH adjusted to 7.1 with 1N NaOH and the volume made up to 1L. Aliquots were dispensed and autoclaved at 121 °C, 15 psi for 15 min. For preservation of stock cultures, glycerol (10% v/v) was added before autoclaving.

Bordet Gengou agar plates

The BG agar base (9 g) was dissolved in 250 ml DW containing glycerol (4% v/v), autoclaved at 121 °C, 15 psi for 15 min., and cooled to 56 °C. Defribinated 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 stored at 4 °C and used within two weeks.

Cyclodextrin liquid (CL) medium (Imaizumi *et al.*, 1983)

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
9. L-cysteine	0.04 g
10. $FeSO_4.7H_2O$	0.01 g
11. Ascorbic acid	0.4 g
12. Nictotinic acid	0.004 g
13. Glutathione	0.15 g
14. Casamino acids	1.0 g
15. Methylbeta cyclodextrin	1.0 g

Ingredients 1-8 , 14 and 15 were dissolved in 800 ml of DW and the pH adjusted to 7.6 with 2.5 N HCl. The volume was made up to 990 ml and autoclaved at 121 °C, 15

psi for 15 min. Ingredients 9-13 were dissolved in DW, filter sterilized and added aseptically to the autoclaved medium.

Appendix 2

Buffer recipes used in the extraction of antigens of *B. pertussis* antigens

0.05M Tris -HCl buffer, pH 8.0

Tris	6.055 g

The Tris was dissolved in 900ml of DW and the pH was adjusted to 8.0 with 5N HCl. The volume was made up to 1L with DW.

0.05M Tris-HCl, pH 8.0 + 1.0M NaCl

Tris	6.055 g
NaCl	58.44 g

The Tris was dissolved in 800ml of DW and the pH was adjusted to 8.0 with 5N HCl. The NaCl was dissolved and the volume made up to 1L.

0.1M Tris-HCL, pH8.0 + 0.5M NaCl + 6M urea

Tris	12.11 g
NaCL	29.22 g
urea	360.36 g

The Tris was dissolved in 200ml of DW and the pH was adjusted to 8.0 with 5N HCl. The NaCl and urea were added and allowed to dissolve over a moderately heated magnetic stirrer. The volume was made up to 1L with DW.

0.1M Tris-HCl, pH 8.5 + 0.5M NaCl

Tris	12.11 g
NaCl	29.22 g

The Tris was dissolved in 900ml of DW and the pH was adjusted to 8.5 with 5N HCl. The NaCl was dissolved and the buffer volume was made up to 1L with DW.

0.1M sodium acetate, pH 4.5 + 0.5M NaCl

sodium acetate	8.203
NaCl	29.22 g

The sodium acetate was dissolved in 900ml of DW and the pH was adjusted to 8.5. The NaCl was dissolved and the volume made up to 1L with DW.

Appendix 3

Layout of a Typical Immunization Experiment

Animal Identification	Preparation	Dose (mg)
Blue head	1	10.0
Blue rump	1	1.0
Blue back	1	0.1
Blue head and rump	2	10.0
Blue head and back	2	1.0
Blue back and rump	2	0.1
Red head	3	10.0
Red rump	3	1.0
Red back	3	0.1
Red head and rump	4	10.0
Red head and back	4	1.0
Red back and rump	4	0.1

A total of 60 mice was divided into groups of 12 per cage and injected with the four preparations at the three doses. Each animal was identified as listed in the table.

Appendix 4

Enzyme-linked immunosorbent assay : buffer recipes

Coating buffer, pH 9.6

0.05M Carbonate buffer, pH 9.6

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

Dissolved in DW and made up to 1L

Washing buffer

Phosphate buffered saline, pH 7.4

NaCl	8.0 g
KH_2PO_4	0.2 g
$Na_2HPO_4.12H_20$	2.8 g

KCl 0.2 g

Salts were dissolved in 900 ml DW and the pH was adjusted to 7.4. Tween 20 and thimerosal were added to 0.05% (v/v) and 0.01% (v/v) respectively and the buffer volume was made up to 1 L.

Incubation buffer

Prepared as wash buffer except the thimerosal was omitted.

Substrate buffer: O-phenylenediamine in citrate/phosphate buffer, pH 5.0

Sunbstrate solutions were prepared as below

0.1 M citric acid 21.01 g

Dissolved in 1 L of DW

0.2 M phosphate ($Na_2HPO_4.2H_20$) 35.6 g

49.0 ml citrate buffer + 51.0 ml phosphate buffer were mixed and the pH checked as 5.0. O-phenelynediamine (34 mg) and 20 µl of hydrogen peroxide was added to the 100 ml of citrate/phosphate buffer and stored in the dark. The solution was prepared just before use and used on the day only.

Appendix 5

Buffers used in SDS-PAGE:

Acrylamide/ bisacrylamide

Acrylamide 30 g

N,N' methylene bisacrylamide 0.8 g

dissolved in 100 ml of DW, filtered and stored at 4 °C.

Lower gel buffer: 1.5 M Tris-HCl, pH 8.8

Tris/HCl 18.1 g

SDS 0.4 g

Dissolve salts in 80 ml of DW and adjust pH to 8.8 with 5N HCl. The volume was made up to 100 ml with DW and store at 4°C.

Upper gel buffer: 0.5M Tris-HCl, pH 6.8

Tris/HCl 6.06 g

SDS 0.4 g

Dissolve salts in 50 ml of DW and adjust pH to 6.8 with 5N HCl. The volume was made up to 100 ml with DW and store at 4°C.

Ammonium persulphate: a 10% (w/v) solution was prepared fresh before use in DW.

Running buffer: Tris-glycine buffer, pH 8.3

Tris	6.06 g
Glycine	28.8 g
SDS	2.0 g

Dissolved in 1.8 L of DW and pH checked and adjusted to 8.3 with 5N HCl. The volume was made up to 2 L with DW.

Solubilising buffer:

Upper gel buffer	25 ml
Glycerol	10 ml
ß-mercaptoethanol	5 ml
SDS	3 g
DW	60 ml
Bromophenol blue	0.01 g

Separating gel:	**12% acrylamide concentration**	**10% acrylamide**
Lower gel buffer	10 ml	10 ml
DW	13.4 ml	16.6 ml
Acrylamide solution	16.6 ml	13.4 ml
Degas above solution then add		
10% ammonium persulphate	200 µl	200 µl
TEMED	20 µl	20 µl

Stacking gel (4.5% acrylamide):

Upper gel buffer	2.5 ml
DW	6.0 ml
Acrylamide solution	1.5 ml
Degas then add	
10% ammonium persulphate	30 µl
TEMED	20 µl

Fixing/Staining solution:

Coomassie blue R250	1.25 g
Methanol	227 ml
DW	227 ml
Glacial acetic acid	46 ml

Destaining solution:

Methanol	50 ml
Glacial acetic acid	75 ml
DW	875 ml

Western blotting:

Transfer buffer

Tris	7.5 g
Glycine	36.0 g
Methanol	500.0 ml

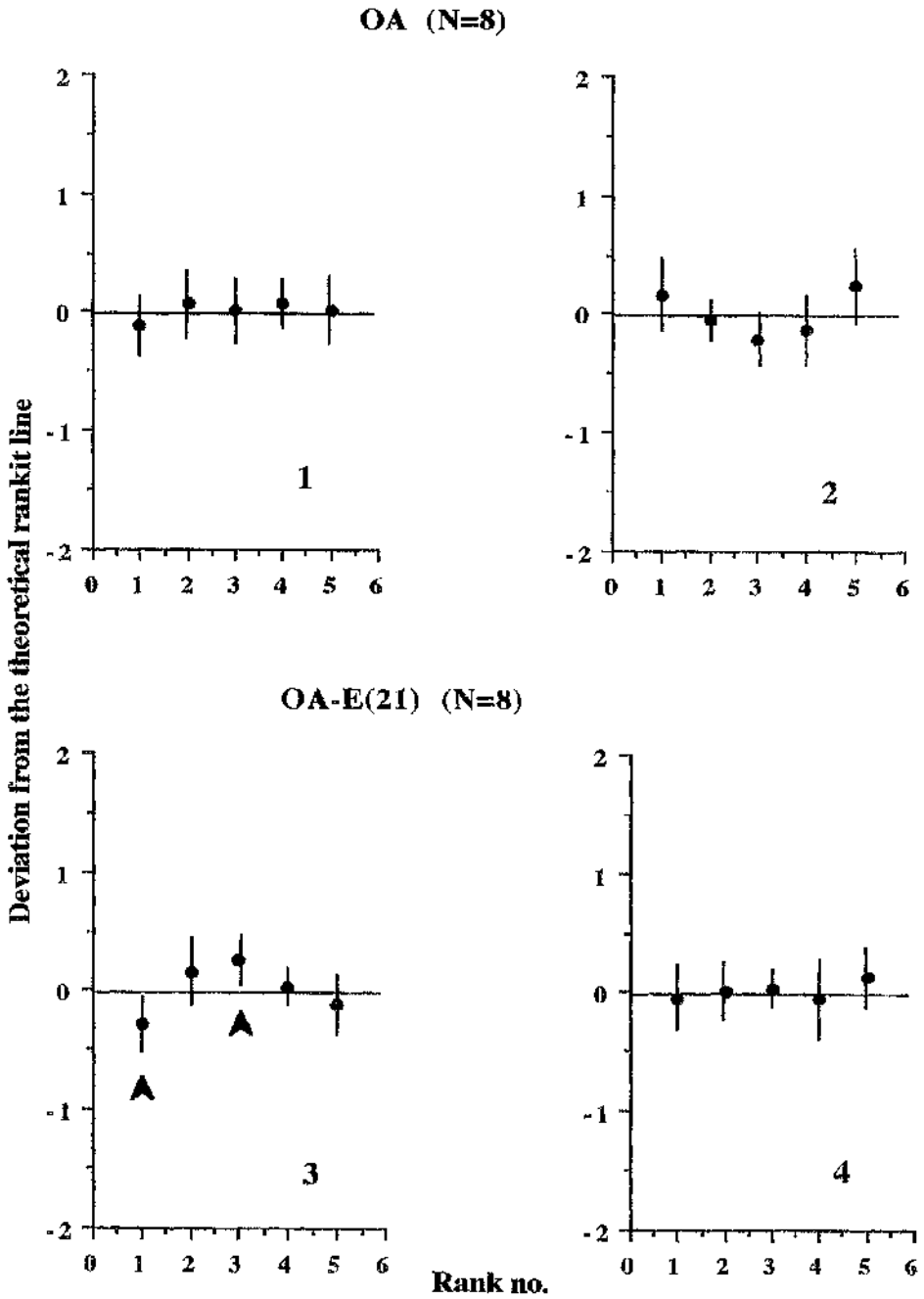
pH was adjusted to 8.3 and the volume was made up to 2.5L

CHO cell clustering assay:

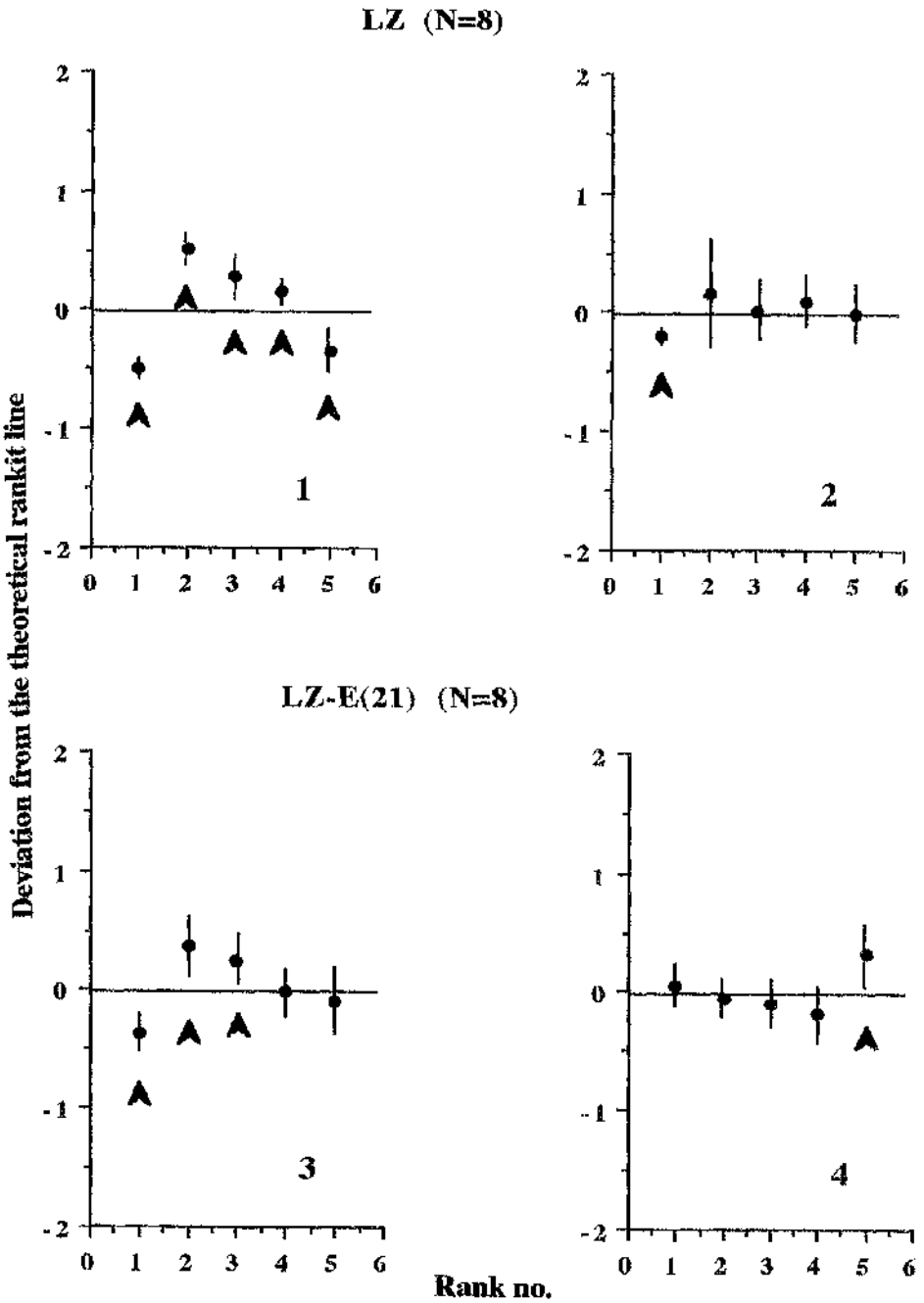
Coomassie blue staining solution

Coomassie blue R250	0.5 g
Methanol	250 ml
DW	250 ml
Glacial acetic acid	35 ml

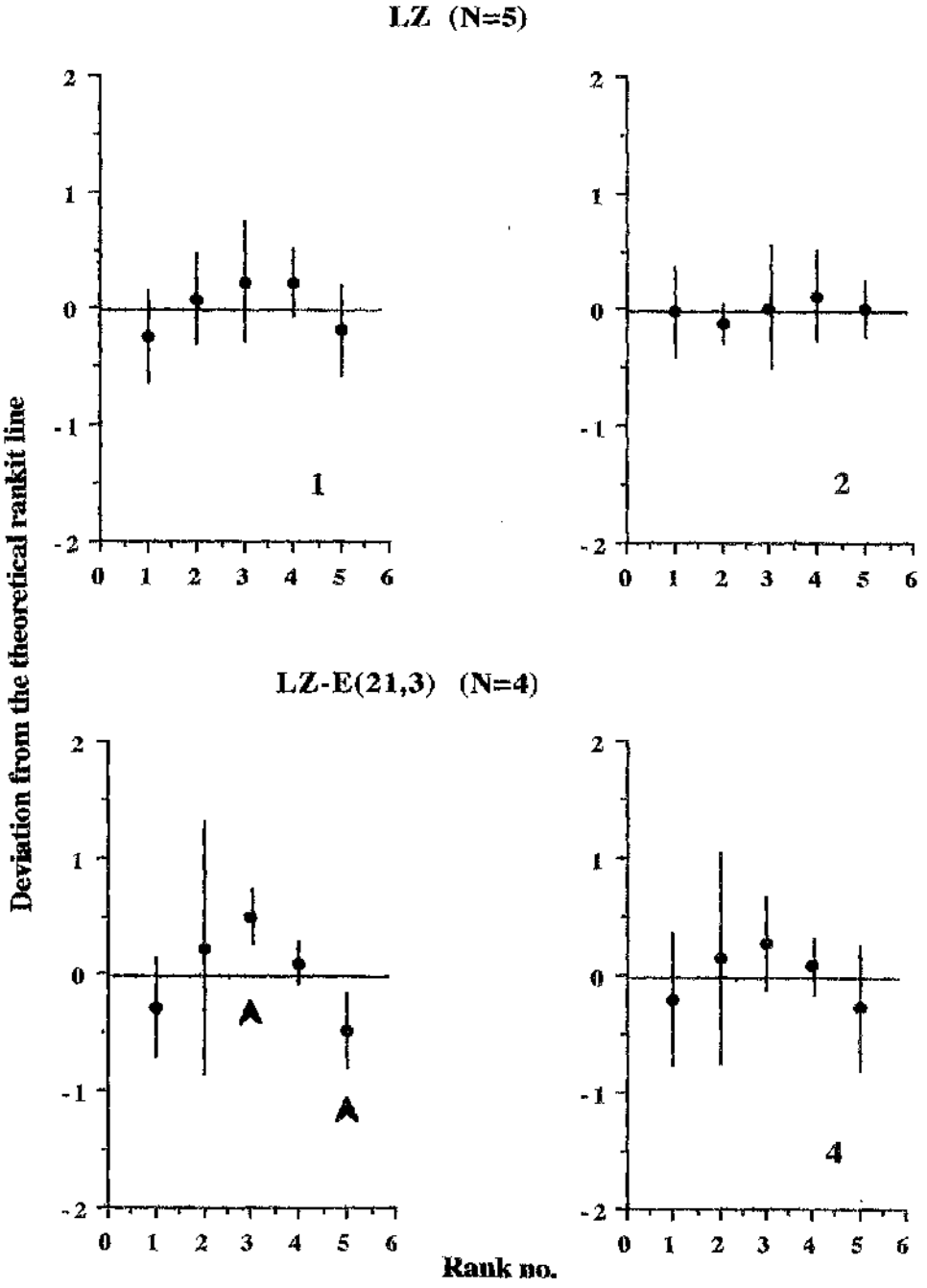
Appendix6 a: Summary plots of rankit analysis on anti-OA ELISA titres from immunization with OA in experiment A and B. The mean vertical deviation from the theoretical line, with 95% CL, is plotted against serum rank no. for each preparation.



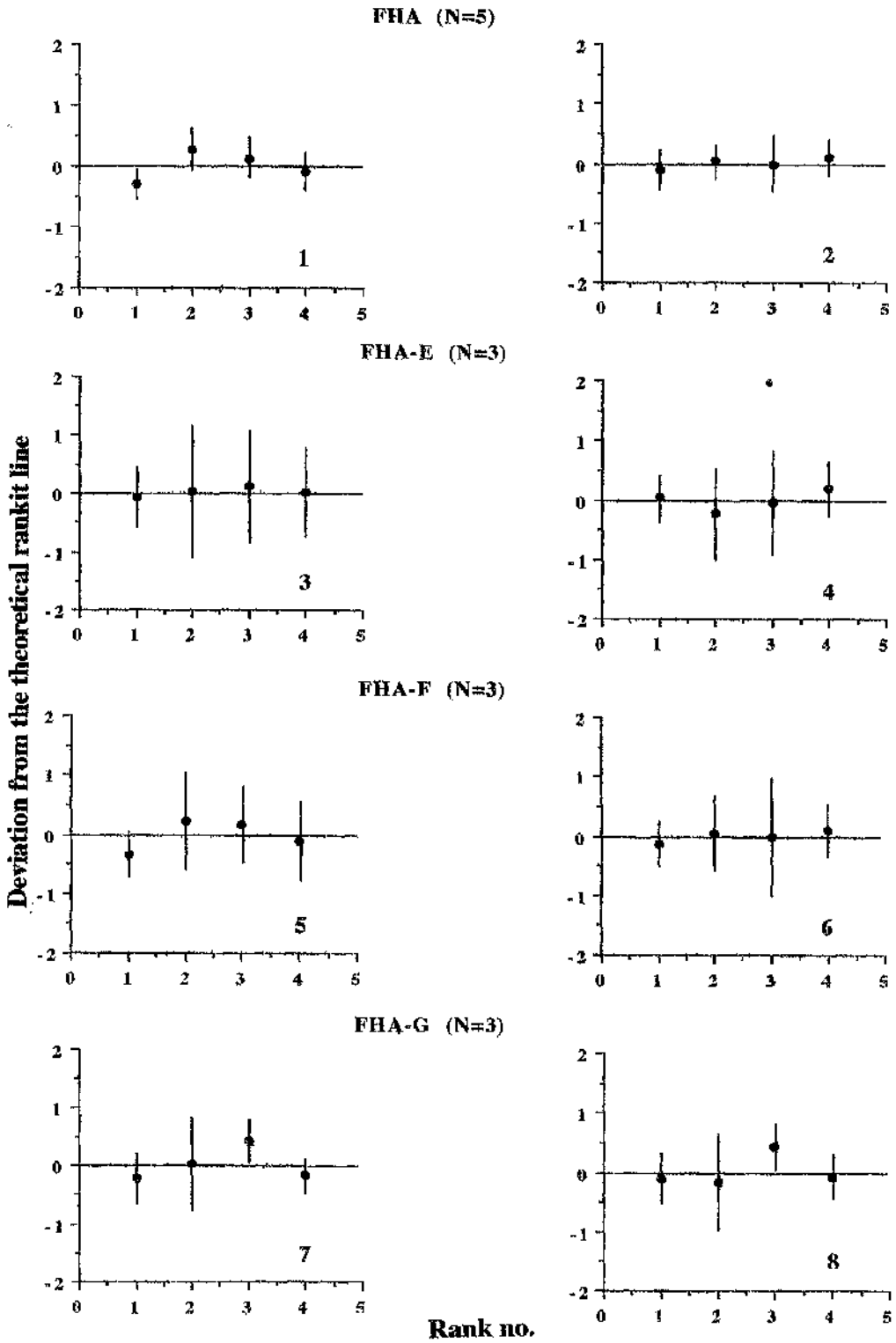
Appendix 6b: Summary plots of rankit analysis on anti-LZ ELISA titres from immunization with LZ in experiment A and B. The mean vertical deviation from the theoretical line, with 95% CL, is plotted against serum rank no. for each preparation.



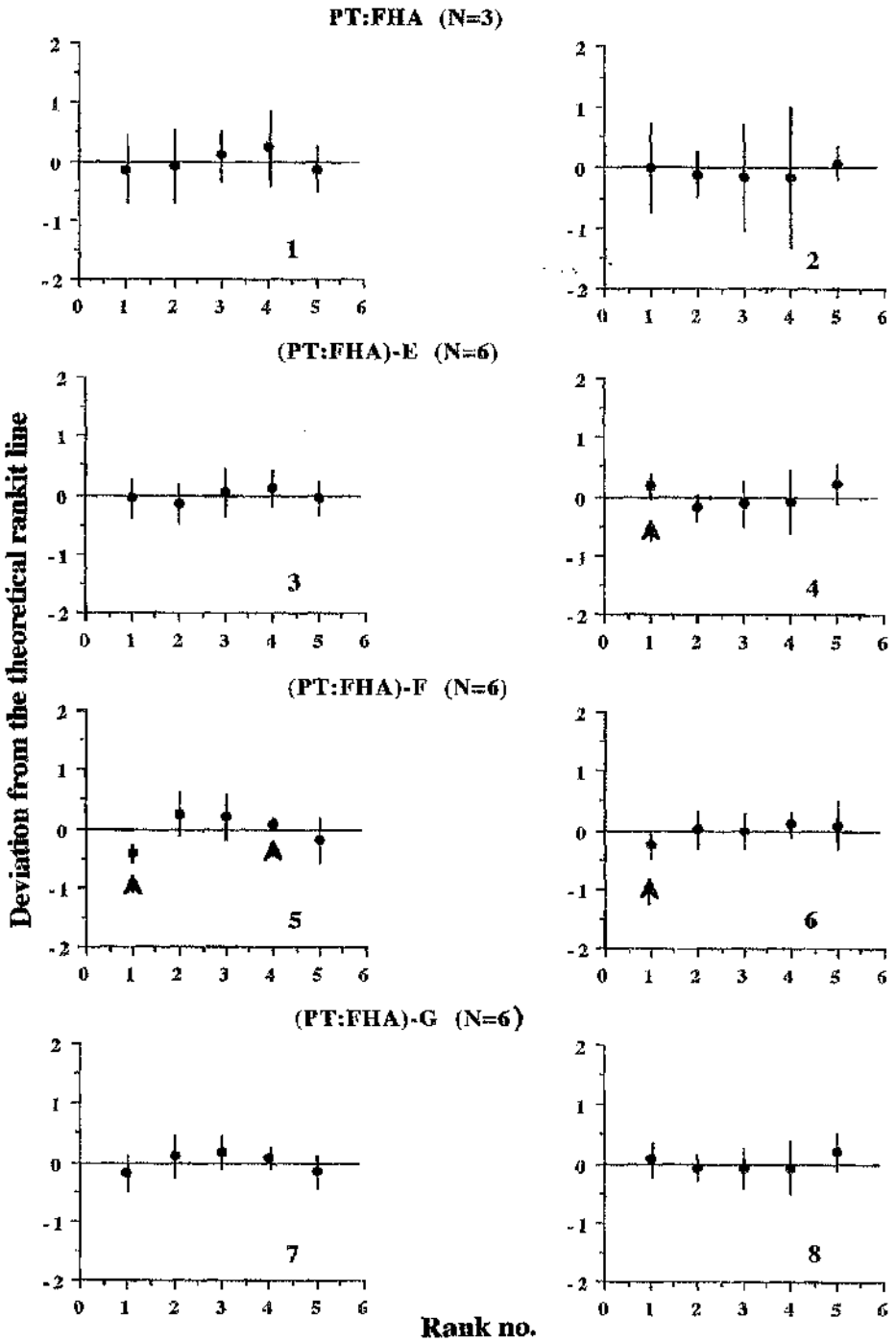
Appendix 6c: Summary plots of rankit analysis on anti-LZ ELISA titres from immunization with LZ in a preliminary experiment. The mean vertical deviation from the theoretical line, with 95% CL, is plotted against serum rank no. for each preparation.



Appendix 6d: Summary plots of rankit analysis on anti-FHA ELISA titres from immunization with FHA in experiment A. The mean vertical deviation from the theoretical line, with 95% CL, is plotted against serum rank no. for each preparation.



Appendix 6e: Summary plots of rankit analysis on anti-FHA ELISA titres from immunization with PT:FHA mixtures. The mean vertical deviation from the theoretical line, with 95% CL, is plotted against serum rank no. for each preparation.



Appendix 6 f: Summary plots of rankit analysis on anti-PT ELISA titres from immunization with PT:FHA mixtures. The mean vertical deviation from the theoretical line, with 95% CL, is plotted against serum rank no. for each preparation.