INACTIVATION OF *BORDETELLA PERTUSSIS* BY RAT LUNG LAVAGE FLUIDS (LLF)

GIAMAL NOURI AL-FELLAH

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Dedication

Dedicated to my parents, my wife and the rest of my family who supported me by their enthusiasm and encouragement in my academic career

DECLARATION

This thesis is the original work of the author except where otherwise stated

Giamal Nouri Al-Fellah

S U M M A R Y

Soluble antibacterial substance(s) in lung lavage fluid (LLF) from healthy normal rats killed phase I *Bordetella pertussis in vitro*. Phase IV *B. pertussis* was insusceptible. LLF that was active towards phase I organisms was obtained from rats of the Sprague Dawley, Lewis, Brown Norway, and Hooded Lister strains. Two strains of *B. pertussis* were mainly used: *B. pertussis* 18-323 for viable count experiments and *B. pertussis* lux for tests with the luminometer. The former experiments were more time consuming but more reliable; luminometry, although rapid, was complicated by substances that affected the luciferase reaction. Electron microscopy showed that LLF caused severe damage to the *B. pertussis* cells, with loss of internal contents

Of the several mammalian species tested, the rat yielded LLF of the highest bactericidal activity (BA) towards *B. pertussis*. The other species LLF were: human, mouse, horse, dog, chicken, rabbit, sheep and calf. Typically rat LLF could be diluted 24 times and still exhibit BA.

For assessment of the BA of LLF, both positive and negative control fluids were used. Normal rat serum, which killed the bacteria rapidly, was used for the former, and phosphate buffered saline (PBS), cyclodextrin liquid (CL) medium and casamino acids (CAA) for the latter. The half-life of *B. pertussis* at 37°C in PBS was 149 min and in CAA 155 min. CL medium allowed growth.

To obtain LLF, four different methods of euthanasia were explored; the highest BA was in LLF obtained by anaesthetising the rats with halothane/ O_2, then heart puncture for blood, and finally cervical dislocation. The least active LLF was obtained after CO_2 euthanasia.

LLF from normal rats was separated by ultracentrifugation at 55,000 g into supernate and surfactant (pellet) fractions. About 95 % of the BA towards *B. pertussis* 18-323 and *lux* was located in the surfactant fraction. No BA towards 18-323 was found in commercially-produced, protein-free artificial surfactant used for alleviating respiratory distress syndrome in premature infants .

Physical, chemical and biochemical treatments of LLF and its fractions suggested that the substance(s) responsible for the BA towards *B. pertussis* 18-323 and lux are

probably long-chain fatty acids, protein and phospholipids. There appeared to be no involvement of antibody, complement and lysozyme in the BA of LLF towards *B. pertussis*. However, the lysozyme in LLF was able to kill and lyse *Micrococcus luteus*.

LLF from rats convalescent after infection with phase I *B. pertussis* tended to have BA lower than that from normal animals. This suggested that one of the toxins of *B. pertussis* might inhibit the synthesis or release of the substance(s) responsible for BA from the secreting cells in the lungs.

B. parapertussis was sensitive to the BA of both surfactant and supernate of LLF from normal rats, while *B. bronchiseptica* was resistant. Other LLF-resistant species were *Staphylococcus aureus*, *Streptococcus pyogenes* and *Escherichia coli*.

B. pertussis 18-323 was strongly sensitive to the BA of long-chain fatty acids, of which the most active was arachidonic (C20:4). The BA of LLF from normal rats could be replicated by a mixture of fatty acids from C14 to C20 at a concentration similar to that found in the LLF itself.

The reduced BA in LLF from convalescent rats might be due to the action of pertussis toxin on type II pneumocytes which are principally involved in production and recycling of surfactant. Further studies on the possible effect of PT on this system might provide valuable insights into pathogenic mechanisms in pertussis.

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LIST OF ABBREVIATIONS

A	Absorbancy
ALEC	Artificial lung expanding compound
BA	Bactericidal activity
BCA	Bicinchoninic acid
BG	Bordet-Gengou
BHIA	Brain heart infusion agar
BSA	Bovine serum albumin
CAA	Casamino acids
CFU	Colony forming units
CL	Cyclodextrin liquid
CLLF	Convalescent lung lavage fluid
CSN	Convalescent supernate
CSUR	Convalescent surfactant
dc	Days of culture
DMβCD	Dimethyl-β-cyclodextrin
DMSO	Dimethyl sulphoxide
DPT	Diphtheria-pertussis-tetanus
DW	Distilled water
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
FA	Fatty acids
FHA	Filamentous haemagglutinin
HEWL	Hen's egg white lysozyme
HLT	Heat-labile toxin
HRP	Horse radish peroxidase
hsc	Hours of subculture
IEP	Inactivation end point
kDa	Kilo Dalton
LLF	Lung lavage fluid
Lum	Luminescence
mv	Millivolt
NCIMB	National Collection of Industrial and Marine Bacteria
NCTC	National Collection Type Cultures
NLLF	Normal lung lavage fluid
NSN	Normal supernate
NSUR	Normal surfactant
OPD	O-Phenylenediamine

PBS	Phosphate buffered saline
PC	Phosphatidylcholine
Ph	Phospholipase
PT	Pertussis toxin
Red	Reduction
SEM	Standard error of the mean
SN	Supernate
SS	Stainer-Scholte
SUR	Surfactant
TEM	Transmission electron microscopy
VC	Viable count
WB	Washing buffer

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INTRODUCTION

BORDETELLA PERTUSSIS

Whooping Cough (Pertussis)

Historical introduction

Whooping cough may historically be of relatively recent origin. The disease subsequently given the name pertussis by Sydenham in 1676 was first mentioned in 1540 by Moulton (Lapin 1943). Linnemann (1979), states that the disease was first described in 1578 by Guillaume de Baillou, otherwise known as Ballonious. The quoted description is as follows (Linnemann 1979): "At the close of summer almost the same diseases prevailed as before ....Principally that common cough ....Serious are the symptoms of this: The lung is so irritated by every attempt to expel that which is causing the trouble, it neither admits the air nor again easy expels it. The patient is seen to swell up and as if strangled holds his breath tightly in the middle of his throat ....For they are without the troublesome coughing for the space of four or five hours at a time, then this paroxysm of coughing returns, now so severe that blood is expelled with force through the nose and through the mouth. Most frequently an upset stomach follows .... For we have seen so many coughing in such a manner, in whom after a vain attempt semi-putrid matter in an incredible quantity was ejected"

Linnemann (1979) also suggests that pertussis was introduced into Europe in the Middle Ages by travellers from other parts of the world. The disease was certainly well recognised by the mid-18th century; it was reported from diverse regions, such as Switzerland (1755), Germany (1769), Brunswick (1770) and Milan (1815). For more detailed historical descriptions see Lapin (1943) and Olson (1975).

Since earlier descriptions of illnesses cannot always be identified in modern terms, some reviewers have suggested that whooping cough (pertussis) appeared as a new infection of man during the last few centuries (Olson, 1975). Other historians have suggested that the disease was only new to Europe in the middle ages, having been imported from other parts of the world (Von Rosenstein, 1960, cited by Linnemann, 1979);

still others have suggested that although it was a common disease, pertussis was not treated by physicians, and thus not mentioned in the medical literature of the times (Creighton, 1894 cited by Linnemann, 1979).

The apparent reason for the disagreement among scholars is that historical descriptions of illnesses characterised predominantly by coughing are not sufficiently detailed to allow either the inclusion or exclusion of *pertussis*. Even Baillou's account does not contain a description of the inspiratory whoop which is the hallmark of the disease; Sticker has concluded that "the historical trail of whooping cough is associated with mental delusions " and one must accept that the exact origins of pertussis cannot be determined (Sticker, 1902, cited by Linnemann, 1979).

Whooping cough has been known in many countries by many names, including Chin cough or Kink cough in English, Coqueluche in French, Kinkhoest in Dutch, Keuchusten in Germany, Kighoste in Danish, or Kikhosta in Swedish (Radbill, 1943 cited by Linnemann, 1979). Also, pertussis is known in Arabic as alsual-aldiki. In English "hooping cough " was generally used until the end of the 19th century but in the 20th, pertussis has gradually replaced "whooping cough " in the medical literature.

Clinical disease

Bordetella pertussis, the causative organism of whooping cough (pertussis) infects only humans, and does not cause disease in animals (Tuomanen *et al.* 1983). It is a world-wide pathogen, and all ages of Man are at risk (Fine, 1988). Its dominance, especially in developed countries during the last several decades, has been in sharp decline largely as a result of intensive vaccination programmes (Wardlaw, 1990).

Pertussis is a highly communicable disease, especially in home exposures (Medical Research Council, 1956); it has an attack rate of > 90 % among unimmunized individuals (Lambert 1965). Also, it was estimated by Fine and Clarkson (1984) and Muller *et al.* (1986) that the vast majority of the world's unvaccinated children contract the infection by the age of 5 years. Nevertheless, the disease is life-threating only in very young infants especially those under 6 months of age (Gordon and Hood, 1951). The latter authors

emphasised that the variable severity of pertussis is influenced by the age and immune status of the patient. It is well established that *B. pertussis* is transmitted by aerosol droplets from active cases.

According to Lapin (1943), after contracting the infectious agent, the incubation time can vary from 6 to 20 days. In the catarrhal stage, the disease manifests itself in the new host as a cold or mild cough, slight conjunctivitis and coryza with little fever (Walker, 1988). After 1-2 weeks the characteristic paroxysmal cough appears. Bouts of coughing, of which there may be an average of about 10 per 24 h (Walker *et al.* 1981) are frequently followed by the inspiratory "whoop" and equally frequently by vomiting; apnoea and cyanotic attacks after a paroxysm are the commonest precipitating reason for hospital admission.

Brain damage through cerebral anoxia or subdural haemorrhage may occur if the paroxysms are sufficiently severe (Cherry, 1984). Whooping cough may last for several months and is not modified by specific therapy (Miller and Fletcher, 1976), although steroids have been advocated for severe cases (Zoumboulakis *et al.* 1973).

Prevention and treatment

For prevention, patients should be isolated from other healthy susceptible children and public places during the 3 weeks after the onset of the paroxysmal stage.

Soon after Bordet and Gengou (1906) isolated *B. pertussis* from the sputum of children with whooping cough, there were attempts by 1914 in the USA to develop a vaccine against the disease (Gordon and Hood, 1951). Some protection in children who received an early vaccine just before a pertussis epidemic in the Faroe Islands was demonstrated by Madsen (1933). Whole-cell pertussis vaccines are suspensions of heat-killed or chemically-killed bacteria with or without adjuvant such as aluminium hydroxide. They may be combined with diphtheria and tetanus toxoids. The UK whole-cell vaccine consists of killed *B. pertussis* cells ($\leq 2 \times 10^{10}$) per single human dose, combined with diphtheria and tetanus toxoids (DPT) adsorbed to aluminium hydroxide and with thiomersal as a preservative (Department of Health, London, 1990). According to Noble *et al.* (1987),

concern with vaccine safety in the middle of the 1970s, caused vaccine uptake to fall from 80 % to 40 % in the UK. Pertussis vaccination is effective for 2-3 years (Medical Research Council, 1959). Sherris *et al.* (1986) reported that among all the world's new born in 1985, 45 % received 3 doses of pertussis vaccine. The degree of protection afforded by whole-cell vaccines against the disease is greater than the protection against infection (Fine and Clarkson, 1987). The adverse effects of whole-cell vaccine range from transient reactions such as high fever, uncontrolled crying and seizures (Cherry *et al.*, 1988) to brain damage (Griffith, 1989).

Because of the adverse effects of the whole cell vaccines mentioned above, attempts have concentrated on producing less reactogenic and more effective vaccines. Acellular vaccines which are derived from cultures of *B. pertussis* and are cell-free preparations containing inactivated pertussis toxin, filamentous haemagglutinin and some of the agglutinogens are being evaluated. The first of the defined acellular pertussis vaccines were developed by Sato *et al.* (1984). For more details see Table 1.

In a field trial in Sweden (1986-1987), a monocomponent pertussis toxoid (PTd) and a two-component PTd + FHA (filamentous haemagglutinin) pertussis vaccine, showed a lower reactogenicity and efficacy than whole-cell vaccines (Ad hoc group, 1988). Despite this disappointing result, vaccines containing PTd with or without other antigens provided good protection against severe disease. Tricomponent acellular pertussis vaccination of 12 month old infants induced T-cell responses specific for the vaccine components (Zepp *et al.* 1996). WHO reported that pertussis caused the death of > 0.5 million among 60 million cases in unvaccinated communities (Muller *et al.*, 1986). Marwick (1995) reported that multi-component vaccines, especially those containing pertactin, are highly efficacious and show few adverse effects.

In a study of effectiveness of antibacterial agents on *Bordetella* organisms localized on ciliated epithelium, the bordetellae were resistant to bacitracin (Rosen *et al.*, 1954) and to penicillin and streptomycin (Ganaway *et al.*, 1965). Also Bass *et al.* (1969) reported that the clinical illness may be aborted or attenuated if any of several antibiotics such as erythromycin, tetracycline, and chloramphenicol are given during the catarrhal stage.

Table 1. Examples of defined acellular pertussis vaccines (Parton, 1994)

Vaccine generation	Composition (Toxoiding process)	Stage of development
1st :	PTd and FHA as main components, co-purified from culture supernate	
	PTd + FHA (+AGGs + other) (Formaldehyde)	Routine use in Japan since 1981
		Licensed as booster in USA, 1991
	PTd + FHA (Formaldehyde)	Swedish phase 3 trials, 1986
2nd:	Individually-purified and chemically-inactivated components	
	PTd (Formaldehyde)	Swedish phase 3 trials 1986
	PTd + FHA + AGGs + pertactin (Glutaraldehyde)	Phase 3 trials
3rd:	Containing recombinant proteins from *B. pertussis* or other organisms	
	Recombinant PTd + FHA + pertactin (Genetic)	Phase 3 trials

The course of pertussis at the paroxysmal stage cannot be altered by introduction of anti-microbial agents (Welkins and Wehrle, 1979). Erythromycin, because of its safety profile in children, was preferred for treatment of early disease and for prophylaxis of exposed, susceptible individuals (Linnemann *et al.* 1975, Altemeir and Ayoub, 1977). The Center for Disease Control and Prevention reported that an erythromycin-resistant *B. pertussis* strain was isolated from a 2-month-old male infant living in Yuma County, Arizona (Lewis, 1995). The same source stated that susceptibility testing at the hospital laboratory suggested that the isolate was resistant to erythromycin but sensitive to trimethoprim- sulfamethoxazole.

Bordetella pertussis

Introduction to the bordetellae

The *Bordetella* organisms are highly communicable, obligatory parasites of the respiratory tract of human, and other warm-blooded animals including birds. So far as is known, they have no reservoirs in the natural environment; nor do they have significant capacity for survival outside of their hosts. *B. pertussis* and until recently *B. parapertussis* were regarded as having a single host species, man. Recently, however, *B. parapertussis* was isolated from healthy and pneumonic lambs (Porter *et al.* 1994). *B. bronchiseptica* has been isolated from different host species- cat, dog, fox, pig, hedgehog, horse, koala bear, monkey, opossum, mouse, rabbit, raccoon, rat, skunk, turkey and occasionally man. *B. avium* has been isolated from turkey, duck, and goose (Kersters *et al.*, 1984).

Recently, two new species have been added to the genus *Bordetella* on the basis of DNA hybridisation studies, DNA base ratio determinations and other genotypic and phenotypic characteristics (Parton, 1996). They are *B. holmesii* and *B. hinzii.* The former was isolated from blood cultures of patients, some of whom were immunocompromised (Weyant *et al.*, 1995). *B. hinzii* was the name given to a *B. avium*-like group of organisms from the respiratory tract of turkeys and chickens (Vandamme *et al.*, 1995). Also, two other strains of *B. hinzii* were isolated from Man, one from sputum and the other from blood of

an AIDS patient.

It is generally thought that *B. pertussis* is a non-invasive organism and can only reside on the ciliated epithelium of the human respiratory tract; but recent evidence suggests that it may have an intracellular stage. This was deduced by recovering viable organisms from gentamicin-treated HeLa cell monolayers and by transmission electron microscopy (Ewanowich *et al.*, 1989). The uptake and intracellular survival of *B. pertussis* in human macrophages was reported by Friedman *et al.* (1992). For more detailed information about the characteristic diseases caused by *Bordetella* species see Table 2.

Bordet and Gengou (1909) described how they isolated their first pure *B. pertussis* (the causative agent of whooping cough) culture from sputum in 1906. Paroxysmal coughing has been the hallmark of this acute bacterial infection of the respiratory tract but occasional instances of similar illness have been attributed to *B. parapertussis* and rarely to *B. bronchiseptica* .

B. parapertussis was first isolated by Bradford and Slavin (1937) and Eldering and Kendrick (1938) from cases of mild whooping cough. *B. bronchiseptica* was obtained by Ferry (1911) and M'Gowan (1911) from the respiratory tract of dogs with distemper and mistakenly identified as the cause of that disease.

The genus *Bordetella* was introduced by Moreno-Lopez (1952), the first three recognised species being: *B. pertussis*, *B. parapertussis,* and *B. bronchiseptica* which had previously been referred to as *Haemophilus pertussis, Bacillus parapertussis* and *Brucella bronchiseptica* (Pittman, 1957, 1974).

The suggestion of including the whole *Bordetella* genus in the family *Brucellaceae* together with *Brucella* and *Alkaligenes* was proposed by Johnson and Sneath (1973), and emphasised the differentiation between the (G + C) content of *Haemophilus* DNA (39-42 mol %) (Hill, 1966) and that of *Bordetella* DNA (61.6 - 69.5 mol %). *B . pertussis, B. parapertussis,* and *B. bronchiseptica* were placed in the same genus because of their similarity in physiology and of a close genetic relationship; See Table 3; for a comparison between *Bordetella* and other similar genera.

Table 2. Characteristics of the diseases caused by the "classic" *Bordetella* species (all transmitted by aerosol).

Characteristic	*Bordetella*			
	pertussis [a]	*parapertussis* [b]	*bronchiseptica* [c]	*avium* [d]
Name of disease	Whooping cough (pertussis)	Atypical whooping cough	Kennel cough Atrophic rhinitis	Infectious coryza Rhinotracheitis Bordetellosis Turkey coryza
Reservoir	Man	Man	Dogs, pigs, and various wild animals	Turkeys, chickens and other birds
Symptoms	Catarrhal, paroxysmal and convalescent stages	As with *B.p*	Nasal atrophy and snout deformation, coughing, sneezing, ocular and nasal discharge, reduced weight gain .	Exudative conjunctivitis sneezing and serious discharge, tracheal rales, reduced feeding.
Site of infection	Cilia of respiratory epithelium	As with *B.p*	Nasal and respiratory epithelium cilia.	Respiratory epithelial cilia.
Carriers	None	None	Dogs, cats, rats, and swine .	Not known.
Immunity	Solid after infection Short-lived with immunization .	As with *B.p.*	As with *B.p.*	Not known.
Duration	Several months	As with *B.p*	Up to 14 weeks	About 12 weeks
Treatment	No effective treatment	As with *B.p*	As with *B.p*	As with *B.p*
Vaccine	Whole cell Acellular	None	Whole cell Acellular	Whole cell

a, b, c and d = Information for each species were gathered from following sources:
a: Connor (1986); Friedman (1988); Pittman and Wardlaw (1981) and Wardlaw and Parton (1988).
b Eldering and Kendrick (1938); Linneman and Perry (1977); Connor (1986); Chen *et al.* (1989).
c Thompson *et al.* (1976); Bemis *et al.* (1977a); McCandlish *et al.* (1978); Goodnow (1980); Rutter (1985) and Papasian *et al.* (1987).
d Hinz and Kunjara (1977); Kersters *et al* (1984) and Simmons *et al.* (1978, 1984).

Morphology, physiology, and growth

The *bordetellae* are small gram-negative cocco-bacilli measuring 0.3 - 0.5 μm x 0.5 - 2.0 μm arranged singly, in pairs or in small groups; upon primary isolation, cells are uniform in size, but in subcultures they become quite pleomorphic, filamentous and thick bacillary forms are common (Wardlaw, 1990). The surface component known as filamentous haemagglutinin (FHA) which was first detected by Keogh *et al.* (1947), when purified consists of filaments with fimbrial dimensions. They are different from the fimbriae that were seen in micrographs of whole cells and identified by Ashworth *et al.* (1982) as the agglutinogens.

The *Bordetella* organisms are strict aerobes with a respiratory metabolism, and not producing H_2S, indole, or acetyl methyl carbinol. Unlike *Haemophilus* species, X- factor (haematin) or V-factor (nicotinamide adenine dinucleotide) are not used by bordetellae species as a nutritional requirements for growth. Lactoferrin and transferrin supported growth of *B. pertussis*, and bordetellin (hydroxamate siderophore) produced by the same organism plays a major role in Fe uptake (Agiato and Dyer, 1992).

Primary isolation of the whooping-cough bacillus was first achieved by Bordet and Gengou (1906) from sputum cultured in a glycerol-potato-extract agar medium, without peptone but containing 50 % defibrinated horse blood. Various solid and liquid media without blood have also been developed for bulk growth of cells, for the production of toxin-containing supernates, and for genetic studies. The amount of blood in Bordet-Gengou medium may be reduced to 15-20 % for easier detection of haemolysis around the colonies; to improve selectivity, penicillin is added to the BG-medium if required (Pittman and Wardlaw, 1981). Also, isolation of *B. pertussis* from patients has been achieved by suction (Herzog and Gaiffe, 1958, cited by Pittman and Wardlaw, 1981)), cough plate (Lautrop, 1960) and nasopharyngeal swab (Kendrick and Eldering, 1969).

Rapidity of growth on Bordet-Gengou medium is such that, with inocula destined to give single colonies of *B. pertussis,* the colonies are still invisible after 24 h at 35 - 37 °C, but start to appear by about 3 days. *B. pertussis* colonies on BG-agar are dome-shaped, pinpoint in size, around 0.5 mm in diameter, and by 5-6 days the diameter

Table 3. Differential characteristics of the genus *Bordetella* and other morphologically and physiologically similar genera (modified from Pittman , 1984).

Characteristics	*Bordetella*	*Alcaligenes*	*Brucella*	*Haemophilus*
Strictly parasitic .	+	-	+	-
Saprophytic	-	+	-	-
Localise on respiratory cilia	+	-	-	-
Strictly aerobic	+	+	+	-
Growth requirement :				
Thiamine	-	-	+	-
Nicotinamide	+	-	-	-
X and V factor	-	-	-	+
Ferment carbohydrates	-	-	-	+
Nitrate reduction	D	D	+	+
Litmus milk alkaline	+	+	-	-
Oxidation of amino acids	+	+	+	-
Tetrazolium reduction	+	-	b	+
Growth on 320 mg/litre				
Potassium tellurite	-	+		-
Citrate utilised	D	+	-	-
PAGE resemblance[c]	-		-	-
Mol % G +C of DNA	61-70 (Tm)	56-70 (Tm)	55-58 (Tm)	38-44 (Tm)

a Symbols : + = positive reduction ; (-) no reaction ; (D) variable

b Blank space indicates no information

c PAGE patterns are distinct for each genus

increases to 2-3 mm, when they are smooth, convex, glistening almost transparent, and pearl-like in appearance.

The growth of bordetellae other than *B. pertussis* may take place on MacConkey's agar; for the primary isolation of *B. bronchiseptica* either blood agar (Thompson *et al.*, 1976) or MacConkey's agar with or without selective ingredients is used. The inhibitory agents which improve selectivity: nystatin, furasolidone and furaltadone were proposed by Farrington and Switzer (1977).

For the culture of *B. avium*, a blood agar containing colombia agar base and 7% defibrinated ox-blood or veal-infusion agar was recommended by Kersters *et al.* (1984).

Growth of *B. parapertussis* is more rapid than *B. pertussis*, visible growth being achieved in 1-2 days to the same colony size as *B. pertussis* at about 3 days. Also a brown discolouration is produced by *B. parapertussis* and is more pronounced on peptone agar or tyrosine agar, as recommended by Lautrop (1960). *B. bronchiseptica* and *B. avium* are the most rapidly growing among *Bordetella* species, their visible colonies being detectable after 24 h on BG-medium. *B. bronchiseptica* on MacConkey's agar gives reddish colonies surrounded by a small red zone with amber underlying medium.

The nutritional requirements of *B. pertussis, B. parapertussis, and B. bronchiseptica* were studied by Proom (1955): Eight amino acids were sufficient for growth, and nicotinamide was the only vitamin needed; starch was necessary for *B. pertussis*. Jebb and Tomlinson (1957), found that glutamic acid and a sulphur-containing amino acid - cysteine, cystine or methionine-together with nicotinic acid were sufficient for a number of strains of *B. pertussis*, and concluded that difficulties in growing the organism were due to its susceptibility to inhibitors in the medium. These inhibitors appeared to be unsaturated fatty acids (Pollock, 1947), colloidal sulphur or sulphides (Proom, 1955) and possibly organic peroxides (Rowatt, 1957). Of these inhibitors, fatty acids may be the most critical. Free fatty acids appear to be formed by *B. pertussis* (Pollock, 1947) suggesting that auto-inhibition may take place. Growth of *B. pertussis* was inhibited by long-chain fatty acid concentrations as low as 1 ppm (Field and Parker, 1979). The other *Bordetella* were not susceptible to the inhibitors of *B. pertussis* that are found in complex media.

Liquid media were developed by Hornibrook (1939, 1940) and Cohen and Wheeler (1946), primarily for vaccine production. They contained salts, amino acids, nicotinic acid and soluble starch. Neither of these media allows growth from small inocula, and when solidified with agar they do not permit single cells to give rise to separated colonies. Starch and albumin because of their affinity for lipids, were included in the original isolation medium of *B. pertussis.* As alternatives to starch (Ungar *et al.*, 1950), albumin (Pollock, 1947), charcoal (Pollock, 1947; Holt, 1962), and anion-exchange resins may be used (Kuwajima *et al.*, 1957, Sutherland and Wilkinson. 1961). More recently, cyclodextrins (Imaizumi *et al.*, 1983 a, b), polyvinyl acetate, polyvinyl alcohol (Greenspan, 1985) and methylcellulose (Nikolajewski *et al.*, 1990) have been proposed, all of which appear to act by adsorbing a growth inhibitor.

Goldner *et al.* (1966), found that glutamic acid and proline were the only amino acids needed for the growth of *B. pertussis.* Stainer and Scholte (1970) developed a chemically defined liquid medium containing glutamic acid, proline, and cystine, together with ascorbic acid, niacin, glutathione, salts and Tris buffer. Although suitable for the bulk growth of cells for vaccine production, Stainer and Scholte medium did not permit growth from small inocula. Imaizumi *et al.* (1983a) overcame this latter difficulty by adding dimethyl-β-cyclodextrin (DMβCD), after which it was solidified with agarose to constitute the first defined medium for *B. pertussis* to allow reliably the growth of separated colonies from dilute inocula. A modified Stainer-Scholte medium was used by Sato *et al.* (1984) to induce *B. pertussis* to release substantial quantities of *pertussis* toxin (PT) and filamentous haemagglutinin (FHA) into culture supernates and thereby facilitate the manufacture of acellular vaccines containing these components.

Because of ammonia formation during growth of the bordetellae, the buffering of culture media is required to prevent excessive rise in pH.

Susceptibility to physical and chemical agents

Exposure of bordetellae to a temperature of 56 °C is lethal within 30 min and the heat-labile toxin is destroyed, after which a suspension potentially suitable, after

standardization, for use as a vaccine may be obtained. Outside the host body, *B. pertussis* in dried droplets was found to survive up to 5 days on glass, 3 days on cloth, and a few hours on paper (Ocklitz and Milleck, 1967, cited by Wardlaw, 1990).

Biochemical activities and metabolism

None of the bordetellae ferments sugars but there are species-associated differences in other biochemical tests (Johnson and Sneath, 1973; Pittman and Wardlaw, 1981; Kersters *et al.*, 1984). Some of their cultural and biochemical features are presented in Table 4.

There have been only a very few basic studies on metabolism. Dobrogosz *et al.* (1979) reported that *B. pertussis, B. parapertussis, and B. bronchiseptica* have the following enzymes that are important in oxygen metabolism: superoxide dismutase, catalase, and peroxidase. McPheat and Wardlaw (1980) showed that the uptake of radiolabelled nicotinic acid and nicotinamide by *B. pertussis* was temperature and energy dependent and required potassium ions; also certain analogues of nicotinic acid acted as specific inhibitors of its uptake by *B. pertussis*, but without inhibiting either the growth of the bacteria in nutrient medium or the ability of washed bacterial suspensions to oxidize glutamate.

Virulence factors and other cellular components

B. pertussis produces a group of toxins, aggressins and adhesins. These virulence factors and cellular components (Table 5) are regarded as important components in bacterial pathogenicity, as follows:

Heat-labile toxin (HLT): Also known as dermonecrotizing toxin, the first reported toxin of *B. pertussis* (Bordet and Gengou, 1909). The toxin is heat-labile, with toxic activity lost upon heating for 10 min at 56 °C (Manclark and Cowell, 1984). The toxin causes inflammation and necrotic skin lesions after subcutaneous injection at low doses in mice but is lethal at high doses (Cowell *et al.*, 1979; Livey and Wardlaw, 1984; and Nakase

Table 4. Differentiation between Bordetella species: *B. pertussis (B.p), B. parapertussis (B.pp), B.bronchiseptica (B.b), B. avium. (B.a), B. hinzii (B.hi)* and *B. holmesii (B.ho)* (modified from Pittman, 1984 ; Parton, 1997).

Characteristics	*B.p*	*B.pp*	*B.b*	*B.a*	*B.hi*	*B.ho*
Growth: colonies visible in (days)	3	1-2	1	1	2	2-3
Peptone agar:						
Phase I	-	+	+	+	?	?
Phase IV	+	+	+	+	?	?
growth	-	+	+	+	+	+
browning	-	+	-	-	-	+
Growth on MacConkey agar	-	+	+	+	+	+
Motile, Peritrichous flagella	-	-	+	+	+	-
β-like haemolysis	+	+	v	v	?	-
Citrate utilization	-	v	+	v	+	-
Nitrate reduction	-	-	+	-	-	-
Oxidase	+	-	+	+	+	-
Urease	-	+	+	-	v	-
Litmus milk alkaline	+	+	+	+	?	?
Major cellular fatty acids*	$C_{16:1w7c}$ $C_{16:0}$	$C_{16:0}$ $C_{17:0\,cyc}$	$C_{17:0cyc}$ $C_{16:1w7c}$	$C_{16:0}$ $C_{17:0cyc}$	$C_{16:0}$ $C_{17:0cyc}$	$C_{16:0}$ $C_{17:0cyc}$
G+C content of DNA (mol%)	67.7-68.9a	68.1-69.0a	68.2-69.5b	61.6-62.6c	65-67	61.5-67

+, positive; -, negative; v, some strains positive, others negative; ?, unknown.
*The number before the colon is the number of carbon atoms and the number after the colon is the number of double bonds; w, double bond position; c, cis isomer; cyc, cyclopropane ring.
a = Johnson (cited by Johnson & Sneath, 1973); b = Type strain; c = Strain 591/ 77 of Hinz.

and Endoh, 1985).

Tracheal cytotoxin (TCT): A glycopeptide which was first discovered by Goldman *et al.* (1982). TCT can caused ciliostasis and specific damage to ciliated cells in hamster tracheal ring cultures and inhibition of DNA synthesis (Goldman and Baseman, 1980). TCT appears to be non-immunogenic (Goldman, 1988). The study by Heiss *et al.* (1993) implicated interleukin-1α, produced by epithelial cells in response to TCT, as a potential intracellular mediator of the primary respiratory cytopathology of pertussis. Heiss *et al.* (1994) have implicated nitric oxide, synthesised in response to IL-1α, as the actual cytotoxic factor

Adenylate cyclase toxin (ACT): The existence of *B. pertussis* ACT was first reported by Wolff and Cook (1973) in studies with whole cell vaccine. ACT is a bi-functional protein with both AC and (weak) haemolytic activities (Coote, 1992). ACT is primarly cell-associated, but may also be extracytoplasmic, and is activated by calmodulin (Wolff *et al.*, 1980). This activation enables the ACT to penetrate a variety of mammalian cells and to cause intoxication by catalysing the production of cyclic adenosine 3',5'-phosphate (cAMP) (Confer and Eaton, 1982). Bourne *et al.* (1974) showed that the inhibition of various phagocytic functions can be due to elevated cAMP.

Studies by Utsumi *et al.* (1978) showed that AC could be recovered from *B. pertussis* cells by urea extraction and that the cyclase activity was quite stable under these conditions. The extract inhibited human polymorphonuclear leukocyte (PMN) functions, including chemotaxis and oxygen consumption.

The production of superoxide by human PMN and alveolar macrophage was inhibited by dialysed AC urea extract (Confer and Eaton, 1982). Also, the AC urea extract caused an accumulation of cAMP to a high levels in PMN, lymphocytes, monocytes, Chinese hamster ovary (CHO) cells, mouse S49 lymphoma cells and isolated rat pituitary cells (Hewlett *et al.*, 1985). The toxin itself is an AC enzyme that enters target cells where it catalyzes the conversion of intracellular ATP to cAMP. In phagocytic leukocytes, this

massive accumulation of cAMP results in paralysis of killing function (Confer and Eaton, 1982; Pearson *et al.*, 1987). Hewlett and Gordon (1988) demonstrated effects of ACT on immune effector cells (Table 5) such as macrophages, monocytes and neutrophils, causing an inhibition of chemotaxis.

Gusio *et al* (1991) showed that active immunization with purified *B. pertussis* AC-HLY or AC (a fragment of the AC-HLY molecule carrying only the adenylate cyclase activity but no toxin activity *in vitro*) protected mice against *B. pertussis* intranasal infection. They reported also that the immunization with AC-HLY or AC significantly shortened the period of bacterial colonization of the mouse respiratory tract.

Pertussis toxin (PT): Among *Bordetella* species virulence factors, PT is produced only by *B. pertussis*. The name of PT was proposed by Pittman (1979), and the toxin was previously known as pertussigen (Munoz and Bergman, 1977), histamine-sensitizing factor (HSF), leukocytosis and lymphocytosis promoting factor (LPF) and islet-activating protein (IAP) (Yajima *et al.*, 1978).

PT may play a major role in the pathogensis of whooping cough (Pittman, 1979; 1984). PT is composed of 6 protein sub-units S1, S2, S3, S4 and S5 in a molar ratio in the native molecule of 1:1:1:2:1 (Tamura *et al.*, 1982). Similar to other toxins that cross cell membranes, PT has an A-B structure. Whereas the A-subunit promoter consists of subunit S1 which is enzyme active as an ADP-ribosyl transferase and NAD-glycohydrolase, the B (binding) portion consists of S2, S3, 2 of S4, and S5; and is responsible for binding PT to specific cell receptors and enabling the enzymatic A portion to reach its site of action in the cell (Katada and Ui, 1982). T cell mitogenic and haemagglutinating activities of PT are due to the B subunit binding to cell surface (as reviewed by Parton, 1997).

Katada and Ui, 1982 showed that the A enzymatic portion catalyzes the ADP-ribosylation of a 41kDa membrane protein (GTP-binding protein, Ni of the membrane adenylate-cyclase system) in a variety of cell types. The ADP-ribosylation of Ni protein inhibits its regulatory involvement in the inhibitory control of mammalian cellular AC function, causing enhanced activity of the mammalian cellular AC (Gilman, 1984). This

increased AC activity in turn causes an increase of cAMP, with consequent disturbance to normal cells. In addition to its toxic activities, PT acts as an adhesin (Tuomanen, 1988) (Table 5). The clinical observations of lymphocytosis, hypoglycemia and impaired rise in serum glucose in response to epinephrine, all effects of PT, were seen in patients with pertussis (Bader-El-Din *et al.*, 1976). *B. pertussis* BP357 (PT deficient) was found to be severely impaired in the ability to cause lethal infection in the infant mouse model (Weiss *et al.* 1984; Weiss and Goodwin, 1989). Weiss and Hewlett (1986) mentioned a group of functions (Table 6) which were inhibited by PT, such as: oxidative burst of neutrophils, migration of monocytes, homing pattern *in vivo* of lymphocytes, degranulation of mast cell and target cells lysis by natural killer cells.

The conversion of PT into toxoids for their use in acellular vaccines was done by treatment with glutaraldehyde (Munoz *et al.*, 1981a, b), formaldehyde (Sato *et al.*, 1984) and carbodi-imide (Christodoulides *et al.*, 1987). Due to this conversion, the immunogenicity was retained while toxicity was lost, although it seems with the formaldehyde treatment that partial reversion may occur (Iwasa *et al.*, 1985).

Mice were passively protected from a lethal aerosol challenge with *B. pertussis* by intraperitoneal injection of anti-PT rabbit hyperimmune sera (Sato *et al.*, 1981). Also, mice immunized with detoxified PT (treated with glutaraldehyde) were protected against intracerebral challenge with *B. pertussis* (Cowell *et al.*, 1982). Sato *et al.* (1984) stated that mice were protected against intracerebral and aerosol challenge with *B. pertussis* by either active immunization with PT-toxoid or passively with anti-PT sera. In a study of the properties of the B oligomer of PT, Nencioni *et al.* (1991) reported that it was able to induce protective immunity in mice but was less potent than molecules containing the S1 subunit also.

The relationship between anti-PT serum titres and long-term immunity to *B. pertussis* infections in children was demonstrated by Granstrom *et al.* (1985).

Filamentous haemaglutinin (FHA): FHA, like PT has *in vitro* haemagglutinating activity. The molecule is highly hydrophobic, and filamentous, and appears in the electron microscope as a fine filament-like protein 2 nm in diameter and 40-100 nm in length. It can agglutinate various types of erythrocytes (Arai and Sato, 1976; Morse and Morse, 1976). The involvement of FHA in the initial attachment and adherence of *B. pertussis* to ciliated epithelial cells of the upper respiratory tract to start infection was suggested by Tuomanen *et al.* (1984); Tuomanen and Weiss, 1985). This suggestion was later confirmed by *in vitro* studies using human WiDr cells (Urisu *et al.*, 1986).

In vitro, both FHA and PT were required for a strong adhesion of *B. pertussis* to the ciliary tufts of human respiratory-tract cells (Tuomanen, 1988). Menozzi *et al.* (1994) demonstrated that FHA contains at least three different binding sites, a feature unusual for bacteria adhesins but similar to features of eukarytic adhesins and extracellular matrix proteins. According to Parton (1997), because of its adhesive and mouse-protective activities, FHA was included as a prime component in acellular pertussis vaccines. Tommaso *et al.* (1991) suggested that subregions of FHA that do not contain sequences that are potentially cross-reactive with self proteins may be sufficient to induce an immune response against the whole protein.

***Fimbriae (Agglutinogens, AGGs)*:** In the early decisive serological work on the genus *Bordetella*, 14 AGGs were described by Eldering *et al.* (1957). Nowdays, only AGGs 1, 2 and 3 of *B. pertussis* are generally recognised and AGGs 2 and 3 form part of the fimbrial structures (Parton, 1997). The role of AGGs in pathogenicity is still unclear. Sato *et al.* (1979) reported that antibodies to AGGs numbers 1 and 2 inhibited attachment of *B. pertussis* to HeLa cells. It was reported that AGGs play no major role in the attachment of *B. pertussis* to the ciliated cells of the human respiratory tract (Tuomanen, 1988). However, monoclonal antibodies to type 2 or type 3 fimbriae blocked the adhesion of *B. pertussis* to Vero cells (Gorringe *et al.*, 1985). Preston (1988) recommended that pertussis vaccine should contain AGGS 1, 2 and 3 to ensure immunization against the major serotypes. In a novel adherence model using tracheal rings removed from baboon monkey,

Funnell and Robinson (1993) demonstrated a functional role (in initial stages of colonization) for the fimbriae of *B. pertussis.*

Endotoxin: The endotoxin of *B. pertussis* has similar biological activities to those of endotoxins from other gram-negative bacteria. *B. pertussis* endotoxin is generally refered to as a lipo-oligosaccharide consists of lipid A and an oligosaccharide core with 2-keto-3-deoxyoctulosonic acid, but it does not have the long-chain polysaccharide O antigen typical of lipopolysaccharide endotoxins (Brodeur *et al.* 1993). Endotoxin properties in general, include pyrogenicity and adjuvanticity, ability to induce antiviral activity, B cell mitogenicity and polyclonal B cell activation (Chaby and Caroff, 1988).

Pertactin (PRN): A 69 kDa outer-membrane adhesin protein was identified by Brennan *et al.* (1988). Leininger *et al.* (1990) reported that PRN and FHA both contain arginine-glycine-aspartic acid (RGD) sequences which may be involved in binding to integrin receptors of mammalian cells. These findings support the idea that PRN and FHA have a role in binding of *B. pertussis* to mammalian cells. As reviewed by Parton (1996), vaccines containing PRN provide protection of mice against aerosol challenge with *B. pertussis* and, when given with FHA, protect against intracerebral challenge.

Other virulence-associated outer-membrane proteins are 1) Bordetella resistance to killing (BrkA), mentioned by Fernandez and Weiss (1994) who found that a mutant deficient in BrkA was less virulent for mice, less adherent and less invasive to HeLa cells, and at least 10-fold more susceptible to killing by normal human serum than the parent cells. 2) Tracheal colonization factor (TCF) which was described by Finn and Stevens (1995). They found that a *B. pertussis* mutant lacking TCF had reduced ability to colonize mouse tracheal cells, compared with the parent strain.

Table 5. The virulence factors and cellular components of the classic Bordetella species: *B. pertussis (B.p)*, *B. parapertussis (B.pp)*, *B. bronchiseptica (B.b)* and *B. avium (B.a)*. (Modified from Wardlaw, 1988; and Parton 1997)

Component	*B. p*	*B. pp*	*B. b*	*B. a*	Probable role in pathogenicity
Toxins :					
Heat-labile toxin	+	+	+	+	Local inflammatory effect
Tracheal cytotoxin	+	+	+	+	Ciliostasis, epithelial cell cytotoxicity
Adenylate cyclase toxin/ Haemolysin	+	+	+	-/?	Interference with immune effector cells/?
Pertussis toxin	+	-	-	-	Adhesin/invasion, interference with immune effector cells
Filamentous haemagglutinin	+	+	+	-	Adhesin/invasion
Pertactin	+	+	+	-	Adhesin/invasion
BrkA	+	?	+	-	Adhesin/invasion, serum resistance
Tracheal colonization factor	+	-	-	-	Adhesin
Endotoxin*	+LOS	+LPS	+LPS	+?	Pyrogenicity
Agglutinogens:					Adhesin/invasion
1, 2, 3,4, 5, 6.	+	-	-	-	
7	+	+	+	?	
8, 9, 10	-	+	+	?	
11, 12	-	-	+	?	
13	+	-	+	?	
14	-	+	-	?	

+ , positive; - , negative; ?, unknown .

* LOS, lipo-oligosaccharide; LPS, lipopolysaccharide

Table 6. Inhibitory effects of adenylate cyclase toxin (ACT) and pertussis toxin (PT) on the functions of immune effector cells. From Weiss and Hewlett (1986).

Toxin	Target cell	Functions inhibited
ACT	Neutrophil	Chemotaxis, phagocytosis, superoxide generation bacterial killing
	Alveolar macrophages	Oxidative burst, bacterial killing
	Monocyte	Oxidative burst, bacterial killing
	Natural killer cells	Target cell lysis
PT	Neutrophil	Oxidative burst
	Monocyte	Migration
	Lymphocyte	Homing pattern *in vivo*
	Mast cell	Degranulation
	Natural killer cells	Target cell lysis

***In vitro* killing of *B. pertussis* by serum**

Serum of humans and many animals possesses a rapid and efficient bactericidal and bacteriolytic activity against a wide variety of Gram-negative bacteria (Taylor, 1983). In fact, any procaryote that presents a lipid bilayer membrane to the external enviroment would appear to be potentially susceptible to complement killing (Taylor, 1983).

Increased phagocytosis of *B. pertussis* in the presence of antibody was reported by Kendrick *et al.* (1937). *In vitro*, specific antisera plus complement are highly bactericidal to *B. pertussis* (Dolby and Standfast, 1961). They suggested that in intracerebrally-infected mice the diffusion of circulating antibodies to the brain (due to blood-brain barrier permeability) may cause the rapid and progressive fall of bacterial viable count. Antisera to *B. pertussis* are bactericidal for some strains of the same bacteria in the presence of complement and lysozyme (Dolby, 1965). She also stated that phase I strains that were virulent for mice by the intracerebral and intranasal routes, are sensitive to the bactericidal effect of antiserum and most mouse avirulent strains are not sensitive. Ackers and Dolby (1972) reported that lipopolysaccharides (lipooligosaccharides) of *B. pertussis* elicit bactericidal antibody active *in vitro* in the presence of complement against serum-sensitive strains.

The protective ability of sera from vaccinated or infected children to protect mice against small, lethal brain infections was correlated with the complement-mediated bactericidal antibody titres of the sera, but this was not related to the state of immunity in the children (Dolby and Stephens, 1973). Strains of *B. pertussis* varied in their ability to elicit (in mice) an antibody bactericidal for an antiserum-sensitive strain of *B. pertussis*, although antibody was usually detectable after only one injection (Dolby and Ackers, 1974).

Viable cell numbers (CFU/ml) of *B. pertussis* strains decreased by 99 % after exposure for 1 h to porcine hyperimmune serum (Byrd *et al.*, 1991). Fernandez and Weiss (1994) showed that the serum component mediating the killing of susceptible *B. pertussis* organisms appears to be complement . Also, they demonstrated that both heat-inactivation and the pretreatment of serum with ethylenediaminetetraacetic acid, which removes divalent cations required for complement activation, resulted in the complete abrogation of serum

killing. There appear to be no studies on the possible BA of respiratory tract secretions on *B. pertussis*.

Animal Models of Pertussis

Introduction

"Animals not only are necessary for determining the etiology of specific infectious diseases and the pathogenicity of particular cultures of bacteria, but are also utilized as a means of isolation, to determine in more detail certain specific pathogenic mechanisms, to maintain species that grow best *in vivo*, to increase pathogenicity, and to produce antibodies" (Warren, 1957). The same investigator mentioned that the bacterial species and the property to be studied determine the choice of the experimental animals.

A great many efforts have been made to reproduce typical human pertussis in laboratory animals. Most of the studies have been with the mouse respiratory infection model, because of its several advantages and this has yielded much knowledge on pathogenesis and immunity in pertussis (Sato and Sato, 1988).

Mice

Only two sites of infection, with a measured volume of standardized bacterial suspension, have been used in the mouse: intranasally into the lung and intracerebrally into the brain (Standfast and Dolby, 1961). These investigators found that the terminal viable count in the lungs and brain was always around 10^8 CFU, with death occurring on the 4th and 5th day after infection (lethal infections).

Intracerebral infection: The intracerebral infection of mice was first suggested by Norton as a result of a successful use of this route with *Salmonella typhi* (Norton and Dingle, 1935). Two different routes of intracerebral infection were used, one through the foramen magnum and the other through the parietal bone (Berenbaum *et al.*, 1960). Multiplication of the bacteria took place in the ciliated layer over the ependyma. No organisms were detected in the lungs, spleen and liver of the cerebrally-infected mice, but

some organisms were found in the blood stream.

Multiplication of *B. pertussis* in the brains of unvaccinated and vaccinated mice occurred at the same rate until day 3 of the infection. Thereafter, with vaccinated animals the infection peak occurred on day 4 with around 10^5 organisms and then the number of bacteria declined until day 5 or 6 and the animals survived (Berenbaum *et al.,* 1960).

Standfast and Dolby (1961) reported that as the cerebrally-infecting dose was lowered from 10^5 to 10 viable organisms, the critical level at which death occured also decreased from $10^{8.5}$ to $10^{7.8}$ organisms respectively. Also as the infecting dose decreased, the time taken for the critical level appeared to increase from 4 to 13 days respectively.

Sublethal infections is not possible in the brain, since regardless of the initial inoculum, colony count numbers consistently increased to 10^8 CFU, with eventual death. Dolby and Standfast (1961) reported the possibility of growth of a single *B. pertussis* cell in the brain to a critical lethal level.

Intranasal infection: Burnet and Timmins (1937) described the intranasal technique for the infection of mice with *B. pertussis.* They demonstrated that intranasal adminstration of *B. pertussis* cultures to anaesthetized mice results in characteristic pneumonic lesions, and in large doses is rapidly fatal. Also by histological sections, they observed an interstitial pneumonia and a typical intense proliferation of bacilli in the mucus lying on the ciliated surface of the bronchial epithelium. The same investigators stated that a significant degree of immunity can be demonstrated after intraperitoneal inoculation of living cultures or formolized vaccines.

Sublethal lung infections were induced in mice with 10^3 CFU. The lung count increased steadily to 10^6 until the 10th to 14th day after infection and then slowly declined over some weeks or months until the lungs became sterile.

Sato *et al.* (1980) introduced a system for the aerosol infection of mice with *B. pertussis* by placing the animals on a mesh screen in a glass chamber fitted with a nebulizer. The animals were exposed to aerosol droplets for 30 min by spraying 0.4 ml of

bacterial suspension per min at a nebulizer pressure of 1.5 kg / cm^2. This system gave a more accurate and reproducible inoculum. From a bacterial concentration of 2 x 10^9 CFU / ml, an initial delivered inoculum of 2 x 10^4 CFU / lung was obtained, which increased to a maximum of 10^7 CFU / lung in 14 days, after which the viable count decreased. Again the lethal dose by this route was found to be 10^8 CFU / lung.

Although *B. pertussis* is not a natural pathogen for mice, valuable basic information about the pathogenesis of human pertussis has been obtained from the studies of respiratory infection in this species. However, the infected mice do not cough and do not pass the disease to other mice. Nevertheless, the mice respiratory infection resembles that in humans in a number of respects:

(1) younger mice have a higher susceptibility to the infection, while adult mice generally do not show severe symptoms; infant or suckling mouse have characteristic symptoms and mortality from the disease. (2) The period of pulmonary infection is of similar duration to that in humans. (3) there are pathophysiological changes such as histamine-sensitization, hypoglycaemia, hyperinsulinaemia, and marked leukocytosis, that persist even after the bacteria have disappeared (Sato and Sato, 1988).

Transposon-insertion mutants deficient in the production of either pertussis toxin (PT) or adenylate cyclase toxin (ACT) are markedly reduced in their virulence for infant mice when given intranasally (Weiss and Goodwin, 1989).

Intratracheal infection: Bradford (1938) observed characteristic lung lesions and hyperleukocytosis after the intratracheal inoculation of mice with suspensions of recently isolated *B. pertussis*. Also he isolated pure cultures of the bacteria from the infected lungs of the mice.

Other species

Infection of chick embryos with *B. pertussis* was done by inoculating the amniotic fluids of the embryonated hen's eggs (Gallavan and Goodpasture, 1937). The infection was concentrated especially on the ciliated epithelial cells of trachea and oesophagus with less

preference to the ciliated epithelial cells of the bronchi and bronchiole.

Primates: Early work using primates as animal model of pertussis was carried out by Inaba (1912, cited from Lapin 1943), when he infected macacus monkeys with freshly isolated *B. pertussis* using a pharyngeal swab. Paroxysmal cough and vomiting were observed within 10 days. Also paroxysmal cough was found with macacus and cebus monkeys challenged with freshly isolated *B. pertussis* either by pharyngeal swab or by injecting into the larynx (Sauer and Hambrecht, 1929). Laboratory examinations revealed *B. pertussis* on the respiratory epithelium of the animal, with leukocytosis and lymphocytosis. Also, the animals which recovered were immune to subsequent injections of various strains of *B. pertussis.*

Young ring-tail monkeys developed a whooping cough after exposure to *B . pertussis* infection by spraying of aerosol (Inaba and Inamori, 1934). Also in 1934, the same investigators reported the transmission of the disease among monkeys, after the animals were infected by spraying a culture of *B. pertussis* into the nares and pharynx, and developed paroxysmal coughing and leukocytosis. Lin (1958) reported that *Macacus cyclopsis* monkeys could be infected with *B. pertussis* strains 18-323, 10-5362 and 23-757 by aerosol and subsequently exhibited a paroxysmal cough with lymphocytosis.

Experimental whooping cough was confirmed in Taiwan monkeys by Huang *et al.* (1962). Rhesus and cynomologus monkeys and marmosets were used for experimental pertussis by Stanbridge and Preston (1974). However, neither rhesus nor cynomologus monkeys showed any signs of illness after pernasal inoculation with 10^{11} organisms. The failure to infect these monkeys might have been due to previous infection with *B. pertussis* or other *Bordetella* species. In marmosets, there are several features of pertussis infection similar to those seen in children with whooping cough, such as catarrh and persistence of colonization of the nasopharynx with *B. pertussis*; however, marmosets failed to produce either the paroxysmal cough or vomiting.

Immunity following reinfection has been demonstrated in monkeys (Sauer and Hambrecht, 1929; Lin, 1958). A possible active immunization by vaccination in Taiwan

monkey was also reported (Huang *et al.* 1962). They also reported that sera from convalescent and vaccinated Taiwan monkys were successfully used for passive immunization of the same species.

Rabbits: The early work of using the rabbit as an animal model of pertussis was by Mallory and his colleagues (1913), when they intratracheally infected animals with *B. pertussis*. In addition to emaciation as a clinical observation, they also observed histological lesions characteristic of pertussis in the ciliated epithelium of the bronchi and bronchioles of the animal. The same investigators however, also stated that their clinical and laboratory observations could have been due to *B. bronchiseptica.*

With rabbits, lymphocytosis within 2-4 days of *B. pertussis* infection (intratracheally) and interstitial mononuclear pneumonia were observed by Sprunt *et al.* (1938). A persistent colonization (9-10 months) of the rabbit nasopharynx and catarrh with no coughing or vomiting was reported by Preston *et al.* (1980).

In addition to their attempts to induce *B. pertussis* infection in rabbits, Mallory *et al.* (1913) also mentioned re-isolation of the same organisms from the respiratory tract of normal puppies 15-25 days after intratracheal infection, with characteristic lesions and spasmodic coughing.

***Rats*:** Hornibrook and Ashburn (1939) reported that the intranasal instillation of live phase I *B. pertussis* into ether-anaesthetised young rats produced a non-specific interstitial broncho-pneumonia, cough-like paroxysms, and recovery of the bacteria from lung homogenates.

Fifty years later, Woods *et al.* (1989) showed that with *B. pertussis* infection using the virulent phase I strain Tohama, the infected ether-anaesthetized adult male Sprague-Dawley rats gave the same clinical and physical changes that was seen in the infected human, including the paroxysmal cough. A reproducible infection, similar to the human disease was produced in the rat with colonization of the lungs, the organisms being recovered at 3 and 7 days after infection. The bacteria were not recovered at days 10 and 14

in lung homogenates but apparently reappeared at 21 days from infection.

The same investigators stated that at day 3, a mild lymphocytic infiltrate was present in the bronchi, with progressive lymphoid hyperplasia peribronchially. By day 7, a necrotizing inflammation of the tracheobronchial mucous membranes, characterized by both mononuclear and polymorphonuclear cells, was noted. Clinical findings in infected rats included hypoglycaemia, circulating lymphocytosis and paroxysms in which air was forcibly expelled from the mouth or nose. No histological changes were noted in rats infected with *B. pertussis* phase III strain Tohama, which was also not recoverable from infected rat lungs after 3 days from inoculation.

The procedure of Wood *et al* (1989) was modified and continued by Wardlaw *et al.* (1993); Parton *et al.* (1994) and Hall *et al.* (1994). Paroxysmal cough, leukocytosis and significant retardation of weight gain were observed with adult Sprague-Dawley rats exposed to a non-lethal infection by phase I *B. pertussis* 18-323 encased in agarose beads and adminstrated intrabronchially. Their procedure differed from that of Woods *et al.* (1989) in several respects, specifically: a) *B. pertussis* strain 18-323 was used instead of Tohama b) low-melting point agarose was employed in place of Ionagar no 2; c) a temperature of 37 °C, instead of 50 °C was taken when suspending the bacteria in the agarose d) a bacterial dose of 10^8 CFU per rat, rather than $5x10^5$ was given; e) Hypnorm/Hypnovel anaesthesia was used in place of ether, as anaesthetic for the operation (although ether was administered for 2 min post-infection for enhancement of coughing). In addition, coughing was quantitated with sound-activated tape recorders (Wardlaw *et al.*, 1993). Young rats (around 200 g) were more responsive to the infection than older animals (330 g).

The same strain of rats produced a paroxysmal cough when infected with either agarose-beads containing phase I *B. pertussis* strains 18-323, Tohama, L84 or transposon-insertion mutant BPM 1809, which lacks only the heat-labile toxin (HLT) (Parton *et al.*, 1994). Phase IV variant of *B. pertussis* strain L84, BP357, a transposon-insertion mutant which is deficient only in pertussis toxin (PT) and *B. parapertussis* were inactive in inducing the cough (Parton *et al.* 1994). They also reported that DPT (whole-cell) vaccine

greatly reduced the incidence of coughing in rats challenged subsequently with phase I *B. pertussis*. Finally, by serological tests, there was confirmation of the PT-positive or -negative status of the strains *in vivo* by the detection of appropriate presence or absence of anti-PT IgG in convalescent sera by ELISA.

Both paroxysmal cough and leucocytosis were at peak levels after 8-10 days of infection (Hall *et al.*, 1994). The same investigators reported a number of a following observations: 1) A 2-min exposure of the rats to ether inhalation after delivery of the beads enhanced the number of the subsequent coughing episodes; 2) Paroxysmal coughing was also enhanced when carrageenan was included in the beads; 3) Moderate amounts of coughing and leucocytosis were achieved in rats infected intranasally with *B. pertussis* suspension. Only low levels of coughing, or no coughing, was detected with untreated rats, or with rats given sterile beads; 4) No cough induction or leucocytosis were observed with rats infected intrabronchially either with heat-killed *B. pertussis* in beads or with live organisms in suspension without beads; 5) Finally, anti-IgG antibodies against both pertussis toxin (PT) and filamentous haemagglutinin (FHA) were detected in the convalescent serum of infected rats.

RESPIRATORY TRACT SECRETIONS (RTS)

In the following text a distinction will be made where possible between whole lung secretions and lung washings. The latter means the diluted lung secretions collected by means of washing (lavage) the lungs with buffers, and the former means undiluted secretions collected from the lungs by suction.

Collection

Whole lung secretions and washings

In the conducting airways, the submucosal glands and surface epithelial (goblet) secretory cells are the major contributors to the mucus secretions (Reid, 1960). Attempts to collect RTS started back in 1882 when Rossbank used blotting paper to dry the exposed

tracheal mucous membrane of cats and dogs during his research into mucus replenishment (cited by Boyd, 1954).

Henderson and Taylor (1910) collected RTS in calcium chloride tubes attached to a tracheal canula. The procedure of postural drainage and bronchoscopic suction was described by Jackson and Jackson (1934). In 1941, Perry and Boyd used a glass tube attached to a tracheal canula to collect the fluid. A special catheter with a cuffed tip was passed into an individual lower lobe of dog lungs. The cuff was inflated and three 100 ml aliquots of isotonic saline were gently instilled and withrawn through the catheter (Finley *et al.*, 1968). The trachea of rats was cannulated with sterile polyethylene tubing in order to collect RTS (Juers *et al.,* 1976). The trachea of rabbits was cannulated with a sterile plastic tube fitted to a three-way stopcock (LaForce *et al.*, 1979). Mice trachea were exposed and cannulated with a blunt 20-gauge needle (Nugent and Fick, 1987).

In order to obtain tracheobronchial washings (TBW) or lung lavage fluids (LLF), different buffer fluids have been used to wash the lungs: isotonic saline for dog lungs (Pfleger and Thomas, 1971) while pig lungs were washed with 150 ml/kg 20 mM Tris (hydroxymethyl) aminomethane- 0.15 M sodium chloride, pH 7.4 (Pruitt *et al.*, 1971). LaForce *et al.* (1979) lavaged rabbit lungs with 50 ml of sterile heparinized saline, whereas, a PBS of pH 7.4 was used to wash rat lungs (Coonrod and Yoneda, 1983). Mouse lungs were washed with 1ml of PBS pH 7.4 (Nugent and Fick, 1987). Finally, Brogden (1992) used PBS, pH 7.2, containing 100 μg of gentamycin per ml to wash the lungs of ewes, a calf and rats.

Processing of the collected lung washings in most cases consisted of centrifugation and accumulation of the cell-free supernate, which was distributed in aliquots, then stored (if it was not used immediately) frozen at -20 or -70°C (Yeager, 1971; Low *et al.*, 1978; Boat and Cheng, 1980).

Lung surfactant

Lung surfactant has been investigated extensively since its discovery by Pattle (1955). Finley *et al.* (1968) fractionated surfactant from dog lung washings fluids by

centrifugation for 20 min at 27,000 g at 0°C. The resulting sediment consisted of 2 layers, the upper white layer (surfactant) and the lower brown layer (mainly blood cells). Surfactant from LLF of pigs was fractionated by passing crude LLF through cheesecloth, the frothy white floating material removed by suction and the lavaged material then centrifuged for 10 min at 1000 g. The dark red pellet was then discarded and the supernate was centrifuged for 20 min at 27,000 yielding an off-white, densely packed pellet (surfactant), which then was resuspended in buffer (Pruitt *et al.*, 1971).

Isolation of surfactant from crude lung lavage (samples containing blood were rejected) of ox, rabbit, rat, and sheep was done by initial centrifugation for 20 min at 300 g to remove cells. This was followed by further centrifugation of the supernate for 60 min at 1000 g and the pellet was resuspended in 21 % (w/v) NaCl. Further centrifugation for 25 min at 1500 g resulted in a three-phase separation into an insoluble floating fraction (surfactant pellicle), a soluble fraction (soluble surfactant), and a small precipitate at the bottom of the tube. The surfactant pellicle was dialysed against water for 48h, freeze-dried and stored under N_2 at - 15 °C (Harwood *et al.*, 1975).

Another method of fractionating surfactant from rabbit crude LLF was by centrifugation for 7 min at 700 g. The deposit containing alveolar macrophages was discarded and the cell-free supernate was recentrifuged for 20 min at 25,000 g at 4 °C. The pellet (surfactant) was reconstituted to its original volume with Hank's balanced solution pH 7.2 (LaForce *et al.*, 1979). Coonrod *et al.* (1984) isolated surfactant by initial centrifugation of crude LLF for 5 min at 160 g to remove leukocytes. The supernate was either concentrated 20-fold by positive-pressure filtration through a 10,000 mol. weight exclusion filter or by centrifugation for 20 min at 40,000 g. The pellet (surfactant) was then resuspended in PBS in one-twentieth the volume, and stored at - 70°C.

Composition

Whole lung secretions and washings

General composition: Human RTS contained approximately 95 % water and 5% solids (Matthews *et al.*, 1963). The water was either free or bound at one of several levels of affinity to macromolecular components, or was trapped within interstices of a gel matrix, formed by polymerization and aggregation of the mucous glycoproteins and other macromolecular components. The 5 % solid content of human RTS included 2-3 % proteins and glycoproteins, 1% lipids, and 1% minerals (Mattews *et al.*, 1963).

The water in the RTS contained inorganic salts, proteins and glycoproteins (Richardson and Phipps, 1981). The respiratory mucus is very complex and includes 95-98 % of water, ions, sugars, amino acids and 1-3 % of proteins, glycoproteins and lipids (Robinson *et al.*, 1989). Variations in the value of respiratory tract fluids component of different material by different investigators is probably due to the method followed for fluid collection and dilution.

***Inorganic components*:** Chloride, sodium, phosphorus, calcium and potassium levels in RTS of various animals were reported by different investigators (Table 7). Boyd (1954) detected chloride in rabbit, cat, and dog LLF at concentrations of 5.2 ± 2.6 mg / ml, and potassium levels in cat RTS of 0.76 mg / ml. The inorganic analysis of human RTS was studied by Matthews *et al.* (1963) and Potter *et al.* (1967). The first investigated the overall chemical composition of pulmonary secretions, whereas Potter *et al.* (1967) studied the ionic environment of secretions from patients with cystic fibrosis and bronchiectasis. The concentrations of sodium and chloride were high in normal secretions compared with potassium and calcium which occurred at much lower concentrations. About 15 % of the monovalent ions and 30 % of calcium were not removed by dialysis, suggesting that there was considerable ion-binding to macromolecular components (Potter *et al.* 1967).

Proteins: Protein concentration in human LLF depended on the method of collection, especially the extent of dilution. This probably explains the different values (Table 8) reported by the following investigators: Matthews *et al.* (1963) found 10.0 ± 3 mg / ml (mean ± SD) protein; while Low *et al* . (1978) reported 0.068 ± 0.029 mg / ml, and Davis *et al.* (1982) got 0.114 ± 0.005 mg/ml. These differences in estimated protein levels in human RTS, by the last two workers compared with Matthews findings, was due to the dilution during sample collection.

Among animal RTS, 0.3 mg/ml of protein was found in the alveolar liquid from the foetal lamb (Adamson *et al.*, 1969); in 1944, Boyd and his group reported protein levels (mg/ml) in rabbit, cat, and a cockerel RTS of 1.04 ± 0.21, 1.36 ± 0.37, and 2.6 ± 0.4 mg/ml respectively. Wardlaw and Stevenson (1984) stated that tracheobronchial washings of mice had a protein concentration approximately equivalent to a 1/50 dilution of serum. Amino acid components of respiratory tract fluids from human and different animal species were reported by Potter *et al.* (1967) and Porter and Wardlaw (1994).

Lysozyme and lactoferrin, another protein which has anti-microbial effects, were reported in RTS (Masson and Heremans, 1973); anti-proteases (Boat and Cheng, 1980); proline-rich polypeptides were noted in bronchial secretion (Bailleul *et al.*, 1977); and glycoproteins (Yeagor *et al.*, 1971; Reasor *et al* ., 1978). Total protein concentrations in various animal TBWs were reported by Porter and Wardlaw (1994) (Table 8).

RTS may contain immune system components. Yeagor (1971) reported that mucous membranes may have their own immune system, thus supporting the previous studies which detected immunoglobulins in human RTS: Keimowitz (1964) reported $ß_{2A}$-globulin and gamma-globulin, while Masson *et al.* (1965) identified IgA and IgG.

Reynolds and Thompson (1973) measured the albumin and immunoglobulin concentrations in rabbits vaccinated with *Pseudomonas aeruginosa* (Table 9). Reynolds and Newball (1974) reported that albumin and immunoglobulins are the major serum proteins present in human LLF, and Reasor *et al* (1978) detected immunoglobulin in cat fluid (Table 10).

Table 7. Inorganic composition (mg/ml unless stated) of human and animal (stated) respiratory tract fluids.

Reference	Inorganic component (mean ± SD)
Boyd (1954)	Cl = 5.2 ± 2.6 (rabbit, cat and dog)
	K = 0.76 (cat)
Matthews *et al.* (1963)	Ca = 0.062 ± 0.02
	P = 0.27 ± 0.16
	Na = 1.65 ± 0.42
	Cl = 1.62 ± 0.6
	K = 0.132 ± 0.054
Potter *et al.* (1967)	Na = 211.1 ± 33.8 mM/L
	K = 16.6 ± 3.4 mM/L
	Ca = 2.45 ±1.11 mM/L
	Cl = 156.7 ± 24.6 mM/L

Table 8. Protein concentrations of human and animal (stated) respiratory tract fluids.

Reference	Protein concentration (mg/ ml) (mean + SEM unless otherwise stated)
Boyd *et al.* (1944)*	1.04 ± 0.21 (rabbit)
	1.36 ± 0.37 (cat)
Matthews *et al.* (1963)*	10.0 ± 3 (mean ± SD)
Adamson *et al.* (1969)*	0.3 (foetal lamb)
Low *et al.* (1978)**	0.068 ± 0.029 (mean ± SD)
Davis *et al.* (1982)**	0.114 ± 0.005 (mean ± SD)
Porter and Wardlaw (1994)**	2.17 ± 0.10
	0.09 ± 0.02 (chicken)
	0.60 ± 0.02 (dog)
	1.29 ± 0.12 (horse)
	1.01 ± 0.03 (mouse)
	0.32 ± 0.02 (rabbit)
	0.77 ± 0.07 (sheep)

* data of respiratory secretions
** data of tracheobronchial washings

Table 9. Albumin and immunoglobulin concentrations[a] in rabbit serum and bronchial secretions after vaccination with *Pseudomonas aeruginosa.* (Reynolds and Thompson, 1973).

Protein (no. of samples)	Bronchial secretions[b] (range)	Serum (range)	Bronchial: serum ratio
Albumin	1.67 ± 0.25	40.8 ± 1.6	0.04
(60)	(0.24-5.0)	(28.0-65.0)	
IgA	2.47 ± 0.20	0.50 ± 0.05	4.9
(64)	(0.9-5.4)	(0.2-1.7)	
IgG	1.79 ± 0.12	13.88 ± 0.66	0.13
(71)	(0.6-4.31)	(4.4-23.0)	
IgM	< 0.1[c]	ND[d]	

a. Concentrations in mg/ml ± SEM; b. Concentrated 100-fold to final 1-ml volume.

c. Detected in 4/73 (5%)samples; d. ND = not done.

Table 10. Immunoglobulins detected in human and animal (stated) respiratory tract fluids.

Reference	Immunoglobulin content (mean + SD)
Reynolds and Newball (1974)	IgA = 0.91 ± 0.11 mg / ml
	IgG = 0.17 ± 0.05 mg / ml
	IgE = 73.2 ± 8.2 ng / ml
Reasor *et al* (1978)	IgG and IgM detected (dog)

Alpha-1-antitrypsin, alpha-1-antichymotrypsin, alpha-1-acidglycoprotein, fibrinogen, ceruloplasmin, haemopexin and several complement components are found in RTS (Boat and Cheng, 1980). Complement titres (Table 11) in serum and bronchial secretions of rabbits vaccinated with *P. aeruginosa* were detected by Reynolds and Thompson (1973). The same investigators reported that even though bronchial lavage fluids appeared free of blood contamination, Wright's-stained differential counts of the cell pellets showed 1 to 5 % red blood cells to be present. They suggested that because of the extreme sensitivity of the complement assay, the low titres detected in bronchial secretions might represent leakage from the pulmonary vasculature due to trauma in lavaging the lungs.

***Carbohydrates*:** concentrations in human RTF were reported by many investigators (Table 12). Yeagor (1971) stated that carbohydrates made up 20-50 % of the nondialysable material in airway fluids.

Lipids: Lipid and phospholipid concentrations in humans and animals were also reported by various investigators (Table 13). Sahu and Lynn (1977) stated that human LLF contains a relatively large amount of lipid, about 40 % of the dried insoluble material.

Lung surfactant

Extracellular surfactant can be harvested from the air spaces by bronchoalveolar lavage and can be further purified by various centrifugation steps (King and Clement, 1972a; King, 1984).

Lipids: Dog surfactant contained about 80-90 % lipid by weight; more than 80 % of the lipid was phospholipid (King and Clements, 1985). Of this phospholipid, the components were phosphatidylcholine (60-70 %), phosphatidylglycerol (5-10 %), phosphatidyl-ethanolamine (5-10 %), and phosphatidylinositol and phosphatidylserine at 3-6 % of total phospholipids.

Table 11. Complement titres detected in serum and bronchial secretions of rabbits vaccinated with *Pseudomonas aeruginosa*. (Reynolds and Thompson, 1973).

Component (no. of samples)	Serum (range)	Bronchial secretions* (range)
C1 (7)	51,000 ± 12,000 (32,258-80,000)	20 ± 5.4 (0-40)
C4 (5)	187 ± 17.5 (152-208)	0**
C6 (9)	526 ± 64 (200-700)	9.3 ± 5.8 (0-51)

* One hundred-fold concentration to 1 ml at 4 °C, immediately after lung lavage and separation of cells.

** Of note was the finding that C4 titres in bronchial secretions diminished approximately 50 % with storage of the fluids for 48 h at - 40 °C. One millilitre of fresh serum diluted to 100 ml in modified Hanks' salt solution (MHS) and rapidly concentrated to its original volume also lost about 50 % of the C4 activity when compared with unaltered serum.

Table 12 . Carbohydrate concentration in human respiratory tract fluids.

Reference	Carbohydrate concentration (mg/ml ± SD unless stated)
Matthews *et al.* (1963) *	9.51 ± 2.18
Potter *et al.* (1967)*	19.3 ± 9.4 mM / ml
Low *et al.* (1978)**	0.008 ± 0.004
Davis *et al.* (1982)**	0.011 ± 0.002 (mean + SEM)

* Respiratory tract secretions

** Tracheobronchial washings

These values were similar to those in pig surfactant (Pruitt *et al.*, 1971), and ox, rabbit, rat and sheep surfactant (Harwood *et al.*, 1975). Shelley *et al.* (1984) detected phospholipids in human and different animal surfactants (Table 14). Coonrod *et al.* (1984) reported the total lipid and fatty acids (FA) content of rat, dog and guinea pig surfactant (Table 15). Klaus *et al.* (1961) suggested that dipalmitoylphosphatidylcholine was the principal surface-active constituent of lung surfactant.

Proteins: Protein comprises approximately 10 % of surfactant by weight (King *et al.*, 1973). King (1974) was the first to analyse the protein component of surfactant from the lavaged lung of dogs by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Two major surfactant-specific proteins appeared to be present: one with a molecular mass of ~ 35 kDa and the second quite hydrophobic protein, with a molecular mass of ~ 11 kDa.

Also, several protein of serum origin such as albumin and immunoglobulin G (IgG) were detected. In other studies, non-specific-surfactant proteins such as secretory immunoglo-bulin A (IgA) (Paciga *et al.*, 1980) and actin (Postle *et al.*, 1985) were detected.

Formation: The formation of surfactant starts during the terminal stages of gestation of the foetus (Farrell and Avery, 1975). One study by Crapo *et al.* (1983), of the cells in the alveolar region of rat, dog, baboon and human, suggested that there is a striking similarity in alveolar cell characteristics among mammals, even those with substantial differences in size and in lung functional characteristics. The air-facing surface(s) of the wall are lined by a continuous layer, one cell thick, of epithelial cells (type I cells). In addition to the type I cells, the alveolar epithelium contains smaller numbers of thicker specialized cells (type II cells).

The type I and type II cells comprise ~ 10 and 12 %, respectively of the total lung cell population. However, ~ 95 % of the total alveolar surface is occupied by the type I cells and ~ 5 % only by the type II pneumocytes (Figure 1). The type II cells contain the characteristic surfactant storage organelles, the lamellar bodies (Figure 2). It was reported that the human lung has about 350 million air pocket (alveoli), with a total absorbing area of

Table 13. Lipid and phospholipid concentrations in human and animal (stated) respiratory tract fluids.

Reference	Lipid and phospholipid concentration* (mg/ml ± SD, unless otherwise stated)
Boyd (1954)*	0.22 (cat)
	0.28 (rabbit)
	0.85 (dog)
Matthews *et al.* (1963)*	8.4 ± 2.7
Potter *et al.* (1967)*	19.3 ± 9.4 mM/L
Low *et al.* (1978)**	0.0778 ± 0.00768 (non-polar lipid)
	0.0441 ± 0.0415 (polar lipid)
	0.00109 ± 0.00033 (phospholipid)
Davis *et al.* (1982)**	0.167 ± 0.0219 (lipid; mean +SEM)
	0.00109 ± 0.00016 (phospholipid; mean + SEM)

* Respiratory tract secretions; ** Tracheobronchial washings

Table 14. Surfactant phospholipid distribution of human and animal species. Percent of total phospholipid (Mean ± SE, n = 6). Data from Shelley *et al.* (1984).

Phospholipids	Human	Cat	Dog	Rabbit	Rat
Phosphatidylcholine	80.5	86.3[a]	81.3	83.6	87.0
	(±1.4)	(± 1.7)	(±1.4)	(±0.8)	(±0.8)
Phosphatidylglycerol	9.1	2.1a	11.1	8.0	8.3
	(±0.4)	(±0.7)	(±1.1)	(±0.5)	(±0.4)
Phosphatidylethanolamine	2.3	3.2	2.0	3.5	0.7
	(±0.8)	(±0.8)	(±0.6)	(±0.4)	(±0.2)
All other phospholipids[b]	8.1	8.4	5.6a	4.9a	4.0a
	(±0.6)	(±1.0)	(±0.3)	(±0.6)	(±0.9)

a. Significantly different from human surfactant with $p < 0.05$.

b. Includes sphingomyelin, phosphatidylinositol (Pl)phosphatidylserine (PS) and lysobis-phosphatidic acid. These phospholipids were determined separately in some samples, and no differences were observed among the species. The amounts of PI ranged from 1.5-2.8 % and PS from 0.2-0 %. while the amounts of the others were more variable.

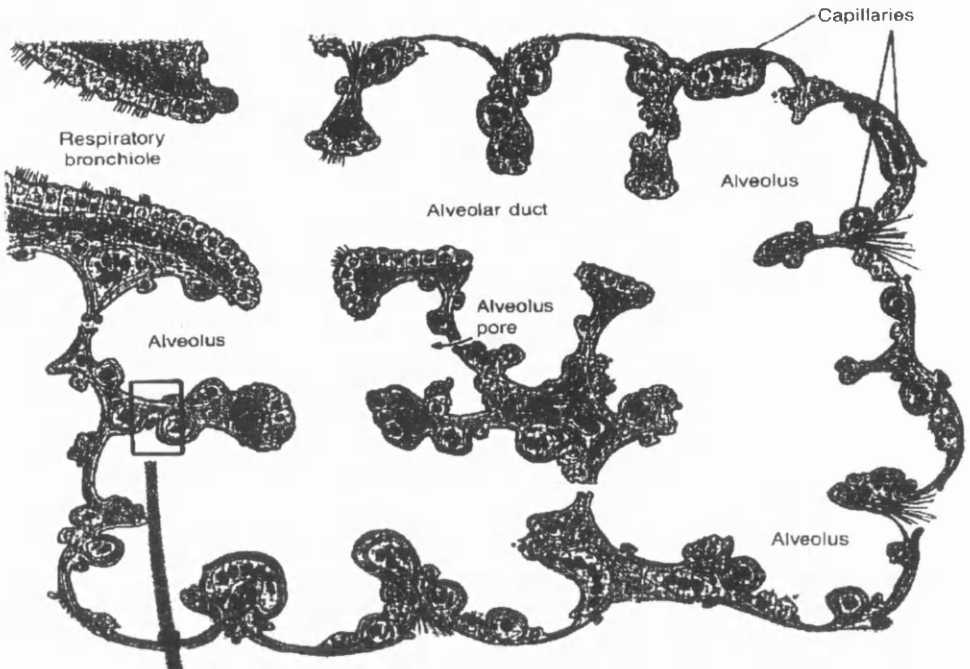
Table 15. Quantitation of fatty acids (FA) in lung lavage of rat, dog and guinea pig by gas liquid chromatography. Data from Coonrod *et al.* (1984).

FFA (% in mixture)	Abbreviation	Rat	Dog	Guinea pig
Lauric	$C_{12:0}$	0.1	-	-
Myristic	$C_{14:0}$	2.8	7.1	2.5
Palmitic	$C_{16:0}$	59.7	33.9	38.3
Palmitoleic	$C_{16:0}$	9.7	7.8	5.4
Stearic	$C_{18:0}$	4.2	11.4	13.8
Oleic	$C_{18:1}$	8.6	32.4	36.8
Linoleic	$C_{18:2}$	8.5	6.1	0.6
Linolenic	$C_{18:3}$	0.3	0	0
Homo-y-linolenic	$C_{20:3}$	0.1	-	-
Arachidonic	$C_{20:4}$	3.6	1.2*	0
Eicosapentaenoic	$C_{20:5}$	0.3	-	-
Docosatetraenoic	$C_{22:4}$	0.2	-	-
Docosapentaenoic	$C_{22:5}$	0.3	-	-
Docosahexaenoic	$C_{22:6}$	0.9	-	-
Unidentified		0.9	0.1	5.4
% Saturated		66.7	52.4	54.5
% Unsaturated		32.4	47.5	40.1
FFA total (µg/animal)		334	665	71.5
Lipid total (µg/animal)		2,121	16,740	4,757
FFA (as % total lipid)		13.2	4.5	0.97

* Data of arachidonic and higher unsaturated FA.

Figure 1. A) Cross section through an area of the respiratory zone of the human lung. There are 18 alveoli, only 4 of which are labelled. Two frequently share a common wall. B) Schematic enlargement of a portion of an alveolar wall. I = alveolar type I cell; II = alveolar type II cell. Data cited from Vander *et al.* (1990).

A.



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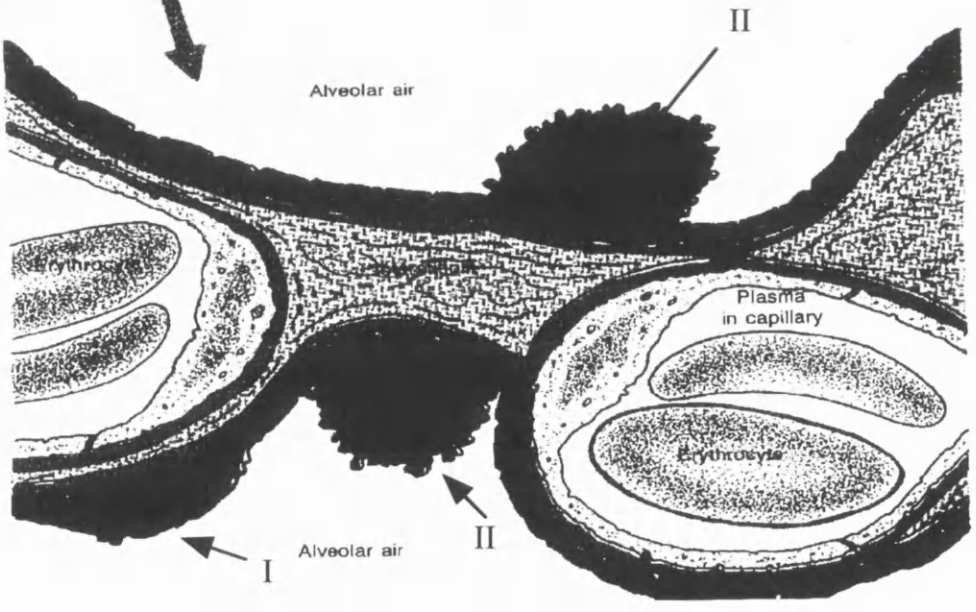


Figure 2. Transmission electron micrograph of lamellar body (plain arrow) contents and the lattice-like tubular myelin (dotted arrow) in the alveolar lining fluids of a rat lung *in situ*, two typical morphological structures of pulmonary surfactant. Data cited from a review of host defence capacities of pulmonary surfactant (Pison *et al.*, 1994).

about 90 square meters - about the surface area of half a tennis court (Moore, 1995).

The dynamic conversions of the various surfactant structures in the alveolar lining fluid were described by Goerk and Clements (1986) as follows: "Surfactant is secreted as lamellar body into the alveolar lining fluid but converted into various macromolecular structures including tubular myelin. How surfactant phospholipids from the variety of structures present in the alveolar fluid move and adsorb to the surface film is not known, but the actual surface film in the lung changes its lipid composition during the dynamic compression to low non-equilibrium tensions on expiration and expansion of the film on inspiration. On inspiration new material adsorbs to the interphase, on expiration some material from the interphase is squeezed out or collapses".

Physiological Activities

The importance of surfactant for lung mechanics has been known since Neergard in 1929, attributed the differences in recoil forces between fluid- and air-filled lungs to the action of surface tension (cited by Pison *et al.* (1994). Also, the presence of a substance which provided very low surface tension in pulmonary oedema fluid and lung tissue was demonstrated by both Pattle (1955) and Clements (1957).

The surface tension of the lung extract at different surface areas *in vitro* was measured by Clements (1957). Brief mention of surfactant functions were listed by Pison *et al.* (1994) as follows: a) modification of surface tension in relation to surface area, b) promotion of lung expansion on inspiration, c) prevents lung collapse on expiration at low transpulmonary pressure associated with normal breathing, and d) balances pulmonary fluids preventing lung oedema formation, and e) stabilizes small airways.

Hause *et al.* (1970) studied pulmonary surfactant of 11 species (mouse, guinea pig, rat, rabbit, sea lion, chicken, dog, man, cow, turtle, and frog), ie. representatives of four vertebrate classes. They stated that the amount of surfactant, estimated by quantitative spreading as a surface film, correlated well with alveolar surface area and with amount of saturated, mainly dipalmitoylphosphatidylcholine in the lung parenchyma. The quantities of other phospholipids did not correlate well with alveolar surface area. King and Clements

(1972b) considered that at least 1 mg dry weight of surfactant material should be present per g weight of lung tissue in order to enable it to cover all alveolar surfaces. This approximate value may vary according to the species studied and the structure of its lungs (Clements, 1971). Govan *et al.* (1991) reported a number of conditions due to absence of surfactant in premature babies leading to a hyaline membrane disease (Figure 3).

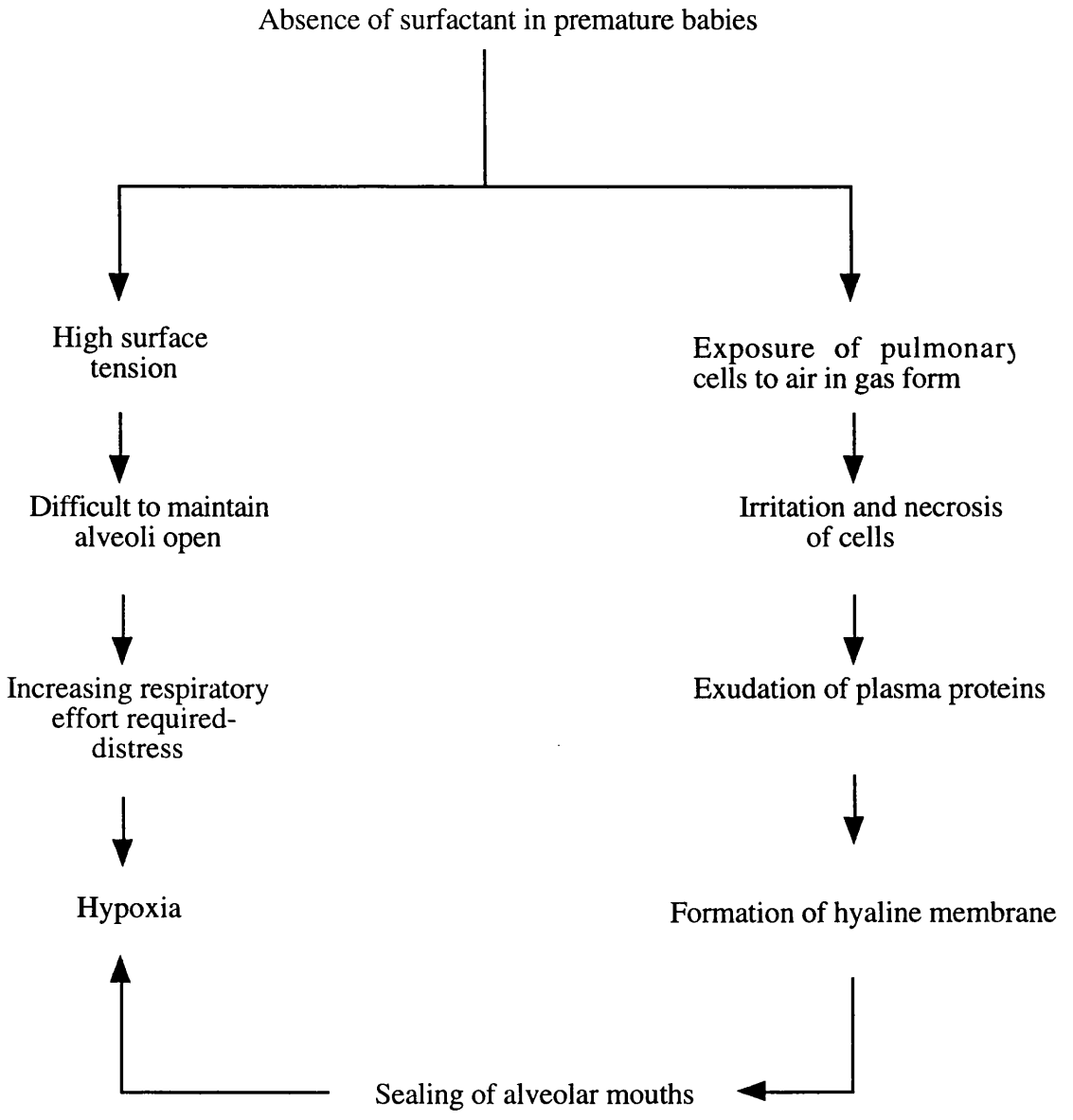
Antibacterial Activities

The defence system of the lung is made up of structural and mechanical mechanisms that are accomplished by miscellaneous non-specific factors, and designated cellular and humoral components (Brain *et al.*, 1977). Many of the antibacterial functions of antibodies are related to their interactions with the components of the complement system (Ishizaka *et al.*, 1965; South *et al.*, 1966). However, current evidence suggests that respiratory secretions do not contain enough of the components of complement to result in complement-mediated responses in the absence of inflammation (Butler *et al.*, 1970; Rossen *et al.*, 1965, ,1966). Opsonization and other antibacterial functions of some classes of antibodies may be denied to IgA (Wilson, 1972). Williams and Gibbons (1972) have suggested that limitations of adherence of bacteria to epithelial surfaces by IgA may also prevent colonization. Wardlaw and Stevenson (1984) reported antibody against LPS of *B. pertussis* strain 18-323 in LLF of convalescent mice, while no antibody was detected in serum from the same animals. No antibody against HLT was detected in either serum or LLF from these animals.

It was reported that nasal secretion contains antibacterial substances such as lysozyme that contributes to lysis of some bacteria and lactoferrin that inhibits the growth of bacteria dependent on iron by chelating soluble iron salts (Rossen *et al.*, 1965).

Biggar and Sturgess (1977) reported that rat alveolar macrophages contain 10-fold-greater intracellular concentrations of lysozyme and release more lysozyme after stimulation than rat blood neutrophils. They also concluded that the greater quantities of lysozyme, both intracellular and released into the extracellular environment by alveolar macrophages,

Figure 3. Absence of surfactant in premature babies and the hyaline membrane disease. This figure was cited from Pathology Illustrated text book edited by Govan *et al.* (1991).



Secondary changes arise as a result of hypoxia - tendency to haemorrhage and disseminated thrombotic episodes.

suggest that this factor may be a mechanism by which alveolar macrophages contribute to pulmonary defences.

Inactivation of staphylococci by alveolar macrophages with preliminary observations on the importance of alveolar lining material have been reported (LaForce *et al.* 1973). Enhancement of bactericidal capacity of alveolar macrophages by human alveolar lining material was reported by Jures *et al.* (1976). They have also confirmed that rat alveolar macrophages do not kill *Staphylococcus aureus in vitro* unless the bacteria have been incubated with rat alveolar lining material before phagocytosis.

LaForce and Boose (1981) described a peptide in normal cell-free rabbit lung lavage fluid (LLF) that faciliates deoxycholate kill of *Escherichia coli.* In another study, Fernandez and Weiss (1996) reported a susceptibility of *B. pertussis* strain BP 338 to a number of antimicrobial peptides.

Coonrod and Yoneda (1983) detected and characterized an anti-pneumococcal factor (s) in alveolar lining material of rats. They concluded that the lipid fraction of rat alveolar lining material affects alveolar macrophage membranes and receptor function. O'Neill *et al.* (1984) stated that human lung lavage surfactant enhances staphylococcal phagocytosis by alveolar macrophages. Other workers were able to show that the lipid fraction of rat alveolar lining material was responsible for the enhancement in killing of *S. aureus* by rat pulmonary alveolar macrophages (O'Neill *et al.*, 1984). Coonrod *et al.* (1984) have attributed the direct bactericidal effects seen in their studies with pneumococci to the high content of free fatty acids in rat alveolar lining material.

Jonsson and coworkers (1986) detected no direct bactericidal effect of healthy nonsmokers human alveolar lining material, nor was there an enhancement of phagocytosis or killing of *S. aureus*, *S. pneumoniae* and *H. influenzae* (ie. common bacterial pathogens of the lung) by pulmonary alveolar macrophages. They also reported in lipid analysis of alveolar lining material that 98 % of the lipid fraction was phospholipid and no free fatty acids were detected. Pulmonary surfactant of sheep induced killing of *Pasteurella haemolytica*, *E. coli* and *Klebsiella pneumoniae* by normal serum (Brogden, 1992).

OBJECT OF RESEARCH

In whooping cough (pertussis), the bacteria remain localized on the ciliated epithelium in the respiratory tract and it is reasonable to suppose that local host defence mechanisms are responsible for bacterial clearance at the onset of the convalescent stage of the disease. Little is known about such mechanisms in the human disease, and the emphasis has been on studying antibodies in convalescent sera, rather than in respiratory tract secretions.

Because of the difficulty of experimental studies in human infants, the coughing-rat model of pertussis has been used here.

The object of the proposed research was to determine the possible antibacterial effect of lung lavage fluids (LLF) from normal and *B. pertussis*- infected rats. Serum from these animals would also be tested in parallel.

The specific questions in the initial phases of the work were:

Q.1. Are lung lavage fluids from normal rats able to kill *B. pertussis* ? Is normal rat serum bactericidal ?

Q.2. Are lung lavage fluids, or serum, from convalescent rats different from lavage fluids and serum from normal animals ?

If the answers to either questions was positive, the project would explore the mechanism(s) of the bactericidal systems. Further question envisaged were:

Q.3. Is activity located in humoral factors or in cellular components (presumably phagocytes) or both ?

Q.4. How do phase I and phase IV *B. pertussis* respond to LLF?

MATERIALS

AND

METHODS

BACTERIA, CULTURE MEDIA AND DILUENTS

Bacteria

B. pertussis strains

A total of 13 strains of *B. pertussis* was used in this investigation. Most of the work was done with strain 18-323, the challenge organism used for assaying the protective potency of pertussis vaccines. This and the other strains are listed in Table 16. It will be noted that both virulent (phase I) and avirulent (phase IV) strains were used; also two vaccine strains, 18-334 and Tohama; a strain isolated from a case of whooping cough in Glasgow; and the strain lux which had luminescence genes inserted. Plate 1 illustrates the colonial appearance of *B. pertussis* strain 18-323 and lux on Bordet Gengou agar, while Plate 2 presents Phase I and Phase IV forms of strain 30042.

All the strains were received as stock cultures frozen in CAA-glycerol (see below) at - 70°C. From time to time fresh samples of these frozen stocks were prepared as required. Cultures were grown on Bordet-Gengou (BG) medium containing 15-20 % (v/v) horse blood, by spreading a portion of frozen inoculum over the surface and incubating for 3 d at 37°C in a closed plastic box with a beaker of water to saturate the atmosphere.

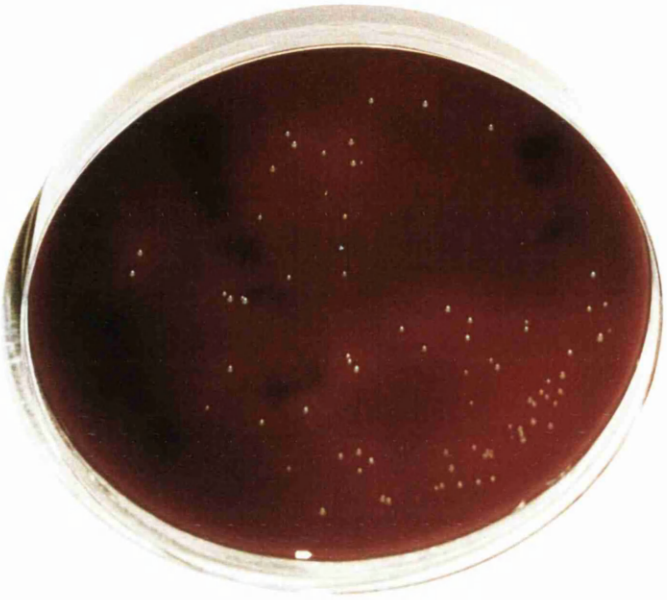
Culture purity was checked by visual inspection and gram stain and initially, by agglutination with a *B. pertussis* hyperimmune rabbit IgG/IgA serum kindly supplied by Dr R. Parton. If these tests were satisfactory, a heavy inoculum (i.e. almost the whole growth on the 3-day plate) was transfered to a fresh BG plate and incubated for 24 h at 37 °C.

With *B. pertussis* lux, the above schedule was modified by having a single-stage, 2-day 37 °C grown culture, which gave satisfactory results. The phase status of *B. pertussis* strains was checked by inoculation on to brain heart infusion agar (BHIA) and incubating for 24 h, or longer, at 37 °C. Phase IV strains gave heavy growth under these conditions while phase I organisms failed to grow.

For storage, a loopful of pure bacterial culture was suspended in 1.6 ml of sterile casamino acids (CAA) and 0.4 ml sterile glycerol (BDH). The mixture was given a few seconds on a whirlimixer (Fisons) and 100 µl aliquots were put into washed, sterilized 2

Plate 1. Colonial appearance of *B. pertussis* on Bordet Gengou agar A) 5-day growth of strain 18-323; B) 5-day growth of strain lux.

A



B

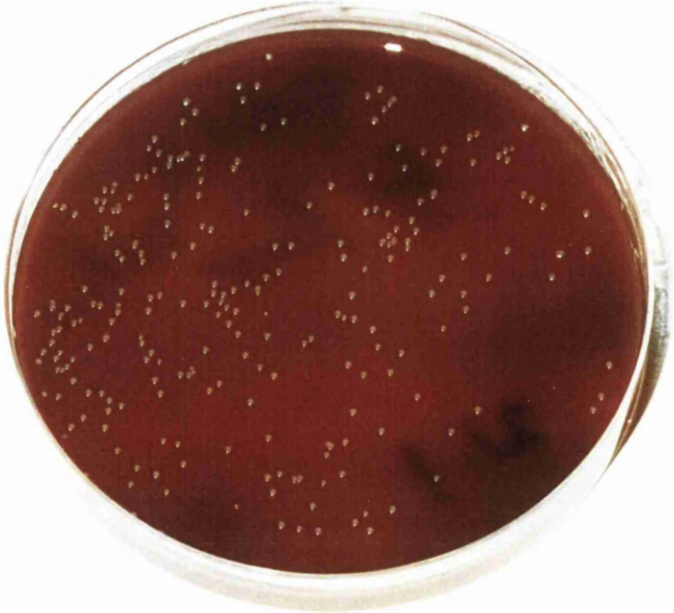
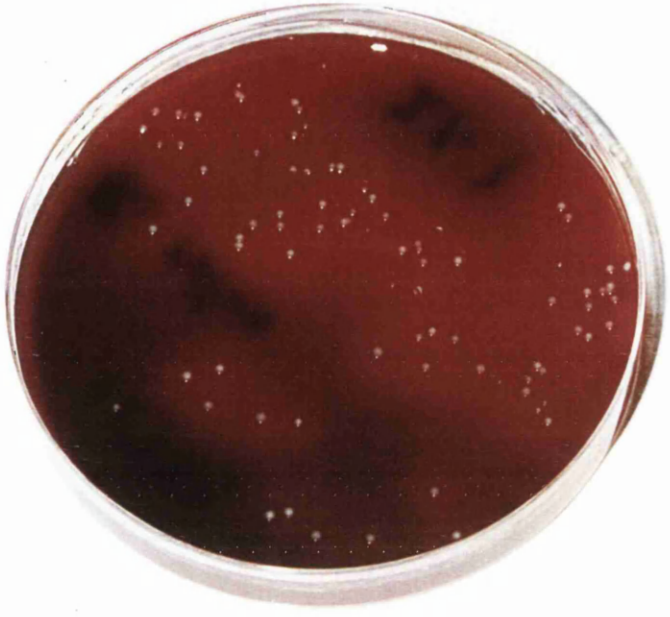


Plate 2. Colonial appearance of *B. pertussis* 30042 on Bordet Gengou agar after 5-day growth of A) Phase I ; B) Phase IV.

A



B

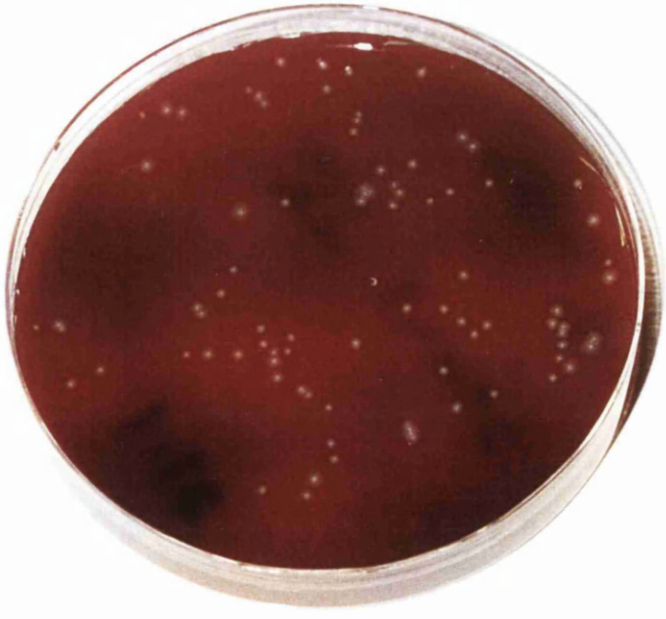


Table 16. Strains of *B. pertussis* (all from departmental stocks) used in this study.

Strain	Phase	Remarks
18-323 A6	I	Standard challenge strain in mouse and rat infection experiments.
18-323 EH1	"	Obtained by passage of 18-323 A6 through rats in 1991.
18-323 EH2	"	Obtained by passage of 18-323 A6 through rats in 1994.
18-334	"	Standard vaccine strain (Connaught Laboratories, Canada).
Tohama	"	Japanese vaccine strain
BP347	"	Transposon mutant vir$^-$ (from Tohama) equivalent to phase IV (originally from A. Weiss).
L-84	"	Lister Institute: virulent strain (originally from J. Dolby)
L-84	IV	Lister Institute: avirulent strain (originally from J. Dolby)
44122/7S	I	Streptomycin-sensitive (originally from P. Branefors)
44122/7R	"	Streptomycin-resistant (originally from P. Branefors)
lux	"	Bioluminescent strain, derived from 44122/7R
77/18319	"	Glasgow pertussis case
30042	"	Originally from J. Dolby
30042	IV	Originally from J. Dolby

ml-volume polypropylene plastic vials with 'O' ring screw-cap (Elkay Lab Products UK Ltd) and stored at - 70 °C.

Other bordetellae

Bordetella bronchiseptica, NCTC 5376, and *B. parapertussis*, NCTC 10520, were received from departmental stock as - 70 °C frozen suspensions and were treated in the same way as *B. pertussis*, except that only a one-stage culture procedure was used. For *B. bronchiseptica*, the incubation time was 24 h at 37 °C and for *B. parapertussis*, 2 d. Culture purity of *B. bronchiseptica* was checked by Gram stain and by ability to grow on BHIA and MacConkey agar, and for *B. parapertussis* also by Gram stain and growth ability on BHIA.

Other bacteria

These were received as plate cultures and were converted into -70 °C glycerol-CAA suspensions as done above for *B. pertussis*, after growth in appropriate media as listed below.

Staphylococcus aureus and *Streptococcus pyogenes* were from departmental stock; *Micrococcus luteus* and *E. coli* Lilly were received from Prof. A. C. Wardlaw and *E. coli* lux WA 803 and *E. coli* lux DH5α were from Dr R. Parton. The culture purity was tested by visual inspection and Gram stain. All the bacteria were grown for 24 h at 37 °C, except *M. luteus* which was grown for 2 d at 30 °C and the culture purity was checked by visual inspection and Gram stain.

M. luteus, this classic, lysozyme-sensitive strain (NCTC 2665) was grown on BHIA. *E. coli* Lilly, a complement-sensitive strain (NCIMB 11888) was grown on BHIA. *E. coli* lux WA 803, a luminous construct was grown on BG agar medium with 25 μg/ml kanamycin. *E. coli* lux DH5α, another luminous construct of a standard laboratory strain was grown on nutrient agar (NA) with 50 μg/ml ampicillin. *S. aureus,* Oxford NCTC 6571 strain was grown on blood agar (BA). *S. pyogenes,* NCTC 5763 was grown on MacConkey agar.

Culture Media

Bordet-Gengou (BG) agar

BG agar medium was prepared by dissolving 20 g of BG agar base (BBL, Becton-Dickinson) in 500 ml of distilled water (DW) containing 1% (v/v) glycerol by steaming at 100°C. It was then sterilized for 15 min at 121°C. After cooling to about 50 °C, 15-20 % (v/v) defibrinated horse blood (Becton-Dickinson, Cowley) was added aseptically, in a laminar flow hood.

The mixture was swirled gently, avoiding the introduction of air bubbles, and about 25 ml portions poured into 90 mm sterile petri-dishes (Sterilin). Solidified BG agar plates were labelled, packed in a sterile plastic sleeve, stored at 4°C, and discarded after three weeks if not used.

For BG with kanamycin, 100 µl of filter sterilized 2.5 % (w/v) kanamycin A (Sigma) in DW was added to 100 ml of cooled (45-50°C) BG agar medium, before adding the horse blood, after which the procedure for plain BG medium was followed.

Other media

The following media were obtained from Oxoid and were dissolved and sterilized according to manufacturer's instruction: Brain heart infusion agar; Nutrient agar; Nurient broth; Blood agar base and MacConkey agar. For blood agar, the melted base in 500 ml portions was cooled to 45-50 °C and 50 ml fresh defibrinated sheep blood (E&O, Laboratories Ltd) added.

Diluents

Phosphate buffered saline (PBS)

At the beginning of the experimental work, PBS was prepared by dissolving 10 PBS tablets (Oxoid) in 1 litre of DW, the pH was checked and found in most cases to be approximately 7.3. The addition of small amount of acid was needed to adjust the pH to 7.2. PBS was sterilized and stored at 4 °C.

However, the PBS which was used in most of the experimental work was prepared by dissolving analytical grade chemicals, 7.99 g NaCl (BDH), 1.196 g Na_2HPO_4 (BDH), 0.212 g KH_2PO_4 (Fisons), and 0.199 g KCl (Fisons) in 1 litre of DW. If necessary, the pH was adjusted to 7.2 by addition of small amounts of alkali or acid. In some experiments, PBS either at pH of 7.3 or 7.4 was used.

Casamino acids (CAA)

CAA 1% (w/v) solution was made by dissolving 10 g casein hydrolysate (Gibco-BRL), 0.1g $MgCl_2$ $6H_2O$ (BDH), 0.016g $CaCl_2$ (Fisons) and 5g NaCl in 1 litre DW. The pH of the mixture was adjusted to 7.1 by addition of NaOH, and the fluid sterilized and stored at 4 °C. It was discarded after two months if not used.

Cyclodextrin liquid (CL) medium

CL-medium was prepared as described by Imaizumi *et al.* (1983a) with minor modification, by dissolving 10.7 g L-glutamate acid (Sigma) [instead of L-glutamic acid sodium salt (BDH) as mentioned by Imaizumi *et al.*, 1983], 2.5 g NaCl, 0.5 g KH_2PO_4, 0.1 g $MgCl_2$. $6H_2O$, 0.02 g $CaCl_2$, 0.2 g KCl (Fisons), 6.25 g Tris (Boehringer Mannheim GmbH), 10 g casein hydrolysate, 0.24 g L-proline (Sigma) and 1 g dimethyl-β-cyclodextrin (J. Shimizu, Teijin Ltd., Tokyo, Japan) in 1 litre of DW. The pH was adjusted at 7.4 with 1 N NaOH. The CL- medium base was sterilised and stored at 4 °C.

A supplement was prepared by dissolving 0.01 g $FeSO_4$ (BDH), 0.04 g L-cysteine, 0.004 g niacin, 0.15 g glutathione (all from Sigma) and 0.4 g ascorbic acid (Lancaster Synthesis Ltd) in 10 ml DW, which then sterilised by passage through a membrane (0.45 μm pore size) filter (Sartorius). It was added to the CL-medium base immediately before use in a volume of 50 μl (instead of 100 μl as mentioned by Imaizumi *et al.*, 1983a) per 10 ml of medium. The supplement was stored at -20 °C, and discarded after two months if not used.

Special cleaning of glassware and plasticware

All 7 ml-volume thin-wall glass Bijoux (L.I.P, Equip & Services Ltd) were immersed in concentrated nitric acid (which was handled with great care by using stainless steel clamp forceps, special eye safety glasses, and latex examination gloves) for 24 h. They were then rinsed ten times with DW, soaked in DW for a further of 24 h, and finally rinsed with DW, to ensure that the acid was completely removed by showing that the rinsing had the same pH as freshly made DW. The glass bijoux were then dried in a hot (about 80 °C) oven for a few hours, sterilized for 15 min at 121 °C, and stored in a covered container away from dust.

Plastic items such as the plastic screw caps of glass bijou bottles, Gilson pipette plastic tips, 1.5-ml volume plastic microfuge tubes (Greiner Labortechnik Ltd) and 2-ml volume screw cap plastic vials were boiled for 30 min in DW. Then they were dried at 80 °C for a few hours, sterilised for 15 min at 121 °C. Latex examination gloves were worn when handling washed glass and plastic ware before and after autoclaving to keep the items clean.

COLLECTION AND PROCESSING OF TEST FLUIDS

Lung Lavage Fluids (LLF) and Serum

Rats

Male and female barrier-reared Sprague Dawley rats "viral antibody free plus status" were obtained from Charles River UK Ltd (Manston Road, Margate) or from Harlan Olac Ltd (Shaws Farm, Backthorn, Bicester). Each batch of rats was certified free from a range of protozoal, viral, and bacterial pathogens including *B. bronchiseptica*, according to the health-status certificate.

Rats were ordered by weight around 150-200 g (Figure 4) equivalent to about 6 to 8 weeks old and were kept in groups of 8-10 in flat bottomed cages with wood shavings as bedding in air filtered rooms. The animals were given free access to diet and water. To provide the cages units with filtered air, prefilters and main filters were used. Air passed

through prefilters to remove particles of > 5 μm and then through main filters to remove particles of > 2 μm size.

Rats either normal, sham-operated, vaccinated and challenged, or convalescent from *Bordetella* pulmonary infection were used as a source of LLF and serum and were made available by Dr E. Hall. The animals at the time of obtaining these fluids were approximately 7-12 weeks old and weighed in the range of 180 - 350 g.

Infected rats (see Appendix 1) were housed as 2 per cage. In addition to Sprague Dawley, Lewis, Brown Norway and Hooded Lister rats were used in this study and were from the same source (Harlan-Olac).

Euthanasia

Various methods (A,B,C,D) of killing the rats before collecting LLF and serum were examined. Of these, method D used in later experiments appeared to be the best.

Euthanasia method (A) consisted in anaesthetising the rats with 2-2.5 % of halothane (RMB Animal Health Ltd, Dagenham) mixed with oxygen for about 3 - 5 min, killing them by transferring them to a 10 litre plastic jar containing CO_2 from a piped source. Blood was then taken by heart puncture followed by LLF collection.

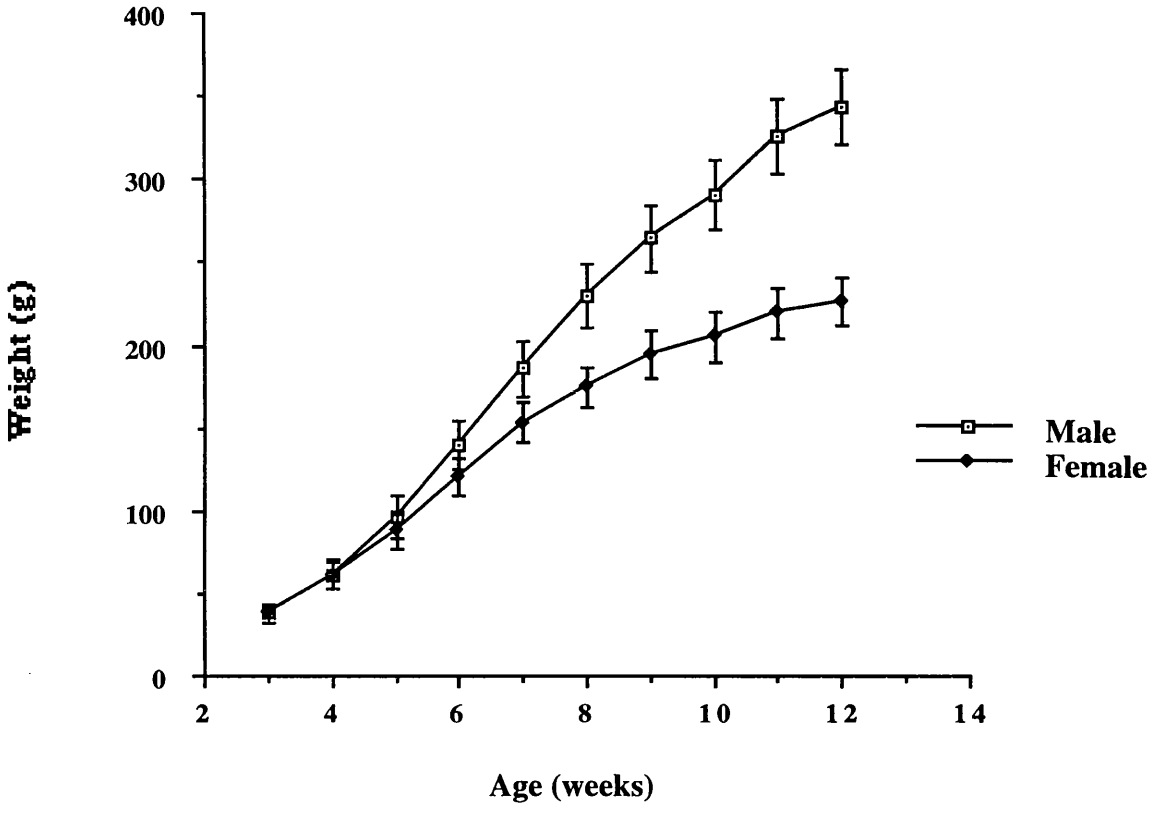
In the second method (B), rats were killed directly with CO_2 without anaesthesia before collection of blood and LLF. In method (C), an overdose of halothane / oxygen was given to the rats for about 15 min as a way of killing followed by blood and LLF collection.

The standard euthanasia method (D) consisted in anaesthetising the rats with halothane / oxygen for about 3 - 5 min. Blood was taken by heart puncture, then the animal was killed by cervical dislocation, after which the LLF was taken. See Table 17 for a summary of the different methods of euthanasia.

Rat serum

Blood was collected by inserting a 0.6 in x 25 gauge needle (Becton Dickinson) on a 10 ml syringe (Becton Dickinson) into the heart to yield about 10 ml blood, which was

Figure 4. Relationship between age and weight of male and female outbred Sprague Dawley rats. Values are means ± standard deviation. (Data from Harlan-Olac)



placed in a sterile thick-wall glass universal bottle (LAB SUPPLIES, University of Glasgow). Pooled bloods were centrifuged (MSE, Mistral 6 L) for 15 min at 2000 rpm at 10 °C. The fluids were filter sterilized (0.45 µm membrane filter, Sartorius) and stored in 1 ml amounts in washed, sterilized 2-ml plastic vials for 24 h at -20 °C, and then transferred to -70 ° C.

Rat LLF

The rats which had just been bled by heart puncture were immediately used as a source of LLF. The animals were placed on the operation table on their backs and fastened with strips of autoclave tape over their legs. About a 3 cm longitudinal incision was made in the throat at the site of the trachea, after which, the trachea was exposed, punctured with sterile 12 cm curved scissors, and 5 ml of sterile PBS injected with a 5 ml-syringe fitted with a special cannula (Figure 5). This was made from a standard 50 mm plastic "yellow tip, Sarstedt" from which 5 mm had been cut at a slant from the narrow end and at 10 mm uniformly around from the wide end.

The PBS was injected and rinsed by drawing up the barrel of the syringe 3 times. The final yield was about 3 ml crude lung washings per animal which were placed in a sterile plastic universal (Sterilin) on ice. Pooled lung washings from several rats were centrifuged for 15 min at 2000 rpm (in later experiments, this was reduced to 500 rpm which is enough for depositing the cells and debris, also to reduce the amount of active material being passed into the deposit fraction in which the bactericidal activity was found) at 10 °C.

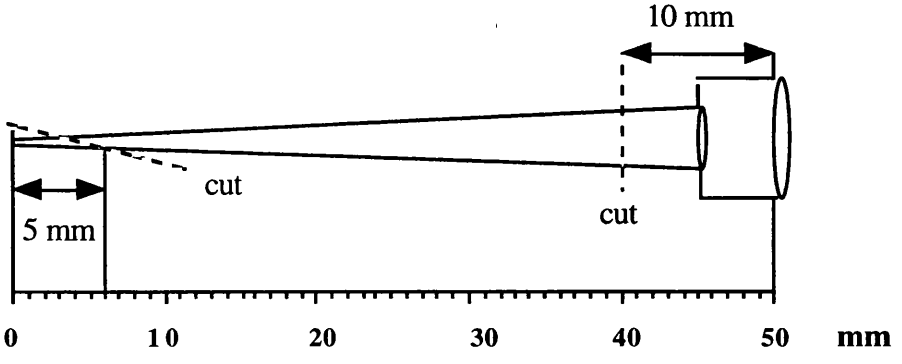
Filtration and storing procedures of the supernate were as with serum. The collection procedures of serum and LLF were done in the same day to avoid the deterioration of the fluids and loss of activity.

The pH of 2 LLF batches number 15 and 34 as a sample from normal and convalescent rats were examined with pH indicator paper (Whatman) and found to be at pH 7.

Table 17. Summary of four different euthanasia methods before collection of rat serum and LLF.

Method	Procedure prior to collection of LLF						
	Halothane/ oxygen	CO_2		Bleeding out by heart puncture before death	Cervical dislocation	Bleeding out by heart puncture after death	LLF batch no.
		under anaesthesia	alone				
A	+	+	-	-	-	+	15,16,17 21,33
B	-	-	+	-	-	+	34,35 37,38.1
C	+	-	-	-	-	+	38.2
D	+	-	-	+	+	-	39,40,41 42,43,45, 55,56,58

Figure 5. Schematic diagram of how a Sarstedt yellow tip was cut with sharp scissors to make a rat tracheal cannula.



Other animal species and human LLF

Other animal species and human LLF were used in this study. The species were mouse, horse, rabbit, sheep, chicken, and dog bronchial lavage specimens and had been stored at -20 °C (Appendix 2). They had been collected by J. Porter during his Ph. D study in 1991 on the growth of *Bordetella* species in LLF and other low-nutrient fluids (Porter and Wardlaw, 1994). One of the human LLF was provided by Dr. Neil Thompson and Dr. Hulk of Glasgow Western Infirmary. This sample was taken from an elderly patient (by bronchial lavage with saline) who had some form of non-infectious lung or bronchial disorder.

In addition to these stored samples, calf LLF was obtained from normal calves provided by Glasgow University Veterinary School. The lungs from the freshly slaughtered animals were removed and the trachea filled with approximately 1 litre of sterile PBS. The lungs were shaken up and down for mixing and then inverted to allow the lavage fluid to drain out. Approximately 450 ml fluid was recovered from each set of lungs. It was centrifuged for 15 min at 2000 rpm and a portion of the supernate was membrane filtered (0.45 μm pore size). This was then stored as 1 ml portions in a sterile 2-ml volume plastic vials for 24 h at -20 °C before transfer to -70 °C for long-term storage.

Long-Chain Fatty Acids and Phosphatidylcholine

Long-chain fatty acids (FA)

These were purchased from Sigma, and consisted of palmitic acid and myristic acids (sodium salts), and the remainder as free acids: elaidic, oleic, palmitoleic, linoleic, arachidonic, petroselinic and pentadecanoic.

Petroselinic and pentadecanoic acids were dissolved by sonication for 3 x 30 sec at 10 % amplitude with a microprobe (Jencons, Sonics & Materials Inc.) in 10 mM NaOH to a FA concentration of 10 mM, which was then diluted in 1 % CAA to a final range of concentrations of 1 mM - 0.1 μM. These were stored in washed and sterilized glass Bijous in volumes of 2.7 ml at -20 °C.

Palmitic and myristic acids were dissolved in methanol (Fisons) and diluted with chloroform (Prolabo) to give 10 mM, which was then further diluted in chloroform to the same final concentrations as above and stored in the same way.

The other FA were dissolved to 10 mM in chloroform, which was then diluted in chloroform to final concentrations of 1 mM - 0.1 μM and stored as before.

When required for bactericidal test, the chloroform solutions were placed in a container of warm water in a fume cupboard, and dried under a slow stream of nitrogen delivered through a plugged pasture pipette with its tip near to the top of the solvent surface. The dried FA were reconstituted with 2.7 ml of 1 % CAA and exposed to brief sonication 3 x 30 sec at 5-10 % amplitude to aid dissolution.

Petroselinic and pentadecanoic acids were taken out from the freezer and left on the bench to warm then dissolved with sonication as above.

Methanol or chloroform solvents without FA, were subjected to the same procedures, for use as control fluids.

Phosphatidylcholine (PC)

PC (Sigma) was obtained in a small vial containing 25 mg in chloroform at 10 mg/ml. This was diluted in chloroform to a final concentrations range of 500 - 15.6 μg/ml (0.59 - 0.02 mM), after which aliquots were dried under nitrogen gas, reconstituted with 1 % CAA to its original volume then exposed to brief sonication 3 x 30 sec at 5-10 % amplitude.

BACTERIAL VIABILITY AND LUMINESCENCE

Standardization of Bacterial Suspensions

For use in bactericidal tests, suspensions of *B. pertussis* and other bacteria were standardized to an absorbancy A = 0.45 at 540 nm in a 1 cm cuvette (as described by Porter *et al.* 1991). In most instances PBS was used as the suspending fluid except in the later

experiments with *B. pertussis* and the other bordetellae when CAA was employed. This was because of better maintenance of viability.

The subsequent dilution (Figure 6) of the standardized suspension then varied according to the bacterial strain and species, and type of test. In viable count experiments the A_{540nm} = 0.45 suspension was diluted between 1/2000 and 1/ 40,000. For luminescence, the standard suspension was used undiluted and at 1/40.

In three experiments, the *B. pertussis* 18-323 was washed by centrifugation according to the method of Porter *et al.* (1991), the other aspects of preparation being unaltered. The washing was done by centrifuging (Biofuge 13, Heraeus Sepatech) 1 ml of A_{540nm} = 0.45 suspension for 15 min at 10500 rpm, the supernatant was discarded and the pellet resuspended in 1 ml PBS and the washing process repeated another 2 times. The washed bacterial suspension was diluted in PBS to 1 in 2000.

Bactericidal Test

Viable count method

Fluids to be tested for bactericidal activity were placed as 480 µl aliquots in washed, sterile 2-ml plastic vials and 20 µl of standardized, diluted bacterial suspension was added and mixed in. A sample of 20 µl was taken immediately and plated onto BG agar medium for the time-zero viable count. The vials were then incubated at 37 °C on a MK IIIB shaker (LH Engineering Co. Ltd) at 110 rpm.

To determine survival and death of bacterial cells at 37 °C, samples of 20 µl were taken from the test mixtures at 15, 30, 60, 120, and 1440 min, and at 2880 min in some experiments. They were plated onto BG agar medium, which was then incubated for 3-5 days at 37 °C, after which colonies were counted.

Luminometry method

***Equipment*:** Luminometer (LKB, Walac 1251) was a bench-top (Figure 7) luminescence photometer intended for the measurement of all types of luminescence.

Figure 6. Scheme of dilutions for preparing a bacterial suspension for bactericidal tests based on viable count (VC) or luminometry (Lum). The dilution of primary thick suspension needed to give an absorbancy of 0.45 at 540 nm was determined by its stepwise addition to PBS or 1 % CAA in a trial cuvette. This dilution was then used to prepare the main dilution series, with sterile precautions .

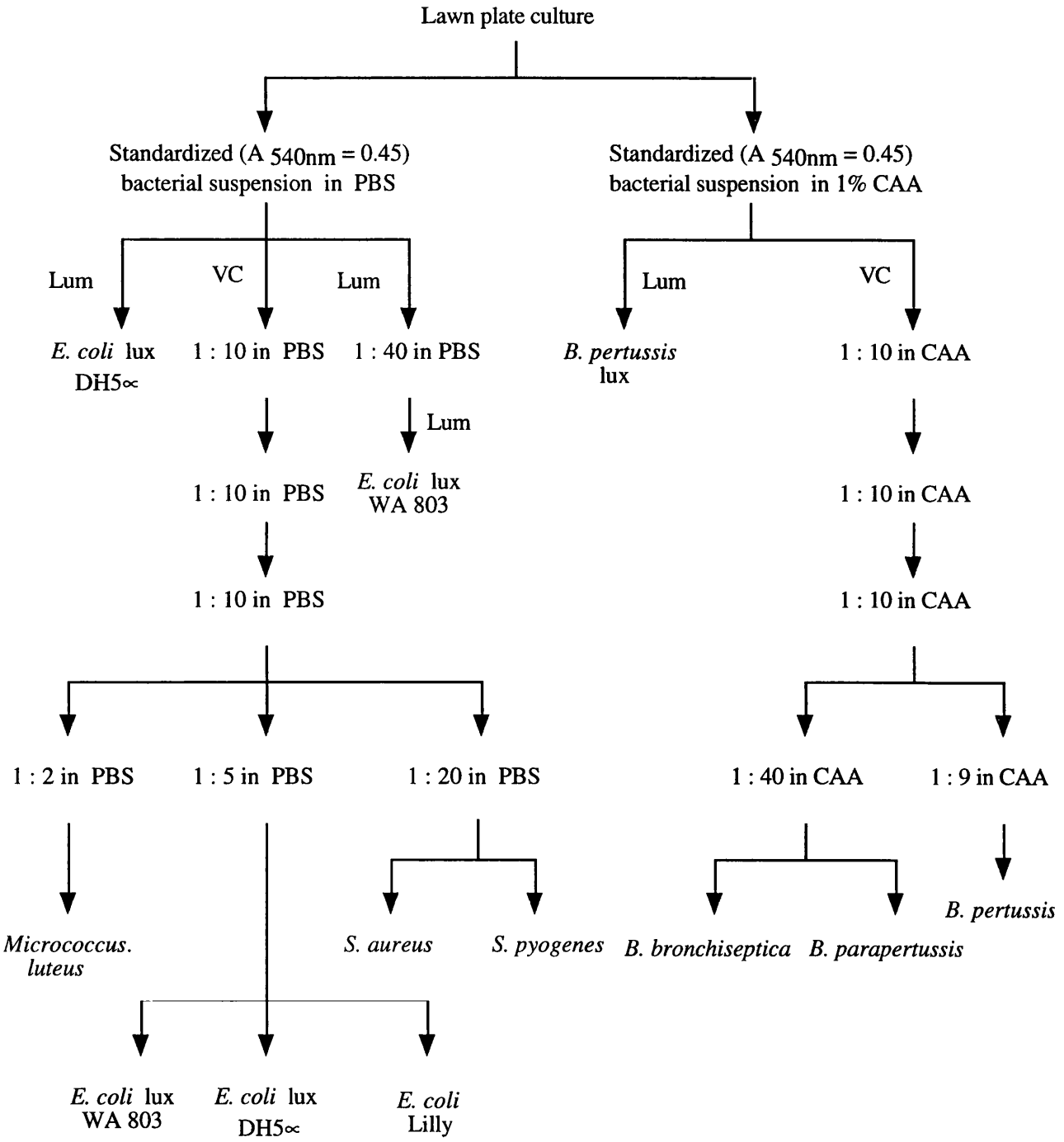
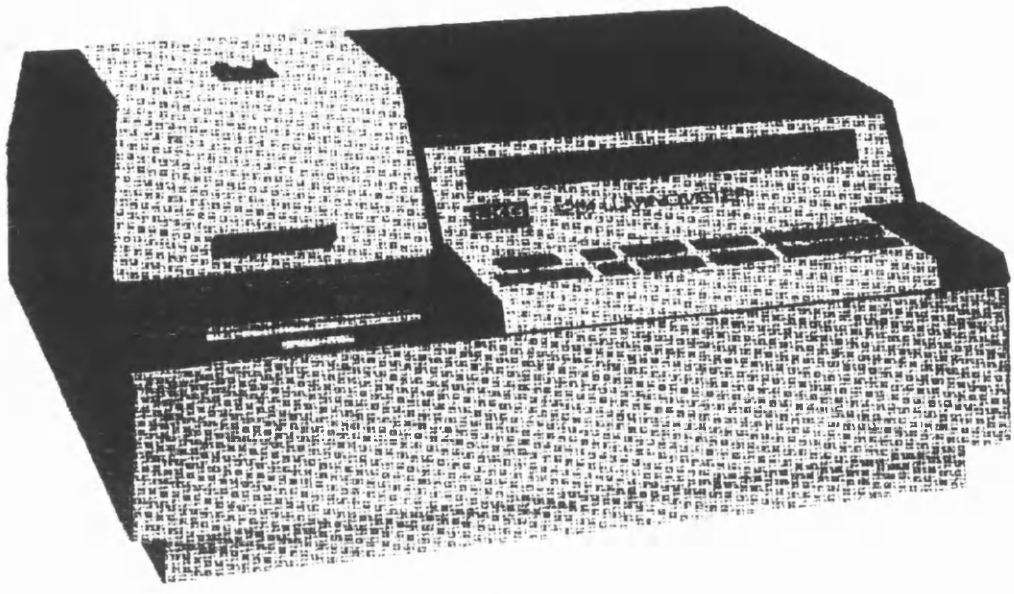
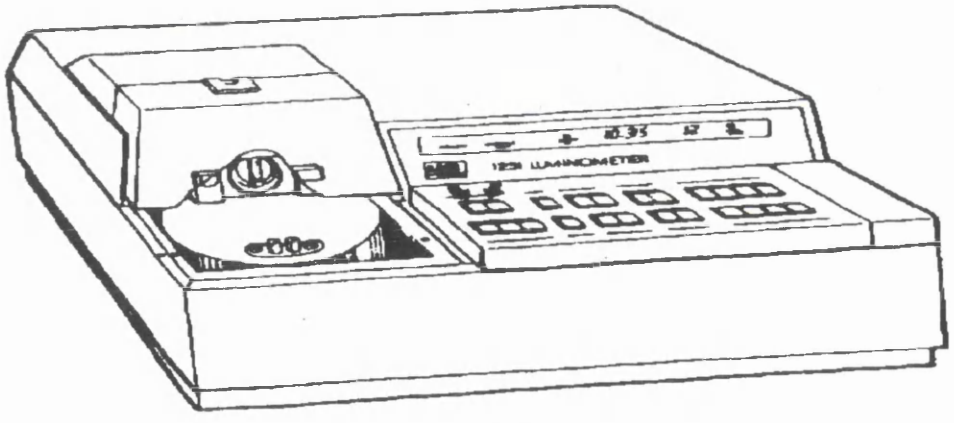


Figure 7. A) General picture of the luminometer; (luminescence photometer); B) Another general picture showing the position of a 25-cuvette capacity measuring chamber; C) Instrument keyboard.

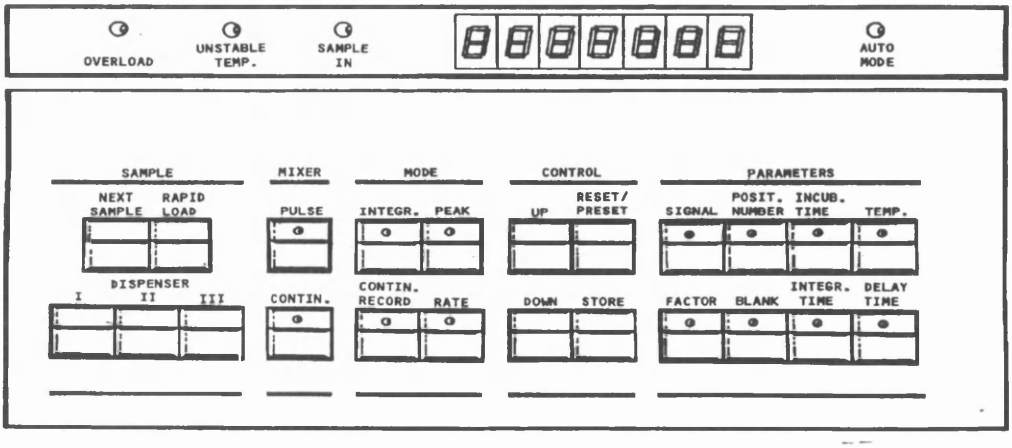
A



B



C



The sample holder was a 25-position carousel-type metal dish, and the addition of reagents was done manually in the measuring chamber. The cover of the sample holder was closed except when applying the sample cuvettes, so as to avoid change of incubation temperature, quick evaporation of aldehyde or accumulating of dust inside the sample holder. The sample was mixed by continuous mixing through a motor which rotated the sample cuvette.

The sample changing and measuring section was used to convey samples to the measuring chamber into which the sample cuvette was raised by elevator and the light emitted from the sample was detected. The photomultiplier detected light accurately at a wavelength of between 300 and 600 nm. The photomultiplier converted the light into an electrical signal which was then amplified and recorded.

***Conduct of test*:** The temperature in the luminometer was adjusted to 37 °C and a PC-computer (IBM) running on Multi-Use software (Bio-OrbitOy, Turku, Finland) was used to control the instrument.

Standardized *B. pertussis lux* (100 µl) was added to 400 µl of test and control fluids in polystyrene luminometer cuvettes (Clinicon). These were incubated in the instrument with mixing for 1 h at 37 °C. To observe luminescence, it was necessary to add highly diluted decanal. A 20 % (v/v) stock solution, prepared fresh, consisted of 100 µl decanal (n-decylaldehyde, Sigma) and 400 µl dimethyl sulphoxide (DMSO, Fisons). This was diluted to 1: 220 in DW at room temperature by adding 10 µl of the 20 % stock solution to 2.190 ml DW. To each test mixture in the luminometer, 20 µl of the 1 : 220 dilution of decanal was added, so that the final amount of the aldehyde was 0.018 µl (or 18 nl) per 0.5 ml test mixture.

The light output in millivolt (mv) from each sample in turn was measured, recorded, saved, and displayed on the computer screen in the form of graph of light output against time and as a Table. Usually, measurement were taken over a 10 min period and the peak value determined. In some experiments the 500 µl test mixture in the 2-ml plastic vials was incubated in a 37 °C water bath (Grant Instruments), then 500 µl of CL-medium was

added to give a final volume of 1 ml. This was then transferred to the luminometer cuvettes, placed in the luminometer at 37 °C and given a complete run for approximately 150 sec, after which 20 µl of decanal solution was added.

In other experiments, the final decanal amount in the 20 µl decanal solution (with DMSO and DW or PBS) was in the range from 0.01 to 0.1 µl. In other groups of experiments the bioluminescence response in the test fluids was detected at 0, 15, 30, 45 and 60 min incubation time, by adding 20 µl decanal solution at each incubation time to all test fluids.

CHARACTERIZATION OF RAT LLF

Fractionation by Centrifugation

Cell-free LLF from either normal or treated rats was placed in a polycarbonate plastic ultracentrifuge tubes (about 10 ml size), and after balancing was spun in an ultracentrifuge (OTD Combi Ultracentrifuge, DU PONT) for 20 min at 29000 rpm (55000 g) at 4°C. The supernate fraction was stored in a washed, sterilized, screw cap 2-ml plastic vial at - 70°C as a 1 ml portion.

The pellet (surfactant fraction) was reconstituted in sterile PBS to its original volume and transfered to a thick-wall glass universal. This was placed on ice and dissolved by sonication using an alcohol-cleaned microprobe kept clear of the glass. The treatment was done with 3 exposures of 2 min at 5-10 % maximum amplitude, after which it was stored as with supernate.

Incorporation of *B. pertussis lux* in agarose ± DM-β-CD

The incorporation (see Appendix 1) of 1 ml of standardized (A_{540nm} = 1.8 in 1 % CAA) *B. pertussis lux* in 1 ml of 4 % (w/v) agarose beads was as described by Hall *et al.* (1994). After this, 2 ml of 1 % CAA with or without 0.1 % (w/v) dimethyl-β-cyclodextrin (DMβCD) was added and the mixture homogenised for 15 sec at low speed. In luminescence (see above) test experiments, 100 µl of *B. pertussis lux* in agarose ± DM-β-

CD, in DM-β-CD only and in DW were added to normal rat surfactant and PBS (as a control).

Artificial surfactant

A protein-free artificial surfactant (artificial lung expanding compound; ALEC pumactant) powder (Britannia Pharmaceuticals Ltd) consisted of dipalmitoylphosphatidyl-choline and phosphatidylglycerol in a ratio of 7 : 3 (Morley *et al.*, 1981). ALEC 100 mg of sterile freeze-dried powder was suspended in 1.1 ml of 0. 85 % (w/v) saline (Oxoid) in DW, and mixed with a whirlimixer.

Biochemical and Serological Analysis

Protein estimation

Total protein content of test samples was estimated according to Smith *et al.* (1985) using bicinchoninic acid (BCA). The protein used as standard was BSA (Sigma) which was vacuum dried at 500 millitor on a freeze-dryer apparatus for 18 h at 20 °C before use. It was dissolved at 20 mg / ml in DW and stored at -20 °C.

Reagent A contained 8% (w/v) anhydrous sodium carbonate (Fisons), 1.6 % (w/v) NaOH (BDH), and 1% (w/v) sodium tartarate (BDH) in 250 ml of DW, pH at 11.25 was adjusted with solid sodium hydrogen carbonate (Fisons) then diluted up to 500 ml as a final volume. Reagent B contained 10 ml of 4 % (w/v) 4,4' dicarboxy-2,2'biquinolin (bicinchoninic acid sodium salt, Sigma) in DW. Reagent C contained 4 % (w/v) $CuSO_4$ $5H_2O$ (BDH) in DW.

The stock BSA was serially diluted in DW to a range of 50 to 3.125 µg / ml and 0.5 ml of standards, blank (DW) and diluted test sample was pipetted in a 7-ml volume plastic bijoux (Sterilin) in triplicate. To this was added 0.5 ml of working solution containing 10 ml of reagent B and 0.4 ml of reagent C. To this mixture an equal volume of reagent A was added.

All samples were incubated for 1 h in a 60 °C water bath, then they were cooled to room temperature, and their absorbancy measured at 562 nm versus a reagent blank. Standard protein concentrations were plotted against absorbancy and the concentrations of the unknowns determined by interpolation on to the standard curve (Figure 8).

Lysozyme detection and estimation

The bactericidal activity test (as above) was done using standardized and diluted *Micrococcus luteus* (ML, sensitive to lysozyme) as a test organism with 10-fold serially diluted LLF from normal and convalescent rats.

PBS, nutrient broth and hen's egg white lysozyme (HEWL, Sigma) at concentrations of 1 µg, 10 µg, and 100 µg / ml (w/v) in PBS were used as a positive and negative bactericidal controls.

The lysozyme content of 2.7 ml neat and diluted test samples in PBS normal and convalescent LLF was determined according to the spectrophotometric method described by Wardlaw and McHenery (1982), by measuring their bacteriolysis effect on 0.3 ml of standardized (A_{540nm} = 4.5) *M. luteus* after a shaking incubation for 30 min at 30 °C. The absorbancy at A = 540 nm of the mixtures together with a diluted HEWL in PBS at 4 µg to 0.125 µg / ml as a standard and PBS as a blank was measured, and plotted against the standard lysozyme concentrations (Figure 9).

Long-chain fatty acids (FA) estimation

FA were determined by the colorimetric assay of Duncombe (1963). Test samples (100 µl) were added to 2.4 ml of chloroform in a 10-15 ml volume glass centrifuge tubes with a suitable stopper, to which a 1.25 ml of a copper reagent [containing 9 volumes of aqueous 1M triethanolamine (Sigma), 1 volume of 1 N acetic acid (Fisons) and 10 volumes of 6.45 % (w/v) cupric nitrate (Sigma) in DW] was added.

The tubes were stoppered and mixed for two min using a whirlimixer, after which they were centrifuged for 5 min at 500 rpm at 10 °C. The supernate (upper phase) was removed carefully by pasteur pipette and discarded, 1.5 ml of chloroform (lower phase)

Figure 8. Standard curve for quantitation of protein. Data are the mean of triplicate tests. Error bars (SEM) were too small to project beyond the points.

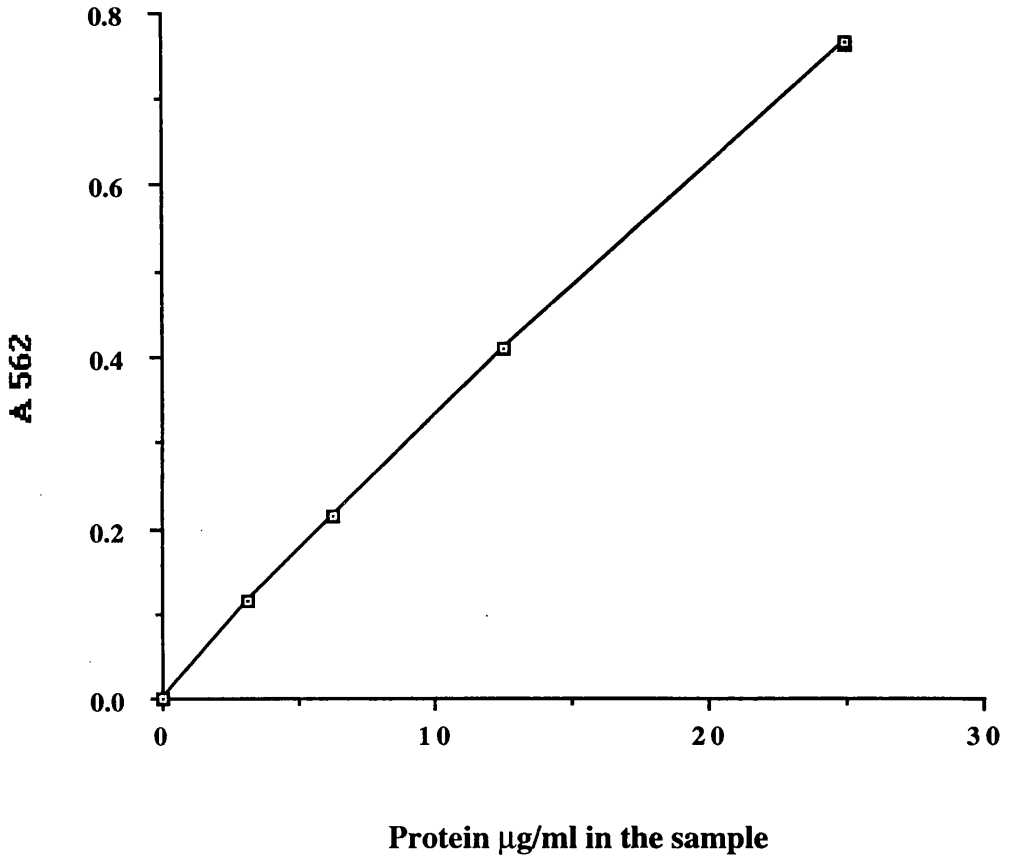
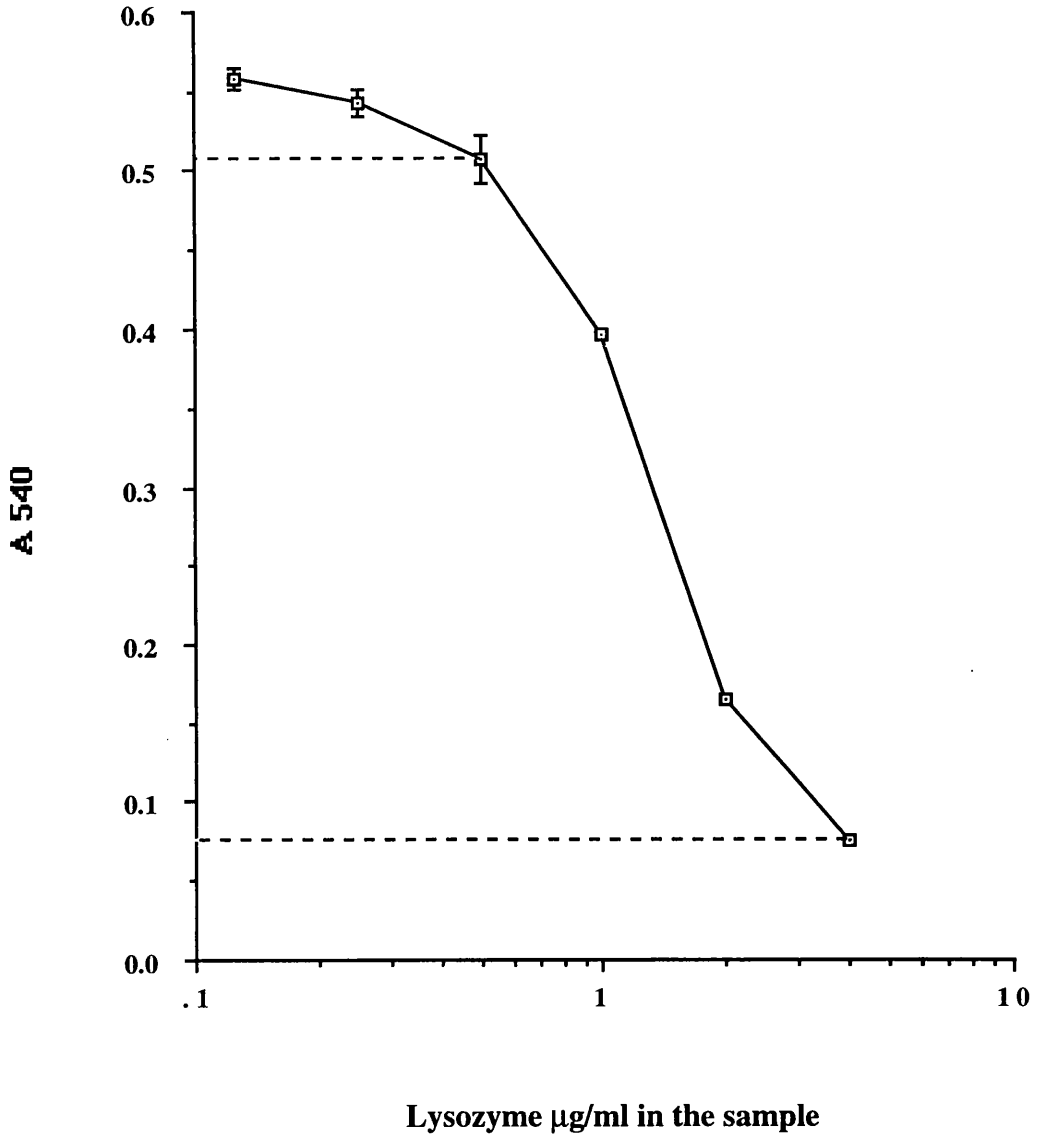


Figure 9. Lysis curve for diluted standard lysozyme. Absorbancy area between the two horizontal dotted lines indicates the region used for assay of unknowns. Data are the mean of duplicate readings and error bars are SEM which when small do not project beyond the points.



was taken cleanly (not to touch the sides of the test tube) with a graduated glass pipette (volac) and delivered into a clean dry test tube. Then 0.25 ml of diethyl dithiocarbamate reagent [containing 0.1 % (w/v) solution of diethyl dithiocarbamic acid (sodium salt, Sigma) in butanol-2 (Riedel-De Haen AG Seelze-Hannover)] was added, after the solutions had been mixed, then the extinction was read at 440 nm in a 1 cm cuvette (lightpath cell) against a blank solution (consisting of 2.5 ml of Analar chloroform and 0.25 ml of diethyl dithiocarbamate reagent) that had been subjected to the same procedure.

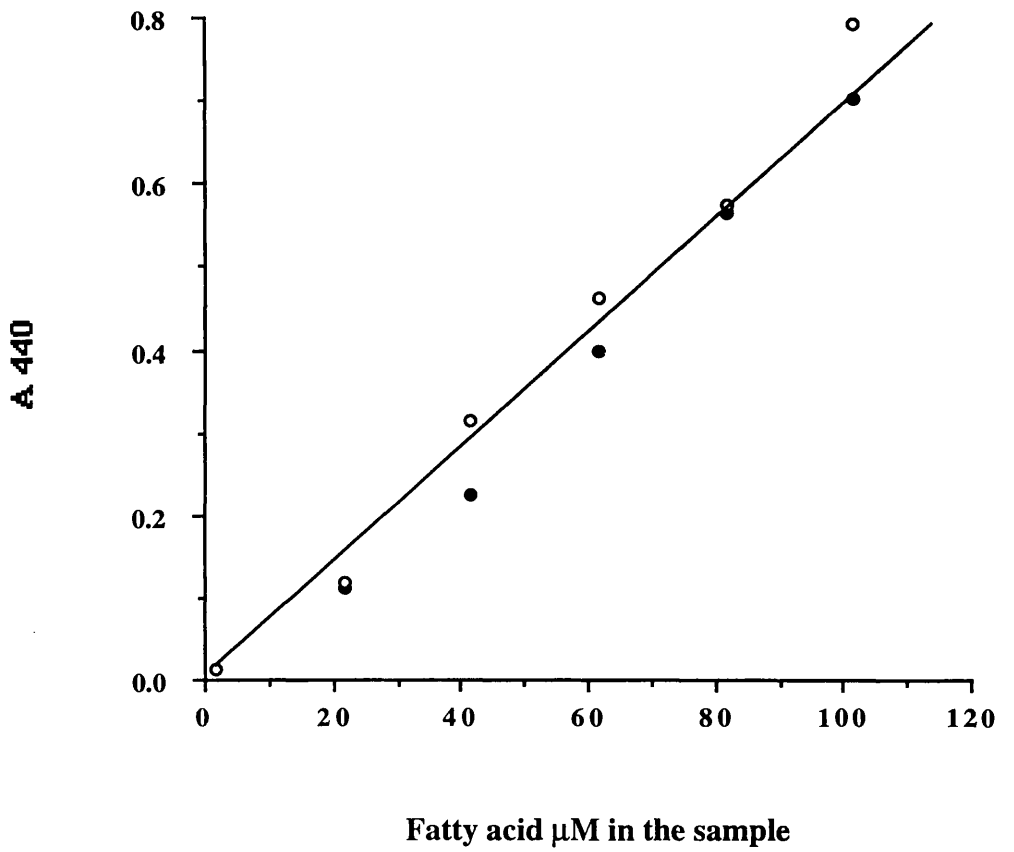
Standard solutions of FA were prepared from a mixture of equal volumes of FA consisting of palmitic, myristic, elaidic, palmitoleic, linoleic, oleic and arachidonic acids. All FA were dissolved in chloroform except palmitic and myristic acids which were dissolved in Analar methanol. The standard FA were made up at a range of 10 - 100 μM and the FA concentration values were obtained from the standard curve (Figure 10).

Antibodies to *B. pertussis*

The detection of antibodies to *B. pertussis* (Bp) sonicated cells, pertussis toxin (PT), and filamentous haemagglutinin (FHA) in normal and convalescent rat LLF and serum was carried out by ELISA assay according to the procedure described by Hall *et al.* 1994. Each well of 96-well U-shape microtitre plate (Nunc Maxisorp, Life Sciences, Paisley) was coated with 0.1 ml of antigen preparation (Appendix 3) diluted in ELISA coating buffer containing Na_2CO_3 1.59 g and $NaHCO_3$ 2.92 g dissolved in 1 litre DW, pH adjusted to 9.6, stored at 4 °C.

B. pertussis whole-cell sonicate stock suspensions were diluted 1 in 1000, FHA antigen was used at 2 μg of protein/ ml, and PT and fetuin (Sigma) used as a sandwich ELISA system at 1 μg /ml. The coated plates were incubated overnight in a moist box at 4 °C, then washed three times with washing buffer (WB) containing NaCl 8g, KH_2PO_4 0.2 g, Na_2HPO_4 1.15 g, and KCl 0.2 g dissolved in 1 litre DW, pH adjusted at 7.4, and Tween 20 (Sigma) added to WB to a final concentration of 0.05 % (v/v).

Figure 10. Standard curve for quantitation of long-chain fatty acids based on duplicate readings.



The plates were blocked with 0.1 ml of 2 % (w/v) bovine serum albumin (Sigma) in WB for 1 h at 37 °C, except in the ELISA for PT in which 0.1 ml of PT 10 μg protein/ml in WB was applied. The plates were washed three times with WB, then 0.1 ml of neat (LLF only) and serially diluted at 10 fold dilution (LLF, serum, and controls at 1:10,1:100 and 1:1000) test fluids in WB were added to duplicate wells.

The plates were incubated for 1h at 37°C in a moist condition, then the plates were washed three times with WB, and 0.1 ml of horse radish peroxidase (HRP)-conjugated goat anti-rat IgG (Sigma) diluted 1 in 5000 in WB, was added to all wells and plates were incubated for 1h at 37 °C in a moist condition, then plates were washed three times with WB. O-phenylenediamine substrate (0.1 ml), which consisted of OPD (Sigma) (34 mg/ ml) and 20 μl of 30 % hydrogen peroxide (Sigma) in 100 ml of citrate-phosphate buffer, pH 5.0 consisted of 51 ml of 0.2 M (35.6 g/l) disodium phosphate and 49 ml of 0.1 M (21.01 g/l) citric acid, was added to all wells.

After placing the plate in the dark for 20 min, the reaction was stopped by adding 50 μl 12.5 % (v/v) H_2SO_4 (Prolabo) in DW to each well. Then the absorbancy was measured at 492 nm in a Titretek Multiscan MC ELISA reader (Flow Laboratories, Hertfordshire).

A reference serum which was used in the ELISA test as a positive control was also prepared by Hall *et al.* (1994) from a hyperimmune pool from two rats immunised intraperitoneally with 1 ml of *B. pertussis* whole-cell sonicate (equivalent to 2 x 10^9 CFU / ml) followed by two further injections at intervals of 3 weeks and bled out 12 days after the last injection. The readings obtained for the reference preparation run on ELISA for the three different antigens were calculated, and enabled reproducibility to be checked. A pool of normal rat serum was used as a negative control, which together with a reference serum were included on every ELISA plate. The mean absorbancy of two sample readings was calculated and plotted against the $\log_{10}$ dilutions of the sample. The dilution at an end-point of $A_{492nm} = 0.5$ was taken as the titre of the test fluid.

Preparation of ELISA antigen and ELISA test for total IgE in LLF fluids were kindly done by E. Hall.

Complement detection

Normal and convalescent rat LLF and serum were tested for bactericidal activity (BA) mediated by complement (Wardlaw, 1962) towards *E. coli* strain Lilly (sensitive to complement) at a final bacterial inoculum of approximately 4700 CFU/ ml. Neat and diluted (1/16 in PBS) guinea pig (GP) serum was taken as a source of complement, nutrient broth and PBS were used as controls. Also inactivated (for 30 min at 56 °C), neat or diluted GP serum was included as a control.

Treatment of LLF

LLF and its supernate and surfactant fractions were exposed to a variety of different physical and chemical treatments in order to characterize the substance responsible for their BA. When dilutions of LLF or its fractions were tested, the end points were taken at 50 % and 90 % reduction of luminescence or viable count, compared with controls. Respectively these end points were designated lum-50 and lum-90, and VC-50 and VC-90.

Physical treatments

Freezing and thawing: Supernate (SN) and surfactant (SUR) fractions of LLF were exposed to freezing and thawing once daily for 10 days by removing them from - 70 °C to room temperature for thawing, after which, they were returned to the -70 °C freezer.

***Dialysis*:** SUR, SN, and PBS were dialysed through a size 1 dialysis tubing (6.3 mm diameter, Medicell International Ltd) which was boiled in DW for 10 min, and then kept in 10 % (v/v) acetic acid in DW, ready for use when required, when it was washed with DW. Then 1 ml of test and control fluids were inserted and the tubing tied at both ends and dialysed against 500 ml of PBS in a beaker with magnetic stirring overnight at 4 °C.

***Ultrafiltration*:** LLF fluids (2 ml) were placed in a microsep centrifugal concentrator (Filtron, Flowgen) with a pore size filter of 30 kDa. This was then placed in a rotor (Sorval SS-34 Rotor) of a fixed angle centrifuge (Sorval RC-5B superspeed refrigerated centrifuge,

DUPONT) which was run for 60 min at 9000 rpm at 20 °C, after which, the filtrate, retentate fluids together with untreated samples were examined on the same day of treatment.

Heat: Test fluids were heated for 30 min at 56 °C in a waterbath or in boiling water for either 5 or 15 min.

Washing in PBS : 1 ml of SUR was placed in a washed, sterile microfuge tube, centrifuged (Biofuge 28 RS, Heraeus SEPATECH) for 20 min at 17000 rpm at 4° C. After which the supernate was discarded and the pellet reconstituted with PBS to its original volume and centrifuged again as before. One and four times washed SUR was made.

Chemical and biochemical agents

pH: SUR, SN and PBS (as a control) were treated with NaOH and HCl (Fisons) at a final concentration of 0.1M of acid or alkali, which then incubated overnight at 4 °C. Then, the pH of test fluids and control was neutralized to approximately pH 7 (detected by Whatman pH indicator paper).

Bentonite: Bentonite (Sigma) treatment was performed according to La Force and Boose (1981). Stock bentonite solution was prepared according to Bloch and Bunim (1959). All glass bottles were washed with DW to ensure that all traces of detergents (used in cleaning glassware) were removed. The suspension of 0.5 g bentonite (Sigma) in 100 ml of DW was homogenised with a Silverson mixer-emulsifier (Silverson Ltd) for 1 min, the homogenising process was repeated after 5 min for another 1 min. The suspension was transferred to a 500-ml washed glass bottle, DW was added to a final volume of 500 ml, shaked and allowed to settle for one hour. Then 120 ml of supernate was distributed into six 30-ml volume thick-walled glass universals, which were centrifuged for 15 min at 1300 rpm. The pellet was discarded, and the supernate was recentrifuged for another 15 min at 1600 rpm. Then the supernate was discarded and the pellet was resuspended to its original

volume in DW, homogenized for 1 min, and kept at room temperature as stock bentonite suspension ready for use and stable as long as six month without losing its adsorptive properties.

Stock bentonite suspension (1 ml) was poured into a tared 1.5 ml volume microfuge tube, centrifuged (Biofuge 13, Heraeus Sepatech) for 10 min at 2300 rpm. The supernate was discarded and the pellet was weighed together with the microfuge tube to know the actual weight of bentonite pellet (found to be approximately 9.9 mg). 900 µl of test fluid was added to the bentonite and the mixture rotated for 30 min at 37 °C. The microfuge tube was then centrifuged for 10 min at 2300 rpm, afterwhich the absorbed supernate together with untreated test fluids were examined.

Charcoal: Activated charcoal (Sigma) was reactivated (Pollock, 1947) as follows: it was made up in a 1 % (w/v) suspension in 10 ml PBS, and centrifuged (Megafuge 1, Heraeus Sepatech) for 10 min at 2000 rpm. Supernate was discarded and the pellet was resuspended and treated twice by boiling in 10 ml of 12 % (v/v) HCl in DW; then the mixture was centrifuged for 10 min at 2000 rpm, the supernate was discarded and the pellet resuspended again in 10 ml PBS, washed in PBS for three times, and the pH was checked and found to be approximately 7.

To a tared microfuge tube, 1 ml of charcoal suspension was poured, centrifuged for 10 min at 2300 rpm, the supernate discarded and pellet together with the microfuge tube was weighed (net weight of charcoal pellet found to be approximately 15 mg), and 0.9 ml of test fluid was added to the pellet. The mixture was rotated for 30 min at 37 °C, then centrifuged for 10 min at 2300 rpm and the test fluid removed.

Serum: Heated (for 30 min 56 °C) normal rat serum was added to the test fluids at a final serum concentration of 0.05 % in the test mixture. This was then incubated for 30 min at 37 °C.

Bovine serum albumin: 1 % (w/v) bovine serum albumin (BSA, Sigma) in DW was added to test fluids at a final concentration of 1 mg/ml, after which, the mixture were incubated for 30 min at 37 °C.

Starch: 1 % (w/v) starch (BDH) was dissolved by boiling for 5 min in PBS and added to test fluids at a final concentration of 1 mg/ml, after which, the mixtures were incubated for 30 min at 37 °C.

Methylcellulose: 1 % (w/v) Methylcellulose (Sigma) in PBS was added to the test fluids at a final concentration of 1 mg/ml, after which the mixtures were incubated for 30 min at 37 °C.

Cyclodextrins: 1 % (w/v) of each of the following cyclodextrins: dimethyl-β-cyclodextrin (Teijin Ltd, Japan), β-cyclodextrin (Sigma) and α-cyclodextrin (Sigma) in PBS was added to the test fluids at a final concentration of 1 mg /ml. The test mixtures were incubated for 30 min at 37 °C.

Enzymes

***Proteases*:** 1.5 % (w/v) in PBS of different proteases were tested: trypsin, chymotrypsin, subtilisin (Protease) and proteinase K (all from Sigma), were added to test fluids at a final enzyme concentration of 1.9 mg/ml in the test mixture, which was then incubated for 30 min at 37 °C.

The proteolytic activity of proteases was checked by incubating 400 µl of proteases with 1 mg hide powder azure (Sigma) and 400 µl of PBS alone with hide powder as a control for 30 min at 37 °C. Also test fluids were incubated with hide powder to detect if there was any enzyme present in them. At the same time, test fluids were incubated with proteases for 30 min at 37 °C, then the mixture was incubated with hide powder for another 30 min at 37 °C, to detect if there were any enzyme inhibitors in the test fluids.

***Phospholipase (Ph)*:** Both Ph A2 and C (Sigma) were suspended in PBS, then added to the test fluids at a final concentration of 1.1 mg/ml for Ph A2 and 0.26 mg / ml for Ph C in the test mixture, which was incubated for 30 min at 37 °C after which the treated and untreated test fluids were examined.

Lipid extraction

Lipids were extracted from freshly prepared SUR from LLF as described by Bligh and Dyer (1959). The SUR pellets from 3 rats (approximately 12 ml LLF) were resuspended in 0.8 ml of PBS, and 3 ml of a mixture of chloroform and methanol at ratio of 1:2 added. The mixture was dispersed with sonication for 1 x 30 sec at 5-10 % amplitude with the microprobe, then 1 ml of chloroform and 1 ml of DW was added to the mixture with sonication after each addition, after which the mixture was centrifuged for 5 min at 900 rpm at 20 °C. The upper phase was discarded, and the inter phase was extracted again following the same procedure. The lower phases (mainly chloroform) from the two extractions were pooled and poured into a tared 7-ml volume thin-wall glass bijoux (which was placed under a source of nitrogen gas in a waterbath containing warm water), dried with a smooth flow of nitrogen gas. The weight of extracted lipids of each SUR pool (4 pools) was measured and found to be 5.75 ± 0.9 mg/ 3 rats. Each pool of extracted lipids was dissolved in 2 ml of chloroform and stored at -70 °C. Then 0.5 ml of extracted lipids in chloroform was dried as before and resuspended in 1 % CAA by sonication for 3 x 30 sec. For bactericidal tests, 240 µl was mixed with 10 µl of *B. pertussis* 18-323 suspension containing 0.8 x 10^3 CFU followed by incubation and plating out.

Column chromatography

Silicic acid column chromatography was done according to the method of Daniels *et al.* (1994). A mini-glass-column of 100 x 6.6 mm with Teflon type tubing and jointers (Whatman International Ltd) was filled with a slurry of a 0.678 g heat-activated (at 125 °C for 4 h) silicic acid (5030 h Silicic acid 325 mesh, Koch- Light Laboratories. Ltd) in 5 ml

chloroform in a 7-ml volume thin-wall glass bijoux, which was placed in a desiccator (for degassing) under a vacuum pressure of 700 mm Hg for 10 min.

Then, 1.8 ml of the degassed silicic acid slurry was applied with a 1-ml graduated glass pipette to the column, the column was drained until the meniscus was about 1 mm above the resin (which was of 4 cm height), then the column was washed with 30 ml of chlorofom, the column was drained again until the meniscus was 1 mm above the resin. After which 1 ml of extracted lipid in chloroform (containing about 2.9 mg lipid) was applied gently and slowly (by the wall of the column without disturbing the resin bed surface) to the column. The column was drained again until the chloroform just entered the resin layer, then 1 ml of chloroform was added and the column was drained again until all the lipids entered the resin bed.

A dry clean sterile thick wall glass universal (as a reservoir) containing 24 ml of chloroform was placed in a position higher than the column connected to the column through a Teflon tube. The solvent was allowed to flow from the reservoir through the column into a dry clean sterile thick-wall glass universal (collection vessel) at a rate not more than 1 ml / min when nearly all the chloroform entered the resin bed.

The column was eluted respectively with 24 ml of acetone and 24 ml of methanol and the collection vessels were changed after each elution. After which each eluate was dried (until the total volume of elution was reduced to about 3 ml) under a smooth current of nitrogen gas in a waterbath containing warm water; then the 3 ml elution was mixed and transferred into a dry, clean, sterile thin-wall glass bijoux, which was well exposed to nitrogen gas for complete dryness. Each dried lipid was dissolved in 1 ml of its original solvent. After which 0.5 ml of each solvent eluate was dried under nitrogen gas and resuspended by sonication for 3 x 30 sec in 2 ml of 1 % CAA. Of this, 240 µl was mixed with 10 µl of *B. pertussis* 18-323 suspension containing 1.1 x 10^3 CFU for BA.

Electron Microscope (EM) Examination

Sample preparation

The component of rat lung SUR, which was fractionated from normal rat LLF was stored overnight at 4 °C. Then 1 ml of prepared SUR was centrifuged for 20 min at 17000 rpm at 4 °C, after which the deposit was examined by transmission electron microscopy (TEM).

B. pertussis 18-323 A6 was cultured on BG-medium agar for 3 days, subcultured for 24 h, suspended in 1 % CAA. The absorbancy was adjusted at either A_{540nm} = 0.45 or 0.9. For *B. pertussis* lux, the bacterial suspension in 1 % CAA was prepared from a 2 days-old lawn plate culture, then the absorbancy was adjusted as with *B. pertussis* 18-323 A6.

Either standardized *B. pertussis* strain 18-323 A6 or lux suspension (200 µl) was added to 400 µl of each test fluids consisting of normal rat SUR, SN, serum or convalescent rat serum, together with PBS and CL-medium as a test control. At time-zero and after 1h incubation at 37 °C, the test samples were then centrifuged for 15 min at 10000 rpm .

Transmission EM (TEM)

The material was fixed in 3 % glutaraldehyde (in 0.2 M phosphate buffer) for 1 h. It was rinsed in 0.2 M phosphate buffer three times for 5 min, and post-fixed in 0.2 % osmium tetroxide for 1 h. The material was then stained in 0.5 uranyl acetate for 1 h, rinsed in DW twice for 5 min, and then embedded in agar, and cut into small pieces for further processing.

The pieces of agar containing the material was dehydrated through a graded ethanol series and embedded in spur resin; sections were cut with a diamond knife on a LKB III microtome at a thickness of 60 nm (silver coloured sections). The sections were picked up on copper grids (300 mesh), and stained in 2 % uranyl acetate (Reynolds, 1963) and lead

citrate (Gibbons and Grimstone, 1960) for 5 min in each. The material was examined on a Zeiss 902 microscope.

RESULTS

-- DEVELOPMENT OF AN EXPERIMENTAL PROTOCOL

Preliminary Experiments

It was not known at the start of this investigation whether lung lavage fluid (LLF) or serum from normal rats would have bactericidal activity (BA) towards *B. pertussis* 18-323. Nor was there information on whether these body fluids when taken from animals that were convalescent from *B. pertussis* pulmonary infection might have heightened activity. A simple experiment was therefore done to test samples of rat LLF and serum, both normal and convalescent, for ability to kill the test strain of *B. pertussis.* As control fluids, cyclodextrin liquid (CL) medium and phosphate buffered saline (PBS) were used. CL medium was chosen because it supports growth of *B. pertussis* from small inocula (Imaizumi *et al.*, 1983), and PBS because it was the fluid used to wash out the rat tracheobronchial tree in order to obtain LLF. The test dose of *B. pertussis* was prepared according to the method of Porter *et al.* (1991) so as to allow direct plating of 20 µl aliquots from the bactericidal test mixtures without further dilution. This was achieved by adjusting a fresh suspension of the bacteria to A_{540nm} = 0.45, followed by a 1/2000 dilution and adding 20 µl to 480 µl of test fluid in Bijoux bottles.

The mixtures were incubated with shaking at 110 rpm at 37°C and 20 µl samples removed at intervals and plated on Bordet-Gengou (BG) medium to determine the viable count. Colonies were counted after 5 days incubation at 37°C. The results of this preliminary experiment are presented in Table 18 and show the following:

1) Convalescent rat serum was the most highly bactericidal of all the fluids tested and gave a viable count of zero in the short time needed to plate out the time zero samples.

2) Next in bactericidal potency were normal rat serum and the normal and convalescent LLF, all of which had killed the inoculum by 120 min.

3) Survival for up to 120 min was satisfactory in both PBS and CL medium, although the latter had an unexplained high viable count at time zero.

4) At the longer incubation times, the bacteria in PBS eventually died out (between 240 and 1440 min) while in CL medium they maintained viability but did not grow. Exactly the same

Table 18. Comparison of bactericidal activity of normal (N) and convalescent (C) rat LLF and serum (S) towards *B. pertussis* 18-323. PBS and CL medium were used as non-bactericidal controls.

Test fluid	Rat batch*	No. of CFU / 20 µl after exposure at 37°C for (min)				
		0	120	240	1440	2880
NLLF	A1	28	0	0	0	nt
CLLF	A2	18	0	0	0	nt
NRS	A1	21	0	0	0	nt
CRS	A2	0	0	0	0	nt
PBS	-	26	24	6	0	0
CL	-	47	26	19	57	44

*For further details on normal fluids see Appendix 9, and for convalescent fluids, Appendix 11.

nt = not tested

experiment was repeated about one week later but with LLF and serum from a different batch of rats. The results (Table 19) were essentially as before except that the CL medium on this occasion permitted actual growth of the *B. pertussis*. The difference between this finding and that in Table 18 where the bacteria merely retained their initial viable count, caused concern. The same batch of CL medium base and CL medium supplement were used on both occasions and the protocol for preparing the inoculum was not varied. Inadequately cleaned glassware was considered a possible explanation and was investigated subsequently as reported below.

Bacterial Suspension, Control Fluids and Test Vessels

Before presenting the detailed results of a large number of tests on the BA of rat LLF, it is convenient to summarize the accumulated information on protocol development and observations on the control fluids. The next few pages therefore describe a variety of control studies during the approximately 36 months of the main experimental work. These control studies comprised: relationship between CFU/ml of the *B. pertussis* suspension and its method of preparation and initial absorbancy; survival of the bacteria in PBS at 37°C; survival and growth of the bacteria in CAA and in CL medium; effect of different containers and effect of different volumes for bactericidal tests; and statistical analysis of the consistency of duplicate counts. The large number of data collected in the above are detailed in Appendices 5 and 6, and presented here as summary charts.

Bordetella pertussis test suspension

During these investigations of the BA of rat LLF, a large number of control tests were done with CL medium to monitor the growth of *B. pertussis* from the small inocula that were used. Altogether 118 such tests were done in which time-zero counts were recorded, from CL medium as well as from the other fluids. In early tests, difficulty was experienced in having a known and reproducible number of viable *B. pertussis* at time zero. Thus the bacterial suspension adjusted to $A_{540nm} = 0.45$ and then diluted by a fixed amount, yielded between as low as 15 and as high as 250 CFU when plated at time zero.

Table 19. A second experiment on the bactericidal activity of normal (N) and convalescent (C) LLF and serum (S) towards *B. pertussis* 18-323. PBS and CL medium were used as non-bactericidal controls.

Test fluid	Rat batch*	No of CFU / 20 µl after exposure at 37°C for (min)				
		0	30	120	1440	2880
NLLF	A2	14	1	0	nt	nt
CLLF	A5	7	0	0	nt	nt
NRS	A2	10	0	0	nt	nt
CRS	A5	0	0	0	nt	nt
PBS	-	13	10	6	0	0
CL	-	14	13	11	185	228

*For further details on normal fluids see Appendix 9, and for convalescent fluids, Appendix 11.

nt = not tested

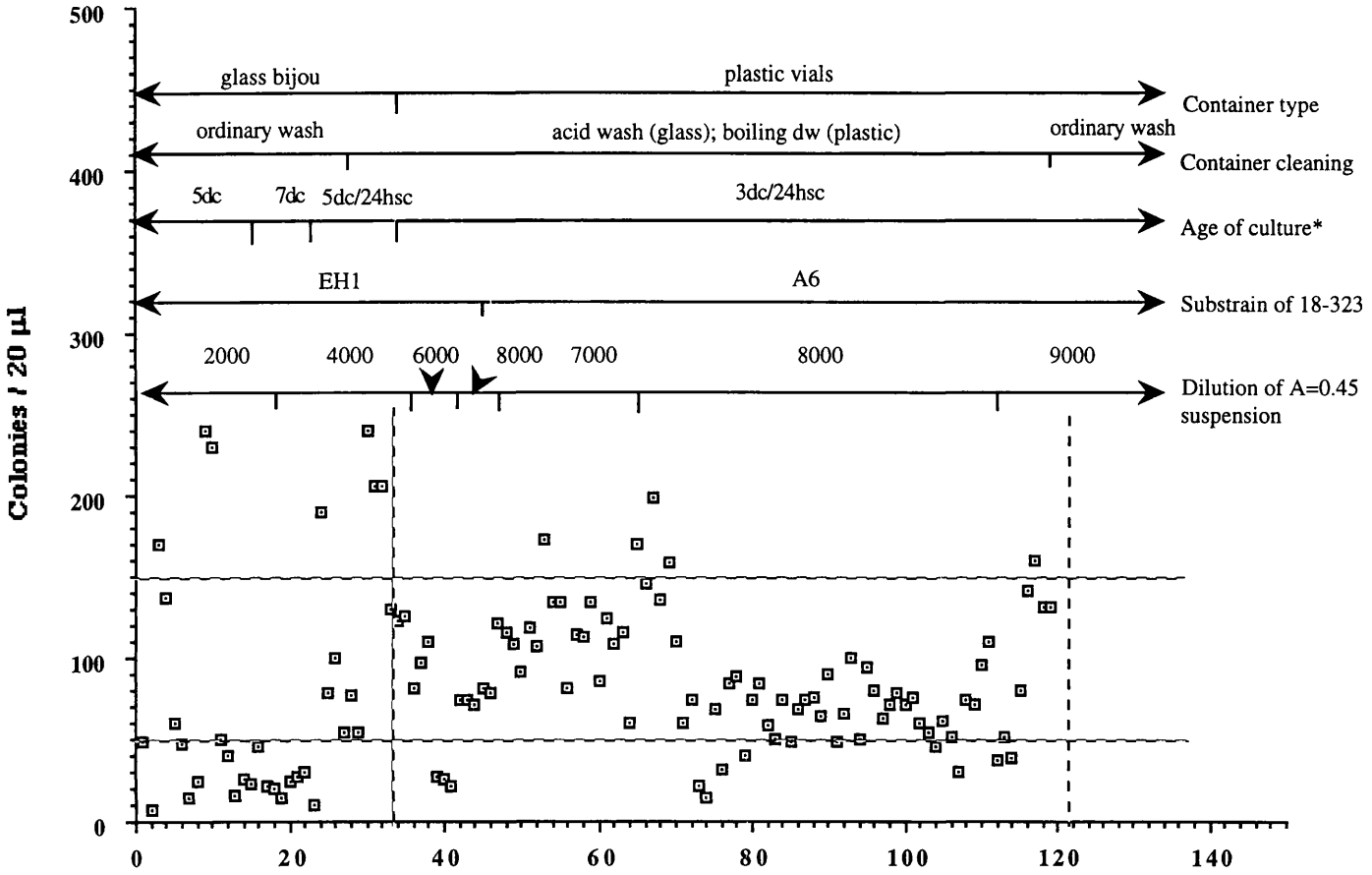
To get better standardization, different culture protocols for the test organisms and different master dilutions were used in the early months of this work. Also, around Experiment Ordinal No. 45, the substrain of *B. pertussis* 18-323 was changed from EH1 to A6.

Summary chart: A summary of these studies in the form of a chart of time-zero viable counts of *B. pertussis* 18-323 from CL medium is presented in Figure 11. It shows that the relatively high variability in time-zero counts in about the first 45 experiments was gradually reduced and brought within closer limits. The top section of the chart summarizes the various changes in experimental protocol that were introduced. Thus at Experiment Ordinal No. 34, the vessels for bactericidal test were changed from glass Bijou to 2-ml plastic vials. Prior to this, at Experiment Ordinal No. 27, extra washing of all glassware (Bijou) and plastic ware was introduced. The Bijous, used for making dilutions etc, were washed in concentrated nitric acid and throughly rinsed in distilled water, while the plastic ware (tips, bijou caps, and plastic vials and caps) were boiled in distilled water. After Experiment Ordinal No. 34 a standard protocol for growing the *B. pertussis* test cultures was adopted and not changed for the rest of the investigation. This consisted in a 3-day primary culture from frozen stock, followed by a heavy inoculum onto a fresh BG plate and incubation for 24 h. This protocol replaced the single-stage 5-day and 7-day cultures and the 2-stage 5 day + 24 h subculture.

Although individually, the above changes may not have had major and reproducible effects, collectively they seemed to improve the consistency of the time-zero counts and to bring most of them into the countable range of 50-150 colonies per plate. Between Experiment Ordinal No. 42 and 104, the master dilution of the A_{540nm} = 0.45 suspension was increased slightly from 1/7000 to 1/9000, in response to rising counts.

Statistical analysis of viable counts: Having developed the above standard conditions, the time-zero counts from Experiment Ordinal No. 74 onwards were summarized in order to obtain an average value of the CFU/ml of *B. pertussis* in a suspension of A_{540nm} = 0.45. The results of 45 such estimates are summarized in the

Figure 11. Summary chart of time-zero viable counts of *B. pertussis* 18-323 from CL medium during approximately three years of experimentation. The upper part of the diagram records the changes in protocol. The two dotted horizontal lines enclose the zone of desired counts. The area between the two dotted vertical lines are the optimum conditions found.



* dc = days of culture;

hsc = hours of subculture

Minitab analysis in Figure 12. The boxplot and histogram present pictorial summaries of the data, indicating a slightly asymmetric distribution around a median of about 70 x 10^8 CFU / ml for an A_{540nm} = 0.45 suspension in a 1 cm cell.

The summary statistics give the more accurate values (x10^8) as :

median	=	71
mean	=	74.01
95 % confidence limits of the mean	=	64.4 , 83.6

Phosphate buffered saline (PBS)

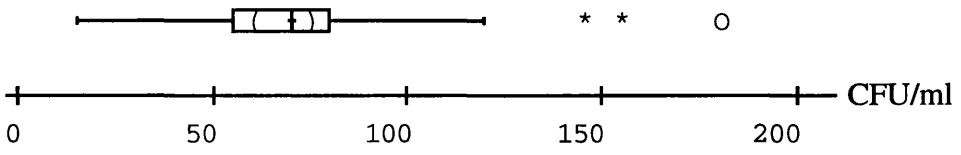
Since PBS was the fluid used to wash out the rat tracheobronchial tree in order to obtain LLF, it was necessary to include PBS as a control fluid in the bactericidal tests. The approximately 800 observations on *B. pertussis* 18-323 survival in PBS during incubation times of 0, 15, 30, 60, 120, and 1440 min are recorded in Appendix 6.

In the early experiments, the PBS was prepared by dissolving 10 tablets (Oxoid) in 1 litre of DW, adjusting to pH 7.2 if necessary, and sterilizing by autoclaving in 10 ml aliquots. However after Experiment Ordinal No. 11, it was decided to prepare the PBS from the individual analytical-grade chemicals and the tablets were no longer used. This change was made to see whether survival of the *B. pertussis* 18-323 during 120 min at 37°C might be improved; minor changes in pH were also introduced, the initial value of 7.2 being adjusted in later experiments to pH 7.4 and finally, pH 7.3.

Summary charts: the previously presented summary chart Figure 11, showed actual colony counts per 20 µl at time zero for each experiment. However to facilitate comparison of results of different experiments, it was decided to express viability as percent survival (Figures 13 and 14). For this purpose the time-zero count from CL medium was taken as 100 % survival. This was done for the first 96 experiments over approximately 36 months, after which the use of CL medium as a control was discontinued (because it was decided to

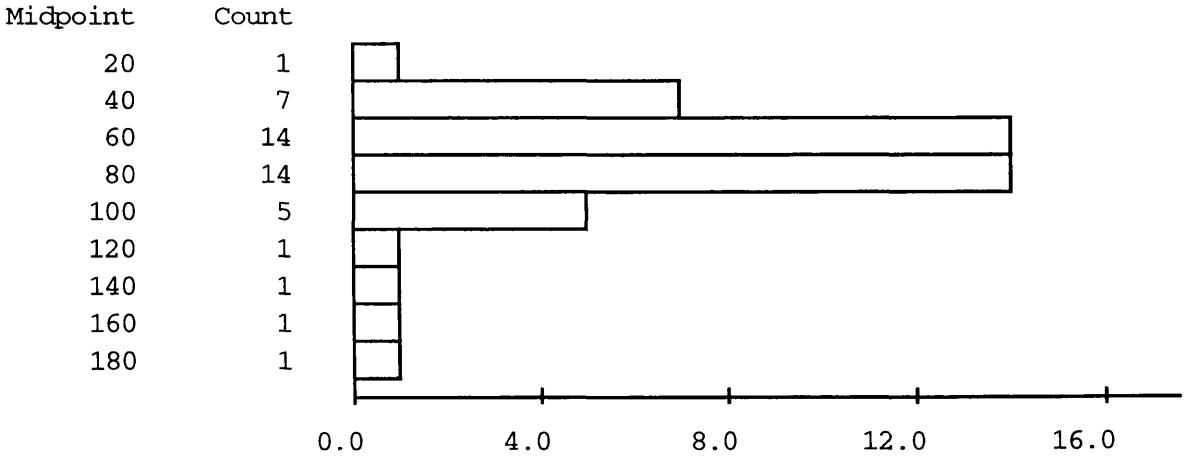
Figure 12. Minitab analysis of 45 observations on the viable count (x 10^8) of *B. pertussis* 18-323 at A $_{540nm}$ = 0.45 in a 1 cm cell.

A. Boxplot



Box	=	Quartiles
Whiskers	=	Range
Vertical line	=	Median
Curved brackets	=	95 % Confidence limits of the median
* * 0	=	Outliers

B. Histogram



C. Summary statistics

N	MEAN	MEDIAN	STD DEV	SEM	95 % CONF LIMTS OF THE MEAN
45	74.0	71.0	31.9	4.8	64.4, 83.6

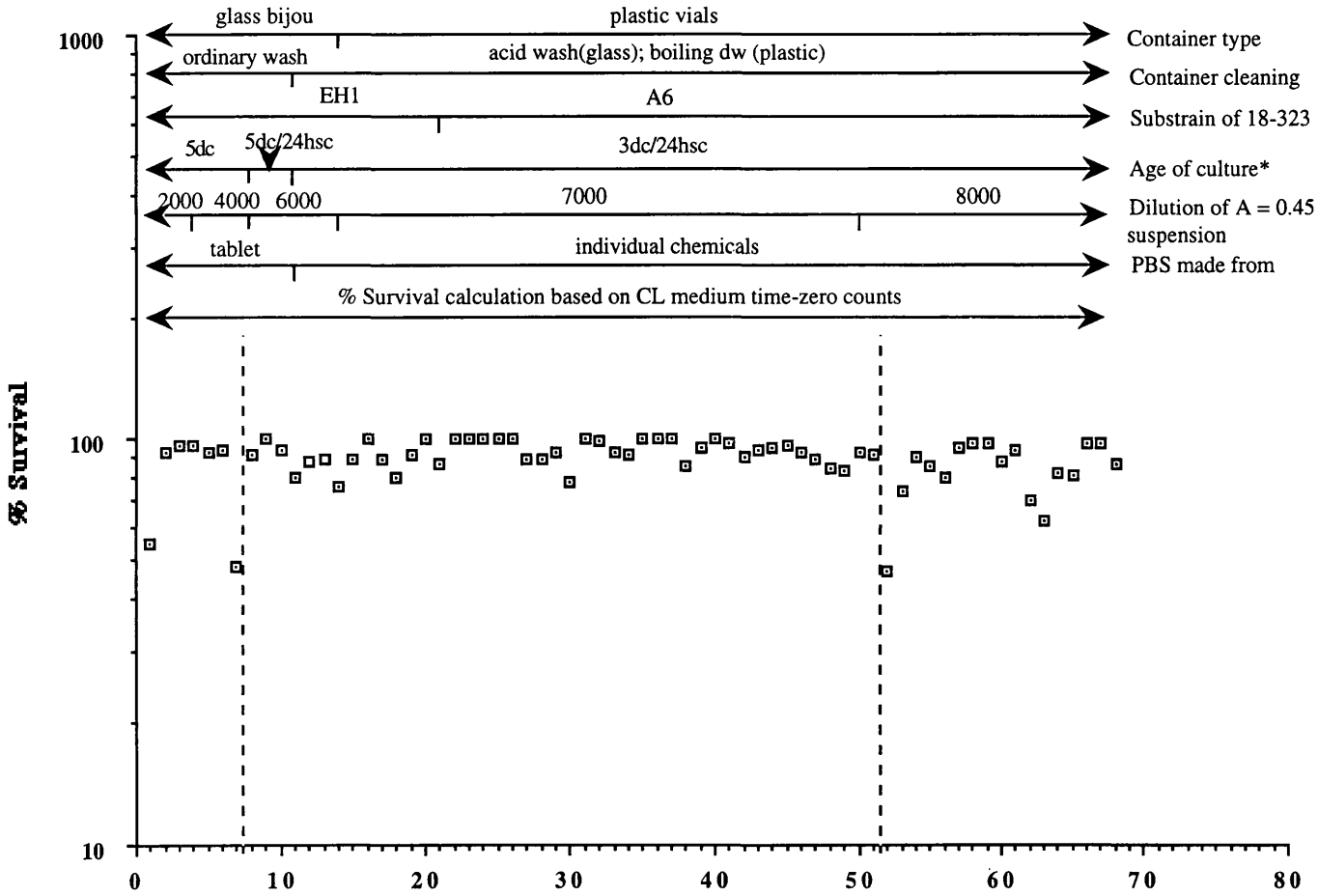
terminate the experiment at incubation time of 120 min). Thereafter, the time-zero count from PBS was taken as indicating 100 % survival. In addition to changes in the method of preparing PBS, there were other alterations in experimental procedure such as those relating to the bacterial suspension and the type of container. Thus Figures 13 and 14 show that none of the experimental manipulations had much consistent effect on the initial bacterial counts in PBS.

Although survival of *B. pertussis* 18-323 after 15, 30, and 60 min at 37°C in PBS was studied in some experiments (see Appendix 6), the standard exposure was 120 min. Many experiments were continued through to 1440 min exposure, but not all of these times were necessarily used in every experiment. These 120 and 1440 min exposures to PBS are summarized in Figures 15 and 16. As with the time-zero summary chart, it is difficult to attribute differences in *B. pertussis* survival to particular changes in protocol. It will be noted that sporadic survival in the region of 5-10 % at 1440 min was sometimes observed.

***Statistical analysis*:** The numerous experiments in which PBS was used as a non-bactericidal control yielded a set of summary statistics of survival of *B. pertussis* 18-323 in this fluid at 37°C (Appendix 6). Figure 17. (A) presents the boxplots of % survival in PBS at each sampling time and show a regular decline in median survival as the time of incubation increased. Although the spread of observations at each time was quite large, as indicated by the width of the boxes and the lengths of the whiskers, the 95 % confidence intervals (of the medians) were mostly quite narrow because of the large number of observations.

At 120 min which eventually was selected as the time for terminating the bactericidal tests, the mean survival in PBS was 56.6 % (n =93) with 95 % confidence limits of 51.3 to 61. 9. The recorded mean percent survival at 1440 min was 1.73 % but this value is not accurate since in many experiments, no viable bacteria were detected. Thus since Minitab does not accept " less than" data, an arbitrary value of 0.3 % survival was inserted to allow statistical evaluation. The lower section B of Figure 17 presents the summary statistics for the percent survival of *B. pertussis* in PBS.

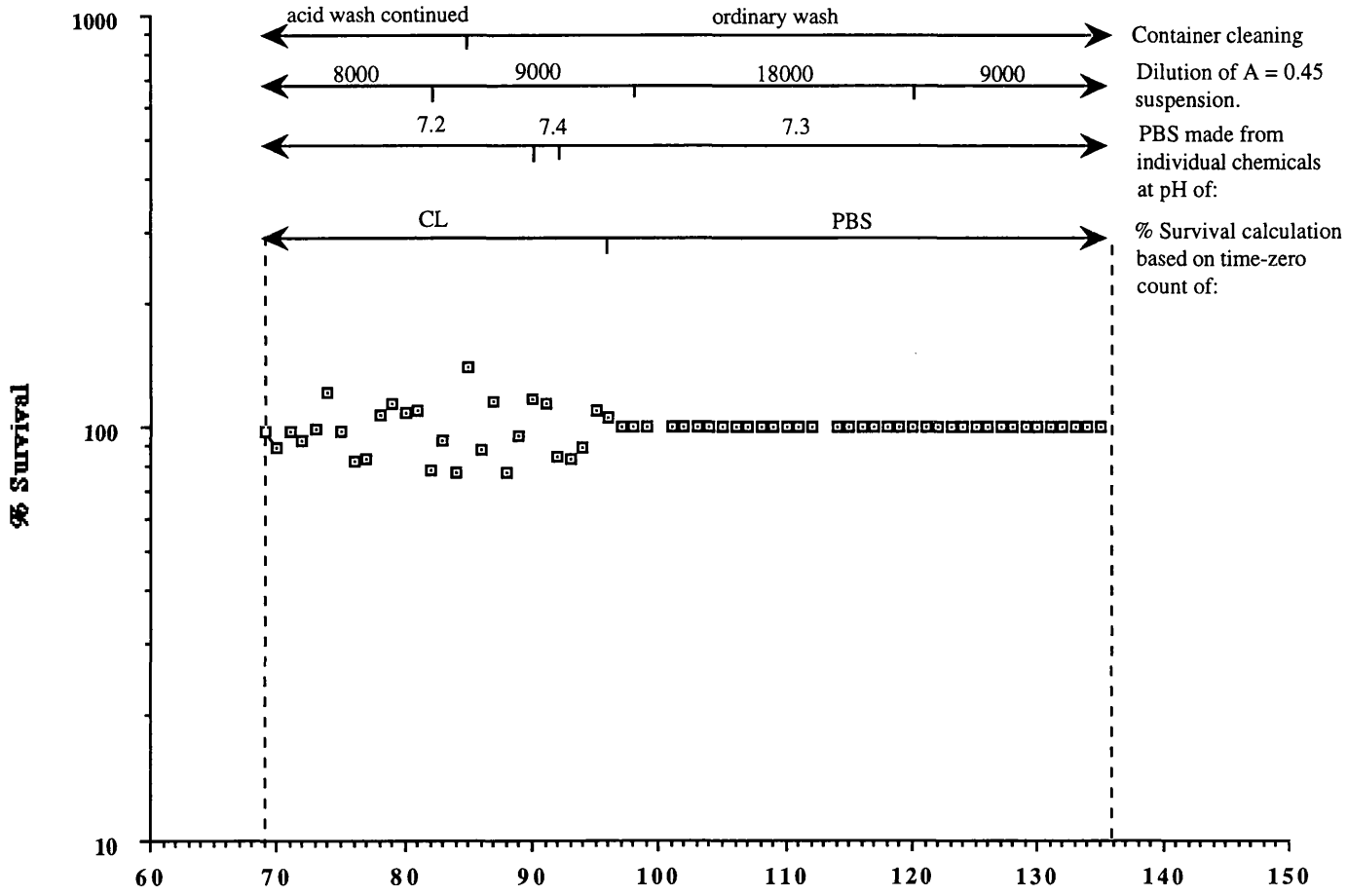
Figure 13. Summary chart of time-zero percent survival (based on time-zero CL counts) of *B. pertussis* 18-323 in PBS during approximately the first two years of experimentation. The upper part of the diagram records the changes in protocol in Experiments Ordinal Number 1- 68. The area between the two dotted vertical lines are the optimum conditions found.



* dc = days of culture;

hsc = hours of subculture

Figure 14. Summary chart of time-zero percent survival of *B. pertussis* 18-323 in PBS during approximately the last year of experimentation. The upper part of the diagram records the changes in protocol. from Experiments Ordinal Numbers 69 - 92 the percent survival was based on time-zero CL counts (as in Figure 13) and from 93 -135 on time-zero PBS counts. The area between the two dotted vertical lines are the optimum conditions found.



* dc = days of culture;

hsc = hours of subculture

Figure 15. Summary chart of percent survival (based on time-zero CL medium counts) at time-120/1440 min of *B. pertussis* 18-323 in PBS during approximately the first two years of experimentation. The upper part of the diagram records the changes in protocol in Experiment Ordinal Numbers 1- 68. The area between the two dotted horizontal lines are the optimum founded conditions.

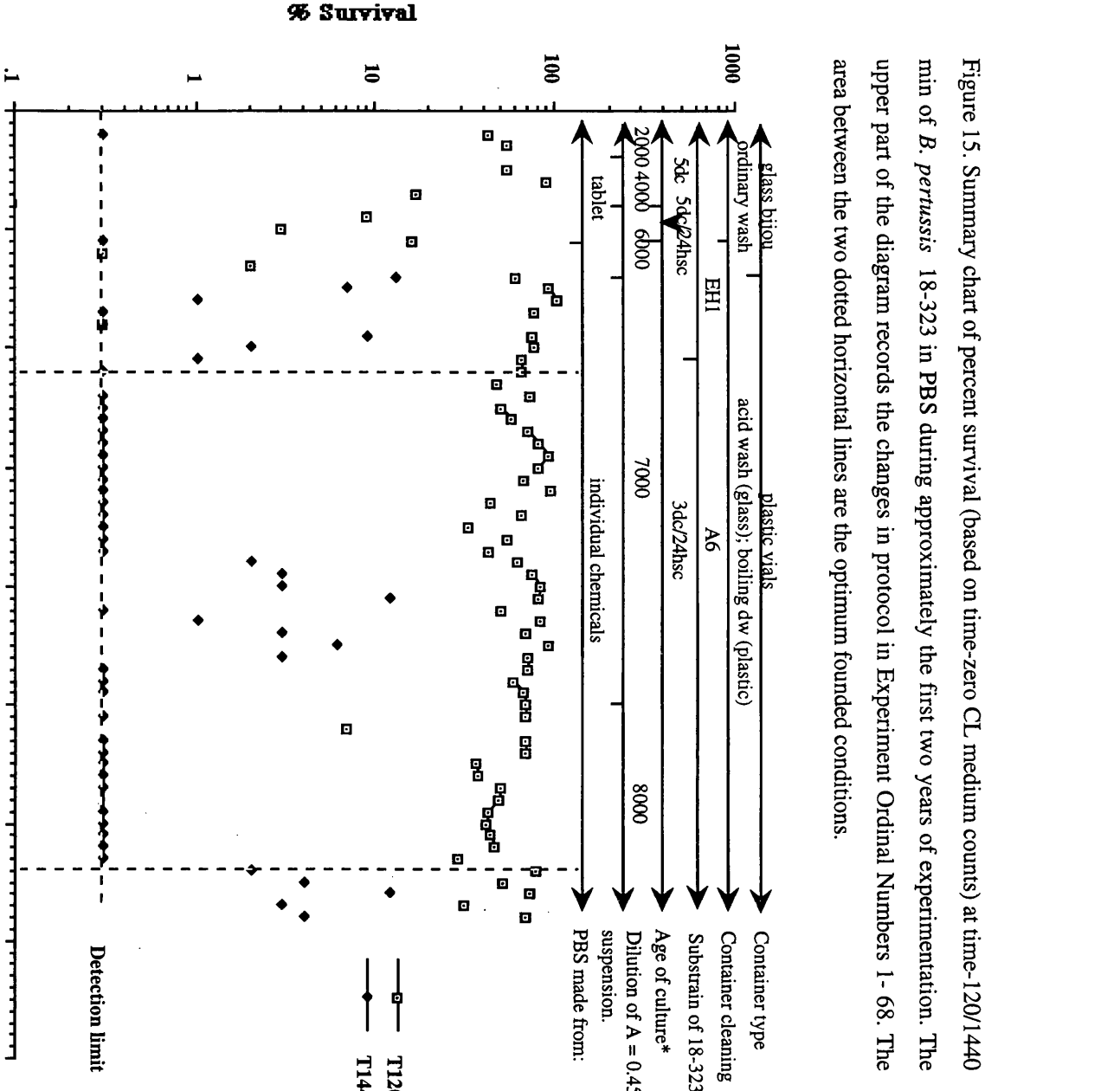


Figure 16. Summary chart of percent survival at time-120/1440 min of *B. pertussis* 18-323 in PBS during approximately the last year of experimentation. The upper part of the diagram records the changes in protocol. In Experiments Ordinal Number from 69 - 92 the percent survival was based on time-zero CL counts (as in Figure 15) and from 93-135 on time-zero PBS counts. The area between the two dotted vertical lines are the optimum conditions found.

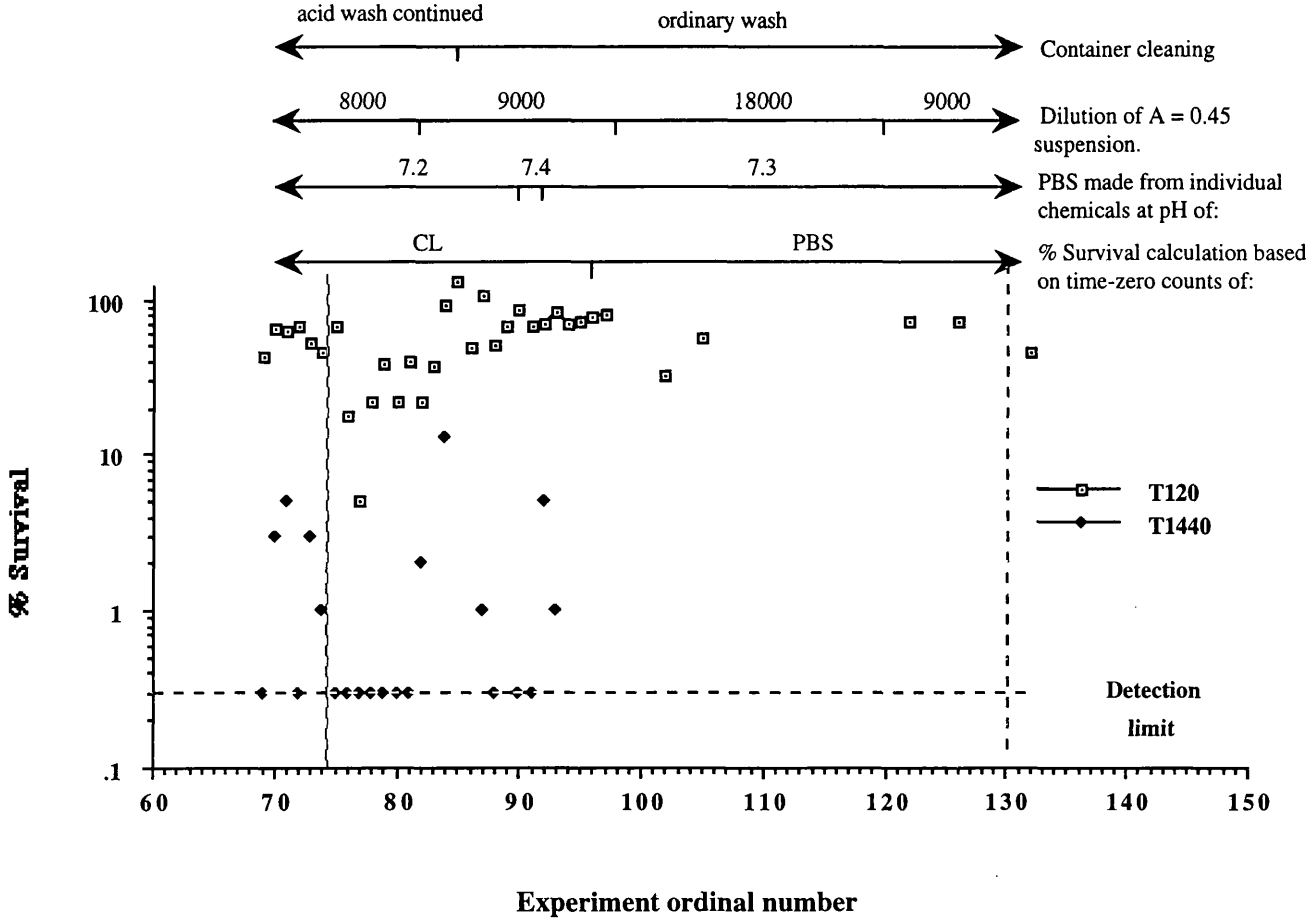
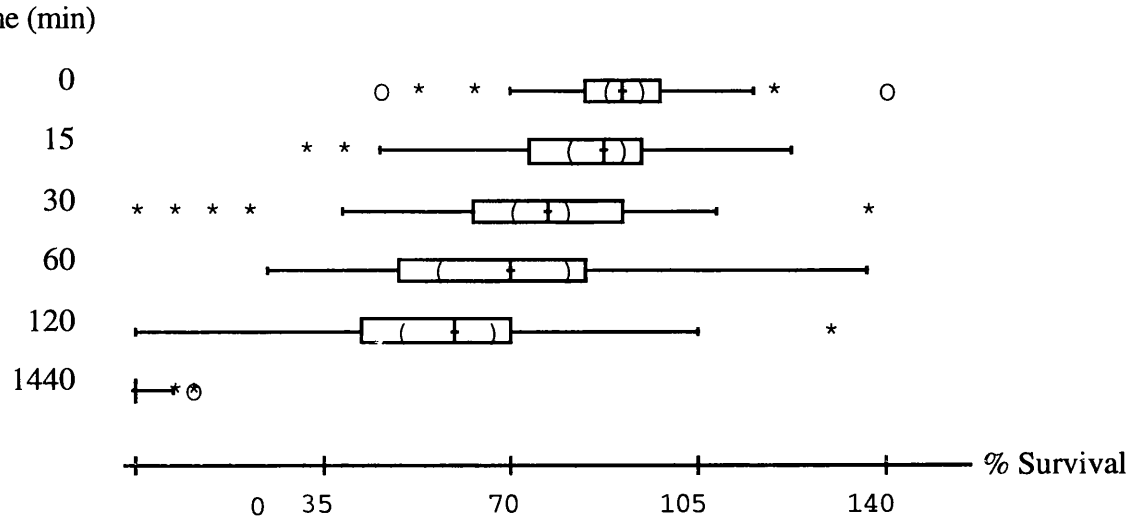


Figure 17: Minitab analysis of percent survival (based on time-zero CL medium counts) of *B. pertussis* 18-323 in PBS during incubation at 37°C for various times.

A). Boxplot



Box = Quartiles
Whiskers = Range
Vertical line = Median
Curved brackets = 95 % Confidence limits of the median
* * 0 = Outliers

B). Summary statistics

TIME (min)	N	MEAN	MEDIAN	STD DEV	SEM	95 % CONF LIMTS OF THE MEAN
0	96	91.7	92.0	13.2	1.4	89.0, 94.4
15	57	83.7	88.0	16.7	2.2	79.3, 88.1
30	85	75.6	78.0	21.6	2.4	70.9, 80.2
60	26	71.3	72.5	27.6	5.4	60.2, 82.5
120	93	56.6	62.0	25.7	2.7	51.3, 61.9
1440	74	1.7	0.0	3.2	0.4	1.0, 2.5

***Quantitative analysis of survival*:** The percent survival data of *B. pertussis* in PBS (Figure 17) are plotted against time in Figure 18. It will be noted that for the first 120 min at 37°C, a strong decrease in percent survival from about 90 to 75 after 30 min was followed by a gradual decrease to about 56 at 120 min.

Casamino acids (CAA)

Casamino acids (CAA) 1 % (w/v) is used as a standard diluent for *B. pertussis* in animal-challenge experiments. It was also used in a majority of my experiments (from Ordinal No. 84, see Appendix 6.) as the suspending medium into which *B. pertussis* 18-323 was scraped from BG plates. It was the diluent for absorbancy standardization and for diluting the standardized suspension prior to its addition to test fluids. In view of the above, CAA was also used periodically as one of the non-bactericidal control fluids, sometimes alone but mostly in parallel with PBS and occasionally alongside CL medium.

Appendix 7 contains the viable count data from incubations of *B. pertussis* 18-323 with CAA for up to 1440 min. These data are recalculated in Appendix 8 as mean % survival, based on the time-zero counts from PBS being taken as 100 %. For the graphic presentation of these results (Figure 19), a straight line connection has been drawn through the time-zero and 30 min points, and the 15 min time has been relatively neglected because there were only 2 observations.

Casamino acids, in its use as a non-bactericidal control fluid, allowed 85% (SEM 3.61, N= 25) survival of *B. pertussis* 18-323 at 60 min, while at the standard incubation time of 120 min, the value was 55.3 % (SEM 11.0, N=7) which is very close to that observed with PBS. In only one out of 5 experiments did *B. pertussis* 18-323 survive for 1440 min in CAA as judged by viable count from 20 µl (Appendix 8). The median % survival is presented as a boxplot and summary statistics in Figure 20. Since for Minitab purposes, almost-confluent plates were arbitrarily taken to have 300 colonies, in all probability the true count was much higher.

Figure 18. Mean (±SEM) percent survival of *B. pertussis* 18-323 during incubation at 37°C in PBS for 120 min. Data are from 96 experiments, the % survival calculation being based on CL time-zero counts.

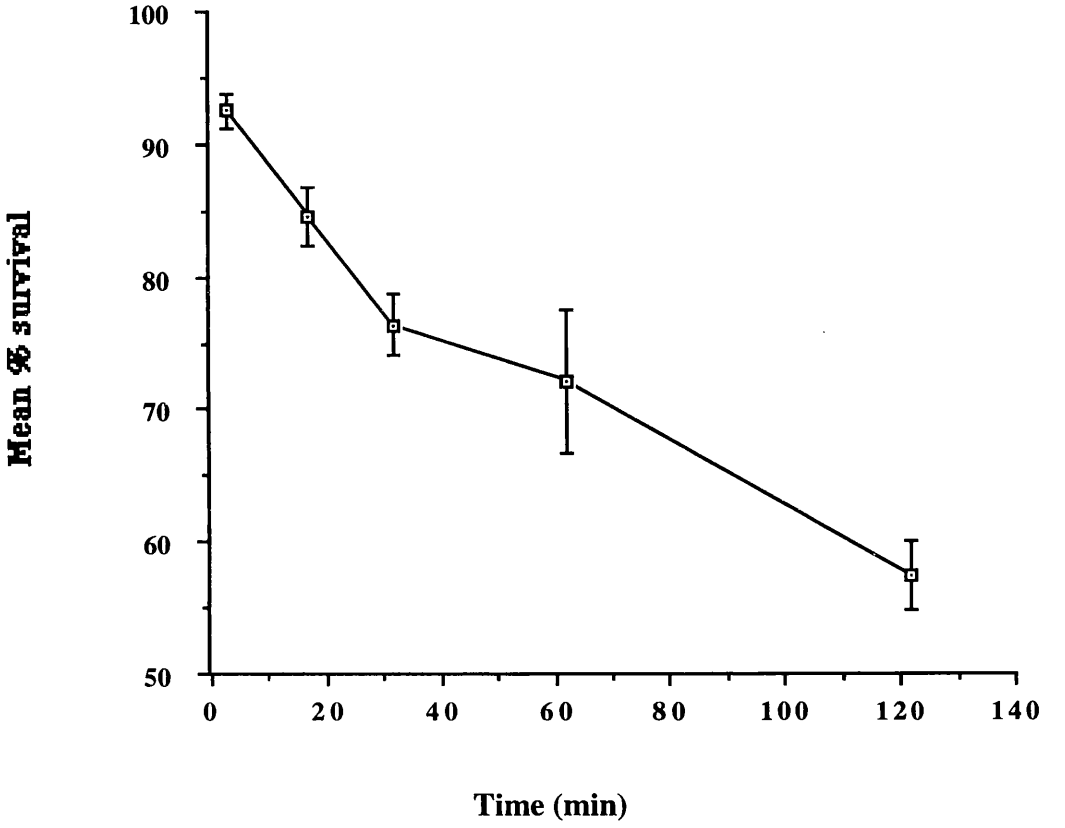


Figure 19. Loss of viability of *B. pertussis* 18-323 during incubation in 1% (w/v) CAA at 37°C. Data are the means (± SEM) of 2 - 25 observations.

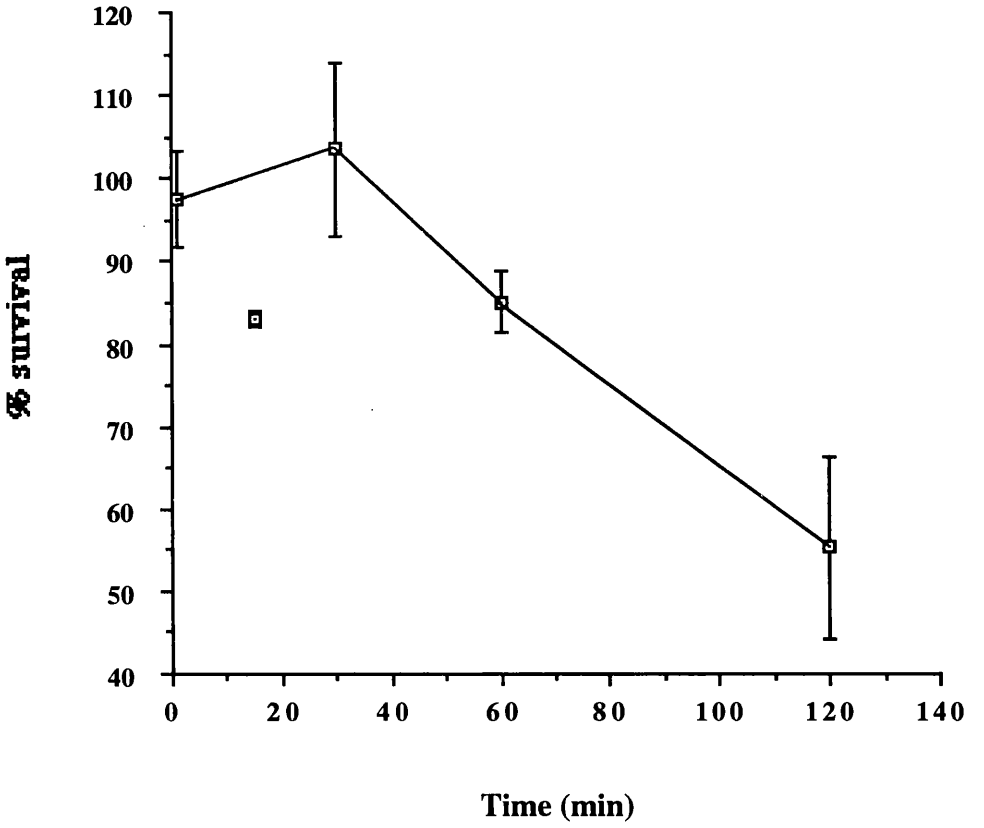
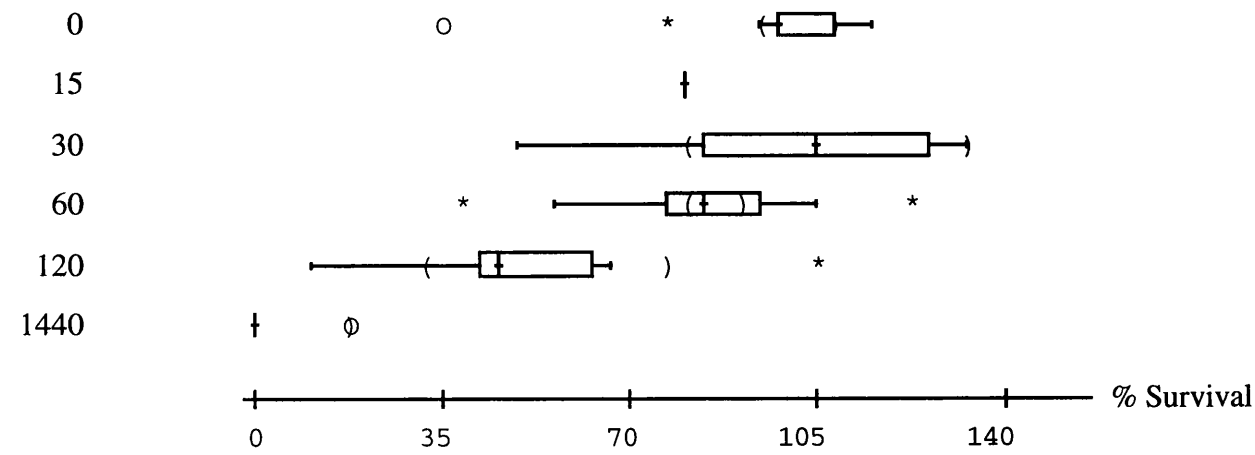


Figure 20. Minitab analysis of percent survival of *B. pertussis* in 1 % CAA during incubation at 37°C for various times. The calculation of % survival was based on PBS time-zero counts.

A). Boxplot



Box	=	Quartiles
Whiskers	=	Range
Vertical line	=	Median
Curved brackets	=	95 % Confidence limits of the median
* * 0	=	Outliers

B). Summary statistics

TIME (min)	N	MEAN	MEDIAN	STD DEV	SEMEAN	95 % CONF LIMTS OF THE MEAN
0	13	97.5	100.0	101.1	5.9	84.6, 110.5
15	2	83.0	83.0	83.0	0.0	83.0, 83.0
30	8	103.5	106.0	103.5	10.4	78.9, 128.1
60	25	85.1	87.0	85.4	3.6	77.6, 92.5
120	7	55.3	47.0	55.3	11.0	28.5, 82.1
1440	5	3.8	0.0	3.8	3.8	-6.8, 14.4

Cyclodextrin liquid (CL) medium

In the present work, CL medium base was prepared, distributed as 10 ml portions in 30 ml glass universals, sterilized, and stored at 4°C. For use, 50 µl of CL supplement was added to make the complete CL medium. The full medium was used as a non-bactericidal control alongside PBS in most of the viable count experiments for approximately the first 2 years of experimentation. The time-zero count in CL medium was used as a base for calculating the % survival for all incubation times and for all test fluids and controls.

***Preliminary experiments*:** In some very early experiments, CL medium as a non-bactericidal control failed to support viability and growth of *B. pertussis* 18-323. It was therefore decided to change the CL medium base by replacing L-glutamic acid sodium salt (BDH), with L-glutamic acid (BDH) and L-glutamic acid (Sigma), These three different batches were tested for their ability to support viability and growth of *B. pertussis* 18-323 in two different experiments (Table 20). In these experiments, it was clearly noticed that the later two batches of CL medium base containing L-glutamic acid from (BDH) and (Sigma) both supported growth and survival of *B. pertussis* 18-323. Also a heavier confluent growth was noticed on a lawn BG plate agar inoculated from CL medium containing L-glutamic acid (Sigma) than the other one (BDH).

After this finding, L-glutamic acid (Sigma) was used in CL medium base for all later experiments. Despite this change in CL medium base, the problem of CL medium not always supporting survival and growth of *B. pertussis* 18-323 still existed. For this reason, further attempts to solve this problem were made. This time, the change was made by adding 50 µl (instead of 100 µl according to Imaizumi *et al.*, 1983a) of CL medium supplement to 10 ml of CL medium base.

The results of two comparative experiments in Table 21 showed that 50 µl of CL supplement was better than 100 µl. From this point onwards, the problem of CL medium as a suitable control fluid was overcome. By all the above-mentioned changes, in both CL medium base and supplement together with other changes concerning type and cleaning of test container, age of bacterial culture, the use of different dilutions and diluent

Table 20. Survival and growth of *B. pertussis* 18-323 in CL medium made with different batches of L-glutamic acid or glutamate. Data of two experiments, each in duplicate.

CL from L-glutamic acid batch*	Mean CFU/20 µl ±SEM after exposure at 37°C for (min)					
	0	15	30	120	1440	2880
A	59±8	50±9	60±9	58±9	240±29	ac
B	50±7	52±8	50±7	59±11	ac	c
C	56±7	51±7	52±12	56±9	ac	c

Batch* A = L-glutamic acid sodium salt (BDH); B = L-glutamic acid (BDH); C = L-glutamic acid (SIGMA); ac = almost confluent, c = confluent

Table 21. Survival and growth of *B. pertussis* 18-323 in CL medium with two different concentrations of CL medium supplement.

Concentration of supplement (µl / 10 ml)	No of CFU/20 µl after exposure at 37°C for (min)						
	0	15	30	60	120	1440	2880
50	52, 30	40, 48	43, 48	43 , 48	51, 36	ac , ac	c , c
100	58 , 36	46 , 53	45 , 40	58 , 36	51 , 41	nt , 52	32 , 20

ac = almost confluent growth, c = confluent growth, nt = not tested.

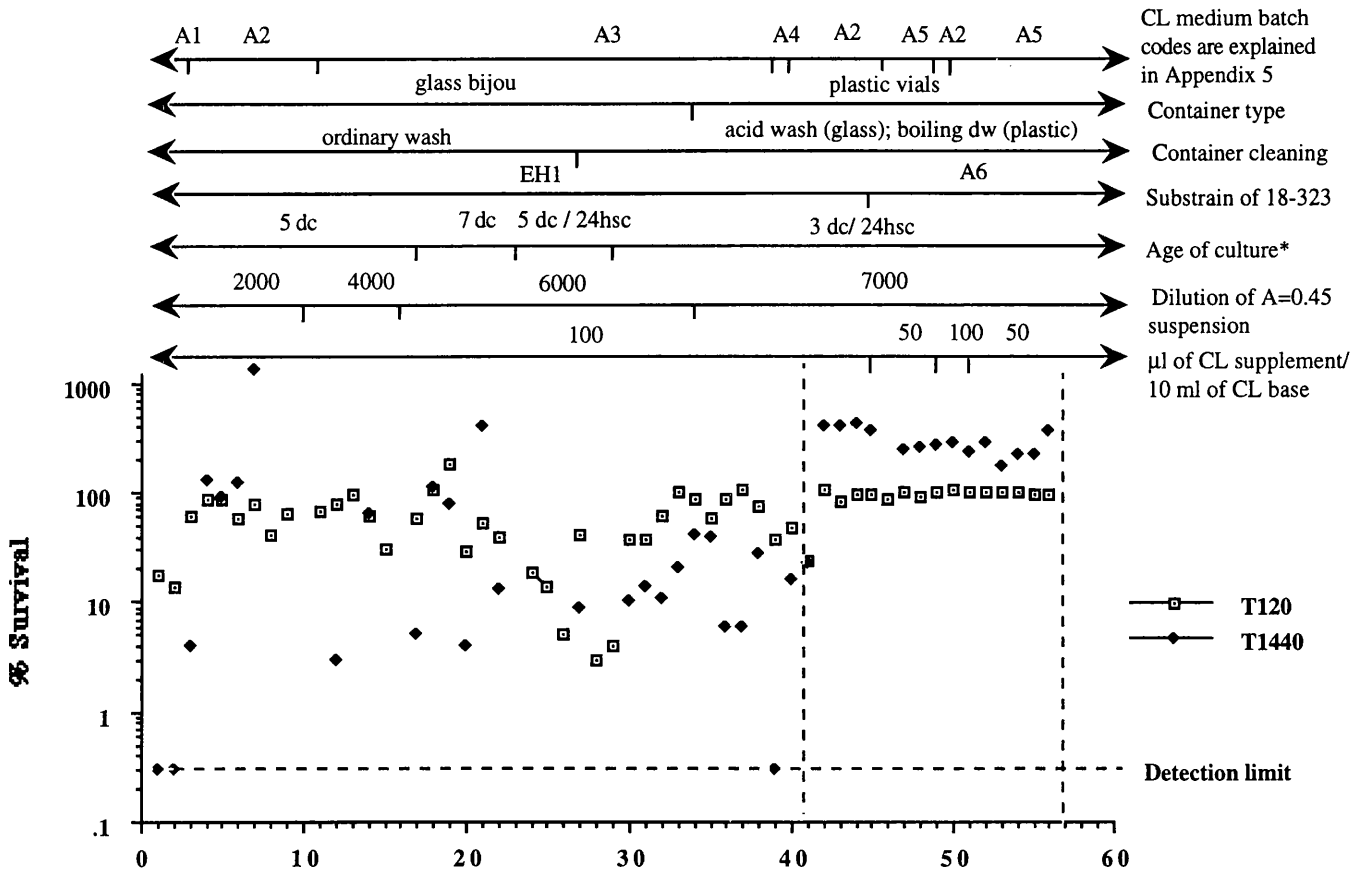
in preparing test suspension, and a different substrain of 18-323 (Appendix 5) CL medium as a non-bactericidal control gave satisfactory results.

***Summary charts*:** The results of 118 experiments where CL medium used as a non-bactericidal control are summarized in Figures 21 and 22 as a percent survival of *B. pertussis* 18-323 after 120 and 1440 min of incubation at 37°C. The arrows on the upper side of the Figures 21 and 22 showed the locations where the different changes were made. From Experiment Ordinal Number 1 up to 41, with a few exceptions, CL medium failed to support the survival and growth of *B. pertussis* 18-323. But, from Experiment Ordinal Number 41 through to 118, CL medium was satisfactory as a non-bactericidal control, by showing no, or very little, reduction in percent survival, and different rates of succesful growth were noticed through a 24 h incubation. This improvment in CL medium action appeared to be due to a combination of changes which were made at an early stage of the experimental work, but not due to one change only.

***Statistical analysis*:** Minitab analysis in Figure 23 of all data of % survival of *B. pertussis* 18-323 in CL medium are summarized as before.

***Survival during incubation*:** The summary data of % survival of *B. pertussis* 18-323 in CL medium from the same 118 experiments is presented in Figure 24 A, B and C. From the figure it is obvious that there was a gradual decrease in mean % survival from 100 % at time zero to about 80 % at 120 min. A 20 % decline mostly occured during the first 41 experiments, during which CL medium was used as a non-bactericidal control before the various problems were overcome. In the later 77 experiments the median survival increased to 95 %.

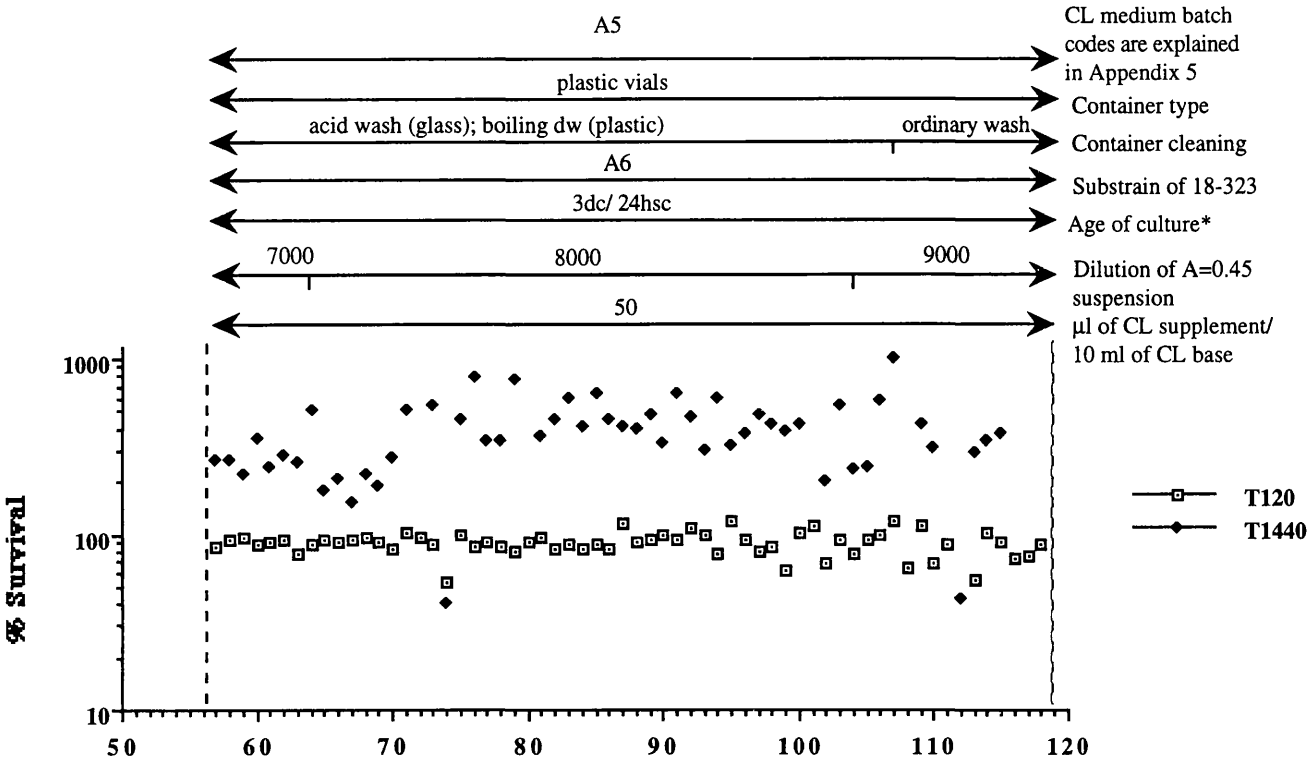
Figure 21. Percent survival (based on time-zero CL medium counts) and growth of *B. pertussis* 18-323 in CL medium after incubation at 37°C for 120 and 1440 min. Data from Experiment Ordinal Numbers 1 - 56. The area between the two dotted vertical lines are the optimum conditions found.



* dc = days of culture;

hsc = hours of subculture

Figure 22. Percent survival (based on time-zero CL medium counts) and growth of *B. pertussis* 18-323 in CL medium after 120 and 1440 min incubation at 37°C. Data from Experiment Ordinal Numbers 57 - 118. The area between the two dotted vertical lines are the optimum conditions found.

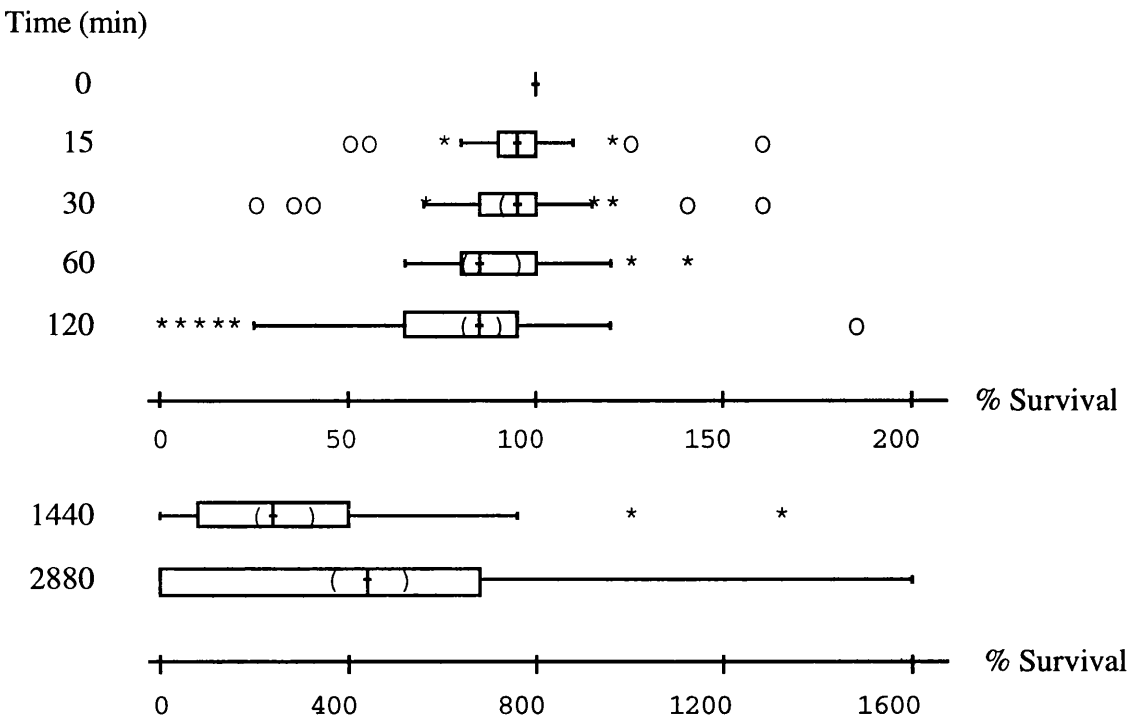


* dc = days of culture;

hsc = hours of subculture

Figure 23: Minitab analysis of percent survival (based on time-zero CL medium counts) of *B. pertussis* 18-323 in CL medium during incubation at 37°C for various times.

A). Boxplot

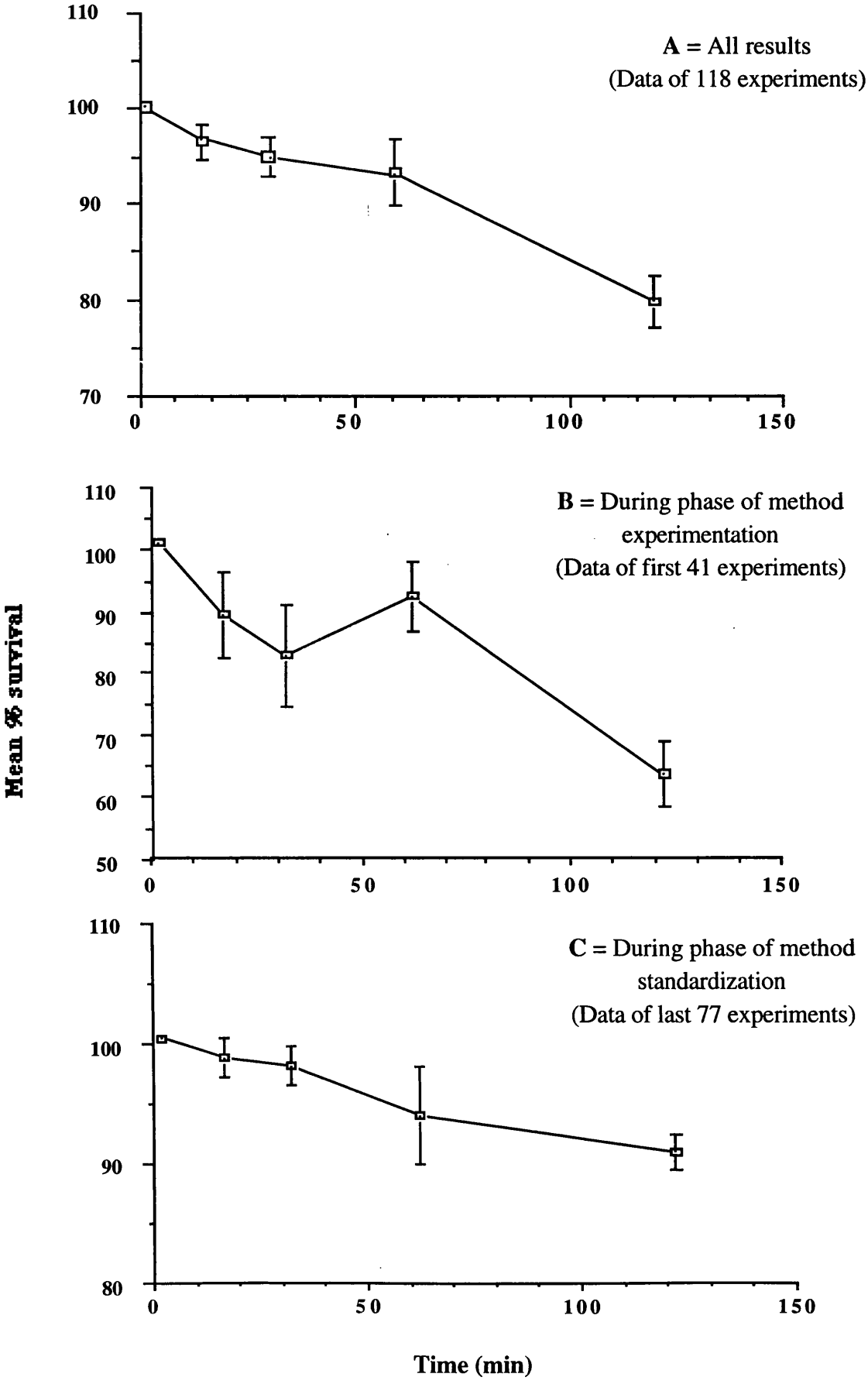


Box = Quartiles
Whiskers = Range
Vertical line = Median
Curved brackets = 95 % Confidence limits of the median
* * 0 = Outliers

B). Summary statistics

TIME (min)	N	MEAN	MEDIAN	STD DEV	SEM	95 % CONF LIMTS OF THE MEAN
0	118	100.0	100.0	0.0	0.0	100.0, 100.0
15	59	96.6	97.0	14.1	1.8	93.0, 100.3
30	89	95.0	96.0	18.8	2.0	91.1, 99.0
60	25	93.1	87.0	17.7	3.6	86.0, 100.6
120	114	79.8	88.0	28.0	2.6	74.6, 85.0
1440	96	286.4	275.5	231.7	23.6	239.4, 333.3
2880	56	482.2	444.0	411.1	54.9	372.0, 592.3

Figure-24. Mean (±SEM) percent survival of *B. pertussis* 18-323 in CL medium up to 120 min at 37°C. Data are presented in relation to method of experimentation and standardization.



Direct comparison of PBS with CAA and CL Medium

In a few experiments a direct comparison was made of the survival of *B. pertussis* 18-323 at 37°C in PBS, CAA and CL medium, tested in parallel. Table 22 shows that loss of viability took place at very similar rates in PBS and in CAA, while in CL medium, the count did not decline appreciably during the first 120 min, and at 1440 min had definitely increased. The figure of 372 % survival in CL medium at 1440 min was slightly lower than usual.

Type and size of test container

At the start of the experimental work, sterile, washed, 7 ml-volume thin-wall glass Bijous with a plastic screw cap were used as the containers for bacterial suspensions and for the test mixtures. However an unexpected reduction in percent survival of *B. pertussis* 18-323 in CL medium and in PBS was found after 120 min of incubation at 37°C in these containers. Attention was then focused on the type and the size of the containers which have a direct contact with the bacterial suspension. These included sterile U-shaped 96-well microtitre plates and sterile washed screw-cap plastic vials (2-ml volume), with 500 µl volumes of test mixture. Sterile, washed 7 ml-volume thin-wall glass Bijoux were the standard containers for the bacterial suspensions of 3 ml volume.

According to the results presented in Table 23, there was an obvious gradual reduction in viability (86 %) of *B. pertussis* 18-323 incubated in PBS after 120 min with the glass bijoux. No reduction in viability was noticed in PBS after 120 min incubation in 2-ml volume plastic vials, whereas a 16.3 % reduction in viability was noticed in PBS after 120 min incubation in U-Shaped 96-well microtitre plates. There was also a reduction (about 67 % after 120 min) in viability of *B. pertussis* 18-323 in CL medium incubated in glass bijoux for 120 min and no growth was detected after 1440 min. In contrast, good survival of the same bacteria was found after corresponding incubation in CL medium in 2-ml volume plastic vials, and good growth was achieved after 1440 min. With a microtitre plate, *B. pertussis* 18-323 showed about 26 and 72 % reduction in viability respectively

Table 22. Percent survival of *B. pertussis* 18-323 in phosphate buffered saline (PBS), casamino acids (CAA) and cyclodextrin liquid (CL) medium.

Test Fluid	Mean % survival ± SEM (No. of experiments) based on PBS time-zero count					
	0	15	30	60	120	1440 min
PBS	100±0	87±2	98±11	93±32	63±26	4±4
	(4)	(2)	(4)	(2)	(4)	(4)
CAA	102±12	84±24	85±13	97±9	50±20	5±5
	(4)	(2)	(4)	(2)	(4)	(3)
CL	113±15	107±7	131±12	104±4	80±18	372±21
	(4)	(2)	(4)	(2)	(4)	(4)

Table 23. Effect of type of container used with the test mixture on the survival and growth of *B. pertussis* 18-323 in PBS and CL medium.

Test fluid	Type of* container	No. of CFU / 20 µl (SEM) after exposure at 37°C for (min) [Percent reduction in viability]				
		0	30	60	120	1440
PBS	Bijou bottle (g)	90 , 86 [0]	32 , 38 [60.2]	15 , 11 [85.2]	12 , 12 [86.4]	0 , 0 [100]
PBS	Vial (p)	62 , 70 [0]	68 , 62 [1.5]	62 , 58 [9.1]	76 , ± 56 [0]	1.0 , 0.0 [99.2]
PBS	Microtitre well (p)	88, 108 [0]	nt -	nt -	80 , 84 [16.3]	0 , 0 [100]
CL	Bijou bottle (g)	84 , 72 [0]	56 , 54 [29.5]	52 , 53 [33.3]	22 , 30 [66.7]	22 , 24 [70.5]
CL	Vial (p)	72 , 88 [0]	74 , 72 [8.8]	67 , 80 [8.8]	72 , 72 [10]	ac [< 275]
CL	Microtitre well (p)	112 , 108 [0]	nt -	nt -	80 , 82 [26.4]	26 , 34 [72.7]

* g = glass, p = plastic; nt = not tested, ac = almost confluent

after 120 and 1440 min of incubation. According to these findings, plastic vials were used as test mixture containers in all further experimental work.

Consistency of duplicate counts

Reference to the various Appendices (5, 6, 7 and 9), in which the full data of bacterial viable counts are recorded, shows that duplicate samples were taken for counting on about one-half of all occasions. To check the consistency of these duplicates, a representative set of N = 65 pairs of counts from LLF (Appendix 9) experiments was chosen for further analysis. Very low (< 15) and very high (> 180) counts were excluded on grounds of reduced reliability. A Chi-square value for each of the 65 pairs of counts was calculated on Minitab and summarized as a histogram in Figure 25. This shows close conformity to expectations on the assumption that the differences between duplicate counts were due to random-sampling fluctuations and there was no detectable additional component of volumetric error. There were 2 out of 65 Chi-square values ≥ 3.84 (the 5 % point of the Chi-square distribution) whereas the expected number is 3.1, a reasonable agreement.

EFFECT OF LLF ON BACTERIAL VIABILITY

Bordetella pertussis 18-323 as Test Organism

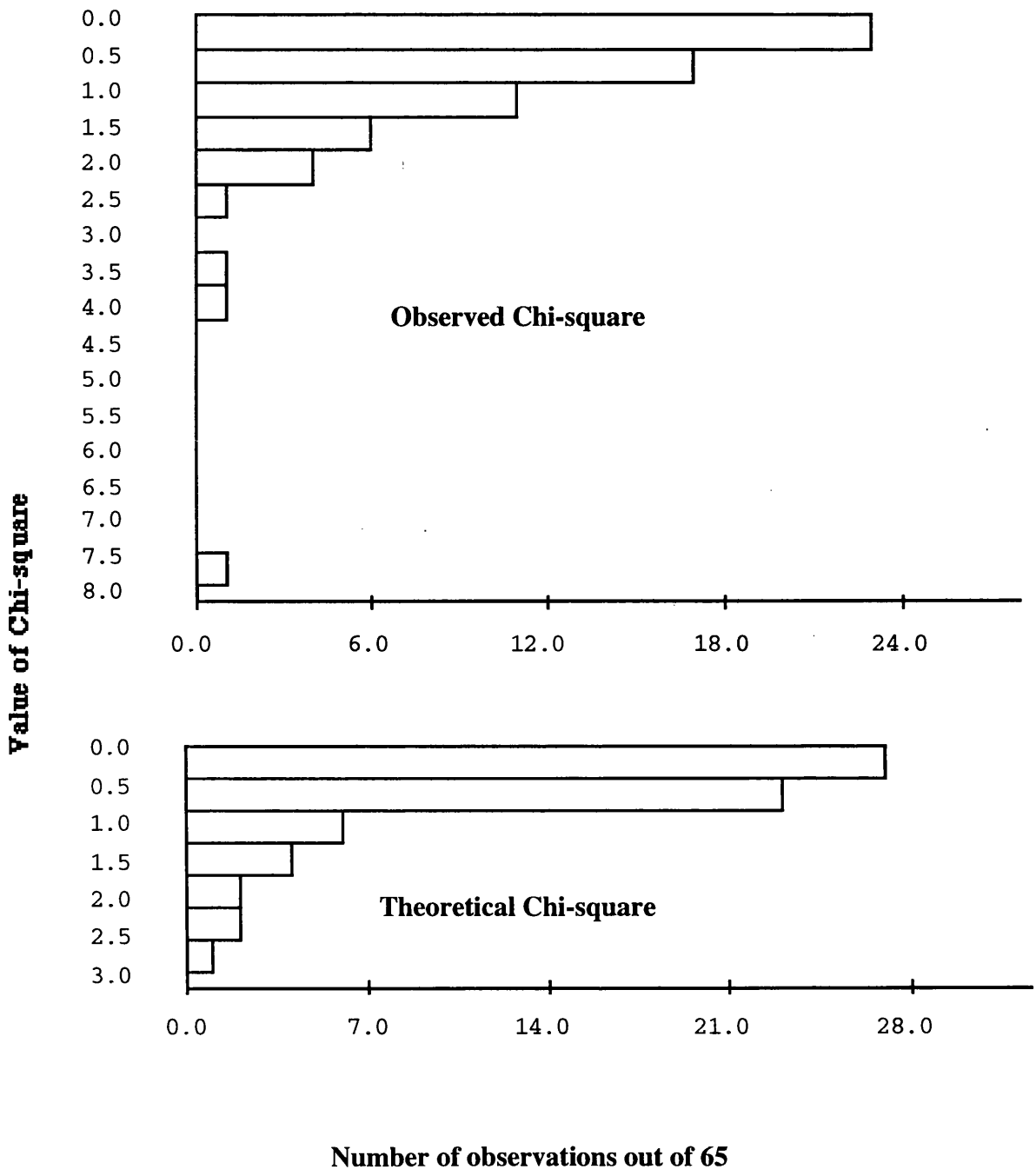
Having established that *B. pertussis* strain 18-323 was killed by normal and convalescent rat lung lavage fluid (LLF), the effect of certain variables was then explored. These included batch-to-batch variation in LLF, method of killing of the rats and different strains of rats.

Batch-to-batch variation of LLF from normal rats

During the course of these studies, four methods of euthanasia were used to kill normal rats prior to obtaining blood and LLF and three of these methods were also applied to convalescent rats. In brief the methods were:

A : Halothane/O_2 anaesthesia followed by CO_2 euthanasia, then bleeding out by heart puncture;

Figure 25. Histograms of Chi-square values from 65 pairs of viable counts from experiments on the bactericidal activity of rat lung lavage fluid (LLF), compared with 65 theoretical Chi-square values for 1 degree of freedom.



B : CO_2-alone euthanasia, then bleeding out by heart puncture;

C : Overdose of halothane / O_2, then bleeding out by heart puncture;

D : Halothane / O_2 anaesthesia, bleeding out by heart puncture followed by cervical dislocation.

Sixteen different batches of LLF from normal rats were tested against *B. pertussis* 18-323 in 90 experiments, of which the detailed results are presented in Appendix 9. The results are re-expressed as the median % survival in Appendix 10, and summarized in Figures 26, 27 and 28 as boxplots.

The boxplots of Figure 26 show that all 5 batches of LLF which had been obtained by euthanasia method A were active, ie. the upper 95 % confidence limit of the median % survival was less than the lower 95 % confidence limit of PBS; also, a progressive loss of viability between 30 and 120 min was apparent. According to the boxplots in Figure 27, little or no BA was found with any of the 4 batches which had been obtained by euthanasia method B, while the only one batch which had been obtained by euthanasia method C was active at 30 and 120 min. In Figure 28, all 6 batches which had been obtained by euthanasia method D were highly active at 120 min and most of them were highly active at 30 min. Most later batches of LLF were collected by euthanasia method D.

***Effect of age of rats*:** LLF from rats aged between 40 and >92 days old, and with mean weights from 126 to 455 g was examined. All samples had been collected by euthanasia method D. The results in Table 24 show very little differences in LLF BA related to age. Thus the LLF from 40 and >92 days old rats both gave complete killing after 30 min. Overall therefore, the age of the rat was not a major determinant of LLF BA.

Convalescent LLF

Experimental variables: With 7 of the batches of Sprague Dawley rats from which normal LLF had been obtained, there was also the opportunity to collect samples of LLF from animals that had been exposed to various infection and/ or vaccination procedures.

Figure 26. Boxplots of bactericidal activity of normal rat LLF (collected by euthanasia method A) towards *B. pertussis* 18-323 after 30 and 120 min incubation. The results are presented in chronological order of LLF batch collection, and the activities of PBS and CL medium are shown at the bottom.

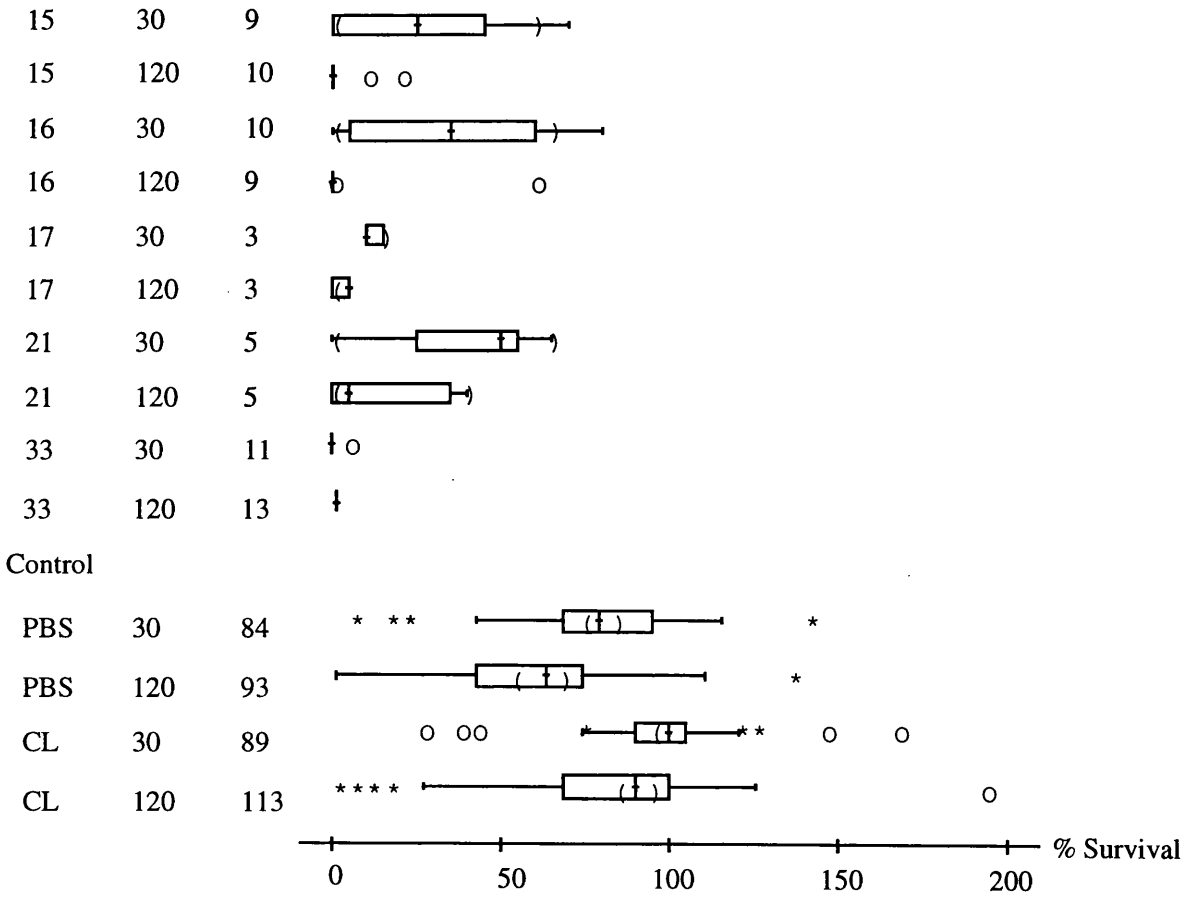


Figure 27. Boxplots of bactericidal activity of normal rat LLF (collected by euthanasia method B, except for 38.2 for which method C was used) towards *B. pertussis* 18-323 after 30 and 120 min incubation. The results are presented in chronological order of LLF batch collection, and the activities of PBS and CL medium are shown at the bottom.

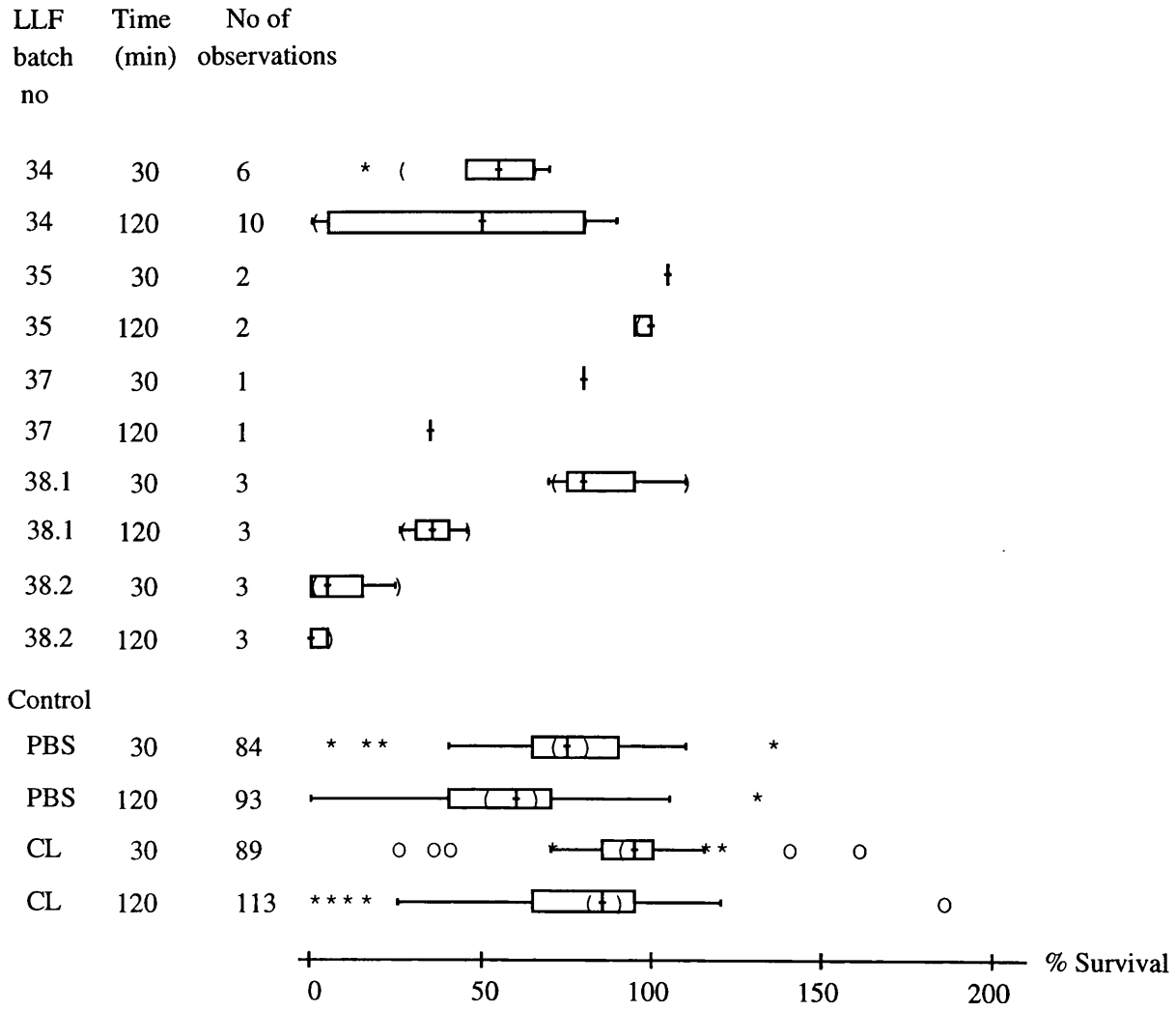


Figure 28. Boxplots of bactericidal activity of normal rat LLF (collected by euthanasia method D) towards *B. pertussis* 18-323 after 30 and 120 min incubation. The results are presented in chronological order of LLF batch collection, and the activities of PBS and CL medium are shown at the bottom.

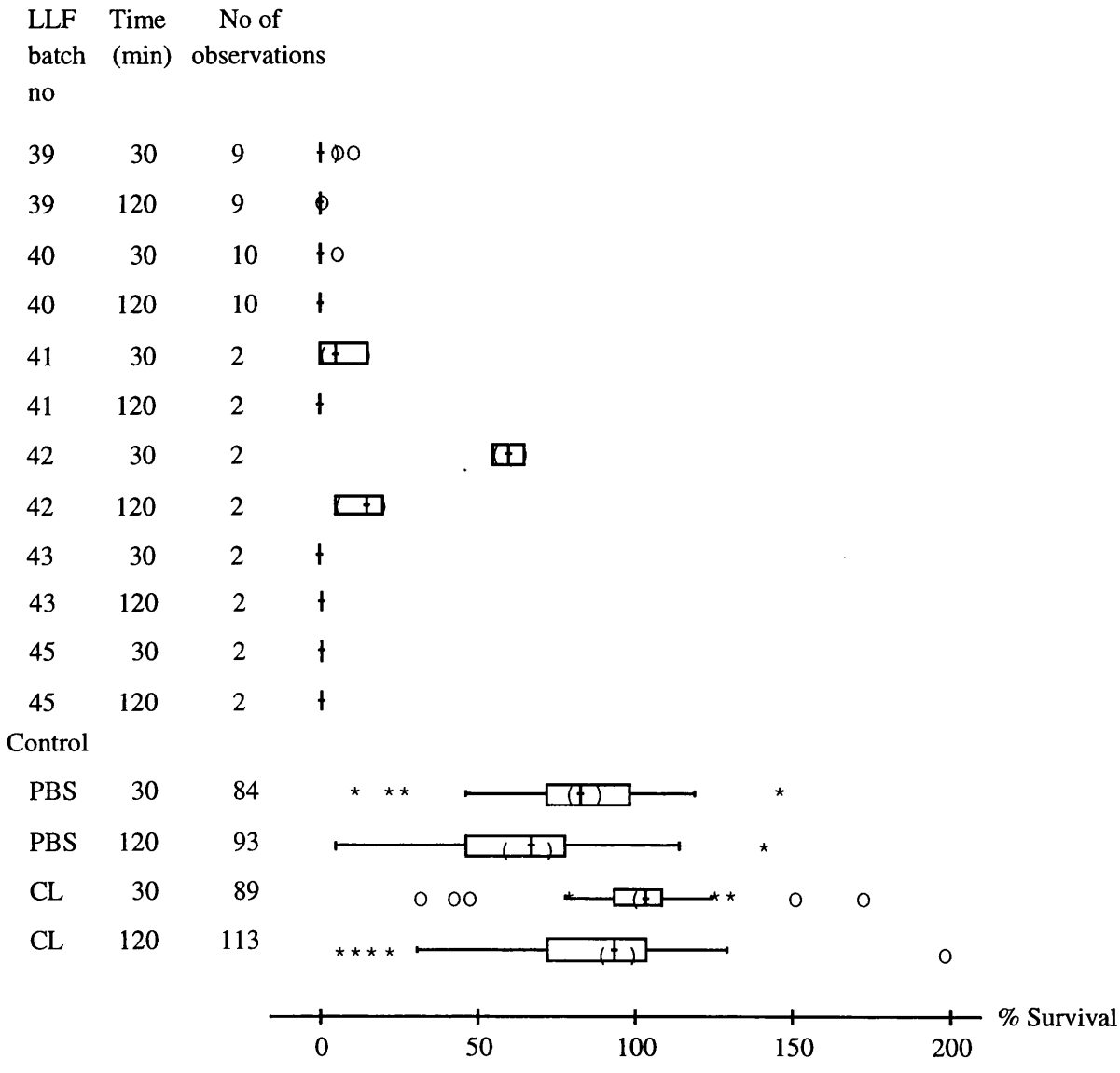


Table 24. Percent survival of *B. pertussis* 18-323 treated with LLF from rats of different ages. Data are the mean of three experiments.

Rat batch/ (No. of rats)	weight mean (SEM)	Age* (days)	Mean (SEM) % survival** after incubation at 37°C for time (min)				
			0	30	60	120	1440
39 (5)	126±4	40	97±12	0	0	0	0
39 (5)	253±2	62	120±14	8±17	7±3	1±1	0
39 (2)	350±6	88	91±3	2±1	0	0	0
40 (15)	355±4	92	98±16	11±6	0	0	0
40 (4)	456±31	>92	94±4	0	0	0	0

* Data derived from Sprague Dawley rats growth chart, which was prepared from growth charts supplied by animal house.

** Based on time-zero PBS counts.

These animals were killed at between 3 and 6 weeks after infection, with LLF from normal control rats being of the same original delivery batch obtained on the same day.

Three different euthanasia methods were used, but on any particular occasion, both normal and convalescent rats were subjected to the same euthanasia procedures. Collection, processing, and storing procedures were the same as with LLF from untreated rats.

All the infection experiments involved the intratracheal administration (under Hypnorm and Hypnovel anaesthesia) of 10^8 *B. pertussis* 18-323 (except in a few experiments where other *B. pertussis* strains were used) in 0.1 ml of agarose bead suspension, except in a few experiments where alginate or carrageenan were used in a place of agarose. As controls, the beads without live *B. pertussis* were given to "sham-operated" rats. In most experiments, after depositing the beads in the lungs, the animals were exposed for 2 min to ether, before being allowed to recover.

For challenge, different *B. pertussis* 18-323 phase I substrains (A6, EH1 and EH2), Tohama, transposon mutants (BP 1809 HLT $^-$, BP 357 PT $^-$), L84 phase IV and *B. parapertussis* strain 10520. In vaccination experiments, the animals were injected intraperitoneally with 0.5 ml of DPT vaccine 21 days before infection. Thus at the time of euthanasia, the rats were usually 9 to 12 weeks of age. Usually, pooled LLF from 8 rats for each group was used.

Results of median % survival of *B. pertussis* 18-323 in convalescent LLF after 30 and 120 min incubation are presented here as 7 figures and explained batch by batch. They were based on between 2 and 17 bactericidal tests. See Appendices 11 and 12 for detailed records. Note that the vaccination and challenge of all these rats were done by my colleagues E. Hall, R. Parton and A. C. Wardlaw.

Effect of B. pertussis challenge: Rats in this batch were divided into two groups, one untreated and the other challenged with *B. pertussis* 18-323 substrain EH1 in agarose beads and then exposed to ether. Euthanasia method B was used before collecting LLF. Bactericidal results in Figure 29 showed a weak or no activity of LLF from untreated rats,

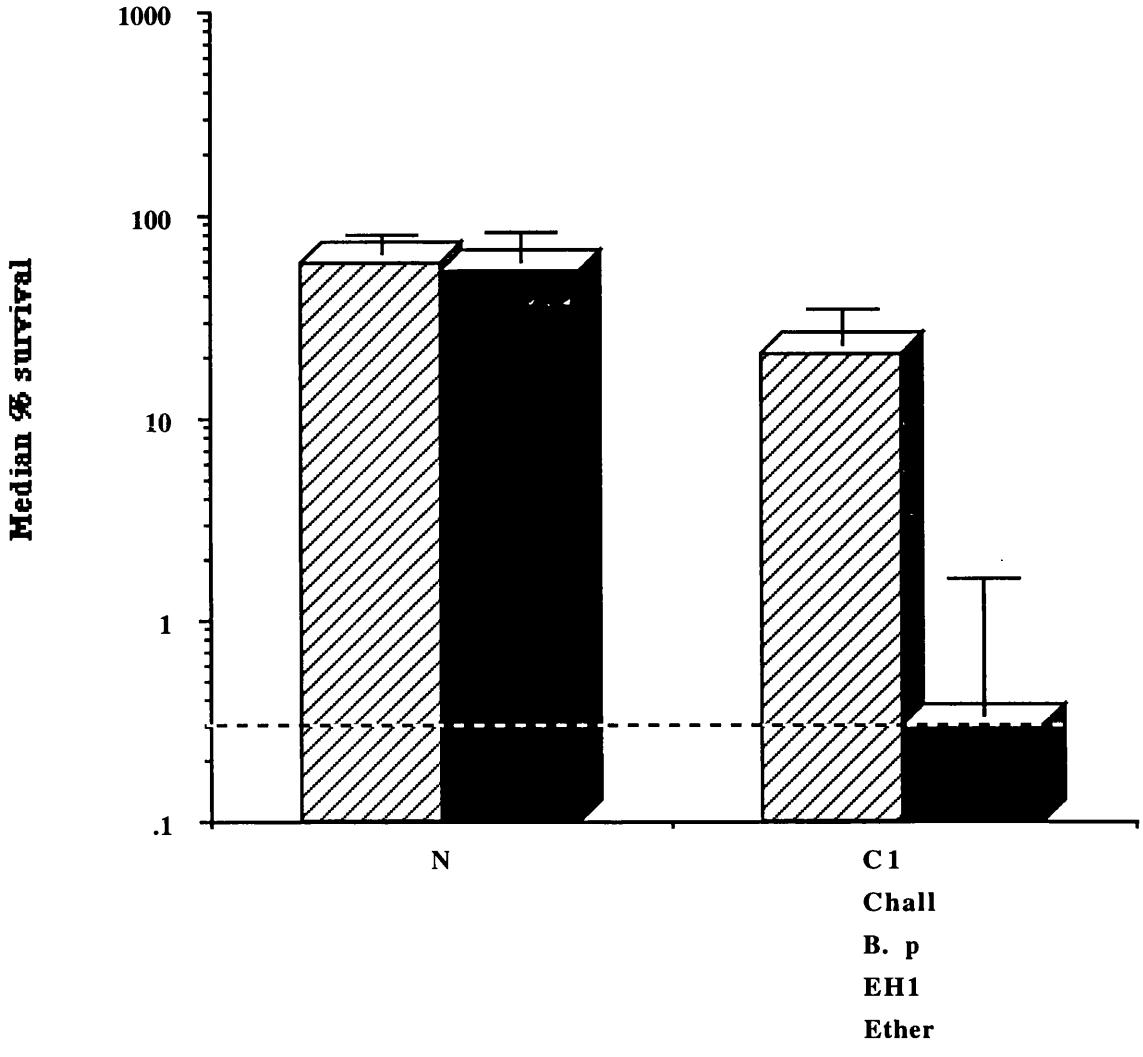
whereas, a high decrease in % survival at 30 min and a complete killing at 120 min were noticed with LLF from convalescent rats.

Effect of rat sex and various B. pertussis strains: The 40 rats in this batch were used to compare the effect of rat sex and phase I and phase IV *B. pertussis* challenge suspension, and the influence of ether. The LLF bactericidal results in Figure 30 show that the normal LLF was more active than any of the LLFs from the convalescent animals. There were, however differences in the latter, in that the LLF from rats challenged with phase IV *B. pertussis* was more bactericidal than that from rats challenged with phase I. No effect of ether was detected.

Effect of agarose and alginate, ± B. pertussis challenge: Five rat groups in this experiment were exposed to euthanasia method D, one untreated, two were sham-operated (one with agarose and the other with alginate) with no bacteria, and the other two were challenged, with *B. pertussis* 18-323 substrain EH2, one in agarose beads and the other in alginate. All treated rats were exposed to ether. BA results of these LLF fluids towards *B. pertussis* 18-323 were presented in Figure 31, which showing a high activity of LLF from untreated and sham-operated rats, and no activity was noticed with LLF from challenged and convalescent rats.

Effect of various B. pertussis challenges (alginate or agarose, ± ether): All treated rats in this experiment were challenged with *B. pertussis* 18-323 substrain EH2, which was in agarose beads for 2 groups and in alginate for the other 2. Also 2 groups were exposed to ether, and the other 2 with no ether treatment. According to the bactericidal results in Figure 32, high BA of LLF from untreated and challenged rats with no ether treatments was observed and weak or no activity with LLF from the other challenged rats which exposed to ether treatment was found.

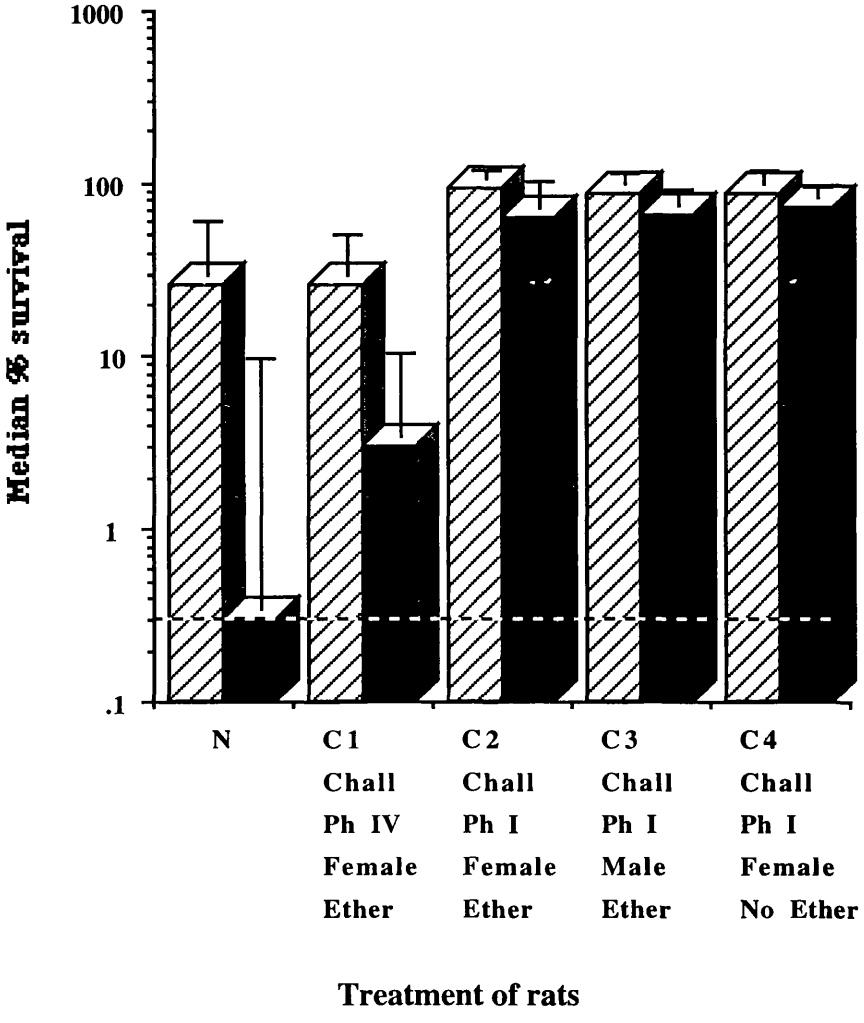
Figure 29: Effect of *B. pertussis* challenge, followed by convalescence, on the bactericidal activity of rat LLF (rat batch no. 34). Diagram shows the median % survival of *B. pertussis* 18-323 after 30 (diagonal line) and 120 (solid block) min at 37°C. Euthanasia of the rats was by method B. The horizontal dotted line indicates the detection limit. Error bars are the upper 95 % confidence limits.



N : Male normal rats.

C1 : Male rats challenged with *B. pertussis* 18-323 substrain EH1 in agarose, with ether and sacrified 4 weeks later.

Figure 30. Effect of rat sex and various *B. pertussis* challenges (Phase I or IV, ± ether) on the bactericidal activity of rat LLF (rat batch no. 15). Diagram shows the median % survival of *B. pertussis* 18-323 after 30 (diagonal line) and 120 (solid block) min at 37°C. Euthanasia of the rats was by method A. The horizontal dotted line indicates the detection limit. Error bars are the upper 95 % confidence limits.



N : Female normal rats.

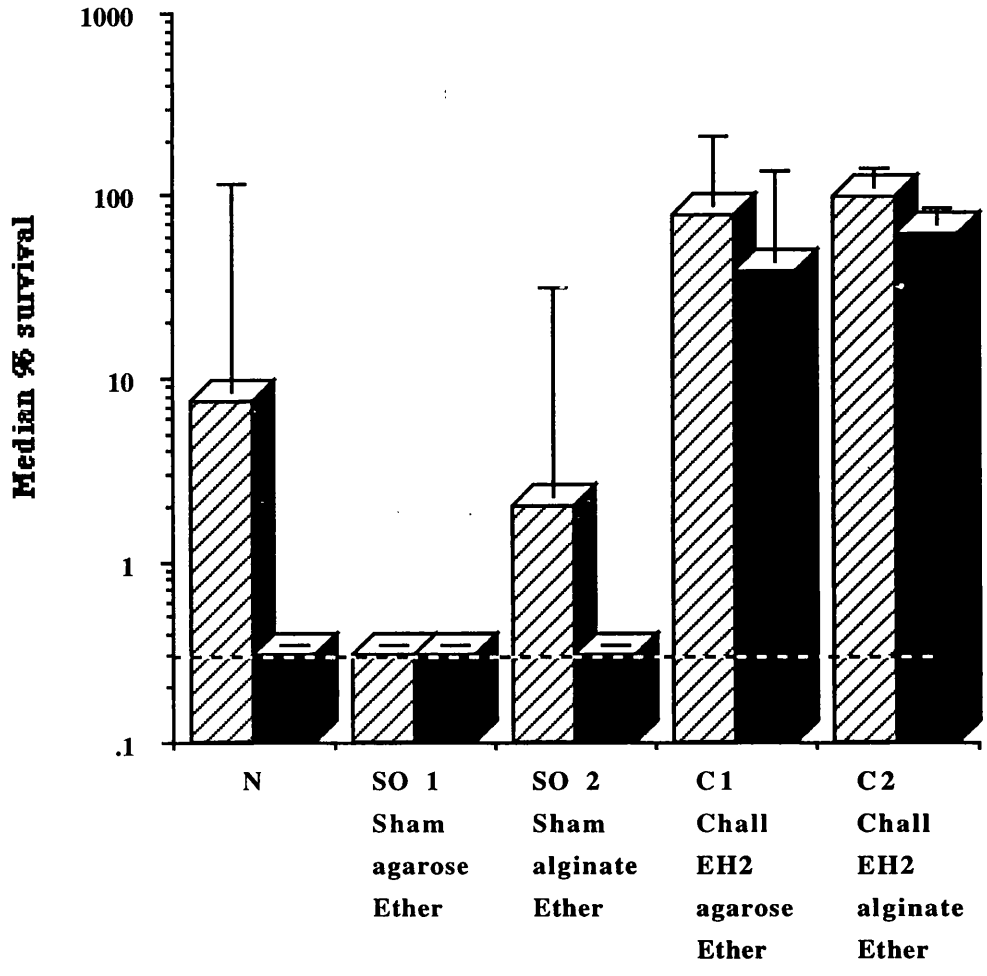
C1 : Female rats challenged with *B. pertussis* L-84 (Phase IV), with ether

C2 : Male „ „ „ „ 18-323 EH1 (phase I), with ether

C3 : Female „ „ „ „ „ „ , with ether

C4 : „ „ „ „ „ „ „ , no ether

Figure 31. Effect of various *B. pertussis* challenges (alginate or agarose) on the bactericidal activity of rat LLF (rat batch no. 41). Diagram shows the median % survival of *B. pertussis* 18-323 after 30 (diagonal line) and 120 (solid block) min at 37°C. Euthanasia of the rats was by method A. The horizontal dotted line indicates the detection limit. Error bars are the upper 95 % confidence limits.



N : Female normal rats.

SO 1 : „ rats given agarose with no bacteria.

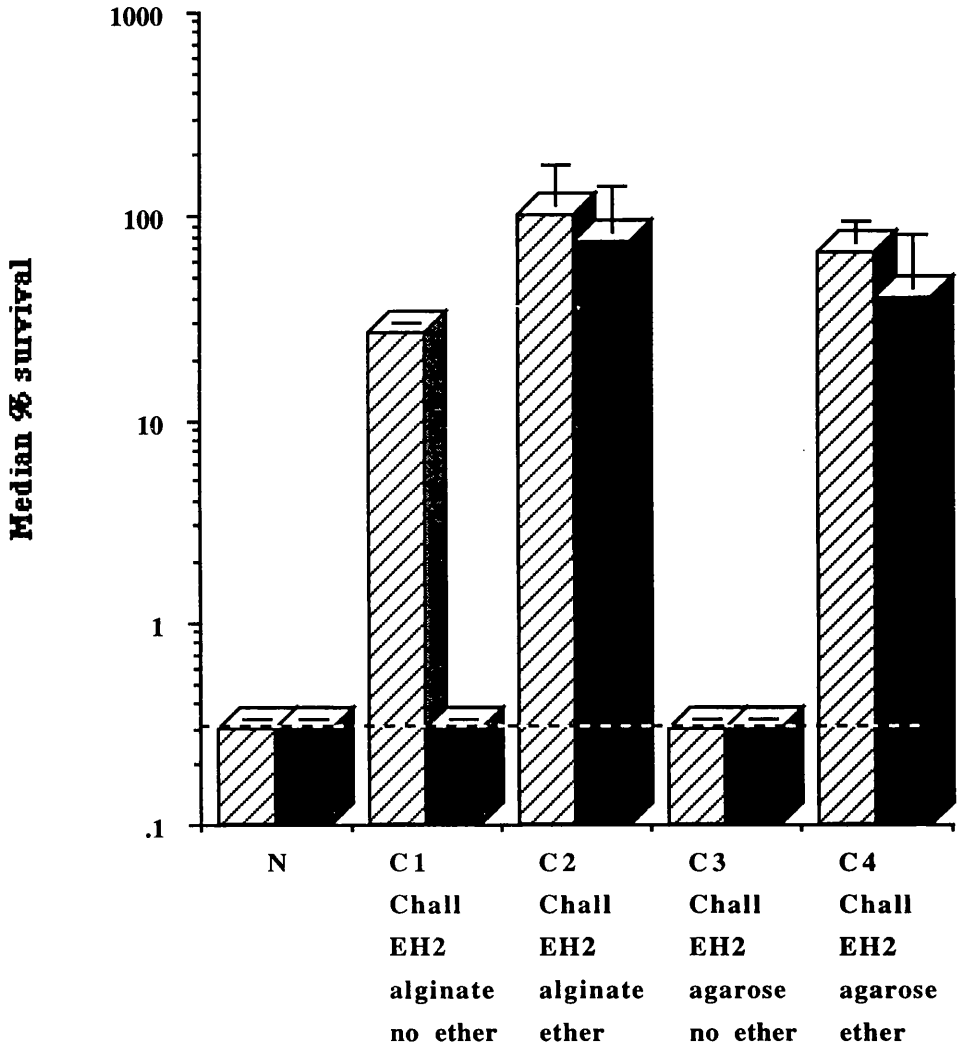
SO 2 : „ „ „ alginate „ „ „

C1 : „ „ challenged with *B. pertussis* 18-323 EH2 in agarose.

C2 : „ „ „ „ „ „ „ alginate.

All treated rats exposed to ether.

Figure 32: Effect of various *B. pertussis* challenges (alginate or agarose, ± ether) on the bactericidal activity of rat LLF (rat batch no. 43). Diagram shows the median % survival of *B. pertussis* 18-323 after 30 (diagonal line) and 120 (solid block) min at 37°C. Euthanasia of the rats was by method D. The horizontal dotted line indicates the detection limit. Error bars are the upper 95 % confidence limits.



N : Female normal rats.

C1 : „ rats challenged with *B. pertussis* 18-323 EH2 in alginate, no ether.

C2 : „ „ „ „ „ „ in alginate, with ether.

C3 : „ „ „ „ „ „ in agarose, no ether.

C4 : „ „ „ „ „ „ in agarose, with ether.

Effect of various Bordetella challenges (± carrageenan): This experiment consisted of five groups of rats. Two groups were challenged with virulent *B. pertussis* strains, one with Tohama and the other with 18-323 substrain A6. Other two groups were challenged with *B. pertussis* transposon mutants, one with BPM 1809 HLT $^-$ and the other with BP 357 PT $^-$. The fifth group was challenged with *B. parapertussis* 10520. All the bacterial inocula were introduced in a mixture of agarose and carrageenan. All treated rats were exposed to ether.

The bactericidal results in Figure 33, shows that all LLF from challenged and convalescent rats with *B. pertussis* HLT $^-$, PT $^-$ and *B. parapertussis* 10520 were active. No activity was found with LLF from rats challenged with Tohama. LLF from untreated rats was less active after 30 min. A low percent survival was achieved after 120 min.

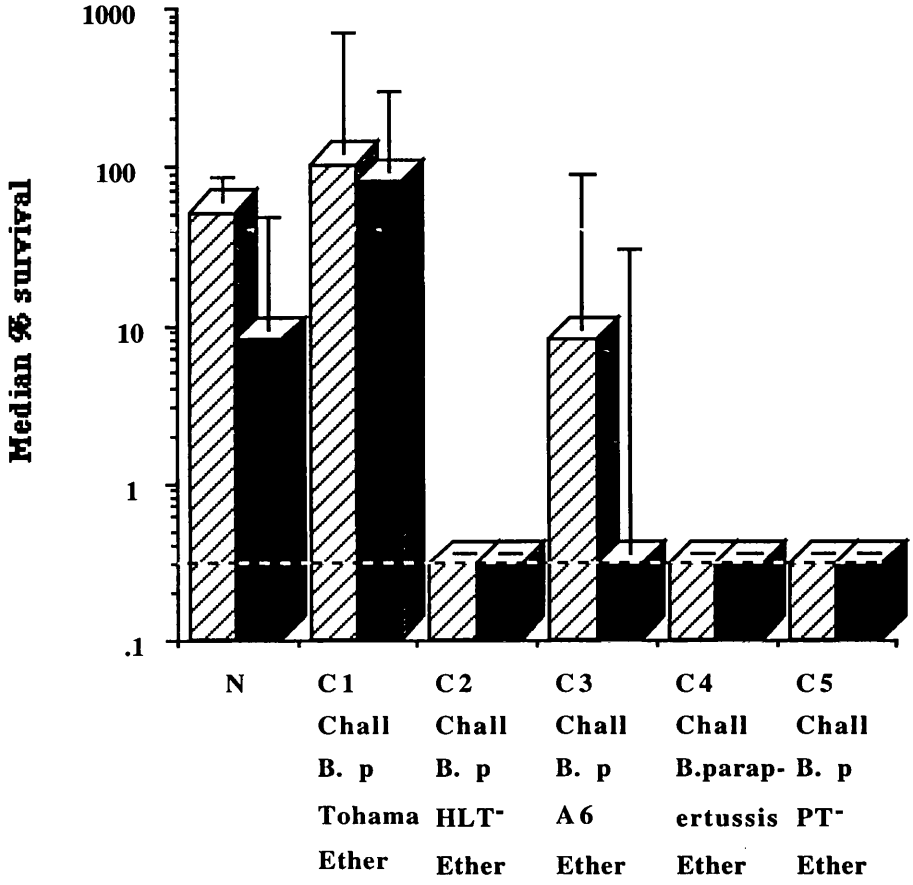
***Effect of vaccination and various challenges*:** Two rat groups of this experiment were vaccinated, one of which was challenged with *B. pertussis* 18-323 substrain A6 and the other challenged with substrain EH1. The other two groups were only challenged, one with *B. pertussis* 18-323 A6 and the other with EH1.

According to the results in Figure 34, the highest BA was noticed with normal , vaccinated and challenged group 1, 2 and convalescent group 2, while weak or no BA was detected with convalescent LLF group no. 1. In all cases, loss of bacterial viability after 120 min was more than that at 30 min.

Effect of sham-operation, vaccination and various challenges: There were no completely normal rats in this batch, since in place of untreated animals, a group of 8 sham-operated rats was included. These had been through the complete tracheotomy and insertion of agarose beads, but the beads were sterile. As shown in Figure 35, the LLF from these animals was very similar to the normal LLF from batch no. 15.

The other animals in this experiments had all been challenged with *B. pertussis* 18-323 EH1, but two of the groups had also been vaccinated 3 weeks before. The experiment

Figure 33. Effect of various *Bordetella* challenges (± carrageenan) on the bactericidal activity of rat LLF (rat batch no. 21). Diagram shows the median % survival of *B. pertussis* 18-323 after 30 (diagonal line) and 120 (solid block) min at 37°C. Euthanasia of the rats was by method A. The horizontal dotted line indicates the detection limit. Error bars are the upper 95 % confidence limits.



N : Male normal rats.

C1 : „ rats challenged with *B. pertussis* Tohama in agarose + carrageenan

C2 : „ „ „ „ „ BPM 1809 HLT - in agarose + „

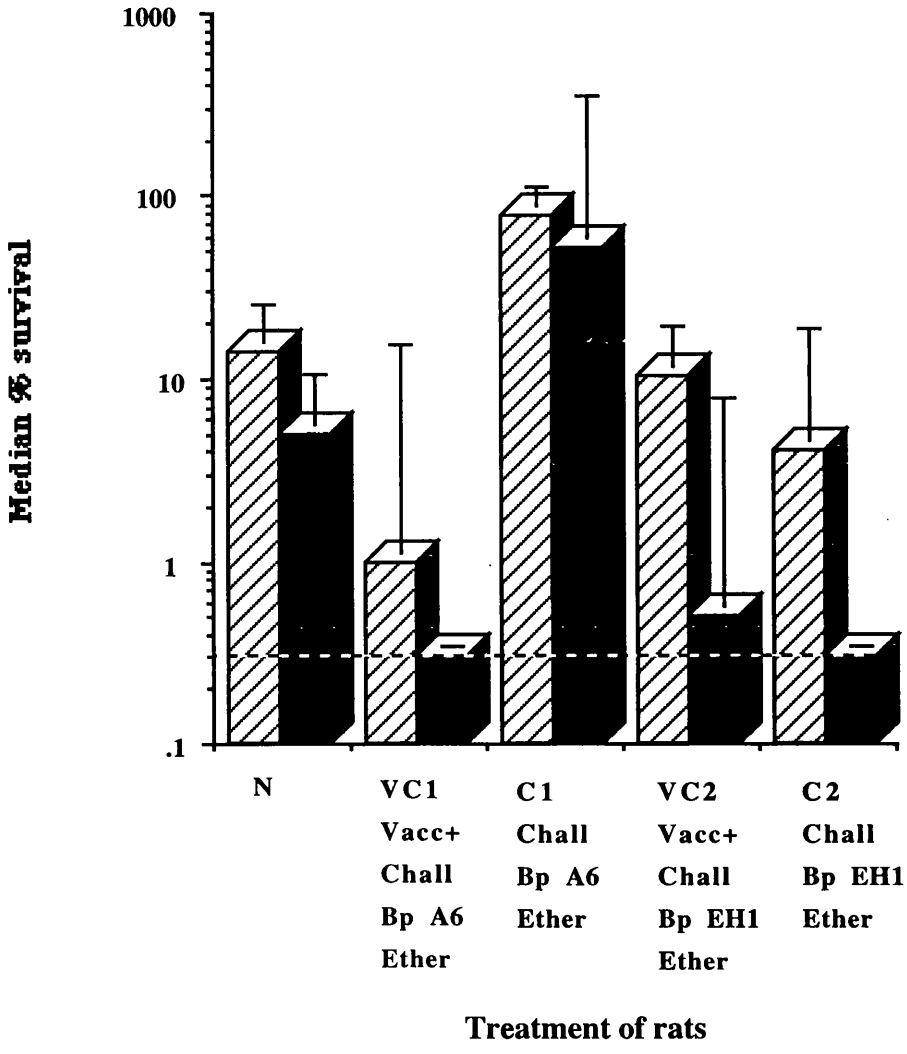
C3 : „ „ „ „ „ 18-323 A6 in agarose + „

C4 : „ „ „ „ *B. parapertussis* 10520 in agarose + „

C5 : „ „ „ „ *B. pertussis* BP 357 PT - in agarose + „

All treated rats were exposed to ether.

Figure 34. Effect of vaccination (V) and various challenges (C) on the bactericidal activity of rat LLF (rat batch no. 17). Diagram shows the median % survival of *B. pertussis* 18-323 after 30 (diagonal line) and 120 (solid block) min at 37°C. Euthanasia of the rats was by method A. The horizontal dotted line indicates the detection limit. Error bars are the upper 95 % confidence limits.



N : Male normal rats.

VC1 : „ rats vaccinated + challenged with *B. pertussis* 18-323 A6 in agarose.

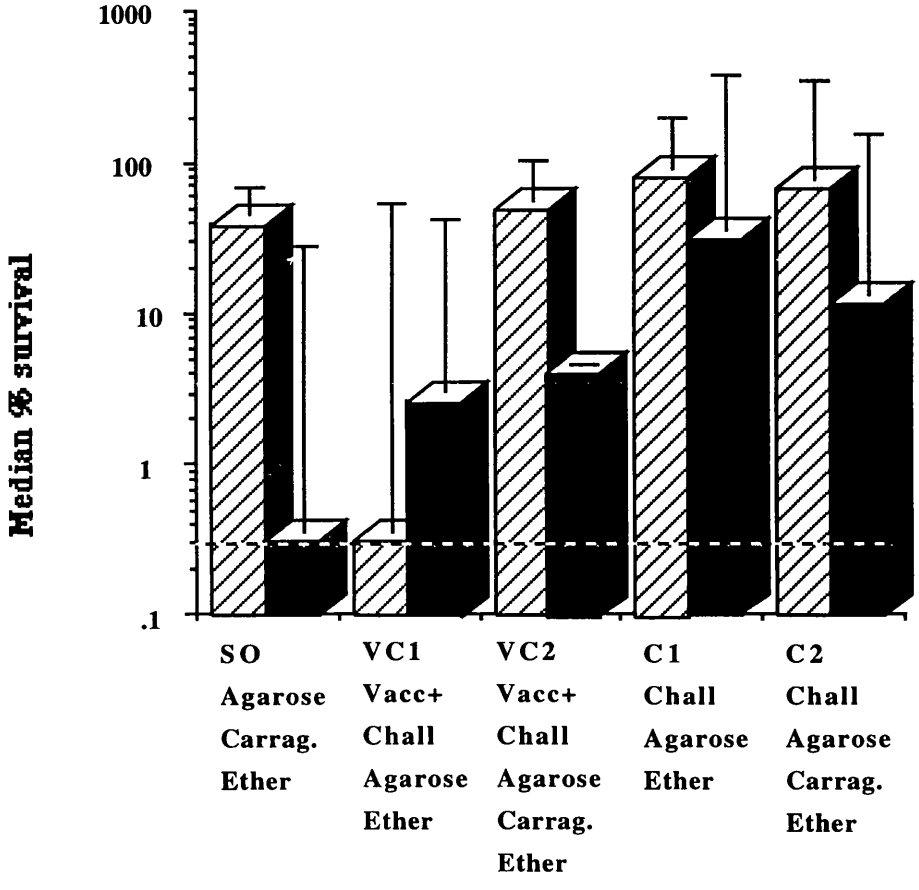
C1 : „ „ challenged with *B. pertussis* 18-323 A6 in agarose.

VC2 : „ „ vaccinated + challenged with *B. pertussis* 18-323 EH1 in agarose.

C2 : „ „ challenged with *B. pertussis* 18-323 EH1 in agarose.

All treated rats exposed to ether.

Figure 35. Effect of sham-operation (SO), vaccination (V) and various challenges (C) on the bactericidal activity of LLF (rat batch no. 16). Diagram shows the median % survival of *B. pertussis* 18-323 after 30 (diagonal line) and 120 (solid block) min at 37°C. Euthanasia of the rats was by method A. The horizontal dotted line indicates the detection limit. Error bars are the upper 95 % confidence limits.



SO : Male rats given agarose and carragenaan only (no bacteria)

VC1 : „ „ vaccinated + challenged with *B. pertussis* 18-323 EH1 in agarose

VC2 : „ „ „ + „ „ „ „ in agarose + carrageenan.

C1 : „ „ challenged with *B. pertussis* 18-323 EH1 in agarose

C2 : „ „ „ „ „ „ „ „ + carrageenan.

All treated rats exposed to ether.

also examined the effect of adding carrageenan to the agarose. The bactericidal results in Figure 35 contain an anomalous observation in that the 120 min survival was apparently higher than the 30 min [Total observations were 17 at time zero, 10 at 30 min and 14 at 120 min].

Summary analysis of effect of experimental treatments on LLF activity: For further analysis of the accumulated results (Appendix 13) of experimental treatment of the rats and the BA of their LLF towards *B. pertussis* 18-323, Figure 36 shows that there was a lot of variation between individual pools of LLF but sex did not appear to be a factor. A general observation was that normal rats gave more bactericidally active LLF than convalescent animals but with much variation between individual pools of LLF, especially from convalescent rats.

Similarly (Figure 37), a lot of variation was also found in the activity of LLF from rats exposed to sham-operation ± ether and also in respect of the euthanasia method. LLF from treated and untreated rats collected after different times after infection showed (Figure 38) also much variation, with the day 23 and 28 samples giving the highest activity. Rats infected with different organisms (Figure 39) also gave LLF with different BAs. LLFs from rats challenged with *B. pertussis* 18-323 substrains (A6, EH1 and EH2) and Tohama showed a lot of variation in their BA.

Of particular interest was the very high activity (low % survival) of fluids from animals infected with the Phase IV, HLT $^{-}$, PT $^{-}$ strains of *B. pertussis* or with *B. parapertussis.*

Summary analysis of euthanasia data

Altogether 43 batches of LLF were collected, of which 16 were from normal (N) and 27 from convalescent (C) animals. Method A of euthanasia was used mainly for early studies and method D in the later work.

Bactericidal tests with *B. pertussis* 18-323 were applied to all different batches of LLF and the results summarised in Table 25 for the 30 min results and Table 26 for the 120

Figure 36. Bactericidal activity of LLF from male and female rats compared with LLF from normal (o) and convalescent (●) animals. Percent survival data were at 30 min.

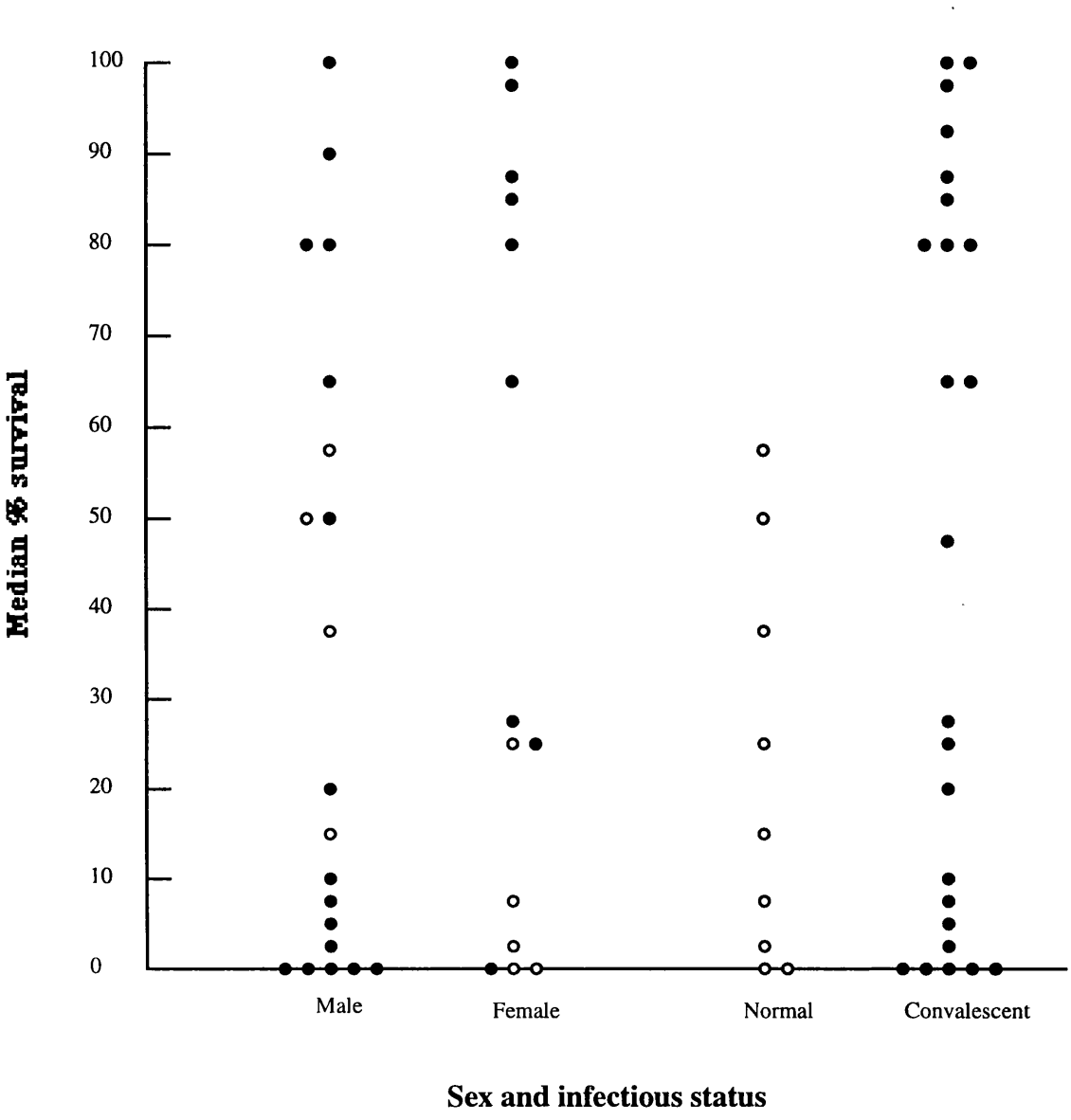
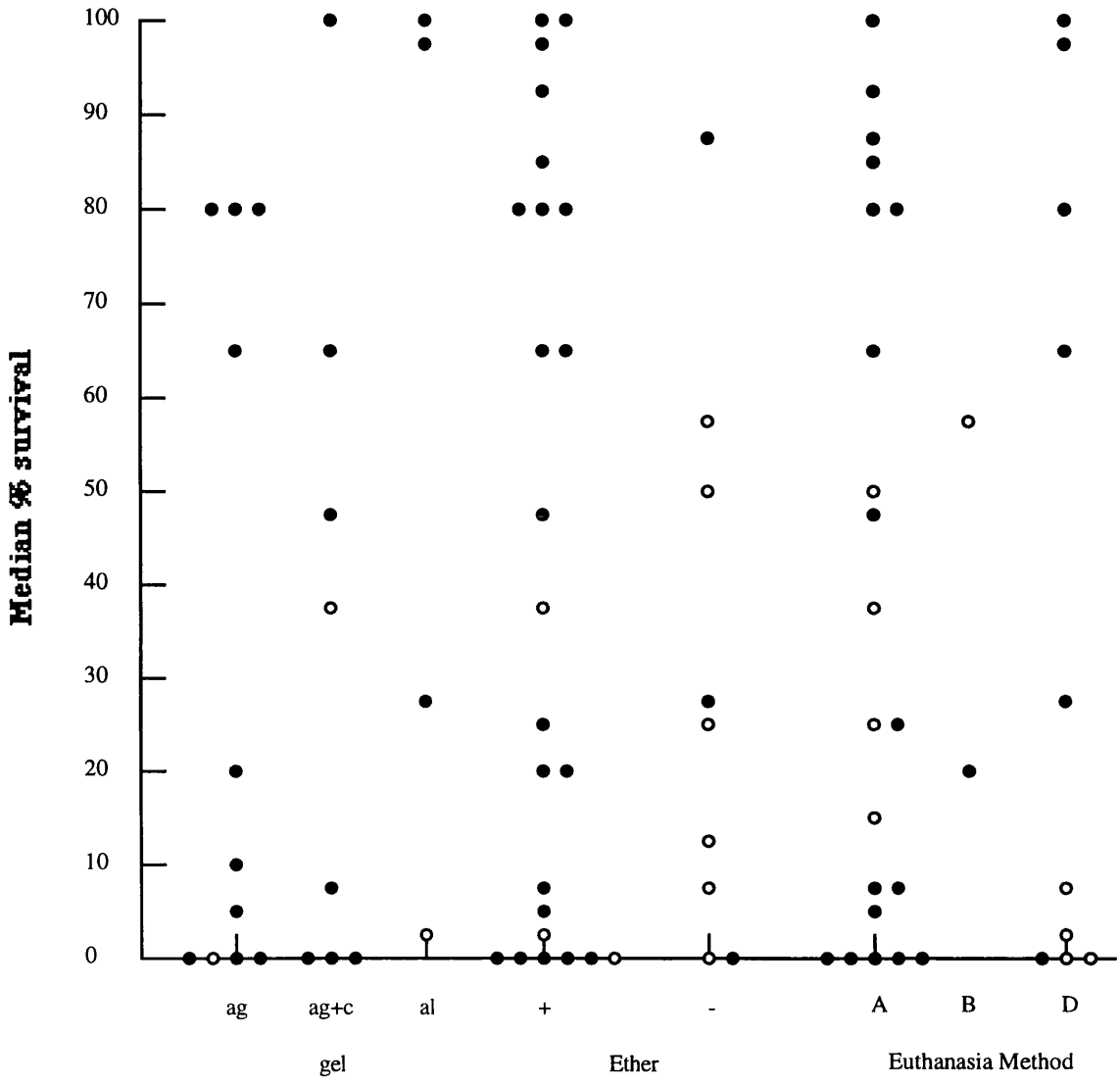


Figure ~~37~~: Effect of sham operation, euthanasia method and ether treatment of rats on the bactericidal activity of their LLF towards *B. pertussis* 18-323. Percent survival data were at 30 min.



ag = agarose
c = carrageenan
al = alginate

See Appendix 6 footnote for euthanasia method A, B and D

o = normal or sham
● = convalescent

Figure 38. Bactericidal activity towards *B. pertussis* 18-323 of LLF collected from normal (o) and convalescent (•) rats at various times after infection or from matched controls. Percent survival data were at 30 min.

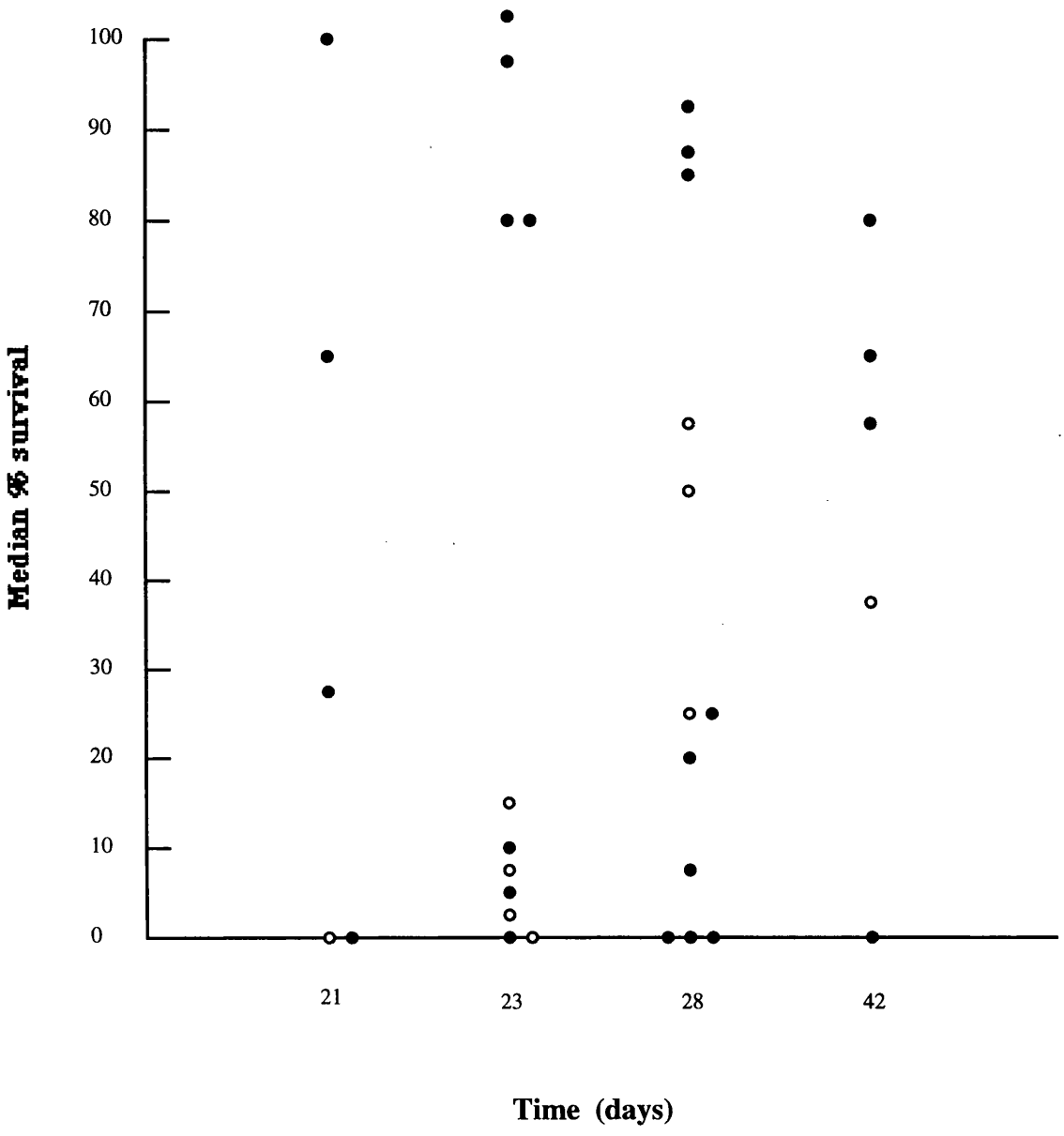
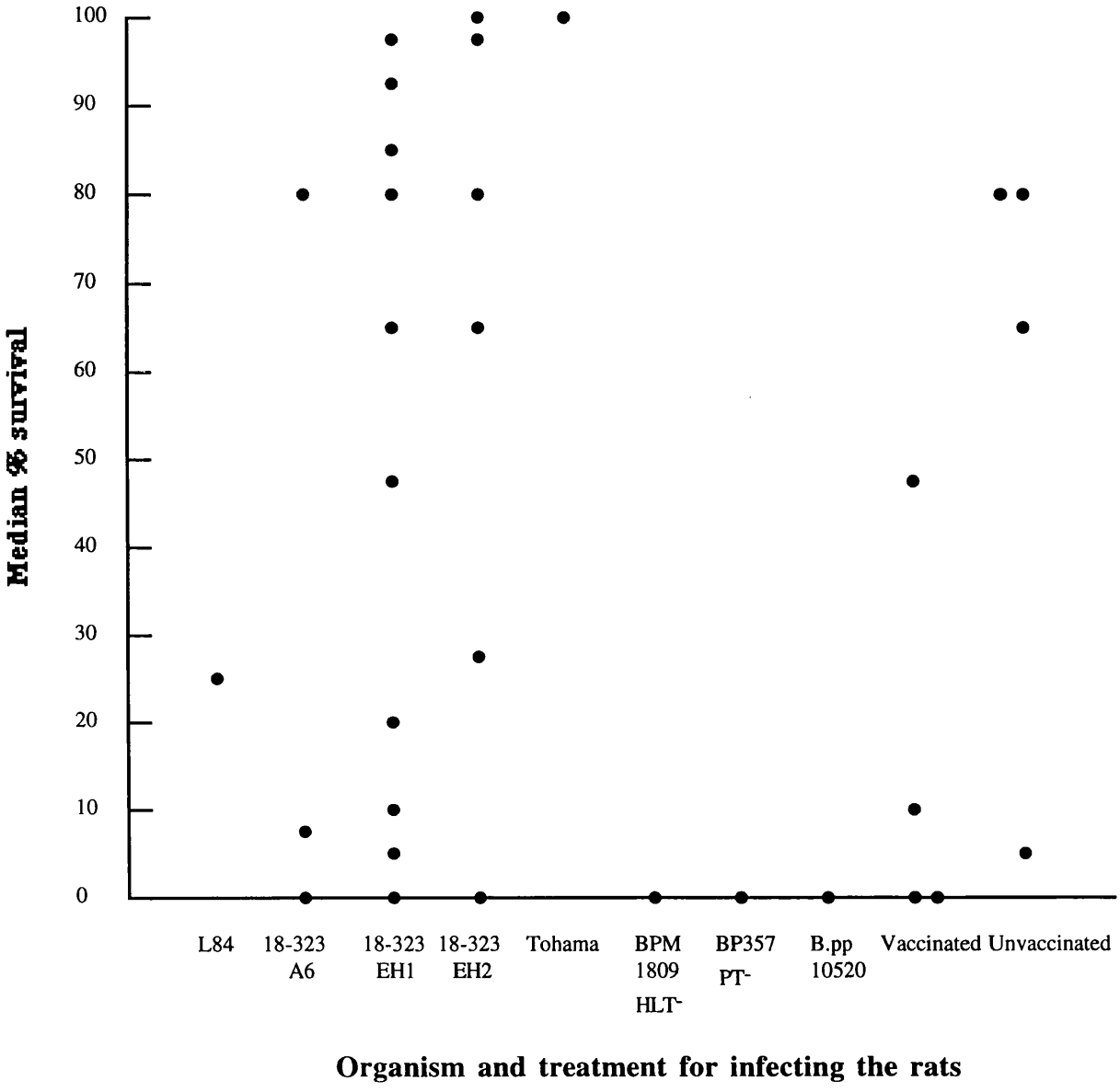


Figure 39. Effect of vaccination and challenge with different organisms on the bactericidal activity of LLF towards *B. pertussis* 18-323 after 30 min incubation.



min. The data are divided into 4 categories of BA, expressed as median % survival. Details of all the individual bactericidal tests may be found in Appendix 9.

Survival of B. pertussis after 30 min: The LLF batches of greatest interest were those that gave complete killing of the bacterial inoculum i.e. a median survival of 0 % after 30 min. Eleven of the 42 batches fell into this highest-activity category. Of these, 5 (1N and 4C) had been obtained by euthanasia method A and 6 (4 N and 2 C) by method D.

In the next highest BA category (median survival 1-20 %) there was a total of 8 batches, of which 5 (1 N and 4 C) had been obtained by euthanasia method A, 1N by method C and 2 (1 N and 1C) by method D.

The table shows that 7 batches of LLF gave median survivals in the range 21-60 % i.e. were of moderate BA. These were distributed as method A : (3 N and 1 C); method B: (1N and 1C); and method D: (1C).

Finally, there were 16 relatively inactive batches of LLF that gave median survival of ≥ 61 % and which were of less use for exploration of BA. These batches were distributed as method A: 8 (0 N and 8 C); method B : 3 (3 N and 0 C); method C none and method D : 5 (1 N and 4 C).

The overall conclusion from these results was that methods A and D of euthanasia were both satisfactory (in giving LLF of a strong BA) for killing the rats before collection of LLF and that there was a suggestion that method D might be the better of the two.

Survival of B. pertussis after 120 min: As with the 30 min results, attention was focused on those LLF batches which gave complete killing of the bacterial inoculum (a median survival of 0 %) at 120 min. Table 26 shows that nineteen of the 42 batches were in this highest-activity category. Of these, 9 (3 N and 6 C) were from rats killed by euthanasia method A, 1 (1 C) by method B, and 9 (5 N and 4 C) by method D.

Nine batches are categorised in the next highest BA (median survival 1-20 %), of which, 7 (2 N and 5 C) had been obtained by euthanasia method A, 1 (1 C) by method C and 1 (1 N) by method D. In the category of moderate BA (a median survival of 21-60),

Table 25:-Summary of percent survival of *B. pertussis* 18-323 incubated for 30 min in different batches of LLF from rats killed by four different euthanasia methods.

Method of euthanasia*	Type of LLF**	Total no. of batches	No. of batches of LLF with median % survival in range: 0	1-20	21-60	≥ 61
A	N	5	1	1	3	0
	C	17	4	4	1	8
	T	23	5	5	4	8
B	N	4	0	0	1	3
	C	1	0	0	1	0
	T	5	0	0	2	3
C	N	1	0	1	0	0
D	N	6	4	1	0	1
	C	8	2	1	1	4
	T	14	6	2	1	5
Total	N	16	5	3	4	4
	C	26	6	5	3	12
	T	42	11	8	7	16

*A : Halothane/O_2 anaesthesia followed by CO_2 euthanasia, then bleeding out by heart puncture;

B : CO_2-alone euthanasia

C : Overdose of halothane / O_2

D : Halothane/O_2 anaesthesia, bleeding out by heart puncture followed by cervical dislocation.

**N = normal, C = convalescent, T = total

there was a total of 7 batches, of which, 2 (2 C) were from rats killed by euthanasia method A, 3 (3 N) by method B and 2 (2 C) by method D.

Finally, seven batches gave a median survival of ≥ 61 %, of these, 4 (4 N) are from rats treated by euthanasia method A, 1 (1 N) by method B and 2 (2 C) by method D.

Taking the results Table 27 at 30 and 120 min where the median survival was 0 %, it was concluded that euthanasia method D was best and accordingly was chosen as the standard method for killing the rats before collection of LLF. However, method A was almost as satisfactory.

Different strains of rats

During the early period of investigating the BA of LLF, only rats of the Sprague Dawley strain were used. Subsequently, there was an opportunity to test other rat strains, namely Lewis, Brown Norway, and Hooded Lister. After killing the animals by euthanasia method D, LLF was obtained from both normal and convalescent animals, with Sprague Dawley rats being tested in parallel. Two batches of LLF from normal rats of each strain were collected.

Normal LLF: The animals were of two different age ranges and their fluids were examined for BA towards *B. pertussis* 18-323 substrain A6. The results (Table 28) show that LLF from the Sprague Dawley rats had typical BA, with some killing apparent at time "zero" and no detectable colonies thereafter. The other three rat strains were very similar, with the LLF from Lewis being the most active (and more active than Sprague Dawley), and Brown Norway the least. The 1 % survival in 6 - 7 week Lewis LLF was exceptionally high, and was never equalled by Sprague Dawley LLF in the whole of this research.

The age of rat from which LLF was obtained had no consistent influence across the strains. Thus with Brown Norway and Hooded Lister, the younger animals yielded the most active fluid, whereas, with Sprague Dawley and Lewis the opposite was observed. The bottom line of Table 28 shows that survival of the *B. pertussis* in PBS was within normal ranges at the different sampling times.

Table 26. Summary of percent survival of *B. pertussis* 18-323 incubated for 120 min in different batches of LLF from rats killed by four different euthanasia methods.

Method of euthanasia*	Type of LLF**	Total no. of batches	No. of batches of LLF with median % survival in range:			
			0	1-20	21-60	≥ 61
A	N	5	3	2	0	0
	C	17	6	5	2	4
	T	22	9	7	2	4
B	N	4	0	0	3	1
	C	1	1	0	0	0
	T	5	1	0	3	1
C	N	1	0	1	0	0
D	N	6	5	1	0	0
	C	8	4	0	2	2
	T	14	9	1	2	2
Total	N	16	8	4	3	1
	C	26	11	5	4	6
	T	42	19	9	7	7

*A : Halothane/O_2 anaesthesia followed by CO_2 euthanasia, then bleeding out by heart puncture;

B : CO_2-alone euthanasia

C : Overdose of halothane / O_2

D : Halothane/O_2 anaesthesia, bleeding out by heart puncture followed by cervical dislocation.

**N = normal, C = convalescent, T = total

Table 27: Comparison between bactericidal activity of LLF obtained by euthanasia methods A and D towards *B. pertussis* 18-323 after 30 and 120 min incubation.

Method of euthanasia*	Type of LLF** Total	Total no. of batches	No. of batches of LLF with median of 0 % survival after incubation time:	
			30	120
A	N	5	1	3
	C	17	4	6
	T	22	5	9
D	N	6	4	5
	C	8	2	4
	T	14	6	9

**N = normal, C = convalescent, T = total

Table 28. Bactericidal activity towards *B. pertussis* 18-323 substrain A6 of LLF* from normal rats of different strains and ages. PBS was used as a non-bactericidal control.

Strain of rat	Age (weeks)	% Survival** in duplicate tests after exposure at 37°C for (min)			
		0	30	60	120
Sprague Dawley	(4 - 5)	46 , 30	0 , 0	0 , 0	0 , 0
	(6 - 7)	24 , 16	0 , 0	0 , 0	0 , 0
Lewis	(4 - 5)	10 , 6	0 , 0	0 , 0	0 , 0
	(6 - 7)	1 , 1	0 , 0	0 , 0	0 , 0
Brown Norway	(4 - 5)	34 , 36	0 , 0	0 , 0	0 , 0
	(6 - 7)	80 , 89	0 , 0	0 , 0	0 , 0
Hooded Lister	(4 - 5)	3 , 3	0 , 0	0 , 0	0 , 0
	(6 - 7)	41 , 32	0 , 0	0 , 0	0 , 0
(PBS control)	-	100 , 100	94 , 77	59 , 52	43 , 22

*LLF pooled from 8 rats of each strain.

** calculation of % survival values was based on PBS time-zero count.

Convalescent LLF: Rats were infected intratracheally with *B. pertussis* 18-323 encased in agarose beads with dimethyl β-cyclodextrin, and the LLFs were obtained at the convalescent stage. In marked contrast to the above results with LLF from normal rats, the corresponding convalescent LLFs were mostly much less active (Table 29). With Sprague Dawley convalescent LLFs of both ages, the results were consistent with those reported previously (Figure 30). Similar to Sprague Dawley were the older Lewis and the younger Hooded lister, As before, with the normal LLFs, Brown Norway convalescent LLF was the most active and at both ages. LLF from the 6 - 7 week Lewis and the 4 - 5 week Hooded Lister had no BA. Indeed the Hooded Lister LLF appeared to act as a growth medium.

To facilitate further comparison of possible rat strain and age effects in BA of LLF, the 30 min results already presented in Tables 28 and 29 are retabulated in Table 30. This highlights the general conclusions that 1) with various ages and strains of rats, normal LLF is more bactericidal than convalescent ; 2) that Brown Norway rats may have LLF that is more active than LLF from the other strains ; and 3) that convalescent Hooded Lister LLF may be the least active. The 30 min incubation time chosen for these comparisons allowed close to 100 % survival of the *B. pertussis* in PBS.

LLF from other animal species and human

LLF was obtained from 7 animal species and from a human patient, and tested for BA towards *B. pertussis* 18-323 substrain A6. One LLF batch from each species was available and was tested against normal rat LLF as a positive control. PBS was the negative control. These studies were not all done on the same occasion and batch nos 15, 33, and 40 of rat LLF were used.

The results in the various sections of Figure 40 show that none of the other animal species yielded LLF as active as that from the normal rat. In fact, the only species where any BA was detected were human (Figure 40 A) and calf (Figure 40 E). LLF with no BA towards *B. pertussis* was obtained from chicken, rabbit, horse, sheep, dog and mouse.

Table 29. Percent survival of *B. pertussis* 18-323 substrain A6 treated with LLF from convalescent rats of different strains and ages. PBS was used as a non-bactericidal control.

Strain of rat	Age (weeks)	% Survival* in duplicate tests after exposure at 37°C for (min)			
		0	30	60	120
Sprague Dawley	(4 - 5)	101, 97	68 , 43	27 , 37	19, 23
	(6 - 7)	82 , 83	18 , 24	6 , 6	8 , 14
Lewis	(4 - 5)	118 , 105	26 , 21	8 , 0	6 , 9
	(6 - 7)	113 , 113	67, 66	108 , 84	83 , 136
Brown Norway	(4 - 5)	5 , 8	0 , 0	0 , 0	0 , 0
	(6 - 7)	53 , 44	0 , 0	0 , 0	0 , 0
Hooded Lister	(4 - 5)	119 , 104	113 , 129	229 , 184	267 , 364
	(6 - 7)	87 , 73	90 , 97	67 , 61	25 , 50
(PBS control)	-	100 , 100	94 , 77	59 , 52	43 , 22

* calculation of % survival values was based on PBS time-zero count.

Table 30. Comparison of percent survival of *B. pertussis* 18-323 substrain A6 after 30 min at 37°C in LLF from normal and convalescent rats of different strains and ages.

Strain of rat	% Survival*			
	Normal		Convalescent	
	4-5	6-7	4-5	6-7 week
Sprague Dawley	0 , 0	0 , 0	68 , 43	18 , 24
Lewis	0 , 0	0 , 0	26 , 21	67 , 66
Brown Norway	0 , 0	0 , 0	0 , 0	0 , 0
Hooded Lister	0 , 0	0 , 0	113 , 129	90 , 97
(PBS control)	94, 77	94, 77	94, 77	94, 77

* calculation of % survival values was based on PBS time-zero count.

Figure 40. Bactericidal activity of different normal animal and human LLF towards *B. pertussis* 18-323 substrain A6. PBS was used as a non-bactericidal control. Data are the mean of 2 to 3 experiments. Error bars are SEM which when small do not project beyond the points.

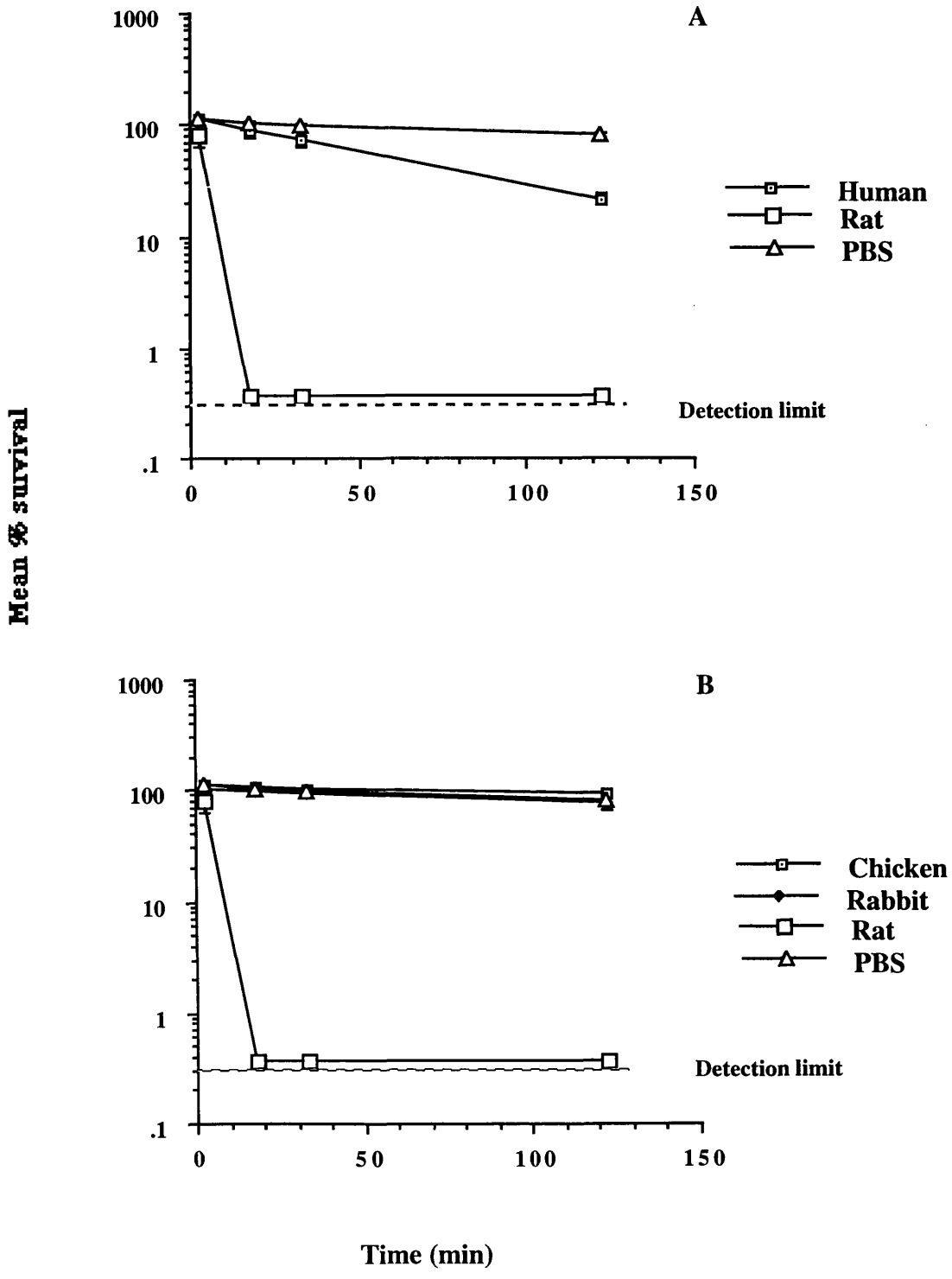
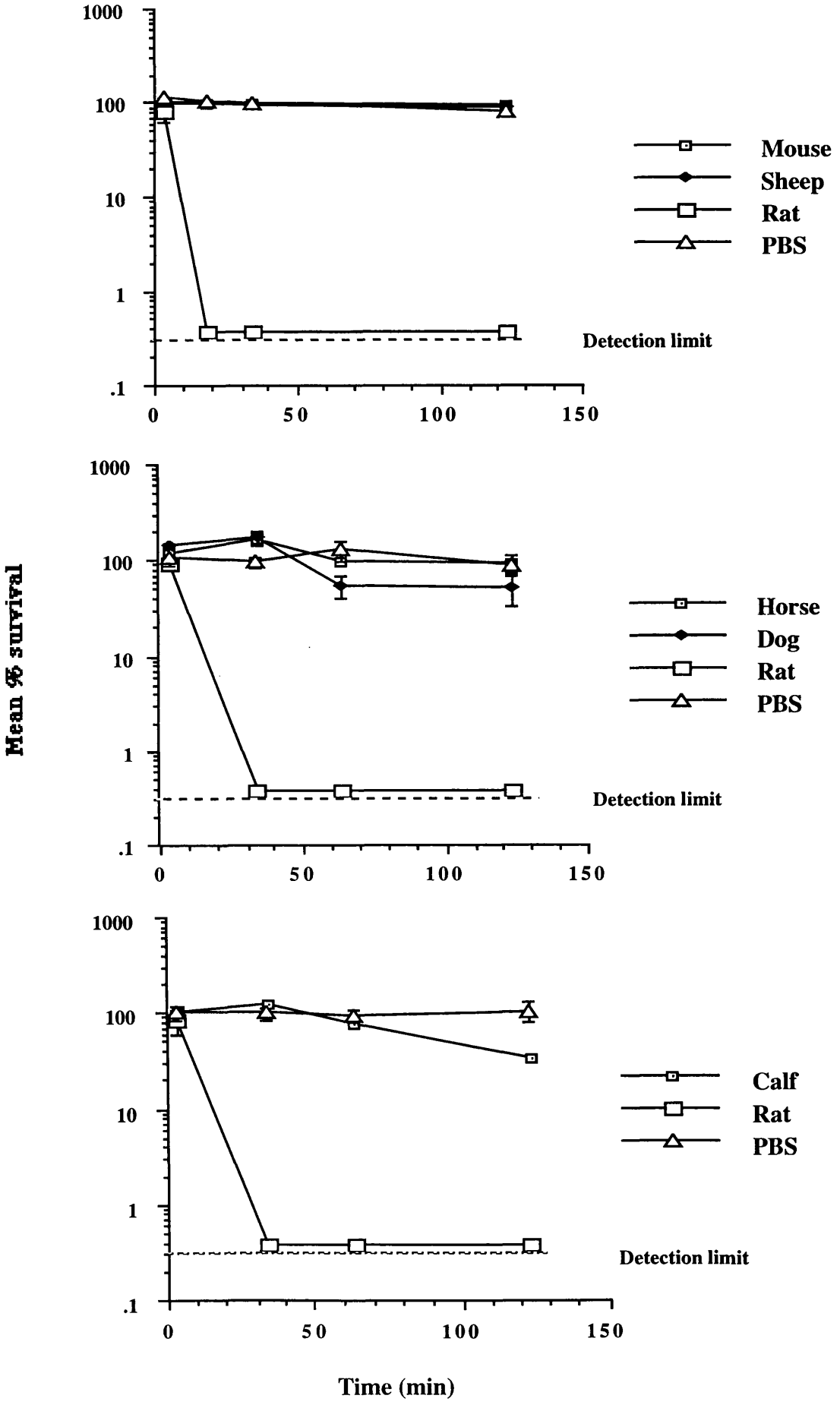


Figure 40 continued



Other Strains of *Bordetella pertussis*

After the confirmation of the BA of normal and treated rats LLF towards *B. pertussis* 18-323 was established, the idea of testing other strains of *B. pertussis* phase I and IV was raised to see whether they have the same sensitivity to the bactericidal effect of LLF from normal and treated rats as with *B. pertussis* 18-323.

Phase I strains, including *B. pertussis* lux

Twenty two experiments were done to test the BA of LLF towards 11 *B. pertussis* phase I strains, the bacterial suspensions being prepared, standardised and diluted as with *B. pertussis* 18-323. PBS was included in all experiments as a non-bactericidal control. All *B. pertussis* strains were tested with normal and convalescent rat (exposed to euthanasia method A) LLF batch no. 15 group 1, except strain 30042 phase I which was tested with LLF from both normal and convalescent rats (exposed to euthanasia method B) batch no. 34.

Convalescent rats batch no. 15 group 1, were challenged with *B. pertussis* L84 phase IV encased in agarose beads and exposed to ether inhalation for 2 min, and LLF then collected 28 days after infection. The convalescent rats batch no. 34, had been challenged with *B. pertussis* 18-323 EH1 phase I incorporated in agarose beads, then the animals were exposed to ether inhalation for 2 min and, LLF obtained 28 days after infection.

It should be noted that the rats of batch no. 34 were exceptional in giving normal LLF that had *less* BA than the fluid from convalescent animal towards both phase I and phase IV test organisms. The only feature of those animals that might be responsible was the collection of LLF by euthanasia method B.

According to the results presented in Figures 41, all *B. pertussis* phase I strains showed between low and moderate loss of viability during incubation in normal rat LLF. With LLF from convalescent rats, the loss of viability was faster. However this particular convalescent LLF had come from rats infected with phase IV *B. pertussis*.

Figure 41. Bactericidal activity of normal (N) and convalescent (C) rat LLF towards different strains of *B. pertussis* phase I, with PBS as the non-bactericidal control. Data are the mean of two experiments with one duplicate of each. Error bars are SEM which when small do not project beyond the points.

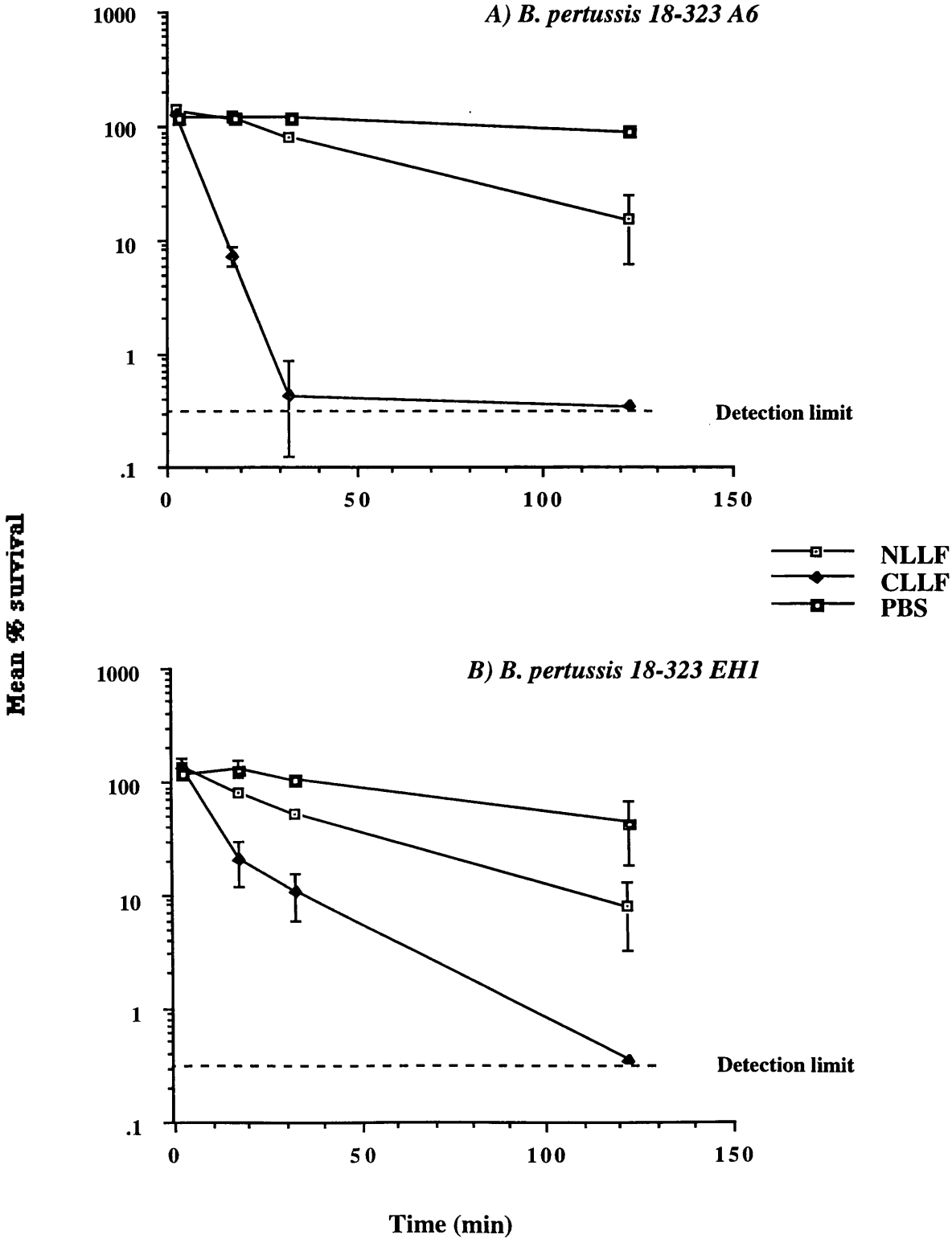


Figure 41. continued

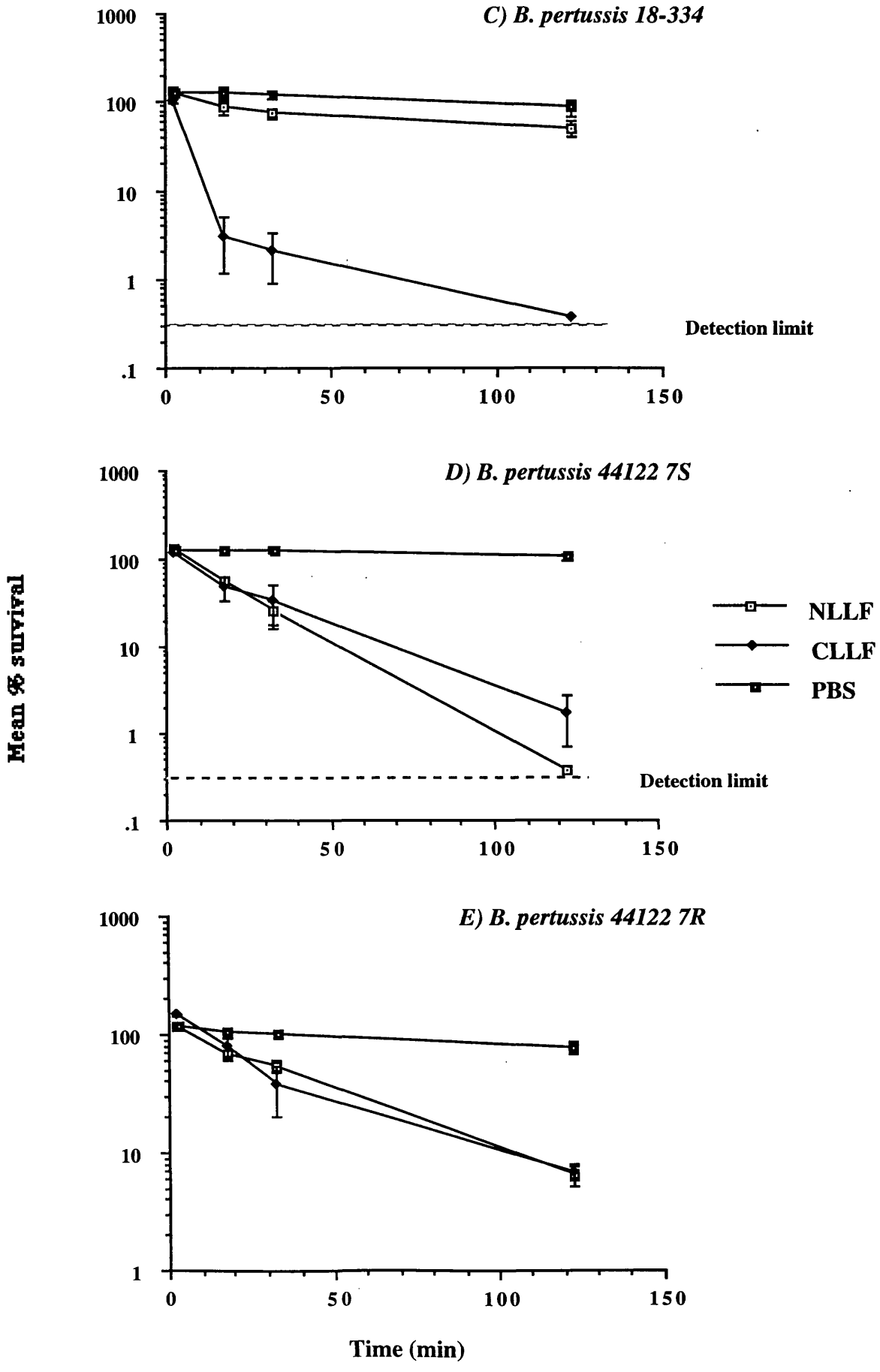


Figure 41. continued

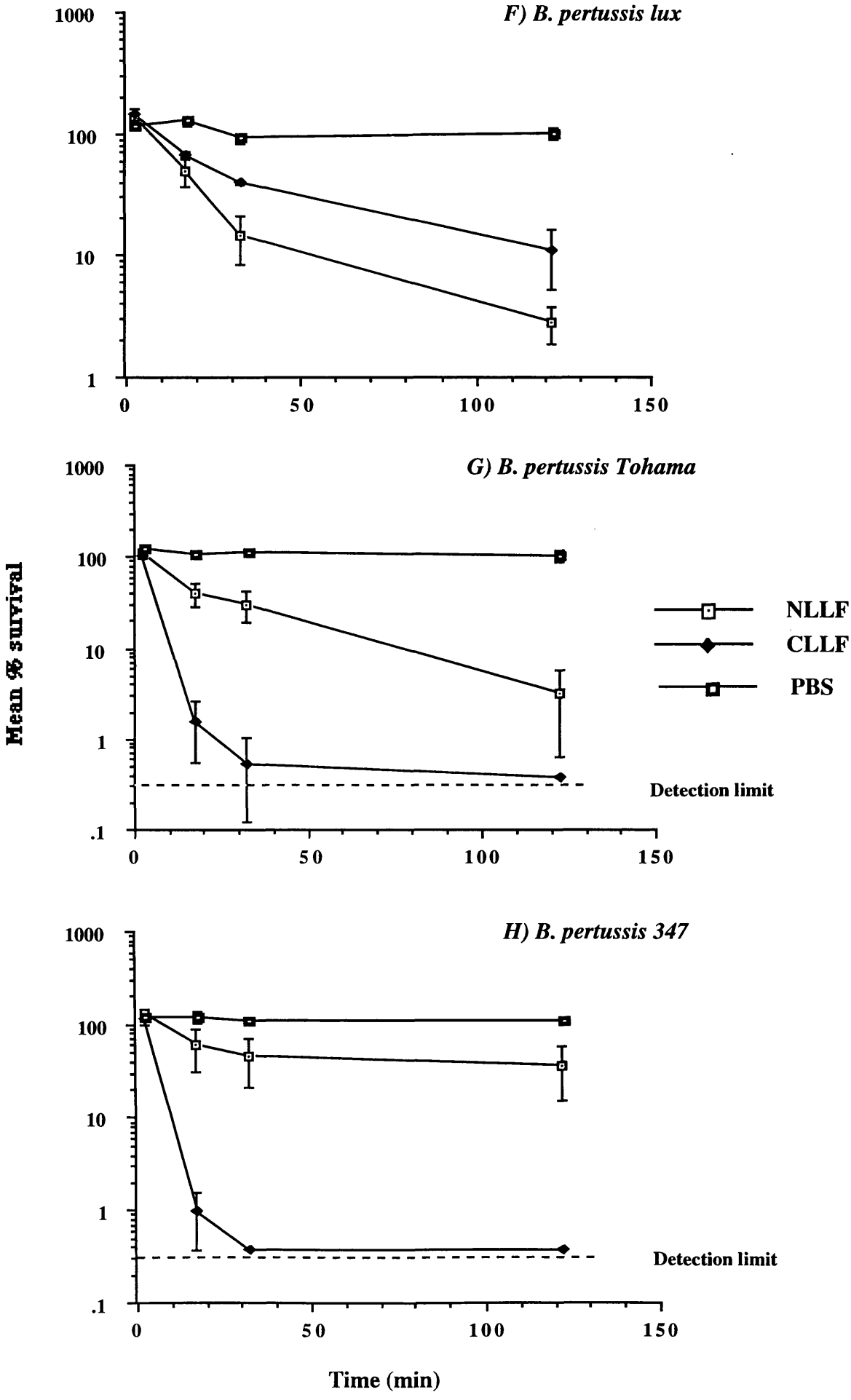


Figure 41. continued

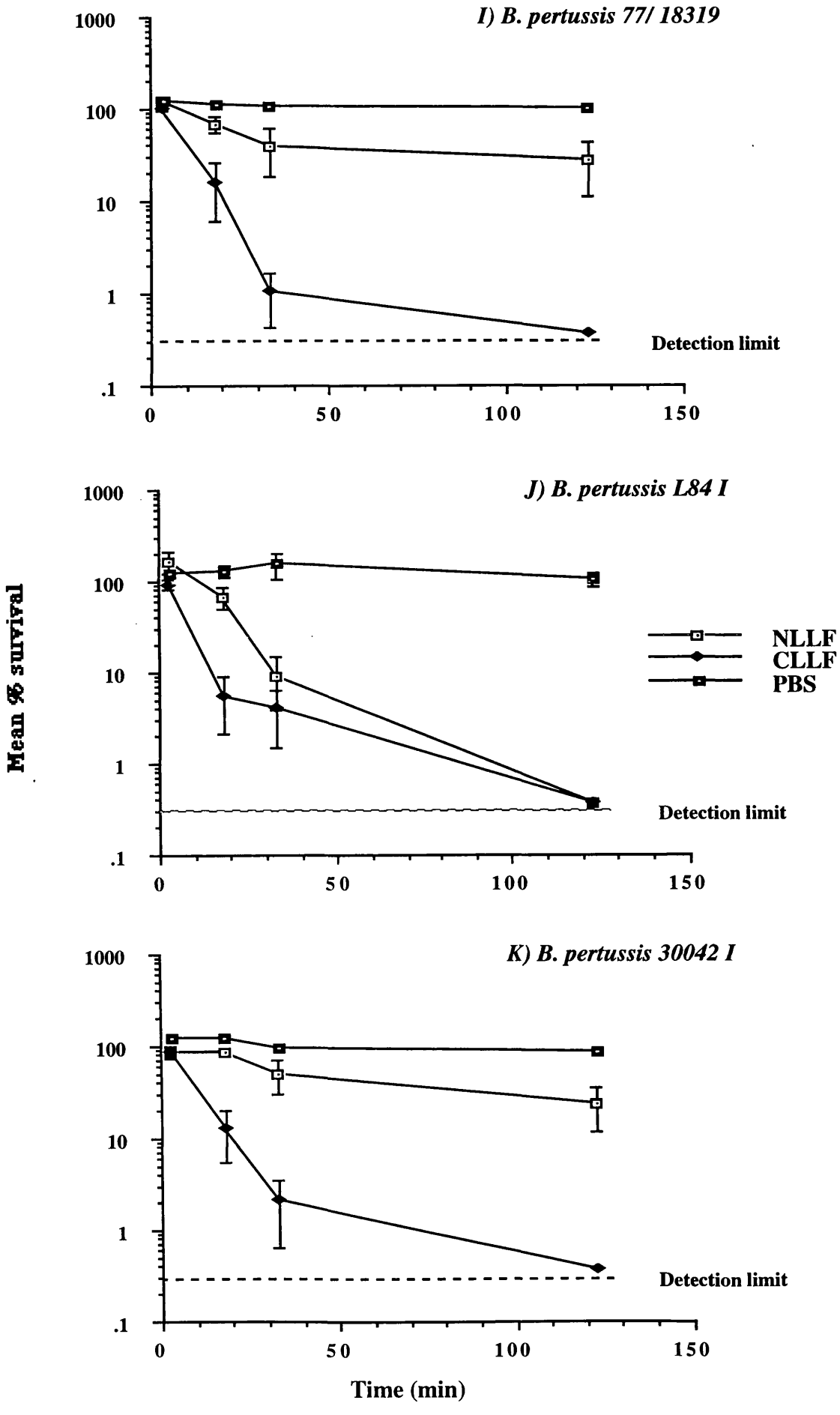


Table 31. Comparison of bactericidal activity of LLF towards *Bordetella pertussis* 18-323 sub-strain A6 and *B. pertussis* lux. CL medium and PBS were used as non-bactericidal controls.

B. pertussis strain	Test fluids	% Survival** after exposure at 37°C for (min)				
		0	30	60	120	1440
18-323 A6	LLF*	119 , 65	0 , 0	0 , 0	0 , 0	0 , 0
	CL medium	141 , 94	131 , 100	125 , 82	147 , 97	406, 394
	PBS	100 , 100	94 , 82	113 , 124	88 , 76	0 , 9
Lux	LLF*	96 , 101	1 , 0	1 , 1	1 , 0	0 , 0
	CL medium	88 , 79	90 , 92	99 , 101	71 , 76	221 , 214***
	PBS	100 , 100	99 , 84	66 , 77	63 , 87	3, 2

LLF* = Batch no. 40

**calculation of % survival was based on PBS time-zero count data.

*** Both of these numbers are the outcome of dividing 300 (no. of cells assumed to be equal to almost-confluent growth) by the PBS time-zero count and multiplying by 100.

Further viable count examination of the BA of LLF from rats (euthanasia method D) batch no. 40 towards *B. pertussis* 18-323 A6 and lux was performed. CL medium and PBS were included as a control fluids. The results in Table 31, showed a complete killing of both *B. pertussis* strains after 30 min of incubation. No or very little loss of viability during 120 min incubation in CL medium was found, and a growth of both strains was achieved after 1440 min. Strain 18-323 grew better in CL medium than lux. Moderate reduction in percent survival of both strains in PBS was observed.

Phase IV strains

Two phase IV strains of *B. pertussis* were used in the following experiments, but because they were tested several months apart, different batches of LLF were used. Also the euthanasia methods with which the LLFs were collected were different.

In the bactericidal tests, the results (Table 32) include PBS as the usual control. The most interesting observation with both of the phase IV strains and both batches of normal LLF was the absence of BA. In fact, the LLFs acted as growth media, in that there was little or no loss of viability at 120 min and an apparent increase in viable count at 1440 min. It should be noted that in contrast with phase I *B. pertussis*, normal LLF of batch no. 15 (c. f. Figure 30) gave little activity compared with the activity of batch no. 34.

The convalescent LLFs were different from the normal fluids collected at the same time in showing moderate to strong BA. The convalescent LLF collected by euthanasia method B was more active than the fluid collected by method A. This result was similar to that previously observed with Phase I *B. pertussis* (Figure 29 and 30). Both phase IV strains slowly lost viability in PBS during 120 min at 37°C, but there were still a few viable cells at 1440 min. Other than batch no. 34, none of the other 11 LLF batches (3 normal and 8 convalescent) which had been tested for their BA towards *B. pertussis* 30042 IV, was active.

Table 32. Survival of *B. pertussis* phase IV strains L84 and 30042 in normal (N) and convalescent (C) rat LLF. PBS was used as a non-bactericidal control. Data of two experiments each with one duplicate.

B. p phase IV strain	Test fluids	Mean (±SEM) % survival* after exposure at 37°C for (min)				
		0	15	30	120	1440
L84	NLLF**	104.8 ± 2.5	102 ± 5.4	98.7 ± 4.4	89.8 ± 6.0	131.9 ± 32.3
	CLLF**	103.5 ± 3.3	93.2 ± 3.4	87.8 ± 3.3	65.5 ± 4.0	3.13 ± 2.5
	PBS	100 ± 0.0	102.9 ± 4.5	91.7 ± 3.2	83.3 ± 4.2	46.2 ± 6.9
30042	NLLF***	98.8 ± 2.9	97.2 ± 3.1	94.4 ± 3.4	90.8 ± 3.7	167.8 ± 12.5
	CLLF***	96.6 ± 2.6	12.2 ± 3.5	3.5 ± 0.5	0.31 ± 0.3	0 ± 0
	PBS	100 ± 0.0	95.8 ± 2.5	95.6 ± 3.9	84.2 ± 1.4	3.6 ± 2.1

*calculation of % survival was based on PBS time-zero counts.

**NLLF batch no. 15, see Appendix 9 footnote for code A_1

**CLLF batch no. 15, see Appendix 11 footnote for code A_1

***NLLF batch no. 34, see Appendix 9 footnote for code A_6

***CLLF batch no. 34, see Appendix 11 footnote for code A_{18}

Comparison of LLF and serum with phase IV

For the comparison of BA of LLF and serum, the single phase IV strain 30042 was used. Also a single batch of rats and a single euthanasia method was used as the source of normal and convalescent LLF and serum. This batch was the same as used in lower half of Table 32. In addition CL medium was used as a further control.

In confirmation of the previous observation, normal LLF (Table 33) acted as a growth medium for phase IV *B. pertussis* and was indistinguishable from CL medium for up to 1440 min. The convalescent LLF was, as before, quite strongly bactericidal but not nearly as active as the two sera. Normal rat serum was so active as to give a low count at zero time, indicating killing during the few minutes of initial sampling. Convalescent rat serum was even more active. PBS was slowly bactericidal during the 120 min of observation.

A summary comparison of survival of *B. pertussis* strain 18-323 A6 Phase I and 30042 IV in LLF and serum from normal and convalescent rats batch no. 34 is presented in Table 34. CL medium and PBS both were used as a control fluids.

Loss of viability of phase I during 120 min incubation in normal LLF and complete killing after 1440 min were found, whereas no reduction in percent survival of phase IV throughout 120 min incubation with a significant growth after 1440 min were recorded. Both *B. pertussis* strains failed to survive in LLF from convalescent rats.

Serum from both normal and infected rats of the same batch was highly bactericidal towards both *B. pertussis* strains. A good survival and growth of both strains in CL medium were noticed. Slow and no reduction of cell viable count respectively of phase I and IV was demonstrated.

Summary comparison of phase I and IV

B. pertussis phase I and IV strains (All strains tested with LLF from rats batch no. 15, except strains 30042 I and IV which were tested with LLF batch no. 34) were compared for bactericidal sensitivity towards LLF from normal and convalescent rats, with PBS as a control.

Table 33: Bactericidal activity of lung lavage fluids (LLF) and serum from normal (N) and convalescent (C) rats batch no. 34 towards *B. pertussis* 30042 IV. PBS and CL medium were used as a non-bactericidal control.

Test Fluids	Mean % survival* ±SEM (No. of experiments) during incubation at 37°C for a time of (min)					
	0	15	30	60	120	1440
NLLF	103.5±4.4	97.3±3.1	100.8±4.4	100.3±4.3	91.0±4.4	168.0±12.5
	(8)	(4)	(8)	(3)	(7)	(4)
CLLF	93.9±1.5	12.3±3.6	5.4±1.2	1.6±0.9	0.27±0.2	0
	(10)	(4)	(10)	(5)	(9)	(4)
NRS	15.3±4.1	0.13±0.1	0.13±0.1	nt	0	0
	(4)	(4)	(4)	-	(4)	(4)
CRS	3.5±0.9	0	0	nt	0	0
	(4)	(4)	(4)	-	(4)	(4)
CL medium	100.8±3	98.5±1.7	99.6±4.3	99.8±7.9	91.9±3.1	170.5±10.6
	(8)	(4)	(8)	(4)	(8)	(4)
PBS	100±0.0	nt	100.8±3.1	93.2±6.2	86.4±7.5	nt
	(6)	-	(6)	(5)	(5)	-

*calculation of % survival was based on PBS time-zero count.

Table 34. Comparison of percent survival between *B. pertussis* 18-323 A6 phase I and *B. pertussis* 30042 phase IV in LLF and serum (RS) from normal (N) and convalescent (C) rats batch no. 34.

Test Fluids	Mean % survival*±SEM (No. of experiments) during incubation at 37°C for a time of (min)					
	B.pertussis 18-323 A6 I			*B.pertussis* 30042 IV		
	30	120	1440	30	120	1440
NLLF	45.0±13.2 (3)	6.7±6.7 (3)	0 (2)	100.8±4.4 (8)	91.0±4.4 (7)	168.0±12.5 (4)
CLLF	24.1±4.7 (7)	1.4±0.6 (7)	0 (2)	5.4±1.2 (10)	0.27±0.2 (9)	0 (4)
NRS	3.0±1.7 (3)	0 (3)	0 (2)	0.13±0.1 (4)	0 (4)	0 (4)
CRS	1.0±1.0 (3)	0 (3)	0 (2)	0 (4)	0 (4)	0 (4)
CL medium	98.6±4.3 (7)	94.7±6.9 (7)	600±0.0** (2)	99.6±4.3 (8)	91.9±3.1 (8)	170.5±10.6 (4)
PBS	93.5±4.1 (6)	76.6±2.5 (6)	nt -	93.2±6.2 (5)	100.8±3.1 (5)	nt -

*calculation of % survival was based on PBS time-zero count data.

** This large number is the outcome of dividing 300 (no. of cells assumed to be equal to almost-confluent growth) by the PBS time-zero count data and multiplying by 100.

Table 35 show that all *B. pertussis* phase I strains showed a 50 % reduction in viability in normal rat LLF as early as 12 min, and as late as 92 min. A 90 % reduction was seen as early as 33 min, and as late as > 120 min. However, the 50 % reduction in viability in convalescent rats LLF was as early as 4 min, and as late as 32 min, and 90 % reduction in the same fluids was as early as 9 min and as late as > 120 min.

For *B. pertussis* phase IV strains, no BA of normal LLF was detected towards either of the two strains (L84 and 30042). While, in convalescent LLF, a 50 and 90 % reduction in viability of strain 30042 IV (tested with LLF of batch no. 34) was achieved respectively after 7 and 21 mins, compared with no BA of LLF from rats batch no. 15 towards strain L84 IV was found. In PBS as a non-bactericidal control, all *B. pertussis* strains (phase I and IV) show a good survival and > 120 min was needed for a 50 and 90 % reduction in viability.

EFFECT OF LLF ON BACTERIAL LUMINESCENCE

Development of Method

Preliminary experiments

For rapid testing, the effect of rat LLF on bacterial luminescence was explored, since experiments could be completed within a few hours instead of waiting 5 days for *B. pertussis* 18-323 colonies to be ready for counting. For this purpose, a standardized (A_{540nm} = 0.45) suspension of *B. pertussis* lux was incubated with test and control fluids for 1 h at 37°C. Then the test mixtures were transfered to the luminometer cuvettes, placed in the luminometer and decanal added. The decanal was added as 20 µl of 0.5 % (v/v) solution in DMSO and PBS so that the volume of the undiluted aldehyde per test was 100 nl. The peak luminescence (light output) in millivolts was then determined over a 10 min period.

Table 36 shows that there was little reduction in bacterial luminescence by either normal or convalescent rat LLF. In contrast, a large reduction in luminescence was produced by normal and convalescent rat serum. In experiment 3 of Table 36, the use of an

Table 35. Summary comparison of percent survival of *B. pertussis* phase I and IV strains incubated in normal and convalescent rat LLF and PBS.

Strain	Phase	Time (min) for killing %					
		Normal		Convalescent		PBS	
		50	90	50	90	50	90
18-323 EH1	I	30	108	10	32	106	>120
18-323 A6	I	55	>120	7	15	>120	>120
18-334	I	92	>120	6	12	>120	>120
44122 7S	I	18	49	14	65	>120	>120
44122 7R	I	24	99	32	96	>120	>120
Lux	I	16	48	24	120	>120	>120
Tohama	I	12	73	4	9	>120	>120
347	I	22	>120	4	9	>120	>120
77/18319	I	22	>120	7	19	>120	>120
L84	I	18	33	4	13	>120	>120
L84	IV	>120	>120	>120	>120	>120	>120
30042	I	29	>120	5	18	>120	>120
30042	IV	>120	>120	7	21	>120	>120

Table 36: Effect of normal (N) and convalescent (C) rat LLF and serum (RS) on the luminescence of *B. pertussis* lux with 100 nl of decanal after 1 h incubation at 37°C. PBS was used as a non-bactericidal control.

Expt no	Age of decanal solution	Luminescence (mv) after 1h incubation in test fluid				
		NLLF	CLLF	NRS	CRS	PBS
1	Fresh	140	140	58	66	200
2	Fresh	160, 180	160, 180	55, 55	55, 55	150, 154
3	> 4 days old	8, 8	14, 14	14, 16	14, 16	8, 10

NLLF & NRS batch no 15, see code A1 in Appendix 9 footnote for more details.

CLLF & CRS batch no 15, see code A1 in Appendix 11 footnote for more details.

old solution of decanal was associated with low luminescence. Also in early experiments there was much unexpected variation in the light output of the same -70°C stock of *B. pertussis* lux when incubated in PBS. For example, the luminosity ranged from 100 mv down to 10 mv for no apparent reason. It was therefore clear that the method for studying death or survival of *B. pertussis* lux in experimental fluids required further development.

Age of bacterial culture

Standardized (A_{540nm} = 0.45) *B. pertussis* lux suspension (in 1 % CAA instead of PBS) of 36, 48, 60, 72 and 84 h old lawn-grown cultures at 37°C were prepared, and 100 µl of each was added to 400 µl of PBS. The experiment, in addition to examining different ages of culture, also had a change in decanal concentration from that used above. Instead of having 100 nl of the aldehyde, 18 nl was used. The test mixtures were incubated for 1 h at 37°C in the luminometer, after which, 20 µl of 0.09 % (v/v) of decanal in DMSO and DW was added (to deliver 18 nl of neat decanal). The highest level of luminescence (Figure 42) of *B. pertussis* lux was from 48 h-grown cells. This age of culture was significantly better than 36 h, or 60 h or older cultures. Accordingly, 48 h-grown cells were used for all later experiments. In 27 observations, *B. pertussis* lux suspended in 1 % CAA survived well in PBS at 37°C for 1 h in that a median % survival of 82 with upper 95 % confidence limit of 87.2 was found.

Bacterial concentration

A series of experiments was done in which the A_{540nm} = 0.45 bacterial suspension was added in volumes from 12.5 to 100 µl to the PBS to give a final volume of 1.0 ml. Luminescence varied in an approximately linear fashion with the log (quantity of luminous bacteria) as shown in Figure 43. In subsequent experiments, 100 µl of the standardized suspension was added to all test and control fluids.

Figure 42. Effect of bacterial culture age on the luminescence of *B. pertussis* lux. Data are the mean (±SEM) of 2 observations in the same experiment.

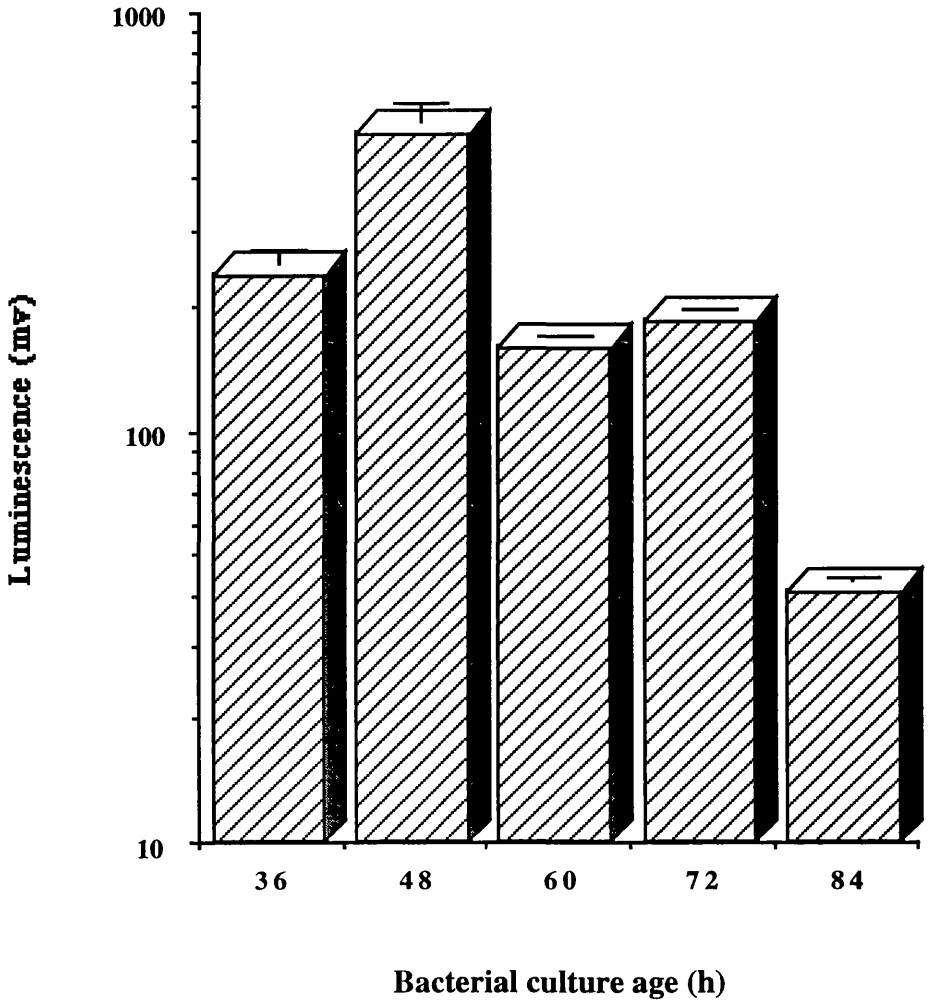
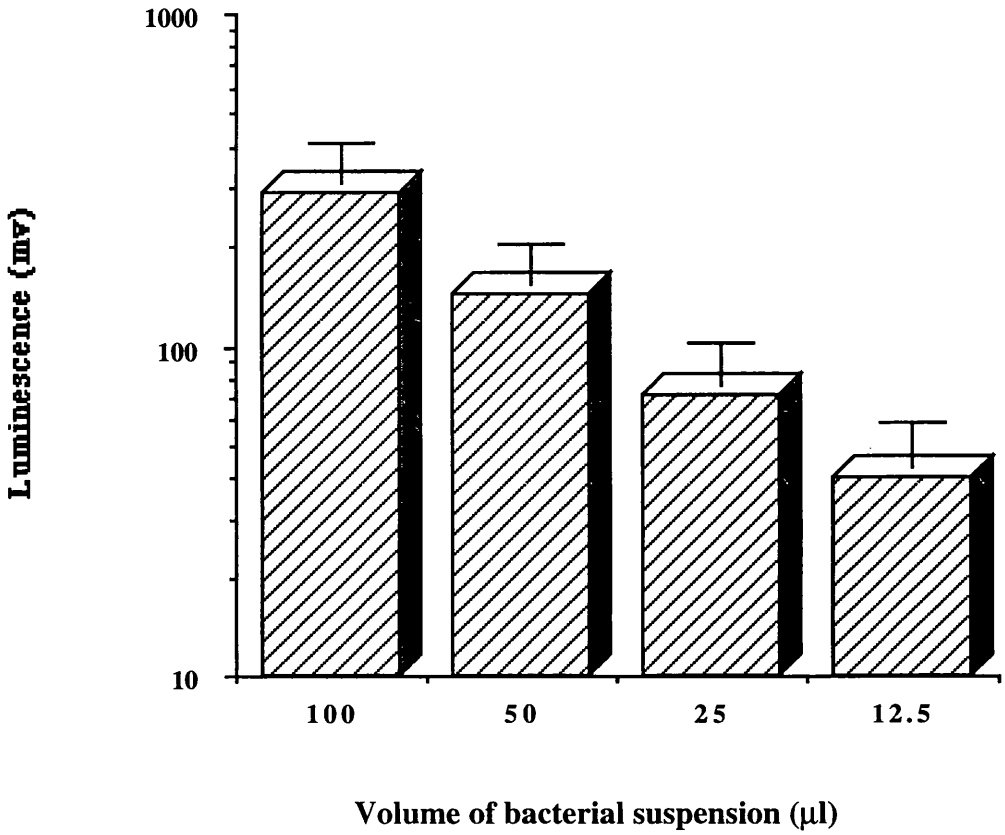


Figure 43. Effect of different *B. pertussis lux* (A_{540nm} = 0.45) volumes incubated in PBS (to give a final volume of 1ml of test mixture) for 1 h on the extent of luminescence. Data are the mean (±SEM) of three experiments.



Decanal concentration and age

A problem in the early experiments was that decanal and DMSO, if used at too high concentrations, appeared to kill *B. pertussis* lux suspended in PBS. This interpretation was reached from tests in which a second dose of decanal (dissolved in DMSO) failed completely to stimulate luminescence.

Therefore, attention was directed at the adjustment of decanal and DMSO concentrations to stimulate strong luminescence and allow frequent addition of decanal at different times without harming the bacteria. For this purpose, two different solutions of 10 % and 20 % (v/v) decanal dissolved in DMSO were prepared after which both mixtures were further diluted in PBS or DW giving a different range of final decanal volumes of 100 to 18 nl, and final DMSO volumes of 900 to 72 nl.

Three of the 10 different dilution schemes tested are described in Table 37. Preparation method A (Figure 44), shows a reduction in luminescence of *B. pertussis* incubated in PBS. Whereas, an increase of light output of bacteria incubated in CL medium was detected.

In method C, an improvement of bacterial luminescence in PBS and CL medium was detected, while, a decrease in 1 % CAA was observed. In preparation method J, no decrease in bacterial luminescence incubated in PBS and an increase in CL medium were found. In 1 % CAA, an improvement was found. Finally it should be noted that method C was the best. All the different dilution schemes are in Appendix 14 and 15. Full results of the effect of frequent addition of decanal (at different volumes) on the luminescence of *B. pertussis* incubated in PBS, CL medium and 1 % CAA at 37°C for various time are in Appendix 16.

Even after these successful suitable final decanal concentrations had been determined, a gradual decrease in light output of *B. pertussis* lux from the same - 70°C stock was noticed in PBS. Attention was focused on the age of the prepared decanal which usually was at about 2, 3 and > 4 days old. An experiment was done testing the bacterial luminescence of *B. pertussis* lux treated with 1 and 48 h prepared decanal. More luminescence from bacteria treated with fresh (1 h old) prepared decanal than from bacteria

Table 37. Three different dilution schemes of 10 and 20 % (v/v) decanal dissolved in dimethyl sulphoxide (DMSO) as the primary solvent and diluted in PBS or DW as the secondary diluent.

Decanal reagent method	Secondary diluent	Final volume (nl) per test	
		Decanal	DMSO
A	PBS	100	900
C	DW	20	180
J	DW	18	72

Figure 44. Effect of three of the ten different dilution schemes for decanal on the luminescence of *B. pertussis* lux when incubated in PBS, CL medium and, with methods C and J, in CAA. Data are the mean of one to three observations. Error bars are SEM which when small do not project beyond the points.

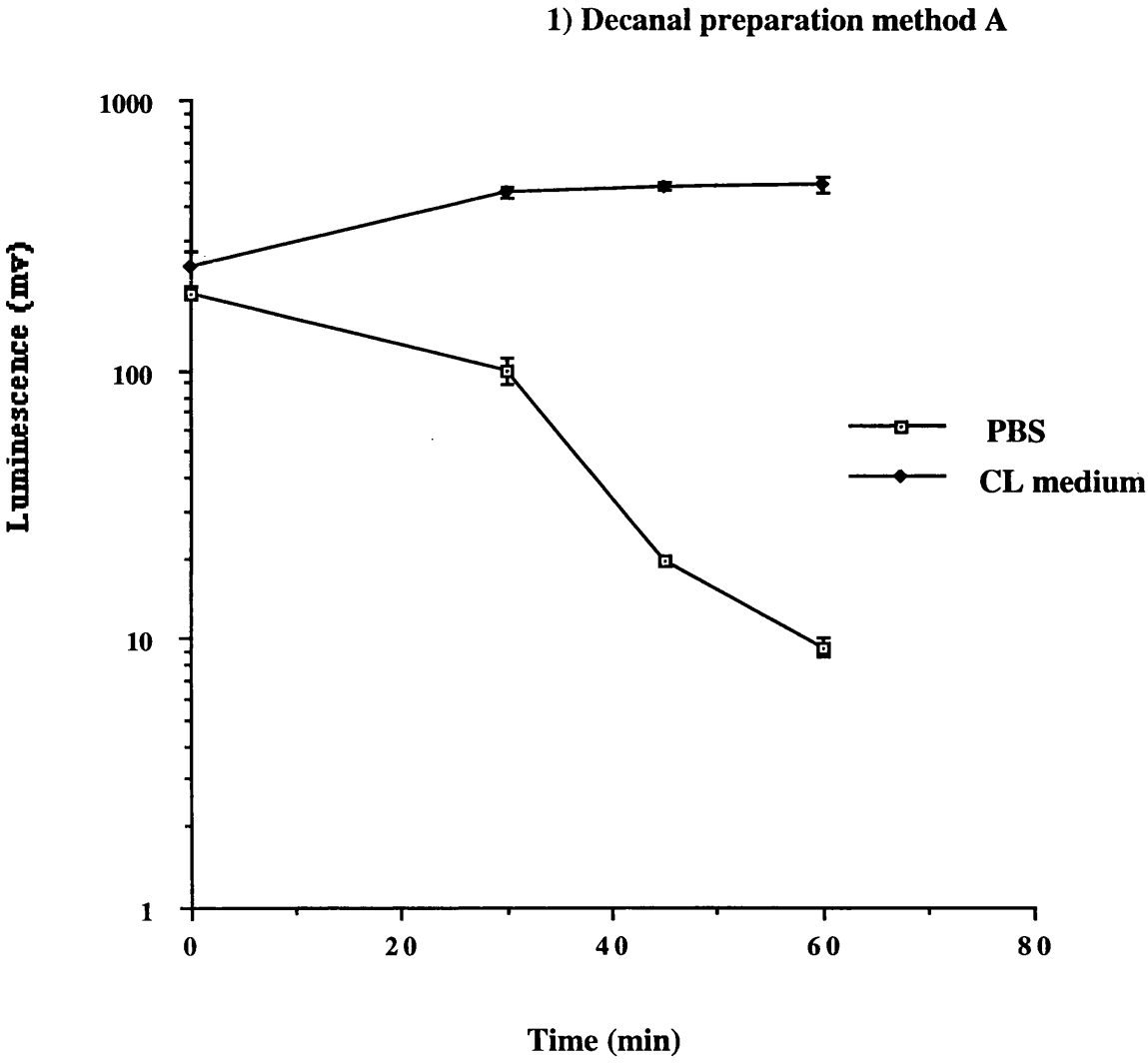
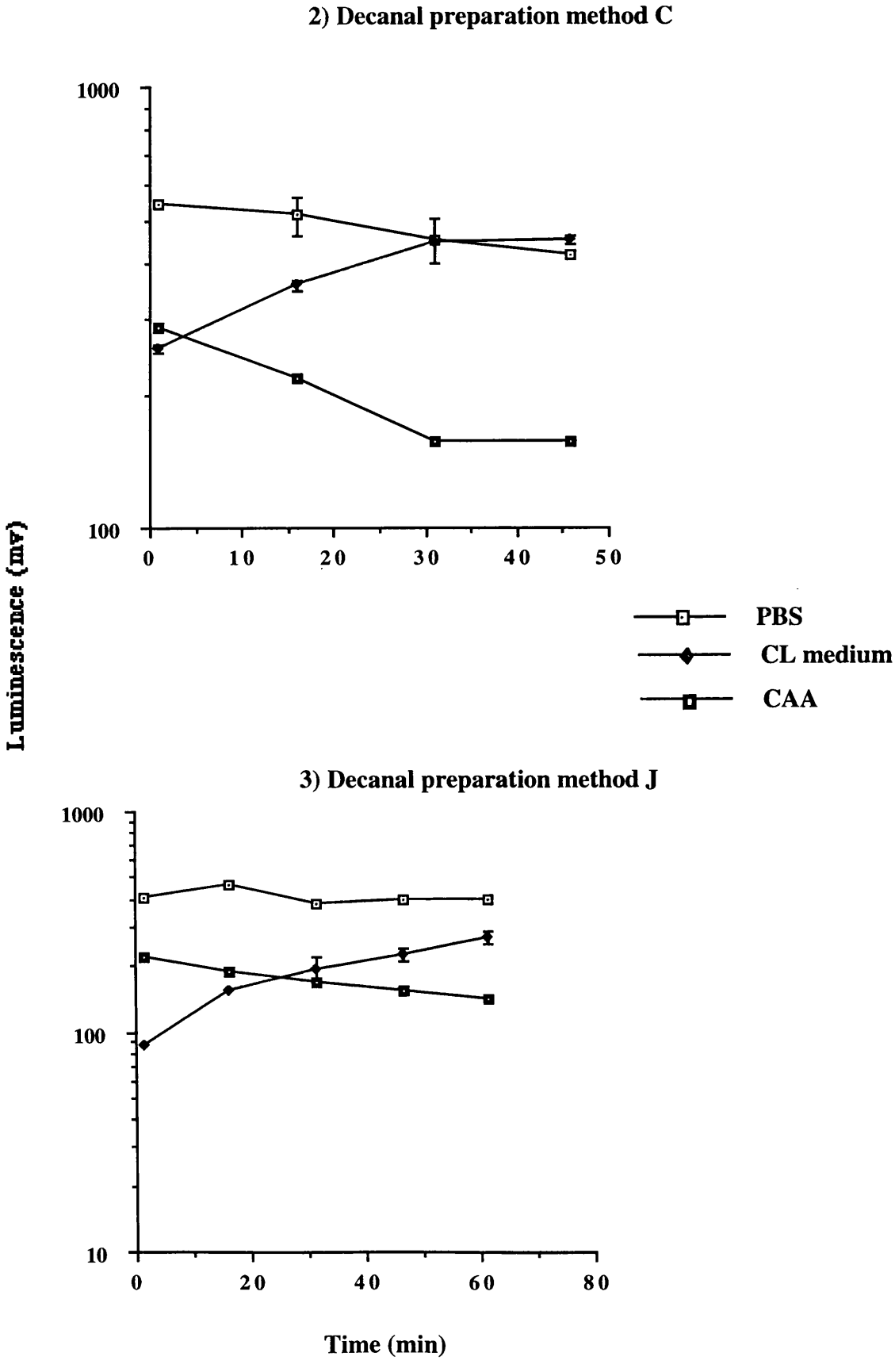


Figure 44. continued



treated with 48 h old prepared decanal was found.

Batch Variation of LLF

Extensive tests by luminometry, similar to those by viable counting with 18-323, were made to find out whether there were differences between LLF samples obtained from different batches of normal and convalescent rats. In collecting these samples, different methods of euthanasia had been used. Thus, thirteen LLF batches from normal rats and 2 from convalescent rats were tested as neat and four-fold dilutions to 1/64 in PBS.

Results of the effect of these different LLF batches on bacterial luminescence are summarised in Table 38, with details in Appendix 17. LLF obtained from rats treated with euthanasia method D were mostly slightly more active than LLF from euthanasia method A. Euthanasia method C was only used on one occasion and although it gave LLF less active than A or D it is difficult to draw firm conclusion. Whereas, no activity was found with Euthanasia method B. There was more BA with LLF from normal rats than from convalescent animals.

The footnote to Table 38 indicates that the use or avoidence of ether, at the time the rats were given their intrabronchial infection with *B. pertussis* did not influence the non-bactericidal activity of the convalescent LLF obtained several weeks later.

B. pertussis Lux Luminescence Compared with 18-323 Viable Counts

Having shown above that LLF reduced the luminescence of *B. pertussis* lux and (in previous section) the viable count of *B. pertussis* 18-323, it was felt necessary to make more direct comparisons. It should be noted that the viability test with *B. pertussis* 18-323 used the bacteria at an initial concentration of 3000 / ml whereas luminometry with *B. pertussis* lux required approximately 10^4-times higher concentration. A series of experiments was done using LLF from 4 different batches of normal rats (exposed to 4 different euthanasia methods) and 2 different batches of convalescent rats (euthanasia method D).

Table 38. Batch to batch variation of LLF from normal (N) and convalescent (C) rats in bactericidal activity towards *B. pertussis* lux.

LLF batch no.	Method of euthanasia	Treatment of rats	Bactericidal activity (µl)* Reduction of luminescence (% IEP)	
			Lum 50	Lum 90
15	A	N	6 , 30	90 , 145
17	A	N	15	285
33	A	N	3 , 37	75 , 245
34	B	N	135 , >400	>400 , >400
35	B	N	>400 , >400	>400 , >400
37	B	N	>400	>400
38.1	B	N	>400 , >400	>400 , >400
38.2	C	N	72 , 83	310 , >400
39	D	N	7 , 12	70 , 205
40	D	N	5 , 20	75 , 207
41	D	N	70 , 205	>400 , >400
42	D	N	>400 , >400	>400 , >400
43	D	N	5 , 25	55 , 107
43	D	C_3	195 , >400	>400 , >400
43	D	C_4	>400 , >400	>400 , >400

* Volume of test fluid required to give either 50 % or 90 % reduction of luminescence (Lum)[(ie. 50 % or 90 % inactivation endpoint [IEP]).

C_3 = No ether treatment, at the time of infection.

C_4 = With ether treatment, at the time of infection.

Table 39 shows that LLF from normal rats was more active than from convalescent animals. Taking the 50 % end points, parallel activity of LLF from normal rats towards both bacteria was observed. ie. the batch 43 was approximately equal to batch 15 in both test systems, and both were more active than batch 38 which, in turn was more active than batch 34. The last-mentioned was in fact inactive, as were the two batches of convalescent LLF. The 90 % end points gave the same pattern of activity, but were shifted to larger volumes eg. batch 15 required a 3 to 4 times greater volume to give a 90 %, compared with a 50 %, end point in the two test systems. Of the 4 euthanasia methods, D and A gave similarly active LLFs from normal rats (except no. 42), while B was much less satisfactory. The single LLF collected by method C had intermediate activity.

CHARACTERIZATION OF RAT LLF

Fractionation by Centrifugation

As a first step in characterizing the substances responsible for the BA of LLF, the fluid (after low-speed deposition of cells) was ultracentrifuged at 29,000 rpm (55,000 g) for 20 min, at 4°C, and the supernate (SN) and deposit (surfactant, SUR) separated. The deposit was dispersed in PBS to the original volume of the LLF and both fractions stored frozen. They were then examined for their BA towards bordetellae and other bacteria.

Viable count experiments

B. pertussis 18-323: A series of tests was done on the SN and SUR fractions for their BA towards *B. pertussis* 18-323. Eight LLF batches from normal rats and two from convalescent rats were used. With fluids from normal rats, Figure 45 shows that the SUR fraction was more bactericidally active than the SN, after both the 30 and 120 min incubations. The longer incubation, as expected, gave greater reduction in % survival of the bacteria in the SN.

Table 39: Comparison of percent reduction of both *B. pertussis* 18-323 A6 and lux incubated in different batches of normal (N) and convalescent (C) rat LLF at 37°C for 60 min. Data are the mean of duplicates in the same experiment.

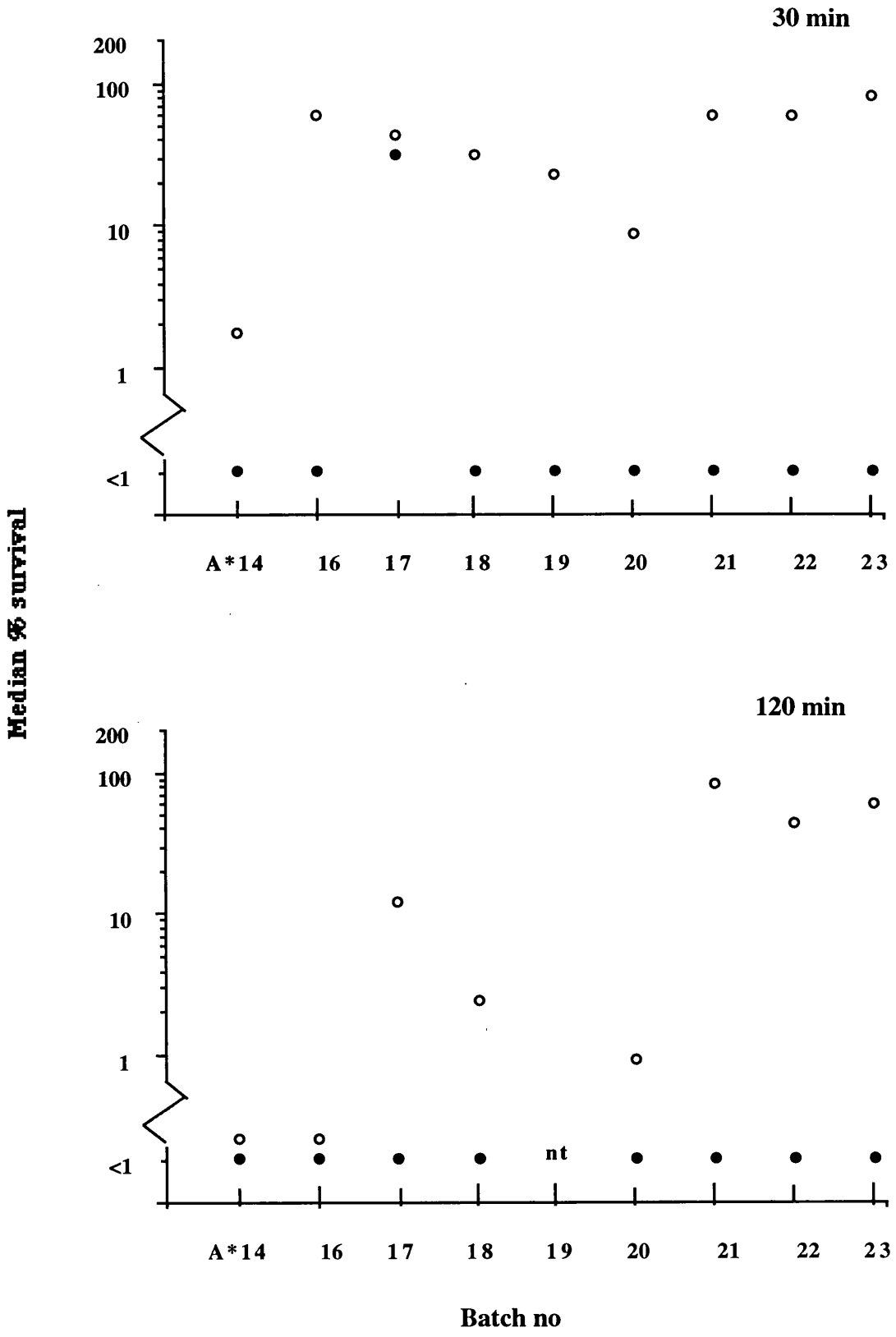
LLF batch no./Method of euthanasia	Treatment* of rat	% Reduction (50 % and 90 %) end point (µl)* after exposure at 37°C for 60 min			
		lux		18-323 A6	
		VC-50	VC-90	VC-50	VC-90
15 /A	N	60	245	25	75
34 /B	N	> 400	> 400	> 400	> 400
38 /C	N	140	> 400	130	245
43 /D	N	45	165	25	65
43 /D	C_3	> 400	> 400	> 400	> 400
43 /D	C4	> 400	> 400	> 400	> 400

* Volume of test fluid required to give either 50 % or 90 % reduction of viable count (VC)[(ie. 50 % or 90 % inactivation endpoint [IEP]).

C_3 = no ether treatment, at the time of infection.

C_4 = with ether treatment, at the time of infection.

Figure 45. Bactericidal activity of normal rat LLF fractions (supernate o and surfactant •) collected by euthanasia method D towards *B. pertussis* 18-323 after 30 and 120 min at 37 °C. Data from 1 to 6 observations at each point.



* For more details see Appendix 9 footnote code number A14, and A16 - 23.

With another batch of rats, the SUR fluids from normal and sham-operated animals were both highly active, whereas little activity was found with the corresponding SNs (Figure 46). Convalescent rats, on the other hand, yielded LLF in which neither the SUR nor the SN fluids had BA. An exception was group C3, whose surfactant showed a strong activity compared with little or no activity in the SN. Normal fluids from rat batch no. A 16 and 18 in Figure 45 are the normal fluids in Figure 46 for comparison with the treated fluids.

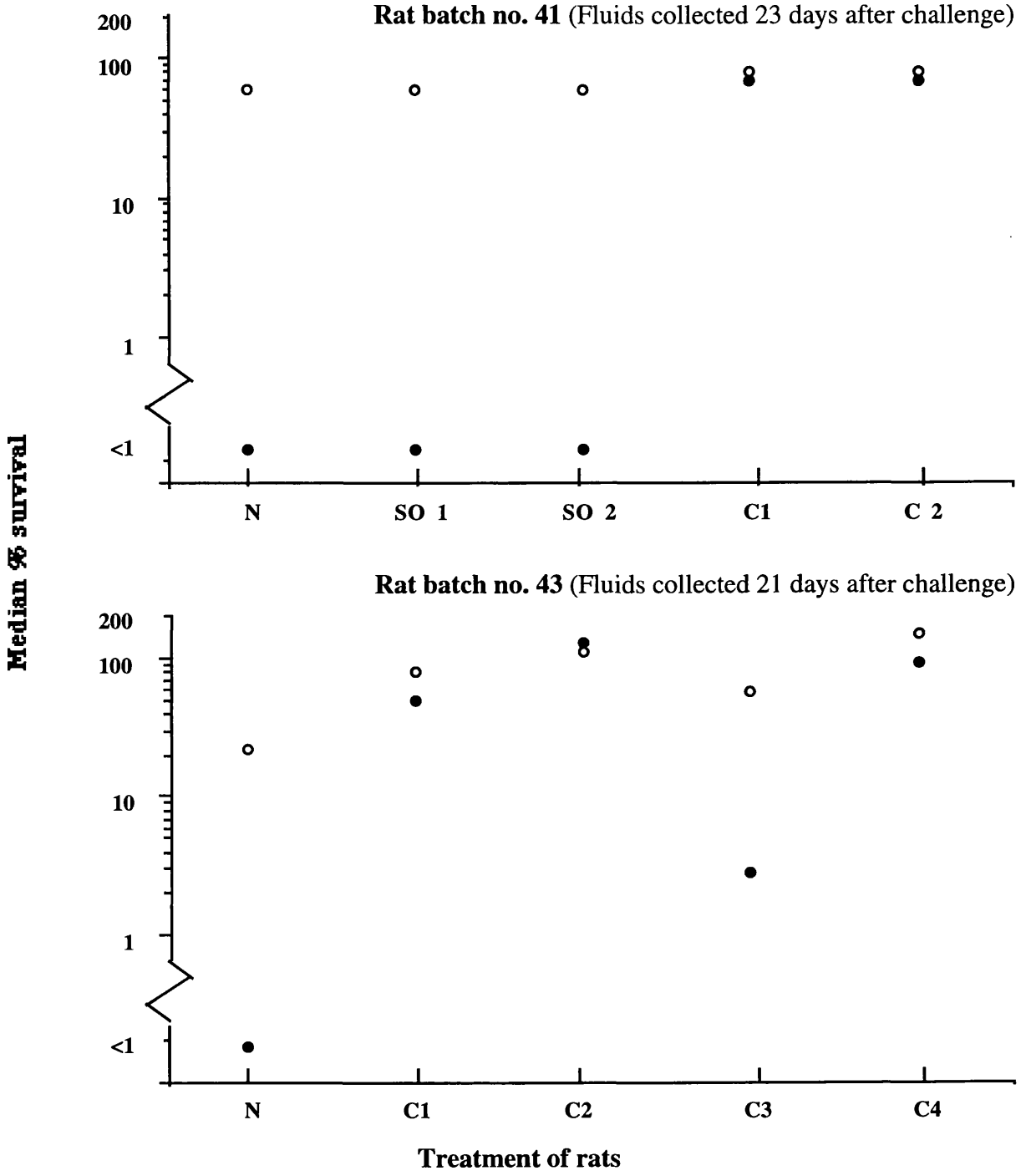
The footnotes in Figure 46 indicate that the sham-operated and the convalescent rats had been exposed to a variety of different procedures at the time of infection with *B. pertussis*. The important observations were that a) sham-operated animals gave LLF fractions that were similar to normal in their BA, and b) that inclusion of *B. pertussis* in the challenge doses gave inactive LLF fractions, except as noted above.

Thus the use of alginate, as against agarose, or the use or non-use of ether had no consistent effects. It should be emphasised that the convalescent LLF had been collected at 21-23 days after infection, indicating that exposure of the rat respiratory tract to live *B. pertussis* caused long-lasting abolition of BA of LLF and/or its fractions.

For more detailed information, the viable count results with *B. pertussis* 18-323 incubated in normal and convalescent SUR and SN at various times are presented in Appendices 18, 20, 22 and 24. The same results expressed as median % survival are in Appendices 19, 21, 23 and 25. It was concluded that the SUR fraction contain most of the BA, whereas, the SN had little activity. Instead of whole LLF, SUR and SN were used as test fluids in all later experiments.

Other bordetellae: Attention was next turned to other bordetellae for their sensitivity to the BA of normal rat surfactant and supernate when tested under similar conditions. *B. pertussis* 18-323 was included for comparison.

Figure 46. Effect of various *B. pertussis* challenges (in alginate or agarose) on the bactericidal activity of rat LLF fractions (supernate○ and surfactant●). Diagram shows the median % survival of *B. pertussis* 18-323 after 30 min at 37°C. Data from 2 to 3 observations at each point.



SO1 : Female rats given agarose with no bacteria, with ether
SO2 : " " " alginate " " " , " "
C1 : " " challenged with *B. pertussis* 18-323 EH2 in agarose, with ether.
C2 : " " " " " " " " alginate, " ".
N : " normal rats.
C1 : " rats challenged with B. pertussis 18-323 EH2 in alginate, no ether.
C2 : " " " " " " " " " , with ether.
C3 : " " " " " " " " " , no ether.
C4 : " " " " " " " " " with ether.

Table 40. Bactericidal activity of surfactant (SUR) and supernate (SN) from rats batch no. 45 towards *Bordetella* species. PBS was used as a non-bactericidal control.

Bordetella species and strain	Test fluid	No. of CFU/20 µl after exposure at 37°C for min			
		0	60	120	1440
pertussis 18-323	SUR	36 , 32	0 , 0	0 , 0	0 , 0
	SN	38 , 32	0 , 0	0 , 0	0 , 0
	PBS	40 , 46	32 , 20	32 , 31	0 , 0
parapertussis 10520	SUR	48 , 57	0 , 0	0 , 0	0 , 0
	SN	68 , 52	0 , 0	0 , 0	0 , 0
	PBS	56 , 42	52 , 42	56 , 49	2 , 0
bronchiseptica 5376	SUR	34,44	33,45	53,40	ac,ac
	SN	60 , 44	36 , 44	44 , 68	c , c
	PBS	43 , 50	30 , 35	37 , 54	ac , ac

c = confluent growth; ac = almost confluent growth.

The tests reported in Table 40 show that *B. parapertussis* was indistinguishable from *B. pertussis* i.e. rapid killing (in less than 60 min) in SUR and SN and (with *B. pertussis*) a slow loss of viability in PBS. In contrast, *B. bronchiseptica* was quite different, in that there was no killing in the LLF fractions and the organism grew in all three test fluids, including PBS. The only difference was in the SN, after 1440 min incubation, when confluent growth was observed. Thus *B. bronchiseptica*, unlike the other two species was able to use the two LLF fractions as growth media (and PBS also).

***Other bacteria*:** Tests similar to the above were made with a few non-bordetella bacteria, namely two gram positive species and 3 strains of *E. coli*. *B. pertussis* 18-323 was included as a control.

With *Staphylococcus aureus* 'Oxford', there was a slow loss of viability in SUR which paralleled the loss of viability in PBS (Table 41). This was possibly due to lack of nutrient. In contrast, the viable count did not decline during 120 min in SN, and the organism subsequently grew, as evidenced by the confluent result at 1440 min. Unlike *S. aureus*, *Streptococcus pyogenes* 5763 grew in both the SUR and SN fractions of rat LLF. However, like the staphylococcus, it lost viability slowly in PBS. The control, *B. pertussis* responses to these test fluids were typical of those reported above.

Tests with 3 strains of *E. coli* were done similarly except that normal rat serum (NRS) was included as an additional test fluid, since *E. coli* Lilly is very sensitive to it. In addition, *B. pertussis* lux was used as a control because two of the *E. coli* strains were lux constructs. They differed in that WA803 requires addition of decanal for luminescence (as does *B. pertussis* lux) whereas *E. coli* lux DH5α is spontaneously luminescent without exogenous decanal.

The results in Table 42 show that the LLF SUR of batch 55 was similar to that of the batch no. 45 reported in Table 41 in being highly bactericidal towards *B. pertussis* 18-323. However, the SN fraction was much less active and allowed about 25 % survival at 120 min. *B. pertussis* lux was much less sensitive than 18-323 in having 100 % survival in SN at 120 min and only about 30 % killing in SUR. Both *B. pertussis* strains showed

Table 41. Bactericidal activity of surfactant (SUR) and supernate (SN) from rat batch no. 45 towards *B. pertussis* and other bacteria. PBS was used as a non-bactericidal control.

Bacterial species and strain	Test fluid	No. of CFU/20 µl after exposure at 37°C for min				
		0	30	60	120	1440
B. pertussis 18-323	SUR	42 , 24	0 , 0	0 , 0	0 , 0	0 , 0
	SN	42 , 44	0 , 0	0 , 0	0 , 0	0 , 0
	PBS	55 , 61	58 , 54	nt , nt	38 , 30	0 , 0
Staphylococcus aureus 'Oxford' 6571	SUR	23 , 20	14 , 19	9 , 10	8 , 7	4 , 2
	SN	20 , 22	14 , 24	15 , 27	27 , 27	c , c
	PBS	18 , 24	12 , 22	16 , 16	15 , 10	2 , 1
Streptococcus pyogenes 5763	SUR	27 , 31	20 , 26	28 , 24	23 , 28	ac , ac
	SN	20 , 18	20 , 18	36 , 26	30 , 50	c , c
	PBS	23 , 21	25 , 17	15 , 12	13 , 13	2 , 0

c = confluent growth; ac = almost confluent growth.

Table 42. Bactericidal activity of surfactant (SUR), supernate (SN), and serum (NRS) from normal rats batch no. 55 towards *B. pertussis* and *E. coli* strains. PBS was used as a non-bactericidal control.

Bacterial species	Test fluids	No. of CFU/20 µl after exposure at 37°C for min			
		0	30	60	120
B. pertussis 18-323	SUR	26, 24	0 , 0	0 , 0	0 , 0
	SN	106 , 80	52 , 62	44 , 34	30 , 32
	NRS	96 , 80	28 , 26	16 , 20	9 , 9
	PBS	76 , 86	76 , 72	86 , 62	52 , 40
B. pertussis lux	SUR	122 , 104	58 , 50	52 , 54	38 , 40
	SN	158 , 148	156 , 126	154 , 146	158,130
	NRS	146 , 138	158 , 122	124 , 120	116 , 120
	PBS	170 , 160	130 , 134	134 , 100	108 , 84
E.coli Lilly	SUR	68 , 88	78 , 68	68 , 66	90 , 56
	SN	82 , 78	64 , 80	ac , ac	c , c
	NRS	28 , 22	0 , 0	0 , 0	0 , 0
	PBS	84 , 90	94 , 106	78 , 94	88 , 62
E.coli luxWA803	SUR	60 , 62	48 , 58	30 , 38	66 , 62
	SN	84 , 64	68 , 64	78 , 104	104 , 124
	NRS	2 , 2	0 , 0	0 , 0	0 , 0
	PBS	54 , 84	48 , 42	36 , 38	36 , 42
E.coli lux DH5α	SUR	74 , 42	34 , 24	16 , 8	10 , 16
	SN	30 , 36	34 , 32	52 , 46	86 , 112
	NRS	24 , 18	0 , 0	0 , 0	0 , 0
	PBS	46 , 48	36 , 46	52 , 46	44 , 48

c = confluent growth; ac = almost confluent growth.

similar loss of viability in PBS, about 45 % at 120 min. With normal rat serum (from the same rats as gave the LLF fraction) *B. pertussis* 18-323 was 90 % killed at 120 min, whereas *B. pertussis* lux showed only about 30 % killing.

The three *E. coli* strains were qualitatively similar to each other in a) being killed rapidly by normal rat serum; b) maintaining, over 120 min, a steady count in PBS, and c) treating the LLF SN as a growth medium. They differed, however, in response to SUR, with DH5α showing about 75 % loss of viability, while the other two strains were without significant loss.

Effect of dilution of normal LLF, surfactant and supernate: LLF and its ultracentrifuge fractions were subjected to dilution assay of their BAs, with the fluids tested from undiluted to 1/64. "Undiluted" means that in the 250 µl test mixture, 240 µl was the test fluid and 10 µl the standard inoculum of bacteria. Dilutions 1/2 to 1/64 were made by dispensing the undiluted fluid in volumes from 120 µl down to 3.75 µl and adjusting the volume to 240 µl with PBS.

After incubation for 1h at 37°C and plating out, the results were expressed as % survival, based on the survival in PBS at that (1 h) time. A typical assay, with both a volume scale and a dilution scale on the abscissa, is presented in Figure 47. Dotted lines have been inserted to enable the 50 % and 90 % killing end points to be read. With this particular batch (no. 55) of LLF, the 50 % end point was at a 1/24 dilution, corresponding to 9.9 µl of undiluted LLF in the test mixture. Likewise, the 90 % killing end point was given by a 1/11 dilution, equivalent to 21 µl in the test mixture.

The same procedure was used to assay the BA of various batches (nos. 45 and 55) of SUR (Figure 48) and SN (Figure 49). With the former, a typical 50 % end point was 1/ 34 (7 µl) while with the latter, a value of 1/1.8 (135 µl) was observed. Because various different batches of LLF, SUR and SN were assayed, due to the work being done at different times, exact estimates of yield of activity in the two fractions are not possible. However, it appeared that SUR could be obtained without significant loss by ultracentrifug-

Figure 47. LLF assay: Mean (±SEM) percent survival of *B. pertussis* 18-323 incubated for 1 h at 37°C in neat and diluted LLF from normal rats batch no. 55. Calculation of % survival was based on PBS time-1h counts of 2 observations at each point. Dotted lines indicates the procedure for reading 50 % (——) and 90 %(------) killing end-ponts.

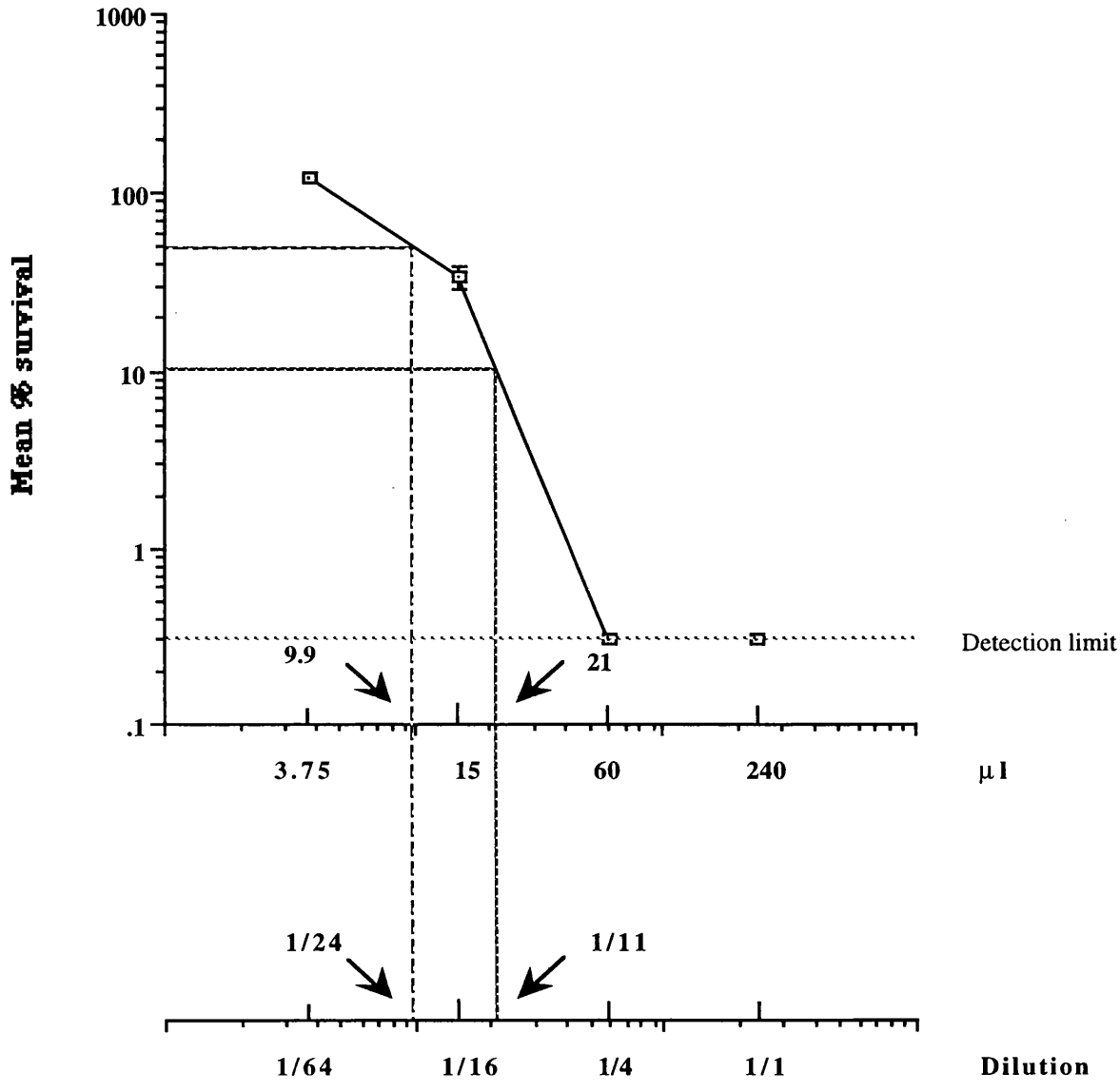


Figure 48. Surfactant assay: Mean (±SEM) percent survival of *B. pertussis* 18-323 incubated for 1 h at 37°C in neat and diluted surfactant fraction of LLF from normal rats batches no. 45 and 55. Calculation of % survival was based on PBS time-1h counts of 12 observations (with 2 batches with very similar results) at each point. Dotted lines indicates the procedure for reading 50 % (.-.-.-.) and 90 % (-------) killing end-ponts.

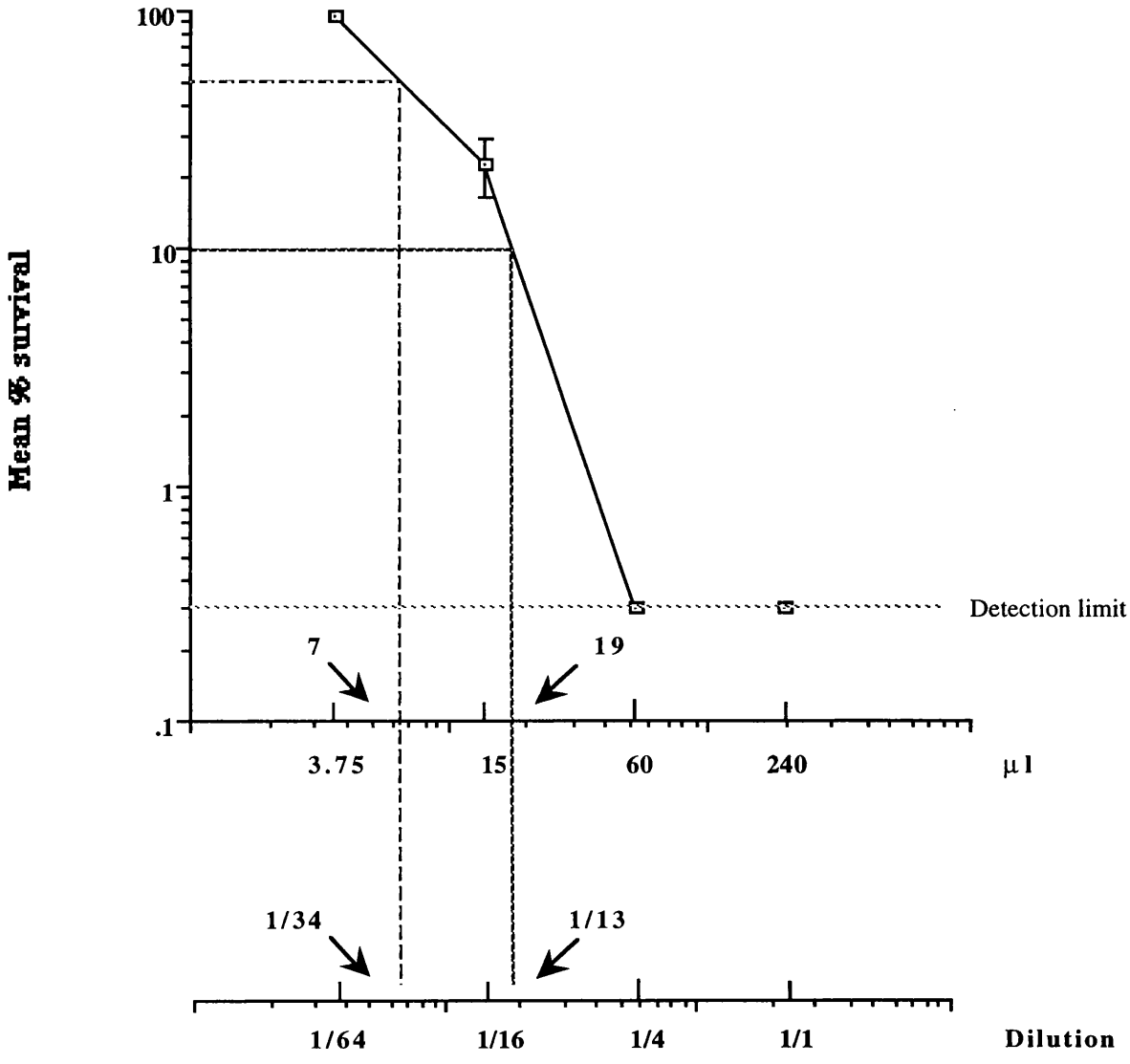
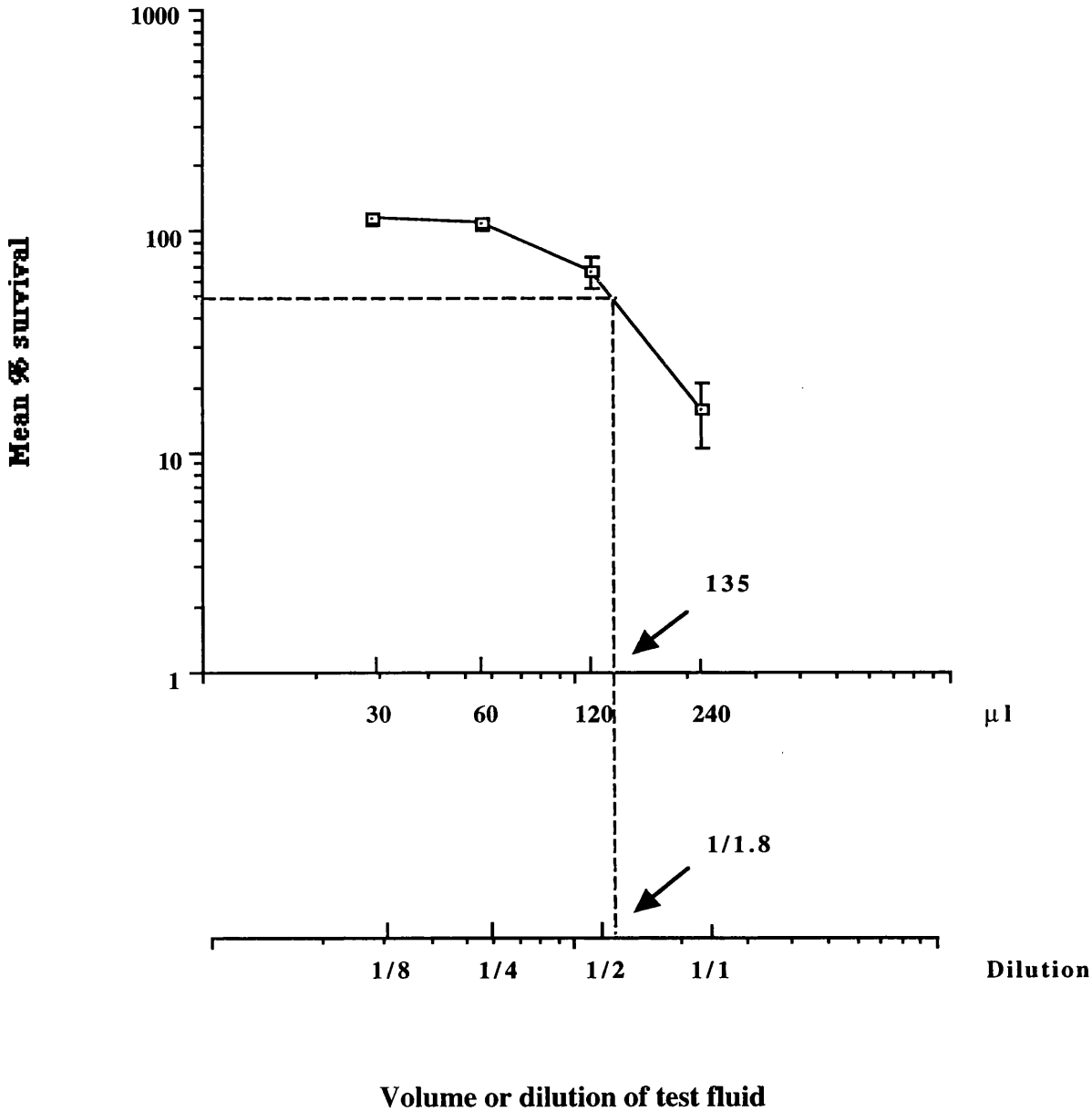


Figure 49. Supernate assay: Mean (±SEM) percent survival of *B. pertussis* 18-323 incubated for 1 h at 37°C in neat and diluted supernate fraction of LLF from normal rats batches no. 45 and 55. Calculation of % survival was based on PBS time-1h counts of 10 observations (with 2 batches with roughly similar results) at each point. Dotted lines indicates the procedure for reading 50 % (- - - - - -) killing end-pont.



ation of LLF and that it was about 20 times more active than SN. This can also be expressed by saying that about 95 % of the initial BA of the LLF was in the SUR and 5 % in the SN.

Effect of membrane filtration on surfactant activity: At the start of the experimental work on LLF, the fluid was sterilized by membrane filtration (0.45 μm pore size). This was done with approximately the first 50 % of all LLF batches collected. However, the filtration was stopped after the experiment about to be described in which the BA of the redispersed SUR fraction was found to be greatly (60- 90 %) reduced by filtration.

These data (Table 43) with 5 preparations of SUR (all from rats batch no 41), show that filtration of SUR removed most of its BA. This was seen with preparations from normal, sham-operated and convalescent animals (the last mentioned having low activity to start with). Although only a few tests were done, it seemed that unfractionated LLF did not lose activity by filtration, at least not to this extent.

Presumably, therefore, the SUR after ultracentrifugation did not disperse sufficiently when resuspended, to allow it to pass through the pores of a 0.45 μm membrane filter. The later batches of (unfractionated) LLF were also not filtered and this did not result in significant background contamination.

Luminescence experiments

***B. pertussis and other lux bacteria*:** SUR and SN of LLF from normal rats batch no. 55 were tested for their effect on the luminescence of *B. pertussis* lux, *E. coli* WA 803 and *E. coli* DH5α. Diluted serum (1/16) was also included as a positive control from the same batch of rats previously used (Table 42).

With SUR, and in a comparison with results in PBS, Table 44 shows that around 80 % reduction in light output was observed with *B. pertussis* lux after 1h incubation, whereas there was no reduction in luminescence of either *E. coli* strain. SN gave a reduction of about 70 % in luminescence of *B. pertussis* lux, whereas no reduction of the

Table 43. Effect of filtration (0.45 µm pore size membrane filter) on the bactericidal activity of surfactant from normal (N), sham-operated (SO) and convalescent (C) rats batch no. 41. CL medium and PBS were used as non-bactericidal controls.

Treatment of rats	Filtration	No of CFU/20 µl after exposure at 37°C for min				
		0	15	30	120	1440
SO1	+	93 , 100	74 , 78	72 , 60	23 , 36	0 , 0
	-	28 , 43	0 , 0	0 , 0	0 , 0	0 , 0
SO2	+	114 , 100	100 , 74	96 , 73	78 , 58	3 , 1
	-	4 , 41	0 , 0	0 , 0	0 , 0	0 , 0
C1	+	124 , 97	104 , 106	118 , 67	96 , 75	3 , 4
	-	82 , 101	99 , 70	84 , 58	4 , 1	0 , 0
C2	+	130 , 104	98 , 100	104 ,92	94 , 86	0 , 1
	-	92 , 99	98 , 73	74 , 68	9 , 17	0 , 0
N	+	110 , 98	112 , 78	120 , 69	66 , 48	3 , 1
	-	8 , 28	0 , 0	0 , 0	0 , 0	0 , 0
PBS		94 , 97	72 , 70	90 , 60	49 , 30	3 , 1
CL medium		95 , 80	90 , 88	110 , 80	114 , 74	ac , ac

SO1 = rats were given agarose; SO2 = rats were given alginate; C1 = rats were challenged with *B. pertussis* 18-323 EH2 in agarose; C2 = rats were challenged with *B. pertussis* 18-323 EH2 in alginate.

light output was found with *E. coli* WA 803, and very high luminescence of *E. coli* DH5α was retained. In serum, about 70 % of *B. pertussis* lux light output was lost, while little decrease of *E. coli* WA 803 luminescence was seen, compared with very high light output of *E. coli* DH5α.

With regard to serum-sensitivity of *E. coli* lux, the two strains gave similar responses in the luminometer, ie. were not killed (Table 44). This was different to the responses (Table 42) when both strains were killed by normal rat serum, however; it should be noted a) that the serum was used undiluted in the viable count experiments, and at 1/16 in luminometery; b) the test doses of bacteria in the luminescence experiments were about 1000-times higher than those for viable counting. If further experiments were to be done, it might be desirable to interchange the serum concentrations and bacterial dose in the two types of test system.

Effect of dilution of normal LLF, surfactant and supernate: Similar to the viable count experiments, dilution assays of the effect of undiluted to 1/64 LLF, SUR and SN on the luminescence of *B. pertussis* lux were performed. A standard bacterial inoculum (100 µl) was added to 400 µl of test fluids to give the undiluted test mixture. Dilutions 1/2 to 1/64 were made as before. After incubation in the luminometer for 1h at 37°C, decanal was added and the light output read and the results were expressed as % survival, based on the survival in PBS at 1h.

A specific assay, with both a volume and a dilution scale, is presented in Figure 36. As with viable count assays, the 50 and 90 % end points were read by interpolation of dotted lines. Results (Figure 50) show that LLF gave a 50 % inactivation end point at a 1/50 dilution equal to 8 µl of undiluted LLF in the test mixture. Correspondingly, the 90 % inactivation end point was achieved by a 1/ 4.8 dilution, approximating to 83 µl in the test mixture.

Table 44. Effect of surfactant (SUR), supernate (SN), and 1 in 16 normal serum (NRS) from rats batch no. 55 on the luminescence of *B. pertussis* lux and *E.coli* lux strains WA 803 and DH5α. PBS was used as a non-bactericidal control. A and B are duplicates.

Bacterial species and strains	Test fluid	Luminescence (mv) after 1h incubation	
		A	B
B. pertussis lux	SUR	17.5	23.6
	SN	33.2	33.9
	NRS	30.7	38.4
	PBS	118	115
E. coli lux WA 803	SUR	151	124
	SN	290	284
	NRS	91.4	105
	PBS	121	127
E. coli lux DH5α	SUR	417	377
	SN	1260	1290
	NRS	1320	1260
	PBS	143	141

The same procedure was applied to assay the effect of various batches (nos. 44 and 45) of SUR (Figure 51) and SN (Figure 52) on bacterial luminescence. With SUR, a typical 50 % end point was at 1/67 dilution (6 µl) whereas, the 90 % end point was at 1/12.5 (32 µl).

With SN, a value of 1/5.1 (79 µl) was obtained for the 50 % end point, while the 90 % end point was at 1/1.4 (290 µl). Because the various different batches of LLF, SUR, and SN were examined at different times, exact comparions of 50 and 90 % inactivation end points are difficult. Despite that, it appeared that SUR had activity about 10 times that of SN.

Effect of ether on the bactericidal activity of LLF and its fractions: An experiment was done with LLF, SUR and SN collected from normal rats (batch no. 58) which had been exposed to ether immediately before euthanasia. This was done to see if recent (after 6 days of treatments) inhalation of ether had an effect on the BA of the fluids towards *B. pertussis* 18-323.

According to the results (Table 45), LLF and SUR from untreated rats showed a high activity, whereas, no activity was found with SN from the same rats.

With the ether-treated rats, the BA of LLF and SUR was not different from the untreated rats, while the activity of the SN was enhanced by the ether treatment. This enhanced activity might have been due to residual dissolved ether in the SN, or to release into the SN of BA from the still-living rat.

***Luminescence of bacteria in agarose with* DMβCD:** Standardized *B. pertussis* lux suspension in 1 % (w/v) CAA was incorporated into agarose gel beads with or without dimethyl-β-cyclodextrin (DMβCD). The preparation was added to PBS or SUR at a final concentration (v/v) of 1/5, and incubated in the luminometer for 1h at 37°C in the usual way. Bacteria without either or both agarose or DMβCD were run in parallel.

Figure 50. LLF dilution assay by luminometry: Mean (±SEM) percent luminescence of *B. pertussis* lux incubated for 1 h at 37°C in neat and diluted LLF from normal rats. Calculation of % luminescence was based on PBS time-1h values of 3 observations at each point. Dotted lines indicates the procedure for reading 50 % (-----) and 90 % (-------) killing end-ponts.

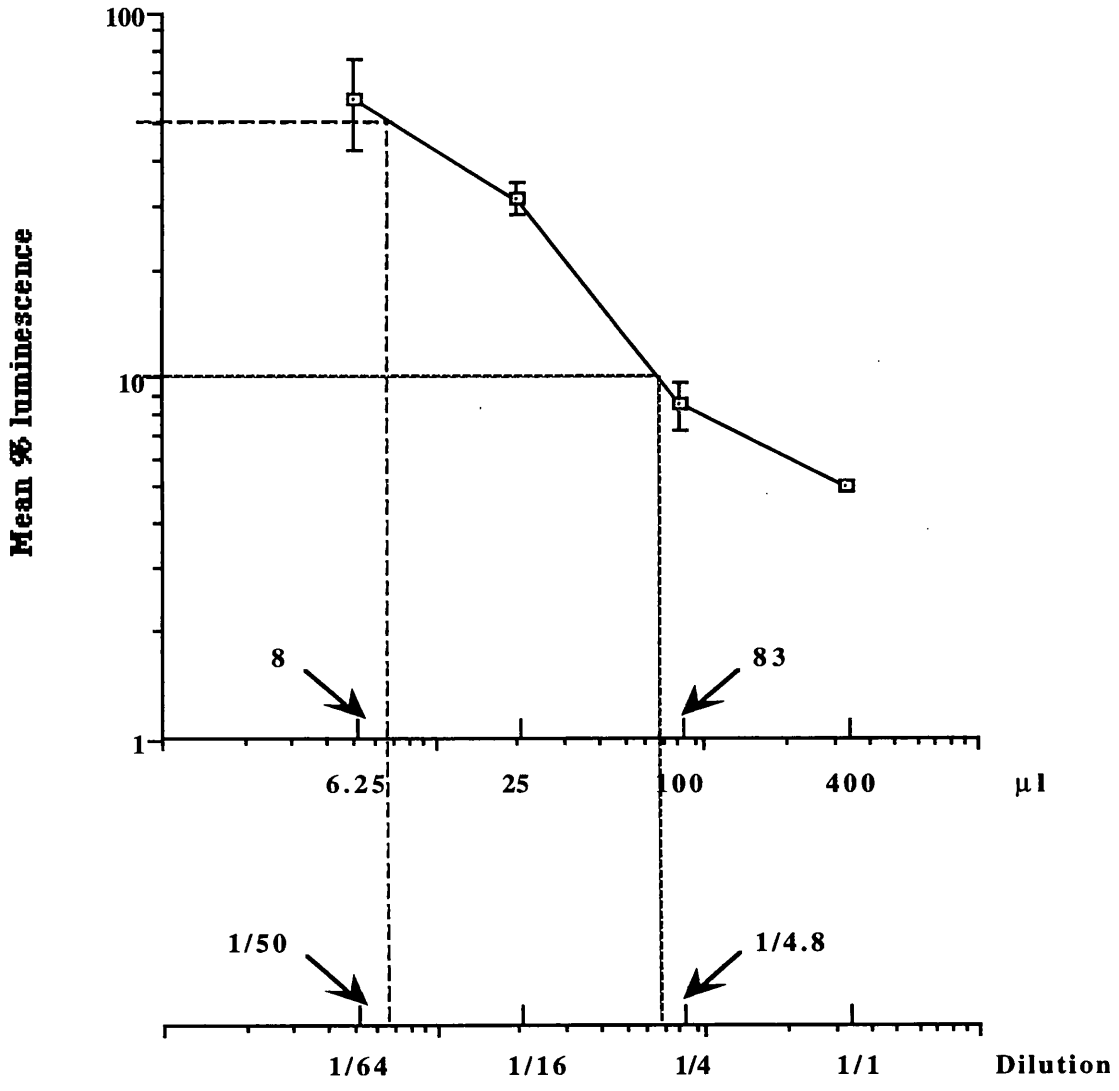


Figure 51. Surfactant dilution assay: Mean (±SEM) percent luminescence of *B. pertussis* lux incubated for 1 h at 37°C in neat and diluted surfactant fraction of LLF from normal rats batches no. 44 and 45. Calculation of % luminescence was based on PBS time-1h values of 7 observations (with 2 batches with very similar results) at each point. Dotted lines indicates the procedure for reading 50 % (.-.-.-.) and 90 % (-------) killing end-points.

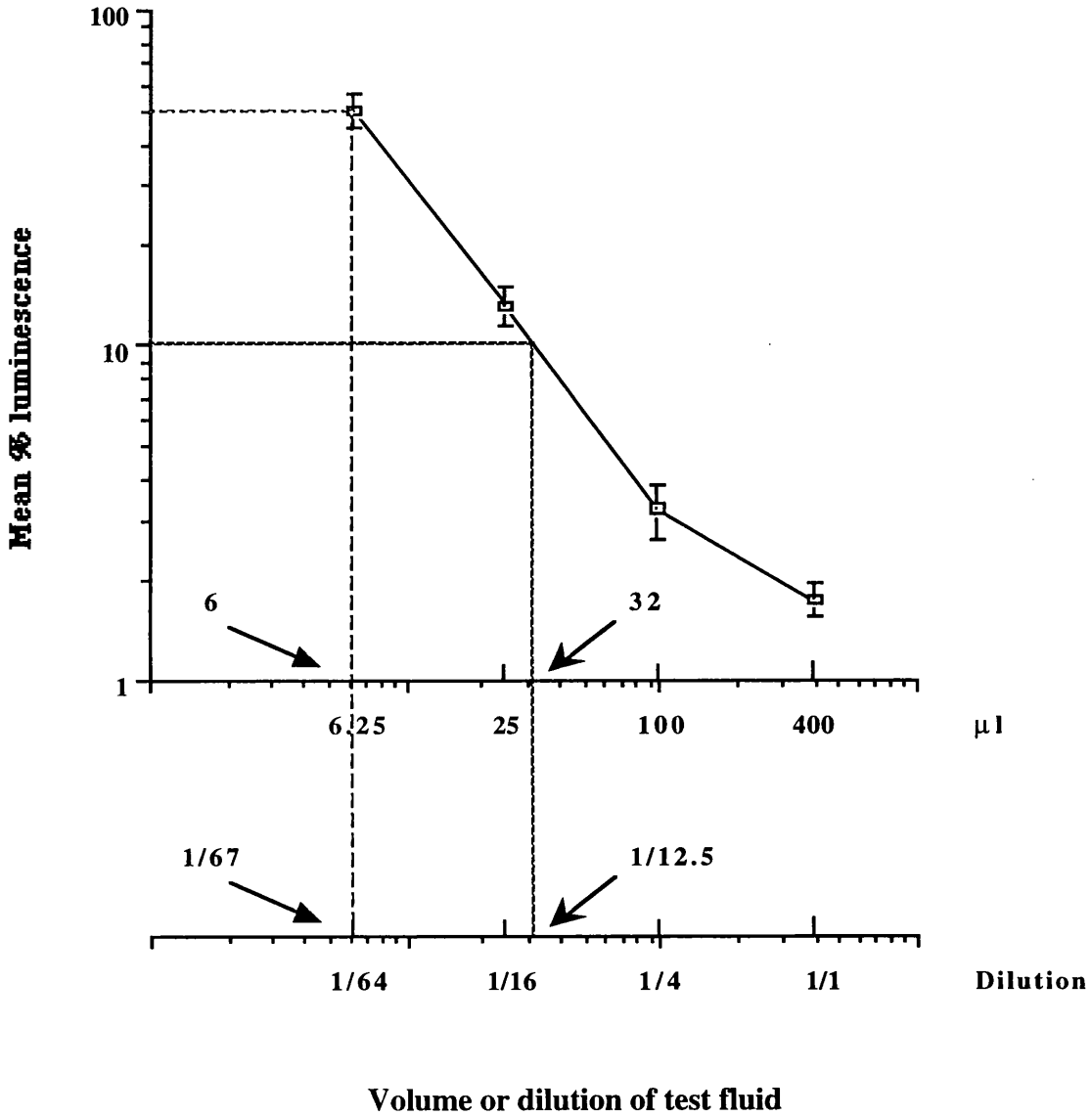


Figure 52. Supernate dilution assay: Mean (±SEM) percent luminescence of *B pertussis* lux incubated for 1 h at 37°C in neat and diluted supernate fraction of LLF from normal rats batches no. 44 and 45. Calculation of % luminescence was based on PBS time-1h values of 7 observations (with 2 batches which very similar results) at each point. Dotted lines indicates the procedure for reading 50 % (- - - - -) and 90 % (———) killing end-points.

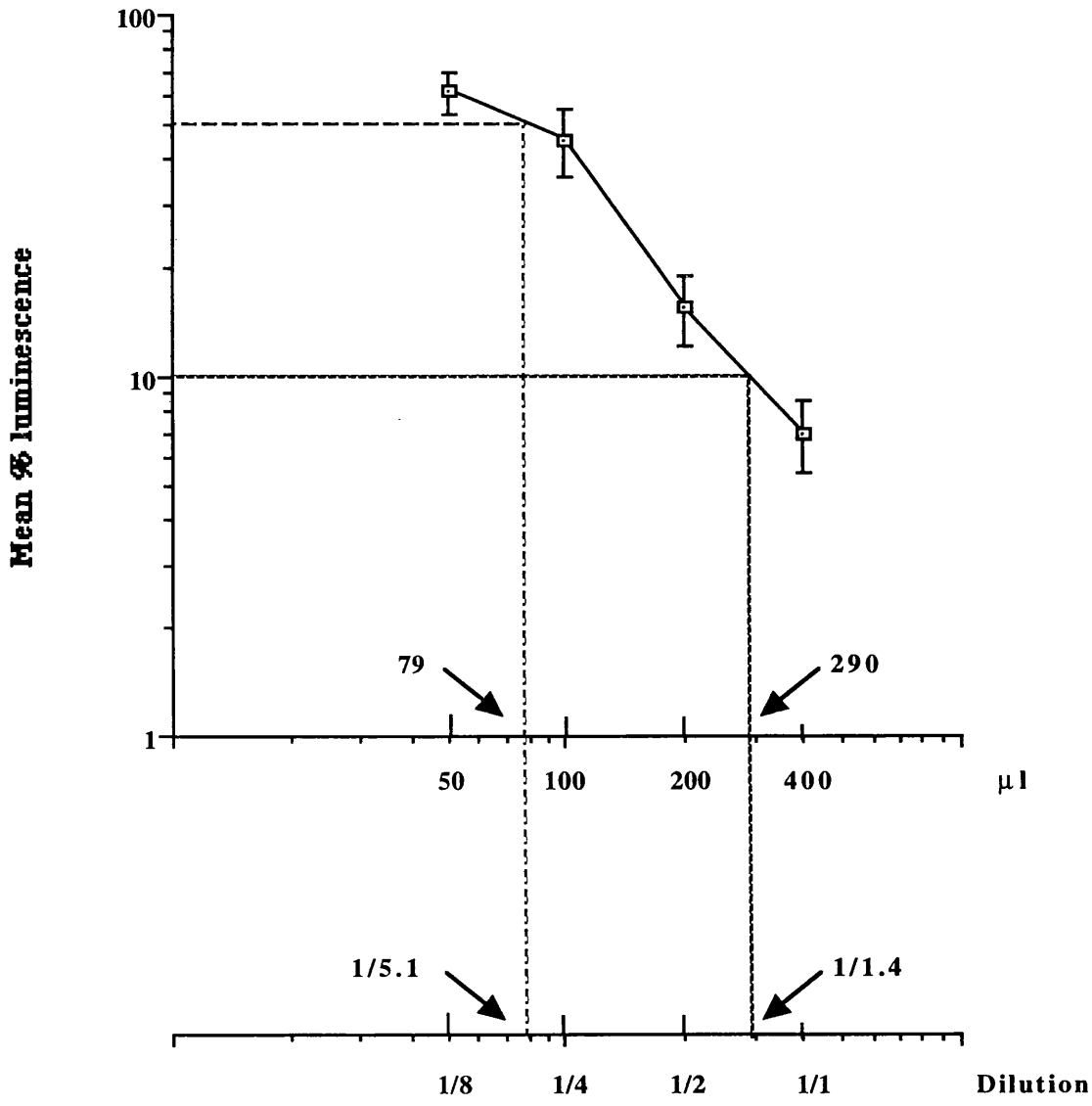


Table 45. Bactericidal activity of LLF, supernate (SN), and surfactant (SUR) from normal rats (batch no. 58) exposed to ether vapour for 2 min immediately before euthanasia method D. *B. pertussis* 18-323 was the test organism and PBS was used as a non-bactericidal control.

Test Fluids	Ether treatment	No. of CFU/20 µl after exposure at 37°C for min			
		0	30	60	120
LLF	-	76 , 80	0 , 0	0 , 0	0 , 0
	+	80 , 86	0 , 0	0 , 0	0 , 0
SN	-	158 , 162	122 , 138	138 , 114	98 , 104
	+	116 , 150	10 , 8	1 , 1	0 , 0
SUR	-	66 , 64	0 , 0	0 , 0	0 , 0
	+	114 , 102	0 , 0	0 , 0	0 , 0
PBS		132 , 158	98 , 102	82 , 98	54 , 76

The results in Figure 53 show :

1) Incorporation of *B. pertussis* lux in agarose beads or addition of DMβCD caused a reduction of bacterial light output. The lowest light output was when both were present.

2) DMβCD cased a reduction in antibacterial effect of SUR, and reduced the luminescence of the bacteria in PBS.

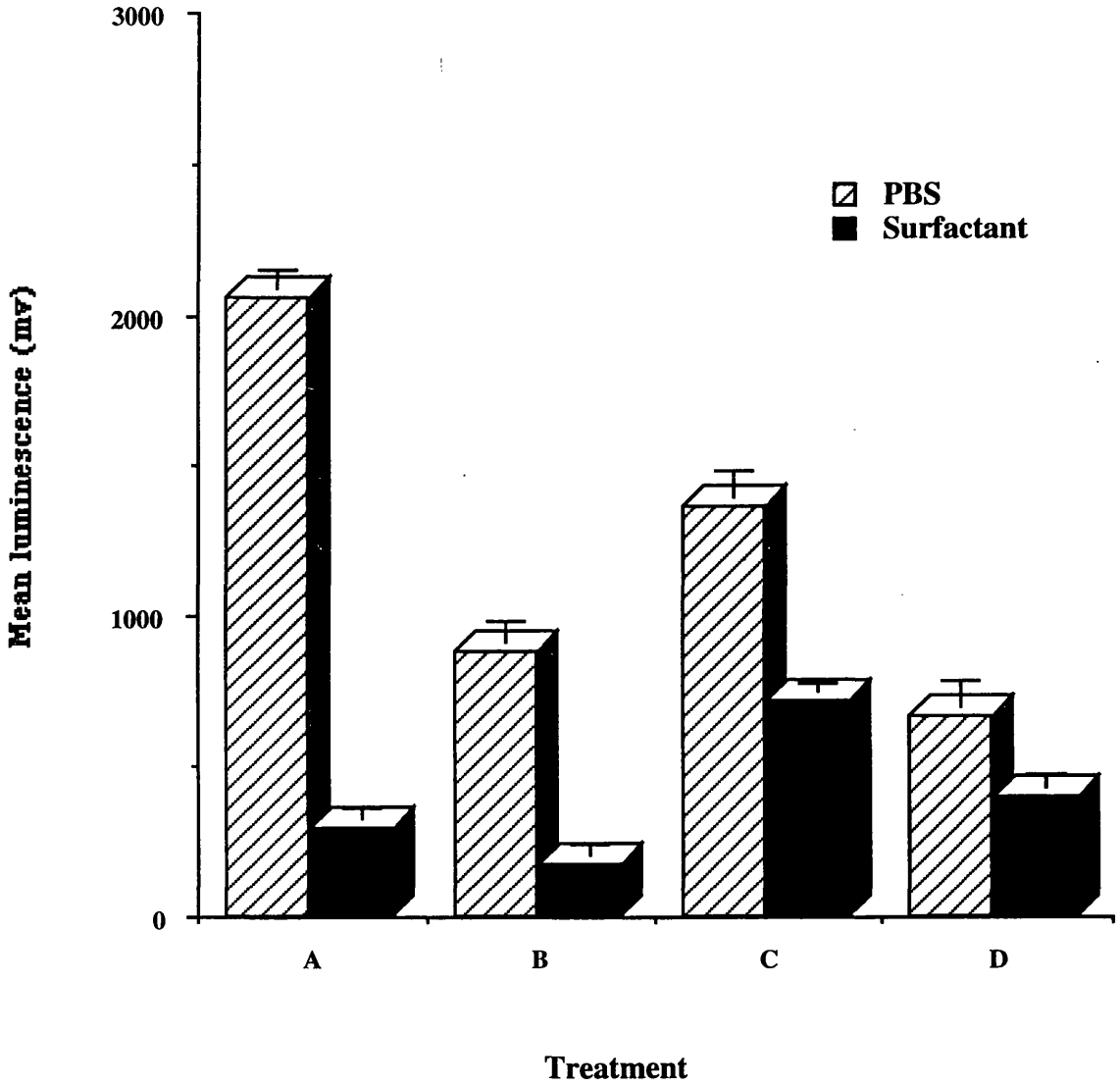
***Measurement of B. pertussis 18-323 half-life in experimental fluids*:** During the course of these studies on BA of rat LLF, its fractions, and the various control fluids, a large number of replicate observation was collected. For example with PBS, there were 96 experiments on the decline in viable count of *B. pertussis* 18-323 at 37°C during 120 min incubation. Similarly, with CL medium, there were 118 experiments, with 1 % CAA 13, and various numbers with the different normal and convalescent rat fluids. All these data were subjected to linear regression analysis with the results given in the 9 diagrams of Figure 54.

It will be seen that with all the fluids, the loss of viability (on a log scale) of *B. pertussis* 18-323 approximated to a straight line when plotted against time on an arithmetic scale.

With each fluid, a regression equation was obtained from which the half-life of *B. pertussis* in each fluid at 37°C was calculated. For convenience of comparison, these parameters are summarized in Table 46.

Theoretically, the intercept of the survival line should be at 2.00 (the $\log_{10}$ of 100 % survival) and most fluids gave parameters close to this figure (column 2 of Table 46). However, the slopes showed much greater variation and, in turn, the half-life values were correspondingly different. The longest half-life, of 386 min, or about 6.5 h, was in CL medium which is well known to support growth of *B. pertussis* from small inocula. A surprizing result was that PBS and 1 % CAA gave similar half-lives of 147 and 155 min respectively. Much shorter half-lives were seen with the bactericidal fluids: normal rat LLF gave 47 min, while normal rat SUR was less than 10 min, an exact value not being obtainable because of the non-linearity of the survival curve. There was longer (70 min)

Figure 53. Sensitivity of *B. pertussis* lux incorporated with or without 4 % (w/v) agarose in beads and 0.1 % (w/v) Dimethyl-β-cyclodextrin (DMβCD) to the antibacterial activity of LLF fraction (Surfactant). PBS was used as a non-bactericidal control. Data are the mean (±SEM) of two observations in the same experiment.



A : *B. pertussis* suspension in 1% (w/v) CAA

B : " " " " + 4 % (w/v) agarose in beads.

C : " " " " + 0.1 % (w/v) DMßCD.

D : " " " " + 4 % (w/v) agarose in beads + 0.1 % (w/v) DMßCD.

Figure 54. Quantitation of survival of *B. pertussis* 18-323 at 37°C in various fluids and the fitting of linear regression lines. Error bars are the 95 % confidence limit of the mean.

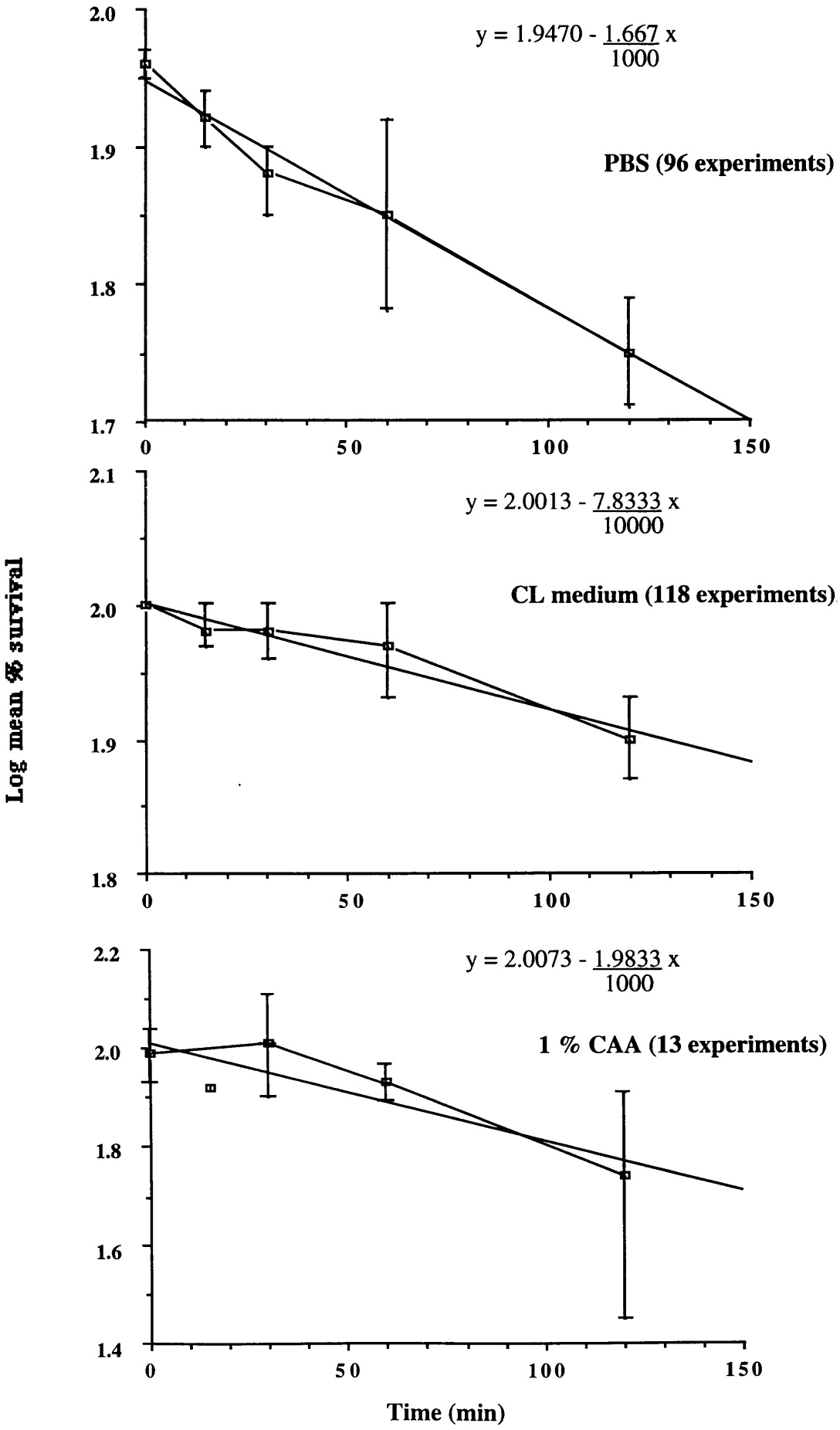


Figure 54. continued

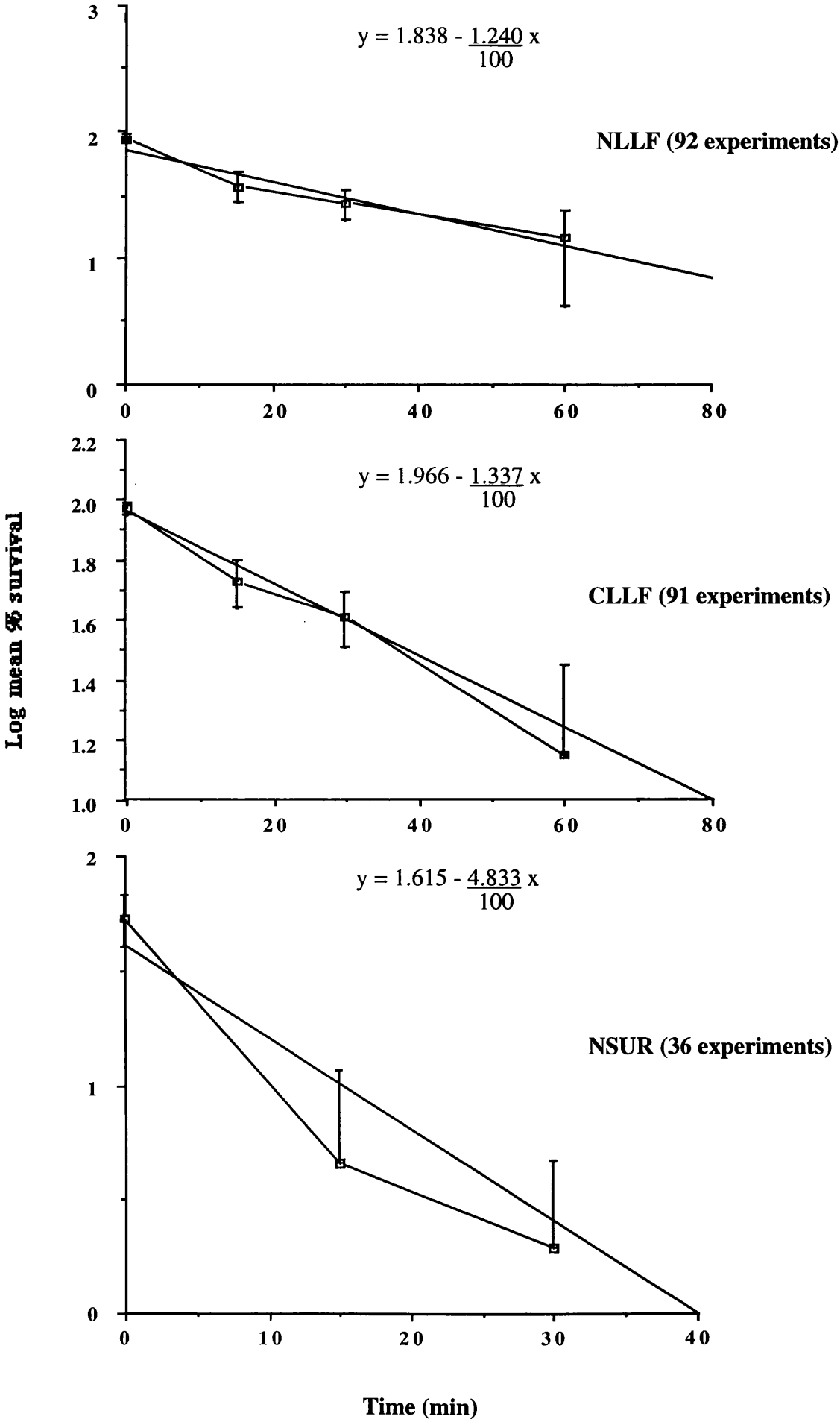


Figure 54. continued

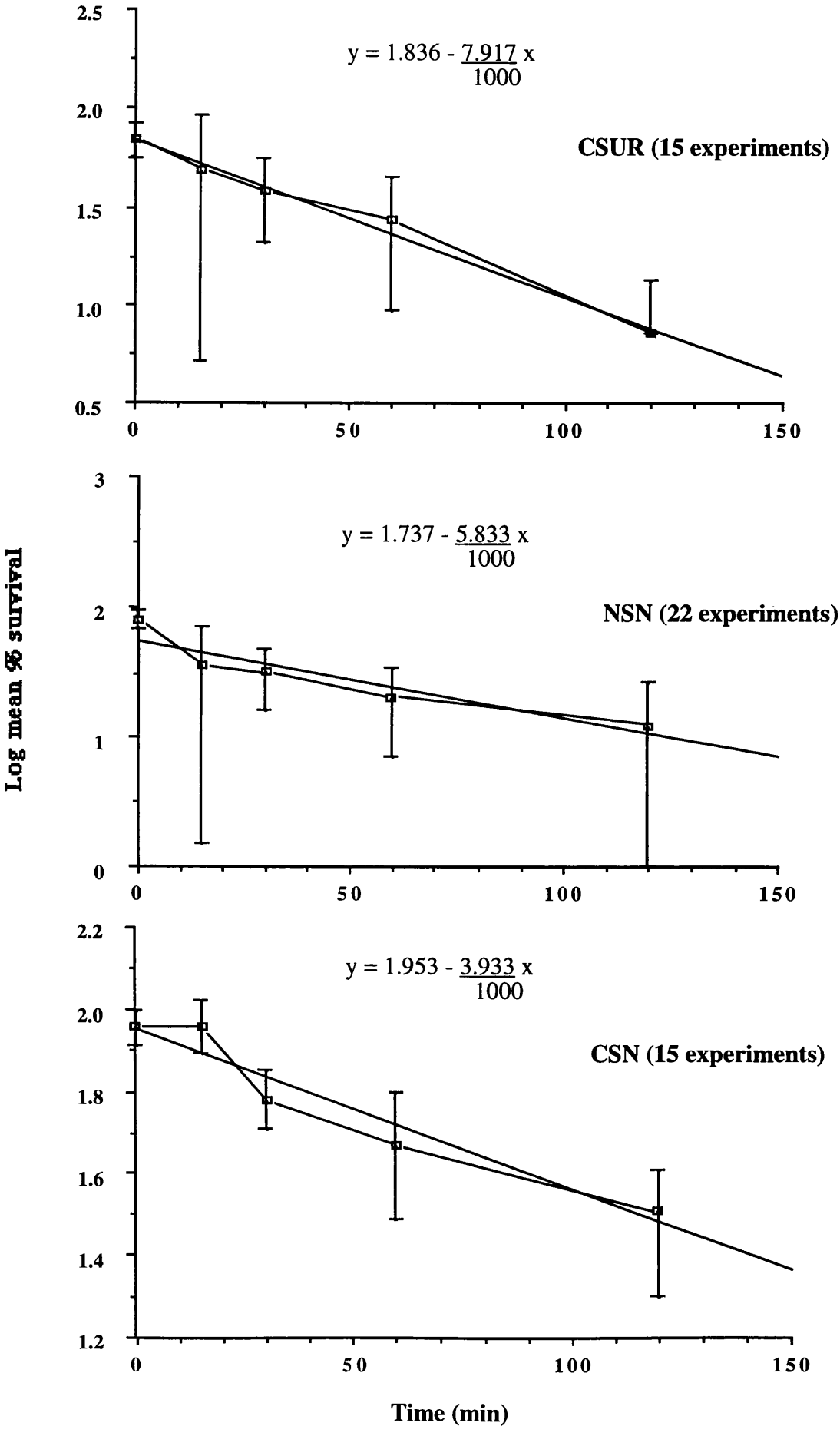


Table 46. Summary of regression parameters and half-life of *B. pertussis* 18-323 at 37°C in various fluids.

Fluid	Intercept	Slope x 1000	Half-life* (min)
PBS	1.9470	- 1.6667	149
1 % CAA	2.0073	- 1.9833	155
CL medium	2.0013	- 0.7833	386
NLLF	1.8380	- 12.400	11.2
CLLF	1.9660	- 13.371	20.0
NSUR	nc	nc	nc
CSUR	1.8363	- 7.9167	17.3
NSN	1.7365	- 5.8333	64.3
CSN	1.9530	- 3.9333	64.6

nc : not calculated because of non-linearity of the relationship.

half-life of bacteria in convalescent rat LLF than in normal LLF, whereas, convalescent rat SUR gave only 17 min. The half-lives of bacteria in normal and convalescent rat SN were indistinguishable.

Artificial surfactant: Commercially prepared artificial surfactant (ALEC) as used to treat premature babies with respiratory destress syndrome, was tested for BA towards *B. pertussis* strains 18-323 and lux. Normal rat SUR, PBS and 0.85 (w/v) saline were included as controls.

Table 47 shows that the artificial surfactant had no bactericidal effect on *B. pertussis* 18-323, compared with the strong bactericidal effect shown by rat SUR. Towards *B. pertussis* lux, both rat and artificial surfactant gave a roughly similar reduction of bacterial luminescence. This could have been due to the bactericidal effect of rat LLF or, in case of the artificial surfactant, to optical interference with luminescence. It is therefore, possible that artificial surfactant may have a slight BA towards *B. pertussis* lux.

Biochemical and Serological Analysis

Since it is well known that lysozyme and free fatty acids have a BA towards some bacteria, LLF and its fractions were analysed for these substances. Also, since the killing of some bacteria is mediated by antibodies and complement, LLF was tested serologically for these components. LLF and its fractions were also assayed for total protein.

Protein determination

LLF and its fractions were assayed for protein by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985) which was chosen because of its high sensitivity. Figure 55 show that LLF collected from convalescent rats had more protein than LLF from normal animals. Euthanasia method B gave LLF with slightly more protein than euthanasia method D. This in turn was higher in protein than LLF of method A. The lowest protein concentration was found with the LLF collected from a single rat batch by method C.

Table 47. Percent survival* of *B. pertussis* 18-323 (viable count) and lux (luminescence) in rat LLF surfactant and artificial surfactant after incubation for 1 h at 37°C.

Dilution	% Survival & luminescence after 1h incubation time at different dilution			
	Rat surfactant		Artificial surfactant	
	Viable count	Luminescence	Viable count	Luminescence
1/1	0 , 0	9	71 , 95	5.2
1/4	0 , 0	44	146 , 185	33
1/16	32 , 42	87	138 , 185	103
1/64	76 , 113	107	125 , 150	132

* % survival calculation was based on PBS time-1h count for rat surfactant and saline time-1h count for artificial surfactant .

Figure 55. Biochemical characterization of LLF in relation to the euthanasia methods A, B, C and D, infectious status and batch number. Clustering of data points is reinforced by area outlining and shadowing. Solid points and shaded areas are for convalescent LLF, plain points and areas for normal LLF. ○ ● (A); △ ▲ (B); □ (C); ◇ ◆ (D).

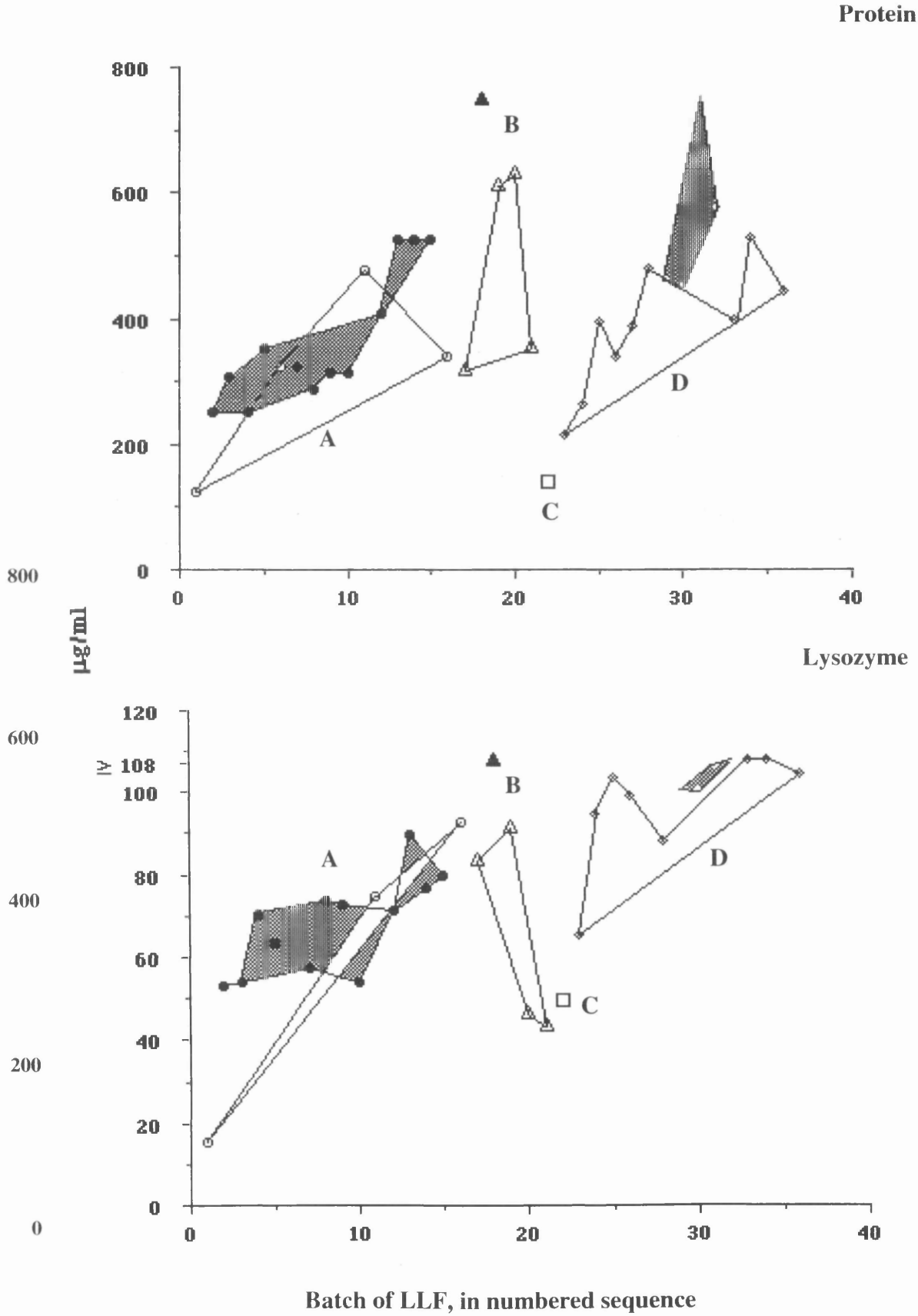
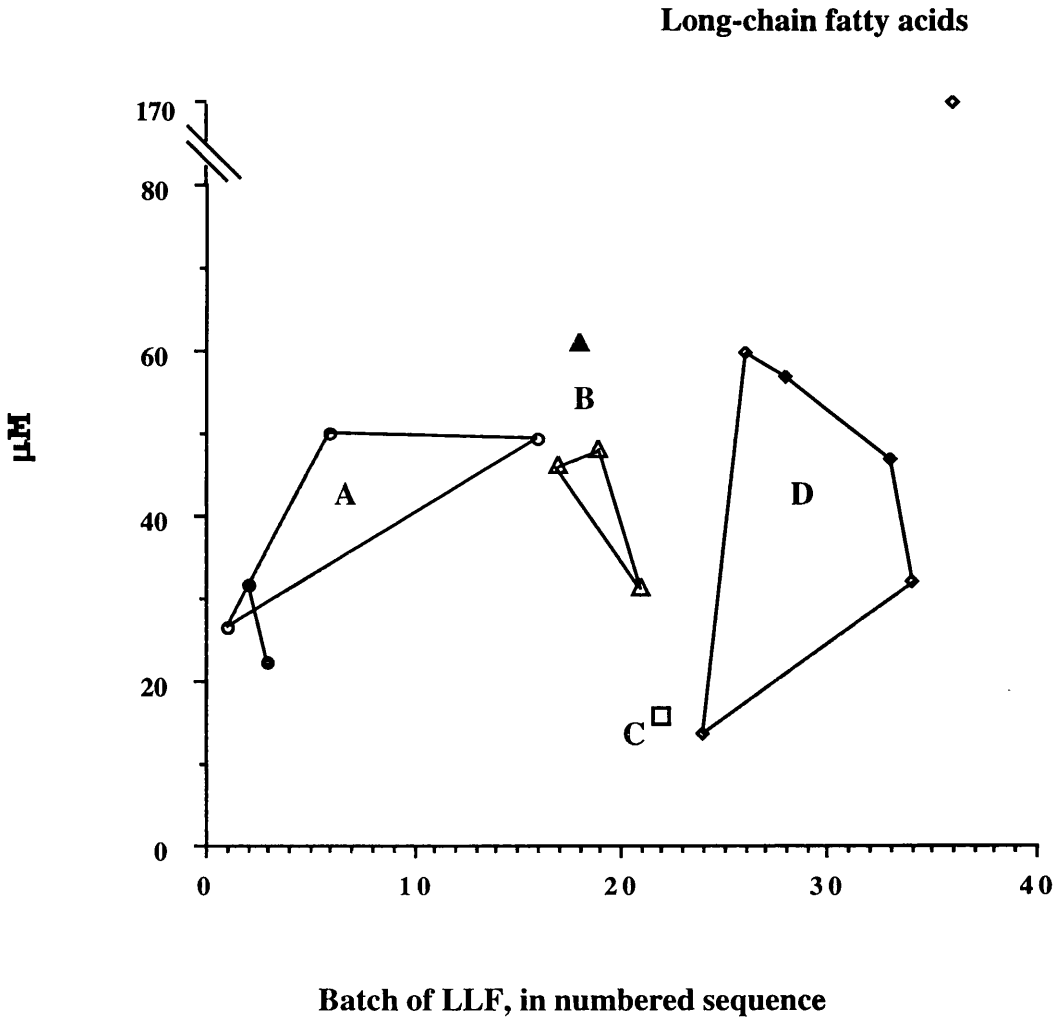


Figure 55. continued.



Nearly similar protein concentrations in LLF from normal and sham-operated rats was found (Table 48), which in turn were lower than the protein concentration in LLF from convalescent rats. Approximately 85 % of LLF protein was in the SN fraction, whereas, only 3 % was in the SUR fraction. Also, less protein was found in membrane (0.45 μm pore size) filtered SUR than in unfiltered material. Appendix 26 has more detailed results.

Lysozyme detection by viable counting

A series of experiments was done to detect whether there was lysozyme in LLF. For this purpose, *Micrococcus luteus* (sensitive to lysozyme) together with *B. pertussis* 18-323 as a test organisms were standardized (A_{540nm} = 0.45), incubated for various time in neat and diluted LLF. Egg hen's white lysozyme at different concentrations, nutrient broth, and PBS were used in all experiments as test controls. Bentonite as adsorbing agent for lysozyme also was applied as a control.

Results (Table 49) revealed that LLF from both normal and convalescent rats had high BA towards *M. luteus*. This activity was higher in LLF from the convalescent animals, but with the exception that *M. luteus* survived better during 1440 min in 1/100 of LLF of convalescent rats than in LLF of normal animals. Complete killing of the bacteria in LLF, at dilutions up to 1:100, from both normal and convalescent rats was observed after 120 min of incubation at 30 °C. The amount of lysozyme in both undiluted test fluids was ≥ 10 μg/ml.

There was gradual loss of viability of *M. luteus* in lysozyme at 1 μg/ ml but complete and rapid killing at 10 μg/ml lysozyme. Incubation of the bacteria in bentonite-treated lysozyme yielded as good survival as in nutrient broth. *B. pertussis* 18-323 was not killed at up to 100 μg/ml lysozyme.

Lysozyme estimation by spectrophotometry

LLF and its fractions were assayed for lysozyme by the spectrophotometric method described by Wardlaw and McHenery (1982). Figure 55 shows that there was more lysozy-

Table 48: Protein concentration in LLF from normal (N) sham-operated (SO) and convalescent (C) rats. The fluid was either membrane (0.45 μm pore size) filtered (F) or unfiltered (UF). Data for supernate and surfactant fractions are also given.

LLF Batch no	Treatment of rats	Protein (μg/ml)±SEM [% yield]			
		LLF	supernate	surfactant	
		F	F	F	UF
37	N	635±10	540±9 [85]	141±1 [22]	nt
40	N	390±3	275±3 [71]	19±1 [5]	-
41	N	480±6	407±4 [85]	10±2 [2]	24±2 [5]
	SO1	460±3	345±3 [75]	11±2 [2]	29±2 [6]
	SO2	450±3	352±2 [78]	9±2 [2]	24±1 [5]
	C1	755±10	670±6 [89]	14±1 [2]	29±2 [4]
	C2	577±9	268±2 [46]	13±2 [2]	24±1 [4]
42	N	400±3	392±2 [98]	nt	36±1 [9]
45	N	445±2	407±3 [[92]	-	52±1 [12]
Median [% yield]		460 [100]	392 [85]	14 [3]	29 [6]

SO1: Female rats given agarose with no bacteria, with ether.

SO2: " " " alginate " " " , " " .

C1 : " " challenged with *B. pertussis* EH2 in agarose, with ether

C2 : " " " " " " "alginate, " "

Table 49. Percent survival of both *Micrococcus luteus* (Ml) and *B. pertussis* (Bp) 18-323 in LLF from normal (N) and convalescent (C) rats. Bentonite treated and untreated egg hens white lysozyme (LZ), PBS and nutrient broth (NB) were used as controls.

Test fluids	Bacteria Species and Temperature (°C)	No. of experiments	Mean % survival* (No. of observations) at incubation time (min)				
			0	30	60	120	1440
NLLF	Ml (30)	12	2±1 (12)	0 (10)	0 (2)	0 (12)	0 (6)
1/10 NLLF	"	5	33±20 (5)	0 (5)	0 (5)	0 (5)	0 (5)
1/100 NLLF	"	5	89±5 (5)	49±17 (5)	59±13 (2)	51±23 (5)	0 (3)
CLLF	"	11	0 (11)	0 (2)	0 (9)	0 (2)	0 (11)
1/10 CLLF	"	5	0 (5)	0 (5)	0 (2)	0 (5)	0 (3)
1/100 CLLF	"	5	77±8 (5)	62±16 (5)	71±0 (2)	24±10 (5)	19±19 (3)
LZ 10 µg/ml	"	4	1±0 (4)	0 (2)	0 (4)	0 (2)	nt
LZ 1 µg/ml	"	9	83±8 (9)	30±10 (8)	54±4 (2)	17±7 (9)	1±1 (6)
LZ 10 µg/ml + bentonite	"	2	105±3 (2)	82±12 (2)	89±12 (2)	96±10 (2)	nt
NB	"	11	101±3 (11)	99±3 (10)	100±7 (2)	93±7 (11)	556±36 (6)
LZ 10 µg/ml	Bp (37)	2	86±4 (2)	83±1(2)	85±1(2)	64±3 (2)	nt
LZ 100 µg/ml	"	2	85±6 (2)	70±7 (2)	67±6 (2)	59±4 (2)	nt
PBS	"	2	100±0 (2)	80±10 (2)	73±3 (2)	56±4 (2)	nt

* % Survival calculations were based on nutrient broth time-zero count for test fluids tested with *M. luteus*, and on PBS time-zero count for fluids tested with *B. pertussis* 18-323.

of LLF collected from rats by euthanasia method B, which was less than that of method D. LLF from only a single batch of normal rats killed by euthanasia method C was similar to method B.

Results in Table 50 shows that almost all (around 100 %) of the LLF lysozyme was in the SN fraction and less than 5 % was in the SUR. There was less lysozyme in membrane (0.45 μm pore size) filtered SUR than in the unfiltered material. For more detailed results see Appendix 27.

Long-chain fatty acids (FA)

The colorimetric assay of Duncombe (1963) was used to estimate FA in LLF and its fractions. Figure 55 show that there were no significant differences in concentration of FA in LLF from normal and convalescent rats. The highest concentration (170 μM) was observed with one out of 6 batches of LLF obtained by euthanasia method D. There were no significant differences between the other 5 LLF batches of euthanasia method D and LLF batches of both method A and B. On the other hand, the single batch of LLF obtained by euthanasia method C gave a low FA concentration (16 μM). The median FA concentration in LLF was 46 μM.

There were variable amounts of FA, with no significant differences, in LLF from normal, sham-operated and convalescent rats (Table 51). Most (44 %) of the LLF FA were detected in the SUR fraction, whereas only 17 % was in the SN fraction (LLF batch no. 45). Also, with 4 other LLF batches, more FA were in the SUR than in the SN. Filtered LLF (batch no. 43) had only about one-half the FA of unfiltered fluids (see footnote).

LLF from species other than the rat were analysed for protein, lysozyme and FA. In addition, the killing of *B. pertussis* by these fluids was determined. Table 52 shows that the highest protein concentration was detected in mouse LLF, whereas the highest lysozyme was found in rat LLF. LLF from human and rats had similar protein and FA concentrations.

Table 50. Yield of lysozyme in normal (N) and convalescent (C) rat LLF, either membrane (0.45 µm pore size) filtered (F) or unfiltered (UF) and in supernate and surfactant fractions after ultracentrifugation.

LLF batch no.	Treatment of rats	Lysozyme (µg/ml)±SEM [% yield]			
		LLF	supernate	surfactant	
		F	F	F	UF
37	N	47±1	45±1 [96]	<5±0 [<11]	nt
40	N	99±1	111±5 [112]	<5±0 [<5]	-
41	N	88±1	99±1 [113]	5±2 [6]	-
41	C1	107±1	>108±0 [>101]	<5±0 [<5]	-
42	N	>108±0	nt	nt	36±1 [>33]
45	N	105±1	107±7 [102]	-	<5±0 [<5]
Median [% yield]		102 [100]	107 [105]	<5 [<5]	<21 [<21]

C1 : Female rats challenged with *B. pertussis* EH2 in agarose, with ether.

Table 51. Long-chain fatty acids (FA) quantitation in filtered (F) and unfiltered (UF) LLF, and in supernate and surfactant fractions from normal (N), sham-operated (SO) and convalescent (C) rats by different method of euthanasia. Data are the means of duplicate observations.

LLF batch no.	Treatment of rats	Method of euthanasia	FA μM (±SEM) in 0.5 ml of		
			Unfractionated LLF	Supernate (% yield)	Surfactant (% yield)
			F	F	NF
15	N	A	26±1	nt	nt
	C1	A	32±0	-	-
	C2	A	22±2	-	-
16	SO	A	50±6	-	-
33	N	A	50±4	-	-
34	N	B	46.0	-	-
	C	B	61±16	-	-
35	N	B	48±4	-	-
38	N	B	31	-	-
	N	C	16±1	-	-
39	N	D	13±1	-	-
40	N	D	60±9	-	-
41	N	D	57±1	-	-
42	N	D	47	-	-
43	N	D	32±0*	-	-
44	N	D	nt	8±2	55±10
45	N	D	170±25	29±1	75±4
				(17)	(44)
50	N	D	nt	19	104±6
55	N	D	-	3±0	91±11
Median			46	14	65

* Unfiltered fluids gave 83±23 μM.

Table 52: Analytical characteristics and bactericidal activity of human and animal LLF towards *B. pertussis* 18-323.

Human and animal LLF	Protein (µg/ml) (±SEM) [no. of experiments]	Fatty acids (µM) (±SEM)	Lysozyme (µg/ml) (±SEM)	Mean (±SEM) % survival* after exposure at 37°C for (min)		
				0	30	120
Human	367±4 [3]	61 [1]	>36±0 [2]	91±5 [3]	61±10 [3]	18±2 [3]
Rat	377±33 [18]	50±11 [12]	75±7 [14]	66±16 [3]	0 [3]	0 [3]
Horse	630±6 [3]	24 [1]	6±1 [2]	95±6 [2]	138±18 [2]	75±17 [2]
Mouse	563±9 [3]	nt [1]	22±1 [2]	84±4 [3]	79±3 [3]	77±3 [3]
Rabbit	212±4 [3]	nt [1]	6±1 [2]	85±4 [3]	76±2 [3]	61±6 [3]
Sheep	322±4 [3]	11 [1]	12±2 [2]	82±0 [3]	76±3 [3]	73±5 [3]
Dog	533±12 [3]	nt [1]	17±1 [2]	119±6 [2]	144±20 [2]	42±15 [2]
Calf	417±4 [3]	26±4 [2]	1±0 [2]	85±10 [2]	100±9 [2]	28±1 [2]

* % Survival based on CL-medium time-zero count.

The lowest protein and lysozyme concentration were found in rabbit LLF. *B. pertussis* 18-323 survived well in the LLF from other than the rat, except that there was gradual decrease in viability in the human and calf fluids.

Detection of antibodies

An ELISA method was used to examine the presence of specific antibody classes IgG and IgA against whole cell sonicate of *B. pertussis* or its active component namely pertussis toxin (PT) and filamentous haemagglutinin (FHA) in rat LLF and serum. Also, ELISA tests for total IgE in LLF were done. Neat and 10-fold dilutions of LLF and serum were obtained from normal, sham-operated (agarose ± carrageenan), vaccinated and challenged (*B. pertussis* L84 IV and / or 18-323 I) rats.

Test fluids from two different rat batches, batch no. 15 (fluids collected 28 days after treatment) and batch no. 16 (fluids collected 42 days after treatment). Rats of these two batches were subjected to euthanasia method A.

As a positive control, a hyperimmune reference serum from two rats was used, and a pool of normal rat serum provided a negative control. Both controls were included in each ELISA plate. Table 53 presents the results of test fluids from rat batch no. 15 and Table 54 for batch no. 16. High titres (Table 53) of IgG to BP sonicate, FHA and PT were observed in serum from convalescent rats. Only very low titres of IgG antibody against the same antigens were found in LLF from the same convalescent rats. No, or very low, titres of IgG and IgA antibodies against the three antigens were observed in either serum or LLF.

Serum and LLF from rats challenged with L84 phase IV gave lower or no titre of antibody of both classes towards respectively Bp sonicate, and FHA or PT. Rats infected with *B. pertussis* 18-323 phase I yielded fluids of a higher titre of antibody of both classes against the same antigen. Serum and LLF from sham-operated (Table 54) rats gave roughly the same titres as with fluids from normal rats (Table 53), while higher titres of both IgG and IgA against the three antigens was found with fluids from vaccinated and challenged animals.

Table 53. Detection of antibodies to BP sonicate, FHA, and PT antibodies in both normal (N) and convalescent (C) rat LLF and serum batch no. 15. Hyperimmune serum from rats injected with *B. pertussis* sonicate was usedas a positive control and normal rat serum as a negative control (Data are the means of duplicate observations).

Test fluid	Rat Treatment	ELISA titre					
		IgG			IgA		
		BP sonicate	FHA	PT	BP sonicate	FHA	PT
LLF	C1	<10	<10	<10	<10	<10	37
	C2	14	18	52	18	<10	26
	C3	<10	14	41	20	<10	26
	C4	<10	<10	16	<10	<10	30
	N	<10	<10	<10	<10	<10	nt
Positive control	-	55000	43000	500	500	360	10000
Negative control	-	<10	<10	<10	<10	<10	5000
Serum	C1	1600	<10	<10	73	<10	nt
	C2	4000	5000	9900	90	<10	nt
	C3	1200	890	5800	55	<10	nt
	C4	3400	1800	3900	60	<10	nt
	N	50	<10	<10	39	<10	nt
Positive control	-	220000	90000	1700	920	19	nt
negative control	-	10	<10	<10	79	<10	nt

C1 = Female rats given *B.pertussis* L84 IV in agarose beads, with ether

C2 = Female rats given *B. pertussis* 18-323 I in agarose beads, with ether

C3 = Male rats given *B. pertussis* 18-323 I in agarose beads, with ether

C4 = Female rats given *B. pertussis* 18-323 I in agarose beads, no ether

Table 54. Detection of antibodies to BP sonicate, FHA, and PT in both sham-operated (SO) and convalescent (C) rat LLF and serum batch no. 16. Hyperimmune serum from rats injected with *B. pertussis* sonicate was used as a positive control and normal rat serum as a negative control (Data are the means of duplicate observation).

Test fluid	Rat Treatment	ELISA titre					
		IgG			IgA		
		BP sonicate	FHA	PT	BP sonicate	FHA	PT
LLF	C1	36	18	<10	<10	<10	18
	C2	30	23	<10	<10	<10	<10
	C3	<10	15	<10	<10	<10	<10
	C4	<10	<10	<10	<10	<10	<10
	SO	<10	<10	<10	<10	<10	17
Positive control	-	30800	37000	120000	500	360	10000
Negative control	-	<10	<10	<10	<10	<10	5000
Serum	C1	nt	nt	nt	nt	nt	nt
	C2	16000	7300	4500	330	17	nt
	C3	3600	1800	900	60	<10	nt
	C4	2500	630	1300	59	<10	nt
	SO	24	<10	<10	60	<10	nt
Positive control	-	170000	100000	<1000	850	26	nt
negative control	-	<10	<10	<10	100	<10	nt

C1 = Male rats given DPT vaccine+*B.pertussis* 18-323 EH1 in agarose beads, with ether.

C2= " rats given DPT vaccine+*B.pertussis* 18-323 EH1 in carrageenan+agarose beads, with ether.

C3 = Male rats given *B.pertussis* 18-323 EH1 in agarose beads, with ether.

C4 = " rats given *B.pertussis* 18-323 EH1 in carrageenan+agarose beads, with ether.

SO = Male rats given agarose beads, with ether.

Infection of unvaccinated rats yielded fluids with lower titres of both IgG and IgA towards the same antigens. The overall conclusion was that serum gave higher titres of IgG and IgA against BP-sonicate, FHA and PT than LLF, except that the LLF IgA titre against FHA was higher than that of serum IgA towards the same antigen. Anti-*B. pertussis* total IgE in undiluted LLF was examined and only very low titres detected.

Complement detection

It is well known that the serum killing of many gram-negative bacteria is mediated by complement, which might act similarly against *B. pertussis* 18-323 in the LLF. Accordingly a series of experiments was done to test for complement. For this purpose *E. coli* Lilly was applied in all experiments due to its sensitivity to complement. As controls, nutrient broth, PBS, untreated, neat, diluted (1/16) and inactivated guinea pig serum (as a source of complement) were used.

Results (Table 55) showed that there was no reduction in viability of *E. coli* Lilly in LLF or its fractions after 120 min of incubation. In serum, complete killing of the bacteria was found after only 15 min incubation. Unfractionated LLF supported the survival and growth of the bacteria, which survived and grew better in both the SN and nutrient broth.

SUR acted similarly as PBS towards *E. coli* Lilly, which was completely killed in both 1/16 and neat guinea pig serum after 30 min of incubation. Heat inactivation of guinea pig serum yielded a good survival and growth of the bacteria in the inactivated serum. Complete killing of the bacteria in diluted guinea pig serum confirmed the high level of complement present in that fluid. It was concluded that LLF and its fractions contained no detectable amount of complement.

Effect of Various Treatments

NLLF and its ultracentrifuged (29, 000 rpm; 55, 000 g) fractions were exposed to various physical, chemical, biochemical and enzyme treatments in order to characterize the substance(s) which might be responsible for the bactericidal effects on *B. pertussis.* In choosing batches of LLF for these studies it was desirable to have batches of the highest

Table 55.-Bactericidal activity of LLF, surfactant (SUR), supernate (SN) and serum (S) from normal (N) and convalescent (C) rats towards *E. coli* Lilly during incubation for 24 h at 37°C. PBS, nutrient broth (NB) and guinea pig serum (GPS) were used as negative and positive controls respectivley.

Test fluids	No. of experiments	Mean % survival* (No. of observation) at incubation time (min)				
		0	15	30	60	120
NLLF	10	102±5 (10)	112±13 (5)	98±6 (9)	137±21 (4)	203±17 (10)
NSUR	2	90±9 (2)	nt	85±9 (2)	77±4 (2)	85±23 (2)
NSN	2	93±6 (2)	nt	83±7 (2)	345±12 (2)	691±24 (2)
NRS	7	10±3 (7)	0 (5)	0 (5)	nt	0 (7)
CLLF	23	95±2 (23)	114±11 (2)	96±3 (22)	147±5 (20)	214±6 (23)
CRS	4	9±5 (4)	0 (2)	0 (2)	nt	0 (4)
GPS	1	117 (1)	nt	0 (1)	0 (1)	0 (1)
1/16 GPS	3	91±11 (3)	0 (3)	0 (3)	nt	0 (3)
In/GPS	1	200 (1)	nt	208 (1)	208 (1)	442 (1)
In 1/16 GPS	1	175 (1)	nt	177 (1)	238 (1)	429 (1)
NB	4	100±0 (4)	100±7 (3)	104±6 (4)	141 (1)	358±34 (4)
PBS	5	100±0 (5)	87±13 (5)	98±5 (5)	111±5 (5)	103±8 (5)

* % survival calculations for all test and control fluids were based on time-zero of nutrient broth, and on PBS time-zero count for PBS only.

activity and obtained by euthanasia method D. Batches nos 44, 45 and 55 met this requirement. These studies used luminometry measurements with strain lux and viability counting with strain 18-323. Table 56, which presents Lum-90 and Lum-50 end points, shows that the SUR fraction of NLLF batches nos. 44 and 45 had about 10 times higher BA towards strain lux as the SN fraction. The low activity in SUR from NLLF batch 50 could not be explained.

Essentially parallel information was obtained with viable counts of strain 18-323, and VC-90 and VC-50 end points (Table 57). For more detailed information, see Appendix 29 and 30.

Physical and chemical treatments of NLLF

In preliminary experiments, three different batches of NLLF were exposed to heating at 56 and 100°C, dialysis and ultrafiltration and acid and alkali treatments. The samples were then tested undiluted for ability to reduce the viable count of *B. pertussis* 18-323 during 30 min at 37°C. The results in Table 58 indicate that the BA was slightly affected by 30 min at 56°C and destroyed to a considerable extent by 5 min at 100°C. The dialysis and ultrafiltration experiments showed that the BA in the LLF was mainly due to factor(s) of high molecular weight.

Finally, the activity was only slightly affected by 18 h exposure to 0.1 M HCl or NaOH at 4 °C. For further details see Appendix 28.

Sonication and washing of surfactant

Since the surfactant (NSUR) fraction of normal LLF contained the major part of the BA, a few experiments were done to determine the best method of preparing this material. In particular, it was necessary to know whether the sonication that was used to disperse the resuspended pellet had a detrimental effect on its activity. Also whether the ultracentrifuge pellet could be resuspended in PBS and recentrifuged, i. e. "washed", in PBS so as to get a cleaner separation from the supernate (NSN) fraction. The effect of these treatments was studied by both luminometry and viable counting as shown in Table 59. It was clear that

Table 56. Comparison of *B. pertussis* lux inactivating potency of 55,000 g supernate and surfactant fractions of normal rat LLF. (original data in Appendix 29).

LLF Batch No.	Luminescence (Lum-90 and Lum-50) end points (µl)* in individual experiments and [Median]			
	Supernate		Surfactant	
	Lum-90	Lum-50	Lum-90	Lum-50
44	350, >400,	120, 165	40, 75,	20, 20
	250, 250	135, 90	55, 50	20, 20
	[300]	[122.5]	[52.5]	[20]
45	175, 265	30, 65	30, 30,	20, 20
	400	70	80, 290	25 , 30
	[265]	[65]	[30]	[22.5]
50	nt	nt	> 400	260

*Volumes of test fluids requred to inactivate 50 % or 90 % of standard inoculum of *B. pertussis* lux.

Table 57. Comparison of *B. pertussis* 18-323 inactivating potency of 55,000 g supernate and surfactant fractions of normal rat LLF. (original data in Appendix 30)

LLF Batch No.	Bactericidal activity (VC-90 and VC-50) end points (µl)* in individual experiments and [Median]			
	Supernate		Surfactant	
	VC-90	VC-50	VC-90	VC-50
55	>240, 132, 165	215, 90, 130	60, 25, 30	30, 8, 15
	160, >240, >240	126, 125, 60	30, 20, 15,	15, 10, 5
	200, 180, >240	120, 90, 150	15, 10, 20	5, 5, 10
	[200]	[125]	[20]	[10]
45	nt	nt	30	20

*Volumes of test fluids requred to inactivate 50 % or 90 % of standard inoculum of *B. pertussis* 18-323.

Table 58: Summary of effect of various treatments of LLF on its bactericidal activity towards *B. pertussis* 18-323 after 30 min of incubation.

Treatment	LLF batch no.	No. of observations	% Survival* [Range]
Nil	33,40,43	8	0 [0-0]
Heat : 56 °C for 30 min	33	2	4.5 [0-9]
Heat : 100 °C for 5 min	33	1	55
Dialysis (retentate)	33	2	0 [0-0]
Ultrafiltration 30 kDa filtrate	43	2	72.5 [54-71]
Ultrafiltration 30 kDa retentate	43	2	0 [0-0]
HCl : 0.1 M, 18 h 4 °C	40	2	2.5 [0-5]
NaOH: 0.1 M, 18 h 4 °C	40	2	8 [3-13]

* % survival calculations were based on CL-medium time-zero count data.

sonication had little deleterious effect but that washing in PBS caused unacceptable losses. From these results and the previously reported adverse effect of membrane filtration (Table 43), a standard procedure emerged for making NSUR. First, the LLF was collected as sterile as possible, ultracentrifuged in clean tubes and the final pellet sonicated, not further washed, and stored in small aliquots at -20 °C for 24 h, then at - 70 °C.

Physical and chemical treatments of NSN and NSUR

The same physical and chemical treatments which were applied to unfractionated NLLF were now given to NSN and NSUR and tested by luminometry with strain lux and by viable count with strain 18-323. Dilution assays were used in both test systems and both 50 and 90 % inactivation end points obtained. Three different batches of NLLF were used for these experiments.

Inactivation of NSN: The results in Table 60 show that the BA of the NSN fraction was partly destroyed at 56 °C and there was no detectable residual activity after heating at 100 °C. Similar extensive inactivation was produced by overnight exposure at 4 °C to either 0.1 M HCl or NaOH. Both the luminometry and the viable count methods gave consistent results with the NSN fractions exposed to these treatments. In contrast 10 cycles of freezing and thawing caused about 50 % inactivation as judge by luminometry while giving an apparent 20 % *increase* in BA by viable counting. A similar pattern of results was obtaind by dialysis, where the retentate fraction had no loss by luminometry but had more than doubled (- 127 % increase) in BA by viable counting. This pattern was again repeated by 18 h storage at 4 °C where there was a small loss in activity by luminometry and 3-fold *increase* by viable count.

It was concluded from these results that NSN contained a non-dialysable, heat-labile antibacterial substance which was also acid- and alkali-labile, and probably therefore protein. The observed *increases* in BA by viable counting with strain 18-323 suggested that the NSN fraction might contain an antibacterial substance in precursor form which could be

Table 59. Effect of sonication and washing (in PBS) of surfactant fraction on its bactericidal activity towards both *B. pertussis* lux and 18-323. The table shows the 50 % endpoints in µl (as lum-50 for luminescence experiments and VC-50 for viable count experiments) and the % inactivation (I). Data are the mean of two experiments.

Treatment*	Activity and effect of treatment			
	Lum-50		VC-50	
	µl	% I	µl	% I
Nil*	30	0	20	0
Sonication	20	-50	20	0
Nil**	260	0	5	0
Washed in PBS once	>400	>35	15	67
Washed in PBS 4 times	>400	>35	35	86

* LLF batch 45 was used in both test systems.

** LLF batch 50 was used for luminometry and batch 55 for viable counts.

activated by freezing and thawing, storage at 4 °C and possible removal of a low molecular weight accessory component by dialysis.

Inactivation of NSUR: Assays of the effects of the various physical and chemical treatments on the NSUR fraction are also presented in Table 60. In both test systems the NSUR was considerably more active than the NSN. In the luminometry system the ratio of NSN/NSUR endpoints ranged from 1.5-fold to 8-fold (average 5-fold), while in the viable counting system the range was from 8.3-fold to 24-fold, with an average of 14-fold. The viable counting results are probably the more reliable for these ratios, since luminometry had various technical problems such as optical quenching due to sample turbidity. Heating at either 56 or 100 °C had much less destructive effect on NSUR than it had on NSN, the 100°C treatment still leaving between 30 and 50 % of the initial activity. The acid and alkali treatments caused about 33 % loss of activity as judged by luminometry and 67 % by viable counting, the latter being taken as the more reliable figure. Neither freezing and thawing nor dialysis had any significant destructive effect on BA of NSUR. The increases in activity produced by some of the treatments when applied to NSN were not seen with NSUR.

It was concluded that the bactericidal substance(s) in NSUR, although also of high molecular weight, was qualitatively different from that in NSN as well as having a higher titre.

Non-enzymic biochemical agents

NSN and NSUR were treated with a variety of biochemical agents choozen for ability to remove lysozyme or growth inhibitors of phase I *B. pertussis*. The list thus included bentonite, charcoal, heated rat serum, bovine albumin serum, starch, methylcellulose and various forms of cyclodextrins. The untreated and treated test fluids were assayed for BA, but only with the viable counting system. This was partly because of complications with luminometry and partly because of shortage of NSN and NSUR samples. The NSN and NSUR fractions were from LLF batches nos. 45 and 55 (as above).

Table 60: Effect of physical and chemical treatments of supernate (NSN) and surfactant (NSUR) fractions of various batches of rat LLF on its bactericidal activity against *B. pertussis* lux and 18-323. The table shows the 50 % endpoints in µl (as Lum-50 for luminescence experiments and VC-50 for viable count experiments) and the % inactivation (I). Data are the mean of two experiments.

Treatment*	Activity and effect of treatment							
	NSN				NSUR			
	Lum-50		VC-50		Lum-50		VC-50	
	µl	% I	µl	% I	µl	% I	µl	% I
Nil*	120	0	125	0	20	0	10	0
Heat : 56°C for 30 min	255	53	>240	>48	30	33	15	33
Heat : 100°C for 15 min	>400	>70	>240	>48	70	71	20	50
Nil*	165	0	120	0	20	0	5	0
0.1 M HCl, 4 °C for 18 h	320	48	>240	>50	30	33	15	67
0.1 M NaOH, 4 °C for 18 h	380	57	180	33	30	33	15	67
4 °C for 18 h	210	21	40	-200	30	33	5	0
Nil*	90	0	60	0	20	0	5	0
Freezing & thawing 10 x	165	45	50	-20	20	0	5	0
Nil**	30	0	125	0	20	0	15	0
Dialysis (retentate)	30	0	55	-127	20	0	20	25

* LLF batch 44 was used for luminometry and batch 55 for viable counts.

** LLF batch 45 was used for luminometry and batch 55 for viable counts.

Inactivation of NSN All of the treatments listed in Table 61 caused at least some loss of the BA of NSN. In most instances the extent of this loss could not be properly quantitated because of the low initial activity of this fraction. Even 30 min incubation at 37°C (a control treatment) caused 30 % inactivation. It was notable that inactivation was caused by such a wide variety of substances, namely: insoluble agents (bentonite and charcoal), high molecular weight substances (heated serum, BSA, starch and methylcellulose) and the small-molecular cyclodextrins.

Inactivation of NSUR: Table 61 also shows that all of the above biochemical treatments (including 30 min at 37 °C control incubation) had a measurable effect on the BA of NSUR, although only a few of them caused essentially complete loss. The highly inactivating agents were charcoal and DMβCD which gave >97 % inactivation of NSUR BA. It was considered significant that unmethylated βCD and αCD were relatively inactive, just as they are ineffective in culture media designed to promote growth of *B. pertussis* from small inocula (Imaizumi *et al.* 1983a).

Protease treatment

To explore a possible role of proteins in the BA of LLF, the fluid was treated with three different proteases at a final concentration of 1.9 mg/ml. Luminometry with *B. pertussis* lux was used to monitor the results (Table 62). There was no significant loss of activity brought about by trypsin, chymotrypsin or subtilisin, leading to the conclusion at that time that proteins in LLF had little involvement. However, the Table shows that the enzymes alone, in control tests, had an apparent enhancing effect on luminescence. This complicates the interpretation of any possible destructive effect of the proteases on proteins in the LLF.

To confirm that the proteases were enzymically active, they were incubated with hide powder azure at 37 °C for 30 min and the release of dye demonstrated. Control experiments showed that there was no detectable amount of protease or protease inhibitors in LLF.

Table 61. Effect of treatment of supernate (NSN) and surfactant (NSUR) fractions of rat LLF batch no. 55 with non-enzymic biochemical agents on its bactericidal activity against *B. pertussis* 18-323. The table shows the 50 % endpoints in µl (as VC-50 for viable count experiments) and the % inactivation (I). Data are the mean of two experiments.

Treatment	Activity and effect of treatment			
	NSN (VC-50)		NSUR(VC-50)	
	µl	% I	µl	% I
Nil	90	0	5	0
Bentonite (11mg/ml)	>240	>63	15	67
Charcoal (16.9 mg/ml)	>240	>63	>240	>98
37 °C for 30 min	130	31	15	67
Nil	130	0	15	0
Heated rat serum (0.05%)	>240	>46	55	73
BSA (1mg/ml)	>240	>46	65	77
Starch (1mg/ml)	215	40	20	25
37 °C for 30 min	190	32	20	25
Nil	150	0	5	0
Methylcellulose (1mg/ml)	>240	>38	15	67
37 °C for 30 min	205	27	15	67
Nil	90	0	8	0
DMβCD (1mg/ml)	>240	>63	>240	>97
βCD (1mg/ml)	>240	>63	16	50
αCD (1mg/ml)	>240	>63	16	50
37 °C for 30 min	>240	>63	16	50

Table 62. Effect of protease treatment of rat LLF batch no. 40 on its effect on luminescence of *B. pertussis* lux after incubation for 1h at 37 °C. Data are single observations.

Test fluid*	% Luminescence
PBS	100
Untreated LLF	3
LLF treated with trypsin	2
LLF treated with chymotrypsin	2
LLF treated with subtilisin	1
Trypsin in PBS	127
Chymotrypsin in PBS	122
Subtilisin in PBS	142

*All enzymes were tested at 1.9 mg/ml final concentration.

Inactivation of NSN: The same protease treatments which had been applied to LLF without significant effect were next used with the NSN fraction. In addition, proteinase K and two phospholipases (to explore phospholipid involvement) were tested, and most samples were assayed for BA in the two test systems. As judged by luminometry, trypsin, chymotrypsin and subtilisin had no destructive effect, while proteinase K caused significant but not complete inactivation (Table 63). This variation in result between the former three proteases and proteinase K is possibly due to differences in their action in hydrolyzing the substrate molecules. Trypsin, chymotrypsin and subtilisin cleave bonds within the peptide chain with varying degrees of specificity for particular amino acyl residues, while proteinase K attacks at different parts of the peptide chain.

Exact comparison of the luminometry and viable counting results is made difficult by the NSN having come from two different batches of LLF. However, it appeared by viable counting that none of the four proteases had much destructive effect on the BA of NSN. Simple incubation at 37 °C appeared to have a slight inactivations effect. Of the two phospholipases, A2 had a destructive effect whereas C did not. With the former however, there was at least 94 % loss of BA in NSUR after treatment.

Inactivation of NSUR: Luminometry experiments (Table 63) indicated that the four proteases caused similar but incomplete destruction of the BA of NSUR. However this was not confirmed by viable counting where the protease treatments for the most part were little different from simple incubation at 37 °C. Possibly proteinase K was slightly more active than the other three. The most significant inactivation was given by phospholipase A2 which was much more destructive (> 94 %) than phospholipase C or any of the proteases. This suggested that the substance(s) responsible for the BA in NSUR might contain, or be associated with, phospholipid.

Table 63. Effect of enzyme treatments of supernate (NSN) and surfactant (NSUR) fraction of rat LLF* on its bactericidal activity towards *B. pertussis* lux and 18-323. The table shows the 50 % endpoints in µl (as lum-50 for luminescence experiments and VC-50 for viable count experiments) and the % inactivation (I). Data are the means of two experiments.

Treatment**	Activity and effect of treatment							
	NSN				NSUR			
	Lum-50		VC-50		Lum-50		VC-50	
	µl	% I	µl	% I	µl	% I	µl	% I
Nil	135	0	215	0	20	0	10	0
Trypsin	135	0	>240	>10	50	60	30	67
Chymotrypsin	135	0	>240	>10	35	43	35	71
Subtilisin	135	0	230	7	50	60	25	60
37 °C for 30 min	155	13	>240	>10	20	0	25	60
Nil	70	0	150	0	25	0	10	0
Proteinase K	230	70	150	0	150	83	35	71
37 °C for 30 min	100	30	205	27	25	0	10	0
Nil	nt	nt	125	0	nt	nt	15	0
Phospholipase A2	-	-	>240	>48	-	-	>240	>94
Phospholipase C	-	-	125	0	-	-	40	63
37 °C for 30 min	-	-	135	7	-	-	20	25

*LLF batch 44 was used by luminometry and batch 55 for viable counts.

** All proteases were used with test fluids at final concentrations of 1.9 mg/ml. Phospholipase A2 was at 1.1 mg/ml and Phospholipase C at 0.26 mg/ml.

Lipid analysis and separation

Preliminary experiments on solvent extraction of NSUR to purify lipids were done by the method of Bligh and Dyer (1959) as modified by Coonrod *et al.* (1984). For this purpose a large batch (84 ml) of LLF from 20 rats was obtained and found to be highly active against *B. pertussis* 18-323 (Table 64). The NSN and NSUR fractions were prepared in the usual way and with results similar to those already reported, i.e. high activity in NSUR and little activity in NSN (Table 64).

Lipid extraction and chromatography: The procedure as described by Coonrod *et al* (1984) involved extraction of NSUR with a 1:2 (v/v) mixture of chloroform and methanol and recovery of the lipids from the lower chloroform layer. NSUR from 12 rats yielded 23 mg lipid, or about 2 mg per animal. Aliquots of the chloroform extract were subjected to silicic acid chromatography as described by Daniela *et al* (1994) using chloroform, acetone and methanol for successive elutions. The results of bactericidal tests (Table 64) show that although only about 40 % of the initial LLF BA was recovered in the initial chloroform layer, the subsequent chloroform eluate from silicic acid was much more active. In fact, this eluate when tested at a dilution equivalent to the original LLF showed over 100 % yield. The procedure was repeated four times with similar results.

Subsequent batchwise elution of the silicic acid with acetone and then with methanol yielded very little BA.

Long-chain fatty acids (FA) and phosphatidylcholine (PC): Previous studies similar to the above by Coonrod *et al* (1984) led to the conclusion that the BA (towards type 25 pneumococci) in NSUR was due to FA. These were associated with neutral lipid, and apparently went into the chloroform layer after chloroform/methanol extraction. In the present work, long-chain (14 C - 20 C) saturated and unsaturated FA and PC were dissolved either in NaOH, chloroform or in a mixture of chloroform and methanol and eventually tested as solutions in 1 % CAA. The PC was included here because of the inactivation reported in Table 63 after phospholipase A2 treatment.

Table 64: Bactericidal activity of normal surfactant, its extracted lipids, and the different solvent eluate of extracted lipids towards *B. pertussis* 18-323. For completeness, the results with unfractionated LLF and ultracentrifuge supernate are also included.

Fraction (from LLF batch no. 55)	No. of observations	50 % end point* (µl)	% recovery of activity from LLF
LLF	2	9.5+1.4	100
Supernate	10	138+17	7
Surfactant	12	8.0±1.4	119
Chloroform/Methanol extract	4	24±0.9	40
Chloroform eluate	4	8.5±1.2	112
Subsequent acetone eluate	4	238±37	4
Subsequent methanol eluate	4	>240±0.0	< 4

* % end points were based on the PBS or CAA 1h-counts with the fractions at concentrations equivalent to undiluted LLF.

Bactericidal tests were done by viable count with 18-323 and luminometry with strain lux. The former method was approximately sixteen times more sensitive (Table 65), perhaps because the test dose of bacteria was much smaller (by a factor of 10^4 CFU).

By the viable count method, the most active FA was the polyunsaturated (4 double bonds) arachidonic (C 20). The least active was the saturated C14 myristic. After arachidonic, the next most active was palmitoleic (C16, one double bond). Unsaturation of the FA was not closely correlated with BA since the saturated C16 palmitic was quite similar to the unsaturated C16 palmitoleic. [Concentration (55 μM) of equal volumes of FA mixture was active towards *B. pertussis* 18-323]. Unlike the FA, phosphatidylcholine had no BA at the highest level tested, 1000 μM.

Morphological effects

Transmission electron microscopic (TEM) examination

Thin sections of variously treated *B. pertussis* 18-323 and lux were examined by transmission electron microscopy to obtain morphological information about the effect of NSN and NSUR.

Included as negative and positive controls respectively were organisms incubated in PBS and CL medium, and in normal and convalescent rat serum. In addition, the ultrastructure of NSUR pellet was examined to see if it contained the lamellar bodies and tubular myelin described by Pison *et al.* (1994).

Surfactant Pellet (Plate 3): This shows the lamellar bodies surrounded by amorphous material and possibly containing the tubular myelin structures described by Pison *et al.* (1994).

Strain 18-323 in CL medium (Plate 4): The samples taken at time zero (A) and after 1h (B) incubation show cells of mainly normal morphology initially, but in B with some some cells losing their internal contents and others appearing irregular in shape.

Table 65. Effect of long-chain fatty acids (FA) and phosphatidylcholine on the viability and luminescence respectively of *B. pertussis* strains 18-323 and lux.

Fatty acid and lipid	Abbreviation*	50 % end point** (µM)		Ratio of endpoints
		18-323	lux	lux/18-323
Myristic	14:0	450,550 (500)	>1000,>1000 (>1000)	>2
Pentadecanoic	15:0	60,66 (63)	nt	-
Palmitic	16:0	25,95 (60)	700,700 (700)	12
Palmitoleic	16:1(9c)	30,30 (30)	550,850 (700)	23
Elaidic	18:1(9t)	95,105 (100)	>1000,>1000 (>1000)	>10
Petroselinic	18:1(6c)	60,60 (60)	nt	-
Oleic	18:1(9c)	55,55 (55)	>1000,>1000 (>1000)	>18
Linoleic	18:2(9c,12c)	50,60 (55)	850,950 (900)	16
Arachidonic	20:4(5c,8c,11c,14c)	15,25 (20)	>1000,900 (>950)	47.5
FA mixture	-	45,65 (55)	900, >1000 (>950)	17
Phosphatidylcholine	-	>1000,>1000 (>1000)	nt	-

* The number of carbon atoms is given first, then the number of double bonds. The positions of the lowest numbered carbon of each double bond and whether the configuration is cis (c) or trans (t) is indicated in parentheses.

** % end points were based on the CAA 1h-counts.

Plate 3. Transmission electron microscopy of a thin section of the 55,000 g pellet (surfactant) fraction of normal rat LLF. X 25,500. Arrows indicate lamellar bodies (dotted) and tubular myelin (plain) structures.

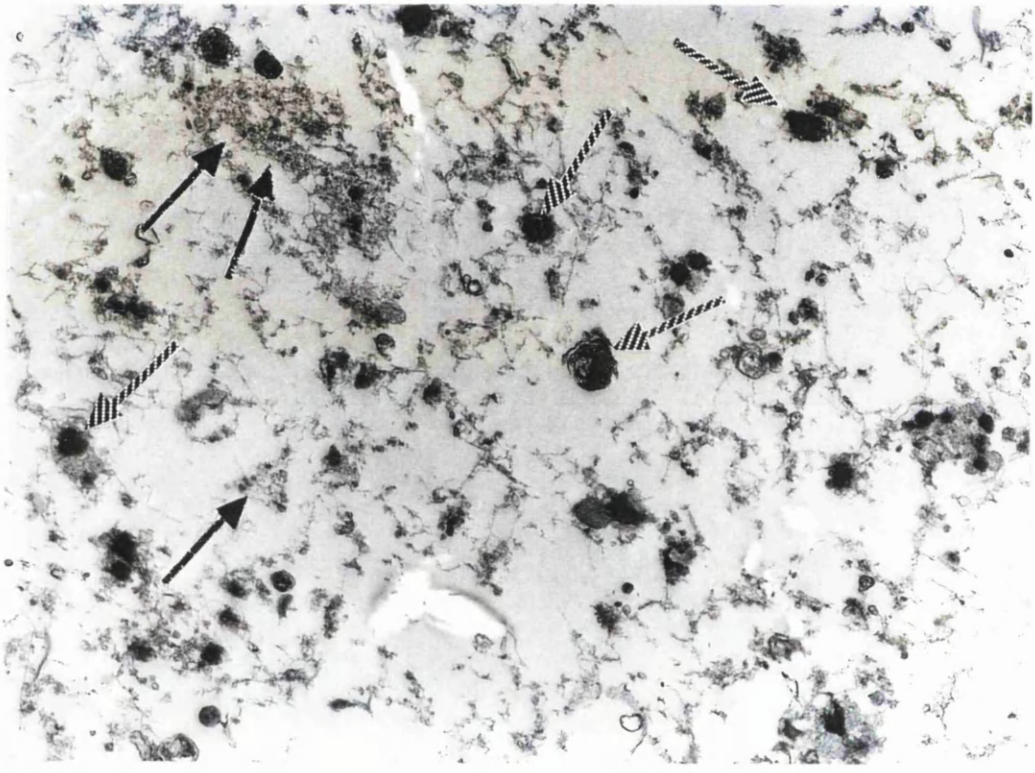
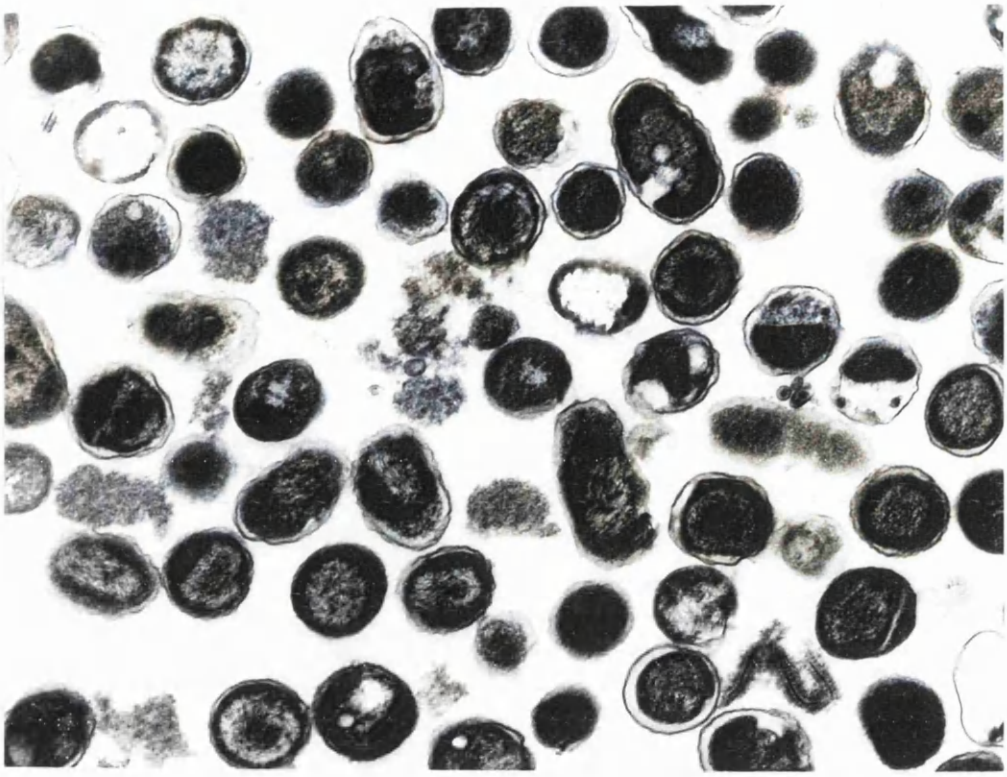
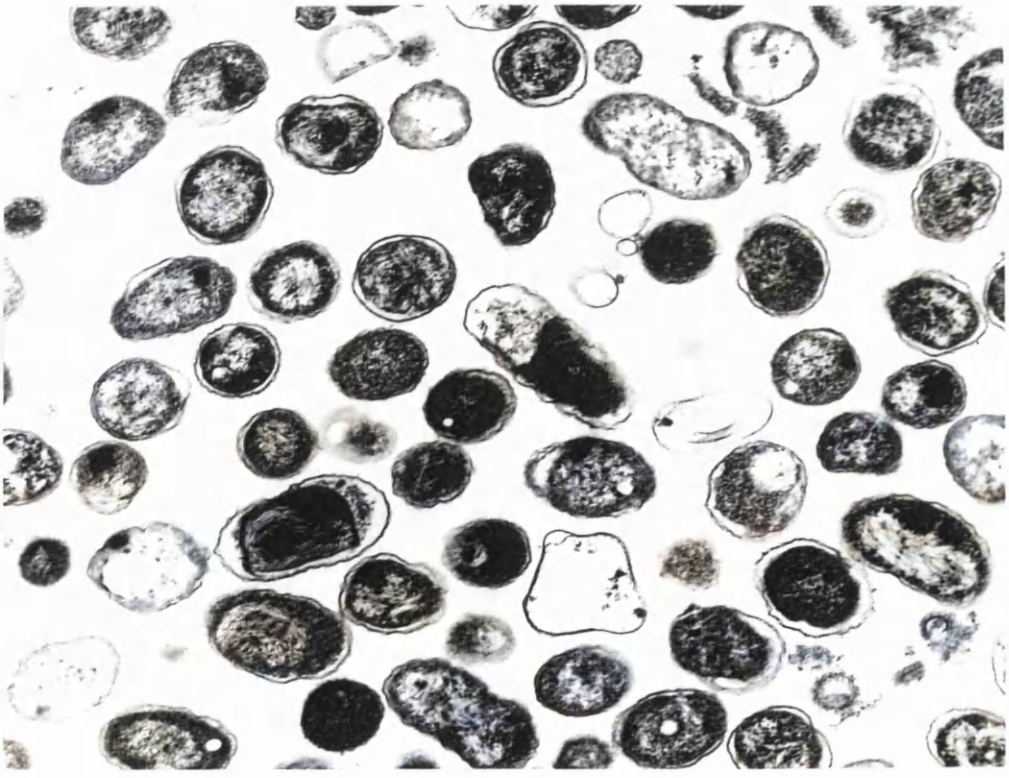


Plate 4. Transmission electron microscopy of thin sections of *B. pertussis* 18-323 A) at time-zero, and B) after incubation for 1h at 37°C in CL medium. X 25, 500.

A



B



Strain 18-323 in PBS (Plate 5): These micrographs were similar to those from CL medium.

Strain 18-323 in serum (Plate 6): In normal rat serum (A) many of the cells were severely damaged, some having lost their internal structures. In convalescent rat serum (B) the damage was even more extensive, with no cells left with normal morphology.

Strain 18-323 in NSN and NSUR (Plate 7): In NSN after 1 h incubation, some *B. pertussis* 18-323 cells (A) kept their normal morphology, a few have lost their internal structure while others showed some cytoplasmic membrane damage. More severe damage was noticed with cells incubated in NSUR (B), which caused cell membrane rupture, irregular cell morphology, and complete disappearance of internal structure for some cells.

Strain lux in PBS (Plate 8): *B. pertussis* lux showed normal intact morphological appearance after time zero (A) and 1h (B) incubation in PBS. An interesting observation was that most of these bacterial cells contained cytoplasmic vacuoles or inclusion bodies which could represent storage granules, which are much more numerous than in strain 18-323. *B. pertussis* lux cells suspension in PBS in this experiment gave the highest value (4000 mv) of light output.

Strain lux in NSN and NSUR (Plate 9): Action of NSN (A) on *B. pertussis* lux after 1 h of incubation resulted in an apparent loosening of the outer membrane and some loss of cell content. It was noticed that most of the cells lost their ability to produce light despite their normal morphology appearance. In NSUR (B), extremely severe damage of the cells and complete loss of internal structure, complete loss of light (cells gave only 15.5 mv, > 99 % light inactivation and rupture of cytoplasmic membrane, irregular appearance of the cell morphology.

Plate 5. Transmission electron microscopy of thin sections of *B. pertussis* 18-323 A) at time-zero, and B) after incubation for 1h at 37°C in PBS. X 25, 500.

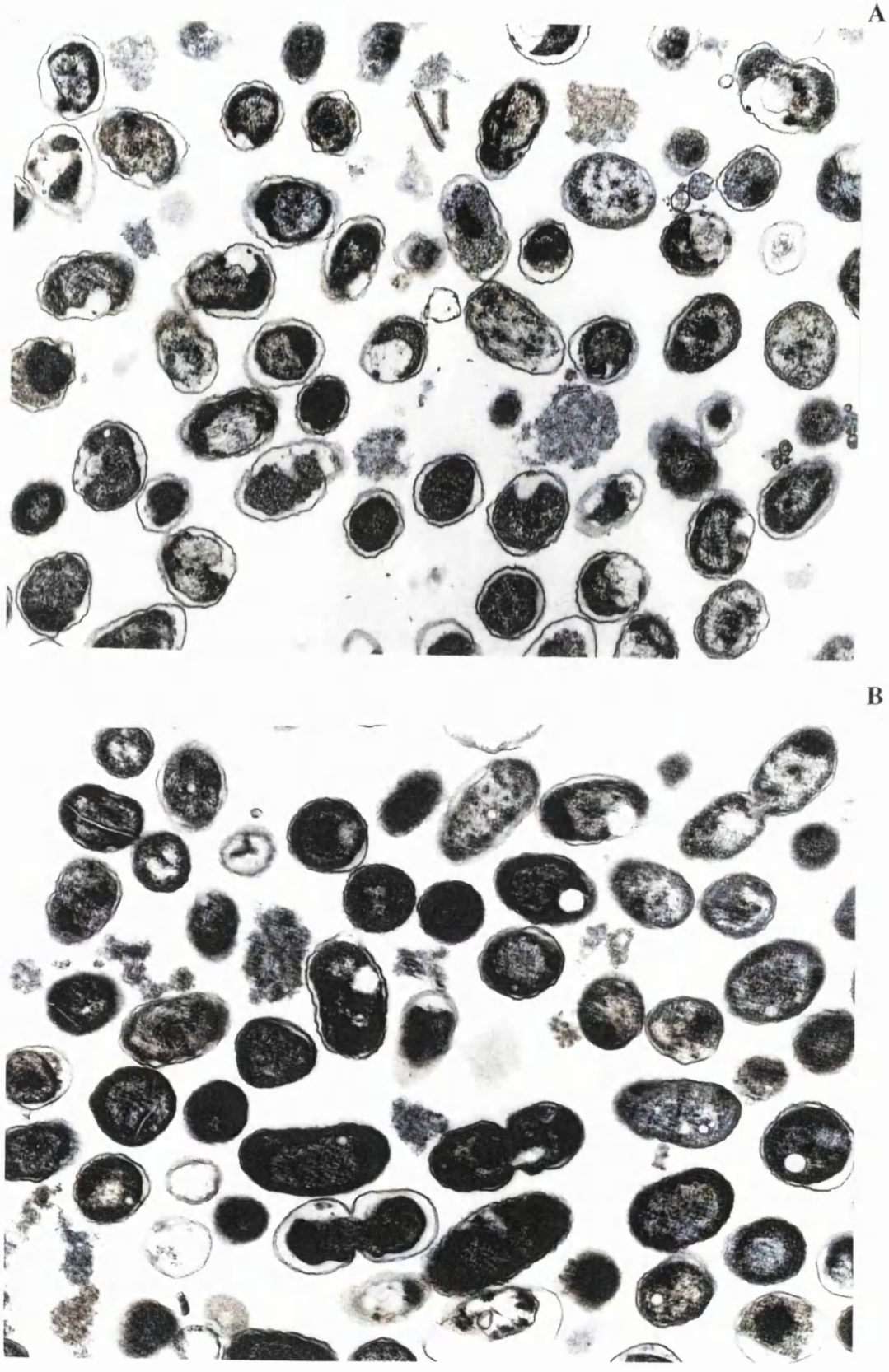
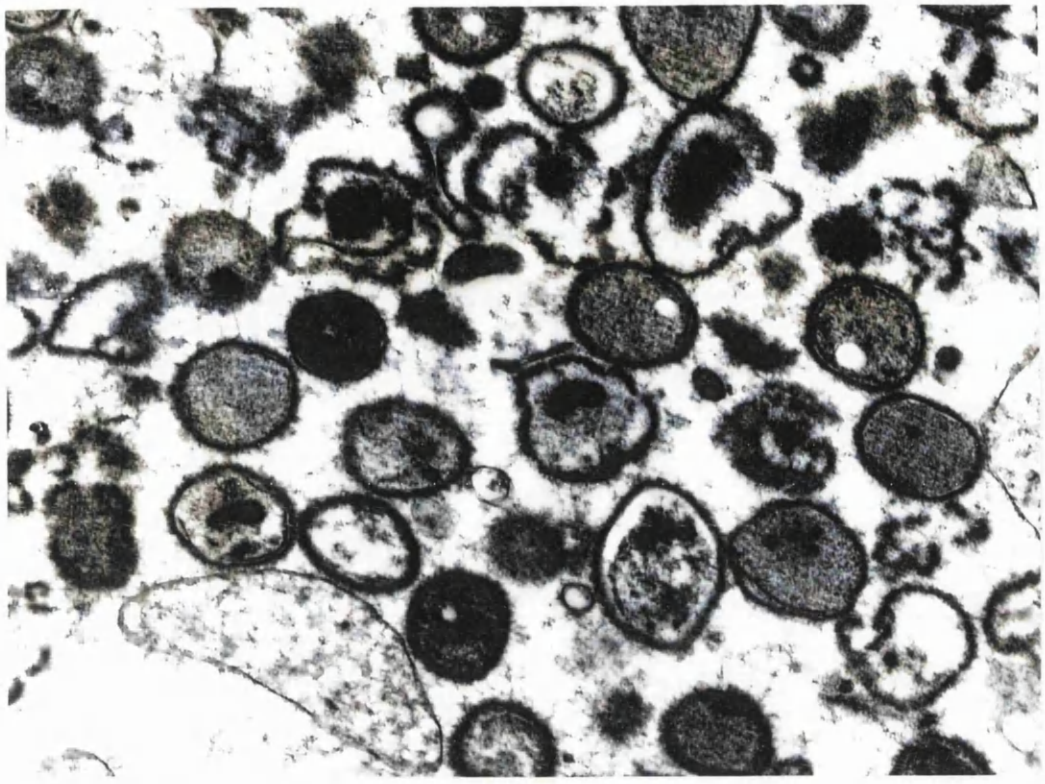


Plate 6. Transmission electron microscopy of thin sections of *B. pertussis* 18-323 after 1h incubation at 37 °C in A) normal ; and B) convalescent rat serum. X 25, 500.

A



B

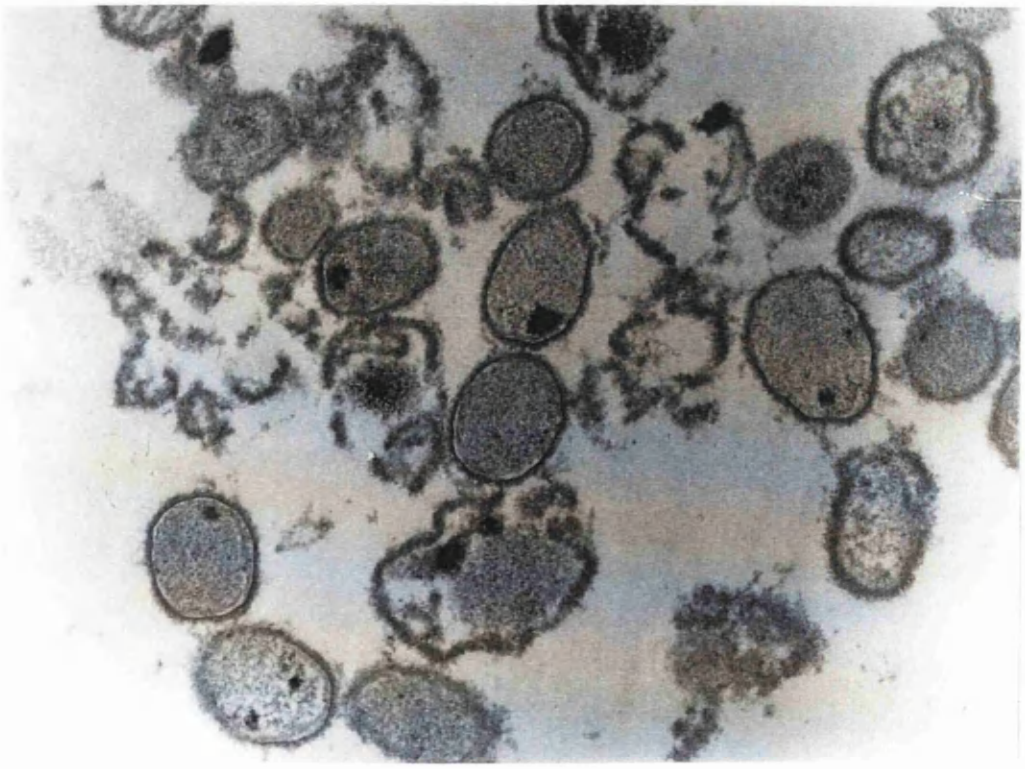


Plate 7. Transmission electron microscopy of thin sections of *B. pertussis* 18-323 after 1 h incubation at 37 °C in A) supernate; B) surfactant . X 25, 500.

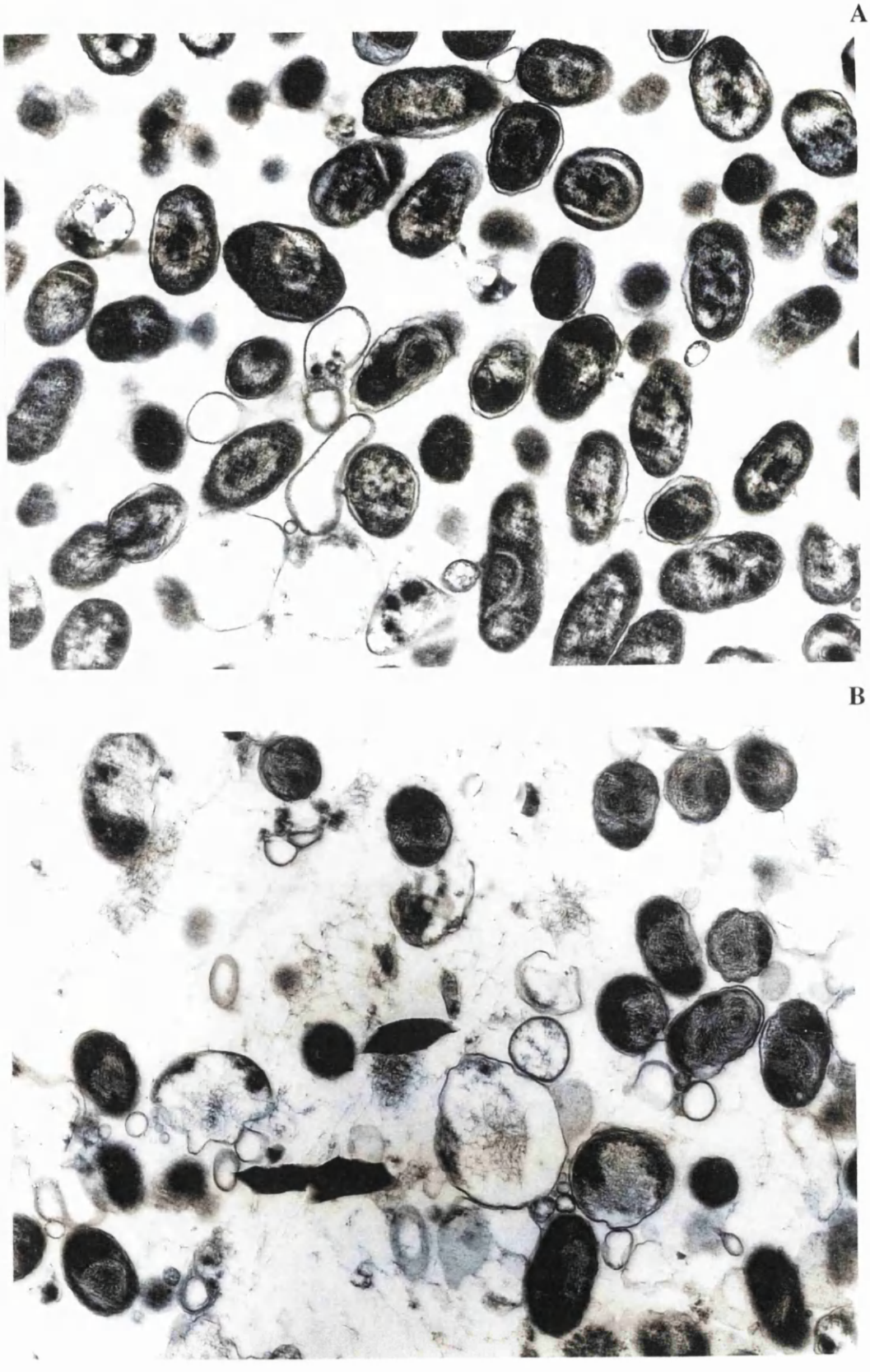


Plate 8. Transmission electron microscopy of thin sections of *B. pertussis* lux A) at time-zero, and B) after incubation for 1h at 37 °C in PBS. X 25, 500.

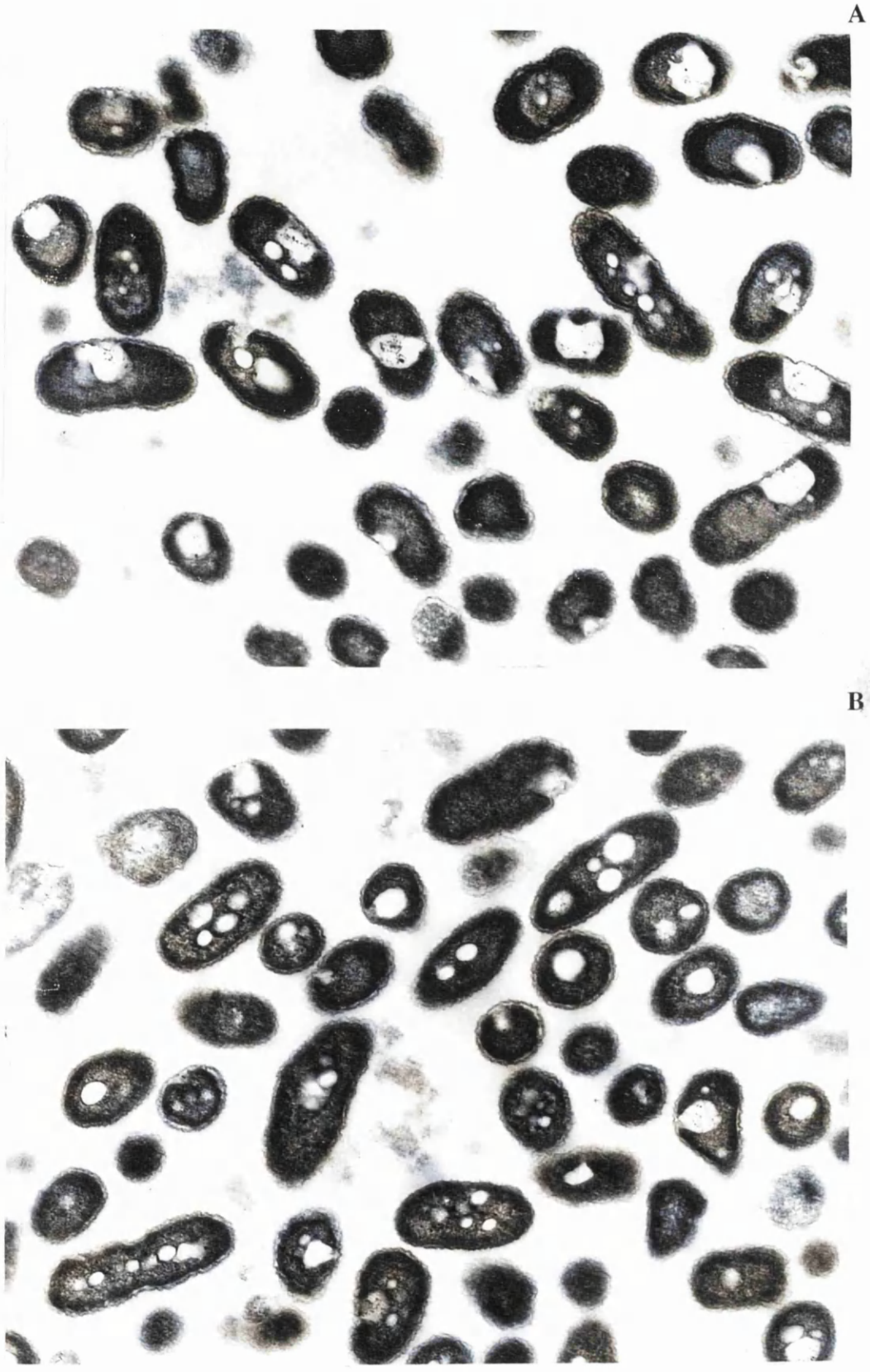
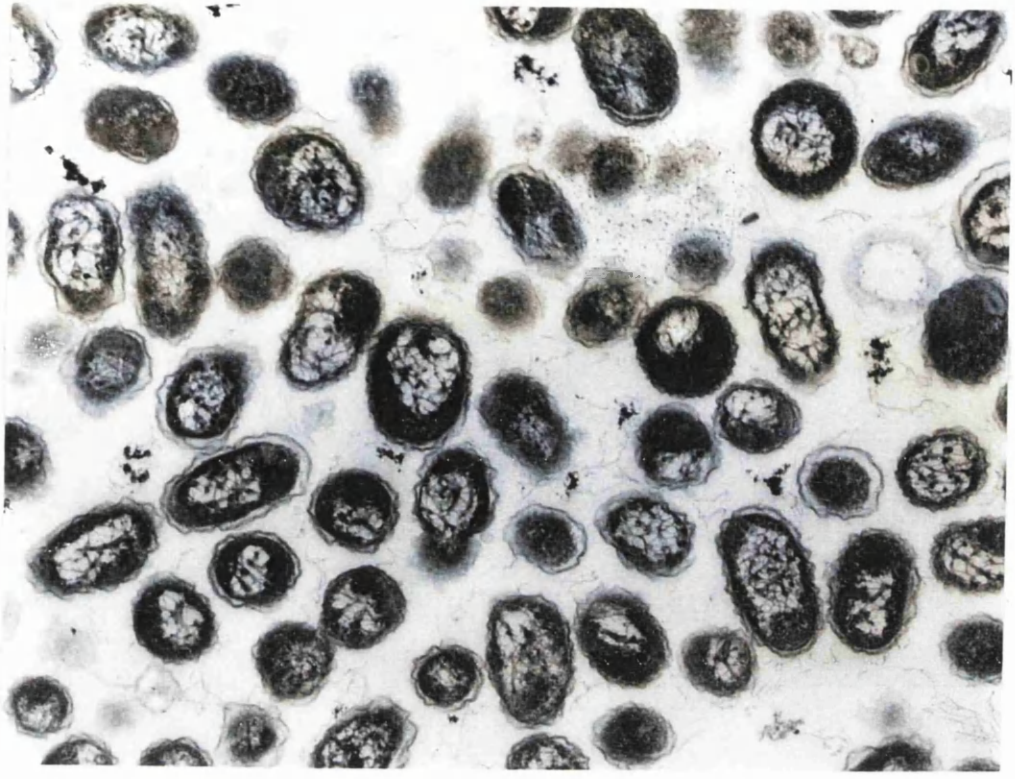


Plate 9. Transmission electron microscopy of thin sections of *B. pertussis* lux after 1 h incubation at 37 °C in A) supernate; B) surfactant. X 25, 500.

A



B

Conclusions briefly, are as follows:

1) *B. pertussis* lux retained normal morphology in PBS during 1 h incubation better than 18-3232 did.

2) *B. pertussis* 18-323 in CL medium showed the same behaviour as in PBS.

3) As judged by TEM changes, NSUR had a greater effect on both *B. pertussis* 18-323 and lux than NSN.

Tests with Phase IV *B. pertussis*

A small number of experiments was done with a single batch of LLF from convalescent rats (exposed to euthanasia method B) batch no. 34 against *B. pertussis* phase IV (strain 30042) with strain 18-323 in parallel. The results in Table 66 show that the convalescent LLF was more active against the phase IV strain than against 18-323. This was not due to loss of viability in the PBS content of the LLF since phase IV viability was retained in the PBS control.

Ultrafiltration experiments (Table 66) showed that the antibacterial factor(s) in LLF towards both *B. pertussis* strains was of molecular weight greater than 10 kDa. However, in the 50 kDa ultrafiltration, the activity appeared in both the filtrate and the retentate.

BA towards both *B. pertussis* strains was destroyed by heat at 56°C and by treatment with trypsin or bentonite. These results are different to those previously reported (Tables 58, 60, 61 and 63) where the activity was partially stable to 56°C and bentonite and unaffected by trypsin. It was concluded that this batch of LLF containing a high molecular weight, heat-labile and a trypsin and bentonite sensitive protein. Further investigation of these interesting preliminary results is desirable.

Table 66. Effect of various physical and biochemical treatments of rat LLF batch no. 34 on its bactericidal activity towards *B. pertussis* 30042 IV and 18-323 I after incubation times of 30 and 120 min. Data from 2 observations except were stated.

Treatment Ultrafiltration/ (pore size of filter)	Mean % survival*±SEM (No. of observations) during incubation at 37 °C for (min)			
	30042 IV		18-323 I	
	30	120	30	120
Nil	5±1 (10)	1±0 (9)	24±5 (7)	1±1
Filtrate (10 kDa)	88±11	41±2 (1)**	66±7	39 (1)
Retentate (10 kDa)	2±0	0 (1)	4±1	1 (1)
Filtrate (50 kDa)	4±1	0	17±4	3±1
Retentate (50 kDa)	2±1	0	14±5	1±1
Heat (56 °C/30 min)	97±1	93±2	117±11	120±5
Trypsin (1.9 mg/ml final)	81±3	72±4	105±5	78±6
Bentonite (11mg/ml final)	93±1	77±3	106±15	79±1
PBS control	100.8+3 (6)	86.4+8 (5)	93.5+4 (6)	76.6+2(5)

*calculation of % survival was based on PBS time-zero count data.

** data of only one observation due to lack of LLF from convalescent rats batch no. 34.

DISCUSSION

-- DEVELOPMENT OF AN EXPERIMENTAL PROTOCOL

Rationale for the Work

The main purpose of this research was to characterise the soluble bactericidal activity (BA) of rat lung lavage fluid (LLF) on *Bordetella pertussis*. Ideally, the work would have been done with LLF from humans, the natural host of *B. pertussis*, but such material is not readily available from healthy subjects. The choice of the rat came from its use as a model of whooping cough (Wardlaw *et al.*, 1993). Thus this rodent species appears to be the only non-primate laboratory animal in which pulmonary infection with *B. pertussis* causes a paroxysmal cough (Hall *et al.*, 1994). For this reason, the coughing rat model of pertussis has been extensively studied in this laboratory. The model involves the bacteria being administrated intrabronchially in fine agarose beads, under anaesthesia, and the coughing being monitored by sound-activated tape recorders. The animals also show lymphocytosis, another cardinal sign of pertussis in man, and they can be protected by immunization with whole-cell pertussis vaccine (Parton *et al.*, 1994). The model is specific for phase-I *B. pertussis*, since phase IV bacteria, *B. parapertussis* and a *B. pertussis* transposon mutant lacking pertussis toxin, failed to induce coughing.

Lung Lavage Fluid

Harvesting of LLF

Since *B. pertussis* in the lungs of live rats must come in contact with respiratory tract secretions, the present work sought to explore the possible BA of these fluids. It was not feasible to obtain undiluted secretions from live animals and a lavage procedure on freshly killed rats was therefore used. The possible role of cellular defence mechanisms in lung lavage fluid (LLF) was not examined in this work, and only the soluble antibacterial activity in the cell-free supernate of centrifuged and filtered LLF was studied.

During the course of the work, four different methods of euthanasia for the rats were applied before collecting LLF, and some differences in antibacterial activity towards *B. pertussis* 18-323 were found. The most satisfactory method (Method D) involved halothane/oxygen anaesthesia, followed by heart puncture to obtain blood and cervical

dislocation. LLF was then collected by lavage of the lungs with PBS through a tracheal cannula. However, even with this method, one batch of LLF out of 6 collected, for no apparent reason, had BA that was too low for intended experimental use.

By contrast, the least satisfactory method (Method B) involved suffocation in a jar of CO_2, followed by heart puncture and lung lavage, but without the need for cervical dislocation. Three out of 4 batches of LLF from normal rats were unsatisfactory when obtained by this method. The other two methods of euthanasia gave intermediate results. Thus method A, which involved halothane/oxygen anaesthesia, killing by CO_2 before heart puncture and lung lavage, yielded two satisfactory batches out of four collected. Euthanasia method C used an overdose of halothane to kill the animals. Only one batch of LLF was collected by this method and although it was satisfactory in BA, the titre was lower than the most active batch of Method D. It should be noted that there no significant differences in the volumes of LLF collected after the different euthanasia methods and that each batch was generally the pooled fluid from at least 8 and, in one occasion, as many as 20 rats. Although not settled definitely, it appeared best to avoid CO_2 during euthanasia in order to obtain LLF with satisfactory *in vitro* BA against *B. pertussis* strains 18-323 and lux.

Although not connected with euthanasia, mention can be made of the use of ether as an adjuvant to anaesthesia during the process of infection of the rats with *B. pertussis*. It was generally assumed that any effect of this treatment would be short lived. Therefore it was surprising to find with one batch of rats that there did appear to be a persistent effect of the ether 6 days later. Thus although the LLFs from normal rats showed no differences due to ether, the ultracentrifuge supernate (SN) fraction had higher BA if obtained from the ether treated rats. BA of surfactant (SUR) fraction from the same animals was not affected.

The only other authors to report on BA of rat LLF are Coonrod and Yoneda (1983), using pneumococci as the target organisms. These authors reported no effect on LLF BA that was due to the method of euthanasia. However, only intraperitoneal pentothal, or halothane inhalation were used before killing by exsanguination or cervical dislocation. It is possible that if CO_2 had been used, a similar result to my observations might have been obtained. The method of euthanasia does not appear to have been widely studied by other

investigators who have collected LLF from various species and for different purposes (Coonrod and Yoneda 1983; Coonrod *et al.*, 1984; LaForce and Boose 1981; O'Neill *et al.*, 1984). The reason that CO_2 euthanasia was used in my study was because Porter and Wardlaw (1994) from this laboratory employed it for getting LLF from mice. They found that mouse LLF did not support the growth of *B. pertussis*, but caused gradual reduction of bacterial viability which was complete after 2 days at 37 °C.

Composition of LLF

According to Matthews *et al.* (1963), the undiluted cell-free respiratory tract secretions from normal humans is composed of 95 % water and 5 % solids. The solids consisted of 2-3 % proteins and glycoproteins, 1 % lipids and 1 % minerals. The mean value for protein was 10 mg/ml, which was about 10 times higher than that reported by Boyd *et al.* (1944) with rabbit and cat secretions, and 30 times higher than found by Adamson *et al.* (1969) with fluid from foetal lambs. With cell-free lavage fluids from human and animal species and where the extent of dilution is uncertain, protein levels from 0.07 to 2.17 mg/ml have been reported (Low *et al.*, 1978; Porter and Wardlaw, 1994). The proteins include lysozyme, lactoferrin, albumin, complement and immunoglobulins (Masson and Heremans, 1973; Reynolds and Thompson, 1973). None of the literature on protein content of LLF dealt with fluids from rats, except that O'Neill *et al.* (1984) stated that protein in rat lung lavage surfactant comprised about 6 % of the material isolated.

In the present study, protein concentration in rat LLF and its fractions was measured with bicinchoninic acid in the presence of alkaline Cu^+ (Smith *et al.*, 1985). This method maintained the small protein-to-protein variation associated with the Lowry (1951) technique but was more sensitive and therefore suitable for the low protein levels expected in LLF. Analysis of 18 batches of normal rat LLF gave an average protein content (± SEM) of 0.4 (± 0.033) mg/ml, which is in the middle of the range (0.07 - 2.17 mg/ml) reported above. This concentration is equivalent in protein to about a 1:220 dilution of rat serum, i.e. somewhat more dilute than the mouse LLF which Wardlaw and Stevenson (1984) reported as being equivalent in protein to a 1: 50 dilution of serum. In rats, the method of euthanasia

did not significantly influence the protein concentration, but LLF from convalescent rats tended to be higher than from normal animals. With normal LLF from 6 other animal species and human, the protein concentrations ranged from 0.212 to 0.630 mg/ml, i.e. similar to the rat fluids and similar to the results with the same batches of these other fluids which were previously analysed by the Lowry method by Porter and Wardlaw (1994).

It is possible that the procedure for collecting LLF could be improved by better standardization of technique and that the content of protein could be increased by more vigorous and longer washing of the respiratory tract.

In 14 batches of normal rat LLF, the mean content of lysozyme, as estimated by the spectrophotometric method of Wardlaw and McHenery (1982) with *Micrococcus luteus*, was 75 µg/ml (SEM = 7 µg/ml). LLF from convalescent rats gave somewhat higher values. The source of this enzyme is probably the alveolar macrophages which especially when activated may secrete lysozyme (Hinman *et al.*, 1980). With alveolar macrophages from healthy human volunteers, Knostan *et al.* (1981) reported extensive release of the enzyme into the culture medium during 48 h *in vitro*. In the present work, the convalescent rats were at 21-42 days, well beyond the peak of infection (which was around day 10), and it is possible that LLF at the peak of infection might have shown greatly elevated levels of lysozyme.

In this study, LLF from the normal rat had the highest level of lysozyme of the various species tested, an observation not found in the literature and worthy of further study. It would be desirable in any such work to use animals of similar health status and to employ similar methods of euthanasia and harvesting of LLF.

Because Coonrod *et al.* (1984) had discovered the importance of FA in the BA of rat LLF towards pneumococci, analysis for these substances was done in the present study. Using gas-liquid chromatography of extracted lipids of the surfactant fraction, these previous authors estimated the total FA in three pools of normal rat LLF as 68, 228 and 706 µg/ animal. In my work FA was estimated, without preliminary lipid extraction, using the colorimetric assay of Duncombe (1963) applied directly to LLF itself. This is based on the formation of the copper salts of fatty acids, which are then estimated with diethyl

dithiocarbamate. The quantitation of the method is linear to 100 μg of FA and sensitive to 10 μg. Twelve batches of LLF from normal rats gave a range of FA levels between 114 and 1496 μg/ rat. The reason for the upper value being about twice of that of Coonrod *et al.* (1984) is not known but could be due to one or more of the following: my assay of FA on unfractionated LLF and possibly that of Coonrod *et al.* (1984) lost some of the FA during extraction processes; interference by protein or other components; the different method of euthanasia used by the other workers and perhaps differences in rapidity of harvesting the LLF. In my studies there was no evidence of systematic differences in the FA content of LLF associated with the different methods of euthanasia.

It was considered important to check whether the rat LLF contained antibodies to *B. pertussis*. A total of 10 batches of LLF was assayed by ELISA for antibodies of the IgG, IgA and IgE classes directed against the following antigens: whole-cell *B. pertussis* (BP) sonicate, pertussis toxin (PT) and filamentous haemagglutinin (FHA). For control purposes, serum samples from the same groups of animals were also assayed. The 10 batches of LLF came from one normal group, one sham-operated group and 8 convalescent groups. These latter consisted of animals that had been vaccinated and then challenged (2 groups), one group challenged with phase IV *B. pertussis* and five groups challenged with Phase I *B. pertussis* encased variously in agarose or carrageenan, with or without the use of ether.

In brief, no significant levels of antibodies were detected in either LLF or serum from the normal or sham-operated rats. However, low but detectable IgG titres were found in the LLF from the vaccinated and phase I-challenged convalescent rats and also from the animals given challenge alone with these organisms. Vaccination was associated with higher titres than challenge alone. Sera from phase I-challenged rats had much higher levels of specific IgG, although if allowance is made for the LLF being equivalent in protein to about a 1:220 dilution of serum, then the serum and LLF IgG titres were quite similar. These above results with sera are similar to those of Hall *et al.* (1994) and Parton *et al.* (1994) for IgG; however these authors did not study LLF. Challenge with phase IV *B. pertussis* induced serum antibodies to BP sonicate but not to FHA or PT, confirming the absence of

these antigens in such bacteria. Variations in challenge procedure such as use of agarose or carrageenan, or use of ether to promote the coughing response did not influence antibody titres.

The IgA titres in convalescent LLF were lower than the IgG, but when multiplied by 220, the titres to BP sonicate in LLF were higher than those in serum. This was not the case with FHA or PT. No IgE antibodies were detected.

To relate these observations to other serological studies with *B. pertussis* is difficult because of the limited amount of research either with LLF or with rats. Thus although there are numerous papers on serological responses of infants to pertussis infection or vaccination (e.g. Ashworth *et al.*, 1983; Ad Hoc Committee, 1988; and Marwick, 1995), ethical constraints have prevented obtaining LLF from babies.

Since complement is a well-known antibacterial factor in serum, it was necessary to test for it in the rat LLF in case it was present. For this purpose the highly complement-sensitive test organism, *E. coli* Lilly, was employed (Wardlaw, 1962). Moreover, Reynolds and Thompson (1973) reported traces of some complement components (C1 and C6) in bronchial secretions in rabbits vaccinated with *Pseudomonas aeruginosa*. The amounts were much less than in serum by 1/2550 for C1 and 1/58 for C6. Other authors have detected individual components of complement in respiratory tract secretions (Boat and Cheng, 1980) although functional complement has not apparently been demonstrated. Weiss (1997) stated that "Underneath the epithelial surface (of the lungs), capillaries supply blood and nutrients to the mucosal layer. In response to inflammatory signals, the tight junctions between epithelial cells of the mucosal surface and between endothelial cells that line the capillaries can quickly and reversibly separate, allowing serum components (including immunoglobulin G [IgG] and complement to exude onto the mucosal surface". Weiss claimed that the intact mucosal surface has about 10 % as much complement as does serum.

In the present work, no functional bactericidal complement was found in LLF from either normal or convalescent rats. Indeed the *E. coli* Lilly grew in the nutrients in both types of bronchial secretion. Rat serum, on the other hand was rapidly bactericidal, as was

guinea pig serum although it was less active than that from the rat. With hindsight it would have been desirable to test the rat serum at a 1:220 dilution to take account of the protein measurements, but this was not done. Also, it would have been interesting to see if individual complement components could be detected as in the work of Reynolds and Thompson (1973). Another potential line of work would be the possible interaction of complement and lysozyme which may occur with other gram negative bacteria (Wardlaw, 1962).

Antibacterial Tests with LLF

Control fluids

At the start of the experimental work, *B. pertussis* suspension in PBS was prepared according to the procedure described by Porter *et al.* (1991) in their study of growth of *Bordetella* species in LLF and other low nutrient fluids. The same workers (1993) observed that *B. pertussis* 18-334 failed to maintain viability in PBS for even 24h at 37° C. However, shorter times were not studied. In the present work (with strain 18-323), 37° C was the standard temperature, but observation times from 15 min to 24 h were explored, with emphasis on periods up to 120 min. Gradual loss of bacterial viability was found in PBS during 120 min incubation with $\geq$ 98 % killing (limited by the volumes plated) after about 24 h. Based on a very large set of observations (N = 135) a half-life of *B. pertussis* 18-323 in PBS was found to be 149 min. There appears to be no published work with which this value could be compared. The 24 h results was nearly the same as those of Porter and Wardlaw (1993), with less extensive killing.

Plastic vessels were used as test container for both studies. Although at the start of this work glass containers were used, but when a rapid loss of *B. pertussis* viability was observed after only 120 min of incubation, which was possibly due to the adherence of bacterial cells to the walls of the test vessel (Gwynn *et al.*, 1981), after which the plastic containers were used throughout the study. All *B. pertussis* strains showed almost the same survival in PBS during 120 min incubation as 18-323, except both L84 and 30042 phase IV showed better survival, which was more pronounced at 24 h.

As a diluent used for suspending the bacterial inoculum and as a non-bactericidal control, 1 % CAA with salts (NaCl, $CaCl_2$ and $MgCl_2.\ 6H_2O$) showed the same behaviour as PBS in allowing only about 55 % survival after 120 min. Despite this similarity in loss of bacterial viability in both PBS and 1 % CAA, the latter was chosen for suspending the initial inoculum. Halperin *et al.* (1992) used PBS containing a casein hydrolysate for transporting secretions collected by nasopharyngeal aspirate. They reisolated the pertussis bacteria from 92 % of clinical specimens held at 4°C for 1 week and from all specimens held at -20 °C (The use of glycerol was not mentioned). These results of Halperin and his group supported the choice of using 1 % CAA as diluent for storing *B. pertussis* at -70 °C, for bacterial suspension (which usually stand on the bench at room temperature for few minutes during experiment) and as a non-bactericidal control. Also, Hall *et al.* (1994) and Imaizumi *et al.* (1983a) used 1 % CAA in preparing bacterial suspensions. CAA is the standard diluent for *B. pertussis* challenge in mouse infection (Dolby and Standfast, 1961). In respect of half-life, *B. pertussis* 18-323 showed roughly a similar half-life of 155 min in CAA as in PBS at 37 °C.

Some difficulties were experienced in using CL-medium, described by Imaizumi *et al.* (1983a) as a growth medium for *B. pertussis* and for PT production. These problems were overcome by a minor modification of the original CL-medium, by reducing the amount of trace nutrients (supplement) by a factor of two ie. Ferrous sulphate from 0.36 µM to 0.18 µM; L-cysteine from 3.3 µM to 1.65 µM; niacin from 0.33 µM to 0.16 µM; glutathione from 4.9 µM to 2.4 µM and L-ascorbic acid from 22.70 µM to 11.35 µM. Also better survival and growth of *B. pertussis* 18-323 in CL-medium containing L-glutamic acid (Sigma, 99 % pure by TLC) than in CL-medium with L-glutamic acid sodium salt (BDH, containing traces amount of chloride, ammonium and lead) especially after 24 and 48 h of incubation (see Table 22). This difference is possibly because of the state of the purity of these chemicals. This modified CL-medium supported the survival (during 120 min of incubation) and growth of *B. pertussis* 18-323 during 24 h of incubation. The differences between this study and Imaizumi *et al.* (1983a) was that they used a 5000-times

higher bacterial concentration at time-zero than I did; they also employed *B. pertussis* strain Tohama instead of 18-323 and an incubation temperature at 35 °C in place of 37 °C.

Porter and Wardlaw (1993) showed a linear rise in the growth of *B. pertussis* strain 18-334 in CL-medium during several days of incubation at 37 °C. The different results obtained with CL-medium between this study (before modification) and Porter and Wardlaw (1993) was possibly because of one or more of: respectively use of glass Bijoux instead of plastic Bijoux; *B. pertussis* strain 18-323 instead of 18-334; lower bacterial inoculum (1/10) at time-zero than they used; unwashed *B. pertussis* suspension in place of washed bacterial cells; and shorter incubation time (min) instead of days. The half-life of *B. pertussis* 18-323 in CL-medium was longer (386 min) than in either CAA or PBS.

Indicators of viability

In the present studies the ability of the *B. pertussis* to form colonies on BG medium was taken as the indicator of bacteria viability. The possibility that the organisms may enter a viable but not-culturable state, as described with other bacteria such as *Salmonella enteritidits* (Roszak *et al.* 1984), *Vibrio cholerae* (Colwell *et al.* 1985) and *Campylobacter jejuni* (Jones *et al.* 1991) was not explored. So far, the viable but non-culturable state has not been reported for *B. pertussis*.

The possibility of using luminescence as an indicator of viability of *B. pertussis* did not exist when the present investigation was being planned. However, during the course of the work *B. pertussis* strain lux became available and was therefore used. The following is a brief outline of the underlying principles: Bacterial luminescence results from the flavin-mediated oxidation of a long chain aliphatic aldehyde catalyzed by the enzyme luciferase. Two of the required substrates for the bioluminescent reaction, reduced flavin mononucleotide ($FMNH_2$) and molecular oxygen, are both readily available in aerobic bacteria ; all that is required to produce light in a recombinant bacterium is the presence of the *lux* operon and a source of long-chain aliphatic aldehyde (Hill *et al.*, 1993)

$$FMNH2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{light}$$

-Luminous bacterial cells in stationary phase emit much less light per cell than those in log phase. Luminescence (light) can be detected by 1) vision ; 2) 35 mm photographic film; 3) X-ray film; and 4) photoelectrically with a luminometer or scintillation counter. *B. pertussis* lux (which was used in this study), was originally derived from *B. pertussis* 44122/7 R a streptomycin-resistant strain (Branefors, 1964). The *lux* (luciferase gene) operon from *Vibrio harveyi* was cloned into phase I *B. pertussis* 44122/7 R (made by McLean, H., Parton, R and Coote, J. G. [unpublished]) , *E. coli* WA 308 and *E. coli* DH5α. The first two of these bacteria required addition of aldehyde in order to produce light. *E. coli* lux DH5α did not require addition of aldehyde for producing light since it also contained the genes for aldehyde production. The detection of luminescence (light) in this study was done by luminometer (luminescence photometer).

B. pertussis strain lux was used in parallel with strain 18-323 for the bactericidal test, because results could be obtained in a few hours instead of several days. However, some difficulties were encountered with the luminescence experiments such as the presence of some compounds (Thore, 1979) such as salts ($CaCl_2$, NaCl and KCl) at a concentration range of 5 and 20 μ mol /ml and BSA (1.04 % w/v) in the test fluids which interfered with the measurement of luminescence. Despite this, the effect of LLF and its fractions on *B. pertussis* lux correlated quite well with the results of the viable count experiments with *B. pertussis* 18-323.

EFFECT OF LLF ON VIABILITY OF *B. PERTUSSIS*

Normal rat LLF

Sixteen batches of LLF were obtained from normal Sprague-Dawley rats of the highest available health status and housed in rooms with a filtered air supply. This latter might be important if dust and microorganisms could influence the composition and activities of lung fluids. As detailed above, the activity of the fluid was influenced by the method of euthanasia and there may also have been other significant variables, such as the weight of the animals although this was not explored in detail. However, there was a

particular batch (39) of normal rats which were sacrificed by the same method (D) of euthanasia, and with the same volume of PBS for lavage, at average weights of 126, 253 and 350 g. The most active LLF came from the smallest animals and the least active from the largest. Whether this was a simple volumetric effect of airways volume in relation to PBS lavage volume, or to a qualitative difference in activity related to age, would require further study. With another batch (40) of rats, the very large animals (455 g average) had more active fluid than animals of mean weight 355 g, indicating the difficulty in drawing conclusions about BA in relation to age separate from other factors.

The only other authors who have investigated the antibacterial activity of rat LLF are Coonrod and colleagues (1983, 1984) with *Streptococcus pneumoniae* type 25. They also used Sprague-Dawley rats in a 'filtered air unit' and observed a rather weak *in vitro* killing effect with LLF from animals weighing 200 g or more. Although my researches with *B. pertussis* and Coonrod's with pneumococci differ in experimental details, I observed significant killing with a 1/24 dilution of normal LLF with 10^3 cells of strain 18-323. While even more highly diluted (1/30) LLF with 10^8 cells of *B. pertussis* lux gave significant loss of luminescence. Coonrod and Yoneda (1983) used 10^8 pneumococci and at best only got 23 % killing with unconcentrated LLF. Even with 20-fold concentrated LLF, in the form of the surfactant fraction, the maximum percentage killing they reported was only 81%. This was accompanied by lysis.

A further possible reason for the LLF of Coonrod and Yoneda (1983) apparently being less active than mine is because of pentothal anaesthesia followed by exsanguination for euthanasia. In the second paper (Coonrod *et al.*, 1984) euthanasia was done with an over dose of fluthane and the author speculated this might increase the BA of the LLF; no relevant data were presented. In my work it was beneficial not to use an over-dose of halothane for killing but to give it as the anaesthetic during exsanguination. The evidence is obviously incomplete but reinforces the view already expressed that the method of euthanasia is an important factor in the subsequent BA of rat LLF.

It would therefore appear that *B. pertussis* is more sensitive to the BA of rat LLF than are pneumococci and that either a viable count test with 18-323 or luminometry with

strain lux- may be used. Indeed it may well be that *B. pertussis* is a specially suitable bacterium for research on BA of rat LLF. A total of 11 strains of phase I and 2 strains of phase IV *B. pertussis* were tested. All the phase I strains had antibacterial sensitivities similar to 18-323 and lux, but the two phase IV strains were resistant to the antibacterial activity of normal rat LLF. This observation is consistent with the possibility that LLF BA is due to FA since phase IV organisms are resistant to these substances while phase I *B. pertussis* is highly sensitive (Pollock, 1947; Field and Parker, 1979; Frohlich *et al.*, 1996; and this study).

For phase IV strains, LLF from normal rats acted as a growth medium as it did with *B. bronchiseptica* and *B. avium,* when Porter and Wardlaw (1994) reported the same observation with other animal species and human LLF.

Normal LLF from other species

Of the eight mammalian species whose LLF was tested for BA towards *B. pertussis*, three (rat, human and calf) gave positive results. Rat was easily the most active but since only one sample of each human and calf LLF was tested, it would be desirable to examine further samples. This is especially so with LLF from humans, the natural host of *B. pertussis*, where LLF may contribute to host defence. The sample of human LLF used here might have had its activity reduced through having been centrifuged at a much higher speed (10000 rpm) than used with my rat samples (500 rpm). This factor should be considered in any further studies with human and animal LLF. With the latter, the method of euthanasia should also be researched. Thus Porter and Wardlaw (1993) used a variety of methods of euthanasia for collecting the LLF for studying its properties as a growth medium for bordetellae. The human LLF they collected and which was used in my work was obtained during bronchoscopy of anaesthetised adults with suspected lung disease.

In the work of Coonrod *et al* (1984) with pneumococci, dog, mice, rabbits and guinea pigs LLF were tested in addition to their main work with the rat they found LLF of dog to be active, mice less so, while no activity was found in LLF from rabbits and guinea pigs.

Inactivation of LLF

To gain information on the nature of the antibacterial substance(s) in LLF, the effect of various physical and chemical treatments was studied with both 18-323 and lux. In general, the activity was stable to most of the treatments applied. Thus it was not affected by heat at 56 °C for 30 min, and there was about only 50 % inactivation during 5 min at 100 °C. Exposure to strong acid and alkali for 18 h at 4 °C had little destructive effect. Likewise treatment with three different proteases caused no loss of activity. All the above observations suggested that the active material in unfractionated LLF did not contain protein. On the other hand, there was evidence of a high molecular weight, or aggregated, factor since the activity was not lost during dialysis; nor did the activity appear in a 30 kDa ultrafiltrate. Very similar observations were made by Coonrod and Yoneda (1983) with their anti-pneumococcal activity in rat LLF. Possibly different antibacterial substance(s) with different modes of action may be present in the rabbit LLF investigated by LaForce and Boose (1981) with *E. coli* as test organism. In this system the LLF was not directly antibacterial but had a differential effect on ability of the bacteria to grow on deoxycholate agar, compared with blood agar. This activity appeared to be enhanced by low-pH treatment.

LLF from *B. pertussis*-convalescent rats

LLF batches from both normal and *B. pertussis*- convalescent rats were studied in this investigation and found to be different in several significant respects. The convalescent samples were taken at between 3 and 6 weeks after the intrabronchial infection, a time at which viable *B. pertussis* 18-323 was no longer recoverable from the rat lungs (Hall *et al.*, 1994). These convalescent animals had been used for experiments by Hall *et al.* (1994).

To help with comparability, LLF was always obtained from normal rats at the same time as the sampling from the convalescents. Considered as normal animals, but kept separate, were those exposed to sham-operation, i.e. the anaesthesia and surgical procedures with administration of agarose beads but without *B. pertussis.* A complication was that the convalescent samples came from animals that had been subjected to a wide

variety of experimental treatments associated with the infection with *B. pertussis.* For example, some animals had been vaccinated before the challenge with 18-323; there was variation in whether or not ether was used as a promoter of subsequent coughing (Hall *et al.*, 1994); there were variations in the bead material, i.e. agarose, agarose + carrageenan, and alginate; and finally there were alternatives to strain 18-323 used for the challenge, notably another phase-I (Tohama), a PT-minus (357) , an HLT-minus (1809), a phase-IV (L-84) and a *B. parapertussis* (10520).

Table 67 summarizes the information from 33 batches of LLF from normal and convalescent rats, together with information on the coughing recorded on between days 8 and 14 and the antibacterial activity expressed as 30 min survival figures. These data were then further analysed to see if there was an association between the exposure of the rats to pertussis toxin (PT), their coughing response and their LLF bactericidal activity (BA). For comparison, PT-unexposed rats were those assumed to have had no recent and significant exposure to active PT if they were in any of the following categories: normal (N), sham-operated (SO), vaccinated and challenged (VC), or infected with *B. parapertussis*, or phase IV or PT-minus *B. pertussis*. On the other hand, the PT-exposed animals were those that were convalescent from infection with phase I *B. pertussis* or with the HLT-minus mutant. The results in bold in Table 67 show that where there was most coughing in each of the treatment groups, the LLF from that group of rats tended to have low BA. The coughing responses, which ranged from zero to 11.4 (average number of paroxysms/ animal), were arbitrarily divided into nil (≤ 0.1) and positive (≥ 0.3) groups, and the percent survivals in the BA tests were divided into 2 categories of low-BA (≥ 50 % survival) and high-BA (≤ 49 % survival).

Table 68 shows that there was a high degree of association between the exposure status of the rat groups to PT and the subsequent coughing and BA of the LLF. Thus in the PT-unexposed groups there was only 1/16 coughing-positive, whereas in the PT-exposed groups there were 17/17. With LLF BA in the PT-unexposed groups there were 2/16 in the low-BA category, compared with the PT-exposed groups where the ratio was 12/17.

Table 67. Comparison of cough induction and % survival of *B. pertussis* 18-323 after 30 min of incubation in the LLF from normal (N), sham-operated (SO), vaccinated and challenged (VC) and convalescent (C) rats.

LLF Batch No.	Treatment	Coughing*/ rat	% Survival** after 30 min of incubation
15	N	0	26
	C-Phase IV	0.1	26
	C-Phase I	0.5	88
	C-Phase I	3.7	84
	C-Phase I	**7.1**	**92**
16	SO	0	38
	VC	0	48
	VC	0.1	0
	C-Phase I	1.0	65
	C-Phase I	**2.1**	**80**
17	N	0	14
	VC	0	1
	VC	0.1	11
	C-Phase I	0.5	4
	C-Phase I	**1.7**	**79**
21	N	0	50
	C-Bpp	0	0
	C-PT -	0.3	0
	C-HLT -	0.5	0
	C-Phase I	1.0	8
	C-Phase I	**1.3**	**101**
34	N	0	59
	C-Phase I	***	21
41	N	0	8
	SO	0	0
	SO	0	2
	C-Phase I	2.8	79
	C-Phase I	**11.4**	**97**
43	N	0	0
	C-Phase I	0.4	100
	C-Phase I	0.9	27
	C-Phase I	3.4	0
	C-Phase I	**3.9**	**66**

*Coughing paroxysms/rat (days 9-14 post-infection); **% survival calculations were based on PBS zero-time count; *** There was definite coughing on day 8 and 11 post-infection, but not counted.

The tentative conclusion may therefore be drawn that rats exposed to intrabronchial infection with a high dose (10^8) of phase I *B. pertussis* develop paroxysmal coughing and show a decline in the BA of their LLF. It should be noted that the coughing and BA observations were unavoidably made at different times, the former being around day 8 after infection, while the LLF was never obtained earlier than day 21 and sometimes as late as day 42. Therefore it is possible that for the observation of maximum decline in BA, the post-infection LLF should also be taken around day 8 when the coughing and the leucocytosis are at their highest levels.

B. pertussis is well known for its ability to interfere with host defence mechanisms. For example, adenylate cyclase toxin (ACT) of *B. pertussis* inhibits the killing function of phagocytic cells (Confer and Eaton, 1982). Tracheal cytotoxin causes ciliostasis and specific damage to ciliated cells and inhibition of DNA synthesis in hamster tracheal rings (Goldman and Baseman, 1980). Pertussis toxin (PT) inhibits (see Table 6) the functions (such as oxidative burst, migration, homing pattern *in vivo*, degranulation and target cells lysis) of immune effector cells such as neutrophils, monocytes, lymphocytes, mast cells and natural killer cells. Therefore my observation of lowered LLF BA in rats recently exposed to *B. pertussis* infection may be part of a general pattern of multiple aggressin activities.

Other *B. pertussis* strains

The sensitivity of other *B. pertussis* phase I strains to the bactericidal activity of convalescent rat LLF was similar to that observed with *B. pertussis* 18-323. LLF from convalescent rats exposed to euthanasia method B gave a strong BA towards *B. pertussis* 30042 phase IV. This result suggested that euthanasia method B had an effect on the LLF by developing a new bactericidal active substance which also active towards *B. pertussis* 18-323. This new substance possibly was synthesised during exposure of rats to CO_2. *B. pertussis* 30042 IV was sensitive only to this batch (34) of LLF, while all other phase I *B. pertussis* strains were insensitive to the bactericidal activity of the same batch.

The average time (half-life) required for 50 % inactivation of the *B. pertussis* 18-323 cells in LLF from normal rats was less (11 min) than that (20 min) observed with LLF

Table 68. Correlation between rat paroxysmal cough and LLF bactericidal activity in relation to exposure to pertussis toxin (PT) during infection with *B. pertussis* (± PT) or *parapertussis* (-PT).

PT-status of rat group	Bactericidal activity: No. of batches			Cough: No. of batches		
	Low	High	No. Low/Total	Nil	Positive	No. Positive/Total
Not exposed to PT	2	14	2/16	15	1	1/16
Exposed to PT	12	5	12/17	0	17	17/17

from convalescent rats (Table 46). This supported the finding above that the *B. pertussis* infection of the rats caused a reduction in BA of their LLF.

Different strains of rat

Experiments with different strain of rat showed that Sprague Dawley were not necessarily characteristic of rats in general in regard to response to *B. pertussis*. For example, the three other rat strain tested gave much less coughing (Wardlaw, Parton and Hall, [unpublished data]) than Sprague Dawley (Table 69), indeed Brown Norway did not appear to cough (Wardlaw, Parton and Hall, unpublished data) at all . Normal LLF from all 4 rat strains had BA and without observed differences between young and old animals. However, remarkable differences between the strains were observed with LLF from convalescent rats. Thus Hooded Lister and to a less extent Lewis, resembled Sprague Dawley in showing much reduced BA. On the other hand, convalescent LLF from Brown Norway rats was just as active as from the normal animals of this strain. It is therefore clear that future work on the BA of rat LLF, especially in relation to *B. pertussis* infection should take account of large differences between rat strains. The possibility that Brown Norway rats are unresponsive to PT merits further study. Finally, Coonrod and Yoneda (1983) found anti-pneumococcal activity in LLF from both Sprague Dawley and Lewis rats.

Tests with phase IV

Approximately 3 batches of normal and 9 batches of convalescent LLF were tested against phase IV *B. pertussis*. Of these, only one convalescent LLF had BA against *B. pertussis* 30042 IV. This batch (no. 34) was exceptional in being the only batch of convalescent LLF obtained by euthanasia method B. might require that the batches be collected with CO_2 euthanasia. It may be noted that towards phase I *B. pertussis* 18-323, this batch had only moderate activity. There was therefore evidence that BA of LLF towards phase IV *B. pertussis* might involve different substances than towards phase I.

Table 69.- Comparison between normal and convalescent* rats of different strains and ages** in cough production and bactericidal activity of their LLF towards *B. pertussis* 18-323.

Strain of rats	Treatment	Coughing/ rat (8 days post infection)		% survival*** after 30 min of incubation	
		Young	Old	Young	Old
Sprague-Dawley	Normal	0	0	0,0	0,0
	Challenged	11.3	2	68,43	18,24
Brown Norway	Normal	0	0	0,0	0,0
	Challenged	0	ND	0,0	0,0
Lewis	Normal	ND	0	0,0	0,0
	Challenged	ND	1.3	26,21	67,66
Hooded Lister	Normal	0	0	0,0	0,0
	Challenged	5	1.3	113,129	90,97

*Challenged with *B. pertussis* 18-323 encased in agarose with dimethyl-β-cyclodextrin); **Young rats = 4-5 weeks old (~ 110 g in weight) ; Old rats = 6-7 weeks old (~ 150 g in weight); *** % survival based on PBS time-zero calculation.

Inactivation studies with this particular batch showed that the BA was removed by ultrafiltration (10 kDa) and destroyed by heat, bentonite and trypsin. It was concluded that the substance(s) responsible for this BA was protein.

SUPERNATE AND SURFACTANT FRACTIONS OF LLF

Distribution of Bactericidal Activity (BA)

B. pertussis and other bacteria

Ultracentrifugation was applied to normal rat LLF to see whether the substance(s) of LLF responsible for the BA is located in supernate (SN) or pellet (SUR) fractions. This followed the method of other workers (Coonrod and Yoneda, 1983; Coonrod *et al.*, 1984; LaForce *et al.*, 1979; LaForce and Boose, 1981) with rat and rabbit LLF. Most of the BA towards *B. pertussis* 18-323 and lux, was found in the SUR fraction of LLF from normal rats. In contrast, the SN fraction showed only moderate or little activity. This is similar to Coonrod and Yoneda (1983) who detected BA in 20-fold concentrated rat SUR towards type 25 *pneumococci* and no activity in 20-fold concentrated SN. The opposite distribution was reported by LaForce and Boose (1981) who demonstrated sublethal damage of *E. coli* when incubated in the SN fraction of rabbit LLF and cultured on deoxycholate agar, and no effect after incubation in the SUR fraction.

In my work, *B. pertussis*, *B. pararpertussis*, *S. aureus* and *E. coli* lux DH5α were all sensitive to the BA of normal rat SUR, while *B. bronchiseptica, E. coli* Lilly, *E. coli* lux WA803, and *S. pyogenes* were insensitive. *M. luteus* was sensitive to the lysozyme in the rat LLF. In the work of Coonrod *et al.* (1984), two other strains of pneumocccus (type 1 and 3) were similarly responsive to type 25 and were sensitive to the BA of rat SUR.

Organisms that were similar in sensitivity to pneumococci were *S. viridans*, *S. pyogenes*, *S. bovis* and 2 unidentified *Bacillus* species. In contrast, various other bacteria were insensitive, such as: *S. aureus*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Proteus vulgaris*, *Serratia marcescens*, *Klebsiella pneumoniae*, *S. fecalis*, *S. durans* and *S. agalactiae*.

To the BA of rat SN, *B. pertussis* and *B. parapertussis* were both sensitive, while *B. bronchiseptica*, *S. aureus*, *S. pyogenes* and *E. coli* were insensitive and grew in SN. Porter and Wardlaw (1994) showed that both *B. bronchiseptica* and *B. avium* survived and grew very well in all animal LLFs (which they tested) and human LLF. They also mentioned that both *B. pertussis* and *B. parapertussis* were not able to grow as well as the two animal *bordetella* species.

Dose-response

Quantitative titration of BA by viable counting with *B. pertussis* 18-323 gave average 50 % endpoints at about 1/24 with LLF, 1/34 with SUR, and only 1/2 with SN. This showed that although both SUR and SN fractions had BA, the SUR had about 17 times as much. There was also a suggestion that the ultracentrifugation used to separate SUR may have removed a BA inhibitor, although this was not specifically investigated. Coonrod *et al.* (1984) also used a dilution assay with a 50 % endpoint, and found that concentrated rat SUR contained much more BA than concentrated SN. Note that in comparing the Coonrod *et al.* (1984) work with mine, they used a different test organism and also a 10^4 times higher dose of bacteria.

Supernate (NSN) Fraction

Inactivation studies were done to characterize the substance(s) in normal rat supernate (NSN) responsible for BA. Most of the work was by viable counting with 18-323 which was found to be more reliable than luminometry with strain lux. Exposure of NSN to a variety of physical, chemical and biochemical agents lead to the conclusions that the BA may be due to 3 different components: protein, phospholipids, and FA. The evidence for protein was the loss of activity by heat, acid, alkali, and bentonite.

Contrary evidence was the fact that the activity was not significantly reduced by treatment with any of 4 different proteases. The evidence for phospholipids was the definite loss of activity by phospholipase A2 while the possibility of FA involvement was suggested by the ability of charcoal to remove the activity. However, other agents that might be

expected to lower the activity by FA removal had relatively small effect. These included serum, BSA, starch, methylcellulose, and cyclodextrins. Thus the strongest evidence was that provided by phospholipase A2, suggesting involvement of phospholipids.

Inactivation of NSN was also studied, although less extensively, by luminometry. Some results so obtained were difficult to integrate with the conclusions from the viable counting studies with 18-323. For example, proteinase K apparently caused significant loss of BA of SN towards lux but not towards 18-323. However, the control test of protease (without NSN) + bacteria showed definitely increased luminescence, which could not be explained. Another discrepancy occurred with the freezing and thawing of NSN. This appeared to enhances the BA of SN towards 18-323 but to decrease it against lux. Further studies would be required to resolve these apparent inconsistencies which were observed on two or more occasions.

There is little published work by other investigators, with which the above may be compared. Coonrod *et al.* (1984) did not report inactivation of NSN fraction in their pneumococcal work because the fraction lacked activity to start with. LaForce and Boose (1981) stated that most of the anti-*E. coli* activity of rabbit LLF was due to high molecular weight heat-stable peptide which was adsorbed by bentonite and sensitive to trypsin.

Surfactant (NSUR) Fraction

Composition

The composition of LLF NSUR has been studied by about more than 10 different groups of investigators such as Pruitt *et al.* (1971); King *et al.* (1974); Harwood *et al.* (1975); Shelley *et al.* (1984); King and Clements (1985) and others. King and Clements (1985) reported that dog SUR contained about 80-90 % lipids and 10 % protein by weight.

In my work, the rat SUR contained 475 µg/ml lipids, whereas protein was estimated to be 58 µg/ml. These figures are equivalent to 85 % lipids and 15 % protein.

Artificial surfactant

ALEC, artificial lung expanding compound (artificial surfactant) together with rat lung SUR were examined for their BA towards both *B. pertussis* 18-323 and lux. Rat lung SUR showed BA towards both *B. pertussis strain* 18-323 and lux. whereas artificial surfactant exhibited no BA towards *B. pertussis* 18-323, although an effect on bacterial luminescence of *B. pertussis* lux was observed.

This decrease in bacterial luminescence was possibly due to the turbidity (which can interfere with light output measurement). The artificial surfactant was composed only of dipalmitoylphosphatidylcholine and phosphatidylglycerol, whereas, rat lung NSUR contains these phospholipids in addition to others, and also lysophospholipids, FA and trace amount of proteins. Coonrod *et al.* (1984) reported that commercially prepared lysophospholipids (specifically palmitoyl and myristoyl lysophosphatidylcholine) acts as a bactericidal agents against some bacteria such as type 25 and 3 pneumococci, *S. viridans* and *S. pyogenes*. Neither FA nor lysophospholipids are present in ALEC, which may explain why ALEC possessed no BA towards *B. pertussis* 18-323.

Effect of incorporation of *B.pertussis* lux in agarose ± DMβCD

The *B. pertussis* 18-323 challenge which was given intratracheally to the different rat strains (see footnote of table 69) was encased in agarose with dimethyl-β-cyclodextrin (DMβCD). An experiment was therefore done to see if DMβCD might protect the bacteria from the BA of the LLF of those rats. *In vitro* tests with *B. pertussis* lux encased in agarose ± DMβCD were incubated in normal rat SUR and in PBS. It was found that a) the bacterial luminescence of *B. pertussis* lux incorporated in agarose was decreased (50 %), this was possibly due to the viscosity status (which can interfere with the measurement of light output) of the test mixture; b) DMβCD by itself caused a 25 % reduction of bacterial luminescence; c) Also the BA of SUR on the bacterial luminescence was reduced by DMβCD, this was possibly due to the inclusion complexes formed by cyclodextrins (Szejtli, 1982, cited by Frohlich *et al.*, 1996) during their binding to FA present in rat SUR.

Inactivation

Inactivation of the BA of the normal rat surfactant (NSUR) fraction was also studied to characterize the substance(s) responsible for their BA towards both *B. pertussis* 18-323 and lux. Exposure of the NSUR to different physical, chemical and biochemical agents suggested that the NSUR also contained three different active components: FA, phospholipids, and protein. Indication of FA was judged by charcoal adsorption, DMβCD, serum and BSA, pH and heat treatments. High and moderate inactivation of the NSUR BA respectively by phosphlipase A2 and C provided evidence for phospholipids. Evidence of protein was concluded from the high reduction of NSUR BA by proteinease K and also the moderate reduction by heat and pH treatment.

Other treatments such as freezing and thawing, dialysis, methylcellulose, starch, bentonite, sonication, and other cyclodextrins were not significantly destructive. On the other hand membrane filteration and washing in PBS removed much activity.

Loss of activity during membrane filteration might be due to the aggregation of the substance(s) responsible for BA during fractionation by ultracentrifugation and therefore the aggregated particles were retained by the filter. This reduction in BA was not observed in cell-free rat LLF before exposure to ultra centrifugation when the fluid could be filtered without loss of activity.

Similar results as with 18-323 inactivation experiments were observed with strain lux, except that no effect of pH and an enhancement (which could not be explained) of the NSUR BA by sonication were observed. Similarly as with NSN, the high inactivation of NSUR BA towards both *B. pertussis* 18-323 and lux by proteinase K could not be explained.

Coonrod and Yoneda (1983) reported that the BA of concentrated rat SUR towards type 25 pneumococci is not affected by heat (56°C), serum (4%), sonication or trypsin. They also stated that the activity was removed by heat (100°C/15min), serum (8 %), 4 times washing in PBS and storing at -70°C for more than 7 d. Thus my results were almost the same as theirs in respect of trypsin, heat, sonication and serum treatments. However, in my work I did not notice any reduction in the SUR BA after storage at - 70 °C.

Pollock (1947) reported that charcoal-treated broth supported growth of *B. pertussis* by removing some inhibitors such as free fatty acids in the culture media. He also stated that albumin is the active fraction of blood which supports the growth of *B. pertussis*. Accordingly it is possible that the effective component in heated rat serum which inactivates the BA of NSUR is albumin. Charcoal and albumin supported the survival and growth of *B. pertussis* better than starch (Pollock, 1947). The same investigator stated that starch was not quite as efficient or reliable with small inocula as charcoal and albumin. Methylcellulose was mentioned by Nikolajewski *et al.* (1990) as one of the adsorbent agents capable of supporting survival and growth of *B. pertussis*. Imaizumi *et al.* (1983a) found that the addition of DMβCD to the medium stimulated cell growth and concluded that the effect was possibly due to the removal of inhibitors such as FA from the medium. Coonrod and Yoneda (1983) concluded that the anti-pneumococcal activity in rat SUR could be attributed to FA. Field and Parker, (1979) mentioned that only small concentrations (1 ppm) of FA are required to inhibit growth of *B. pertussis* strain 114.

It is possible that the BA of the test fluids is due to phospholipids since phospholipase A2 abolished the BA of NSUR. However, phospholipase A2 catalyses the hydrolysis of the ester bond at the position (2) of glycerophospholipids to form a free fatty acid and lysophospholipid. The excessive release of FA due to the action of phospholipase A2 would be expected to increase the BA of the test fluids rather than reduce it.

Coonrod and Yoneda (1983) reported that phospholipid extracts of rat surfactant were active against pneumococci.

Lipids and long chain fatty acids (FA)

The effect of FA acids (0.0001 - 1 mM) and phosphatidylcholine on the viability and luminescence respectively of *B. pertussis* strains 18-323 and lux was studied. It was found that *B. pertussis* 18-323 was sensitive to arachidonic, palmitoleic, oleic, linoleic, palmitic, petroselinic, pentadecanoic, elaidic, and myristic acid. No sensitivity was found with phosphatidylcholine. *B. pertussis* lux was not sensitive to any FA or phosphatidyl-choline.

Generally, the measured FA concentration in normal rat LLF was found to be at a median of 46 μM. On the other hand the concentration of FA mixture which had a BA and were able to kill 50 % of *B. pertussis* 18-323 inoculum was 55 μM. Therefore it was concluded that most of the LLF FA were in the active state.

Field and Parker (1979) reported that *B. pertussis* strain 114 (phase not mentioned) showed extreme sensitivity towards unsaturated fatty acids (0.001- 0.1 mM) such as petroselinic, oleic, elaidic, palmitoleic, linolenic, arachidonic and linoleic. They also stated that the same organism was less sensitive to saturated fatty acids (0.001 - 1000 mM) such as myristic, pentadecanoic and palmitic acids. The work of Pollok (1947), Field and Parker (1979) and Frohlich *et al.* (1996) concentrated on studying *B. pertussis* growth inhibitors whereas my study dealt with the direct BA of rat LLF towards the same bacteria.

The BA towards *B. pertussis* 18-323 was discovered in the chloroform fraction after lipid extraction of rat SUR with chloroform-methanol. Chromatography on silicic acid of the chloroform active fraction with chloroform, acetone and methanol, showed that the antibacterial activity was eluted with the neutral lipids in chloroform. Similarly Coonrod *et al.* (1984) recovered the anti-pneumococcal activity in the chloroform (neutral lipids) fraction after lipid extraction from rat surfactant. They also detected all of the activity with neutral lipids fraction after elution in chloroform after silicic acid chromatography. When rechromatographed on silicic acid with hexane, hexane-chloroform, and chloroform, they found that the BA was eluted with FA. They stated that the BA was confined to the FA fraction which was established by Thin-layer chromatography.

Ultrastructural changes

TEM examination of the effect of rat SUR on cells of *B. pertussis* 18-323 and lux after 1h incubation showed a severe damage to the bacterial cells, disruption of the cytoplasmic membrane and disappearance of internal structure. Very similar results were given by Coonrod and Yoneda (1983) who reported the effect of 20-fold concentrated rat SUR on pneumococci type 25 after 30 min incubation and they stated that the pneumococci

showed a large cell wall defect with exudation of protoplasm. They also detected a bacterial "ghosts" with almost no internal structure.

Direct examination of the SUR pellet itself by transmission electron microscopy (TEM) showed small numbers of lamellar bodies. Jonsson *et al.* (1986) also detected lamellar bodies in human surfactant by direct examination with TEM. Coonrod and Yoneda (1983) reported that lamellar bodies purified by discontinuous sucrose density gradient from rat SUR were strongly bactericidal active against peumococci type 25. In this study, this batch of SUR (from rat batch no. 58) from which lamellar bodies were detected by TEM also showed very high BA towards *B. pertussis* lux. Thus further studies on the BA of rat SUR should pay attention to the concentration of lamellar bodies in the preparations.

CONCLUSIONS AND PERSPECTIVES

These studies have shown that normal rat LLF fractions (NSN and SUR) contained antibacterial active substance(s) towards *B. pertussis* 18-323. Preliminary evidence suggests that the responsible substances are protein, phospholipids and fatty acids. BA of LLF from convalescent rats was found to be lower than with LLF from normal animals. The reduction in BA was possibly due to the effect of a bacterial toxin (such as PT) on the antibacterial substance(s) itself or on the pneumocyte cell type II. Another substance of protein nature and with BA towards both phase IV *B. pertussis* strain 30042 and phase I *B. pertussis* strain 18-323 was found in LLF from convalescent rats exposed to euthanasia method B (mainly killing by CO_2). It was not known whether this active substance(s) was originally located in the lung and which was enhanced during exposure to CO_2 inhalation, since this active substance(s) was not discovered in other LLF from convalescent rats exposed to other methods of euthanasia (A, C and D).

Possible future work would be to characterize these antibacterial substance(s), to gain more knowledge about the behaviour of *B. pertussis* in the lung and how it can protect itself from the host defence mechanisms in LLF.

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A P P E N D I C E S

Appendix 1. Infection of rats with *B. pertussis* and related bacteria (information provided by Dr. E. Hall).

Materials for the Operation

The materials, equipment, and utensils are listed below:

12cm forceps; 12 cm curved scissors; 12 cm artery forceps; 10 cm Alms retractor (optional); Swann-Morton scalpel holder for no.10 blades (All were obtained from Vet Drug Co); 1x 22g curved bead tipped needles (IMS, Dane Mill Broadhurst Lane, Congleton Cheshire); dish towel to lay instruments on (several small operating towels were provided to place on top of the rat); several pairs of latex examination gloves for the surgeon; cotton wools wabs in glass petri dishes; electric warming mat; animal clippers (size 40) with blade in them; Dilute hibitane in sqeezy bottle or (Quik Prep) chlorohexidine / alcohol spray; scalpel blades (Swann Morton); 1ml syringe; 21 gauge needle; anaesthetic and bottle of sterile distilled water for dilutions; scissors for opening sterile packs; disinfectant wipes (Southern Hygiene Co Ltd); anaesthetic ether and glass beaker with cotton wool; universal racks (to prevent spillage of bacteria / bead mix); incubator set at 32 °C for warming the animals post-operatively; suturing materials (1) (Chromic cat gut , 2 metric with 20 mm curved needle (Ethicon product no. W 658); suturing Materials (2) (Prolene, 3 metric, with 60 mm straight needle (Ethicon product no. w 8623). Both suturing materials no.(1) and no.(2) were purchased from Vet Drug Co, Falkirk.

Bacterial Challenge Suspension

Bordetella pertussis strain 18-323 (NCTC 10739, Phase I) was grown on Bordet-Gengou (BG) Agar containing defibrinated horse blood 20 % (v/v). The growth from plates incubated for 24 h at 37°C was suspended in casamino acids (CAA) solution to give $2x10^9$ CFU/ml. Ten-fold serial dilutions were made in CAA and 0.1-ml volumes were plated on BG medium to provide viable counts.

The undiluted suspension was either used directly for infecting the rats, or more commonly, was first incorporated into agarose beads.

Agarose Beads

All materials and equipment were sterilised by either autoclaving or disinfected by immersion in ethanol followed by rinsing in sterile water.

Low-gelling-temperature agarose (Sigma) 2 % (w/v) in PBS was melted at 100°C, transfered to a water bath at 37°C and allowed to cool. Meanwhile, 200 ml of liquid paraffin (Hays Chemicals Distribution Ltd, Glasgow), previously warmed to 37°C, was transferred to a 600-ml beaker and mixed with a Silverson mixer-emulsifier at the lowest speed setting.

Appendix 1. continued

Five ml of the bacterial suspension at $2x10^9$ CFU/ml were mixed with 5 ml of the agarose, rapidly transferred to a 10-ml syringe fitted with a 22 g x 1.5 in needle and expelled dropwise into the paraffin. After mixing for 5 min, the paraffin was placed on ice and mixing continued for a further 5 min during which time the agarose droplets solidified into beads of c. 100 μm diameter. This mixing procedure was done in a microbiological safety cabinet. The beads were deposited by centrifugation at 7000g at 4°C for 20 min, washed 3 times in CAA by further centrifugations under the same conditions, primarly to remove the paraffin oil, and resuspended to 5 ml of CAA.

The process of bead preparation, from harvesting of the challenge suspension, generally took c. 2-3 h and the beads containing *B. pertussis* were used within 2 h of preparation.

Alginate Beads

Alginate beads were prepared by dropping a 4 % (w/v) solution of sodium alginate (Sigma) in DW (8 ml) into a mixture of CAA (8 ml) and 50 mM calcium chloride (8 ml) with constant swirling. Large spherical beads which formed immediately sank to the bottom and the excess liquid was decanted off leaving 1 volume of supernate : 2 volume of beads. The mixture was homogenised for 30 sec followed by centrifugation at 500 g for 5 min. The supernate was decanted and the bead mixture resuspended with 1 volume CAA to 2 volumes of alginate beads. This process was completed within 30 min. The alginate slurry was much thicker than the agarose.

For control purposes.i.e "sham" operations, agarose and alginate beads were prepared in the same way but without incorporation of bacteria. In several experiments, carrageenan (carrageenan lambda, type IV; Sigma) was incorporated into the agarose beads at a final concentration of 1 % (w/v). In other experiments, heat killed bacteria were used in place of live cells. This was done by heating the standardised bacterial suspension in a water bath at 56°C for 40 min after which 0.1 ml was plated out on BG medium as a check on sterility.

Vaccination

Each rat was given a single human dose (0.5 ml) of adsorbed diphtheria-tetanus-pertussis (DPT)-vaccine containing killed whole *B. pertussis* (Wellcome Trivax-AD) by intraperitoneal injection and challenged 3 weeks later with the usual infecting dose of *B. pertussis* strain 18-323.

Appendix 1. continued

Anaesthesia

The anaesthetic used for surgery was an injectable combination of Hypnorm (midazolam hydrochloride; Janssen Pharmaceuticals Ltd, Grove, Oxford) and Hypnovel (fentanyl citrate and fluanisone; Roche Products Ltd, Welwyn Garden City) mixed with water in 1: 1: 2 proportions and adminstrated intraperitoneally at a dose rate of 3.3 mg/kg, eg 0.3 ml per rat was sufficient. This type of anaesthetic is described as medium term and has the rat immobilised in 10 min and unconscious in 20 min, with the effect lasting 30-60 min. Some variation in senstivity had been experienced and occasionally an extra 0.1ml or more had been given to maintain unconsciouness; the operation was started as soon as the rat become unconscious. For the rats of the size of 150-175 g, the administration of anaesthetic was considered a two-persons task (the handler and the injector), the handler carefully picked up the rat round the upper body between the neck and abdomen so that the front legs were crossed over, the back legs were secured with the other hand and by holding the rat in a horizontal position, the second person (injector) injected 0.3 ml of the anaesthetic intraperitoneally using a 1ml syringe and 21 gauge 1 inch needle.

Operative Procedure

The immune status to *B. bronchiseptica* was detected by ELISA, using a serum sample obtained from each rat tail by veni-puncture before the operation. All the autoclaved materials, utensils and other items required are listed above. The warming mat and the incubator for post-operative recovery were turned on two hours before the operation. With sterile gloves, the instruments were arranged on the dish towel, meanwhile the anaesthetised rat was placed on its back and shaved round the neck region, it was then taped to the warm mat and the upper chest and throat were cleaned with disinfectant, the excess fluid was wiped off with sterile cotton wool, and an operating towel placed over the rat with the opening revealing the area to be operated upon.

Using a sterile scalpel a midline incision about 3 cm long was made in the lower part of the neck, the subcutaneous tissue revealed was picked up with the forceps and divided through using the scalpel to cut only intially. Blunt dissection was continued with the forceps and scissors or artery forceps to expose the trachea, which looks like a clear tube with spiral rings round it.

At this point the retractor might prove useful in holding the tissues, meanwhile an assistant drew up 0.2 ml of air followed by 0.1 ml of the bead/*B.pertussis* mix into a 1 ml syringe and attached the bead-tipped needle. Using a fine 21 gauge x1 inch needle, a hole was punctured in the trachea through which the bead-tipped needle was fed and angled down to the left hand side (The whole length should go in without resistance) and the

Appendix 1. continued

0.1ml inoculum delivered. The wound in the skin was sutured with a single cat gut stitch (brown in colour) subcutaneously in the middle of the wound. Prolene (blue in colour) was used for 3 stitches to draw the outer skin together starting 1 mm from the edge of the wound to allow drainage, These stitches were tied very loosely to allow for swelling, the justification for using two layers of stitching was that the rats nibble the outer prolene in 1-2 days and leave the cat gut which decomposed in about 8 days.

Ether was administered after the operation to each rat for 2 min since it was considerd benefical to the induction of coughing. After the operation, the rats were left to recuperate in an incubator at 32 °C for approximately one hour, after which they were transferred to cages of (two rats per cage) and kept in a room designated for them. Appropriate labels were attached to the cages, and entry to the room was restricted to authorised person only who had to wear face masks.

The rats were inspected daily for several days after the operation by the person legally responsible for them.

Appendix 2. Collection of LLF from animals other than rats (based on information from Dr. J. Porter).

Mouse: LLF were from adult mice, and each mouse was killed in a jar containing CO_2 and pinned out on its back on a board. A scalpel was used for removing the fur and the skin from the throat, The trachea was exposed by making an incision through the tissue covering it, and a hole pierced in the trachea with dissection scissors. A cannula of diameter approximately 1 mm, with a 2 ml syringe attatched to the end was inserted into the trachea and tied in with fine thread and 1.5 ml of PBS passed into the lungs. After 10-15 sec the fluids was sucked out and transferred to a 30 ml plastic Universal. The LLF collected from 20-30 mice on the same day was pooled, except that any with visible blood contamination was discarded.

The pooled collected washings were spun in a laboratory bench centrifuge (MSE minor 'S') at 10, 000 rpm for 15 min, afterwhich the supernate was filter-sterilized through a 0.45 μm filter (Gelman Science) into 1.2 ml alquots dispensed into microcentrifuge tubes. The tubes were stored at -20°C as were the pellets from the centrifugations.

Horse: Approximately 90 ml of LLF was obtained by Professor A.C.Wardlaw at Glasgow University Veterinary School from a 16-year old Arab stallion with a spinal weakness which had had to be put down. The fluid was centrifuged at 10, 000 rpm for 15 min. Some of the supernate was stored in bulk before sterilization. The remainder was filter-sterilized (as with mouse LLF) into 3 x 20 ml portions in 30 ml plastic Univerals, and in 1.2 ml aliquots in microcentrifuge tubes and stored at -20 °C.

Rabbit: LLF was similarly collected as for mouse LLF except that the rabbits were killed by an overdose of Sagittal (1-1.5 ml). The trachea cannulated with larger cannula (diameter of 2-3 mm), and 40 ml of PBS was used to wash the lungs. Centrifugation, sterilization, and storage of the LLF and cell pellets were as before.

Sheep: LLF was obtained from Glasgow University Veterinary School. The lungs from freshly slaughtered normal sheep were removed and rinsed with approximately 100 ml of PBS, the first rinsings being separated from the rinses 2 and 3. The PBS was poured into the sheep trachea and removed after 10-15 sec. Centrifugation, sterilization, and storage of the LLF and cell pellets were as before. Only the rinse 2 was used in this study.

Chicken: Two-year old black-spotted chickens were provided by The Royal Hospital for Sick Children (Glasgow), and the collection procedure was used for lung washing similar to that for mouse, and approximately 20-30 ml of fluid was obtained. Centrifugation, sterilization, and storage of the LLF and cell pellets were as before.

Appendix 2. continued

Dog: A 14 kg mongrel bitch was provided by a Glasgow University Veterinary School, and was used for obtaining LLF. The dog was premedicated with acetylpromazine, anaesthetized with 5% (w/v) sodium thiopentone and maintained with halothane/nitrous oxide/oxygen during a 4 h operation, and killed with an injection of barbiturate. The trachea had previouly been incubated with a leak-proof cannula and approximately 200 ml of sterile PBS was poured in within 1-2 min of death. The fluid was then recoverd by tilting the operating table and letting the fluid run out under gravity. Centrifugation, sterilization, and storage of LLF and cell pellets were as before.

The pH of all animal LLF and human bronchial lavage (which were provided by John Porter), was measured previously by using Johnson's test paper, and found in all cases to be approximately 7.

Appendix 3. ELISA of rat LLF and sera (information provided by Dr. E. Hall).

Materials

Antigens for ELISA used in this study were prepared by Hall *et al.* (1994) during a study on cough production, leucocytosis and serology of rats infected intrabronchially with *B. pertussis*. The antigen was a whole-cell sonicate from *B.pertussis* 18-323 grown overnight on BG agar plates at 37 °C, after which the bacterial culture was harvested in 1 % CAA, centrifuged at 7000 g for 15 min at 4 °C, suspended in ELISA coating buffer to a concentration equivalent of 2 x 10^9 cfu / ml. Cells were disrupted by sonication (MSE sonicator; 4-5 μm amplitude, 20 kHz) on ice, for 30 sec with intervals of 30 sec for cooling, for a total of 10 min, then frozen at -20 °C ready for use when needed.

Pertussis toxin (PT) and filamentous haemagglutinin (FHA) were prepared from *B. pertussis* transposon-insertion mutants Bp 353 and Bp 357, which were respectively deficient in the production of FHA and PT. The bacteria were grown in CL medium; PT and FHA were extracted from the grown culture supernates by dye-ligand chromatography and the protein content of the antigens was estimated.

Appendix 4. Code for details of experimental conditions (Excluding the test fluids that applies to Appendices 5, 6, 7, 9, 11, 18, 20 , 22 and 24)
Code A: See footnote on pages 312 for CL medium, 316 for PBS, 317 for CAA, 321 for NLLF and 326 for CLLF.

Code B: Substrain of *B. pertussis* 18-323 and culture conditions.

Code number	Substrain	Culture (d)	Subculture (h)
1	A6	2	15
2	A6	2	48
3	EH1	3	0
4	EH1	3	24
5	A6	3	24
6	EH1	4	0
7	EH1	5	0
8	EH1	5	24
9	A6	5	24
10	EH1	5	48
11	EH1	6	0
12	EH1	7	0

Code C: Type of container
1- Thin walled glass bijou
2- Screw cap plastic vials (2 ml volume size)
3- U-shape microtitre plate
4- Glass conical flask (size of 100 ml volume)

Code D: Washing of vessels (both glass and plastic)
1- Ordinary wash
2- Acid wash for glass bijou and boiling in DW for plastic ware

Code E: Preparation of the bacterial suspension (A540 = 0.45 in diluent)

Code number	Diluent	Washing yes(Y)/No(N)	Dilution	Inoculum (µl)	Total volume (µl)
1	PBS	N	1 : 2000	20	500
2	PBS	N	1 : 4000	20	500
3	PBS	N	1 : 6000	20	500
4	CL	N	1 : 6000	20	500
5	PBS	N	1 : 7000	20	500
6	PBS	N	1 : 7000	12	300
7	PBS	N	1 : 8000	20	500
8	PBS	N	1 : 8000	12	300
9	PBS	N	1 : 8000	10	250
10	PBS	N	1 : 9000	12	300
11	PBS	N	1 : 9000	10	250
12	CAA	N	1 : 9000	20	500
13	CAA	N	1 : 9000	12	300
14	CAA	N	1 : 9000	10	250
15	CAA	N	1 : 18000	10	250
16	PBS	Y	1 : 2000	12	300
17	PBS	N	1 : 9000	20	500
18	DW	N	1 : 2000	20	500

Appendix 5. Survival (colony count) data on *B. pertussis* strain 18-323 in CL medium at 37 °C. A and B are duplicates; Expt code is explained in the Appendix 4 and footnote.

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)													
		0		15		30		60		120		1440		2880	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	$A_1B_7C_1D_1E_1$	49								9		0			
2	$A_1B_7C_1D_1E_1$	7								1		1			
3	$A_1B_7C_1D_1E_1$	170								105		6		1	
4	$A_2B_7C_1D_1E_1$	137								120		175		300	
5	$A_2B_7C_1D_1E_1$	60								53		54		180	
6	$A_2B_7C_1D_1E_1$	47								26		57		44	
7	$A_2B_7C_1D_1E_1$	14				13				11		185		228	
8	$A_2B_7C_1D_1E_1$	24				26				10					
9	$A_2B_7C_1D_1E_1$	240		190		185				150					
10	$A_2B_7C_1D_1E_1$	230		240		230		230							
11	$A_2B_7C_1D_1E_1$	50								34					
12	$A_3B_7C_1D_1E_2$	38	41			39	34	39	34	37	27	0	2	0	0
13	$A_3B_7C_1D_1E_7$	16		20				13		15					
14	$A_3B_7C_1D_1E_7$	26								16		17		250	
15	$A_3B_{11}C_1D_1E_2$	23				10				7					
16	$A_3B_7C_1D_1E_1$	45				41									
17	$A_3B_{12}C_1D_1E_{18}$	22								13		1			
18	$A_3B_{12}C_1D_1E_{18}$	20								21		22		0	
19	$A_3B_{12}C_1D_1E_{18}$	15								28		12		0	
20	$A_3B_{12}C_1D_1E_{18}$	24								7		1		0	
21	$A_3B_{12}C_1D_1E_{18}$	27								14		110		300	
22	$A_3B_{12}C_1D_1E_{18}$	30								12		4		0	
23	$A_3B_{12}C_1D_1E_{18}$	10													
24	$A_3B_8C_1D_1E_2$	160	200							40	34				
25	$A_3B_8C_1D_1E_2$	68	88			62	70			10	11				
26	$A_3B_8C_1D_1E_2$	100	140	86	100	40	36			5	5				
27	$A_3B_{10}C_1D_2E_3$	52	58			68	64			24	22	5	5	5	4
28	$A_3B_8C_1D_2E_2$	74	80	40	42	20	24			1	3				
29	$A_3B_8C_1D_2E_2$	50	60	32	30	22	16			4	0				
30	$A_3B_4C_1D_2E_2$	235	245							100	80	14	31		
31	$A_3B_8C_1D_2E_2$	200	210							80	70	20	46		
32	$A_3B_5C_1D_2E_2$	200	210							120	124	20	26		
33	$A_3B_9C_1D_2E_2$	140	120							120	140	22	30		
34	$A_3B_4C_2D_2E_3$	118	128							110	102	44	58	2	1
35	$A_3B_4C_4D_2E_3$	126	112							70	76	50	50	300	300
36	$A_3B_4C_2D_2E_3$	80	82							70	72	3	6	0	0
37	$A_3B_4C_2D_2E_4$	98	96							96	108	8	4	0	0
38	$A_3B_4C_3D_2E_7$	112	108							80	82	26	34	6	5
39	$A_3B_4C_2D_2E_7$	24	30	22	30	20	20			10	10	0	0	0	0
40	$A_4B_4C_2D_2E_7$	22	28	20	24	34	28			14	10	4	4	0	0
41	$A_2B_4C_2D_2E_7$	20	24	18	26	24	26			16	24	8	2	0	0
42	$A_2B_5C_2D_2E_7$	70	78	80	74	88	84			86	72	300	300	600	600
43	$A_2B_4C_2D_2E_5$	80	70	80	66	66	64			68	58	300	300	300	300

Appendix 5. continued

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)													
		0		15		30		60		120		1440		2880	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B
44	$A_2B_5C_2D_2E_5$	70	72	70	68	72	70			62	72	300	300		
45	$A_2B_5C_2D_2E_5$	82	80	86	80	80	84			74	80	300	300	34	40
46	$A_2B_5C_2D_2E_5$	74	84	72	68	70	68			68	70				
47	$A_5B_5C_2D_2E_5$	120	124	120	128	120	124			120	114	300	300	600	600
48	$A_5B_5C_2D_2E_5$	120	112	114	122	110	118			100	110	300	300	600	600
49	$A_5B_5C_2D_2E_5$	106	110	110	100	106	104			110	106	300	300	600	600
50	$A_2B_5C_2D_2E_5$	90	94	100	94	98	90			102	96	250	280	340	360
51	$A_2B_5C_2D_2E_5$	120	116	118	112	116	116			114	120	260	280		
52	$A_5B_5C_2D_2E_5$	106	108	110	102	108	106			102	110	300	300		
53	$A_5B_1C_2D_2E_5$	172	174	170	178	170	174			174	168	300	300		
54	$A_5B_2C_2D_2E_5$	130	140	130	136	132	130			140	130	300	300	600	600
55	$A_5B_5C_2D_2E_5$	140	130	130	136	130	130			130	134	300	300	600	600
56	$A_5B_5C_2D_2E_5$	80	84	88	82	74	76			80	80	300	300	600	600
57	$A_5B_5C_2D_2E_5$	114	116	100	110	110	110			98	98	300	300	600	600
58	$A_5B_5C_2D_2E_5$	116	110	110	100	110	110			100	110	300	300	600	600
59	$A_5B_5C_2D_2E_5$	140	130	141	125	140	130			130	130	300	300	600	600
60	$A_5B_5C_2D_2E_5$	92	80	84	84	80	84			80	70	300	300	600	600
61	$A_5B_5C_2D_2E_7$	124								110		300			
62	$A_5B_5C_2D_2E_7$	108								100		300		600	
63	$A_5B_5C_2D_2E_7$	116		112		102				90		300		600	
64	$A_5B_5C_2D_2E_7$	60		61						52		300		600	
65	$A_5B_5C_2D_2E_{16}$	170		170		164				160		300		600	
66	$A_5B_5C_2D_2E_7$	146		142		140				130		300		600	
67	$A_5B_5C_2D_2E_{16}$	198		198		190				186		300		600	
68	$A_5B_5C_2D_2E_7$	136		132		132				130		300		600	
69	$A_5B_5C_2D_2E_{16}$	158		144		142				140		300		600	
70	$A_5B_5C_2D_2E_7$	110		100		100				90		300		600	
71	$A_5B_5C_2D_2E_{17}$	60		58		60				62		300			
72	$A_5B_5C_2D_2E_{17}$	74		72		72				70					
73	$A_5B_5C_2D_2E_{17}$	22		20		20				19		120		130	
74	$A_5B_5C_2D_2E_7$	15		14		12				8		6			
75	$A_5B_5C_2D_2E_7$	68		68		66				66		300			
76	$A_5B_5C_2D_2E_7$	32		36		34				34		300			
77	$A_5B_5C_2D_2E_7$	84		88		86				78		300		600	
78	$A_5B_5C_2D_2E_7$	88		84		82				74		300		600	
79	$A_5B_5C_2D_2E_7$	40		39		32				32		300		600	
80	$A_5B_5C_2D_2E_7$	75				66				68					
81	$A_5B_5C_2D_2E_7$	84				82				81		300			
82	$A_5B_5C_2D_2E_7$	58				62				56		300			
83	$A_5B_5C_2D_2E_7$	50				48				44		300			
84	$A_5B_5C_2D_2E_7$	74				70				60		300			
85	$A_5B_5C_2D_2E_7$	48				46				42		300			
86	$A_5B_5C_2D_2E_7$	68		58		64				55		300		600	
87	$A_5B_5C_2D_2E_7$	74		70		74				84		300		600	

Appendix 5. continued

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)													
		0		15		30		60		120		1440		2880	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B
88	A5B5C2D2E7	76		77		77				68		300		600	
89	A5B5C2D2E7	64		49		54				60		300		600	
90	A5B5C2D2E7	90		108		88				87		300		600	
91	A5B5C2D2E7	48		46		56				44		300		600	
92	A5B5C2D2E7	66		64		80				72		300		600	
93	A5B5C2D2E7	100		108		100				98		300		600	
94	A5B5C2D2E7	50		41		52				38		300		600	
95	A5B5C2D2E7	95		90		110				114		300		600	
96	A5B5C2D2E7	80		88		80				74		300		600	
97	A5B5C2D2E7	63				56		58		50		300			
98	A5B5C2D2E7	71				58		60		60		300			
99	A5B5C2D2E7	78	80			54	58	58	48	48	50	300	300		
100	A5B5C2D2E7	74	70			75	73	73	80	74	73	300	300		
101	A5B5C2D2E7	76				78		80		84					
102	A5B5C2D2E7	60				72		58		40		120			
103	A5B5C2D2E7	55				79		64		51		300			
104	A5B5C2D2E11	45				33		33		35		130			
105	A5B5C2D2E11	62				64		51		57		150			
106	A5B5C2D2E14	52		40		43		43		51		300			
107	A5B5C2D1E14	30		48		48		42		36		300			
108	A5B5C2D1E13	75	73			60	60	66	58	44	50				
109	A5B5C2D1E13	76	68			84	94	98	84	82	72	300	300		
110	A5B5C2D1E13	92	100			86	90	80	86	60	70	300	300		
111	A5B5C2D1E13	118	102			108	112	98	84	98	96				
112	A5B5C2D1E13	32	42					46	44	43	49	12	20		
113	A5B5C2D1E13	50	52			47	42	43	41	28	27	140	154		
114	A5B5C2D1E13	32	45			34	42	40	28	47	33	130	134		
115	A5B5C2D1E13	88	72			72	74	67	80	72	72	300	300		
116	A5B5C2D1E13	146	136			138	128	114	122	90	112				
117	A5B5C2D1E13	170	150			132	142	118	120	120	120				
118	A5B5C2D1E13	136	128			110	122	106	126	100	130				

Note: to allow entry of numbers in the data base, confluent growth is tabulated as " 600"; semi-confluent as "300"' while counts in the region of 200 are to be understood as only approximate.

Appendix 5. continued

Code A : Variation in formulation of CL medium

Code number	Glutamic Acid (a) or Na Glutamate (b)		DMβCD (g / l)	Vitamin supplement (µl /10 ml)
	Source	Form		
1	(BDH)	(b)	0.1	100
2	(Sigma)	(a)	1.0	100
3	(BDH)	(b)	1.0	100
4	(BDH)	(a)	1.0	100
5	(Sigma)	(a)	1.0	50

For the explanation of codes B, C, D and E, see Appendix 4.

Appendix 6. Survival (colony count) data on *B. pertussis* strain 18-323 in PBS at 37 °C. A and B are duplicate; Expt code is explained in the Appendix 4 and footnote.

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
1	$A_1B_7C_1D_1E_1$	26								24		0	
2	$A_1B_7C_1D_1E_1$	13				10				6		0	
3	$A_1B_7C_1D_1E_1$	230		205		180				130			
4	$A_1B_7C_1D_1E_1$	220		215		210		220					
5	$A_1B_7C_1D_1E_2$	46								27			
6	$A_1B_7C_1D_1E_7$	15		12				60		14			
7	$A_1B_{11}C_1D_1E_7$	11				10		7		4			
8	$A_1B_7C_1D_1E_1$	41				27		62					
9	$A_1B_{12}C_1D_1E_{18}$	22								2			
10	$A_1B_8C_1D_1E_2$	68	78			52	54	24	21	2	2		
11	$A_2B_{10}C_1D_2E_3$	45	43			34	38			8	9	0	0
12	$A_2B_8C_1D_2E_2$	74	62	34	28	12	14			0	0	0	0
13	$A_2B_8C_1D_2E_2$	50	48	24	28	12	12			1	0		
14	$A_2B_4C_2D_2E_3$	92	96							72	76	15	17
15	$A_2B_4C_2D_2E_3$	70	74							80	68	8	4
16	$A_2B_4C_2D_2E_4$	106	100							102	108	2	0
17	$A_2B_4C_3D_2E_3$	88	108							80	84	0	0
18	$A_2B_4C_2D_2E_7$	18	22	30	18	18	18			0	0	0	0
19	$A_2B_5C_2D_2E_5$	70	64	76	60	70	76			56	52	8	6
20	$A_2B_4C_2D_2E_5$	78	92	84	84	66	76			62	66	3	1
21	$A_2B_5C_2D_2E_5$	60	64	66	64	50	68			44	48	2	0
22	$A_2B_5C_2D_2E_5$	86	78	82	80	70	80			54	52	0	0
23	$A_2B_5C_2D_2E_5$	80	78	52	58	60	64			40	34		
24	$A_2B_5C_2D_2E_5$	126	130	120	124	120	114			100	84	0	0
25	$A_2B_5C_2D_2E_5$	118	110	80	74	60	68			60	54	0	0
26	$A_2B_5C_2D_2E_5$	110	100	104	100	90	96			58	62	0	0
27	$A_2B_5C_2D_2E_5$	84	80	88	90	80	76			68	60	0	0
28	$A_2B_5C_2D_2E_5$	100	110	110	118	108	110			90	100	0	0
29	$A_2B_5C_2D_2E_5$	100	98	102	98	98	98			100	94	0	0
30	$A_2B_1C_2D_2E_5$	130	140	140	140	130	148			140	136	6	4
31	$A_2B_2C_2D_2E_5$	140	148	130	140	130	140			110	90	0	0
32	$A_2B_5C_2D_2E_5$	130	136	130	130	120	130			120	130	0	0
33	$A_2B_5C_2D_2E_5$	78	72	80	80	66	58			46	26	0	0
34	$A_2B_5C_2D_2E_5$	110	100	100	100	98	86			86	60	0	0
35	$A_2B_5C_2D_2E_5$	114	110	100	110	70	60			40	34	0	0
36	$A_2B_5C_2D_2E_5$	140	136	130	134	94	90			4	4	0	0
37	$A_2B_5C_2D_2E_5$	126	124	82	80	76	70			1	3	0	0
38	$A_2B_6C_2D_2E_5$	68	78	68	60	62	60			50	56	2	2
39	$A_2B_5C_2D_2E_7$	118								90		4	
40	$A_2B_5C_2D_2E_7$	110								90		3	
41	$A_2B_5C_2D_2E_7$	113		102		96				92		14	
42	$A_2B_5C_2D_2E_7$	54		50						30		0	
43	$A_2B_5C_2D_2E_{16}$	160		156		156				140		1	

Appendix 6. continued

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
44	$A_2B_5C_2D_2E_7$	138		120		110				100		5	
45	$A_2B_5C_2D_2E_{16}$	190		186		184				180		12	
46	$A_2B_5C_2D_2E_7$	126		124		120				97		4	
47	$A_2B_5C_2D_2E_{16}$	140		120		118				110		0	
48	$A_2B_5C_2D_2E_7$	92		88		80				64		0	
49	$A_2B_5C_2D_2E_{17}$	50		46		50				40		0	
50	$A_2B_5C_2D_2E_{17}$	68		64		64				50			
51	$A_2B_5C_2D_2E_{17}$	20		18		18				18		0	
52	$A_2B_5C_2D_2E_7$	7		5		1				1			
53	$A_2B_5C_2D_2E_7$	50		48		46				46		0	
54	$A_2B_5C_2D_2E_7$	34		30		28				24		0	
55	$A_2B_5C_2D_2E_7$	76		60		52				32		0	
56	$A_2B_5C_2D_2E_7$	70		66		52				34		0	
57	$A_2B_5C_2D_2E_7$	38		37		28				20		0	
58	$A_2B_5C_2D_2E_7$	73				40				36			
59	$A_2B_5C_2D_2E_7$	82				70				35		0	
60	$A_2B_5C_2D_2E_7$	60				44				28		0	
61	$A_2B_5C_2D_2E_7$	47				37				22		0	
62	$A_2B_5C_2D_2E_7$	52				42				34		0	
63	$A_2B_5C_2D_2E_7$	32				24				14		0	
64	$A_2B_5C_2D_2E_7$	56		54		51				52		1	
65	$A_2B_5C_2D_2E_7$	60		70		66				38		3	
66	$A_2B_5C_2D_2E_7$	74		72		70				55		9	
67	$A_2B_5C_2D_2E_7$	62		39		34				20		2	
68	$A_2B_5C_2D_2E_7$	78		100		82				61		4	
69	$A_2B_5C_2D_2E_7$	47		46		34				20		0	
70	$A_2B_5C_2D_2E_7$	59		69		73				43		2	
71	$A_2B_5C_2D_2E_7$	98		89		84				62		5	
72	$A_2B_5C_2D_2E_7$	46		34		44				33		0	
73	$A_2B_5C_2D_2E_7$	94		72		90				49		3	
74	$A_2B_5C_2D_2E_7$	97		70		60				36		1	
75	$A_2B_5C_2D_2E_7$	62				64		64		43		0	
76	$A_2B_5C_2D_2E_7$	58				42		35		14		0	
77	$A_2B_5C_2D_2E_7$	60	72			30	34	20	24	4	4	0	0
78	$A_2B_5C_2D_2E_7$	72	80			52	52	36	36	16	16	0	
79	$A_2B_5C_2D_2E_7$	86				68		50		30		0	
80	$A_2B_5C_2D_2E_7$	65				32		36		13		0	
81	$A_2B_5C_2D_2E_7$	60				54		33		22		0	
82	$A_2B_5C_2D_2E_{11}$	35				27		12		10		1	
83	$A_2B_5C_2D_2E_{11}$	57				42		32		23			
84	$A_2B_5C_2D_2E_{14}$	40		34		52		50		48		7	
85	$A_2B_5C_2D_1E_{14}$	42		37		41		26		39		0	
86	$A_2B_5C_2D_1E_{13}$	66	63			56	46	44	50	34	38		
87	$A_2B_5C_2D_1E_{13}$	82	84			78	80	82	80	76	78	1	0

Appendix 6. continued

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
88	$A_2B_5C_2D_1E_{13}$	84	64			60	72	50	60	48	50	0	0
89	$A_2B_5C_2D_1E_{13}$	100	110			96	84	94	87	76	70		
90	$A_4B_5C_2D_1E_{13}$	40	46			20	32	32	31	0	0		
91	$A_4B_5C_2D_1E_{13}$	55	61			58	54	38	30	0	0		
92	$A_4B_5C_2D_1E_{12}$	32	34			28	30	36	42	28	26	3	0
93	$A_3B_5C_2D_1E_{12}$	62	70			68	62	62	58	56	76	1	0
94	$A_3B_5C_2D_1E_{13}$	120	132			120	140	120	108	94	104		
95	$A_3B_5C_2D_1E_{13}$	150	160			140	150	130	122	110	120		
96	$A_3B_5C_2D_1E_{13}$	120	128			106	112	104	110	104	96		
97	$A_3B_5C_2D_1E_{13}$	120	136			126	132	116	126	96	110		
98	$A_3B_5C_2D_1E_{15}$	64	72					62	62				
99	$A_3B_5C_2D_1E_{15}$	58	78					70	84				
100	$A_3B_5C_2D_1E_{15}$							80	90				
101	$A_3B_5C_2D_1E_{15}$	90	96					80	82				
102	$A_3B_5C_2D_1E_{15}$	99	83			78	76	49	51	22	36		
103	$A_3B_5C_2D_1E_{15}$	76	82					80	66				
104	$A_3B_5C_2D_1E_{15}$	61	59					50	74				
105	$A_3B_5C_2D_1E_{15}$	86	76			76	72	86	62	40	52		
106	$A_3B_5C_2D_1E_{15}$	76	72					60	72				
107	$A_3B_5C_2D_1E_{15}$	70	56					48	46				
108	$A_3B_5C_2D_1E_{15}$	70	62					60	62				
109	$A_3B_5C_2D_1E_{15}$	54	50					44	38				
110	$A_3B_5C_2D_1E_{15}$	42	52					54	58				
111	$A_3B_5C_2D_1E_{15}$	34	40					20	30				
112	$A_3B_5C_2D_1E_{15}$	32	46					36	34				
113	$A_3B_5C_2D_1E_{15}$							30	24				
114	$A_3B_5C_2D_1E_{15}$	28	40					26	26				
115	$A_3B_5C_2D_1E_{15}$	36	40					34	44				
116	$A_3B_5C_2D_1E_{15}$	28	30					30	38				
117	$A_3B_5C_2D_1E_{15}$	22	22					22	24				
118	$A_3B_5C_2D_1E_{15}$	34	38					24	28				
119	$A_3B_5C_2D_1E_{15}$	11	17					13	11				
120	$A_3B_5C_2D_1E_{14}$	94	100					82	100				
121	$A_3B_5C_2D_1E_{14}$	84	90					64	74				
122	$A_3B_5C_2D_1E_{14}$	80	88			74	78	68	60				
123	$A_3B_5C_2D_1E_{14}$	70	80					86	78				
124	$A_3B_5C_2D_1E_{14}$	146	116					96	86				
125	$A_3B_5C_2D_1E_{14}$	90	90					54	62				
126	$A_3B_5C_2D_1E_{14}$	70	76			68	60	66	56	52	52		
127	$A_3B_5C_2D_1E_{14}$	108	102					108	112				
128	$A_3B_5C_2D_1E_{14}$	84	100					82	90				
129	$A_3B_5C_2D_1E_{14}$	110	96					74	80				
130	$A_3B_5C_2D_1E_{14}$	72	82					78	80				
131	$A_3B_5C_2D_1E_{14}$	86	78					60	70				

Appendix 6. continued

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
132	$A_3B_5C_2D_1E_{14}$	158	152			136	104	105	101	74	66		
133	$A_3B_5C_2D_1E_{14}$	74	68					70	84				
134	$A_3B_5C_2D_1E_{14}$	82	60					56	72				
135	$A_3B_5C_2D_1E_{14}$	112	108					98	74				

Note: Counts in the region of 200 are to be understood as only approximate.

Code A : Variation in formulation of PBS

Code number	PBS from tablet [BDH] (a) or individual chemicals (b)	pH
1	a	7.2
2	b	7.2
3	b	7.3
4	b	7.4

For the explanation of codes B, C, D and E, see Appendix 4.

Appendix 7. Survival (colony count) data on *B. pertussis* strain 18-323 in 1 % (w/v) casamino acids (CAA) at 37 °C. A and B are duplicates; Expt code is explained in the Appendix 4 and footnote.

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
1	$AB_7C_1D_1E_1$	17						11		7		0	
2	$AB_7C_1D_1E_1$	57						48		26		0	
3	$AB_7C_1D_1E_1$	48						33		23		0	
4	$AB_{12}C_1D_1E_{18}$	8											
5	$AB_{10}C_1D_2E_3$	58	72			30	26			7	7	0	0
6	$AB_5C_2D_2E_{14}$	40		43		47		42		36			
7	$AB_5C_2D_1E_{14}$	30		25		40		37		32		6	
8	$AB_5C_2D_1E_{15}$	82	70					70	70				
9	$AB_5C_2D_1E_{15}$	110	86					84	82				
10	$AB_5C_2D_1E_{15}$	78	98					94	88				
11	$AB_5C_2D_1E_{15}$	96	90					85	63				
12	$AB_5C_2D_1E_{15}$							14	18				
13	$AB_5C_2D_1E_{14}$	74	80			70	62	32	52	46	46		
14	$AB_5C_2D_1E_{14}$	94	134					102	94				
15	$AB_5C_2D_1E_{14}$							92	94				
16	$AB_5C_2D_1E_{14}$							62	58				
17	$AB_5C_2D_1E_{14}$							72	78				
18	$AB_5C_2D_1E_{14}$							68	74				
19	$AB_5C_2D_1E_{14}$							196	176				
20	$AB_5C_2D_1E_{14}$							202	188				
21	$AB_5C_2D_1E_{14}$							58	54				
22	$AB_5C_2D_1E_{14}$							74	76				
23	$AB_5C_2D_1E_{14}$							76	64				
24	$AB_5C_2D_1E_{14}$							114	128				
25	$AB_5C_2D_1E_{14}$							138	112				
26	$AB_5C_2D_1E_{14}$							100	112				
27	$AB_5C_2D_1E_{14}$							168	120				
28	$AB_5C_2D_1E_{14}$					137	141						
29	$AB_5C_2D_1E_{14}$					106	100						
30	$AB_5C_2D_1E_{14}$					54	64						
31	$AB_5C_2D_1E_{14}$					66	66						

Note: Counts in the region of 200 are to be understood as only approximate.

Code A: 1% (w/v) casamino acids was prepared by dissolving 10g Casein Hydrolysate (Gibco), 0.1g $MgCl_2$ $6H_2O$, 0.016g $CaCl_2$, and 5g NaCl in 1000 ml distilled water and the PH of the mixture was adjusted at 7.1 with NaOH, sterilized at 121°C for 15 minutes, stored at 4°C ready for use, and discarded after two months if not used.

For the explanation of codes B, C, D and E, see Appendix 4.

Appendix 8. Percent survival (based on PBS time-zero counts) of *B. pertussis* strain 18-323 in 1 % (w/v) casamino acids (CAA) after exposure at 37 °C for various times and in relation to Experiment Ordinal Number.

Expt ordinal no.	% Survival after exposure at 37 °C for time (min)					
	0	15	30	60	120	1440
1	100			65	42	0
2	100			80	46	0
3	98			67	47	0
4	36					
5	118		51		13	0
6	77	83	90	81	69	
7	100	83	133	123	107	19
8	112			103		
9	100			85		
10	95			98		
11	118			94		
12				88		
13	105		90	58	63	
14	109			93		
15				107		
16				58		
17				97		
18				87		
19				85		
20				92		
21				79		
22				106		
23				403		
24				70		
25				88		
26				96		
27				83		
28			124			
29			84			
30			134			
31			122			
Median	**100**	**83**	**106**	**87**	**47**	**0**

Appendix 9. Survival (colony count) data on *B. pertussis* strain 18-323 in normal rat LLF at 37 °C. A and B are duplicates; Expt code is explained in the Appendix 4 and footnote.

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
1	$A_1B_7C_1D_1E_1$	62						0		0		0	
2	$A_1B_7C_1D_1E_1$	4				5							
3	$A_1B_7C_1D_1E_1$	28								0		0	
4	$A_2B_7C_1D_1E_1$	14				1				0			
5	$A_2B_7C_1D_1E_1$	28				18				18			
6	$A_2B_7C_1D_1E_1$	172		155		44		0					
7	$A_2B_7C_1D_1E_1$	220		165		120		71					
8	$A_2B_7C_1D_1E_2$	24								0			
9	$A_2B_7C_1D_1E_8$	21		0				0		0			
10	$A_2B_{11}C_1D_1E_2$	17				0		0		0			
11	$A_2B_7C_1D_1E_1$	40				4		0					
12	$A_2B_8C_1D_1E_2$	73	87			74	59	47	60	2	0		
13	$A_2B_8C_1D_1E_2$	110	100	110	96	66	74			0	0		
14	$A_2B_8C_1D_2E_2$	94	94	53	65	40	30			0	0		
15	$A_2B_8C_1D_2E_2$	44	36	40	24	14	18			0	0		
16	$A_1B_4C_2D_2E_8$	22	32	10	14	6	8			0	0	0	0
17	$A_1B_5C_2D_2E_6$	82	64	54	52	62	34			0	0	0	0
18	$A_1B_4C_2D_2E_6$	80	90	64	66	42	42			12	10	0	0
19	$A_1B_5C_2D_2E_6$	80	78	80	70	40	38			18	14	0	0
20	$A_1B_5C_2D_2E_6$	100	80	0	0	0	0			0	0	0	0
21	$A_1B_5C_2D_2E_6$	54	60	0	0	0	0			0	0	0	0
22	$A_3B_5C_2D_2E_6$	100	110	30	22	25	17			8	4	0	0
23	$A_3B_5C_2D_2E_6$	80	74	20	18	16	16			8	4	0	0
24	$A_1B_5C_2D_2E_6$	100	110	4	4	0	0			0	0	0	0
25	$A_1B_5C_2D_2E_6$	66	70	1	0	0	0			0	0	0	0
26	$A_6B_1C_2D_2E_5$	180	160	170	162	120	110			90	110	2	2
27	$A_6B_2C_2D_2E_5$	188	190	110	114	110	110			90	90	1	0
28	$A_6B_5C_2D_2E_6$	120	130	100	110	100	90			10	4	0	0
29	$A_3B_5C_2D_2E_6$	86	70	12	4	8	8			1	1	0	0
30	$A_5B_5C_2D_2E_6$	114	110	32	30	7	5			0	0	0	0
31	$A_7B_5C_2D_2E_6$	130	116	118	120	120	120			110	116	0	0
32	$A_5B_5C_2D_2E_6$	100	110	0	0	0	0			0	0	0	0
33	$A_5B_6C_2D_2E_6$	20	28	0	0	0	0			0	0	0	0
34	$A_5B_5C_2D_2E_8$	80								0		0	
35	$A_6B_5C_2D_2E_8$	110								100		3	
36	$A_5B_5C_2D_2E_8$	18								0		0	
37	$A_6B_5C_2D_2E_8$	90								88		2	
38	$A_5B_5C_2D_2E_8$	56		0		0				0		0	
39	$A_6B_5C_2D_2E_8$	116		112		106				102			
40	$A_5B_5C_2D_2E_8$	20		0		0				0		0	
41	$A_6B_5C_2D_2E_8$	64		62						46		2	
42	$A_5B_5C_2D_2E_{16}$	158		0		0				0		0	

Appendix 9. continued

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
43	$A_5B_5C_2D_2E_8$	110		0		0				0		0	
44	$A_5B_5C_2D_2E_{16}$	112		0		0				0		0	
45	$A_5B_5C_2D_2E_8$	120		0		0				0		0	
46	$A_5B_5C_2D_2E_{16}$	60		0		0				0		0	
47	$A_5B_5C_2D_2E_8$	40		0		0				0		0	
48	$A_6B_5C_2D_2E_{17}$	58		30		10				0		0	
49	$A_6B_5C_2D_2E_{17}$	60		56		34				0			
50	$A_8B_5C_2D_2E_{17}$	65		62		60				26			
51	$A_6B_5C_2D_2E_{17}$	22		21		13				4		0	
52	$A_{10}B_5C_2D_2E_8$	97		3		0				0		0	
53	$A_9B_5C_2D_2E_8$	96		92		65				24		0	
54	$A_{10}B_5C_2D_2E_8$	74		34		6				2		0	
55	$A_9B_5C_2D_2E_8$	84		98		70				42		0	
56	$A_{10}B_5C_2D_2E_8$	44		26		10				3		0	
57	$A_9B_5C_2D_2E_8$	51		52		44				14		0	
58	$A_{10}B_5C_2D_2E_8$	76				50				33			
59	$A_{10}B_5C_2D_2E_8$	98				88				64		0	
60	$A_{10}B_5C_2D_2E_8$	72				43				18		0	
61	$A_{11}B_5C_2D_2E_8$	73		0		0				0		0	
62	$A_{12}B_5C_2D_2E_8$	60		29		7				1		0	
63	$A_{13}B_5C_2D_2E_8$	58		1		0				0		0	
64	$A_{11}B_5C_2D_2E_8$	74		1		0				0		0	
65	$A_{12}B_5C_2D_2E_8$	91		48		7				0		0	
66	$A_{13}B_5C_2D_2E_8$	74		3		0				0		0	
67	$A_{11}B_5C_2D_2E_8$	36		0		0				0		0	
68	$A_{12}B_5C_2D_2E_8$	68		2		0				0		0	
69	$A_{13}B_5C_2D_2E_8$	40		0		0				0		0	
70	$A_{14}B_5C_2D_2E_8$	76		11		0				0		0	
71	$A_{15}B_5C_2D_2E_8$	60		0		0				0		0	
72	$A_{14}B_5C_2D_2E_8$	90		14		0				0		0	
73	$A_{15}B_5C_2D_2E_8$	86		0		0				0		0	
74	$A_{14}B_5C_2D_2E_8$	34		0		0				0		0	
75	$A_{15}B_5C_2D_2E_8$	42		0		0				0		0	
76	$A_{16}B_5C_2D_2E_8$	88		4		0				0		0	
77	$A_{16}B_5C_2D_2E_8$	92		40		12				0		0	
78	$A_{14}B_5C_2D_2E_8$	32				0				0		0	
79	$A_{14}B_5C_2D_2E_8$	58				0				0		0	
80	$A_{14}B_5C_2D_2E_8$	68	72			4	5	3	0	0	0	0	0
81	$A_{18}B_5C_2D_2E_8$	50	40			0	0	0	0	0	0	0	0
82	$A_{18}B_5C_2D_2E_8$	92				0		0		0		0	
83	$A_{17}B_5C_2D_2E_9$	80				33		14		14		0	
84	$A_{17}B_5C_2D_2E_9$	68				38		23		5		0	
85	$A_{20}B_5C_2D_2E_{11}$	15				0		0		0		0	

Appendix 9. continued

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
86	$A_{20}B_5C_2D_2E_{11}$	40				0		0		0		0	
87	$A_4B_5C_2D_2E_{14}$	56		22		14		2		4			
88	$A_4B_5C_2D_1E_{14}$	38		22		15		6		1			
89	$A_4B_5C_2D_1E_{13}$	66	46			1	1	0	0	0			
90	$A_4B_5C_2D_1E_{13}$	90	88			42	52	32	38	24	30	0	0
91	$A_4B_5C_2D_1E_{13}$	74	80			58	52	38	48	46	32	0	0
92	$A_{13}B_5C_2D_1E_{13}$	38	22			0	0	0	0	0	0	0	0

Note: Counts in the region of 200 are to be understood as only approximate.
*All normal Sprague Dawley (SD) rat lungs were washed with 5ml (except rat batch no. 37 and 38 respectively their lungs were washed with 15 and 10 ml sterile PBS) sterile PBS (pH7.2 - 7.4), pooled rat LLF fluids kept in ice then centrifuged at 2000 rpm for 15 minutes, afterwhich the pellet 1(mainly macrophages and monocytes) was discarded, and the supernatant was membrane filtered (this step is stopped from batch no. 42), distributed in a washed sterile 2-ml volume plastic vials as about a 1ml portions, stored in -20°C for 24 h, then transferred into -70°C for long term storage ready for use when its needed, that was the standard procedure for obtaining LLF, except were stated.

Code A: Different batches of normal rats from which LLF was collected

Code number	Rat Batch* no.	Sex	Weight (SEM)	Method of ** euthanasia
1	15	F	230 ± 4	A
2	16	M	374 ± 6	A
3	17	M	286	A
4	21	M	190	A
5	33	M	-	A
6	34	M	296 ± 0	B
7	35	F	-	B
8	37	-	350	B
9	38.1	M	175 ± 4	B
10	38.2	M	175 ± 4	C
11	39S	F	126 ± 4	D
12	39M	M	253 ± 2	D
13	39L	M	350 ± 6	D
14	40L	M	355 ± 4	D
15	40XL	M	456 ± 31	D
16	41	F	170 ± 3	D
17	42	-	-	D
18	43	F	220 ± 3	D
19	44	-	182±9	D
20	45	F	270 ± 12	D
21	50	M	313±3	D
22	55	F	171± 3	D
23	58	F	182± 4	D

For the explanation of codes B,C,D and E see Appendix 4.

Appendix 9. continued

* All batches of rats were normal (untreated) except rat batch no. 16, which was sham-operated (LGT agarose beads + carrageenan given intratracheally then rats exposed to ether. S= small; M = medium; L = large; XL = extra large.

**Summary of four different euthanasia methods immediately prior to collection of rat LLF

Method	Procedure prior to collection of LLF					
	Halothane/ oxygen	CO_2		Bleeding out by heart puncture before death	Cervical dislocation	Bleeding out by heart puncture after death
		under anaesthesia	alone			
A	+	+	-	-	-	+
B	-	-	+	-	-	+
C	+	-	-	-	-	+
D	+	-	-	+	+	-

Appendix 10. Batch to batch variation in bactericidal activity of normal rat LLF towards *B. pertussis* 18-323.

Batch* code	No. of experiments	Median % survival [Range](No. of observations) at incubation time (min)					
		0	15	30	60	120	1440
A1	11	100 [57-100] (11)	23.5 [0-95] (8)	26 [0-71] (9)	0 [0] (1)	0 [0-20] (10)	0 [0-0] (10)
A2	12	92.5 [28-100] (12)	64 [0-98] (6)	37.5 [0-84] (10)	0 [0-62] (6)	0 [0-64] (9)	0 [0] (1)
A3	3	86 [66-95] (3)	16 [10-21] (3)	14 [10-17] (3)	nt - -	5 [1-5] (3)	0 [0-0] (3)
A4	5	108 [76-127] (5)	57.5 [42-73] (2)	50 [1-65] (5)	20 [0-49] (5)	8 [0-41] (5)	0 [0-0] (2)
A5	13	57 [17-97] (13)	0 [0-27] (11)	0 [0-5] (11)	nt - -	0 [0-0] (13)	0 [0-0] (13)
A6	10	97 [81-107] (10)	87 [50-103] (8)	58 [17-70] (6)	nt - -	53 [0-91] (10)	1 [0-3] (9)
A7	2	109 [103-115] (2)	105 [104-106] (2)	106 [106-106] (2)	- - -	100 [97-103] (2)	0 [0-0] (2)
A8	1	88 [88 - 88] (1)	84 [84 - 84] (1)	81 [81 - 81] (1)	nt - -	35 [35 - 35] (1)	nt - -
A9	3	109 [95-128] (3)	111 [105-130] (3)	80 [74-110] (3)	nt - -	35 [27-48] (3)	0 [0-0] (3)
A10	3	110 [84-115] (3)	39 [4-65] (3)	7 [0-25] (3)	nt - -	2 [0-8] (3)	5 [0-0] (3)
A11-13	9	91 [75-100] (9)	0 [0-53] (9)	0 [0-11] (9)	nt - -	0 [0-2] (9)	0 [0-0] (9)
A14,15	10	85 [51-100] (10)	0 [0-15] (6)	0 [0-6] (10)	0 [0-0] (2)	0 [0-0] (10)	0 [0-0] (10)
A16	2	104 [93-115] (2)	27 [4-50] (2)	7.5 [0-15] (2)	nt - -	0 [0-0] (2)	0 [0-0] (2)
A17	2	129 [124-133] (2)	nt - -	62 [55-69] (2)	32.5 [23-42] (2)	16 [9-23] (2)	0 [0-0] (2)
A18	2	92 [63-121] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)
A20	2	49 [33-121] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)

* see Appendix 9 footnote for explanation of batch code.

Appendix 11. Survival (colony count) data on *B. pertussis* strain 18-323 in convalescent rat LLF at 37 °C. A and B are duplicates; Expt code is explained in the Appendix 4 and footnote.

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
1	$A_2B_7C_1D_1E_1$	4				7							
2	$A_2B_7C_1D_1E_1$	18								0		0	
3	$A_5B_7C_1D_1E_1$	7				0				0		0	
4	$A_5B_7C_1D_1E_1$	38				22				14			
5	$A_5B_7C_1D_1E_1$	225		1		0		0					
6	$A_5B_7C_1D_1E_1$	190		0		0		0					
7	$A_5B_7C_1D_1E_2$	30								0			
8	$A_5B_7C_1D_1E_8$	24		0				0		0			
9	$A_5B_{11}C_1D_1E_2$	21				0		0		0			
10	$A_5B_7C_1D_1E_1$	44				0		0					
11	$A_5B_8C_1D_1E_2$	180	200							0	0		
12	$A_5B_8C_1D_1E_2$	110	130	44	38	0	0			0	0		
13	$A_5B_8C_1D_2E_2$	100	98	52	56	38	34			4	6		
14	$A_1B_4C_2D_2E_8$	32	22	2	0	1	0			0	0	0	0
15	$A_1B_5C_2D_2E_6$	78	66	4	2	1	0			0	0	0	0
16	$A_1B_4C_2D_2E_6$	78	74	22	28	10	16			0	0	0	0
17	$A_1B_5C_2D_2E_6$	68	70	4	6	0	0			0	0	0	0
18	$A_1B_5C_2D_2E_6$	80	84	26	32	26	32			8	12	0	0
19	$A_2B_5C_2D_2E_6$	82	86	80	80	70	80			48	50	1	0
20	$A_3B_5C_2D_2E_6$	82	88	80	78	70	74			66	62	4	2
21	$A_4B_5C_2D_2E_6$	80	86	80	78	70	78			62	50	0	0
22	$A_1B_5C_2D_2E_6$	80	74	36	26	38	26			7	3		
23	$A_2B_5C_2D_2E_6$	72	76	78	74	70	72			48	52		
24	$A_3B_5C_2D_2E_6$	64	60	76	70	60	70			42	48		
25	$A_4B_5C_2D_2E_6$	66	62	68	64	74	70			62	58		
26	$A_9B_5C_2D_2E_6$	100	110	10	8	0	0			0	0	0	0
27	$A_{10}B_5C_2D_2E_6$	104	108	100	98	90	98			38	38	0	0
28	$A_{11}B_5C_2D_2E_6$	110	114	36	38	15	11			0	0	0	0
29	$A_{12}B_5C_2D_2E_6$	94	100	10	14	6	2			0	0	0	0
30	$A_9B_5C_2D_2E_6$	98	104	24	14	4	0			0	0	0	0
31	$A_{10}B_5C_2D_2E_6$	102	96	92	90	92	94			82	84	0	0
32	$A_{11}B_5C_2D_2E_6$	100	92	36	42	12	12			2	0	0	0
33	$A_{12}B_5C_2D_2E_6$	90	84	21	13	8	4			0	0	0	0
34	$A_5B_5C_2D_2E_6$	94	90	88	84	60	62			20	28	0	0
35	$A_6B_5C_2D_2E_6$	92	88	78	70	54	50			4	4	0	0
36	$A_7B_5C_2D_2E_6$	104	92	100	98	80	87			10	8	0	0
37	$A_7B_5C_2D_2E_6$	100	94	96	90	54	48			2	2	0	0
38	$A_5B_5C_2D_2E_6$	92	98	70	78	80	76			20	34	0	0
39	$A_6B_5C_2D_2E_6$	94	96	90	82	60	66			6	2	0	0
40	$A_7B_5C_2D_2E_6$	96	90	80	76	82	78			46	52	1	1
41	$A_8B_5C_2D_2E_6$	88	92	90	86	78	72			18	20	0	0
42	$A_1B_5C_2D_2E_6$	110	118	100	108	70	80			16	14	0	0
43	$A_2B_5C_2D_2E_6$	120	112	110	112	100	118			90	180	0	0

Appendix 11. continued

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
44	$A_3B_5C_2D_2E_6$	106	112	100	108	106	100			80	74	8	4
45	$A_4B_5C_2D_2E_6$	114	100	110	100	100	98			98	92	2	1
46	$A_1B_5C_2D_2E_6$	68	74	42	40	38	36			6	6	0	0
47	$A_2B_5C_2D_2E_6$	72	76	74	74	72	74			70	72	1	1
48	$A_3B_5C_2D_2E_6$	74	78	70	70	70	70			70	72	6	4
49	$A_4B_5C_2D_2E_6$	70	72	70	74	70	70			66	70	0	0
50	$A_{18}B_1C_2D_2E_5$	170	168	70	60	70	60			3	5	0	0
51	$A_{18}B_2C_2D_2E_5$	182	170	56	40	16	14			2	2	0	0
52	$A_{18}B_5C_2D_2E_6$	124	130	50	40	34	40			6	6	0	0
53	$A_{18}B_5C_2D_2E_8$	68								2		0	
54	$A_{18}B_5C_2D_2E_8$	60								0		0	
55	$A_{18}B_5C_2D_2E_8$	110		32		6				0		0	
56	$A_{18}B_5C_2D_2E_8$	42		31						0		0	
57	$A_{18}B_5C_2D_2E_{17}$	60		36		20				0			
58	$A_{18}B_5C_2D_2E_{17}$	62		58		28				0			
59	$A_{18}B_5C_2D_2E_{17}$	22		4		2				0			
60	$A_{18}B_5C_2D_2E_8$	68				22				0			
61	$A_{18}B_5C_2D_2E_8$	88				13				0		0	
62	$A_{18}B_5C_2D_2E_8$	64				0				0		0	
63	$A_{19}B_5C_2D_2E_8$	54		6		0				0		0	
64	$A_{20}B_5C_2D_2E_8$	89		0		0				0		0	
65	$A_{21}B_5C_2D_2E_8$	111		94		83				30		0	
66	$A_{22}B_5C_2D_2E_8$	111		112		94				58		0	
67	$A_{19}B_5C_2D_2E_8$	96		0		0				0		0	
68	$A_{20}B_5C_2D_2E_8$	82		9		3				0		0	
69	$A_{21}B_5C_2D_2E_8$	100		72		56				36		0	
70	$A_{22}B_5C_2D_2E_8$	88		65		76				50		0	
71	$A_{23}B_5C_2D_2E_8$	75		54		16				0		0	
72	$A_{24}B_5C_2D_2E_9$	62		66		57				42		0	
73	$A_{25}B_5C_2D_2E_9$	66		6		0				0		0	
74	$A_{26}B_5C_2D_2E_9$	82		62		40				22		0	
75	$A_{23}B_5C_2D_2E_9$	74		55		15				0		0	
76	$A_{24}B_5C_2D_2E_9$	88		89		57				43		0	
77	$A_{25}B_5C_2D_2E_9$	90		6		0				0		0	
78	$A_{26}B_5C_2D_2E_9$	65		59		35				23		0	
79	$A_{13}B_5C_2D_2E_{14}$	47		27		32		30		27			
80	$A_{14}B_5C_2D_2E_{14}$	49		7		0		0		0			
81	$A_{15}B_5C_2D_2E_{14}$	54		22		4		0		0			
82	$A_{16}B_5C_2D_2E_{14}$	46		0		0		0		0			
83	$A_{17}B_5C_2D_2E_{14}$	58		0		0		0		0			
84	$A_{13}B_5C_2D_1E_{14}$	37		37		42		29		24			
85	$A_{14}B_5C_2D_1E_{14}$	36		0		0		0		0			
86	$A_{15}B_5C_2D_1E_{14}$	30		5		0		0		0			
87	$A_{16}B_5C_2D_1E_{14}$	23		2		0		0		0			

Appendix 11. continued

Expt ordinal no.	Expt code	CFU/20µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
88	$A_{17}B_5C_2D_1E_{14}$	28		0		0		0		0			
89	$A_{15}B_5C_2D_1E_{13}$	62	56			1	0	0	0	0	0		
90	$A_{15}B_5C_2D_1E_{13}$	79	94			60	52	34	28	10	12	0	0
91	$A_{15}B_5C_2D_1E_{13}$	94	102			62	56	56	48	26	30	0	0

Note: Counts in the region of 200 are to be understood as only approximate.
*All treated Sprague Dawley (SD) rats (exposed to the same methode of euthanasia as with untreated rats, see Appendix 6) lungs were washed with 5 ml sterile PBS (pH7.2-7.4), pooled rat LLF fluids kept in ice then centrifuged at 2000 rpm for 15 minutes, after which the pellet 1(mainly macrophages and monocytes) was discarded, and the supernatant was membrane filtered (this step is stopped from batch no 42) distributed in a washed sterile 2-ml volume plastic vials as about a 1ml portions, stored in -20°C for 24 h, then transferred into -70°C for long term storage ready for use when its needed, that was the standard procedure for obtaining LLF, except were stated.

Code A: Different batches of normal rats* from which LLF was collected

Code no.	Rat Batch/ group no.	Sex /(Weight) (M/F)/(SEM)	Treatment			
			Sham Operated	Vaccination	Challenge	Ether
1	15/1	F/ 220± 4	(a)	-	{[a], (28)}	+
2	15/2	F/ 227± 3	(a)	-	{[b], (28)}	+
3	15/3	M/ 325± 7	(a)	-	{[b], (28)}	+
4	15/4	F/ 227± 5	(a)	-	{[b], (28)}	-
5	16/1	M/ 377±6	(a)	+	{[b], (42)}	+
6	16/2	M/ 347 ±10	(a/b)	+	{[b], (42)}	+
7	16/3	M/ 371±4	(a)	-	{[b], (42)}	+
8	16/4	M/ 363±7	(a/b)	-	{[b], (42)}	+
9	17/1	M/ 275	(a)	+	{[c], (23)}	+
10	17/2	M/ 390	(a)	-	{[c], (23)}	+
11	17/3	M/ 275	(a)	+	{[b], (23)}	+

Appendix 11. continued

Code no.	Rat Batch/ group no.	Sex /(Weight) (M/F)/(SEM)	Treatment			
			Sham Operated	Vaccination	Challenge	Ether
12	17/4	M/ 280	(a)	-	{[b], (23)}	+
13	21/1	M/ 184	(a/c)	-	{[d], (28)}	+
14	21/2	M/ 186	(a/c)	-	{[e], (28)}	+
15	21/3	M/ 186	(a/c)	-	{[c], (28)}	+
16	21/4	M/ 186	(a/c)	-	{[f], (28)}	+
17	21/5	M/ 184	(a/c)	-	{[g], (28)}	+
18	34/1	M/ 268±6	(a)	-	{[b], (28)}	+
19	41/1	F/ 171±3	(a)	-	{[nil], (23)}	+
20	41/2	F/ 171±3	(b)	-	{[nil], (23)}	+
21	41/3	F/ 177±2	(a)	-	{[h], (23)}	+
22	41/4	F/ 176±2	(b)	-	{[h], (23)}	+
23	43/1	F/ 227±2	(b)	-	{[h], (21)}	-
24	43/2	F/ 225±5	(b)	-	{[h], (21)}	+
25	43/3	F/ 217±3	(a)	-	{[h], (21)}	-
26	43/4	F/ 219±3	(a)	-	{[h], (21)}	+

*Treatment:1) Sham Operated (SO) of rats consisting of: LGT-agarose (a), Alginate (b) and Carrageenan (c).
Appendix 8. continued.
2) Vaccination: 0.5 ml of DPT per rat was given intraperitoneally 21 days before challenge (infection), + = vaccinated, - = not vaccinated.
3) Challenge: {0.1 ml of 10^8 cfu/ rat of *B. pertussis* L84 IV [a], (day of obtaining serum and LLF after treatment)} 18-323 EH1[b], 18-323 A6 [c], Tohama [d], BPM 1809 HLT $^-$ [e], *B. parapertussis* 10520 [f], *B.pertussis* BP 357 PT $^-$ [g] and 1.3 x 10^8 cfu/ rat18-323 EH2[h]
4) Ether : Some rats exposed to ether inhalation (cotton wool saturated with ether in a small glass beaker which placed close to the rats nose) for 2 min after treatment operation. + = rats exposed to ether, - = rats not exposed to ether.
For the explanation of codes B, C, D and E, see Appendix 4.

Appendix 12. Comparison of batch to batch variation in bactericidal activity of LLF from normal (N), sham-operated (SO), vaccinated (V), and variously challenged (C) rats (treated with different euthanasia method) towards *B. pertussis* 18-323.

Batch* code	Treatment of rat	No. of experiment	Median % Survival [range] (No. of observation) at incubation time (min)					
			0	15	30	60	120	1440
A1	N	11	100 [57-100] (11)	23.5 [0-95] (8)	26 [0-71] (9)	0 [0] (1)	0 [0-20] (10)	0 [0-0] (10)
A1	C	8	97 [66-100] (8)	34 [4-88] (8)	26 [0-63] (8)	nt - -	3 [0-13] (8)	0 [0-0] (7)
A2	C	6	81.5 [38-101] (6)	95 [69-99] (4)	92 [68-100] (5)	nt - -	63 [0-73] (5)	0.5 [0-1] (4)
A3	C	4	85 [71-105] (4)	90 [65-98] (4)	84 [65-89] (4)	nt - -	65.5 [57-79] (4)	5 [4-5] (3)
A4	C	4	86 [66-102] (4)	86.5 [67-98] (4)	87.5 [65-91] (4)	nt - -	72.5 [64-81] (4)	0 [0-1] (3)
A2**	SO	12	92.5 [28-100] (12)	64 [0-98] (6)	37.5 [0-84] (10)	0 [0-62] (6)	0 [0-64] (9)	0 [0] (1)
A5	VC	17	98 [45-100] (17)	34 [0-78] (7)	0 [0-82] (10)	0 [0-0] (5)	3 [0-100] (14)	0 [0-0] (2)
A6	VC	3	83 [83-103] (3)	69 [69-93] (3)	48 [48-70] (3)	nt - -	4 [4-4] (3)	0 [0-0] (3)
A7	C	2	96 [91-101] (2)	88 [85-92] (2)	80 [73-87] (2)	nt - -	30.5 [8-53] (2)	0.5 [0-1] (2)
A8	C	2	94 [90-98] (2)	91 [86-96] (2)	64.5 [47-82] (2)	nt - -	11.5 [2-21] (2)	0 [0-0] (2)
A3	N	3	86 [66-95] (3)	16 [10-21] (3)	14 [10-17] (3)	nt - -	5 [1-5] (3)	0 [0-0] (3)
A9	VC	2	86.5 [86-87] (2)	11.5 [7-16] (2)	1 [0-2] (2)	nt - -	0 [0-0] (2)	0 [0-0] (2)
A10	C	2	86 [85-87] (2)	79.5 [78-81] (2)	78.5 [77-80] (2)	nt - -	51.5 [31-72] (2)	0 [0-0] (2)
A11	VC	2	87.5 [83-92] (2)	32 [30-34] (2)	10.5 [10-11] (2)	nt - -	0.5 [0-1] (2)	0 [0-0] (2)
A12	C	2	77.5 [75-80] (2)	12.5 [10-15] (2)	4 [3-5] (2)	nt - -	0 [0-0] (2)	0 [0-0] (2)

Appendix 12. continued

Batch* code	Treatment of rat	No. of experiment	Median % Survival [range] (No. of observation) at incubation time (min)					
			0	15	30	60	120	1440
A4	N	5	108 [76-127] (5)	57.5 [42-73] (2)	50 [1-65] (5)	20 [0-49] (5)	8 [0-41] (5)	0 [0-0] (5)
A13	C	2	106.5 [90-123] (2)	87.5 [52-123] (2)	101 [62-140] (2)	77 [58-97] (2)	66 [52-80] (2)	nt - -
A14	C	2	107 [94-120] (2)	6.5 [0-13] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)	nt - -
A15	C	5	102 [80-119] (5)	29.5 [17-42] (2)	8 [0-78] (5)	0 [0-54] (5)	0 [0-29] (5)	0 [0-0] (2)
A16	C	2	82.5 [77-88] (2)	3.5 [0-7] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)	nt - -
A17	C	2	102.5 [93-112] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)	nt - -
A6	N	10	97 [81-107] (10)	87 [50-103] (8)	58 [17-70] (6)	nt - -	53 [0-91] (10)	1 [0-3] (9)
A18	C	13	94 [55-100] (13)	35.5 [18-78] (8)	21 [0-38] (10)	nt - -	0 [0-4] (13)	0 [0-0] (11)
A16	N	2	104 [93-115] (2)	27 [4-50] (2)	7.5 [0-15] (2)	nt - -	0 [0-0] (2)	0 [0-0] (2)
A19	SO	2	88.5 [57-120] (2)	3 [0-6] (2)	0 [0-0] (2)	nt - -	0 [0-0] (2)	0 [0-0] (2)
A20	SO	2	98.5 [94-103] (2)	5.5 [0-11] (2)	2 [0-4] (2)	nt - -	0 [0-0] (2)	0 [0-0] (2)
A21	C	2	121 [117-125] (2)	94.5 [90-99] (2)	78.5 [70-87] (2)	nt - -	38.5 [32-45] (2)	0 [0-0] (2)
A22	C	2	113.5 [110-117] (2)	99.5 [81-118] (2)	97 [95-99] (2)	nt - -	62 [61-63] (2)	0 [0-0] (2)
A18	N	2	92 [63-121] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)	0 [0-0] (2)
A23	C	2	130 [125-135] (2)	95 [90-100] (2)	27 [27-27] (2)	nt - -	0 [0-0] (2)	0 [0-0] (2)
A24	C	2	131.5 [103-160] (2)	136 [110-160] (2)	99.5 [95-104] (2)	nt - -	74 [70-78] (2)	0 [0-0] (2)

Appendix 12. continued

Batch* code	Treatment of rat	No. of experiment	Median % Survival [range] (No. of observation) at incubation time (min)					
			0	15	30	60	120	1440
A25	C	2	137 [110-164] (2)	10.5 [10-11] (2)	0 [0-0] (2)	nt - -	0 [0-0] (2)	0 [0-0] (2)
A26	C	2	127 [118-137] (2)	105 [103-107] (2)	65 [64-67] (2)	nt - -	39 [37-42] (2)	0 [0-0] (2)

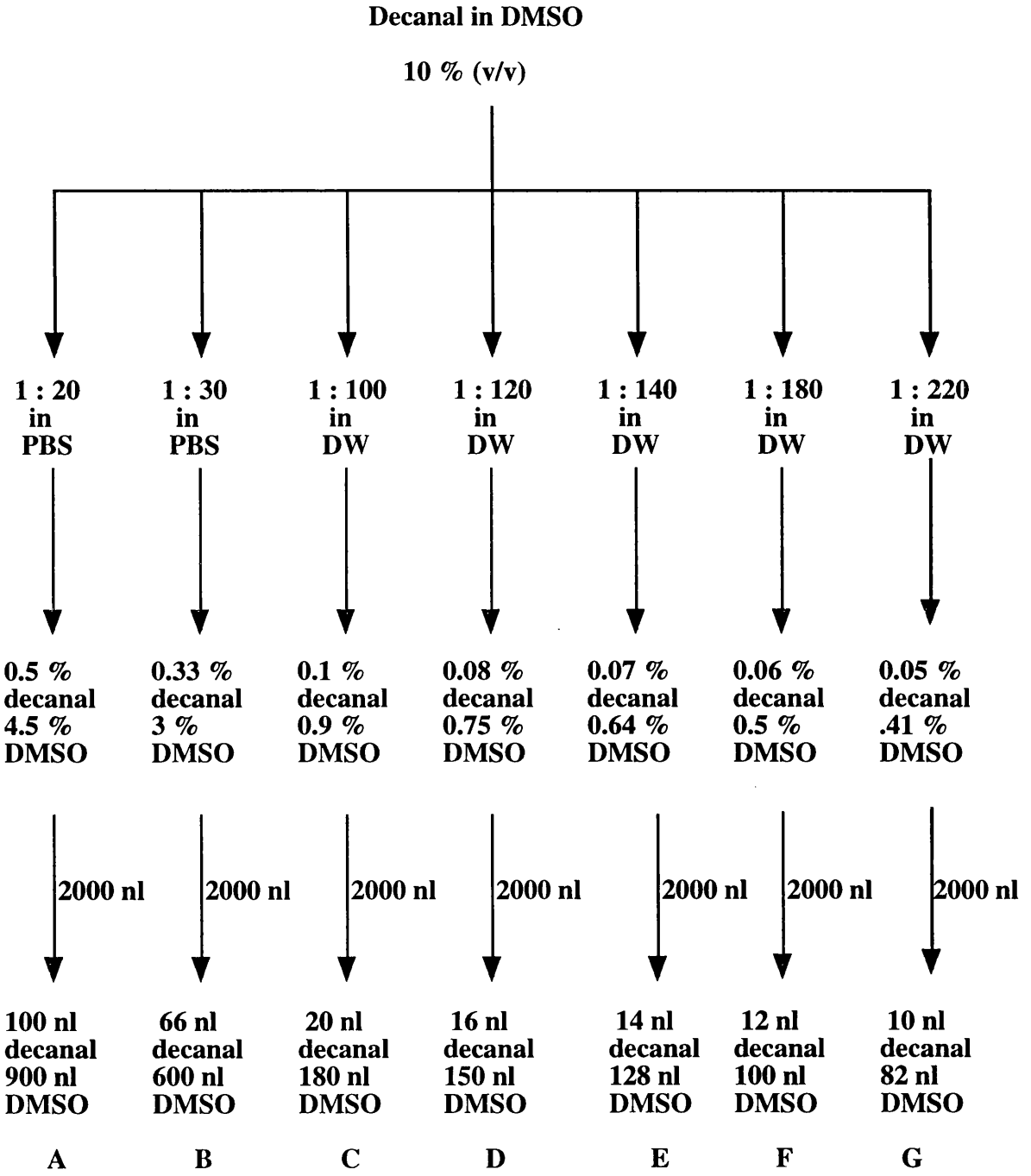
* For batch code, see Appendix 9 footnote for normal rats and Appendix 11 footnote for treated animals.
A2** For this sham-operated rats see Appendix 9 footnote.

Appendix 13. Influence of experimental treatment, and batch to batch variation, of rats on bactericidal activity of LLF obtained about 3-6 weeks later.

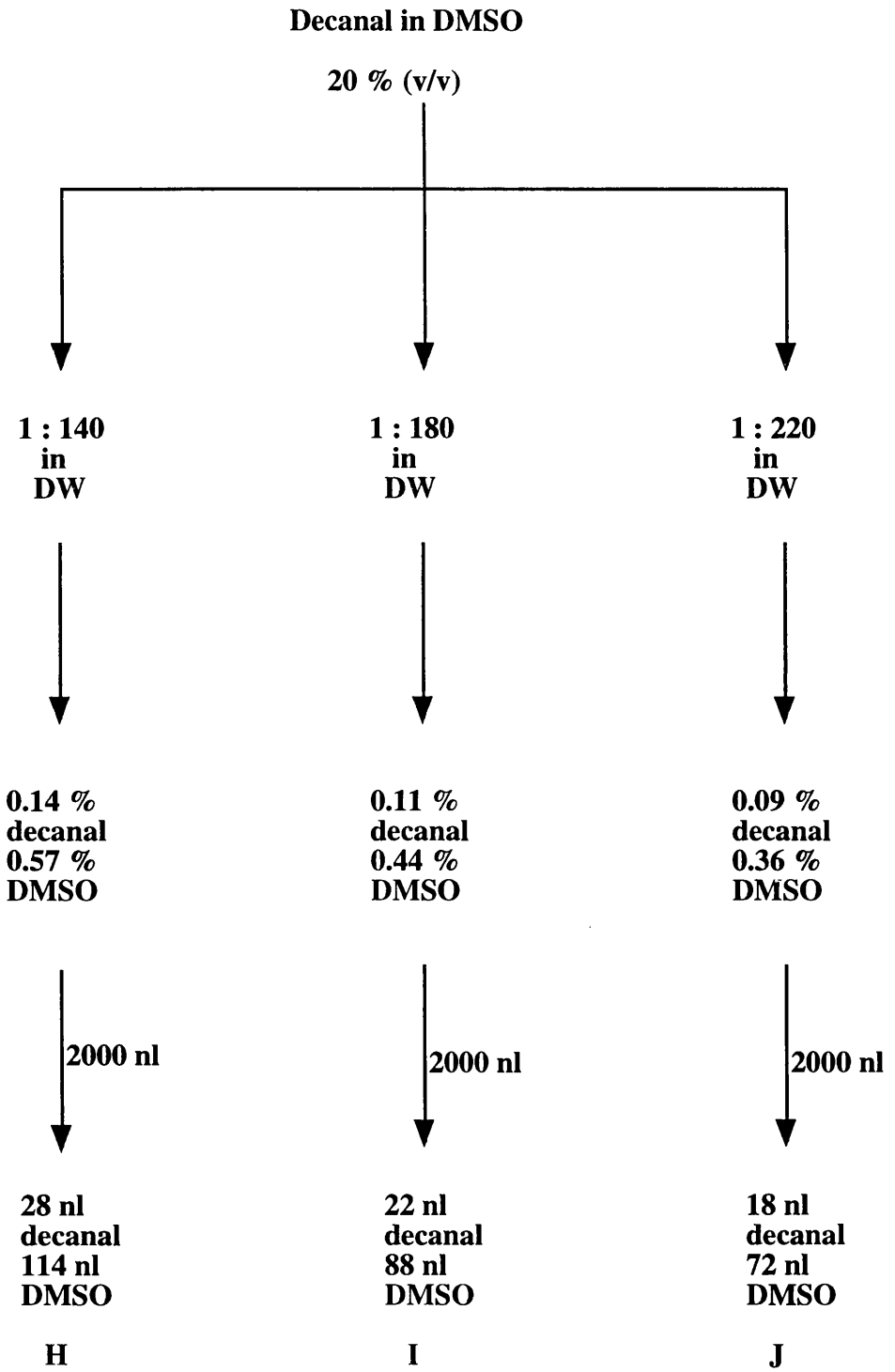
Rat batch No./ [Sex] (N or C)	*B.pertussis* (*Bp*) *B.parapertussis* (*B.pp*)	V	BM	E	C	ME	% survival* after time (min) incubation	
							30	120
15 [F](N)	-	-	-	-	0	A	26	0
15 [F](C)	L84 IV	-	-	+	0.1	A	26	0
15[M](C)	18-323 EH1	-	-	+	7.1	A	92	62
15[F](C)	18-323 EH1	-	-	+	3.7	A	84	66
15[F](C)	18-323 EH1	-	-	-	0.5	A	88	73
16[M](SO)	-	-	ag+c	+	0	A	38	0
16[M](C)	18-323 EH1	+	ag	+	0.1	A	0	3
16[M](C)	18-323 EH1	+	ag+c	+	0	A	48	4
16[M](C)	18-323 EH1	-	ag	+	2.1	A	80	31
16[M](C)	18-323 EH1	-	ag+c	+	1.0	A	65	12
17[M](N)	-	-	-	-	0	A	14	5
17[M](C)	18-323 A6	+	ag	+	0	A	1	0
17[M](C)	18-323 A6	-	ag	+	1.7	A	79	52
17[M](C)	18-323 EH1	+	ag	+	0.1	A	11	1
17[M](C)	18-323 EH1	-	ag	+	0.5	A	4	0
21[M](N)	-	-	-	-	0	A	50	8
21[M](C)	Tohama	-	ag+c	+	1.3	A	101	66
21[M](C)	375 HLT $^-$	-	ag+c	+	0.5	A	0	0
21[M](C)	18-323 A6	-	ag+c	+	1.0	A	8	0
21[M](C)	B.pp (10520)	-	ag+c	+	0.3	A	0	0
21[M](C)	375 PT $^-$	-	ag+c	+	0	A	0	0
34[M](N)	-	-	-	-	**	B	58	53
34[M](C)	18-323 EH1	-	ag	+	0	B	21	0
41[F](N)	-	-	-	-	0	D	8	0
41[F](SO)	-	-	ag	+	0	D	0	0
41[F](SO)	-	-	al	+	0	D	2	0
41[F](C)	18-323 EH2	-	ag	+	2.8	D	79	39
41[F](C)	18-323 EH2	-	al	+	11.4	D	97	62
43[F](N)	-	-	-	-	0	D	0	0
43[F](C)	18-323 EH2	-	al	-	0.9	D	27	0
43[F](C)	18-323 EH2	-	al	+	0.4	D	100	74
43[F](C)	18-323 EH2	-	ag	-	3.4	D	0	0
43[F](C)	18-323 EH2	-	ag	+	3.9	D	65	39

Col 1: M=male; F = female; N = normal; C = challenged; SO = sham-operated
Col 2:Substrain and transposon of *B. pertussis* and *B. parapertussis* (Bpp).
Col 3: Vaccination status (V); Column 4: Bead material (BM), ag=agarose,al=alginate, ag+c= agarose+carrageenan; Column 5: Ether (E); Column 6: Coughing (C)/ per rat after 7-14 days after infection; Column 7: Method of euthanasia (ME).*% survival calculations were based on PBS time-zero count; **There was a number of coughing on day 8 and 11 post-infection, but not counted.

Appendix 14. Seven different dilution schemes of 10 % (v/v) decanal in dimethyl sulphoxide (DMSO).



Appendix 15. Three different dilution schemes of 20 % (v/v) decanal in dimethyl sulphoxide (DMSO).



Appendix 16. Effect of frequent addition of decanal (at different volumes) on the luminescence of *B. pertussis* lux after incubation in PBS, CL medium and CAA at 37°C for various times.

Test fluid & Method*	Final volume in test mixture (nl) **Decanal** *DMSO*	Mean (± SEM) luminescence in mv (No. of observations) of *B.pertussis* lux after exposure at 37°C for (min)				
		0	15	30	45	60
PBS	**100**	190±0.0	nt	100±11.5	19.3±0.7	9.3±0.67
A	*900*	(3)	-	(3)	(3)	(3)
CL	**100**	240±35.1	nt	453.3±21.9	476.7±14.5	486.3±33.7
A	*900*	(3)	-	(3)	(3)	(3)
PBS	**66**	206±4.0	nt	126.7±3.3	73.3±6.7	30±5.8
B	*600*	(3)	-	(3)	(3)	(3)
CL	**66**	230±20	nt	465±55	500±20	535±5
B	*600*	(2)	-	(2)	(2)	(2)
PBS	**20**	520±10	490±50	430±50	400±0.0	nt
C	*180*	(2)	(2)	(2)	(2)	-
CL	**20**	245±5.0	340±10	425±5.0	430+10	nt
C	*180*	(2)	(2)	(2)	(2)	-
CAA	**20**	275	210	150	150	140
C	*180*	(1)	(1)	(1)	(1)	(1)
PBS	**17**	420	340	260	240	260
D	*150*	(1)	(1)	(1)	(1)	(1)
CL	**17**	80	120	90	120	120
D	*150*	(1)	(1)	(1)	(1)	(1)
CAA	**17**	200	200	150	150	140
D	*150*	(1)	(1)	(1)	(1)	(1)
PBS	**14**	370	460	360	420	410
E	*128*	(1)	(1)	(1)	(1)	(1)
CL	**14**	80	125	120	160	165
E	*128*	(1)	(1)	(1)	(1)	(1)
CAA	**14**	210	230	165	165	150
E	*128*	(1)	(1)	(1)	(1)	(1)
PBS	**11**	164	178	140	132	110
F	*100*	(1)	(1)	(1)	(1)	(1)

Appendix 16. continued

Test fluid & Method*	Final volume in test mixture (nl) **Decanal** *DMSO*	Mean (± SEM) luminescence in mv (No. of observations) of *B.pertussis* lux after exposure at 37 °C for (min)				
		0	15	30	45	60
CL	**11**	38	54	64	75	82
F	*100*	(1)	(1)	(1)	(1)	(1)
CAA	**11**	112	104	70	78	110
F	*100*	(1)	(1)	(1)	(1)	(1)
PBS	**9**	200	190	180	200	200
G	*82*	(1)	(1)	(1)	(1)	(1)
CL	**9**	45±5.0	67.5±2.5	82±4.0	87.5±2.5	99±11
G	*82*	(2)	(2)	(2)	(2)	(2)
CAA	**9**	130	140	118	110	110
G	*82*	(1)	(1)	(1)	(1)	(1)
PBS	**29**	175	170	150	130	170
H	*114*	(1)	(1)	(1)	(1)	(1)
CL	**29**	51.5±3.5	69.5±5.5	84±6.0	91±7.0	96±4.0
H	*114*	(2)	(2)	(2)	(2)	(2)
CAA	**29**	135	98	78	60	65
H	*114*	(1)	(1)	(1)	(1)	(1)
PBS	**22**	178	120	165	145	145
I	*88*	(1)	(1)	(1)	(1)	(1)
CL	**22**	45±5.0	47.5±0.5	58±2.0	68±0.0	79±5.0
I	*88*	(2)	(2)	(2)	(2)	(2)
CAA	**22**	120	78	76	75	70
I	*88*	(1)	(1)	(1)	(1)	(1)
PBS	**18**	370	420	350	360	360
J	*72*	(1)	(1)	(1)	(1)	(1)
CL	**18**	80±0.0	140±0.0	175±25	205±15	245±15
J	*72*	(2)	(2)	(2)	(2)	(2)
CAA	**18**	200	170	155	140	130
J	*72*	(1)	(1)	(1)	(1)	(1)

* methods of decanal preparation A, B, C, D, E, F, and G presented in Appendix 14, and for methods of decanal preparation H, I and J see Appendix 15.

Appendix 17. Comparison of batch to batch variation of LLF from normal (N) and convalescent (C) rats (treated with different euthanasia methods) in their effect on luminescence of *B. pertussis* lux.

Experiment particulars	Mean % Luminescence [range] (No. of observations) after 1h incubation time at different dilution / µl						Inactivation end point **90 %** *50 %* µl
	1/1 400	1/2 200	1/4 100	1/8 50	1/16 25	1/64 12.5	
15 N	5	nt	9.5	nt	46	104	**100**
A (2)	[3 - 7]	-	[8 - 11]	-	[35 - 37]	[104]	*25*
	(2)	-	(2)	-	(2)	(1)	
17 N	5	nt	33	nt	49	nt	**295**
A (1)	[5]	-	[33]	-	[49]	-	*30*
	(1)	-	(1)	-	(1)	-	
33 N	5	nt	10	nt	44	133	**100**
A (2)	[3 - 7]	-	[6 - 15]	-	[27 - 61]	[133]	*25*
	(2)	-	(2)	-	(2)	(1)	
34 N	108	nt	94.5	nt	129	nt	**> 400**
B (2)	[30 - 186]	-	[54 - 135]	-	[119 - 139]	-	*> 400*
	(2)	-	(2)	-	(2)	-	
35 N	169.5	nt	173	nt	158	nt	**> 400**
B (2)	[117 - 222]	-	[113 - 233]	-	[100 - 217]	-	*> 400*
	(2)	-	(2)	-	(2)	-	
37 N	108	nt	106	nt	110	nt	**> 400**
B (1)	[108]	-	[106]	-	[110]	-	*> 400*
	(1)	-	(1)	-	(1)	-	
38.1 N	108.5	nt	150	nt	141	nt	**> 400**
B (2)	[56 - 161]	-	[108 - 193]	-	[90 - 193]	-	*> 400*
(2)	-	(2)	-	(2)	-		
38.2 N	9.5	nt	37	nt	95	150	**385**
C (2)	[8 - 11]	-	[30 - 44]	-	[81 - 109]	[150]	*75*
	(2)	-	(2)	-	(2)	(1)	
39 N	5	nt	7.5	nt	25	69	**80**
D (2)	[5 - 5]	-	[7 - 8]	-	[17 - 33]	[69]	*10*
	(2)	-	(2)	-	(2)	(1)	
40 N	5	nt	11	nt	35	26	**120**
D (2)	[5 - 5]	-	[7 - 15]	-	[21 - 49]	[26]	*20*
	(2)	-	(2)	-	(2)	(1)	
41 N	23	nt	45	nt	129	105	**> 400**
D (2)	[12 - 34]	-	[24 - 66]	-	[92 - 167]	[105]	*90*
	(2)	-	(2)	-	(2)	(1)	
42 N	159	nt	112.5	nt	110.5	nt	**> 400**
D (2)	[157 - 161]	-	[88 - 137]	-	[71 - 150]	-	*> 400*
	(1)	-	(1)	-	(1)	-	
43 N	5	nt	7	nt	33	80	**80**
D (2)	[5 - 5]	-	[5 - 10]	-	[17 - 49]	[80]	*20*
(2)	-	(2)	-	(2)	(1)		
43/3 C	40	nt	84.5	nt	98	113	**> 400**
D (2)	[26 - 54]	-	[67 - 102]	-	[81 - 115]	[113]	*320*
	(2)	-	(2)	-	(2)	(1)	
43/4 C	157	nt	144	nt	125	nt	**> 400**
D (2)	[140 -174]	-	[140 - 149]	-	[121 - 130]	-	*> 400*
	(2)	-	(2)	-	(2)	-	

* Rat batch no; A, B, C, and D = methods of euthanasia (see Footnote of Appendix 9) ; ( ) = no of experiments

Appendix 18. Survival (colony count) data on *B. pertussis* 18-323 in normal rat LLF fraction (surfactant) at 37°C. A and B are duplicates; Expt code is explained in the Appendix 4 and footnote.

Expt ordinal no.	Expt code	CFU/ 20 µl after exposure at 37°C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
1	$A_{14}B_5C_2D_2E_8$	70		2		0				0		0	
2	$A_{15}B_5C_2D_2E_8$	74		19		0				0		0	
3	$A_{14}B_5C_2D_2E_8$	110		0		0				0		0	
4	$A_{15}B_5C_2D_2E_8$	98		11		0				0		0	
5	$A_{14}B_5C_2D_2E_8$	42		0		0				0		0	
6	$A_{15}B_5C_2D_2E_8$	30		0		0				0		0	
7	$A_{16}B_5C_2D_2E_8$	8		0		0				0		0	
8	$A_{16}B_5C_2D_2E_8$	28		0		0				0		0	
9	$A_{14}B_5C_2D_2E_8$	46				0		0		0		0	
10	$A_{14}B_5C_2D_2E_8$	56	56			0	0	0	0	0	0	0	0
11	$A_{18}B_5C_2D_2E_8$	22	33			0	0	0	0	0	0	0	
12	$A_{18}B_5C_2D_2E_8$	80				0		0		0		0	
13	$A_{17}B_5C_2D_2E_9$	100				22		7		0		0	
14	$A_{18}B_5C_2D_2E_9$	49				0		0		0		0	
15	$A_{17}B_5C_2D_2E_9$	72				20		2		0		0	
16	$A_{18}B_5C_2D_2E_9$	30				0		0		0		0	
17	$A_{20}B_5C_2D_1E_{13}$	98	100			0	0	0	0	0	0		
18	$A_{20}B_5C_2D_1E_{13}$	36	32			0	0	0	0	0		0	0
19	$A_{20}B_5C_2D_1E_{13}$	42	24			0	0	0	0	0	0	0	0
20	$A_{20}B_5C_2D_1E_{13}$	22	16			0	0	0	0	0	0		
21	$A_{21}B_5C_2D_1E_{13}$	68	82			0	0	0	0	0	0		
22	$A_{22}B_5C_2D_1E_{15}$							0	0				
23	$A_{22}B_5C_2D_1E_{15}$	26	24			0	0	0	0	0	0		
24	$A_{22}B_5C_2D_1E_{15}$							0	0				
25	$A_{22}B_5C_2D_1E_{15}$							0	0				
26	$A_{22}B_5C_2D_1E_{15}$							0	0				
27	$A_{22}B_5C_2D_1E_{15}$							0	0				
28	$A_{22}B_5C_2D_1E_{15}$							0	0				
29	$A_{22}B_5C_2D_1E_{15}$							0	0				
30	$A_{22}B_5C_2D_1E_{15}$							0	0				
31	$A_{22}B_5C_2D_1E_{14}$							0	0				
32	$A_{22}B_5C_2D_1E_{14}$							0	0				
33	$A_{22}B_5C_2D_1E_{14}$		35	31		0	0	0	0		0	0	
34	$A_{21}B_5C_2D_1E_{14}$							0	0				
35	$A_{21}B_5C_2D_1E_{14}$							0	0				
36	$A_{22}B_5C_2D_1E_{14}$							0	0				
37	$A_{23}B_5C_2D_1E_{14}$		66	64		0	0	0	0		0	0	

Note: All rats anaestheized with Halothane about 3-5 minutes, then killed by heart puncture (during taking of blood) and cervical dislocation, afterwhich LLF was collected and fractionated (ultracentrifugation 29000 rpm) in the same day into supernate and surfactant. For the explanation of code A see Appendix 9 footnote, and for codes B, C, D and E, see Appendix 4.

Appendix 19 . Batch to batch variation in bactericidal activity of normal rat LLF fraction (surfactant) towards *B. pertussis* 18-323.

Batch* code	No. of experiments	Median % survival [Range](No. of observations) at incubation time (min)					
		0	15	30	60	120	1440
A14	8	91	3.5	0	nt	0	0
		[60-112]	[0-29]	[0-0]	-	[0-0]	[0-0]
		(8)	(6)	(6)	-	(6)	(6)
A16	2	21.5	0	0	nt	0	0
		[8-35]	[0-0]	[0-0]	-	[0-0]	[0-0]
		(2)	(2)	(2)	-	(2)	(2)
A17	2	149	nt	36.5	8	0	0
		[131-167]	-	[36-37]	[4-12]	[0.0]	[0.0]
		(2)	-	(2)	(2)	(2)	(2)
A18	3	55	nt	0	0	0	0
		[39-83]	-	[0.0]	[0.0]	[0.0]	[0.0]
		(3)	-	(3)	(3)	(3)	(3)
A20	4	78.5	nt	0	0	0	0
		[13-95]	-	[0.0]	[0.0]	[0.0]	[0.0]
		(4)	-	(3)	(4)	(4)	(2)
A21	2	57	nt	0	0	0	nt
		[57]	-	[0]	[0.0]	[0]	-
		(1)	-	(1)	(2)	(1)	-
A22	12	38	nt	0	0	0	nt
		[31-45]	-	[0.0]	[0.0]	[0.0]	-
		(2)	-	(2)	(12)	(2)	-
A23	1	100	nt	0	0	0	-
		[100]	-	[0]	[0]	[0]	-
		(1)	-	(1)	(1)	(1)	-

* See Appendix 9 footnote for explanation of batch code.

Appendix 20. Survival (colony count) data on *B. pertussis* 18-323 in sham-operated and challenged (C) rats LLF fraction (surfactant) at 37 °C. Expt Code is explained in the footnote.

Expt ordinal no.	Expt code	CFU/ 20 µl after exposure at 37°C for time (min)					
		0	15	30	60	120	1440
1	$A_{19}B_5C_2D_2E_8$	28	0	0		0	0
2	$A_{20}B_5C_2D_2E_8$	4	0	0		0	0
3	$A_{21}B_5C_2D_2E_8$	82	99	84		4	0
4	$A_{22}B_5C_2D_2E_8$	92	98	74		9	0
5	$A_{19}B_5C_2D_2E_8$	43	0	0		0	0
6	$A_{20}B_5C_2D_2E_8$	41	0	0		0	0
7	$A_{21}B_5C_2D_2E_8$	101	70	58		1	0
8	$A_{22}B_5C_2D_2E_8$	99	73	68		17	0
9	$A_{23}B_5C_2D_2E_9$	66		22	22	0	0
10	$A_{24}B_5C_2D_2E_9$	74		66	22	0	0
11	$A_{25}B_5C_2D_2E_9$	70		3	0	0	0
12	$A_{26}B_5C_2D_2E_9$	70		46	54	18	0
13	$A_{23}B_5C_2D_2E_9$	77		32	23	0	0
14	$A_{24}B_5C_2D_2E_9$	86		64	51	40	0
15	$A_{25}B_5C_2D_2E_9$	57		0	0	0	0
16	$A_{26}B_5C_2D_2E_9$	78		49	44	19	0

Note: All rats anaestheized with halothane for about 3-5 minutes, then killed by heart puncture (during taking of blood) and cervical dislocation, after which LLF was collected and fractionated (Ultracentrifugation 55,000 g) in the same day into supernate and surfactant.

For the explanation of code A see Appendix 11 footnote, and for codes B, C, D and E, see Appendix 4.

Appendix 21. Comparison of batch to batch variation in bactericidal activity of normal (N), sham-operated (SO), and various challenges (C) rats (treated with euthanasia method D) LLF fraction (surfactant) towards *B. pertussis* 18-323.

Batch* code	Treatment of rat	No. of experiment	Median % Survival [range] (No. of observation) at incubation time (min)					
			0	15	30	60	120	1440
A16	N	2	21.5	0	0	nt	0	0
			[8-35]	[0-0]	[0-0]	-	[0-0]	[0-0]
			(2)	(2)	(2)	-	(2)	(2)
A19	SO	2	41.5	0	0	nt	0	0
			[29-54]	[0-0]	[0-0]	-	[0-0]	[0-0]
			(2)	(2)	(2)	-	(2)	(2)
A20	SO	2	27.5	0	0	nt	0	0
			[4-51]	[0-0]	[0-0]	-	[0-0]	[0-0]
			(2)	(2)	(2)	-	(2)	(2)
A21	C	2	106	96	80.5	nt	2.5	0
			[86-126]	[88-104]	[73-88]	-	[1-4]	[0-0]
			(2)	(2)	(2)	-	(2)	(2)
A22	C	2	110.5	97.0	81.5	nt	15.0	0
			[97-124]	[91-103]	[78-85]	-	[9-21]	[0-0]
			(2)	(2)	(2)	-	(2)	(2)
A18	N	3	55	nt	0	0	0	0
			[39-82]	-	[0-0]	[0-0]	[0-0]	[0-0]
			(3)	-	(3)	(3)	(3)	(3)
A23	C	2	125	nt	47.5	39.5	0	0
			[[110-140]	-	[37-58]	[37-42]	[0-0]	[0-0]
			(2)	-	(2)	(2)	(2)	(2)
A24	C	2	139.5	nt	113	83	36.5	0
			[123-156]	-	[110-116]	[73-93]	[0-73]	[0-0]
			(2)	-	(2)	(2)	(2)	(2)
A25	C	2	110.5	nt	2.5	0	0	0
			[104-117]	-	[0-5]	[0-0]	[0-0]	[0-0]
			(2)	-	(2)	(2)	(2)	(2)
A26	C	2	129.5	nt	83	85	32	0
			[117-142]	-	[77-89]	[80-90]	[30-35]	[0-0]
			(2)	-	(2)	(2)	(2)	(2)

See Appendix 11 footnote for explanation of batch code.

Appendix 22. Survival (colony count) data on *B. pertussis* in normal rat LLF fraction (supernate) at 37 °C. A and B are duplicates. Expt code is explained in the Appendix 4 and footnote.

Expt ordinal no.	Expt code	CFU/ 20 µl after exposure at 37 °C for time (min)											
		0		15		30		60		120		1440	
		A	B	A	B	A	B	A	B	A	B	A	B
1	$A_{14}B_5C_2D_2E_8$	84		44		29				0		0	
2	$A_{15}B_5C_2D_2E_8$	63		2		0				0		0	
3	$A_{14}B_5C_2D_2E_8$	112		60		20				0		0	
4	$A_{15}B_5C_2D_2E_8$	88		5		0				0		0	
5	$A_{14}B_5C_2D_2E_8$	60		2		0				0		0	
6	$A_{15}B_5C_2D_2E_8$	26		0		0				0		0	
7	$A_{16}B_5C_2D_2E_8$	84		66		70				0		0	
8	$A_{16}B_5C_2D_2E_8$	106		91		38				0		0	
9	$A_{14}B_5C_2D_2E_8$	60				22		18		2		0	
10	$A_{14}B_5C_2D_2E_8$	62	72			30	28	18	18	0	0	0	0
11	$A_{18}B_5C_2D_2E_8$	60	72			11	5	3	2	0	0	0	0
12	$A_{18}B_5C_2D_2E_8$	96				36		30		8		0	
13	$A_{17}B_5C_2D_2E_9$	70				37		24		3		0	
14	$A_{18}B_5C_2D_2E_9$	72				26		13		0		0	
15	$A_{17}B_5C_2D_2E_9$	88				50		29		10		0	
16	$A_{18}B_5C_2D_2E_9$	62				5		8		4		0	
17	$A_{20}B_5C_2D_1E_{13}$	100	116			2	4	0	0	0	0		
18	$A_{20}B_5C_2D_1E_{13}$	38	32					0	0	0	0	0	0
19	$A_{20}B_5C_2D_1E_{13}$	42	44			0	0	0	0	0	0	0	0
20	$A_{20}B_5C_2D_1E_{13}$	110	120			50	30	16	10	4	4		
21	$A_{21}B_5C_2D_1E_{13}$	88	94			90	110	94	120	112	118		
22	$A_{22}B_5C_2D_1E_{15}$							26	24				
23	$A_{22}B_5C_2D_1E_{15}$	106	80			52	62	44	34	30	32		
24	$A_{22}B_5C_2D_1E_{15}$							16	14				
25	$A_{22}B_5C_2D_1E_{15}$							0	0				
26	$A_{22}B_5C_2D_1E_{15}$							0	0				
27	$A_{22}B_5C_2D_1E_{15}$							0	0				
28	$A_{22}B_5C_2D_1E_{15}$							7	5				
29	$A_{22}B_5C_2D_1E_{15}$							1	1				
30	$A_{22}B_5C_2D_1E_{14}$							4	4				
31	$A_{22}B_5C_2D_1E_{14}$							10	16				
32	$A_{23}B_5C_2D_1E_{14}$	158	162			122	138	138	114	98	104		

Note: All rats anaestheized with halothane for about 3-5 minutes , then killed by heart puncture (during taking of blood) and cervical dislocation, after which LLF was collected and fractionated (ultracentrifugation 55,000 g) in the same day into supernate and surfactant.

For the explanation of code A see Appendix 9 footnote, and for codes B, C, D and E, see Appendix 4.

Appendix 23. Batch to batch variation in bactericidal activity of normal rat LLF fraction (supernate) towards *B. pertussis* 18-323.

Batch* code	No. of experiments	Median % survival [Range](No. of observations) at incubation time (min)					
		0	15	30	60	120	1440
A14	6	103.5 [52-127] (6)	26.5 [0-67] (6)	2 [0-44] (6)	nt - -	0 [0-0] (6)	0 [0-0] (6)
A16	2	110.5 [88-133] (2)	91.5 [69-114] (2)	61 [48-74] (2)	nt - -	0 [0-0] (2)	0 [0-0] (2)
A17	2	138.5 [117-160] (2)	76.5 nt -	46.5 [62-91] (2)	nt [40-53] (2)	11.5 [5-18] (2)	0 [0-0] (2)
A18	4	116.5 [92-126] (4)	nt - -	27 [9-47] (4)	18.5 [4-39] (4)	3.5 [0-11] (4)	0 [0-0] (4)
A20	4	89.8 [82-98] (4)	nt - -	10.3 [0-28] (3)	2.3 [0-9] (4)	0.8 [0-3] (4)	0 [0-0] (2)
A21	1	69 [69] (1)	nt - -	76 [76] (1)	81 [81] (1)	87 [87] (1)	nt - -
A22	10	115 [115] (1)	nt - -	70 [70] (1)	9.5 [0-48] (10)	38 [38] (1)	nt - -
A23	1	100 [100] (1)	nt - -	81 [81] (1)	79 [79] (1)	63 [63] (1)	nt - -

For explanation of batch code see Appendix 9 footnote.

Appendix 24. Survival (colony count) data on *B. pertussis* 18-323 in sham-operated and challenged, and challenged rats LLF fraction (supernate) at 37°C. Expt code is explained in the Appendix 4 and footnote.

Expt ordinal no.	Expt code	CFU/ 20 µl after exposure at 37°C for time (min)					
		0	15	30	60	120	1440
1	$A_{19}B_5C_2D_2E_8$	90	72	52		3	0
2	$A_{20}B_5C_2D_2E_8$	78	64	42		1	0
3	$A_{21}B_5C_2D_2E_8$	114	110	92		62	0
4	$A_{22}B_5C_2D_2E_8$	97	108	94		64	0
5	$A_{19}B_5C_2D_2E_8$	114	97	62		29	0
6	$A_{20}B_5C_2D_2E_8$	84	85	52		18	0
7	$A_{21}B_5C_2D_2E_8$	124	86	72		42	0
8	$A_{22}B_5C_2D_2E_8$	116	89	70		44	0
9	$A_{23}B_5C_2D_2E_9$	85		57	32	7	0
10	$A_{24}B_5C_2D_2E_9$	78		63	52	38	1
11	$A_{25}B_5C_2D_2E_9$	82		28	28	13	0
12	$A_{26}B_5C_2D_2E_9$	80		64	67	40	0
13	$A_{23}B_5C_2D_2E_9$	77		50	35	9	0
14	$A_{24}B_5C_2D_2E_9$	85		64	77	53	3
15	$A_{25}B_5C_2D_2E_9$	73		36	25	3	0
16	$A_{26}B_5C_2D_2E_9$	80		68	60	59	0

Note: All rats anaestheized with halothane for 3-5 minutes, then killed by heart puncture (during taking of blood) and cervical dislocation, after which LLF was collected and fractionated (ultracentrifugation 55000 g) in the same day into supernate and surfactant.
For the explanation of code A see Appendix 11 footnote, and for codes B, C, D and E, see Appendix 4.

Appendix 25. Comparison of batch to batch variation in bactericidal activity of normal (N), sham-operated (SO), and variously challenged (C) rats (treated with euthanasia method D) LLF fraction (supernate) towards *B. pertussis* 18-323. Data of two observations.

Batch* code	Treatment of rat	Median % Survival [range] at incubation time (min)					
		0	15	30	60	120	1440
A16	N	110.5 [88-133]	91.5 [69-114]	61 [48-74]	nt -	0 [0-0]	0 [0-0]
A19	SO	119 [95-143]	98.5 [76-121]	66.5 [55-78]	nt -	19.5 [3-36]	0 [0-0]
A20	SO	93.5 [82-105]	86.5 [67-106]	54.5 [44-65]	nt -	12 [1-23]	0 [0-0]
A21	C	137.5 [120-155]	112 [108-116]	93.5 [90-97]	nt -	59 [53-65]	0 [0-0]
A22	C	123.5 [102-145]	112.5 [111-114]	93.5 [88-99]	nt -	61 [55-67]	0 [0.0]
A18**	N	116.5 [92-126]	nt -	27 [9-47]	18.5 [4-39]	3.5 [0-11]	0 [0-0]
A23	CI	141 [140-142]	nt -	93 [91-95]	58.5 [53-64]	14 [12-16]	0 [0-0]
A24	CI	142.5 [130-155]	nt -	110.5 [105-116]	113.5 [87-140]	79.5 [63-96]	3.5 [2-5]
A25	CI	135 [133-137]	nt -	56 [47-65]	46 [45-47]	13.5 [5-22]	0 [0-0]
A26	CI	112.5 [80-145]	nt -	115.5 [107-124]	110.5 [109-112]	87 [67-107]	0 [0-0]

*See Appendix 9 footnote for normal rat LLF and Appendix 11 footnote for LLF from treated animals; **Data of four observations.

Appendix 26. Protein concentration of membrane (0.45 μm pore size) filtered (F) and unfiltered (UF) LLF, supernate and surfactant collected from normal (N), sham-operated (SO) and convalescent rats by different method of euthanasia. Data are the mean of triplicates in the same experiment.

LLF batch no.	Treatment of rats	Method of euthanasia	Protein μg/ml (±SEM) in			
			Unfractionated LLF	Supernate	Surfactant	
			F	F	F	UF
15	N	A	125±5	nt	nt	nt
	C1	A	250±0	-	-	-
	C2	A	308.±4	-	-	-
	C3	A	250±0	-	-	-
	C4	A	352±2	-	-	-
16	SO	A	322±4	-	-	-
	C1	A	323±2	-	-	-
	C2	A	288±2	-	-	-
	C3	A	315±5	-	-	-
	C4	A	315±5	-	-	-
17	N	A	477±7	-	-	-
	C1	A	408±6	-	-	-
	C2	A	527±7	-	-	-
	C3	A	527±7	-	-	-
	C4	A	527±7	-	-	-
33	N	A	338±4	-	-	-
34	N	B	320±3	-	-	-
	C	B	752±6	-	-	-
35	N	B	613±6	nt	nt	nt
37	N	B	635±10	540±9	141±1	-
38	N	B	355±3	nt	nt	-
	N	C	140±3	-	-	-
39S	N	D	215±3	-	-	-
39M	N	D	263±2	-	-	-
39L	N	D	395±6	-	-	-
40L	N	D	338±2	223±13	29±0	-
40xL	N	D	390±3	275±3	19±1	-
41	N	D	480±6	407±4	10±2	24±2
	SO1	D	460±3	345±3	11±2	29±2
	SO2	D	450±3	352±2	9±2	24±1
	C1	D	755±10	670±6	14±1	29±2
	C2	D	577±9	268±2	13±2	24±1
42	N	D	400±3	392±2	nt	36±1
43	N	D	529±6*	nt	-	nt
44	N	D	nt	483±2	-	146±0
45	N	D	445±2	407±3	-	52±1
50	N	D	nt	353±4	-	59±0
55	N	D	-	345±8	-	58±0
Median			390	353	14	33
yield (%)			0	91	4	8

* Unfiltered fluids gave 550±0 μg/ml

Appendix 27. Lysozyme concentration of filtered (F) and unfiltered (UF) LLF, supernate and surfactant collected from normal (N), sham-operated (SO) and convalescent (C) rats by different method of euthanasia. Data are the mean of triplicates in the same experiment.

LLF batch no.	Treatment of rats	Method of euthanasia	LZ µg/ml (±SEM) in			
			Unfractionated LLF	Supernate	Surfactant	
			F	F	F	UF
15	N	A	16±1	nt	nt	nt
	C1	A	53±2	-	-	-
	C2	A	54±0	-	-	-
	C3	A	70±3	-	-	-
	C4	A	64±1	-	-	-
16	C1	A	57±1	-	-	-
	C2	A	74±1	-	-	-
	C3	A	73±3	-	-	-
	C4	A	54±3	-	-	-
17	N	A	76±3	-	-	-
	C1	A	72±1	-	-	-
	C2	A	90±2	-	-	-
	C3	A	77±1	-	-	-
	C4	A	80±1	-	-	-
33	N	A	93±1	-	-	-
34	N	B	84±3	-	-	-
	C	B	>108±0	-	-	-
35	N	B	92±1	-	-	-
37	N	B	47±1	45±1	< 5±0	nt
38	N	B	44±1	nt	nt	-
38	N	C	49±0	-	-	-
39S	N	D	65±2	-	-	-
39M	N	D	95±3	-	-	-
39L	N	D	104±1	-	-	-
40	N	D	99±1	111±5	< 5±0	-
41	N	D	88±1	99±1	< 5±0	-
41	SO1	D	101±4	108±5	nt	-
41	SO2	D	101±5	97±5	-	-
41	C1	D	107±1	> 108	< 5±0	-
41	C2	D	> 108±0	103±5	nt	-
42	N	D	> 108±0	nt	-	36±1
43	N	D	> 108±0	-	-	nt
44	N	D	nt	106±1	-	12±0
45	N	D	105±1	107±7	-	< 5±0
50	N	D	nt	110±2	-	< 5±0
55	N	D	-	96±2	-	< 5±0
Median			80	107	< 5	< 5

Appendix 28. Effect of various treatments of rat LLF on their bactericidal activity towards *B. pertussis* 18-323. Data are the mean of 2 observations.

Treatment of LLF/ LLF batch no.	Mean % survival* [Range] at incubation time (min)				
	0	15	30	60	120
nil	28.50	0	0	nt	0
33	[22 - 35]	[0 - 0]	[0 - 0]	-	[0 - 0]
Dialysis (retentate)	81.50	12	0	nt	0
33	[80 - 83]	[11-13]	[0 - 0]	-	[0 - 0]
PBS	86	74.5	71.5	nt	62
	[74 - 98]	[74 - 75]	[65 - 78]	-	[54 - 70]
nil	40.50	0	0	nt	0
33	[33 - 48]	[0 - 0]	[0 - 0]	-	[0 - 0]
56°C for 30 min	86.50	30.50	4.50	nt	0
33	[83 - 90]	[27 - 34]	[0 - 9]	-	[0 - 0]
100°C for 5 min	72	59.50	55	nt	4.50
33	[63 - 81]	[50 - 69]	[55]**	-	[0 - 9]
PBS	93.50	85.50	83	nt	64.5
	[90 - 97]	[83 - 88]	[83]**	-	[50 - 79]
nil	66.5	nt	0	0	0
40	[51 - 82]	-	[0 - 0]	[0 - 0]	[0 - 0]
HCl: 0.1 M, 18 h 4°C	77.5	nt	2.50	0	0
40	[56 - 99]	-	[0 - 5]	[0 - 0]	[0 - 0]
NaOH: 0.1 M, 18 h 4°C	92.5	nt	25	8	1
40	[92 - 93]	-	[15 - 35]	[3 - 13]	[0 - 2]
At 4°C	64.5	-	0	0	0
40	[44 - 85]	-	[0 - 0]	[0 - 0]	[0 - 0]
PBS	90	-	80.5	75.5	44
	[82 - 98]	-	[59 - 102]	[49 - 102]	[20 - 68]
nil	62.5	-	0	0	0
43	[54 - 71]	-	[0 - 0]	[0 - 0]	[0 - 0]
Filtrate/30 kDa	121.5	-	72.5	51.50	26
43	[114 - 129]	-	[54 - 91]	[43 - 60]	[22 - 30]
Retentate/30 kDa	85.5	-	0	0	0
43	[77 - 94]	-	[0 - 0]	[0 - 0]	[0 - 0]
PBS	105.5	-	72	50	22.5
	[103 - 108]	-	[70 - 74]	[49 - 51]	[22 - 23]

* % survival calculations were based on CL medium time-zero count data.
** This was a single observation

Appendix 29. Effect of various treatment on the bactericidal activity of supernate (SN) and surfactant (SUR) fractions of LLF towards *B. pertussis* lux (% Luminescence calculations were based on PBS time-1h count data).

Experiment @ particulars	Mean % luminescence [range] (No. of observations) after 1h incubation time at different dilution / µl						Inactivation end point **90 %** *50 %* µl
	1/1 400	1/2 200	1/4 100	1/8 50	1/16 25	1/64 12.5	
SN 44 A (2)	7.50 [6 - 9]	18.0 [13 - 23]	56.0 [46 - 66]	69.50 [66 - 73]	nt -	nt -	**350** *120*
SN 44 B_1 (2)	17.50 [13 - 22]	65.50 [56 - 75]	79.50 [71 - 88]	89.0 [83 - 95]	nt -	nt -	**>400** *255*
SN 44 B_2 (2)	74.50 [68 - 81]	67.0 [66 - 68]	76.0 [74 - 78]	63.5 [51 - 76]	nt -	nt -	**>400** *>400*
SUR 44 A (2)	1.0 [1 - 1]	nt -	2.5 [2 - 3]	nt -	10.50 [8 - 13]	52 [43 - 61]	**40** *20*
SUR 44 B_1 (2)	2.0 [2 - 2]	nt -	8.0 [5 - 11]	nt -	44.5 [28 - 61]	77.0 [64 - 90]	**100** *30*
SUR 44 B_2 (2)	10.50 [4 - 17]	nt -	33.5 [11 - 56]	nt -	73.0 [47 - 99]	86.50 [79 - 94]	**>400** *70*
SN 44 A (2)	15.0 [10 - 20]	34.5 [34 - 35]	77.0 [76 - 78]	80.5 [45 - 116]	nt -	nt -	**>400** *165*
SN 44 C_1 (2)	34.5 [34 - 35]	71.0 [57 - 85]	95.0 [83 - 107]	107 [88 - 126]	nt -	nt -	**>400** *320*
SN 44 C_2 (2)	43.0 [34 - 52]	88.0 [85 - 91]	111 [109 - 113]	122 [118 -127]	nt -	nt -	**>400** *380*
SN 44 D (2)	26.50 [26 -27]	48.50 [42 - 55]	85.50 [77 - 94]	97.0 [74 - 120]	nt -	nt -	**>400** *210*
C_1/PBS	1.0*	-	-	-	-	-	
C_2/PBS	105*	-	-	-	-	-	
D/PBS	94*	-	-	-	-	-	
SUR 44 A (2)	2.50 [1 - 4]	nt -	5.50 [2 - 9]	nt -	19.0 [10 - 28]	60.0 [59 - 61]	**75** *20*
SUR 44 C_1 (2)	1.5 [1 - 2]	nt -	12.0 [11 - 13]	nt -	38.50 [36 - 41]	76.50 [76 - 77]	**140** *30*
SUR 44 C_2 (2)	7.0 [4 - 10]	nt -	14.0 [8 - 20]	nt -	36.0 [29 - 43]	71.0 [66 - 76]	**285** *30*

Appendix 29. continued

Experiment particulars	@	Mean % luminescence [range] (No. of observations) after 1h incubation time at different dilution / µl					Inactivation end point **90 %** *50 %* µl
	1/1 400	1/2 200	1/4 100	1/8 50	1/16 25	1/64 12.5	
SUR 44 D (2)	2 [1 - 3]	nt -	6 [3 - 9]	nt -	17.50 [12 - 23]	62.50 [57 - 68]	**75** *30*
C_1/PBS	1.0*	-	-	-	-	-	
C_2/PBS	104*	-	-	-	-	-	
D/PBS	93*	-	-	-	-	-	
SN 44 A (2)	2.50 [2 - 3]	13.50 [10 - 17]	76.50 [69 - 84]	89.5 [79 - 100]	nt -	nt -	**250** *135*
SN 44 E_1 (2)	4.50 [2 - 7]	20.50 [16 - 25]	68.0 [61 - 75]	94.0 [78 - 110]	nt -	nt -	**310** *135*
SN 44 E_2 (2)	6.0 [4 - 8]	18.50 [11 - 26]	74.0 [72 - 76]	100 [98 - 102]	nt -	nt -	**330** *135*
SN 44 E_3 (2)	3.0 [2 - 4]	13.50 [7 - 20]	73.0 [66 - 80]	102 [97 - 107]	nt -	nt -	**255** *135*
SN 44 F (2)	4.0 [3 - 5]	28.0 [17 - 39]	76.0 [75 - 77]	92.50 [88 - 97]	nt -	nt -	**320** *155*
SUR 44 A (2)	1.5 [1 - 2]	nt -	3.0 [3 - 3]	nt -	16.0 [15 - 17]	67.0 [61 - 73]	**55** *20*
SUR 44 E_1 (2)	2.0 [2 - 2]	nt -	13.50 [13 - 14]	nt -	67.50 [65 - 70]	94.0 [92 - 96]	**160** *50*
SUR 44 E_2 (2)	2.0 [2 - 2]	nt -	7.0 [6 - 8]	nt -	51.50 [47 - 56]	83.50 [74 - 93]	**95** *35*
SUR 44 E_3 (2)	2.0 [1 - 3]	nt -	10.50 [7 - 14]	nt -	71.0 [57 - 85]	83.0 [77 - 89]	**125** *50*
SUR 44 F (2)	1.5 [1 - 3]	nt -	3.5 [3 - 4]	nt -	18.0 [18 - 18]	58.0 [51 - 65]	**60** *20*
E_1/PBS	97 [91 - 103]	- -	- -	- -	- -	- -	
E_2/PBS	107.5 [103 - 112]	- -	- -	- -	- -	- -	
E_3/PBS	121.5 [105 - 138]	- -	- -	- -	- -	- -	
F/PBS	92.0	-	-	-	-	-	

Appendix 29. continued

Experiment particulars	@	Mean % luminescence [range] (No. of observations) after 1h incubation time at different dilution / µl						Inactivation end point **90 %** *50 %* µl
		1/1 400	1/2 200	1/4 100	1/8 50	1/16 25	1/64 12.5	
SN 44 A (2)		6.0 [4 - 8]	10.50 [10 - 11]	42.50 [37 - 48]	59.5 [44 - 45]	nt -	nt -	**250** *90*
SN 44 G (2)		14.0 [13 - 15]	36.0 [33 - 39]	70.0 [68 - 73]	84.0 [78 - 90]	nt -	nt -	**>400** *165*
SUR 44 A (2)		1.5 [1 - 2]	nt -	1.5 [1 - 2]	nt -	16.0 [8 - 24]	48.0 [39 - 57]	**50** *20*
SUR 44 G (2)		1.5 [1 - 2]	nt -	2.5 [2 - 3]	nt -	11.5 [9 - 14]	49.50 [49 - 50]	**40** *20*
SN 45 A (2)		4.0 [1 - 7]	7.0 [3 - 11]	15 [7 - 23]	27.5 [16 - 39]	nt -	nt -	**175** *30*
SN 45 H (1)		5.0 [5]	9.0 [9]	14.0 [14]	27.0 [27]	nt -	nt -	**210** *30*
SUR 45 A (2)		1.5 [1 - 2]	nt -	2.5 [1 - 4]	nt -	9.0 [3 - 15]	35.0 [23 - 47]	**30** *20*
SUR 45 H (2)		3.0 [1 - 5]	nt -	5.50 [1 - 10]	nt -	9.50 [2 - 17]	31.0 [19 - 43]	**30** *20*
SN 45 A (3)		5.0 [3 - 7]	11.33 [8 - 14]	26.3 [13 - 49]	49.7 [34 - 77]	nt -	nt -	**265** *65*
SN 45 I (3)		1.33 [1 - 2]	1.67 [1 - 2]	2.0 [2 - 2]	7.33 [2 - 11]	nt -	nt -	**50** *<0**
SN 45 F (3)		8.33 [4 - 12]	14.0 [9 - 19]	34.33 [25 - 40]	61.0** [56 - 66]	nt -	nt -	**375** *80*
SUR 45 A (3)		2.33 [2 - 3]	nt -	2.33 [2 - 3]	nt -	6.67 [6 - 7]	27.33 [21 - 38]	**30** *20*
SUR 45 I (3)		1.67 [1 - 2]	nt -	7.67 [2 - 12]	nt -	39.0 [11 - 54]	67.3 [45 - 79]	**100** *30*
SUR 45 F (3)		2.0 [2 - 2]	nt -	3.0 [3 - 3]	nt -	10.0 [9 - 12]	51.0* [51]	**40** *20*
I/PBS (3)		1.33 [1 - 2]	- -	- -	- -	- -	- -	
SN 45 A (2)		9.0 [7 - 11]	14.0 [14 - 14]	25.0 [23 - 27]	54.0 [52 - 56]	nt -	nt -	**400** *70*
SN 45 J (2)		16.0 [11 - 21]	51.50 [49 - 54]	113 [108 - 118]	137 [137 - 137]	nt -	nt -	**>400** *230*

Appendix 29. continued

Experiment particulars	@	Mean % luminescence [range] (No. of observations) after 1h incubation time at different dilution / µl						Inactivation end point **90 %** *50 %* µl
		1/1 400	1/2 200	1/4 100	1/8 50	1/16 25	1/64 12.5	
SN 45 F (2)		10.0 [8 - 12]	20.0 [20 - 21]	36.0 [36 - 36]	nt -	nt -	nt -	**>400** *100*
SUR 45 A (2)		2.0 [2 - 2]	nt -	5.50 [5 - 6]	nt -	17.0 [16 - 18]	67.0 [63 - 71]	**80** *25*
SUR 45 J (2)		13.0 [13 - 13]	nt -	56.50 [55 - 58]	nt -	124 [118 - 130]	139.5 [131 - 148]	**>400** *150*
SUR 45 F (2)		2.0 [2 - 2]	nt -	5.50 [5 - 6]	nt -	20.0 [19 - 21]	nt -	**80** *25*
J/PBS (2)		169.50 [169 - 170]	- -	- -	- -	- -	- -	
F/PBS (2)		96.50 [94 - 99]	- -	- -	- -	- -	- -	
SUR 45 A (2)		5.50 [5 - 6]	nt -	19.50 [16 - 23]	nt -	45.0 [40 - 50]	50.0 [50 - 50]	**290** *30*
SUR 45 K (2)		9.0 [8 - 10]	nt -	20 [16 - 24]	nt -	29.50 [21 - 38]	43.50 [39 - 48]	**400** *20*
SUR 50 A (4)		27 [19 - 35]	nt -	75.75 [65 - 83]	nt -	94.25 [82 - 110]	102.25 [94 - 108]	**>400** *260*
SUR 50 L_1 (4)		62.5 [43 - 67]	nt -	88.75 [70 - 99]	nt -	95.25 [85 - 105]	102.25 [97 - 110]	**>400** *>400*
SUR 50 L_2 (4)		96.5 [81 - 103]	nt -	101.5 [95 - 105]	nt -	97.75 [71 - 112]	99 [74 - 110]	**>400** *>400*

* This was a single observation,**Mean of 2 experiments, @ Rat batch no, ( ) = no. of experiments, A = untreated test fluid, B_1 = heat at 56 °C for 30 min, B_2 = heat at 100 °C for 15 min, C_1 = HCl (0.1 M final), C_2 = NaOH (0.1 M final), D = test fluid at 4°C for overnigth, E_1 = trypsin (2mg/ml final), E_2 = chymotrypsin (2mg/ml), E_3 = subtilisin (2mg/ml), F = test fluid at 37°C for 30 min, G = Freezing and thawing of test fluids, H = dialysed test fluids, I = test fluids treated with bentonite, J = proteinase K (2mg/ml final), K = sonicated surfactant, L_1 = surfactant washed in PBS one time, L_2 = surfactant washed in PBS four times.

Appendix 30. Effect of various treatment on the bactericidal activity of supernate (SN) and surfactant (SUR) fractions of LLF from normal rats (batch no. 55, euthanasia method D) towards *B. pertussis* 18-323. Data are of two observations for each treatment. (% survival calculations were based on PBS time-1h count data).

Experiment particulars	@	Mean % survival [range] after 1h incubation time at different dilution / µl						Inactivation end point **90 %**
		1/1	1/2	1/4	1/8	1/16	1/64	*50 %*
		240	120	60	30	15	7.5	µl
SN		16	52	52	66.5	nt	nt	**> 240**
A		[11-21]	[45-59]	[45-59]	[65-68]	-	-	*125*
SN		70.5	101.5	117.5	150.5	nt	nt	**> 240**
B1		[59-82]	[91-112]	[100-135]	[136-165]	-	-	*> 240*
SN		135	123.5	130.5	101.5	nt	nt	**> 240**
B2		[105-165]	[100-147]	[114-147]	[91-112]	-	-	*> 240*
PBS		104.5	-	-	-	-	-	-
B1		[91-118]	-	-	-	-	-	-
PBS		107.5	-	-	-	-	-	-
B2		[[88-127]	-	-	-	-	-	-
SUR		0	nt	0	nt	12	130.5	**20**
A		[0-0]	-	[0-0]	-	[12-12]	[123-128]	*10*
SUR		0	nt	0	nt	46	100	**30**
B1		[0-0]	-	[0-0]	-	[38-54]	[85-115]	*15*
SUR		0	nt	15	nt	61.5	107.5	**75**
B2		[0-0]	-	[15-15]	-	[38-85]	[100-115]	*20*
PBS		119	-	-	-	-	-	-
B1		[115-123]	-	-	-	-	-	-
PBS		134.5	-	-	-	-	-	-
B2		[127-142]	-	-	-	-	-	-
SN		4.5	51.5	117.5	130.5	nt	nt	**200**
A		[4-5]	[45-58]	[108-127]	[125-136]	-	-	*120*
SN		65.5	79	131.5	122	nt	nt	**> 240**
C1		[58-73]	[67-91]	[108-155]	[117-127]	-	-	*> 240*
SN		34.5	70	74.5	143.5	nt	nt	**> 240**
C2		[27-42]	[67-73]	[58-91]	[142-145]	-	-	*180*
SN		0	17.5	22	65.5	nt	nt	**140**
D		[0-0]	[17-18]	[17-27]	[58-73]	-	-	*40*
PBS		82.5	-	-	-	-	-	-
C1		[73-92]	-	-	-	-	-	-

Appendix 30. continued

Experiment particulars	@	Mean % survival [range] after 1h incubation time at different dilution / µl						Inactivation end point **90 %** *50 %* µl
	1/1 240	1/2 120	1/4 60	1/8 30	1/16 15	1/64 7.5		
PBS C2	74 [73-75]	- -	- -	- -	- -	- -		- -
PBS D	100.5 [83-118]	- -	- -	- -	- -	- -		- -
SUR A	0 [0-0]	nt -	0 [0-0]	nt -	9 [5-13]	67 [63-71]		**15** *5*
SUR C1	0 [0-0]	nt -	0 [0-0]	nt -	48 [39-57]	101 [92-110]		**30** *15*
SUR C2	0 [0-0]	nt -	0 [0-0]	nt -	36.5 [26-47]	90 [83-97]		**30** *15*
SUR D	0 [0-0]	nt -	0 [0-0]	nt -	6.5 [3-10]	89.5 [79-100]		**15** *5*
PBS C1	nt -	- -	- -	- -	- -	- -		- -
PBS C2	95.5 [84-107]	- -	- -	- -	- -	- -		- -
PBS D	89.5 [[79-100]	- -	- -	- -	- -	- -		- -
SN A	32 [29-35]	138 [122-154]	123 [117-130]	142 [135-150]	nt -	nt -		**> 240** *215*
SN E1	68.5 [67-70]	139 [113-165]	154 [138-170]	153.5 [150-157]	nt -	nt -		**> 240** *> 240*
SN E2	121 [117-125]	166.5 [146-187]	123.5 [122-125]	174.5 [174-175]	nt -	nt -		**> 240** *> 240*
SN E3	40.5 [33-48]	126 [117-135]	170.5 [158-183]	134 [125-143]	nt -	nt -		**> 240** *230*
SN F	100 [96-104]	148.5 [139-158]	134.5 [104-165]	147 [133-161]	nt -	nt -		**> 240** *> 240*
PBS E1	164.5 [142-187]	- -	- -	- -	- -	- -		- -
PBS E2	151 [135-167]	- -	- -	- -	- -	- -		- -

Appendix 30. continued

Experiment particulars	@	Mean % survival [range] after 1h incubation time at different dilution / µl					Inactivation end point **90 %** *50 %* µl
	1/1 240	1/2 120	1/4 60	1/8 30	1/16 15	1/64 7.5	
PBS E3	131.5 [117-146]	- -	- -	- -	- -	- -	- -
PBS F	127.5 [125-130]	- -	- -	- -	- -	- -	- -
SUR A	0 [0-0]	nt -	0 [0-0]	nt -	5 [3-7]	86.5 [83-90]	**15** *10*
SUR E1	0 [0-0]	nt -	6.5 [6-7]	nt -	90.5 [81-100]	120.5 [108-133]	**60** *30*
SUR E2	0 [0-0]	nt -	11.5 [6-17]	nt -	99 [78-120]	121 [102-140]	**70** *35*
SUR E3	0 [0-0]	nt -	0 [0-0]	nt -	106 [92-120]	113.5 [94-133]	**45** *25*
SUR F	0 [0-0]	nt -	0 [0-0]	nt -	86 [72-100]	96 [72-120]	**45** *25*
PBS E1	97 [94-100]	- -	- -	- -	- -	- -	- -
PBS E2	115.5 [108-123]	- -	- -	- -	- -	- -	- -
PBS E3	93 [86-100]	- -	- -	- -	- -	- -	- -
PBS F	87.5 [78-97]	- -	- -	- -	- -	- -	- -
SN A	16 [11-21]	52 [45-59]	52 [45-59]	66.5 [65-68]	nt -	nt -	**> 240** *60*
SN G	28 [27-29]	31 [27-35]	38.5 [36-41]	82.5 [59-106]	nt -	nt -	**> 240** *50*
PBS G	101.5 [91-112]	- -	- -	- -	- -	- -	- -
SUR A	0 [0-0]	nt -	0 [0-0]	nt -	12 [12-12]	130.5 [123-128]	**15** *5*
SUR G	0 [0-0]	nt -	0 [0-0]	nt -	4 [4-4]	104 [100-108]	**10** *5*

Appendix 30. continued

Experiment particulars	@	Mean % survival [range] after 1h incubation time at different dilution / µl					Inactivation end point **90 %** *50 %* µl
	1/1 240	1/2 120	1/4 60	1/8 30	1/16 15	1/64 7.5	
PBS G	138 [138-138]	- -	- -	- -	- -	- -	- -
SN A	0 [0-0]	67 [67-67]	111 [80-142]	83.5 [67-100]	nt -	nt -	**160** *125*
SN H	22.5 [20-25]	40 [33-47]	45 [40-50]	86 [80-92]	nt -	nt -	**> 240** *55*
PBS H	86 [80-92]	- -	- -	- -	- -	- -	- -
SUR A	0 [0-0]	nt -	0 [0-0]	nt -	54.5 [50-59]	122.5 [112-133]	**30** *15*
SUR H	0 [0-0]	nt -	0 [0-0]	nt -	106 [94-118]	114.5 [111-118]	**30** *20*
PBS H	49 [39-59]	- -	- -	- -	- -	- -	- -
SN A	4 [4-4]	24 [22-26]	111 [99-124]	123 [113-133]	nt -	nt -	**180** *90*
SN I1/Ben	75 [67-83]	97.5 [91-104]	105 [95-115]	110 [107-113]	nt -	nt -	**> 240** *> 240*
SN I2/Char	106.5 [93-120]	94.5 [85-104]	109 [105-113]	87 [85-89]	nt -	nt -	**> 240** *> 240*
SN F	2 [1-3]	69.5 [63-76]	107 [107-107]	129.5 [109-150]	nt -	nt -	**190** *130*
PBS I1/Ben	118 [99-137]	- -	- -	- -	- -	- -	- -
PBS I2/Char	94.5 [85-104]	- -	- -	- -	- -	- -	- -
PBS F	94.5 [89-100]	- -	- -	- -	- -	- -	- -
SUR A	0 [0-0]	nt -	0 [0-0]	nt -	0 [0-0]	110.5 [100-121]	**10** *5*
SUR I1/Ben	0 [0-0]	nt -	0 [0-0]	nt -	43 [36-50]	77 [75-79]	**30** *15*

Appendix 30. continued

Experiment particulars	@	Mean % survival [range] after 1h incubation time at different dilution / µl					Inactivation end point **90 %** *50 %* µl
	1/1 240	1/2 120	1/4 60	1/8 30	1/16 15	1/64 7.5	
SUR I2/Char	134 [125-143]	nt -	159 [143-175]	nt -	135.5 [129-142]	149.5 [142-157]	**> 240** *> 240*
SUR F	0 [0-0]	nt -	0 [0-0]	nt -	31 [29-33]	102 [71-133]	**30** *15*
PBS I1/Ben	148.5 [133-164]	- -	- -	- -	- -	- -	- -
PBS I2/Char	131 [129-133]	- -	- -	- -	- -	- -	- -
SN A	19.5 [14-25]	66 [57-75]	121.5 [121-122]	139.5 [130-149]	nt -	nt -	=PK =Pk
SN I3/Meth.Cell	56 [52-60]	87.5 [79-96]	115 [111-119]	132.5 [128-137]	nt -	nt -	**> 240** *> 240*
SN F	39.5 [39-40]	91 [83-99]	145 [122-168]	92.5 [87-98]	nt -	nt -	=PK =Pk
PBS I3/Meth.Cell	134 [125-143]	- -	- -	- -	- -	- -	- -
SUR A	0 [0-0]	nt -	0 [0-0]	nt -	0 [0-0]	110.5 [100-121]	**10** *5*
SUR I3/Meth.Cell	0 [0-0]	nt -	0 [0-0]	nt -	71 [46-96]	73.5 [64-83]	**30** *15*
SUR F	0 [0-0]	nt -	0 [0-0]	nt -	31 [29-33]	102 [71-133]	**30** *15*
PBS I3/Meth.Cell	41.5 [33-50]	- -	- -	- -	- -	- -	- -
SN A	19.5 [14-25]	66 [57-75]	121.5 [121-122]	139.5 [130-149]	nt -	nt -	**> 240** *150*
SN E4/Pk	4.5 [3-6]	105 [99-111]	92.5 [90-95]	142.5 [133-152]	nt -	nt -	**210** *150*
SN F	39.5 [39-40]	91 [83-99]	145 [122-168]	92.5 [87-98]	nt -	nt -	**> 240** *205*
PBS E4/Pk	113.5 [108-119]	- -	- -	- -	- -	- -	- -

Appendix 30. continued

Experiment particulars	@	Mean % survival [range] after 1h incubation time at different dilution / µl					Inactivation end point **90 %** *50 %* µl
	1/1 240	1/2 120	1/4 60	1/8 30	1/16 15	1/64 7.5	
SUR A	0 [0-0]	nt -	0 [0-0]	nt -	12 [9-15]	98 [93-103]	**20** *10*
SUR E4/Pk	0 [0-0]	nt -	3 [3-3]	nt -	100.5 [97-104]	125.5 [124-127]	**50** *35*
SUR F	0 [0-0]	nt -	0 [0-0]	nt -	33 [31-35]	96.5 [96-97]	**20** *10*
PBS E4/Pk	100.5 [86-115]	- -	- -	- -	- -	- -	- -
SN A	0 [0-0]	12.5 [9-16]	113.5 [90-137]	136.5 [136-137]	nt -	nt -	**132** *90*
SN I4/DMßCD	95 [95-95]	151 [147-155]	127.5 118-137]	138.5 [132-145]	nt -	nt -	**> 240** *> 240*
SN I5/ßCD	105.5 [100-111]	126.5 [95-158]	120.5 [109-132]	144 [109-179]	nt -	nt -	**> 240** *> 240*
SN I6/ßCD	121 [100-142]	123 [114-132]	116 [95-137]	133.5 [109-158]	nt -	nt -	**> 240** *> 240*
SN F	98.5 [86-111]	128.5 [121-136]	109 [100-118]	147.5 [127-168]	nt -	nt -	**> 240** *> 240*
PBS I4/DMßCD	107 [82-132]	- -	- -	- -	- -	- -	- -
PBS I5/ßCD	84 [68-100]	- -	- -	- -	- -	- -	- -
PBS I6/αCD	92.5 [90-95]	- -	- -	- -	- -	- -	- -
PBS F	105.5 [95-116]	- -	- -	- -	- -	- -	- -
SUR A	0 [0-0]	nt -	0 [0-0]	nt -	15 [10-20]	93.5 [77-110]	**25** *8*
SUR I4/DMβCD	60.5 [58-63]	nt -	92 [84-100]	nt -	108.5 [89-128]	101.5 [87-116]	**> 240** *> 240*
SUR I5/ßCD	1.5 [0-3]	nt -	4.5 [3-6]	nt -	76.5 [63-90]	87 [77-97]	**50** *16*

Appendix 30. continued

Experiment particulars	@	Mean % survival [range] after 1h incubation time at different dilution / µl					Inactivation end point **90 %**
	1/1	1/2	1/4	1/8	1/16	1/64	*50 %*
	240	120	60	30	15	7.5	µl
SUR	0	nt	10	nt	72	nt	**70**
I6/αCD	[0-0]	-	[8-12]	-	[63-81]	-	*16*
SUR	0	nt	0	nt	74	84	**35**
F	[0-0]	-	[0-0]	-	[61-87]	[68-100]	*16*
PBS	81.5	-	-	-	-	-	-
I4/MßCD	[73-90]	-	-	-	-	-	-
PBS	78.5	-	-	-	-	-	-
I5/ßCD	[74-83]	-	-	-	-	-	-
PBS	93.5	-	-	-	-	-	-
I6/αCD	[93-94]	-	-	-	-	-	-
PBS	85	-	-	-	-	-	-
F	[73-97]	-	-	-	-	-	-
SN	0	90	101.5	115	nt	nt	**165**
A	[0-0]	[80-100]	[53-150]	[100-130]	-	-	*130*
SN	156.5	130	160	96.5	nt	nt	**> 240**
I7/BSA	[93-220]	[80-180]	[140-180]	[93-100]	-	-	*> 240*
SN	41.5	111.5	121.5	130	nt	nt	**> 240**
I8/Starch	[33-50]	[110-113]	[113-130]	[120-140]	-	-	*215*
SN	93.5	131.5	133.5	148.5	nt	nt	**> 240**
I9/IRS	[87-100]	[130-133]	[87-180]	[107-190]	-	-	*> 240*
SN	36	81.5	93.5	161.5	nt	nt	**> 240**
F	[35-37]	[73-90]	[87-100]	[153-170]	-	-	*190*
PBS	138.5	-	-	-	-	-	-
I7/BSA	[107-170]	-	-	-	-	-	-
PBS	111.5	-	-	-	-	-	-
I8/Starch	[73-150]	-	-	-	-	-	-
PBS	143.5	-	-	-	-	-	-
I9/RIS	[87-200]	-	-	-	-	-	-
PBS	100	-	-	-	-	-	-
F	[80-120]	-	-	-	-	-	-
SUR	0	nt	0	nt	27	100	**30**
A	[0-0]	-	[0-0]	-	[24-30]	[100-100]	**15**

Appendix 30. continued

Experiment particulars	@	Mean % survival [range] after 1h incubation time at different dilution / µl					Inactivation end point **90 %**
	1/1	1/2	1/4	1/8	1/16	1/64	*50 %*
	240	120	60	30	15	7.5	µl
SUR	0	nt	45	nt	98	94.5	**120**
I7/BSA	[0-0]	-	[38-52]	-	[93-103]	[89-100]	*65*
SUR	0	nt	0	-	72	107	**35**
I8/Starch	[0-0]	-	[0-0]	-	[66-78]	[100-114]	*20*
SUR	0	nt	37.5	nt	89.5	92.5	**115**
I9/IRS	[0-0]	-	[34-41]	-	[89-90]	[89-96]	*55*
SUR	0	nt	0	nt	68.5	71.5	**35**
F	[0-0]	-	[0-0]	-	[52-85]	[69-74]	*20*
PBS	118	-	-	-	-	-	-
I7/BSA	[110-126]	-	-	-	-	-	-
PBS	74	-	-	-	-	-	-
I8/Starch	[59-89]	-	-	-	-	-	-
PBS	120	-	-	-	-	-	-
I9/RIS	[110-130]	-	-	-	-	-	-
PBS	71.5	-	-	-	-	-	-
F	[67-76]	-	-	-	-	-	-
SN	0	67	111	83.5	nt	nt	**160**
A	[0-0]	[67-67]	[80-142]	[67-100]	-	-	*125*
SN	80.5	84	114	116	nt	nt	**> 240**
J1/Pho(A2)	[53-108]	[75-93]	[108-120]	[100-133]	-	-	*> 240*
SN	0	64	62.5	122.5	nt	nt	**160**
J2/Pho(C)	[0-0]	[53-75]	[58-67]	[120-125]	-	-	*125*
SN55	0	103	129	102	nt	nt	**170**
F	[0-0]	[73-133]	[125-133]	[87-117]	-	-	*135*
PBS	85	-	-	-	-	-	-
J1/Pho(A2)	[83-87]	-	-	-	-	-	-
PBS	106	-	-	-	-	-	-
J2/Pho(C)	[87-125]	-	-	-	-	-	-
PBS	60	-	-	-	-	-	-
F	[12-108]	-	-	-	-	-	-
SUR	0	nt	0	nt	54.5	122.5	**30**
A	[0-0]	-	[0-0]	-	[50-59]	[112-133]	*15*

Appendix 30. continued

Experiment particulars	@	Mean % survival [range] after 1h incubation time at different dilution / µl					Inactivation end point **90 %** *50 %* µl
	1/1 240	1/2 120	1/4 60	1/8 30	1/16 15	1/64 7.5	
SUR J1/Pho(A2)	85.5 [65-106]	nt -	111.5 [106-117]	nt -	139.5 [129-150]	185.5 [183-188]	**> 240** *> 240*
SUR J2/Pho(C)	0 [0-0]	nt -	20 [18-22]	nt -	144 [117-171]	142 [129-156]	**90** *40*
SUR F	0 [0-0]	nt -	0 [0-0]	nt -	89 [72-106]	111 [100-122]	**30** *20*
PBS J1/Pho(A2)	117.5 [111-124]	- -	- -	- -	- -	- -	- -
PBS J2/Pho(C)	134 [129-139]	- -	- -	- -	- -	- -	- -
PBS F	128 [112-144]	- -	- -	- -	- -	- -	- -
SUR A	0 [0-0]	nt -	0 [0-0]	nt -	0 [0-0]	56.5 [51-62]	**10** *5*
SUR K1/wash 1	0 [0-0]	nt -	0 [0-0]	nt -	43 [37-49]	91.5 [84-99]	**20** *15*
SUR K2/wash 4	0 [0-0]	nt -	19 [16-22]	nt -	59 [54-64]	68.5 [68-69]	**90** *35*
SUR* L1/Sonicated	0 [0-0]	nt -	2 [2-2]	nt -	64.5 [59-70]	94 [85-103]	**40** *20*
SUR* L2/Unsonic	0 [0-0]	nt -	0 [0-0]	nt -	55 [45-65]	92 [79-105]	**30** *20*

* Surfactant batch no. 45, @ /A = untreated test fluid, B_1 = heat at 56°C for 30 min, B_2 = heat at 100°C for 15 min, C_1 = HCl (0.1 M final), C_2 = NaOH (0.1 M final), D = test fluid at 4°C for overnigth, E_1 = trypsin (2mg/ml final), E_2 = chymotrypsin (2mg/ml), E_3 = subtilisin (2mg/ml), E_4 = proteinase K (2mg/ml final), F = test fluid at 37°C for 30 min, G = freezing and thawing of test fluids, H = dialysed test fluids, I_1 = test fluids treated with bentonite, I_2 = charcoal, I_3 = methyl cellulose, I_4 = DMβCD, I_5 = βCD, I_6 = αCD, I_7 = bovine serum albumin (BSA) , I_8 = =starch, I_9 = inactivated rat serum (IRS); J_1= Phospholipase A2, J_2 = Phospholipase C, K_1 = Surfactant washed in PBS one time, K_2 = Surfactant washed in PBS four times, L_1 = Sonicated surfactant, L_2 = Unsonicated surfactant.