UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

MICROBIOLOGY

THE EFFECTS OF GROWTH CONDITIONS ON THE EXPRESSION OF VIRULENCE DETERMINANTS OF *BORDETELLA PERTUSSIS*

by Andrew Richard Gorringe

1988

ACKNOWLEDGEMENTS

I gratefully acknowledge the help of the following of my colleagues at CAMR: Dr. L.I. Irons and Dr. L.A.E. Ashworth for their advice and guidance during this research, Mr. A.R. Blake and Mr. G.R.G. Moody for their practical help in the laboratory. Special thanks are due to Dr. A. Robinson for his constant help and encouragement throughout the work presented here. Thanks are also due to Dr. J.E. Heckels for his supervision from the University of Southampton.

Work presented in this thesis has been included in the following publications:

ROBINSON, A., GORRINGE, A.R., IRONS, L.I. & KEEVIL, C.W. (1983). Antigenic modulation of Bordetella pertussis in continuous culture. FEMS Microbiology Letters 19, 105-109.

ROBINSON, A., GORRINGE, A.R. & KEEVIL, C.W. (1983). Expression of virulence determinants in Bordetella pertussis and Neisseria gonorrhoeae. In: Continuous Culture 8 pp.22-37. Edited by A.C.R. Dean, D.C. Ellwood and C.G.T. Evans. Chichester: Ellis Horwood.

GORRINGE, A.R., ASHWORTH, L.A.E., IRONS, L.I. & ROBINSON, A. (1985). Effect of monoclonal antibodies on the adherence of Bordetella pertussis to Vero cells. FEMS Microbiology Letters 26, 5-9.

ROBINSON, A., ASHWORTH, L.A.E., GORRINGE, A.R. & IRONS, L.I. (1985). Progress in the development of acellular pertussis vaccines. In Bacterial protein toxins. Proceedings of the Second Symposium on Bacterial Toxins, Wepion, Belgium pp. 389-396. Edited by P. Falmagne. Berlin: Fischer Verlag.

ASHWORTH, L.A.E., ROBINSON, A., IRONS, L.I., DOWSETT, A.B., GORRINGE, A.R. & WILTON-SMITH, P. (1986). Fimbriae of Bordetella pertussis. In: Protein-Carbohydrate Interactions in Biological Systems: The Molecular Biology of Microbial Pathogenicity pp.291-293. Edited by D.L. Lark. FEMS Symposium No. 31. London: Academic Press.

ROBINSON, A., DUGGLEBY, C., GORRINGE, A.R. & LIVEY, I. (1986). Antigenic variation in Bordetella pertussis. In: Antigenic Variation in Infectious Diseases. Special publication of the Society for General Microbiology Volume 19 pp. 147-161. Edited by T.H. Birkbeck and C.W. Penn.

Oxford: IRL Press.

GORRINGE, A.R., IRONS, L.I. & ROBINSON, A. (1986). The effect of Meβcyclodextrin on the stability of Bordetella pertussis filamentous haemaglutinin. Microbe '86. XIV International Congress of Microbiology, Manchester.

GORRINGE, A.R., IRONS, L.I., ASHWORTH, L.A.E. & ROBINSON, A. (1986). Bordetella pertussis: The disease and vaccine prospects. In: Proceedings of Symposium, Trends in Respiratory Infection, Beaumaris, Anglesey pp. 25-29. Basingstoke: The Modern Medicine Group for Lilly Industries Limited.

IRONS, L.I. & GORRINGE, A.R. (1988). Pertussis toxin: Production, purification, molecular structure and assay. In: Pathogenesis and Immunity in Pertussis pp. 95-120. Edited by A.C. Wardlaw and R. Parton. Chichester: John Wiley.

CONTENTS

LIST OF FIGURES. PAGE

LIST OF TABLES. PAGE

ABREVIATIONS.

AGG	- Agglutinogen.
CA	- Charcoal agar.
CAMR	- Centre for Applied Microbiology and Research.
CD	- 2,6-O-dimethylβ cyclodextrin.
CL	- Medium of Imaizumi et al. (1983b) without CD.
cAMP	- Cyclic adenosine monophosphate.
DTP	- Diptheria - tetanus - pertussis vaccine.
FHA	- Filamentous haemagglutinin
HA	- Haemagglutination.
HLT	- Heat labile toxin.
HRP	- Horse radish peroxidase.
i.c	- Intracerebral.
LPF	- Lymphocytosis promoting factor.
LPS	- Lipopolysaccharide.
McAb	- Monoclonal antibody.
MEM	- Minimal essential medium.
OD	- Optical density at 550nm.
OMP	- Outer membrane protein.
PBS	- Phosphate buffered saline pH 7.2.
SDS-PAGE	- Sodium dodecyl sulphate polyacrylamide gel electrophoresis.
SS	- Medium of Stainer and Scholte (1971).
TCT	- Tracheal cytotoxin.
Tris	- Tris(hydroxymethyl)methylamine.
TSBA	- Tryptone soya broth agar.
WHO	- World Health Organisation.

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

MICROBIOLOGY

Doctor of Philosophy

THE EFFECTS OF GROWTH CONDITIONS ON THE EXPRESSION OF VIRULENCE DETERMINANTS OF *BORDETELLA PERTUSSIS*

by Andrew Richard Gorringe

The expression of virulence determinants by *Bordetella pertussis* was studied during growth in batch and continuous culture. The addition of 2,6-O-dimethyl β cyclodextrin (CD) reduced the lag phase of batch cultures, increased the maximum culture density in shaken cultures and increased production of lymphocytosis promoting factor (LPF) and filamentous haemagglutinin (FHA). CD was not a nutrient but in its presence glutathione was no longer required.

Purified FHA was stable when incubated statically but lost activity on shaking. CD had a stabilising effect on FHA incubated with shaking, suggesting that increased yields of FHA could be due to this stabilising effect of CD.

Virulent X-mode *B. pertussis* were maintained in continuous culture. CD caused an increase in culture density in continuous culture indicating that an inhibition of growth was acting in its absence. Continuous culture was not suitable for vaccine production as it did not allow maximum expression of vaccine components and there was a possibilty of selection of phase IV organisms. A fermentation process suitable for vaccine production was developed using a stainless steel fermenter and medium containing CD.

Antigenic modulation of *B. pertussis* produced C-mode variants lacking LPF, FHA, fimbriae, X-mode specific outer membrane proteins and adenylate cyclase. C-mode cells had a reduced ability to adhere to Vero cells and reduced hydrophobicity. Antigenic modulation was reversible in continuous culture and the rate of loss of X-mode components was approximately equal to the theoretical washout rate for a number of dilution rates, indicating a common mechanism of control of expression. The addition of CD to C-mode medium partially overcame the repression of X-mode components.

Chapter 1. Introduction.

1.1 Pertussis.

1.1.1 A short history.

The first written description of pertussis or whooping cough disease appeared in 1578 when an epidemic occurred in Paris (Olson, 1975). No previous descriptions of this disease have been found and the next records date from almost a century later when it appears that whooping cough was endemic in Europe (Lapin, 1943). The term pertussis was first used by Sydenham in 1670 (per very severe; tussis cough) and this designation is now prefered by many to whooping cough since not all patients with the disease whoop (Lapin, 1943, Olson, 1975).

Bordet is credited with first describing the bacteria associated with the disease when, in 1900, he observed numerous coccobacilli in respiratory secretion collected from his infant daughter suffering from whooping cough. The bacteria occured in such abundance and purity that their association with the infection could hardly be doubted (Olson, 1975). Bordet's name remains associated with the organism as a result of extensive studies subsequently published together with Gengou. Bordet and Gengou were able to culture the organism succesfully on artificial media, using secretions from Bordet's son who had the disease. In a series of articles they described the medium, which is still in use, and characterised the morphology, culture characteristics, virulence and antigenicity of the organism (Lapin, 1943).

In 1908 and 1909, Klimenko (cited by Lapin, 1943) reported that he had produced whooping cough in monkeys and puppies by inoculation with Bordetella pertussis. There are other reports of animal models of infection, but it was Williams (1914) who isolated pure cultures of B. pertussis from many cases of whooping cough and confirmed the etiological significance of the B. pertussis. The MacDonalds (MacDonald & MacDonald, 1933) provided direct evidence for B. pertussis as the causative agent, in what is to us an astounding manner, by inoculating all four of

their children with the organism, two of them having been immunised with killed organisms. Seven days after challenge both unimmunised children developed typical pertussis and B. pertussis was isolated from each of them.

Bordetella parapertussis, isolated by Eldering & Kendrick (1938) has been associated with the pertussis syndrome and, when implicated, the infection usually takes the form of a mild case of pertussis. Although apparently quite rare in the U.K. and the U.S.A., B. parapertussis contributes more significantly to pertussis outbreaks in other parts of the world (Olson, 1975).

Bordetella bronchiseptica can cause the pertussis syndrome but this is rare. B. bronchiseptica is, however, the cause of respiratory tract infections in many animals such as kennel cough in dogs and infectious atrophic rhinitis in pigs (Goodnow, 1980).

There are still doubts that all cases of pertussis are caused by one of the three Bordetella species. Other agents, in particular adenoviruses, have been implicated (Olson, 1975).

The genus Bordetella was proposed by Lopez in 1952 to include the three Bordetella species. Members of the genus are minute coccobacilli (0.2-0.3 μm by 0.5-1.0 μm) arranged singly or in pairs or rarely in short chains. B. bronchiseptica is motile possessing flagella, whilst B. pertussis and B. parapertussis are not. They are chemoorganotrophs with a respiratory metabolism and do not ferment sugars. The optimal growth temperature is 35-37°C, with no growth under anaerobic conditions (Pittman, 1974). Kloos et al. (1981) showed, using DNA hybridisation techniques, that the members of the genus had very close DNA relatedness.They suggested reconsidering the Bordetella species as representing different subspecies belonging to a single species. Musser et al. (1986) studied allelic variation in Bordetella in structural genes encoding 15 enzymes by multilocus enzyme electrophoresis. They also found that genetic diversity of the factors studied was relatively limited compared with

many other pathogenic bacteria.

Antigenically deficient forms of B. pertussis can be produced by two distinct mechanisms: (i) successive subculture in vitro can cause a loss of virulence known as phase variation (section 1.3.4) and (ii) changes in the growth environment can cause a reversible transition known as antigenic modulation (section 1.3.3). Virulent organisms are known as phase I or X-mode and degraded forms, which lack all the known virulence determinants of the B. pertussis, phase IV or C-mode.

1.1.2 The disease in man.

1.1.2.1 Disease characteristics.

Whooping cough is a highly communicable, acute infection of the respiratory tract. After an incubation period of about 10 days, the catarrhal stage begins and lasts for one to two weeks. This is followed by the paroxysmal stage with the characteristic cough that can last from one to six weeks. The convalescent or decline period has a duration of one to six weeks before the child returns to normal (Munoz & Bergman, 1977).

At the catarrhal stage the disease cannot be clinically differentiated from other respiratory tract infections and the paroxysmal coughing is not seen. With the onset of the second stage the coughing typically develops into paroxysms which are more severe at night. The paroxysmal cough is characteristic, consisting of from 5 to 20 forceful, hacking, successive coughs, allowing little time for breathing. Saliva and mucus stream from the nose and mouth. As the final cough appears to clear offending secretions or mucus from the upper airway, air rushes into the lungs against a still narrowed glottis and the patient can whoop. During these episodes vomitting is frequent (Olson, 1975). In children with a history of allergic disorders a more severe and protracted disease is observed and frequently persistent bronchial asthma follows an attack of whooping cough (Lapin, 1943).

It has long been known that the severity of pertussis is age-related. This was shown by the data of Miller & Fletcher (1976) from the 1974-75 outbreak in England and Wales. They found that 60% of cases in infants under 5 months were hospitalised, 28% of the cases in the 6-11 month age group and only 9% of 1-2 year olds. Children of 5 years and over had only a 2% hospitalisation rate.

1.1.2.2 Epidemiology.

The World Health Organisation (WHO) estimates that the number of cases of whooping cough in the world to be 60 million annually, with half a million to one million deaths. Reliable statistics on numbers of cases do not exist as in many countries whooping cough is not a notifiable disease. Also, in third world countries the great majority of cases are not likely to be reported, either due to deficiences in the reporting system, or because the patient does not come into contact with the health services. Even in England and Wales it is estimated that only 10-20 % of all cases are reported (Clarkson & Fine, 1985). In Europe annual incidence rates of reported cases range between 0.2 to 62 per 100 000 population (Muller & Leeuwenburg, 1985). However in the prevaccination era it was regarded as an ubiquitious disease (Pollock et al., 1984) and the WHO estimates that in countries without a vaccination programme, 80% of surviving children will acquire pertussis in the first five years of life (Muller & Leeuwenburg, 1985).

Transmission of B. pertussis probably occurs by droplet infection, through direct contact with organisms coughed up by an infected person. The period of infectiousness lasts from the beginning of the catarrhal stage to 2-3 weeks after the onset of the paroxysmal stage (Lapin, 1943). It is not known whether a carrier state exists, which could act as a disease reservoir. B. pertussis has been isolated from individuals with respiratory disease but no charactistic cough and in other instances, from asymptomatic subjects. Whether this

represents a true carrier state or is a transient infection in a partially immune host is not known (Broome et al., 1981).

Reported case fatality rates for hospitalised cases range from 0.5 per thousand in England and Wales to nearly 14% in Uganda (Bwibo, 1971). Mortality is greater in cases below the age of one year and greatest in the first few months of life (Pollock et al., 1984)

In England and Wales whooping cough occurs in epidemic waves every 3-4 years. This epidemic pattern has been remarkably constant in spite of considerable fluctuations in vaccine uptake. This is thought to be because the total number of susceptibles has remained constant, the effect of vaccination being to reduce the incidence of infection and the disease (Fine & Clarkson, 1984). This pattern of epidemics with a three year interval has also been seen in a study in Kenya (Muller et al., 1984).

1.1.2.3 Pathogenesis.

The pathogenesis of pertussis can be considered as having four principal stages; (i) adhesion of the organism to the respiratory tract, (ii) growth and multiplication, (iii) production of local disease effects, and (iv) the production of systemic disease effects (Robinson et al.,1985b). Once the bacteria are adhering to the respiratory tract the other stages may overlap considerably, with systemic effects continuing long after initial growth and colonisation have terminated. These stages in pathogenesis are discussed below. The possible involvement of B. pertussis components is discussed in section 1.1.3.

(i) Adhesion to the respiratory tract. B. pertussis is a non-invasive organism which colonises the ciliated mucosa of the upper respiratory tract where it must attach to overcome muco-ciliary clearance. Affinity of the organism for cilia has been inferred from several studies involving human cells (Tuomanen & Hendley, 1983; Tuomanen et al.,1983) and animal organ cultures (Muse et al.,1977;

Matsuyama; 1977 and Opremcak & Rheins, 1983). Adhesion is probably a multifactoral process and several protein antigens are thought to play a role. The role of carbohydrates and the mechanisms by which B. pertussis penetrates the mucus layer are not known. The initial attachment of B. pertussis may be mediated by fimbriae which would overcome the repulsive forces that occur when the bacteria and receptor cell approach (Jones & Isaacson, 1983). After the organism has become attached other bacterial components may reinforce attachment and ensure close contact with host cells, thus providing an ideal environment for growth.

(ii) Growth and multiplication. For this stage of the infection the organism must resist the non-specific clearance mechanisms such as mucociliary flow and coughing. It is assumed that the supply of nutrients, particularly amino acids, at the mucosal surface is sufficient to allow growth. Local tissue damage, once induced, may well augment this supply. The organism must overcome any nutrient deficiences to maintain its X-mode phenotype, since a change to the antigenically deficient C-mode would cause a loss of adhesins (Burns & Freer, 1982).

(iii) Local disease. Pertussis is a localised infection and septicaemia is not produced by the organism. The infection can produce the same microscopic effects in animals (Collier et al., 1977; Muse et al.,1977) as in man (Mallory & Horner, 1912). These include attachment to cilia, ciliostasis, reduction in size and the number of cilia and extrusion of ciliated cells from the epithelium to the lumen. Thus it appears that B. pertussis causes extrusion and loss of the very cells to which it attaches. Bacteria are seen in phagocytic cells but no B. pertussis are found in or between the cells of the epithelium or the submucosa (Manclark & Cowell, 1984).

Attempts to obtain an animal model of human pertussis have failed to produce any reproducible tissue damage of the nasal or tracheal epithelium despite prolonged

colonisation of the upper respiratory tract (Preston et al., 1980; Ashworth et al., 1982b).

(iv) Systemic disease. Although the organism produces only a local infection, metabolites of B. pertussis escape from the site of infection and produce systemic effects. The major systemic effects of pertussis are largely attributable to the lymphocytosis promoting factor (LPF, see later) (Pittman, 1979). These include lymphocytosis, effects on glucose homeostasis and possible neurotoxicity and vascular permeability changes (Munoz & Bergman,1977). The importance of endotoxin in these effects is not known but it is presumed that fever is caused by endotoxin (Manclark & Cowell, 1984). The paroxysmal cough is seen as a complex response to the local tissue damage and to the neurological effects of LPF. The brain damage associated with pertusis infection may be triggered by hypoxic episodes caused by coughing, disturbances in glucose metabolism or the direct neurological effects of LPF (Wardlaw & Parton, 1983b; Olson, 1975). A survey of hospitalised cases of pertussis in Glasgow (Walker et al., 1981) showed a high incidence of complications during the disease. The percentage incidence of complications were: chest complications, 26%; prolonged apnoea/collapse, 5%, convulsions, 4%, otitis media, 6%, subconjunctival haemorrhage. 3%; rectal prolapse or hernia. 0.3%. There were 4 deaths in the survey in 647 cases.

1.1.3 Components of B. pertussis: properties and role in pathogenesis.

There are many biologically active components of B. pertussis that are involved with the stages of the pathogenesis of the disease outlined in section 1.1.2.3. The role(s) of these virulence factors in pathogenesis have mostly involved the study of isolated purified components which may be presented in a different manner than in the intact organism. Thus the roles assigned to the individual components are based on indirect rather than direct evidence.

(a) Lymphocytosis promoting factor (LPF).

This is one of the two haemagglutinins produced by B. pertussis and it is probably the major toxin involved in the pathogenesis of pertussis. It is also known as pertussis toxin or pertussigen although these terms could lead to confusion as other toxins are produced by B. pertussis. In addition, LPF is also known as histamine sensitising factor (HSF), islets activating protein (IAP) and LPF-haemagglutinin, depending on which of its biological activities are being measured. In this thesis the term LPF will be employed. As implied by its many names, LPF produces a wide range of biological effects when nanogram doses are injected into laboratory animals. These include an enhanced leukocytosis causing an increased leukocyte count with a predominance of small lymphocytes. It has been shown that the lymphocytosis was not due to the multiplication of the lymphocytes but rather to the prevention of the recirculation of these cells through the lymphoid tissue, once released from these organs. LPF produces enhanced (200-fold) senstivity of mice to histamine and a number of other vasoactive compounds. Mice vaccinated with LPF can have a 200-fold increase in sensitivity to histamine. It causes enhanced insulin secretion and adjuvanticity, especially stimulating IgE production. LPF causes laboratory animals to have increased susceptibility to anaphylactic shock, it is mitogenic for T-cells in vitro and causes enhanced induction of experimental allergic encephalomyelitis and other autoimmune diseases (Wardlaw & Parton, 1983a; Munoz et al., 1981b; Munoz, 1985).

LPF can be isolated from either the cells or supernatant of B. pertussis cultures. The precise cellular location and the mechanism of release of LPF remains to be elucidated and may depend on the growth conditions. Imaizumi et al. (1983b) have shown that the addition of 2,6-O-dimethyl β cyclodextrin to the growth medium can enhance the amount of LPF that can be detected in the culture supernatant. LPF appears as spherical particles of

about 6 nm diameter when observed in the electron microscope (Arai & Sato, 1976).

The affinity of LPF for sialoproteins has allowed its purification on columns of haptoglobin-Sepharose (Irons & MacLennan, 1979) and fetuin (Sekura et al., 1983). It has also been purified from absorption and elution from columns of Affigel blue (Sekura et al., 1983) and from affinity columns made from anti-LPF antibodies (Arai & Munoz, 1981).

The structure of LPF has been extensively studied. It is a hexamer with a molecular weight of 117 000 composed of five dissimilar units, which are named in order of their molecular sizes: S1 (28 000), S2 (23 000), S3 (22,000), S4 (11 700) and S5 (9 300) (Ui et al., 1985). An A-B subunit structure similar to some other bacterial toxins has been proposed for LPF (Tamura et al., 1982). The A (active) -protomer is composed of subunit S1 and the B (binding) -oligomer composed of the other 5 subunits. The subunits are assembled so that S1 is readily dissociable from the residual pentamer which is an association product of two dimers; S2 plus S4 (dimer 1, D1) and S3 plus S4 (dimer 2, D2) linked by means of S5 (Tamura et al., 1982).

There have been many recent reports concerning the mechanism of action of LPF on different cell systems at a molecular level. Katada et al. (1982) observed that LPF enhanced adenylate cyclase activity of rat C6 glioma cells leading to increased cAMP levels. This was subsequently found to be a result of the ADP-ribosylation of a mammalian membrane protein which was distinct from the protein ADP-ribosylated by cholera toxin (Katada & Ui, 1982). It appears that cholera toxin acts on the the stimulatory arm of adenylate cyclase regulation (N_s) and LPF acts on the inhibitory arm (N_i) by transfering ADP-ribose from NAD to N_i. Normally N_i suppresses the adenylate cyclase, but when ADP-ribosylated, N_i loses this regulatory function. This leads to an enhanced receptor-mediated activation of membrane adenylate cyclase

and a receptor-mediated accumulation of intracellular cAMP (Tamura et al., 1983). Using cell-free membrane systems from C6 cells the A-protomer was found to be the active ADP-ribosylating unit of LPF (Katada et al., 1983). The B-oligomer was responsible for binding to the cell surface and allowing the A-protomer to reach its site of action within the cell. It is also the B-oligomer that has mitogenic activity for lymphocytes (Tamura et al., 1983). Thus the biological activities of LPF may be accounted for by the mitogenic action of the B-oligomer as well as the ADP-ribosyltransferase activity of the A-protomer.

The properties of chemically modified LPF (Nogimori et al., 1986a) and of hybrid toxins, prepared from modified and unmodified monomers and dimers (Nogimori et al., 1986b) have shown that dual mechanisms are involved in the diverse biological activities of the toxin. The stimulation of lymphocytes is due to firm (divalent) binding of the B-oligomer to the cell surface. The free amino groups in the lysine residues in D2 may be involved in this type of binding (Nogimori et al., 1984). The other mechanism is the ADP-ribosylation of the N_i by the A-protomer. This is transported across the plasma membrane to its target sites within the cells as a result of binding by the B-oligomer. D1 appears to play a more important role than D2 in this binding which was not affected by chemical modification of the lysine amino groups in the LPF molecule (Nogimori et al., 1986a&b).

The LPF gene has been cloned and sequenced (Nicosia et al., 1986; Locht & Keith, 1986). It was found to occur on a DNA fragment of 4.7 kilobases which contained the genes coding for the 5 subunits (Nicosia et al., 1986).

The role of LPF in the pathogenesis of clinical pertussis is not known but it is considered to be a major toxin of the disease in the child (Wardlaw & Parton, 1983a). Some of the effects of injecting LPF into laboratory animals are similar to the symptoms of clinical pertussis, e.g. hypoglycaemia, lymphocytosis, enhanced immune response and neurological changes (Munoz & Bergman,

1977; Munoz et al., 1981b).

(b) Filamentous haemagglutinin (FHA). The other haemagglutinin of B. pertussis is known as filamentous haemagglutinin and it appears as fine filaments 2nm in diameter and 40-100 nm in length when examined in the electron microscope (Arai & Sato, 1976; Morse & Morse, 1976). FHA has been purified from static liquid culture supernatants (Arai & Munoz, 1979; Ashworth et al., 1982a) and from extracts of cells grown on agar medium (Askelof et al., 1982; Irons et al., 1983). However, Imaizumi et al. (1984) have shown that when 2,6 O-dimethyl-βcyclodextrin was added to the culture medium FHA could be purified in greatly increased quantities from supernatants of shake flask and stirred fermenter cultures. FHA can be purified from culture supernatants by column chromatography on spheroidal hydroxylapatite with elution at pH 7.0 with increasing ionic strength. Passage through haptoglobin-Sepharose or fetuin-Sepharose removes small amounts of contaminating LPF and there is a final gel filtration through Sepharose 6B to ensure that the preparation is free of any LPF (Sato et al., 1983). FHA can be distinguished from LPF by haemagglutination assays performed in tubes (Irons & MacLennan, 1979).

The physicochemical properties of purified FHA have not been as extensively studied as for LPF. The molecular weight of FHA isolated from strain Tohama of B. pertussis has been estimated to be 133 000 by sucrose density gradient ultracentrifugation (Arai & Sato, 1976). FHA purified from liquid supernatants has been found to be heterogeneous when examined by SDS-PAGE. It has been shown to have major polypeptides of molecular weight 220 000, 127 000 and 95 000 (Irons & MacLennan, 1979, Robinson et al., 1981) ,or 126 000 (Arai & Sato, 1976), and 160 000, 115 000 and 90 000 (Cowell et al., 1981). This heterogeneity has been examined by electrophoretically transferring the polypeptides separated by SDS-PAGE onto nitrocellulose paper and reacting them with different monoclonal antibodies to FHA (Irons et al., 1983). Various

polypeptides of molecular weight between 220 000 and 58 000 carried the same epitope indicating that they are probably derived from the highest molecular weight polypeptide (220 000). The heterogeneity of FHA preparations was found to be dependent on the strain of B. pertussis and the growth conditions used.

It was originally thought that FHA was derived from cell surface fimbriae (pili) and it was widely known as fimbrial haemagglutinin or haemagglutinating pili (Sato et al., 1974; Morse & Morse, 1976). The fimbrial origin of FHA was seemingly confirmed by Sato et al. (1981) when antibody that was supposedly specific for FHA was shown to react with B. pertussis fimbriae. It was suggested that this fimbrial haemagglutinin played an attachment role in the pathogenesis of the disease (Sato et al., 1981).

Ashworth et al. (1982a), however, concluded that FHA was not fimbrial in origin. This conclusion was based on studies that showed that there were differences between the degree of fimbriation and the amount of FHA extracted from cells produced in shaken and static cultures. They showed that the dimensions of FHA and fimbriae differed in the electron microscope. Fimbriae appear as long filaments 5 nm in diameter whilst FHA filaments appear 2 nm in diameter. Also fimbriae would label with polyclonal antibody to purified agglutinogen 2 but not with monoclonal or polyclonal antibodies to FHA. Furthermore, the molecular weight of FHA as determined by SDS-PAGE was very much higher than the subunit molecular weights of fimbriae of other organisms (about 20 000). Ashworth et al. (1982a) suggest that the labelling of FHA antibodies in previous work (Sato et al., 1981) may have been due to the presence of contaminating serotype-specific agglutinins in the antiserum.

The morphology of B. pertussis cells, fimbriae and FHA was studied by Blom et al. (1983) by electron microscopy. They proposed a model for the subunit structure of B. pertussis fimbriae which states that the short filamentous structures present in purified FHA originate from

fimbriae. This is obviously in disagreement with the findings of Ashworth et al. (1982a) which do not appear to have been considered by Blom et al.. FHA has been shown by immuno-electron microscopy to occur in aggregates on the surface of formaldehyde-fixed B. pertussis cells (Ashworth et al., 1986). FHA has also been shown to be a surface protein by Parker et al. (1986) using radioiodination and fluorescent labelling with monoclonal antibodies.

The probability that FHA is not fimbrial in origin does not preclude it still being involved with mediating attachment of B. pertussis to mammalian cells and FHA has been shown to be important in adherence of B. pertussis to human respiratory epithelial cells (Urisu et al., 1986).

(c) Agglutinogens. The agglutinogens of B. pertussis can be defined as surface antigens that stimulate the production of antibodies which cause bacterial cell agglutination. These antibodies were used in the original scheme for serotyping the genus Bordetella (Andersen, 1953). This scheme was later extended by Eldering et al. (1957). Smooth strains of B. pertussis have a common heat stable O antigen and one or more heat labile agglutinogens. Agglutinogens 1 and 7 are found in all strains of B. pertussis and agglutinogen 7 is common to all species of the genus. Different strains of B. pertussis may also possess agglutinogens 2 to 6 in various combinations. Agglutinogens 2 and 4 tend to occur together, as do agglutinogens 3 and 6, and it is now generally believed that there are three major agglutinogens (1, 2 and 3). It is not certain whether all these agglutinogens exist as separate components and it is thought that agglutinogens 4, 5 and 6, if they exist at all are probably minor antigens (Robinson et al., 1985b).

It has been observed that pure cultures of B. pertussis can display heterogeneity in the serotypes of the constituent organisms (Cameron, 1967; Stanbridge & Preston, 1974b; Bronne-Shanbury & Dolby, 1976; Preston et al., 1982). Starting with single colony isolates of defined serotype, Standbridge and Preston (1974b) detected

upon serial subculture, variants that had independently either lost or gained agglutinogens 2 or3. Factor 1 was always retained. There is evidence that this phenomenon also occurs *in vivo*, both in experimental animal models of infection (Standbridge and Preston, 1974a; Preston *et al*., 1980) and in the child (Preston and Standbridge, 1972). It remains to be determined whether the serotype of an organism, as defined by possession of agglutinogens 2 and 3, is a stable characteristic or if it is subject to variation, perhaps at a frequency as high as 10^{-3} or 10^{-4} per generation (Standbridge & Preston, 1974b).

Agglutinogen 2 has been purified from a sonicate of cell walls of a serotype 1,2 strain of *B. pertussis* by DEAE cellulose and Sepharose 6B chromatography (Ashworth *et al*., 1982a) and by mechanical shearing and by successive precipitations (Zhang *et al*., 1986). The purified protein in both studies gave a single band on SDS-PAGE of molecular weight of about 22 000. A rabbit antibody against this protein, when used in immune electron microscopy, labelled fimbriae on serotype 1,2 organisms (Ashworth *et al*., 1982a). An extension of this work (Ashworth *et al*., 1986) showed that purified agglutinogen 3 had a slightly smaller subunit molecular weight than agglutinogen 2. This was also noted by Fredriksen *et al*. (1986). Purified agglutinogen 2 and 3 have been found to be fimbrial in appearance when examined by electron microscopy and monoclonal antibodies to agglutinogens 2 and 3 labelled fimbriae in a serotype-specific fashion in immune electron microscopy (Ashworth *et al*., 1986). Lee *et al*. (1986) studied fimbriae from *B. bronchiseptica* and *B. pertussis*. They found that *B. bronchiseptica* fimbriae were similar to *B. pertussis* fimbriae in morphology and in the molecular size and antigenic structure of their subunits. However, the intact fimbriae of *B. bronchiseptica* and *B. pertussis* appeared to have weak serological cross-reactivity.

The nature of agglutinogen 1 is not known. It has been separated from mouse protective antigen and histamine

sensitising factor by starch block electrophoresis but this has not been characterised by modern electrophoretic methods (Ross & Munoz, 1971). The fimbrial nature of agglutinogens 2 and 3 strengthens the case that they are important in mediating attachment to the host in pertussis disease as fimbriae of other bacteria have a known role in attachment.

(d) Outer membrane proteins (OMP). Major OMP of a number of Gram-negative bacteria have been recognised as important in pathogenesis and the immune response of the host has been shown to be directed against specific OMP (Loeb & Smith, 1982; Zak et al., 1984). Parton & Wardlaw (1975) showed that the SDS-PAGE profile of B. pertussis was dependent on the phenotype mode of the organism. X-mode, virulent, organisms were found to have two proteins of a molecular weight of about 30 000 which were not found in avirulent, C-mode cells. These observations have subsequently been confirmed by other workers and other high molecular weight proteins found (Robinson & Manchee, 1979; Ezzell et al., 1981a; Robinson & Hawkins, 1983). Six polypeptides are now recognised as specific for phase 1 B. pertussis with molecular weights of 90 000, 86 000, 82 000, 33 000, 31 000 and 30 000. There are also differences in surface-exposed proteins of X- and C-mode B. pertussis (Redhead, 1983). No specific functions have, however, been ascribed to the X-mode specific proteins.

(e) Heat labile toxin (HLT). HLT, also known as dermonecrotic toxin or leinotoxin, was first described in 1909 but still very little is known about its chemistry, immunology and role in pathogenesis (Wardlaw & Parton, 1983a). The toxin is also produced by virulent strains of B. bronchiseptica and B. parapertussis. It is rapidly inactivated by heating to 56°C or by treatment with a number of chemicals including formaldehyde. The precise location of the toxin is not known but it is thought to be intracellular (Cowell et al., 1979).

HLT has been very difficult to purify (Livey & Wardlaw, 1984), partly due to its lability, although there

are several reports of the preparation of purified HLT (Nakase et al., 1969; Sekiya et al., 1982; Nakase & Endoh, 1986). HLT is a protein and its molecular weight has been determined by gel filtration to be 89 000 (Livey & Wardlaw, 1984) and 102 000 (Nakase & Endoh, 1986). Nakase & Endoh (1986) showed it to consist of two polypeptides of 30 000 and 24 000 molecular weight. The pure toxin or crude cell lysates are lethal to mice and produce marked dermonecrosis in guinea-pigs or infant mice and purified toxin produces atrophy of the spleen in certain strains of mice (Sekiya et al., 1982). Parton (1986) showed that the corticosteroids prednisolone and meclofenamate inhibit the dermonecrosis produced in mice. He suggested that these may be of benefit in treatment of the disease effects of HLT.

The mechanism by which HLT induces dermonecrosis and splenic atrophy is largely unknown but Nakase & Endoh (1986) have demonstrated that HLT inhibits an activity of an Na^+-K^+ ATPase and this induces constriction on vascular smooth muscle arising from a depolarisation of the muscle fibre. They propose the atrophy of the spleen is the result of the action of HLT on vascular smooth muscle of the splenic arterioles. HLT has also been shown to suppress the formation of antibody (Sekiya, 1983) and this is considered to result from splenic insufficiency due to its reduced blood supply rather than direct action of HLT on antibody forming cells.

Although HLT is a potent toxin present in all virulent strains of Bordetella its function in pathogenesis is unknown.

(f) Adenylate cyclase. The adenylate cyclase produced by B. pertussis has unusual properties which may indicate it has a role in pathogenesis. A significant proportion of the enzyme is extra-cytoplasmic (Hewlett & Wolff, 1976) and the activity of the enzyme is markedly enhanced by calmodulin, a protein unique to eukaryotic cell systems (Wolff et al., 1980). There are two modes of calmodulin action; a high affinity Ca^{2+}-dependent process and a low

affinity Ca^{2+}-independent process (Kilhoffer et al., 1983). It was thought that B. pertussis adenylate cyclase purified from culture supernatants was a single protein of molecular weight 70 000 (Hewlett & Wolff, 1976). However, Hewlett et al. (1986) purified adenylate cyclase from urea extracts of cells and found that it consisted of a second, high molecular weight component in addition to the 70 000 molecular weight protein. It was only the larger fraction that demonstrated aden ylate cyclase toxin activity, that is, the ability to increase cyclic AMP levels in S49 target cells. This two component structure for adenylate cyclase was confirmed by Kessin & Franke (1986) who partially purified the enzyme.

Confer & Eaton (1982) found that culture supernatants or extracts of B. pertussis inhibit the activity of macrophages. It has also been shown that B. pertussis extracts possessing adenylate cyclase activity have the ability to elicit increased cyclic AMP levels in target cells (Slungaard et al., 1983; Hanski & Farfel, 1985). They postulated that adenylate cyclase enters the target mammalian cells causing unregulated cyclic AMP production which impairs cellular functions. Shattuck & Storm (1985) showed that it is not the cyclic AMP that is taken up by the target cells but it is the bacterial enzyme that is taken up and not the calmodulin-enzyme complex. This unregulated cyclic AMP production is thought to contribute to the impaired host defences seen in Bordetella infections. Weiss et al. (1984) demonstrated that transposon mutants of B. pertussis, deficient in adenylate cyclase and haemolysin, were avirulent in an infant mouse infection model.

(g) Haemolysin. B. pertussis, when grown on Bordet-Gengou agar containing blood, produces zones of haemolysis around areas of growth. The study described above using transposon mutants deficient in adenylate cyclase and haemolysin (Weiss et al., 1984) indicates that haemolysin may have role in pathogenesis. Mutants deficient in haemolysin only had reduced virulence in the

infant mouse infection model but the reduction in virulence was more pronounced in mutants that lacked both adenylate cyclase and haemolysin.

(h) Lipopolysaccharide (LPS). The LPS of B. pertussis has many properties in common with LPS from other Gram negative bacteria in that it is heat stable, antigenic, pyrogenic and toxic (Wardlaw & Parton, 1983a). Le Dur et al. (1980) isolated two distinct LPS types by hydroxylapatite chromatography in the presence of SDS. Similar results have been obtained by examination of LPS by SDS-PAGE and this has been related to phenotypic variation (Peppler, 1984). Chemical analysis of LPS has shown that it is composed of two polysaccharides and two distinct lipids (lipid A and lipid X) (Le Dur et al., 1980; Ayme et al., 1980).

Again, a definite role for LPS in the pathogenesis of pertussis has not been elucidated but it could contribute to fever and local damage.

(i) Tracheal cytotoxin (TCT). TCT has been isolated from B. pertussis culture supernatants as a small glycopeptide containing diaminopimelic and muramic acids (Goldman et al., 1982) which indicates that it probably originates from the cell envelope. TCT mimics completely the ciliated cell pathology characteristic of pertussis. TCT also causes a dose dependent inhibition of DNA synthesis in cultured hamster trachea epithelial cells, which provides an assay for its measurement during purification (Goldman & Herwaldt, 1986).

1.2 Vaccination against pertussis.

1.2.1 A history of immunisation against pertussis.

Soon after it was demonstrated that the causative agent of pertussis could be cultured from clinical materials there was much research to determine the best method of growing B. pertussis so that a vaccine could be produced. Cultures for early vaccines were grown on Bordet-Gengou (BG) agar and the cells were inactivated by a variety of physical and chemical means. Vaccines essentially fell into one of three groups. Those that were effective in preventing disease, those that were too toxic for human use and those that had low toxicity but seemed to have no effect in preventing the disease (Manclark & Cowell, 1984). The problem was that there was no means for testing for efficacy before use.

Madsen (1933) reported on the results of a clinical trial in the Faroe Islands during the whooping cough epidemic of 1923-24. The proportion with disease was the same in both the unvaccinated and vaccinated groups, but the disease was milder and there were fewer deaths in the group that had received vaccine. A second study in 1929 showed more encouraging results. In the unvaccinated group 98% contracted the disease, whereas 75% were protected from disease by the vaccine.

Kendrick, in the USA, began working on pertussis in the early 1930's. She prepared a vaccine using freshly isolated strains of B. pertussis grown on BG medium containing sheep blood and the vaccine concentrates were inactivated by storage at 4°C with thiomersal. A clinical trial began in 1934 and it was shown that the subjects who received the vaccine had significantly fewer cases of pertussis than were reported in the unimmunised control group (Kendrick & Eldering, 1936). At the same time another study (Doull et al., 1936) showed no significant protection from disease by a pertussis vaccine. Kendrick & Eldering realised there was a need to measure the content and potency of vaccines so that results obtained in a

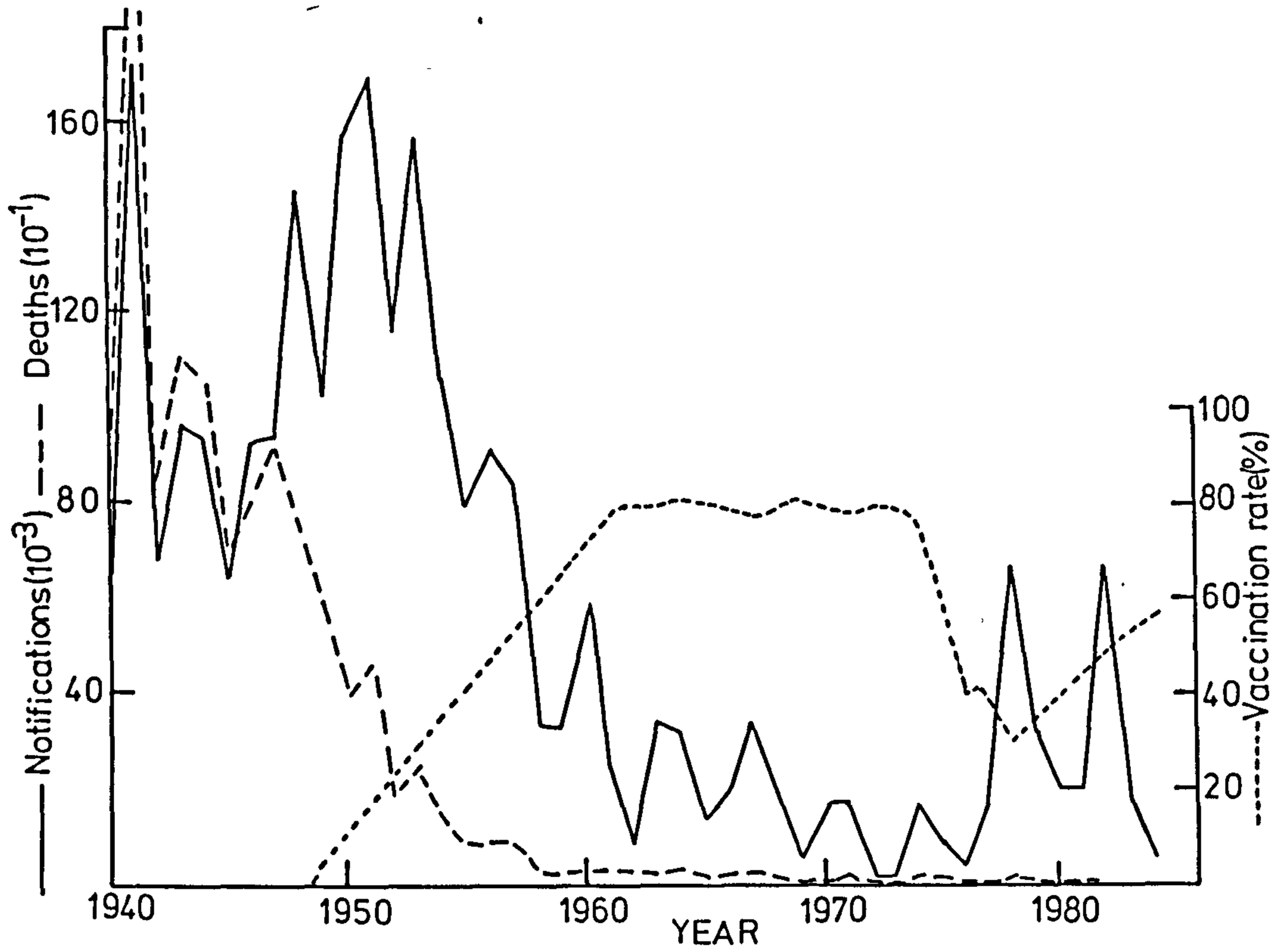
clinical trial might be comparable. This resulted in the concept of Opacity Units as a measure of bacterial content (Manclark & Cowell, 1984), They also developed the mouse intracerebral (i.c.) challenge test for vaccine potency and additional trials performed in the late 1930's and 1940's related clinical efficacy to the potency of vaccine as measured in the mouse test (Kendrick et al., 1947).

Vaccines standardised with the mouse (i.c.) test were included in the field trials in England carried out by the Whooping Cough Immunisation Committee of the Medical Research Council (MRC). The results of the MRC trials (MRC, 1951, 1956) showed that the clinical efficacy of pertussis whole cell vaccines correlated with laboratory potency as measured in the mouse intracerebral challenge test. It has been official policy to recommend pertussis vaccination in the U.K. since that time (Committee on Safety of Medicines and the Joint Committee on Vaccination and immunisation, 1981).

1.1.2 Vaccine acceptance rates and the incidence of pertussis in the U.K..

Vaccine acceptance rates and the numbers of notifications and deaths due to pertussis in England and Wales are shown in fig. 1. It can be seen that there are three phases of the history of pertussis in the U.K.. During the first pre-vaccination phase, which ended in the 1950's, pertussis was a common and dreaded disease of childhood with a high annual mortality. In the second phase, from the mid 1950's, there was a marked reduction in the incidence of pertussis. Whether this reduction was due to the introducton of mass immunisation in the 1950's, and/or simply due to improvements in socioeconomic conditions, nutrition and health care has been the subject of much discussion (Dick, 1975; Bassili & Stewart, 1976). In lesser developed countries in recent years the mortality rate has been aproximately 2% (Cook, 1979) which is similar to that in Britain and the USA many decades ago. In the 1978-79 epidemic in Britain, there were 36

Fig. 1. Vaccine acceptance rates and the numbers of notifications and deaths due to pertussis in England and Wales.



Data compiled from Communicable Disease Reports, PHLS Communicable Disease Surveillance Centre, Colindale.

deaths among 102 000 notifications, a rate of 0.035% (Miller et al., 1982).

The most recent phase followed considerable adverse publicity concerning the incidence of brain damage that was alleged to be related to pertussis vaccination. Public concern over vaccine safety was reflected in a marked reduction in vaccine acceptance rates nationally. Thus, 70-80% of children completed a course of pertussis vaccination by the end of their second year in 1964-74, but only 30% did so in 1978. This reduction in vaccine uptake coincided with three epidemics of pertussis, in 1978-79, in 1981-82 and in 1985-86, which were the most serious since the inception of mass vaccination. Recently vaccine acceptance rates have begun to rise gradually, encouraged by a report from the Department of Health and Social Security Joint Committee on Vaccination and Immunisation (1981) and a vaccination publicity campaign.

These recent epidemics in the U.K. have provided an opportunity to assess the efficacy of the vaccine statistically. The inverse relationship between vaccine acceptance rates and the incidence of pertussis nationally strongly suggests that the vaccine is effective. Various studies have shown that the vaccine is effective, with estimates of efficacy reaching over 90% (Robinson et al., 1985b).

1.2.3 Adverse reactions to whole cell pertussis vaccines.

Vaccination against pertussis is regarded as an effective and beneficial public health measure and it is recommended in the U.K. (DHSS, 1981). The disease is still serious, especially in the very young and it is deemed that the benefits of protection by vaccination outweigh the risks associated with the vaccine. However, certain infants are excluded from vaccination if they display the following contraindications: history of seizures, convulsions or cerebral irritation or a history or family history of epilepsy, any febrile illness until the patient is fully recovered or any previous reaction to a pertussis

vaccine dose (Joint Committee on Vaccination and Immunization, 1977).

The toxic side effects of vaccination are of major concern in any vaccination programme and form the other side of the risk and benefit debate in the usage of the vaccine. Reaction to pertussis vaccines can be considered under two main headings:

(a) Mild and moderate reactions. These are the transient reactions which are no lasting cause for concern. Such reactions are fairly common and include pain, swelling and redness at the site of injection, fever and irritability.

(b) Severe reactions. These are severe neurological reactions and include persistent screaming, shock, collapse, febrile and non-febrile convulsions, permanent brain damage and death. The problem in assessing the incidence of these severe neurological reactions is made difficult by their low frequency and by the lack of a specific pertussis vaccine syndrome. Also, as already stated, vaccines are not identical and are therefore likely to differ in reactogenicity. Thus data from different areas using different vaccines may not be comparable and comparison with data from early studies may not be applicable if methods of manufacture and the strains used have changed. Also, some of the reactions attributed to pertussis vaccine may have arisen from other undefinable causes and became implicated with the vaccine spuriously due to a co-incidence of timing. As the incidence of spasms, convulsions and seizures is relatively high in children of vaccination age, some reactions will occur purely fortuitously within 72 h of vaccination and be wrongly attributed to the vaccine (Griffith, 1978).

Because of concern over the numbers of possible neurological disorders following pertussis vaccination, estimated by Stewart (1977; 1979) to be between 1:10 000 and 1:60 000, the National Childhood Encephalopathy Study (NCES) was set up to assess the risk of severe

neurological reactions to vaccines and to identify other factors involved in the development of such disorders. The main conclusions of the NCES were that:

(a) The great majority (over 95%) of cases of neurological disorder were not associated with recent (within 28 days) immunisation with pertussis vaccine.

(b) The overall modal age of onset of disease with permanent sequelae was in the range 4-10 months, coincidently the age for primary immunisation with pertussis vaccine.

(c) Such illnesses occur more frequently than predicted within seven days of diptheria-tetanus-pertussis (DTP) vaccination.

(d) The estimated attributable risk of a serious neurological disorder within 7 days following DTP vaccination in previously normal children was 1 in 110 000 immunisations (95% confidence limits, 1 in 44 000 to 1 in 360 000 immunisations). The corresponding figures for permanent (1 year later) brain damage was 1 in 310 000 (95% confidence limits, 1 in 54 000 to 1 in 5310 000) immunisations. The NCES report states that permanent brain damage is a very rare event and attribution of a cause in individual cases is precarious (DHSS, 1981).

It has been claimed that pertussis vaccine can produce a mild fever that may precipitate a neurological disorder in a predisposed child, who may have suffered from such an outcome from fever mediated by any cause e.g. the common cold (Griffith & Freestone, 1981). Thus the pertussis vaccine may be merely hastening an inevitable response. Data from the NCES show that this is likely to be the case for infantile spasms following pertussis vaccine (Bellman et al., 1983). Pollock & Morris (1983) concluded from a seven year study of disorders attributed to vaccination that there was no convincing evidence that DTP vaccination caused major neurological damage in children although it may sometimes provoke a febrile convulsion.

1.2.4 Acellular pertussis vaccines.

1.2.4.1 Reasons for development of acellular pertussis vaccines.

It is generally thought that the current whole cell pertussis vaccine provides a moderate to high level of protection but that in a very small number of children it may provoke a severe neurological reaction. In spite of the belief that the benefits of using the current vaccine outweigh the risks (DHSS, 1981), there are still reasons for the development of an acellular pertussis vaccine.

(i) It is known that there is some inherent toxicity in the whole cell pertussis vaccine and that it commonly produces mild reactions. It has also been implicated in the rare triggering of neurological disorders so it is of great importance to develop a completely non-toxic pertussis vaccine.

(ii) There is concern that the degree of protection provided by the whole cell vaccine is not as solid as would be wished, particularly in the case of home contacts (PHLS, 1982). Fine & Clarkson (1982) have shown from epidemiological studies that the vaccine may protect principally against the symptoms of the disease, rather than the infection, and that this may lead to vaccinated chidren becoming asymptomatic carriers of the disease. A defined acellular vaccine would be designed to protect against both the infection and the disease.

(iii) It is now over thirty years since the introduction of mass immunisation against pertussis and in that time there have been considerable advances made in immunology, biochemistry and the understanding of microbial pathogenicity. It is now possible to identify the important antigens of B. pertussis which can induce a protective immune response and to purify these components for inclusion in a vaccine. Using purified components it should be possible to produce vaccines of defined composition which are constant in protective potency and in lack of toxicity.

(iv) A new acellular pertussis vaccine is required to

restore public confidence in pertussis vaccination that was lost because of reports of adverse reactions to the existing pertussis vaccine. The restoration of widespread confidence would allow vaccine aceptance level to rise to those that are required for effective herd immunity to the disease.

1.2.4.2 Components of *B. pertussis* for inclusion in acellular pertussis vaccines.

There is nearly universal agreement that LPF is an important protective antigen and that appropriately detoxified LPF should be a component of an acellular vaccine (WHO, 1985). It has been claimed that LPF is the major i.c. protective antigen of *B. pertussis* and that antiserum raised against LPF will passively protect mice (Munoz *et al*., 1981a). However purified native LPF is non-protective in the i.c. test and it is only protective when its toxicity is destroyed by toxoiding (Munoz *et al*., 1981b; Robinson & Irons, 1983). Toxoided LPF is also protctive in the mouse intranasal respiratory infection model (Robinson *et al*., 1986a).

FHA is also considered to be a candidate for inclusion due to its low toxicity and protective activity against animal infections. However FHA rigorously purified of LPF is not protective in the i.c. test (Munoz *et al*., 1981a) but mixtures of FHA and LPF were found to be highly protective (Robinson & Irons, 1983). FHA is protective in the mouse intranasal test (Robinson *et al*., 1986a) and antibodies to FHA protect rabbits and the protection correlated with level of IgA antibody in nasal washings (Ashworth *et al*., 1982b).

Opinion is divided whether serotype specific agglutinogens should be included. The change in the prevalent serotype after the introduction of vaccination in Britain and the recent reversion when immunisation declined indicates that protection is, at least in part, serotype specific (Preston, 1986). It is also the case that protective activity of agglutin ogen 2 and 3

preparations in the i.c. test is greatly enhanced by the presence of added LPF (Robinson & Irons, 1983). Agglutinogens are also protective in the mouse intranasal infection model (Robinson et al., 1986a).

Extracts of B. pertussis containing X-mode specific OMP are highly protective in the mouse i.c. and intranasal infection model but again, treatment to abolish LPF activity also reduces protective potency in the i.c. test, which can be restored by readdition of LPF (Robinson & Hawkins, 1983; Robinson et al., 1986a). It has been postulated that this enhancement of protection in the mouse i.c. test by LPF is due to the LPF altering the permeability of the blood brain barrier in the mouse, allowing access of antigens or antibodies and immune cells into the brain (Robinson & Irons, 1983). LPF can also enhance the protective effect of various antigens in the mouse intranasal infection model but preparations devoid of active LPF, that are non-protective in the i.c. test, can still protect against respiratory infections with B. pertussis (Robinson et al., 1986a).

A different line of thought on vaccine components is given by Novotny et al. (1986) who show mouse intracerebral protective activity of Bordetella adenylate cyclase and they state that adenylate cyclase is a "mandatory component of any future subcellular vaccine against Bordetella species".

There is aggreement, however, that a future component vaccine should contain little or no endotoxin (LPS).

1.2.4.3 Early vaccines.

A number of acellular pertussis vaccines have been proposed since the development of the whole cell vaccine. The first acellular vaccine was developed by Pillemer, who extracted sonicated B. pertussis cells and absorbed this onto human erythrocyte membranes (stromata). This stromata-protective-antigen complex was effective in protecting mice in the Kendrick test. It was included in the M.R.C. clinical trials in the 1950's but it

unfortunately caused too many untoward reactions (Parish, 1965).

An acellular pertussis vaccine was prepared from a salt extract of B. pertussis cells (Weihl et al., 1963) and this was produced from 1962 to 1977 by the Eli Lilly company as a component of their DPT Adsorbed vaccine (Manclark & Cowell, 1984). A similar vaccine was developed by Wyeth Laboratories Inc., which was protective in Kendrick test and a limited clinical trial of this vaccine showed it to be antigenic but reaction rates did not differ markedly from whole cell vaccines (Manclark & Cowell, 1984). Helting & Blackkolb (1981) proposed extraction of cells with urea-salt followed by adsorption onto a mineral carrier. This vaccine was later withdrawn from clinical trial (Robinson, et al., 1985b). These vaccines are still closely related to the whole cell vaccine and are no real advantage over the existing vaccine as they are not defined.

1.2.4.4 The Japanese acellular pertussis vaccine.

The Japanese acellular vaccine (Sato et al., 1984), which contains mainly toxoided LPF and FHA, is prepared from the culture supernatant of B. pertussis. The culture supernatant is treated with ammonium sulphate and a crude extract of the haemagglutinins is extracted from the precipitate using phosphate buffer containing 1M sodium chloride.This crude extract is fractionated by a sucrose density gradient to obtain a preparation of haemagglutinins practically free of endotoxin. The preparation is treated with formalin to destroy the activities that cause leukocytosis promotion and histamine sensitisation and aluminium hydroxide or phosphate is added as adjuvant. According to the Japanese workers (Sato et al., 1984) the new vaccine has an acceptable level of potency, as measured in their modified mouse potency assay, but it contains less than one tenth of the specific toxicities associated with the whole cell vaccine (leukocytosis, histamine sensitisation and endotoxicity).

However, when the Japanese vaccine was assayed by a standard mouse potency test it was found to be less potent than the whole cell vaccine (WHO, 1985).

The Japanese vaccine has been termed by them the Precipitated Purified Pertussis Vaccine and it has been used, mixed with diphtheria and tetanus toxoids, in mass immunisation of mainly two year-old children in Japan since the autumn of 1981 and by 1984 $15x10^6$ doses had been given (Kimura & Hikino, 1986). It should be noted that the efficacy of the vaccine is still under study although results of small scale household contact studies have yielded vaccine efficacy estimates ranging from 78%-92% in children of 1 year or older (Noble et al., 1987).

Since the pertussis component of the vaccine is rigorously toxoided with formalin and the vaccine contains much less bacterial mass and endotoxin than the whole cell vaccine, it would be expected to be less reactogenic when injected into children. Comparisons of whole cell and acellular vaccine have shown that the latter does indeed induce fewer febrile reactions and less erythema and induration than the whole cell vaccine (Kimura & Hikino, 1986). This has been confirmed in recent phase II trials of the Japanese vaccine carried out in Sweden (Olin, 1986).

It is implied from the name "Precipitated Purified Pertussis Vaccine" that a single vaccine has been studied and used in Japan, but this is not the case (WHO, 1985). Although the six licensed manufacturers employ a prescribed manufacturing procedure, there are differences not only between the vaccines produced but also variations from lot to lot which are inherent in the production method. Some formulations contain only toxoided LPF and FHA and no detectable agglutinogens, whereas other formulations contain appreciable amounts (10%) of agglutinogens and other unidentified antigens in addition to toxoided LPF and FHA. The ratio of LPF:FHA also varies between vaccines from 1:1 to 1:9. These differences in vaccine formulation clearly complicate the evaluation of

clinical trial data, especially as it seems that no record has been made of the formulation used for any or all of the vaccinations received by each subject (WHO, 1985). Nevertheless, this is the first acellular pertussis vaccine to be used in a mass immunisation programme and much useful data on its reactogenicity and efficacy has been obtained.

1.2.4.5 The CAMR acellular vaccine.

The CAMR vaccine can be considered as being a further development in pertussis vaccines as it has a defined content of purified antigens. The CAMR acellular vaccine consists of separately purified and detoxified LPF, FHA and fimbriae (Agglutinogens 2 and 3). Purified FHA and agglutinogens 2 and 3 sometimes contain traces of LPF so all three antigens are independently toxoided and are blended together in the presence of alhydrogel or aluminium phosphate (Robinson et al., 1985a). It has been proposed that a single human dose of vaccine will contain 10 ug each of detoxified LPF, FHA and agglutinogens 2 and 3 (U.K. Patent application No. 8601279, January 1986).

The Porton acellular vaccine had protective activity in the mouse intracerebral challenge test although the protective potency was considerably less than the whole cell vaccine (Robinson et al., 1985a). An alternative potency test is the mouse intranasal protection test (Robinson et al., 1986a) in which mice are immunised and then challenged 14 days later by intranasal instillation of live B. pertussis. Both the acellular and the whole cell vaccines dramatically reduced the number of B. pertussis oganisms recovered from the lungs of immunised mice (Robinson et al., 1986a). The CAMR vaccine has been tested in adult volunteers and had low reactogenicity and produced good serum antibody responses to vaccine antigens, measured by ELISA (Rutter et al., 1988). Phase II trials involving children are planned.

The defined acellular vaccine should have several advantages over the whole cell vaccine. It is of defined,

constant antigenic content and it has reduced toxicity when examined by the mouse weight gain, histamine sensitisation or lymphocytosis promoting tests. Both the acellular and the whole cell vaccine induce high levels of antibodies to FHA in mice, but the acellular vaccine produces higher titres of antibodies in mice to LPF and to agglutinogens 2 and 3. It is hoped that the acellular vaccine will induce high antibody levels to these antigens in the child and thus protect against both the infection and the disease.

1.3 *In vitro* growth of *B. pertussis*.

B. pertussis is a fastidious, slow growing organism that is difficult to grow in culture. The development of an acellular pertussis vaccine has required large volumes of culture containing LPF, FHA and agglutinogens in sufficient quantity to purify. The growth of *B. pertussis* and expression of virulence determinants has therefore been studied.

1.3.1 The development of growth medium.

The existence of *B. pertussis* was known for some years before it was succesfully grown on artificial media. Bordet & Gengou (1906) developed a solid medium containing a glycerol-potato extract and 50% blood. This was the only medium for the isolation and continued cultivation of *B. pertussis* until Hornibrook (1939) described a liquid medium containing amino acids, cysteine and starch. With the introduction of Hornibrook's and related media, the growth requirements of *B. pertussis* were investigated and various inhibitors of growth identified. The development of early solid and liquid media is reviewed by Rowatt (1957b).

(a) Solid medium. Workers attempting to develop media other than Bordet-Gengou (B-G) for the growth of *B. pertussis* did not realise that cells grown on, for example, nutrient agar were antigenically different from fresh isolates. Leslie & Gardner (1931) showed that freshly isolated strains were antigenically distinct from cells grown on simplified media, these cells being phase IV or degenerate strains (see section 1.3.4). Several media were devised to replace B-G agar (Silverthorne & Cameron, 1942; Dawson *et al*., 1951) but none proved superior.

Pollock (1947) showed that blood in the medium could be replaced by serum, serum albumin or charcoal and that isolates maintained on such media retained phase I characteristics. Liquid media solidified with agar were

not found suitable for growth of B. pertussis unless blood was added (Verway et al., 1949; Mazloum & Rowley, 1955). Parker (1976) developed a solid medium incorporating agarose for use in genetic studies of B. pertussis where a clear medium is required. A solid medium with charcoal and 10% defibrinated horse blood has been developed by Oxoid (CM119) and this, together with B-G agar are the most widely used solid media for the growth of B. pertussis.

(b) Liquid medium. The first successful liquid medium was that of Hornibrook (1939) and contained hydrolysed casein, starch, salts, cysteine and yeast extract. Most subsequent liquid media are based on this. In a later modification Hornibrook (1940) replaced the yeast extract with nicotinamide. The medium was further modified by Cohen & Wheeler (1946) by increasing the buffering capacity, adding ferrous and copper sulphates and increasing the starch content. They also added casamino acids (Difco) to replace the hydrolysed casein. This was similar from batch to batch and allowed more uniform cultures to be produced. Sutherland & Wilkinson (1961) and Morse & Bray (1969) described modifications to Cohen and Wheeler medium which contained either anionic exchange resin or charcoal. Several recipes for chemically defined media have been devised (Jebb & Tomlinson, 1955, 1957; Wilson, 1963; Goldner et al., 1966). All these media were very complex and no defined medium was widely used until the medium of Stainer & Scholte (1971). This was a much simplified medium giving improved growth yields, containing proline, glutamic acid, cystine, salts, tris buffer, ascorbic acid, nicotinic acid and glutathione.

1.3.2 Growth requirements and effect of toxic compounds.

(a) Growth requirements. B. pertussis does not oxidise or ferment any sugars (Rowatt, 1957b). Its major metabolic activity is the oxidation of amino acids to give carbon dioxide and ammonia. Rowatt (1955) observed that B. pertussis oxidises glutamate, serine, alanine, proline and aspartic acid. The uptake of amino acids by B. pertussis

was studied by Vajdic et al. (1966). They showed the primary importance of glutamic acid for the growth of B. pertussis as it was rapidly utilised during exponential growth. Other amino acids were taken up when the glutamic acid acid was exhausted and the culture entered stationary phase. Cysteine was included in the medium of Hornibrook (1939) and it was demonstrated to be necessary for growth but it could be replaced by cystine or glutathione but not by methionine. This was confirmed by Proom (1955). Hornibrook (1940) showed that nicotinamide was an essential growth factor for B. pertussis and this or nicotinic acid have been included in all subsequent media.

(b) Effect of toxic compounds. Growth of B. pertussis is affected by several growth inhibitors present in the culture medium. These include fatty acids, sulphur and peroxide (Rowatt, 1957b). Pollock (1947) suggested that the function of the blood in B-G medium was to overcome the inhibition of growth by fatty acids. He found that the blood could be replaced by starch, albumin or charcoal. One of the most important factors in Hornibrook's medium was the inclusion of starch. It was originally thought that this was metabolised by the organism but Ungar et al. (1950) showed that the starch was not used and Rowatt (1957b) suggested that it may adsorb fatty acids. Sutherland & Wilkinson (1961) used anion exchange resin in place of starch to adsorb fatty acids and they claim that this medium solidified with agar provided comparable growth with B-G medium. More recently, Field & Parker (1979) studied the inhibitory effects of fatty acids on growth of B. pertussis in defined medium. They found that B. pertussis was affected by saturated fatty acid but it was particularly sensitve to unsaturated fatty acids and that 0.005mM oleic, elaidic or petroselinic acids completely inhibited growth.

Proom (1955) showed that B. pertussis would not grow in medium containing autoclaved cysteine as colloidal sulphur and sulphides formed on autoclaving inhibited growth. Improved growth was obtained when cysteine was

omitted from the medium before autoclaving and filter sterilised cysteine added subsequently (Rowatt, 1957a). A third inhibitor of growth, probably peroxide formed on autoclaving, was described by Rowatt (1957a) and this was neutralised by adding lysed red blood cells, haemin or filtered ferrous sulphate.

Novotny & Cownley (1979) claimed that B. pertussis was extremely sensitive to high quality stainless steel, especially when grown in defined medium. They suggest that this may be due to manganese present in the steel being inhibitory to the growth of B. pertussis. This was refuted by Cameron et al. (1985). They showed that equivalent growth of B. pertussis could be obtained in stainless steel and glass vessels.

1.3.3 Antigenic modulation.

Antigenically deficient forms of B. pertussis can be produced by two distinct mechanisms: (a) changes in the growth environment can cause a reversible antigenic transition, termed antigenic modulation and (b) successive subculture in vitro can cause a loss of virulence known as phase variation (section 1.3.4).

Lacey (1960) reported that changes in the growth medium could cause a reversible loss of virulence characteristics in B. pertussis. Growth on medium containing NaCl produced virulent organisms, the X-mode phenotype, whereas growth on medium containing $MgSO_4$ gave organisms with the avirulent C-mode phenotype. Lacey called this reversible change antigenic modulation. He found that some ions favoured X-mode growth and others favoured C-mode organisms. An intermediate I-mode was observed under conditions intermediate between those leading to X- and C-mode growth (Lacey, 1960). However this has not been reported by other workers and it may be that populations tested were intermediate whilst the individual organisms were not.

Subsequently Pusztai & Joo (1967) observed that a similar antigenic modulation could be induced by

increasing the concentration of nicotinic acid (NA) in the medium.

1.3.3.1 Changes observed during antigenic modulation.

During antigenic modulation several components of B. pertussis are lost (table 1). It appears that the changes observed during antigenic modulation caused by $MgSO_4$ are very similar to those caused by an increased nicotinic acid (NA) concentration, except that Pusztai & Joo (1967) reported a different serological response. McPheat et al. (1983) reported that NA modulation resulted in a loss of agglutinogens 2 and 3, but an increase in agglutinogen 1. It has also been reported that antigenic modulation induced by NA does not produce a complete loss of HLT (Livey et al., 1978).

1.3.3.2 Environmental conditions affecting antigenic modulation.

Lacey (1960) determined the modulating potential of a range of salts assessed in different ratios. X-mode growth was favoured by lithium, sodium, potassium and ammonium cations, and chloride, bromide, iodide and nitrate anions. For C-mode growth the most effective cations were magnesium, strontium and calcium especially in combination with sulphate, selenate, butyrate or glutarate as the anion. The I-mode was observed with X-mode cations with lactate, malate, fumarate and several other anions. Lacey also observed that C-mode growth was obtained when the B-G plates were incubated at 25°C. Brownlie et al. (1985a) examined the effect of various inorganic and organic salts on adenylate cyclase activity, histamine sensitising activity (HSA) and X-mode specific polypeptides. They found $MgSO_4$, Na_2SO_4, sodium lactate, sodium succinate, sodium butyrate and sodium caprylate all caused a loss of these X-mode characteristics, but there was never a case where one of these was lost independently of the others.

Pusztai & Joo (1967) reported that antigenic modulation was induced by increasing the concentration of

Table 1. Factors and attributes lost by *B. pertussis* during antigenic modulation.

Property.	References.
LPF.	Parton & Wardlaw (1975); Idigbe *et al*. (1981).
Agglutinogens.	McPheat *et al*. (1983).
HLT.	Livey *et al*. (1978); Idigbe *et al*. (1981).
X-mode specific polypeptides	Wardlaw *et al*. (1976); Idigbe *et al*. (1981); Schneider & Parker, (1982); Ezzell *et al*. (1981a); Dobrogosz *et al*. (1979).
Adenylate cyclase.	Parton & Durham, (1978); Hall *et al*. (1982); McPheat *et al*. (1983).
Haemolysin.	Lacey (1960).
Cytochrome d629.	Dobrogosz *et al*. (1979); Ezzell *et al*. (1981b)
Adhesion.	Burns & Freer, (1982); Redhead (1985).

NA in the growth medium from 0.05 to 0.5 g/l. Schneider & Parker (1982) examined several pyridines and compounds resembling pyridines for their ability to affect modulation. They found that 6-chloronicotinic acid and quinaldic acid were more effective modulating stimuli than NA on a molar basis. 2-chloronicotinic acid and isoniazid on the other hand interfered with NA induced modulation and were termed antimodulators. Modulation cannot be induced by increasing the concentration of nicotinamide (nicotinic acid amide) in the growth medium (Wardlaw et al., 1976).

1.3.4 Phase variation.

Upon repeated subculture in vitro B. pertussis strains may lose the same virulence-associated antigens lost in antigenic modulation. This process is termed phase variation and in contrast to antigenic modulation it is not freely reversible in response to changes in the growth environment. The avirulent variant produced by phase variation has an increased resistance to certain antibiotics and fatty acids (Dobrogosz et al., 1979; Field & Parker, 1979; Peppler & Schrumpf, 1984; Bannatyne & Cheung, 1984). The increased resistance to fatty acids may explain the ability of avirulent variants to grow on media which do not support the growth of virulent phase organisms (e.g. media not supplemented with charcoal, blood, starch or cyclodextrin). The avirulent variants form flat colonies on B-G agar in contrast to the domed colonies of the virulent organism.

The process of phase variation has not been adequately defined. Leslie & Gardner (1931) classified four distinct stages: I (as isolated) and II being toxic to guinea pigs and III and IV being relatively non-toxic. Later work (Lawson, 1939; Flosdorf et al., 1941; Standfast, 1951; Kasuga et al., 1953 and 1954) showed that the degradation process was not so simply defined. Lawson (1939) proposed that there were many more intermediates, depending on the strain and environmental conditions, and that the phase

changes proceeded gradually, making them difficult to distinguish. More recently, Parker (1976) proposed that only the terms "fresh isolate", "intermediate strain" and "degraded" strain should be used to describe phase variation. However the phase I/phase IV classification is still in common usage to denote the extremes of the phase types.

It is unclear how the transition from the virulent to the avirulent form of B. pertussis proceeds. One view is that it is a multistep process with the various virulence factors being lost in either a random (Standfast, 1951; Parker, 1976, 1979) or in a ordered way (Leslie & Gardner, 1931; Goldman et al., 1984). Alternatively traits may be lost and regained in a single step event (Weiss & Falkow, 1984).

Weiss & Falkow (1984) used increased tolerance to erythromycin as a virulent phase marker to select B. pertussis colonies that lacked haemolysin, LPF and FHA. The proportion of these avirulent forms in the virulent phase population was estimated to be between 10^{-3} and 10^{-4}, depending on the strain. Weiss & Falkow (1984) found this change to be reversible as reversion to the haemolytic form was observed. These isolates had also regained LPF, FHA and sensitivity to erythromycin. This reversion seemed influenced by environmental factors as only certain batches of medium favoured the selection of virulent organisms, which may be why this has not been seen by others (Peppler, 1982; Goldman et al., 1984). Weiss & Falkow (1984) stated that this reversible variation appeared to be a single step event and this was further confirmed by the observation that a single insertion of transposon Tn5 at a particular site on the B. pertussis chromosome caused the loss of not only LPF, FHA and haemolysin but also adenylate cyclase and HLT.

It appears then, that the loss of virulence factors from B. pertussis can occur in a single step, reversible process although there may be other ways in which avirulent variants arise. The complete loss of virulence

factors does not appear to be concomitant with the aquisition of the ability to grow on nutrient agar as the avirulent Tn5 mutant of Weiss & Falkow does not grow on nutrient agar. Thus growth on nutrient agar seems to be an additional attribute aquired in a process distinct from the loss of virulence characteristics.

1.3.5 Growth in continuous culture.

In the natural environment bacteria are subjected to a wide variation in the availability of essential nutrients. However, it is nearly always the case that natural environments are nutrient limited. Consequently it is of great interest to study the response of populations of microorganisms to nutrient limited conditions. Experimentally such conditions can be imposed on microbial populations in a flow-controlled continuous culture system. The structural and functional changes in microbes on exposure to low nutrient environments have been reviewed by Harder & Dijhuizen (1983).

The study of pathogenic microorganisms in low nutrient environments is thought to be important as growth in a nutrient rich environment may produce organisms incomplete in determinants of virulence (Brown & Williams, 1985). The specific conditions in vivo, e.g. limiting nutrients and host defence mechanisms, may produce the conditions for optimal expression of virulence determinants. This is seen in that bacteria often lose virulence by growth in vitro, which is restored by animal passage (Smith, 1977). The limiting nutrients in the host that are thought to be important in influencing the pathogen include phosphate, zinc, magnesium and iron (Brown & Williams, 1985). Of these iron has been the most studied.

Iron is an essential nutrient that plays a role in the metabolism of almost all living cells, including bacteria. However, in animals iron is typically unavailable to bacteria. At neutral pH and in aerated systems, iron exists primarily as insoluble polymeric hydroxides (Bullen, 1981). Also many body fluids, including mucosal

secretions contain the iron-binding protein transferrin and/or lactoferrin, both of which have an extremely high association constant for Fe^{3+} and are normally only partially saturated. The amount of free iron in equilibrium with these proteins is of the order of 10^{-18} M, which is too low for bacterial growth (Griffiths et al., 1983). To overcome this many bacteria synthesise high affinity iron-transport systems. These consist of low molecular weight iron chelating agents (siderophores) and receptor proteins involved in the uptake and transport of the siderophore-iron complexes across the bacterial membrane (Griffiths, 1983). Iron is a ubiquitous contaminant of most ingredients of culture media, therefore iron deprivation in vitro requires special procedures involving either depletion of iron by treatment with an iron exchange resin (Brown et al., 1984), or by restriction of available iron by adding a biological or synthetic chelator (Griffiths, 1983). Iron limitation in continuous culture has been studied with Pseudomonas aeruginosa and an altered penicillin-binding-protein profile was observed (Turnowsky et al., 1983).

In addition to phenotypic variability, a pathogen growing in continuous culture is also subjected to genotypic selection. Under the selective pressure of continuous culture, avirulent genotypes often come to predominate because they do not have the burden of producing antigens not required in vitro. Thus chemostat culture of pathogens has sometimes led to the irreversible production of avirulent cultures of no practical value in vaccine manufacture (Manchee et al., 1980). This has been seen for Brucella abortus (Hauschild & Pivnick, 1961), Pasteurella pestis (Pirt et al., 1961), Neisseria gonorrhoeae (Brookes & Sikyta, 1967) and for B. pertussis (Van Hemert, 1974).

Continuous culture is defined here as the process in which, by continuous addition of medium and simultaneous withdrawal of culture fluid, a so-called "steady state" is established (Van Hemert, 1974). The theoretical basis for

this method of culture was first described by Monod (1950) and by Novick & Szilard (1950) and the theory of the chemostat with later modifications is described by Tempest (1970). The main points are the following:

(a) In the steady state, the dilution rate D (i.e. the replacement per hour, divided by the culture volume) becomes equal to the exponential growth rate u (i.e. 1/N dN/dt, where N is the bacterial density). Thus:

$$D = u\ (h^{-1})$$

(b) From this relationship of dilution rate with the doubling time (t_d) is easily deduced:

$$D = \frac{\ln 2}{t_d} = \frac{0.69}{t_d}$$

(c) The system is self-regulating; if the bacterial concentration increases, the medium (or more precisely the limiting factor) is further depleted, resulting in a decrease in the growth rate. This results in a decrease in the bacterial population, until a steady state is again reached. In the reverse case, the growth will increase until a factor in the medium becomes limiting and a steady state is achieved. The maximal dilution rate obtainable theoretically is the one at which the growth rate is at its maximum in the environment provided. A further increase in the dilution rate will cause the culture to wash out.

1.3.5.1 Growth of B. pertussis in continuous culture.

Van Hemert (1974) described attempts to use continuous culture to produce B. pertussis for use as a cellular vaccine. This was not found to be succesful for two reasons. Firstly, some cells adhered to the walls and tubes in the growth vessel and formed clumps of lysed cells that turned dark in colour. This resulted in a final cell suspension containing dark particles that could not be seperated from the bulk, lowering the quality of the product. Secondly, they found that the ability of the organism to produce protective antigen was lost after

several weeks cultivation and as the test results were only known after four weeks, an inferior product was being made in the meantime.

Successful continuous cultivation of B. pertussis was reported by Novotny & Cownley (1979). They state that cultures were were grown under glutamate / proline limited conditions and that levels of agglutinogens gradually increased until a steady state was achieved at a culture time of 8 - 10 days at D = 0.1 h^{-1}. Mouse protective activity reached a peak that coincided with the maximum agglutinogen titre and then declined. They also found that if the dilution rate of the culture was quickly increased the culture would washout and could not be recovered by reducing the dilution rate as the bacteria were dead.

More recently, Jagicza et al. (1985) describe a technique for the production of B. pertussis for vaccine use by continuous culture. The maximum mouse protective activity of the culture was obtained if it was maintained at pH 7.9 - 8.0 and they found that protective activity was maintained for at least 170 h. Further studies are needed on the expression in continuous culture of B. pertussis components now known to be important in pathogenesis and protection.

1.4 Cyclodextrin.

1.4.1 Chemistry and properties.

The cyclodextrins, sometimes called Schardinger dextrins or cycloamyloses, are a series of oligosaccharides produced by the action of the amylase of Bacillus macerans on starch. Cyclodextrins are composed of α-(1,4)-linkages of a number of D-glucopyranose units arranged in a doughnut-shaped ring. The number of glucose units is designated by the Greek letter α for 6, β for 7, γ for 8 etc. and these cyclodextrins form molecules with cavities of 0.45, 0.70 and 0.85 nm respectively (Bender & Komiyama, 1978).

Cyclodextrins have been extensively studied as they form inclusion complexes with a variety of smaller molecules. The inclusion complexes of cyclodextrins, which are formed in the solid state and in solution, consist of guest molecules held in the cavity of the macrocycle of the host cyclodextrin. The interaction is stabilised by Van der Waals forces and to a lesser extent by dipole-dipole interactions. Inclusion complexes in aqueous solution are thought to be further stabilised by hydrophobic interactions, i.e. by the tendency of the solvent water to push hydrophobic solutes of suitable size and shape into the hydrophobic cavity, in order to attain the minimum energy of the system (Bender & Komiyama, 1978; Casu et al., 1979). Methylation of cyclodextrin greatly increases its solubility (Szejtli, 1983) and it has been shown to increase the strength of inclusion complexes formed in aqueous solution, possibly because the methyl groups provide a hydrophobic lining for the cavity, favouring hydrophobic interactions (Casu et al., 1979).

1.4.2 Effect of cyclodextrins on B. pertussis.

The effect of cyclodextrins on the growth of B. pertussis was evaluated by Imaizumi et al. (1983a). Various cyclodextrins and soluble starch were incorporated

into Stainer and Scholte (1971) medium solidified with agar and the ability of these media to support growth of B. pertussis from small inocula was determined. The efficacy sequence was as follows: MeβCD > MeαCD > αCD > βCD. Soluble starch, γCD and MeγCD were not effective at allowing growth, even with a large inoculum. MeβCD was found to be the most effective for supporting the growth of B. pertussis when added to Stainer and Scholte agar. This medium gave the same efficiency of plating and growth rates as found on B-G agar. Also, with the addition of MeβCD, growth inhibition by fatty acids such as oleic or palmitic acid was overcome and normal growth observed.

Imaizumi et al. (1983b) also studied the effect of MeβCD on the growth and expression of pertussis toxin, LPF, by B. pertussis in liquid media. MeβCD was seen to stimulate growth, reducing the lag time of the cultures and it greatly enhanced LPF production. They claim MeβCD enhanced LPF production 100 times in Stainer and Scholte medium compared with the LPF produced in MeβCD-free medium in 48 h shake cultures. A modification of Stainer and Scholte medium in which the concentration of glutathione and ascorbic acid was increased and 10 g/l casamino acids was added to the medium was also described. This further enhanced growth and LPF production in the presence of MeβCD. It was demonstrated that the purified LPF obtained was biochemically and biologically identical to that produced from MeβCD-free static cultures.

Subsequently, Imaizumi et al. (1984) described that the addition of MeβCD to liquid culture medium enabled FHA to be purified from supernatants of shaken cultures. Previously this was not possible (Arai & Munoz, 1979). They suggest that this makes FHA production for incorporation in an acellular vaccine more convenient as previously this was only possible using numerous shallow, static liquid cultures.

Recently Aoyama et al. (1986) have reported that the addition of the antibiotic cephalexin to the cyclodextrin solid medium of Imaizumi et al. (1983a) produces a

selective medium for *B. pertussis* that can be used for clinical isolation of the organism.

Chapter 2. Materials and methods.

2.1 Strains of B. pertussis and growth of the organism.

2.1.1 Strains. Strain Wellcome 28 (serotype 1,2,3, obtained from Dr. P. Novotny, Wellcome Research Laboratories, Beckenham, Kent) was used for all experiments except where stated. A phase IV variant and a transposon Tn5-induced FHA$^-$ mutant were also used, both derived by Dr. I. Livey. All Strains were stored in freeze-dried vials at 4°C. The bacteria were freeze-dried in medium consisting of 7.5ml 10% bovine serum albumin, 2.5ml nutrient broth and 1.5ml 50% glucose.

2.1.2 Media.

Solid medium. B. pertussis was grown on charcoal agar plates (CA, Oxoid CM119), containing 10% (v/v) defibrinated horse blood.

Liquid media. Several liquid media were used for the growth of B. pertussis Throughout this study.

A:The medium of Sato et al. (1974). Formula:

	g/l
Casamino acids	10.0
Tris	6.07
NaCl	2.5
Soluble starch	1.5
KH_2PO_4	0.5
$MgCl_2.6H_2O$	0.4
DL-Glutamic acid	0.2
Niacinamide (nicotinic acid amide)	0.03
Cysteine	0.03
$CaCl_2$	0.01
$CuSO_4.7H_2O$	0.75mg
Glutathione	0.1
$FeSO_4.7H_2O$	0.1

The soluble starch was dissolved separately by warming to

60°C in 50ml H_2O and then added to the remaining ingredients with the exception of glutathione and $FeSO_4.7H_2O$. The medium was then made up to volume with distilled H_2O, adjusted to pH 7.2 by the addition of concentrated HCl and sterilised by autoclaving at 121°C for 30 min. The glutathione and $FeSO_4.7H_2O$ were prepared together as a 1% solution and filter sterilised. 0.1ml of this solution was added asceptically to each 100ml of medium before use.

B:The defined medium of Sainer & Scholte (1971). The modification of Imaizumi et al. (1983b) was used, designated SS medium. Formula:

	g/l
Sodium glutamate	10.7
L-Proline	0.24
NaCl	2.5
KH_2PO_4	0.5
KCl	0.2
$MgCl_2.6H_2O$	0.1
$CaCl_2$	0.02
Tris	6.1
L-Cystine	0.04
Nicotinic acid	0.004
$FeSO_4.7H_2O$	0.01
Glutathione	0.15
Ascorbic acid	0.02

The medium was made up to volume with distilled H_2O and adjusted to pH 7.6 with concentrated HCl and filter sterilised.

C:The medium of Imaizumi et al. (1983b). This was a modification of SS medium, designated CL medium. The formulation is changed to contain 10 g/l casamino acids and the concentrations of glutathione and ascorbic acid were increased to 0.15 and 0.4 g/l respectively.

2.1.3 Growth of B. pertussis.

2.1.3.1 Agar plates. Freeze-dried bacteria were

reconstituted by aseptically adding 0.5ml sterile distilled H_2O to each vial, spreading this over the surface of two CA plates and incubating at 35°C in a humid atmosphere for 48h. Plates were subcultured after 48h growth. The culture from each vial was never subcultured more than 4 times to ensure no reversion to an avirulent phase IV variant.

2.1.3.2 Shake flasks. B. pertussis was grown in 100ml Sato's, SS or CL medium, in 250ml conical flasks. Each flask was inoculated by scraping the growth from 50% of the area of a 24-48h CA plate with a sterile loop and adding this loopful of bacteria to each flask. Flasks were incubated on an orbital shaker (180 rpm) at 35°C (New Brunswick Psychrotherm, New Jersey, USA).

2.1.3.3 Thompson bottles. Larger volumes of B. pertussis were grown in 300ml volumes of liquid medium per 2.5l Thompson bottle. Thompson bottles were inoculated with 5-10ml of 24h culture from a shake flask containing appropriate medium. Thompson bottles were then incubated either statically or on a gently reciprocating shaker at 35°C.

2.1.3.4 Continuous culture. B.pertussis was grown in continuous culture in LH 500 series chemostats (LH Fermentation Ltd., Stoke Poges). Glass 1l vessels, with a working volume of 500ml, were used. The stainless steel top was replaced with one made of teflon and the projections into the vessel were made of glass. This was because it has been reported that stainless steel might be inhibitory to the growth of B. pertussis (Novotny & Cownley,1979). The chemostat pH was monitored using a steam-sterilizable pH electrode (Russell pH Ltd., Auchtermuchty, Fife) and oxygen was monitored using a galvanic oxygen electrode (Uniprobe Instruments Ltd., Cardiff).Aeration was by passing sterile air (approx. 200cm^3/min) across the head space of the chemostat and by

vortex mixing provided by a magnetically coupled impellor. The percent oxygen saturation in the chemostat was controlled by a proportional band controller regulating the stirrer speed. The pH was controlled to 7.6 by automatic addition of 1M HCl. The medium flow rate was controlled by a peristaltic pump (Watson-Marlow Ltd., Falmouth) and the culture volume was maintained at 500 ml by a second peristaltic pump drawing culture away from a weir.

The chemostat was inoculated by adding 50ml of a 24h shake flask culture of B. pertussis in Sato's or CL+CD (1g/l CD) via the sample port. The culture was then allowed to grow for 6h before the pumps were switched on to a slow dilution rate ($D=0.05h^{-1}$). After 24h the dilution rate was adjusted to that required for the experiment and the chemostat left for at least 10 generation times to achieve a steady state. To maintain X-mode growth in the chemostat it was necessary to replace the medium reservoir with one containing fresh medium every 3 or 4 days.

2.1.3.5 Growth of B. pertussis in LH 500ml batch fermenter. The LH 500 series chemostat was used for batch culture of B. pertussis. The vessel was inoculated with sufficient B. pertussis, from a 24h CL shake flask, to give an initial concentration of 10^9 organisms ml^{-1}, determined from a total count made using a Thoma counting chamber. The pH was controlled to 7.6 by automatic addition of 1M HCl and the oxygen saturation of the culture was maintained at 20% by automatic adjustment of the stirrer speed.

2.1.3.6 LH 2000 Series fermenter. The LH 2000 Series fermenter was fitted with a stainless steel vessel of 12l total volume, 8l working volume. The fermenter featured in situ sterilisation and dissolved oxygen control by stirrer speed. Oxygen saturation was measured by a polarographic oxygen electrode (Ingold Ltd.). Foam control was by

automatic, timed addition of antifoam. After sterilisation the vessel was filled with 8l of medium and inoculated with 2x100ml 24h CL+CD shake flask cultures.

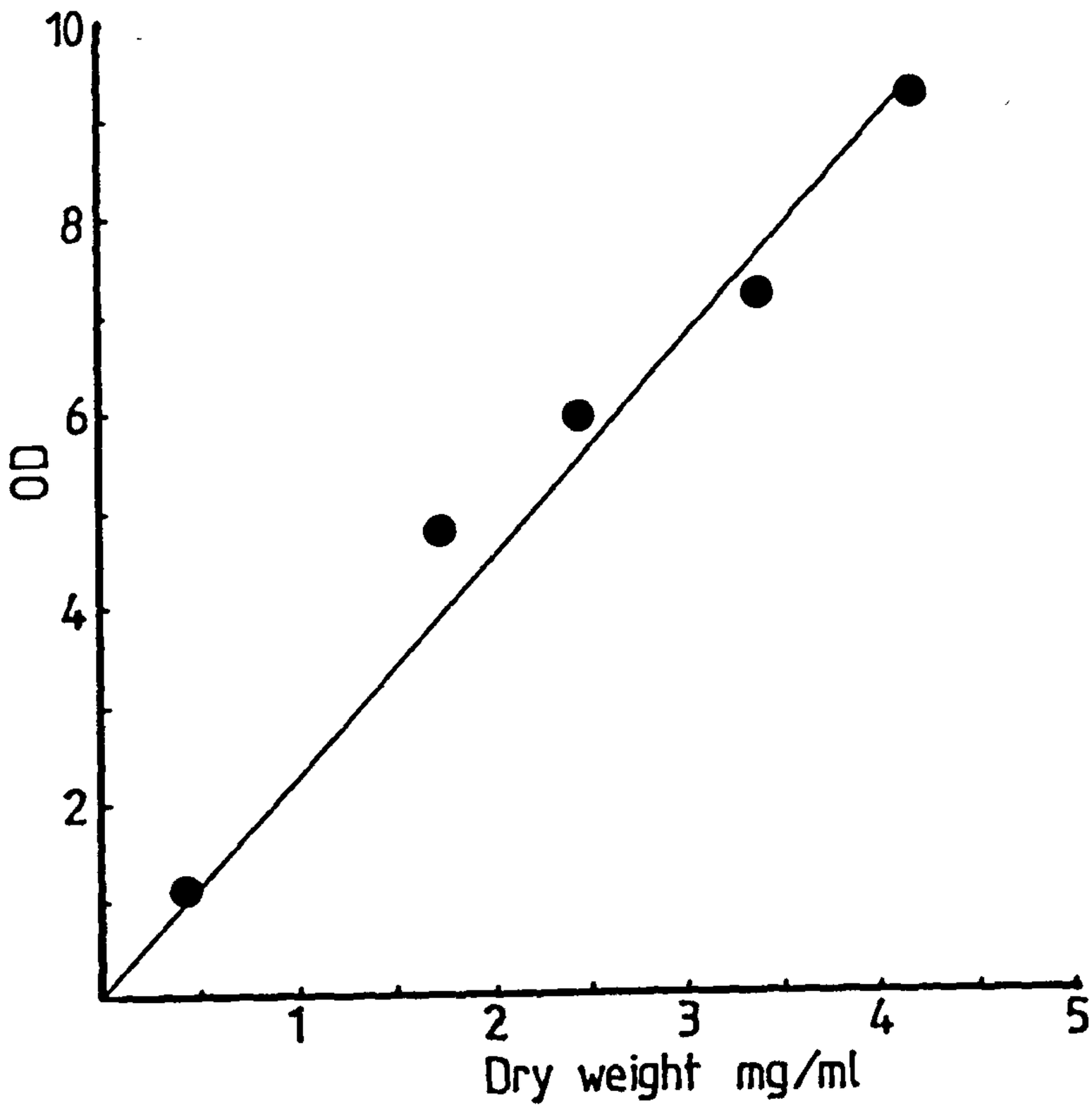
2.1.4 Viable counts of B. pertussis. These were performed using the method of Miles & Misra (1938). Serial dilutions of B. pertussis cultures were made in 1% casamino acids solution and from each dilution 6 drops were placed on duplicate CA plates using a 32 drop/ml Pasteur pipette. CA plates were incubated for 1 week at 35°C in a humid atmosphere and the colonies counted.

2.1.5 Optical density. Optical density (OD) measurements were made on a Pye Unicam SP500 spectrophotometer at a wavelength of 550nm. The relationship between OD and dry weight is shown in fig. 2.

2.1.6 Culture purity. Culture purity was routinely monitored by observing Gram-stained smears from cultures at x1000 by light microscopy. In addition 0.2ml of each inoculum flask was spread over the surface of a tryptone soya broth agar plate (TSBA; 3% Oxoid tryptone soya broth, 1.5% agar). Phase I B. pertussis will not grow on this medium but many common contaminants will. These plates were observed after 24h incubation at 35°C.

2.1.7 Culture harvesting using the Millipore Pellicon. The Pellicon is a tangential-flow, ultrafiltration device, consisting of a positive displacement pump and a Pellicon cassette holder and membrane. Flow of liquid to be processed is recirculated across the membrane surface in a sweeping motion. This sweeping action helps to keep material retained by the membrane from creating a layer on the filter surface. Materials smaller than the pore size or nominal molecular weight cutoff are able to pass through the membrane, whilst materials larger than the pore size or molecular weight cutoff do not pass through the membrane and are concentrated. The Pellicon was used

Fig. 2. The relationship between optical density at 550 nm and dry weight of bacteria per ml of B. pertussis culture.



with a membrane with a nominal molecular weight cutoff of 10 000 and an area of 0.5ft².

Culture or supernatant was recirculated across the membrane with an inlet pressure of 20-30 lb in² and a filtrate pressure of 10 lb in² and it was concentrated up to 1/10 of its original volume. The cells and supernatant proteins with a molecular weight exceeding 10 000 were retained and concentrated as the low molecular weight components passed though the membrane. The concentrated cells and supernatant were then centrifuged and processed for vaccine manufacture.

2.2 Haemagglutination assay.

The haemagglutinating activity of B.pertussis supernatants and extracts was determined by the ability to agglutinate goose red blood cells,as described by Irons & MacLennan (1979). A suspension of goose red cells was prepared by washing goose blood three times in PBS and then making a 0.5% v/v suspension in PBS from the pellet of red cells. Assays were performed in 96 well disposable trays with V-shaped wells (Linbro, Flow Laborories Ltd.). To each well on the tray was added 50 µl of PBS. 50ul of sample was added to well 1 and then serially diluted through 50µl for 12 doubling dilutions. 50µl of freshly prepared 0.5% goose red blood cells were then added to each well and the red cells were allowed to settle. The HA titre was taken as the number of doubling dilutions in which the red cells were agglutinated.

2.3 ELISA antigen assays.

Sandwich-type assays were performed to determine LPF and FHA in culture supernatants and extracts of B. pertussis. Competition assays were used to determine AGG2 and FHA on on the bacterial surface. The monoclonal antibodies (McAb) used were provided by Dr. L.A.E. Ashworth, CAMR.

Reagents:

Coating buffer - 0.05M carbonate buffer pH 9.6.

Wash buffer - PBS containing 0.1% Tween 20 as a

blocking agent.

Substrate - 3,3',5,5'-tetramethyl benzidene (TMB). This was dissolved in dimethyl sulphoxide (10 mg/ml). 100 µl of this solution was added to each 10 ml of 0.05M acetate buffer pH 6.0 required and hydrogen peroxide (20 volume) added to 0.05%.

2.3.1 LPF and FHA assays. Flat-bottomed 96 well plates (Nunc immunoplate 1) were coated overnight at room temperature with McAb L10 or F2 at 2µg/ml in coating buffer for LPF or FHA assays respectively. Serial dilutions of samples were made in 96 well plates (Sterilin M29A) and transferred to the corresponding wells of washed, antibody-coated plates which were covered and shaken for 2h at room temperature. Plates were then washed and 100µl of appropriate antibody conjugate (L4HRP or F3HRP) was added to each well and shaking continued for another 2h, after which the plates were washed and 100µl of freshly prepared substrate added to each well. The blue colour was allowed to develop for 15min, after which the reaction was stopped by adding 25µl of 1M sulphuric acid The resulting yellow colour was measured using a Titertek Multiscan ELISA reader (Flow Laboratories Ltd.) with a 450nm filter.

Standard LPF and FHA of known concentration were titrated within each assay and sample concentrations were calculated by the ratio of sample 50% end point dilution divided by the standard 50% end point dilution, multiplied by the standard concentration.

2.3.2 FHA competition assay. Flat-bottomed 96 well plates (Dynatech M129A) were coated overnight with purified FHA (1µg/ml in coating buffer). Serial dilutions of cell suspensions were made in separate plates and an equal volume of F8HRP conjugate (100ng/ml) was added to each well. Plates were shaken for 3 min at room temperature and then dilutions were transferred to corresponding wells of the washed, antigen-coated plates which were covered and shaken for 2h at room temperature. Plates were then washed and substrate

PAGE NUMBERING AS ORIGINAL

added as above.

2.3.3 AGG2 assay. Nunc immunoassay plates were coated overnight with a homogenate supernatant of strain Wellcome 28 at 1µg/ml in coating buffer. Serial dilutions of cell suspensions were made in separate plates and an equal volume of AGG2 McAb (diluted 1 in 20) was added and incubated with shaking at room temperature for 30min. The dilutions were then transferred to the washed antigen-coated plates and shaken at room temperature for 2h. The plates were then washed and rabbit anti-mouse conjugate (RAM HRP) at 0.5µg/ml in 10% foetal calf serum added to each well. The plates were again shaken at room temperature for 2h after which the plates were washed and substrate added as above.

2.4 In vivo assay of LPF.

LPF was determined by measuring lymphocytosis in vivo using the method of Morse & Morse (1976). Cells were harvested by centrifugation, washed in water, dispersed in PBS and held at -20°C until required. Cells were diluted to 0.5-1.0 mg/ml protein (determined by an automated Lowry process), heated at 56°C for 10min and briefly sonicated. The LPF content of the cells was then determined by intravenous injection of 0.2 ml cells into 5-week-old Porton mice. After 3 days, the white blood cell counts of these mice, determined using an Improved Neubauer counting chamber, were compared with those of control mice injected with PBS.

2.5 Electron microscope observation of fimbriae.

Bacteria were examined for the presence of fimbriae by electron microscopy (Ashworth et al., 1982a). Washed bacterial suspensions were applied to Formvar film-coated electron microscope specimen grids (200-mesh copper) and negatively stained with 1% phosphotungstic acid pH 7.2. The grids were then carbon coated before being examined in a Philips EM300 electron microscope operated at 80 KV.

2.6 Fluorescence assay for CD.

The ability of CD to greatly enhance the fluorescence of the fluorescent dye 2-p-toluidinylnaphthalene-6-sulphonate (TNS) was observed by Kondo et al. (1976) and used to quantify cyclodextrins in solution. This method was modified to assay CD in culture supernatants and in mixtures of FHA+CD.

Sample aliquots were diluted in distilled water to 5ml. TNS (Sigma) was dissolved in methanol (10mg in 10ml) and 0.1ml added to each 5ml of test solution just prior to measurement of fluorescence. TNS solutions were prepared freshly for each assay. Samples were placed in quartz glass 10mm cuvettes and fluorescence determined using a Perkin-Elmer LS-5 luminescence spectrophotometer at an excitation wavelength of 366nm and an emission wavelength of 460nm. The concentration of CD in samples was determined by comparison of fluorescence with a standard curve for CD (0 - 0.1 mM CD) obtained for each assay. Background fluorescence of control samples without CD were subtracted from test samples

2.7 SDS-PAGE.

Bacteria were harvested by centrifugation, washed in water and dispersed in water to 1-2 mg/ml protein. The cells were then added to 0.25 vol. SDS-mercaptoethanol (10% w/v SDS, 10% v/v mercaptoethanol, 50% v/v glycerol 0.3M Tris pH 6.8) and heated to 100°C for 5min in a water bath before electrophoresis on a polyacrylamide gel. The polyacrylamide gels were made as follows:

Resolving gel (12.0% acrylamide, 0.1% bisacrylamide)

66ml	2x resolving gel buffer (0.75M Tris, pH8.8)
44ml	36% acrylamide, 0.3% bisacrylamide
22ml	H_2O
1ml	10% ammonium persulphate
0.1ml	TEMED

Before addition of the persulphate and the TEMED the solution was degassed. The complete gel mixture was poured into the casting apparatus and overlayed with butanol to

form a flat top to the gel. When the gel was set the butanol was washed off and the gel covered with stacking gel made as below:

Stacking gel (4% acrylamide, 1% bisacrylamide)

6ml	2x stacking gel buffer (0.25 Tris pH8.0, 0.2% SDS)
6ml	40% sucrose, 8% acrylamide, 2% bisacryamide
100µl	10% ammonium persulphate
10µl	TEMED

Samples (50µl) were applied to the slots formed by a comb in the stacking gel and the gel was electrophoresed with a constant current of 80 mA. Gels were then stained with Coomassie blue R250 (0.05% w/v in 25% v/v isopropyl alcohol, 10% glacial acetic acid) overnight and then for 4h in 0.025% Coomassie blue in 10% v/v isopropyl alcohol, 10% v/v glacial acetic acid. Gels were then destained in repeated changes of destainer (40% methanol, 10% glacial acetic acid).

2.8 Cyclic AMP assay.

The assay was performed using the kit produced by Amersham International p.l.c.. The assay was based on the competition between unlabelled cyclic AMP (cAMP) and a fixed quantity of tritium labelled adenosine 3', 5' cyclic phosphate for binding to a protein that has a specific affinity for cAMP. The amount of labelled protein-cAMP complex formed is inversely related to the amount of unlabelled cAMP in the sample. Thus measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated. Separation of bound from unbound cAMP is achieved by adsorption of free cAMP on to coated charcoal, followed by centrifugation.

The assay was performed in disposable centrifuge tubes which were kept in an ice/water bath throughout the assay. Standard cAMP (50 µl) from 1-16 pmol per tube was added to a series of tubes and 50 µl of each unknown was added to additional assay tubes. Labelled cAMP (50 µl) was added to each tube followed by adding 100 µl of binding protein solution to each standard and unknown tube. The tubes were then vortex mixed for 5 seconds and incubated at 0°C for 2

h. Ice-cold charcoal suspension (100 µl) was then added to all tubes. After a further vortex mixing the tubes were centrifuged at 11 600 xg for 5 min. Then supernatant (200 µl) was withdrawn from each tube and added to 5 ml scintillant for counting. The blank for the assay was 150 µl of 0.05M Tris 4mM EDTA pH 7.5 buffer plus 50 µl labelled cAMP treated with charcoal as above and the counts per minute (cpm) determined on the supernatant. Binding in the absence of unlabelled cAMP was determined by placing 50 µl Tris EDTA buffer, 50 µl labelled cAMP and 100 µl binding protein in a tube, treating with charcoal and measuring the cpm obtained.

To produce a standard curve and use this to determine cAMP concentrations in unknowns the data was processed as follows: The blank cpm was subtracted from each value and the ratio,

$$\frac{\text{binding in absence of unlabelled cAMP cpm - blank } (C_0)}{\text{standard or unknown cpm - blank } (C_x)}$$

This ratio was plotted against the known concentration of the standards and from the C_0/C_x value of each unknown the concentration of cAMP was read from the curve.

2.9 Cell surface hydrophobiciy.

This was determined by a modification of the method of Rosenberg et al. (1983). Bacteria were harvested by centrifugation and resuspended in PBS to an absorbance of 1.0 at 550nm (Unicam SP500 spectrophotometer). Various volumes of octane (50- 600µl) were added to 3.5ml of bacterial suspension in round-bottom test tubes (15mm diameter). The mixtures were then agitated on a vortex mixer for 1 min. Tubes were then left to stand for 15 min and the percentage of cells remaining in the aqueous phase was determined by removing this phase with a Pasteur pipette and measuring its adsorbance at 550nm.

2.10 Vero cell adherence assay.

The method was modified from that of Sato et al. (1981). Vero cells were harvested from confluent growth in Roux flasks and diluted in fresh minimal essential medium containing 10% foetal calf serum (MEM) to 10^5 cells ml^{-1}, based on a total cell count obtained using an Improved Neubauer counting chamber. This suspension (2ml) was added to 3.5cm diameter Petri dishes (Nunc), each containing a cleaned, sterile glass coverslip (22x22mm) and incubated in a sealed container for 18h at 35°C. The coverslips with adherent Vero cells were washed once with fresh MEM before addition of bacterial suspension. Bacteria grown under various conditions were harvested by centrifugation, resuspended in MEM and adjusted to 10^7 ml^{-1}, based on a total count made in a Thoma counting chamber. Bacterial suspensions were incubated with the Vero cells for 2h and then washed with 3 changes of fresh MEM to remove any non-adherent bacteria. The Vero cells on the coverslips were then fixed in 95% ethanol, stained for 10 min with crystal violet and mounted in DPX (George Gurr, London).

The number of bacteria around the periphery of a sample of Vero cells was counted microscopically (x1000). Two replicates of 40 cells were counted for each condition and a mean and standard deviation (S.D.) of the pooled results calculated. Mean values were compared by the student's t test with those of controls in each assay.

2.11 Preparation of B. pertussis homogenate extracts.

B. pertussis cultures were harvested by centrifugation and the cells resuspended in TSMA buffer (0.05M Tris, 0.15M NaCl, 0.05M $MgCl_2$ pH 7.4, containing 0.1% sodium azide), 5g in 100ml and homogenised for 15 min using a Silverson homogeniser. The homogenates were centrifuged (9 000 rpm, 30 min) and supernatants were concentrated by a 30% ammonium sulphate precipitation overnight. The precipitates were recovered by centrifugation and extracted with Tris/NaCl buffer (0.05M Tris, 0.15M NaCl, pH7.4) and analysed by SDS-PAGE.

2.12 Preparation of Empigen BB extracts of B. pertussis cell envelopes.

Envelopes were prepared by Braun homogenisation and differential centrifugation and then suspensions of envelopes in water (at 10mg/ml protein) were extracted by shaking orbitally with 1.2% Empigen BB (Marchon Div., Albright and Wilson, Whitehaven, England) in an equal volume of 0.1M sodium phosphate buffer, pH8.0 for 90 min at 35°C (Robinson & Manchee, 1979).

Chapter 3.

Effects of conditions of culture on growth and expression of virulence determinants in B. pertussis.

B. pertussis is a fastidious, slow growing organism that is difficult to grow in culture (see 1.3). It is very sensitive to several growth inhibitors present in the culture medium, such as fatty acids, sulphur and peroxide (Rowatt, 1957b). Various substances, e.g. starch and charcoal, have been used to try to overcome these inhibitory effects (Hornibrook, 1939; Pollock, 1947) but the growth produced can still be variable. Growth of B. pertussis for use in whole cell vaccines often follows empirical methods based on experience (Griffith, 1978), without knowledge of the factors that influence growth. For the production of an acellular vaccine, defined, reproducible growth is required with consistent expression of the virulence determinants to be purified.

The aims of this study were to more fully define the factors that effect growth of B. pertussis in batch and continuous culture and to determine the effects of these conditions on the expression of virulence determinants, especially LPF and FHA. The effects of the medium additive 2,6-O-dimethylβ-cyclodextrin (CD) which has been reported to stimulate growth and expression of LPF and FHA by B. pertussis (Imaizumi et al., 1983a & b; 1984), were also investigated.

3.1 Batch cultures.

3.1.1 Effects of different SS medium formulations on growth of B. pertussis.

The defined medium of Stainer & Scholte (1971) has been modified by several subsequent workers by substituting cysteine for cystine (Novotny & Brookes, 1975) and using a reduced concentration of tris (Hewlett & Wolff, 1976). B. pertussis was grown in four different formulations of SS medium to determine the effects of

these changes and to decide which medium should be used subsequently.

B. pertussis was grown in SS medium containing cystine or cysteine (0.05 g/l) and containing 1.52 or 6.0 g/l tris to determine the effect of these modifications on growth. After 48 h growth 5x100 ml shake flask cultures were pooled and the OD, pH and wet weight of cells produced, were measured (table 2). Similar growth was produced in all four media, although cultures with the lower concentration of tris rose to a higher pH. A similar yield of wet weight of cells was produced in each medium.

The substitution of cystine for cysteine and the reduced concentration of tris did not significantly effect growth and subsequently medium containing cystine and 6.0 g/l tris was used.

The effect of growth with and without glutathione is shown in section 3.1.2c.

3.1.2 The effects of CD on growth and expression of virulence determinants.

It has been reported that the addition of CD to liquid growth medium enhanced LPF expression 100 times more than that in CD-free medium, in 48h shake cultures (Imaizumi et al., 1983b). The addition of CD also enhanced FHA production by B. pertussis about 100 times more than in CD-free medium (Imaizumi et al., 1984). These claims have been investigated with B. pertussis grown under various conditions to determine whether this degree of enhancement of LPF and FHA production is due to CD or to other conditions specific to their culture techniques.

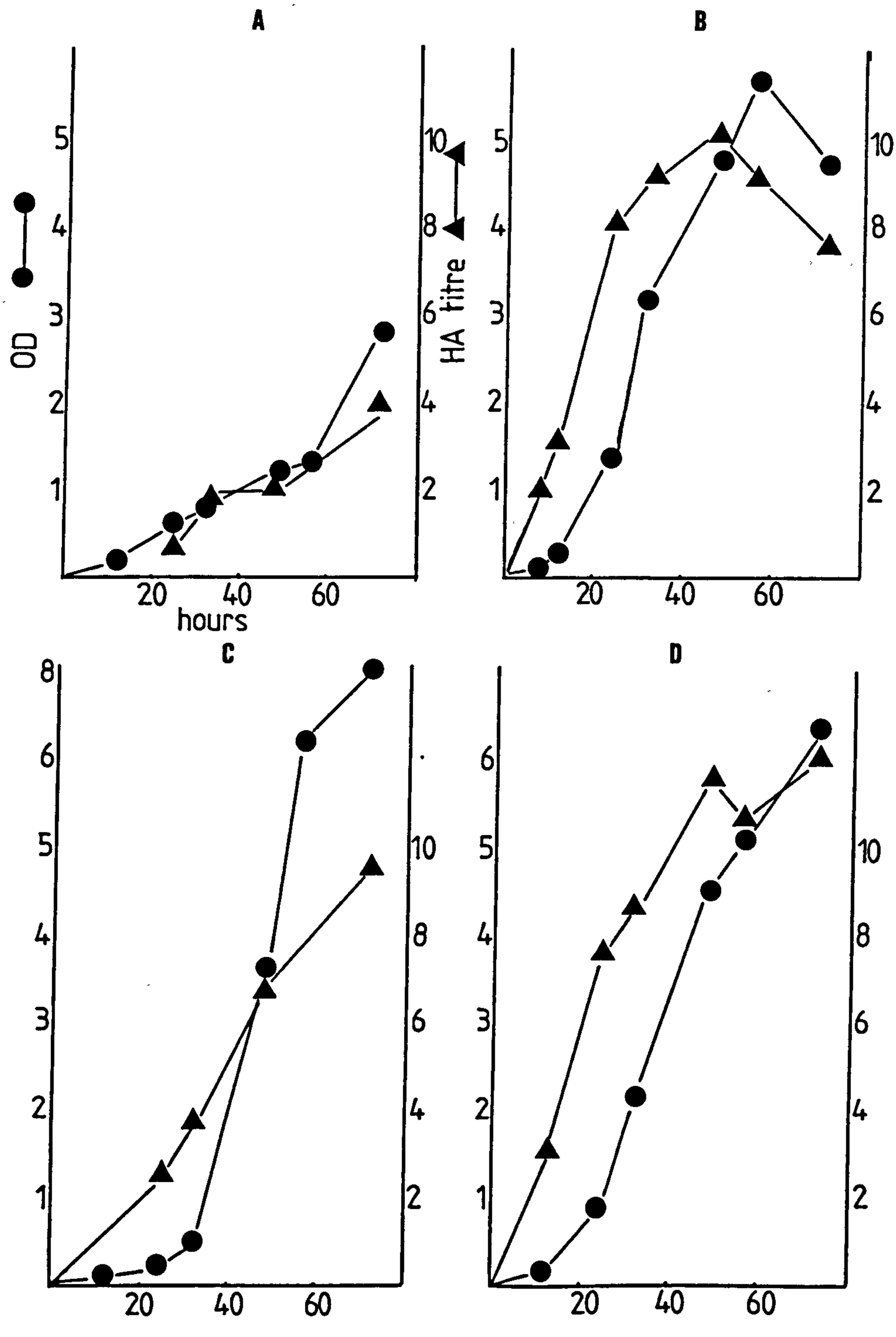
(a) In shake flask cultures.

B. pertussis was grown in shake flasks in SS and CL medium with and without CD (fig. 3). In SS medium the maximum OD achieved was 2.8, with a HA titre of 4. It can be seen that the maximum growth achieved using SS medium was less than that obtained using the same medium in section 3.1.2c. Growth of B. pertussis in SS medium was

Table 2. Growth of B. pertussis in SS medium containing either cysteine or cystine and either high or low levels of Tris.

	48h pool of 5x100ml shake flasks		
Medium	OD	pH	g wet wt. cells/500ml
1 Cysteine (.05g/l) Tris (6.0g/l)	3.97	8.3	2.7
2 Cysteine (.05g/l) Tris (1.25g/l)	4.41	8.5	2.5
3 Cystine (.05g/l) Tris (6.0g/l)	4.11	8.35	2.8
4 Cystine (.05g/l) Tris (1.52g/l)	4.88	8.55	3.1

Fig. 3. Growth and haemagglutination titre of B. pertussis cultures grown in A, SS; B, SS+CD; C, CL and D, CL+CD media.



generally found to be very variable in shake flask and Thompson bottle cultures. In SS+CD medium the maximum OD was 5.8 and the maximum HA titre 11. Thus both growth and HA activity were greatly promoted in SS medium by the presence of CD. In CL medium the maximum OD was similar with and without CD but with CD the lag time of the culture was reduced. Also the HA titre in the CD medium was higher and produced earlier in the culture.

Therefore CD greatly improved the growth in SS medium and reduced the length of the lag phase observed in CL medium. It also enhanced production of B. pertussis haemagglutinins in these media.

(b) The effect of CD on Thompson bottle cultures incubated statically and with shaking.

A common method for the large scale growth of B. pertussis is using many Roux or Thompson bottles and cultures are grown with static incubation. Static incubation is used as it has been reported that FHA was not produced in shaken cultures (Arai & Munoz, 1979) and even if FHA was produced in a static culture, the haemagglutinating activity rapidly diminished when the culture was transferred from a static to a shake culture. Also less FHA is extractable from B. pertussis cells grown in shaken Thompson bottle cultures than from Thompson bottle cultures with static incubation (Ashworth, et al., 1982a). Static and shaken Thompson bottle cultures of B. pertussis were compared for growth and production of LPF and FHA. The effect of CD on these cultures was also determined.

Static Thompson bottle cultures were grown using SS medium in the presence and absence of 1 g/l CD for 120h (table 3). The OD and pH of the cultures and HA titre, LPF and FHA concentrations of the supernatant were determined. There was very little difference between static cultures grown in either medium, with both cultures reaching a maximum OD of approximately 3 and maximum LPF and FHA concentrations of approximately 3 and 8 μg/ml

Table 3. Growth of B. pertussis in statically incubated Thompson bottles.

	OD	pH	HA titre	LPF supt. µg/ml	FHA supt. µg/ml
Static 120h	2.86(.25)	8.41(.04)	6.3(.5)	2.8(.8)	8.9(2.0)
Static + CD 120h	3.19(.38)	8.32(.1)	6.2(.4)	3.2(.9)	8.1(1.8)

Results are the mean of 6 Thompson bottle cultures.

Figures in parenthesis = Standard deviation.

respectively.

Growth in static Thompson bottles was compared with the growth and production of LPF, FHA and agglutinogen 2 by B. pertussis in shaken Thompson bottles in 5 media (table 4). The medium of Sato et al. (1974), which contains casamino acids and starch was used. This was until recently the medium of choice for liquid culture growth of B. pertussis. The defined SS medium and the modification of Imaizumi et al. (1983b), which included the addition of casamino acids, were also used with and without 1 g/l CD.

Sato's medium produced poor growth and poor expression of the antigens measured. SS and CL media produced similar maximum culture densities, The maximum densities produced by cultures containing CD were slightly higher, with CL+CD medium giving the highest culture density.

The maximum LPF concentration in the supernatant for SS cultures was 3.5 µg/ml (produced at 48 h), whereas for SS+CD cultures the maximum was 8.7 µg/ml (also produced at 48 h). CL medium cultures produced a maximum LPF concentration approximately twice that of SS medium. Cultures grown in CL+CD medium gave a mean LPF concentration in supernatants of 11 µg/ml. This was produced at 58 h, 10 h later than the maxima for the other media used.

No FHA was detected in supernatants from Sato's or SS medium, except at 72 h. The highest level of FHA seen was in CL+CD 72 h supernatant (3.7 µg/ml). FHA was detected on cells from all cultures by competition ELISA. SS and Sato's medium cultures showed similar levels of cellular FHA and these were more than doubled in SS+CD medium with 4.3 µg/ml cell-bound FHA measured at 58h. This value was further improved with CL medium but the highest cell-bound FHA levels were measured in CL+CD medium cultures, up to 8.8 µg/ml culture.

The concentration of agglutinogen 2 on the surface of cells was also measured by a competition ELISA. Concentrations of agglutinogen 2 in excess of 10 µg/ml

Table 4. The effects of CD on growth of B. pertussis in Thompson bottles incubated with shaking.

OD of cultures.

Medium	24h	48h	58h	72h
Sato's	0.73 (.02)	2.18 (.2)	1.82 (.1)	1.64 (.04)
SS	1.02 (.1)	4.66 (.3)	3.53 (.1)	3.16 (.2)
SS+CD	1.44 (.2)	5.01 (.4)	4.52 (.3)	3.94 (.2)
CL	1.36 (.1)	4.44 (.6)	4.89 (.2)	4.11 (.3)
CL+CD	1.19 (.1)	5.48 (1.0)	6.00 (.3)	5.62 (.4)

LPF in supernatant, µg/ml.

Medium	24h	48h	58h	72h
Sato's	0.1 (.02)	0.2 (.1)	0.1 (.04)	0.03 (.02)
SS	0.1 (.05)	3.5 (.7)	1.8 (.8)	0.7 (.3)
SS+CD	1.9 (.6)	8.7 (1.8)	5.8 (1.6)	5.2 (2.6)
CL	0.6 (.5)	6.7 (2.2)	4.9 (1.1)	4.2 (.9)
CL+CD	1.6 (.2)	6.3 (2.9)	11.0 (1.1)	8.0 (0)

FHA in supernatant, µg/ml.

Medium	24h	48h	58h	72h
Sato's	ND	ND	ND	ND
SS	ND	ND	ND	0.2 (.1)
SS+CD	ND	0.04	ND	0.3 (.7)
CL	ND	0.2 (.1)	0.2 (.1)	1.1 (.4)
CL+CD	ND	3.1 (.7)	1.9 (1.1)	3.7 (2.4)

FHA on cells, µg/ml.

Medium	24h	48h	58h	72h
Sato's	0.7 (.1)	1.3 (.3)	ND	ND
SS	0.6 (.1)	1.7 (.4)	ND	1.2 (.7)
SS+CD	2.2 (.8)	3.6 (1.3)	4.3 (1.3)	3.7 (1.4)
CL	0.8 (.4)	5.7 (2.1)	3.0 (.5)	1.3 (.7)
CL+CD	0.2 (.1)	7.3 (2.5)	6.2 (.4)	8.8 (3.0)

Agglutinogen 2 on cells, µg/ml.

Medium	24h	48h	58h	72h
Sato's	3.9 (.8)	6.3 (.5)	10.2 (3.9)	10.2 (3.9)
SS	5.2 (.4)	13.5 (1.1)	19.9 (7.3)	18.8 (3.6)
SS+CD	16.9 (9.8)	30.8 (6.9)	40.6 (4.9)	19.7 (3.4)
CL	4.1 (3.4)	19.2 (9.9)	23.6 (6.5)	10.5 (3.2)
CL+CD	5.9 (4.6)	12.7 (3.8)	31.4 (0)	16.7 (5.8)

Results are the mean of 3 replicate cultures grown in each medium.
Figures in parenthesis = standard deviation.

were measured for all the media used. The highest level attained was using SS+CD medium (40.6 ug/ml). Agglutinogen 2 appears to be to be produced in greater amounts than LPF or FHA so this will not be the limiting constituent in a vaccine containing equal quantities of LPF, FHA and agglutinogens.

Cells harvested at 48 h from each medium were homogenised and the cell-free homogenates analysed by SDS-PAGE (fig. 4). Agglutinogens are present for all culture conditions but the greatest yields occur in the 48 h cultures. It can be seen that the different media cause varying expression of surface proteins by B. pertussis with the high molecular weight FHA proteins more distinct in homogenates from cultures containing CD.

To summarise, the effects of CD are to increase the maximum culture density achieved in shaken Thompson bottle cultures and LPF in culture supernatants was approximately doubled by the presence of CD in the growth medium. FHA was either not detected in CD-free cultures or only detected in very low concentrations. CD enabled FHA to be produced by B. pertussis in shaken cultures and for CL medium containing CD there was a 15-fold enhancement of FHA in the supernatant at 48h, although the concentration was still less than that obtained with static cultures. Levels of agglutinogen2 and cell-bound FHA were also enhanced by the presence of CD.

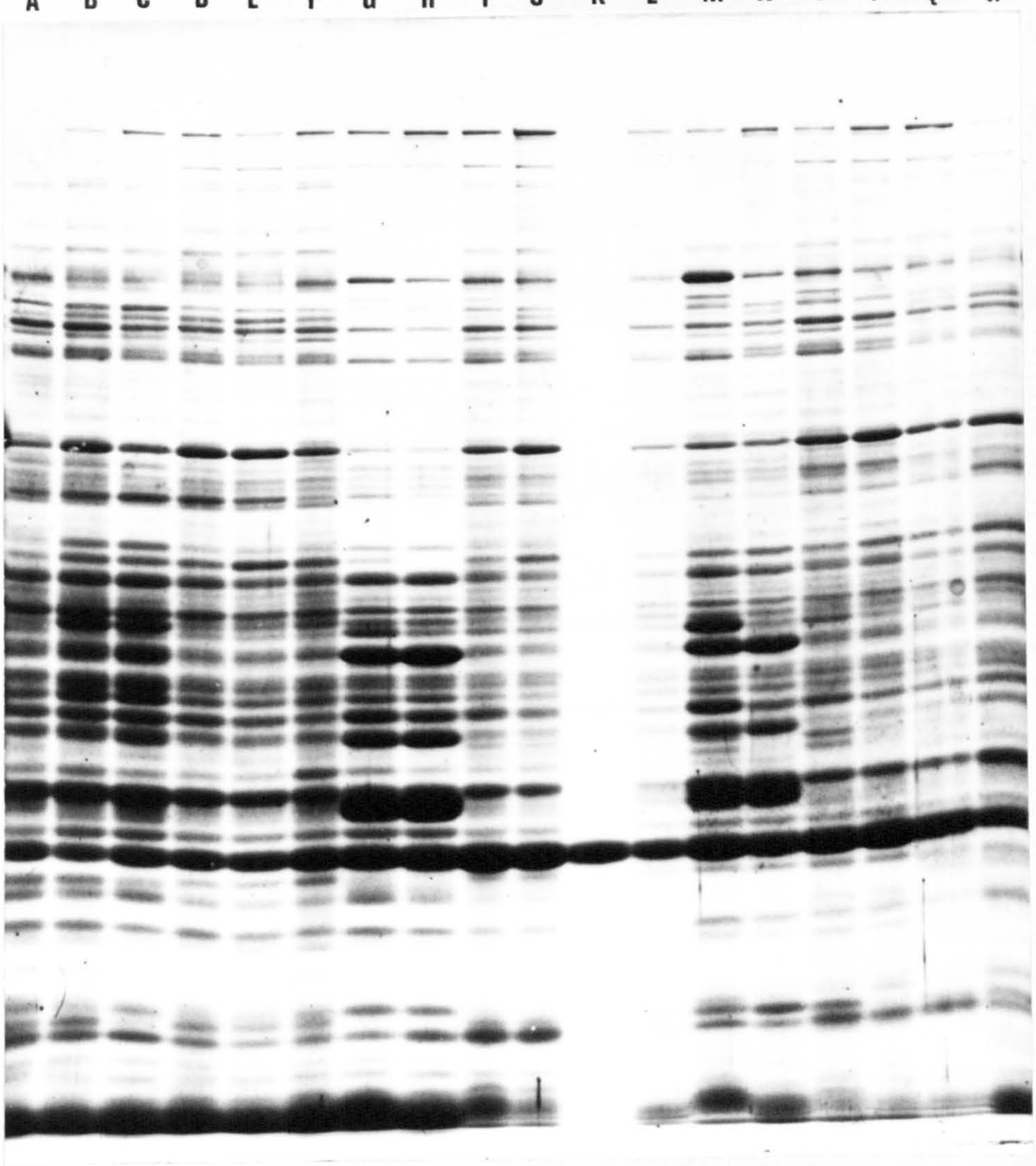
The enhancement of LPF and FHA produced by CD was not as great as that reported by Imaizumi et al. (1983b, 1984). This has also been found by Perera et al. (1986). The explanation for this is not clear.

(c) Effect of CD on growth in SS and CL medium with and without glutathione.

Glutathione has been reported to be an essential medium constituent for growth in a defined medium (Stainer & Scholte, 1971) and Imaizumi et al. (1983b) state that it is essential for the production of LPF. It was therefore determined whether the presence of CD altered this

Fig. 4. SDS-PAGE of homogenates of B. pertussis grown in Sato's, SS, SS+CD, CL and CL+CD media.

A : Sato's 24 h.
B : SS "
C : SS+CD "
D : CL "
E : CL+CD "
F : Sato's 48 h.
G : SS "
H : SS+CD "
I : CL "
J : CL+CD "
K : Agg. 2+3
L : Sato's 72 h.
M : SS "
N : SS+CD "
O : CL "
P : CL+CD "
Q : CL+CD 48 h.
R : CL+CD 24 h.


A B C D E F G H I J K L M N O P Q R

requirement for glutathione and if glutathione was essential for production of LPF and FHA

Therefore, B. pertussis was grown in SS medium, with and without glutathione in the presence and absence of CD, and also in CL+CD medium with and without glutathione (table 5). Cultures were sampled at 48 and 72 h and the OD, HA titre and LPF and FHA concentrations were measured. There was very little growth in SS medium without glutathione and no LPF or FHA were detected. However this medium containing CD allowed some growth at 48 h (OD = 0.5) and profuse growth (OD = 3.67) at 72 h, with levels of 3.6 µg/ml LPF and 25.4 µg/ml FHA in the supernatant. This growth was better than that achieved in SS medium containing glutathione which provided for only poor growth at this level of inoculum (10^9 organisms ml^{-1}). SS medium + glutathione + CD produced much greater growth at 48 h than the same medium without glutathione, with much higher levels of LPF and FHA measured. The comparison of CL+CD medium with and without glutathione showed slightly reduced growth and expression of LPF and FHA at 48 h without glutathione but very similar values at 72 h.

In SS medium B. pertussis has a requirement for glutathione but with CD it will grow in its absence. Glutathione makes little difference to growth and LPF and FHA production in CL+CD medium, so it seems that glutathione is not a requirement for LPF production in the presence of CD. Vajdic et al. (1966) observed that glutathione was the primary source of organic sulphur for B. pertussis in a defined medium, its use corresponding with the start of exponential growth. They also observed that the concentration of cystine in the medium did not begin to decrease until the onset of the stationary phase of the culture. It appears that CD alters the nutrient requirements of the organism so that its organic sulphur needs can be obtained from cystine and that B. pertussis is not capable of this in the absence of CD. The statement of Imaizumi et al. (1983b) that glutathione is essential for the production of LPF is not true if CD is present.

Table 5. The effect of different SS formulations +/- CD on the growth of B. pertussis in shaken flask cultures.

			µg/ml		
48h	OD	HA titre	LPF supt.	FHA supt.	FHA cells
SS no glut.	0.003	0	0	0	0
SS no glut.+CD	0.57(.02)	1.5	0.15	0.6(.1)	0.4(.1)
SS +glut.	0.06(.07)	0.6	0	0	0
SS +glut.+CD	3.28(.03)	7.0	3.8(.7)	9.9(.7)	4.5(.8)
CL+CD no glut.	3.02(.18)	6.0	3.0(.5)	17.6(1.9)	5.0(1.2)
CL+CD +glut.	3.62(.10)	6.7	4.5(.3)	35.9(9.7)	10.0(4.2)
72h					
SS no glut.	0.006	4.0	0	0	0
SS no glut.+CD	3.67(.06)	7.0	3.6(.1)	25.4(6.8)	8.0
SS +glut.	1.08(.74)	6.0	0.6(.7)	0.5(.7)	1.3
SS +glut.+CD	4.35(.20)	7.7	2.9(.1)	26.6(2.9)	6.5
CL+CD no glut.	5.13(.31)	8.0	2.4(.5)	82.6(38.1)	6.5
CL+CD +glut.	5.23(.55)	8.0	2.3(.8)	53.0(.9)	6.5

3.1.3 The concentration of CD in culture supernatants.

It was not known whether the CD included in culture media was metabolised by B. pertussis or if it remained in the supernatant. Therefore supernatants from Thompson bottle cultures of B. pertussis in SS+CD medium (1 g/l CD), incubated statically and with shaking, were assayed for CD using the TNS fluorescence assay. Results presented in table 6 show that the concentration of CD in the culture supernatant did not change from its initial concentration in the medium. This indicates that it is not a nutrient for B. pertussis but modifies the growth environment to favour growth and expression of haemagglutinins.

3.1.4 Growth of B. pertussis in 500 ml batch fermenters.

There is a discrepancy between the previous results, obtained in shaken Thompson bottle cultures on the effect of CD on production of LPF and FHA, and the results of Imaizumi et al. (1983b & 1984). It was therefore necessary to determine the effects of CD on B. pertussis in the more defined conditions provided by a small, stirred fermenter. This was also necessary as a first stage in developing a fermentation process for the growth of B. pertussis on a larger scale.

Cultures were grown in an LH 500 series 500ml fermenter as described in section 2.1.3.5.

(a) Effect of increasing concentrations of CD.

Previous growth experiments in shaken flasks and Thompson bottles have used one concentration of CD (1 g/l). The effect of increasing concentrations of CD on growth and expression of LPF and FHA was determined.

Growth curves obtained by growing B. pertussis in the LH 500 Series batch fermenter with CL medium containing increasing amounts of CD are shown in fig. 5. For CL medium the lag phase of growth occurs over the first 35 h of the culture, at the level of inoculum used. With 2 g/l CD the lag phase was less than half that time and the

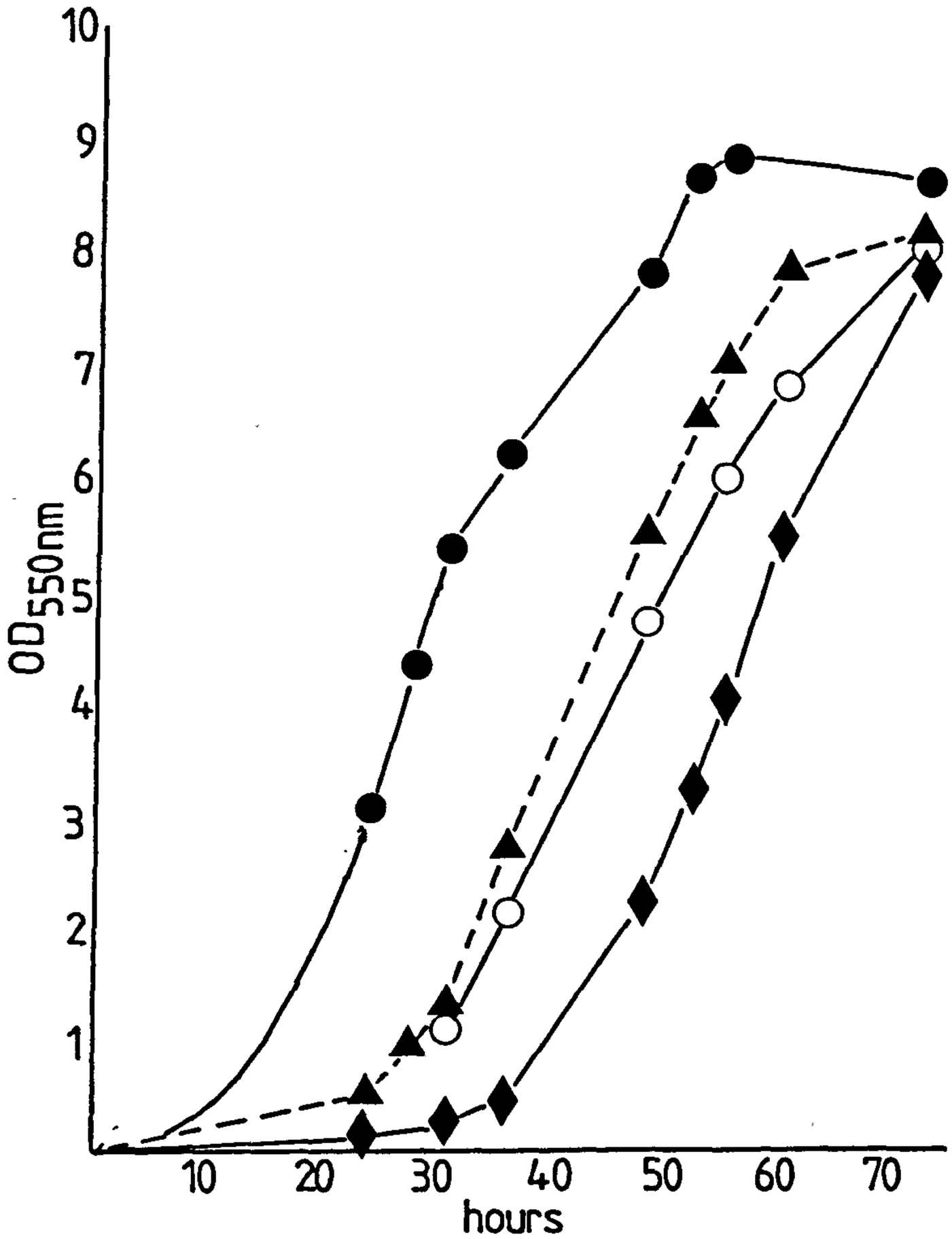
Table 6. Concentration of CD in supernatant from SS+CD Thompson bottle cultures determined by TNS fluorescence assay.

	OD	CD g/l
5 day static culture	3.19 (0.38)	1.05 (0.03)
48 h shaken culture	3.72 (0.07)	1.16 (0.1)

Results are the mean of 6 replicate cultures.

Figures in parenthesis = standard deviation.

Fig. 5. Growth of B. pertussis in 500 ml fermenter in CL medium with ♦, 0; ○, 0.05; ▲, 0.5 and ●, 2.0 g/l CD.



organism entered the exponential phase of growth approximately 25 h before the CL medium culture. The intermediate CD concentrations produced an intermediate effect in shortening the lag phase of growth. All cultures achieved approximately the same final OD which was higher than that observed in shaken flask or Thompson bottle cultures. This may be because the fermenter cultures were not oxygen limited as may occur in the shaken growth vessels. In the fermenter the percent O_2 in the culture was maintained at 20% by automatic control of the stirrer speed.

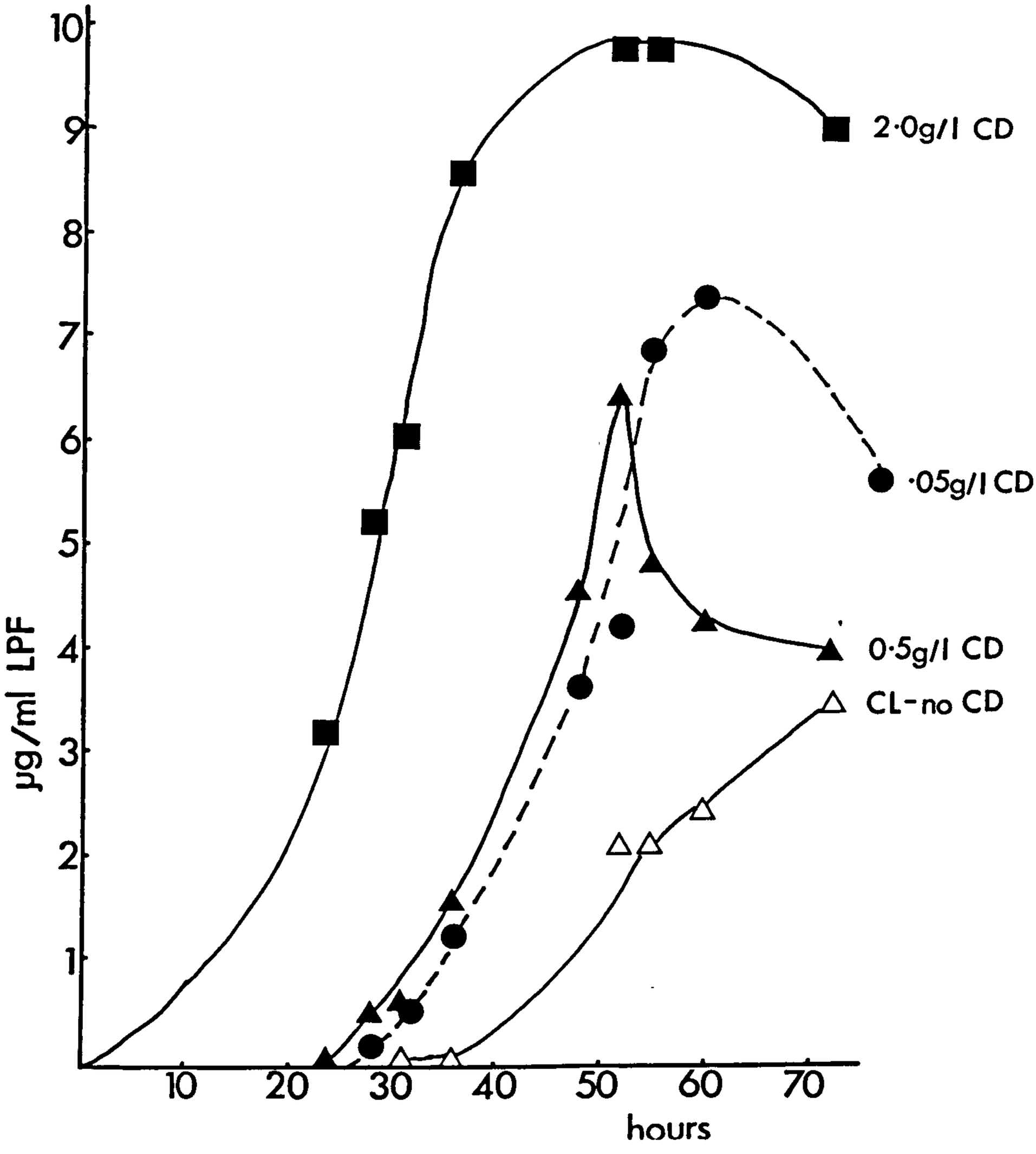
The concentrations of LPF in the supernatants from these cultures are shown in fig. 6. With medium containing 2 g/l CD the LPF concentration increases at an earlier culture time and the maximum LPF concentration was more than three times the maximum obtained in the absence of CD. The lower concentrations of CD caused a smaller increase in the expression of LPF. After the LPF concentration had reached its peak in the CD cultures there followed a decline in the LPF detected.

The CD did not increase the maximum culture density as occurred in the shaken flasks and Thompson bottles. However the CD acts to reduce the length of the lag phase of the cultures and to increase the expression of LPF. This occurred at all the concentrations of CD tested but the greatest effects were with the highest concentration of CD.

(b) Effect of pH control and CD on the expression of FHA.

During the growth of <u>B. pertussis</u> the pH rises and the highest FHA concentrations coincide with this elevated pH. So the expression of FHA was determined in 500 ml fermenter cultures with and without pH control. This would show whether the release of FHA into the supernatant was favoured by high pH or if the high pH was incidental and that FHA was mainly produced by stationary phase cells. Cultures were grown with and without CD to determine if

Fig. 6. Effect of concentration of CD on supernatant LPF measured by ELISA in 500 ml fermenter.



this also affects expression of FHA.

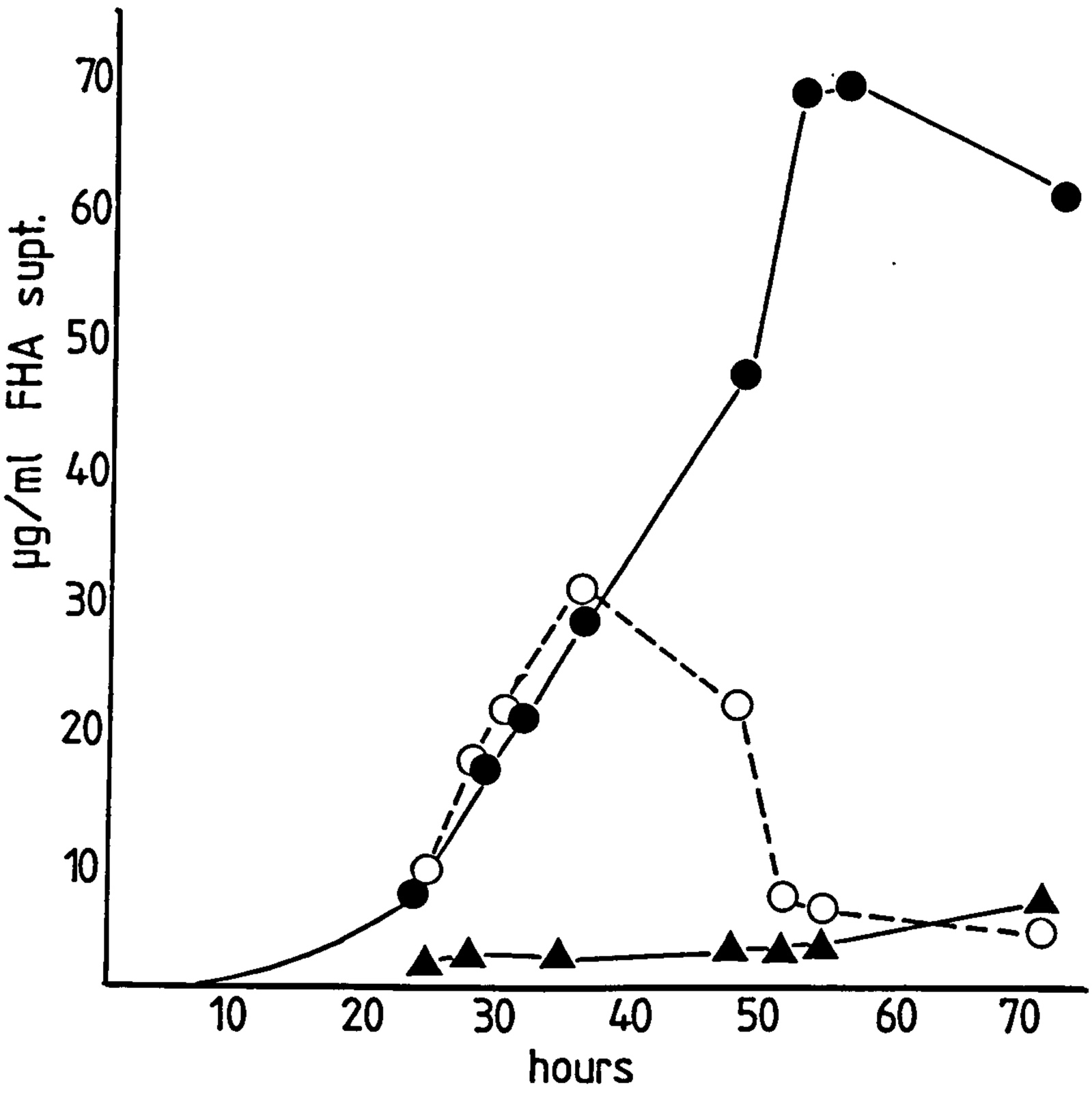
The growth and expression of LPF shown in figs. 5 and 6 were obtained from cultures where the pH was controlled to 7.6 by automatic addition of 1 M HCl. The expression of FHA during growth in the 500 ml batch fermenter is shown in fig. 7. For the culture containing 2.0 g/l CD with the pH controlled to 7.6, there was a peak of FHA concentration in the supernatant (30 µg/ml) at 35 h, after which the FHA concentration dropped to less than 10 µg/ml by a culture time of 50 h. Without pH control the expression of FHA in the supernatant of a 2.0 g/l CD culture followed a similar initial pattern. However, the concentration did not peak at 35 h and then reduce, but it carried on rising to a maximum value of 70 µg/ml at 55 h. With no CD or pH control very little FHA was detected in the supernatant at any time during growth.

Very little FHA is produced in stirred fermenter cultures by B. pertussis in the absence of CD, with or without pH control. CD greatly enhances the expression of FHA and with uncontrolled pH the FHA concentration continues to increase as the culture reaches a more alkaline pH. The effect of CD to enhance expression of FHA is even greater than that seen for LPF and its use allows stirred fermenter cultures to be a source for the purification of FHA. In conjunction with CD, culture pH is an important factor for the expression of FHA, being enhanced by the high pH produced by the products of the organism's metabolism. For subsequent fermenter cultures medium containing CD and no pH control were used.

3.2 Growth of B. pertussis in continuous culture.

It is known that the nature of the growth environment and the growth rate can have a profound effect on the bacterial surface (Ellwood & Robinson, 1981). Previous experiments have compared growth in batch cultures which have variable growth rates. The continuous culture system was therefore used to study the growth of B. pertussis at a constant growth rate and under nutrient limited

Fig. 7. Effect of CD and pH control on supernatant FHA measured by ELISA in 500 ml fermenter. ▲, no CD, no pH control; ○, 2.0 g/l CD, pH controlled to 7.6; ●, 2.0 g/l CD, no pH control.



conditions. Nutrient limited conditions could be expected to be the natural state of the organism in vivo (Brown & Williams, 1985) and so it was attempted to produce growth limited by an essential amino acid (cystine) and by iron, a nutrient known to occur in body fluids in low concentrations (Griffiths, 1983).

3.2.1 Effects of nutrient limited conditions on B. pertussis.

(a) Cystine.

It was attempted to achieve cystine limited conditions for B.pertussis grown in continuous culture using SS medium at D = 0.1 h^{-1}. After 96 h growth with SS medium the cystine concentration in the medium was reduced by 50% (table 7). From a culture time of 120 to 192 h the culture density fell slightly and then between 168 and 192 h it fell rapidly to an OD of 0.61. The OD of the culture then increased, reaching a value of 2.85 at 264 h. It can be seen that the concentrations of LPF and FHA in the supernatant were reduced to 0 µg/ml when the rapid drop in OD occurred. The concentrations did not return to previous values when the culture density subsequently increased. The concentrations of proline and cystine in the culture supernatants were below the level of detection by amino acid analysis. Analysis of whole cells by SDS-PAGE (data not shown) demonstrated that there was a loss of phase I proteins when the culture density increased under reduced cystine conditions, indicating that the culture had degraded to the phase IV variant.

When B. pertussis was grown in this stressed condition, i.e. under a nutrient limitation, this produced a population of phase IV variants which have simpler growth requirements. The cystine limited conditions appeared to favour the genotypic change to phase IV variants in addition to being selective for phase IV organisms as the loss of phase I characteristics occurred at a faster rate of phase variation than seen on nutrient rich agar media (10^{-5} - 10^{-7} per generation).

Table 7. Effects of cystine limited conditions on growth of B. pertussis in continuous culture.

Medium	Time (h)	OD	LPF supt. (µg/ml)	FHA supt. (µg/ml)	Proline (mM)	Cystine (mM)
SS	24	0.86	-	-		
	48	1.48	-	-		
	72	1.94	-	-		
	96	1.83	0.6	0.4	(2.5)*	(0.09)*
50% Cystine						
	120	1.83	0.3	0.4	<0.1	<0.03
	144	1.64	0.1	0.4	"	"
	168	1.56	0.4	0.8	"	"
	192	0.61	0	0		
	264	2.85	0	0		

*before addition to chemostat.

(b) Iron. It is known that human body fluids contain very little free ionic iron and that this is insufficient for normal bacterial growth (Bullen, 1981). Many bacteria have mechanisms for acquiring iron in competition with the host. It was attempted to determine if B. pertussis would grow under iron limited conditions and if any proteins could be detected which were involved in iron binding and not expressed under conditions of iron excess.

After 120 h growth in the chemostat at D = 0.1 h^{-1}, SS medium containing no added iron was connected. There was no reduction in culture density (table 8). There was also no reduction in culture density with medium containing 0.1 g/l of the iron chelator desferal. When the desferal concentration was doubled, at a culture time of 456 h, the culture density began to drop. At 504 h the OD was 2.02 and by 528 h the culture was begining to wash out. Whole cells taken from the chemostat were analysed by SDS-PAGE (fig. 8). Cells grown in the presence of desferal showed loss of the X-mode specific proteins and the cells appeared to be degrading to phase IV variants.

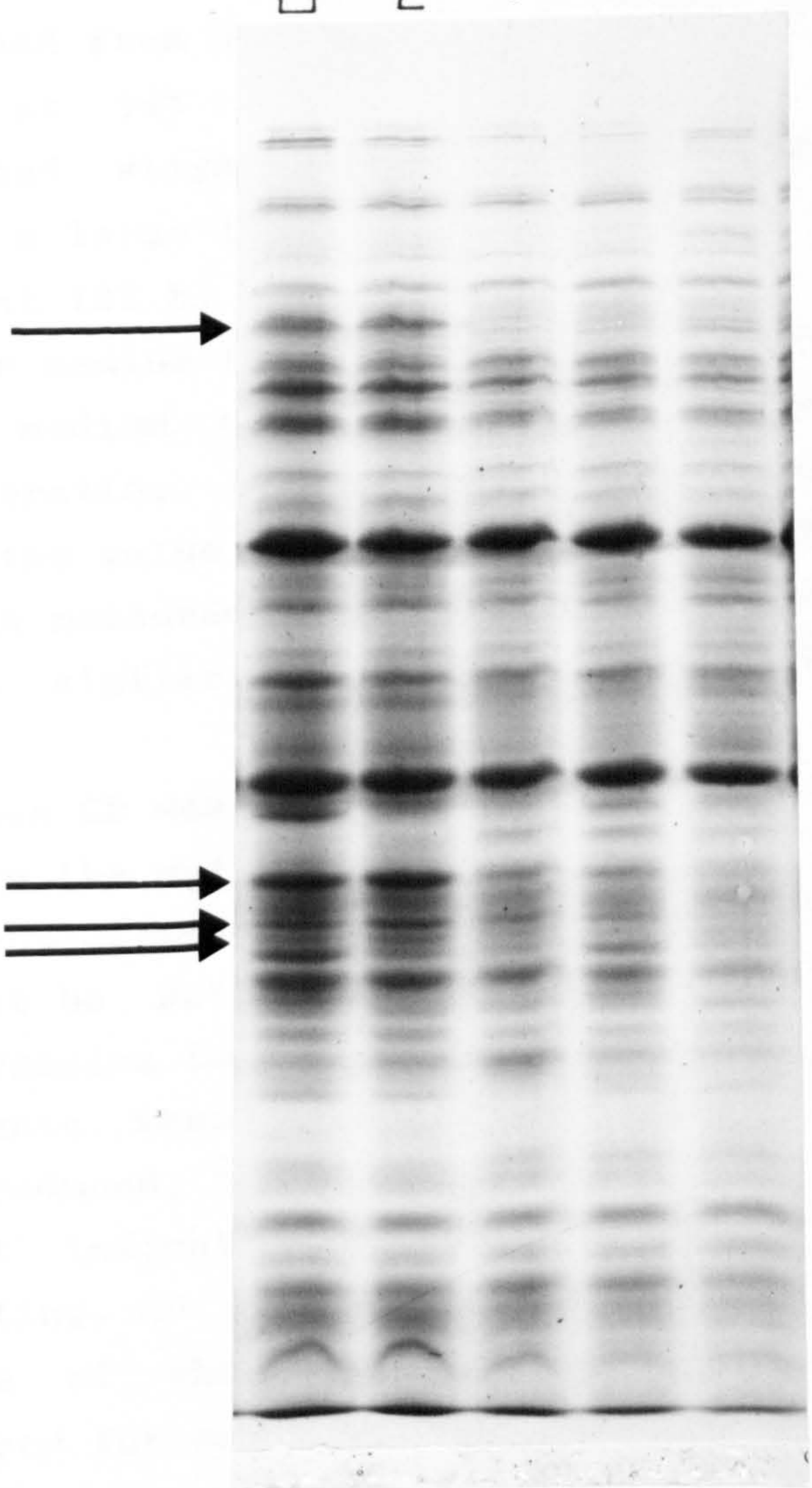
B. pertussis can grow in medium with no added iron, gaining sufficient from iron contaminating other medium constituents. As in cystine limited conditions, when the organism was stressed under iron restricted conditions the population reverted to the avirulent phase IV form. However, the phase IV organisms were no more successful in growing under these conditions and the culture washed out and did not grow when the pumps were switched off, making it a batch system.

When B. pertussis was subjected to an unfavourable growth environment, e.g. a nutrient limitation in vitro, it changed to the avirulent phase IV form. Phase IV organisms have simpler growth reqirements, as evidenced by their ability to grow on nutrient agar and their increased resistance to the effects of toxic fatty acids (Dobrogosz et al. 1979; Field & Parker, 1979) and the organisms do not have the burden of producing a range of

Table 8. Effect of iron limitation on the growth of B. pertussis in continuous culture

Medium	Culture time	OD	Fe supt.(ppm)
SS	24	1.32	0.3
"	48	1.55	
SS -no added Fe	120	1.52	
"	144	1.57	
"	168	1.63	
"	192	1.86	
"	216	1.72	
"	240	1.61	
"	288	2.28	
SS +0.1g/l desferal	312	2.07	
"	336	2.26	0.1
"	360	3.08	
"	384	2.70	<0.1
SS +0.2g/l desferal	456	2.62	"
"	480	2.52	"
"	504	2.02	"
"	528	0.67	"
Pumps off	552	0.13	"
"	576	0.19	

Fig. 8. SDS-PAGE of B. pertussis cells grown in continuous culture under iron limited conditions



complex virulence determinents.

3.2.2 Effects of CD on growth of B. pertussis in continuous culture.

The effects of CD on culture density and expression of LPF and FHA by B. pertussis growing at a dilution rate of 0.1 h^{-1} are given in table 9. At a culture time of 96 h the OD of the culture was 1.10. When SS medium containing 0.5 g/l CD was connected to the chemostat the culture density gradually increased until at 192 h the OD was 2.01. The concentration of LPF in the supernatant also increased from 0.5 µg/ml without CD to a maximum of 2.9 µg/ml at 144 h. The FHA in the culture supernatant increased slightly but the FHA measured on the cells showed a large increase from 0.7 µg/ml to a maximum of 3.7 µg/ml at 192 h.

The medium in the chemostat was changed at 264 h to SS+CD medium containing 50% of the original cystine concentration. After this the culture density fell to below the value it was before the addition of CD. The LPF and FHA measured in the culture supernatant also fell to levels similar to those obtained before the addition of CD.

When CD was added to the growth medium for continuous culture the culture density increased, indicating that a nutrient limitation was not operating in the system. The CD must be acting to remove a growth inhibition, allowing the organism to grow maximally, utilizing the available nutrients. When the concentration of cystine in the medium was reduced, the culture density fell by a proportional amount indicating that cystine limitation was now operating. CD increased the LPF in the supernatant in excess of the increase in culture density. This also occurred for cellular FHA. FHA is detected in low levels in the supernatant and CD appears to make little difference. This may be because FHA appears to be released into the supernatant to the greatest degree by stationary phase cells and in a continuous culture system the

Table 9. Effects of CD on B. pertussis in continuous culture.

Conditions	Culture time (h)	OD	LPF supt. µg/ml	FHA supt. µg/ml	FHA cells µg/ml
D=0.1, SS medium	96	1.10	0.5	0.04	0.7
+0.5 g/l CD	120	1.33	2.2	0.03	0.7
"	144	1.40	2.9	0.1	3.0
"	168	1.47	2.7	0.02	3.0
"	192	2.01	2.6	0.3	3.7
"	264	1.92	1.4	0.1	2.5
" 50% cystine	288	1.20	0.4	0.1	3.0
" "	312	0.90	0.5	0.06	3.0
" "	336	-	1.0	0.08	4.0

population is kept in exponential phase.

3.3 Summary.

The growth of B. pertussis was studied in batch and continuous culture systems. In batch cultures the pH increased during exponential and stationary phase growth. This has been noted by previous workers (Rowatt, 1957b; Lane, 1968; Van Hemert, 1974). The maximum expression of LPF in the supernatant occurred at the end of the exponential and the beginning of the stationary phase of growth, also found by Sato et al. (1974), Sekura et al. (1983) and Bellalou & Relyveld (1984). In the absence of CD FHA was only detected in static liquid cultures, whilst in shaken liquid cultures it was almost completely absent from the supernatant in aggreement with the finding of Arai & Munoz (1979).

It was possible to maintain the growth of virulent, phase I B. pertussis in continuous culture. The maintainance of mouse protective activity of B. pertussis cultures in continuous culture has been demonstrated using complex media (Van Hemert, 1974; Jagicza et al., 1985) and using defined media (Novotny & Cownley, 1979) but this study is the first to show the maintainance of defined virulence components (LPF, FHA, certain outer membrane proteins). The response of B. pertussis to cystine or iron limited conditions was to change to the avirulent phase IV variant. Phase IV organisms were able to grow under the cystine limited conditions but not in the presence of the iron chelator desferal.

The effects of CD on B. pertussis cultures were observed. The addition of CD to the growth medium reduced the length of the lag phase of growth and increased the maximum culture density in shaken flask or Thompson bottle cultures. The maximum culture density was not increased in stirred fermenter cultures but a higher density was achieved by all cultures even in the absence of CD. This may be because the fermenter cultures were not oxygen limited, as may occur in shaken culture vessels and the CD

may allow greater growth under these oxygen limited conditions. CD was also seen to affect the expression of virulence determinants. The expression of LPF was increased but not to the extent reported by Imaizumi et al. (1983b). The concentrations of LPF achieved here are similar to those achieved by Perera et al. (1986). CD had a greater effect on the expression of FHA in shaken Thompson bottle cultures and particularly in stirred fermenter cultures. In the presence of CD FHA was detected in the supernatant and the expression of FHA in fermenter cultures was also affected by the culture pH. The high pH that occurred in stationary phase cultures appeared to favour release of FHA into the supernatant and the concentration of FHA declined during stationary phase if the culture was maintained at the initial pH. The nutrient requirements of B. pertussis were also altered by CD with B. pertussis no longer having an obligate requirement for glutathione as it presumably could obtain its organic sulphur from the cystine in the medium, which does not occur in the absence of CD (Vajdic et al., 1966).

CD affected the growth of B. pertussis in continuous culture. When CD was added to the medium the culture density increased. This suggested that there was no nutrient limitation acting in the system but that there is a growth inhibition, possibly due to toxic fatty acids present in the medium. It may be that CD removes the growth inhibition and allows the organism to fully utilise the available nutrients. When the concentration of cystine was reduced in the presence of CD the culture density was reduced, thus cystine limitation was now operating.

Chapter 4.

8 l scale growth of B. pertussis.

Having studied the growth of B. pertussis in small scale vessels it was necessary to determine the effects of conditions of culture in larger (8-10l) fermenters. Large volumes of B. pertussis culture are required for producing pertussis vaccine and cultures must also contain the required antigens in sufficient quantity for purification. Pertussis vaccine manufacturers tend to adhere to "time honoured" cultural practices without defining the conditions that make for effective growth and yield of required antigens. According to Griffith (1978), Wellcome pertussis vaccine has been produced on solid medium containing starch for the last 25 years with little variation in procedure and only one change in the production strain. Sato et al. (1984) describe the Japanese acellular vaccine as being produced in liquid medium in many large Roux bottles. Both these methods are highly labour intensive and expensive but are continued with because of their reliability. The growth of B. pertussis in stainless steel fermenters has been found difficult (Novotny & Cownley, 1979) but if this method could be made succesful it would be a great advantage in vaccine production.

Growth of B. pertussis for initial production of the CAMR acellular vaccine was performed using shaken Thompson bottles (approximately 60 Thompson bottles per 20l batch). Inoculating and harvesting cultures grown in this way, adhering to the principles of Good Manufacturing Practice, was very time consuming, especially as these operations were performed in an enclosed class III safety cabinet. This was to prevent exposure of the product to outside contamination.

Various fermenters were evaluated for the growth of B. pertussis. These included a Porton 10l and a Porton 20l enclosed fermenter (Evans et al., 1971), a Bioengineering 10l fermenter and an LH 2000 series fermenter with a

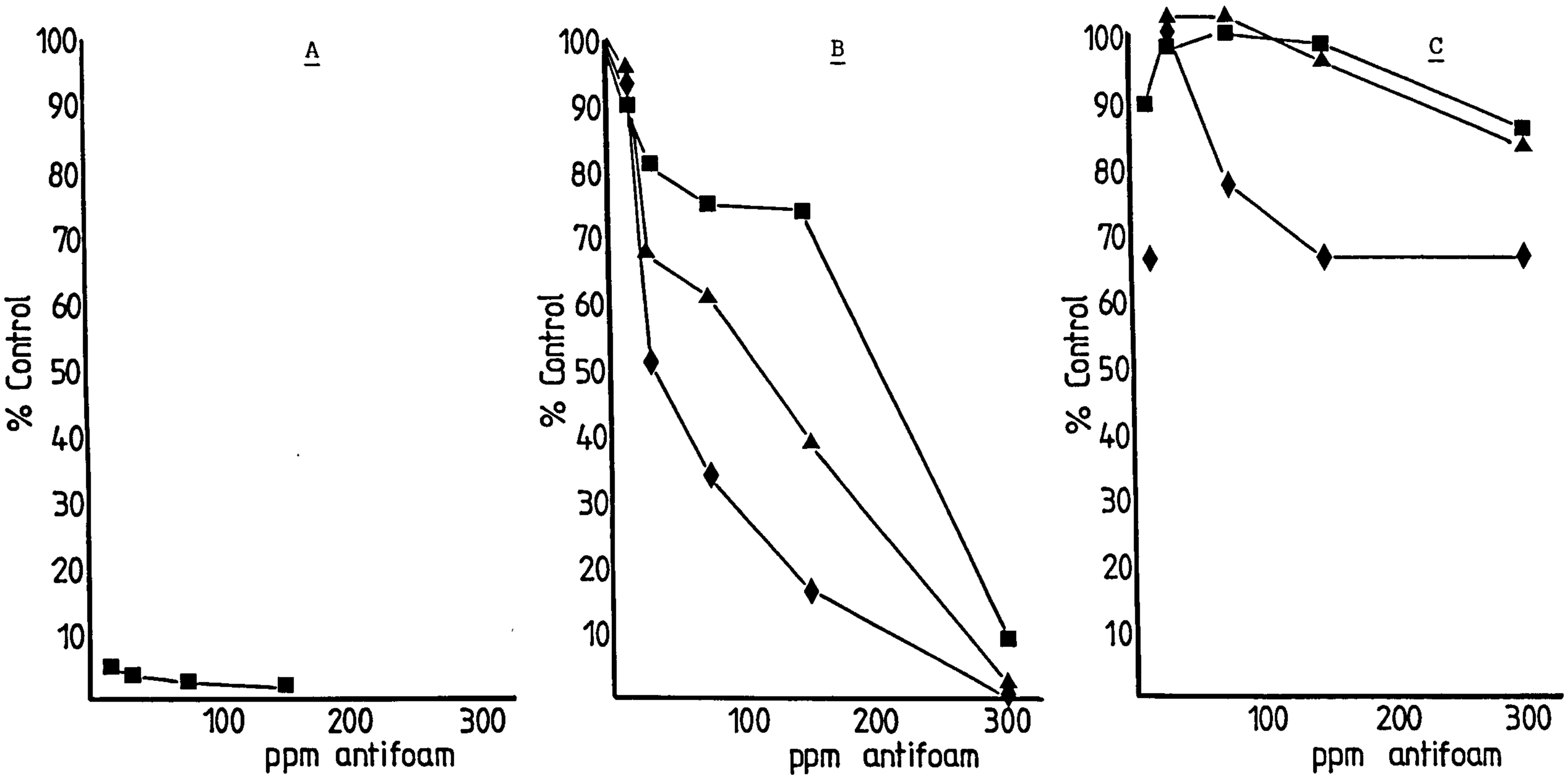
working volume of 8l. The LH 2000 series fermenter was chosen to study the growth of B. pertussis as it enabled control of both pH and oxygen tension in the culture. With all the fermenters there was a problem with the culture foaming and blocking the air-out filter, terminating the culture. Initially, PPG antifoam was used to overcome this problem but after addition of PPG the fermenters contained a mass of lysed bacteria floating on the surface of the medium and the cultures were dead. It was therefore essential to find an antifoaming agent that could be used to control foaming in B. pertussis cultures.

4.1 The effect of antifoaming agents on growth of B. pertussis.

Initial experiments indicated that B. pertussis was very sensitive to the addition of antifoaming agents during growth. Therefore the effects of the following antifoaming agents were determined; polypropylene glycol (PPG, K.W. Revai Chemicals Ltd.) and RD and M30 silicone emulsions (Dow Corning Ltd.).

The effect of PPG, silicone RD and M30 emulsion antifoams on growth and expression of LPF and FHA was determined in shake flask cultures in CL+CD medium. Growth and production of LPF and FHA were expressed as a percentage of control values with no antifoam (fig. 9). No growth was observed with even the lowest concentration of PPG used. Silicone RD emulsion caused a marked inhibition of growth (75% of control at 75 ppm RD emulsion and 10% of control at 300 ppm RD emulsion). Inhibition of expression of LPF and FHA in the supernatant was greater than the inhibition of growth by silicone RD emulsion (FHA reduced to 50% of control by 25 ppm RD emulsion). M30 silicone emulsion caused a much smaller inhibition of growth (82% of control at 300 ppm M30 emulsion) and expression of LPF was inhibited to a similar degree. Expression of FHA was inhibited slightly more, reduced to 68% of control by 150 ppm M30 emulsion, but this was still a smaller inhibition than caused by RD silicone emulsion.

Fig. 9. Effect of antifoaming agents on growth and expression of LPF and FHA. A: PPG, B: Silicone, RD emulsion, C: Silicone, M30 emulsion. ■ , OD_{550nm}; ▲ , LPF Supt.; ♦ , FHA Supt.



Silcone RD and M30 emulsions can be used to suppress foaming in B. pertussis cultures but for maximum growth and expression of LPF and FHA it is important to use as sparingly as possible. M30 silicone emulsion,which is described as being suitable for pharmaceutical applications was the least toxic to B. pertussis.

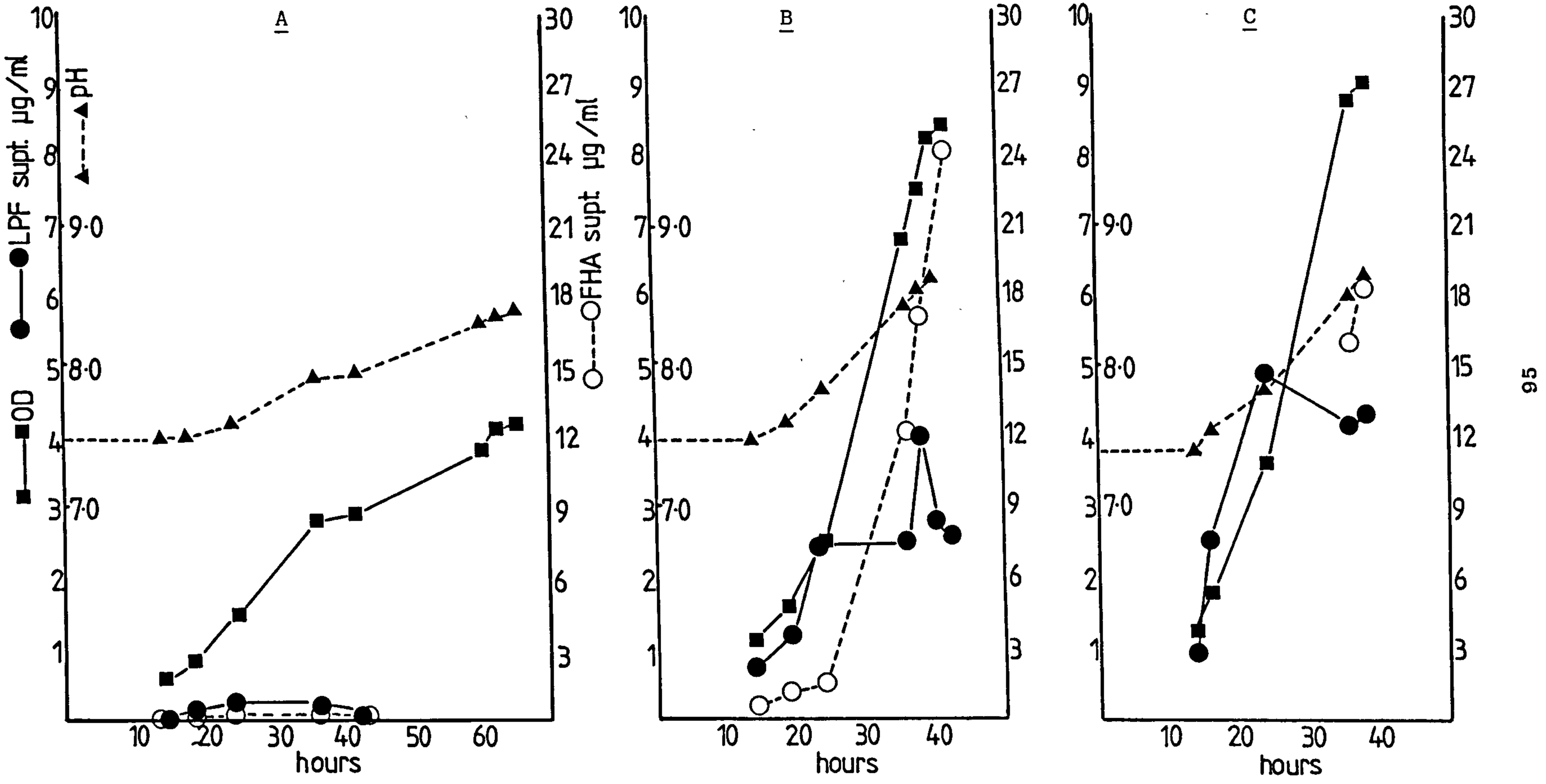
4.2 Growth of B. pertussis in the LH 2000 Series fermenter.

4.2.1 Effect of CD on growth of B. pertussis in the LH fermenter.

As CD enhanced growth and expression of LPF and FHA in glass 500ml stirred fermenters its effect was determined in the 8l stainless steel LH fermenter.

B. pertussis was grown in the LH fermenter using an oxygen saturation set point of 20%. Silicone RD emulsion antifoam was used on automatic timed addition from a culture time of 24 h to the end of the growth. The growth and expression of LPF and FHA obtained in medium containing 0, 1 and 2 g/l CD is shown in fig. 10. Without CD poor growth was observed, the maximum OD only reaching 4 after over 60h. LPF and FHA were present in the supernatant between 20 and 40 h, but concentrations were only just within the limit of detection of the assays. This was a general result that poor growth occured in fermenter in the absence of CD, even with a larger inoculum (data not shown). The use of medium containing 1 and 2 g/l CD produced simliar growth but with a slightly higher maximum OD in the 2 g/l culture.The maximum LPF concentration in the supernatants of the 1 and 2 g/l CD cultures were 4.0 and 5.0 µg/ml. The maximum LPF concentration in the 2 g/l CD culture was achieved approximately 15 h earlier at a culture time of 24 h. The FHA concentration in the supernatant was similar for each of the CD cultures with slightly the higher amount seen in the 1 g/l CD culture.

Fig. 10. Growth of B. pertussis in LH 8 l fermenter with CL medium containing: A, 0 g/l CD; B, 1 g/l CD; C 2 g/l CD.



4.2.2 Concentration of CD in fermenter supernatants during growth.

The concentration of CD was determined in supernatants from Thompson bottle cultures of B. pertussis and was found to remain unchanged. However, higher culture densities were obtained in the 8 l fermenter so the concentration of CD in the supernatant was determined during growth to see if it also remained unchanged. Results presented in table 10 show no loss of CD during fermentation, even with the high culture density achieved. This confirms that CD does not serve as a nutrient in the medium.

4.2.3 Effect of aeration on growth of B. pertussis in the LH fermenter.

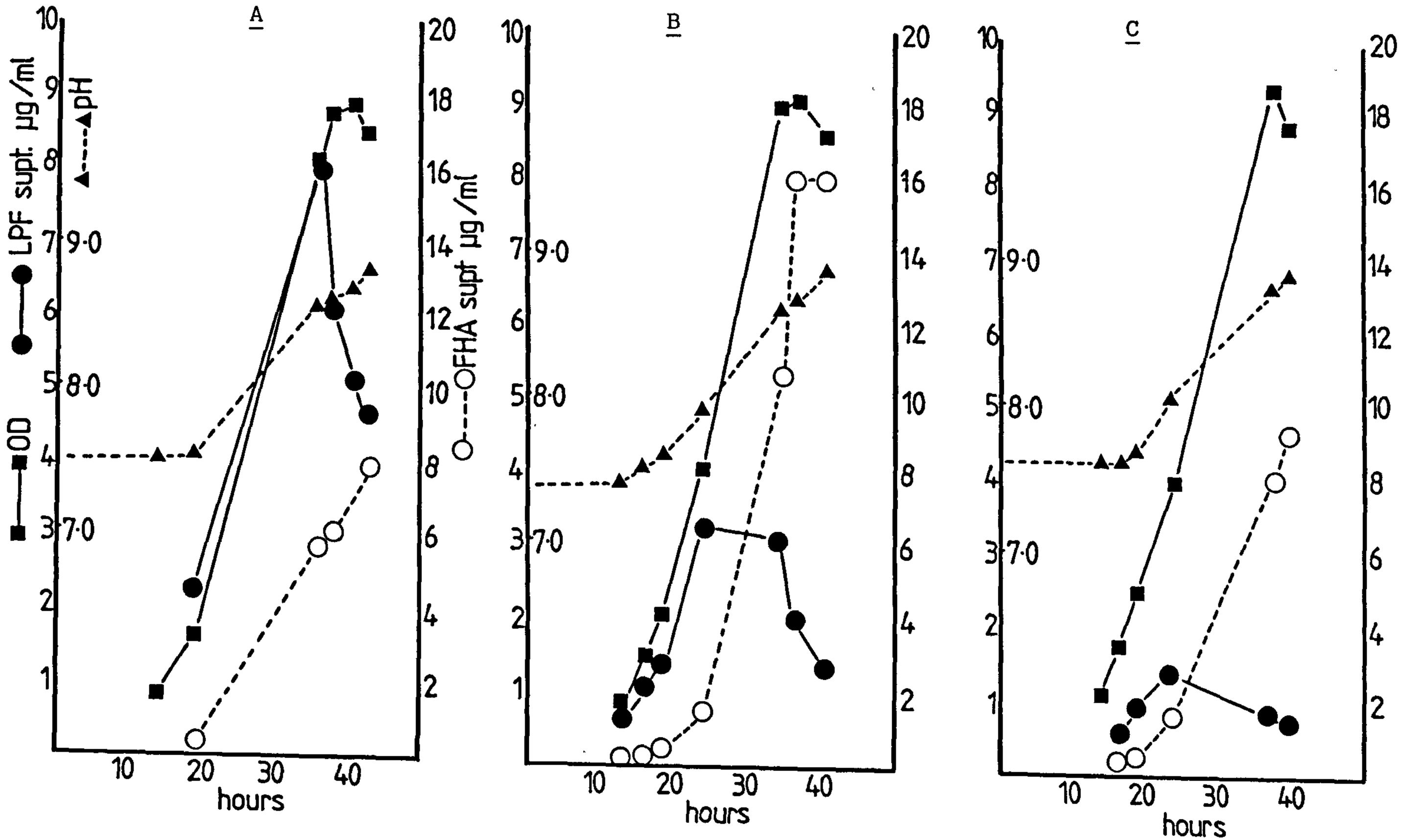
In the absence of CD, FHA is produced by B. pertussis in highest concentration in static Thompson bottle cultures. This may be because the degree of aeration in statically incubated Thompson bottles is favourable for FHA production. Lane (1968) reported that B. pertussis cultures grown with a high degree of aeration were less toxic to mice than cultures grown at lower levels of aeration, suggesting that the degree of aeration is important for maximum expression of virulence determinants. The facility of the LH 2000 fermenter to control the O_2 saturation in the culture allowed the effect of aeration on growth of B. pertussis to be studied.

Growth and expression of LPF and FHA by B. pertussis with % O_2 set points of 5, 20 and 50%, in 1 g/l CD medium, are shown in fig. 11. Growth curves were very similar at all three % O_2 set points, indicating that none of these O_2 concentrations were limiting for growth. The expression of LPF in the supernatant was favoured by the lower O_2 set point, with LPF maximum concentrations of 8.0, 3.2 and 1.3 at O_2 set points of 5, 20 and 50%. FHA concentrations were similar at O_2 set points of 5 and 50%, with the highest amount (16.0 µg/ml) detected in the 20% O_2 set point

Table 10. Concentration of CD in LH 81 fermenter CL+CD supernatant determined by TNS fluorescence assay.

Time (h)	OD	CD g/l
16	0.73	0.92
19	1.12	0.88
23	1.12	0.89
28	0.84	0.97
40	6.00	0.97
42	7.15	1.05
44	8.78	1.04
47	9.10	1.05

Fig. 11. Growth of B. pertussis in LH fermenter at O_2 set points A, 5%; B, 20%; C, 50%.



culture.

The effects of other aeration regimes on growth of B. pertussis were also studied. Fig. 12A shows the effect of top aeration only. In this case the baffles were removed from the vessel so that a slight vortex was formed on stirring and air was passed across the head space of the fermenter. After inoculation of the fermenter the % O_2 quickly fell below that measurable by the O_2 electrode. It can be seen that these conditions provided insufficicent aeration as almost no growth was observed in over 120 h.

The effect of sparged aeration but with a constant stirrer speed of 300 rpm on growth and expression of LPF and FHA is shown in fig. 12B. Again, the % O_2 reading fell soon after inoculation to below the limit of detection of the electrode. The rate of increase in culture density was slower than that obtained when the % O_2 was controlled by automatic regulation of the stirrer speed, the culture reaching a maximum OD of 6.4 at 68 h. The increase in LPF and FHA lagged behind the increase in OD but all reached maximum values at the same time. The maximum LPF concentration was 5.4 µg/ml and the maximum concentration of FHA was 23.5 µg/ml.

B. pertussis cultures were grown with an O_2 set point of 20% for the first 36 h of the culture and then the sparge aeration was turned off and the stirrer speed reduced to a constant 200 rpm. The effect of this on expression of LPF and FHA is shown in fig. 13. This culture produced high levels of LPF and FHA with the LPF concentration increasing slightly when the air was turned off. Turning the air off had the greatest effect on the concentration of FHA in the supernatant. It increased from 35 µg/ml at 36 h to 45 µg/ml at 42 h. The culture density had achieved its maximum at 36 h and quickly fell after this time when the air was turned off. It appears that the combination of low oxygen concentration and gentle mixing was favoured expression of FHA in the supernatant.

From these cultures grown in the LH 81 fermenter it

Fig. 12. Growth of B. pertussis in LH fermenter. A; With top aeration. B; With sparged aeration and a constant, slow stirrer speed.

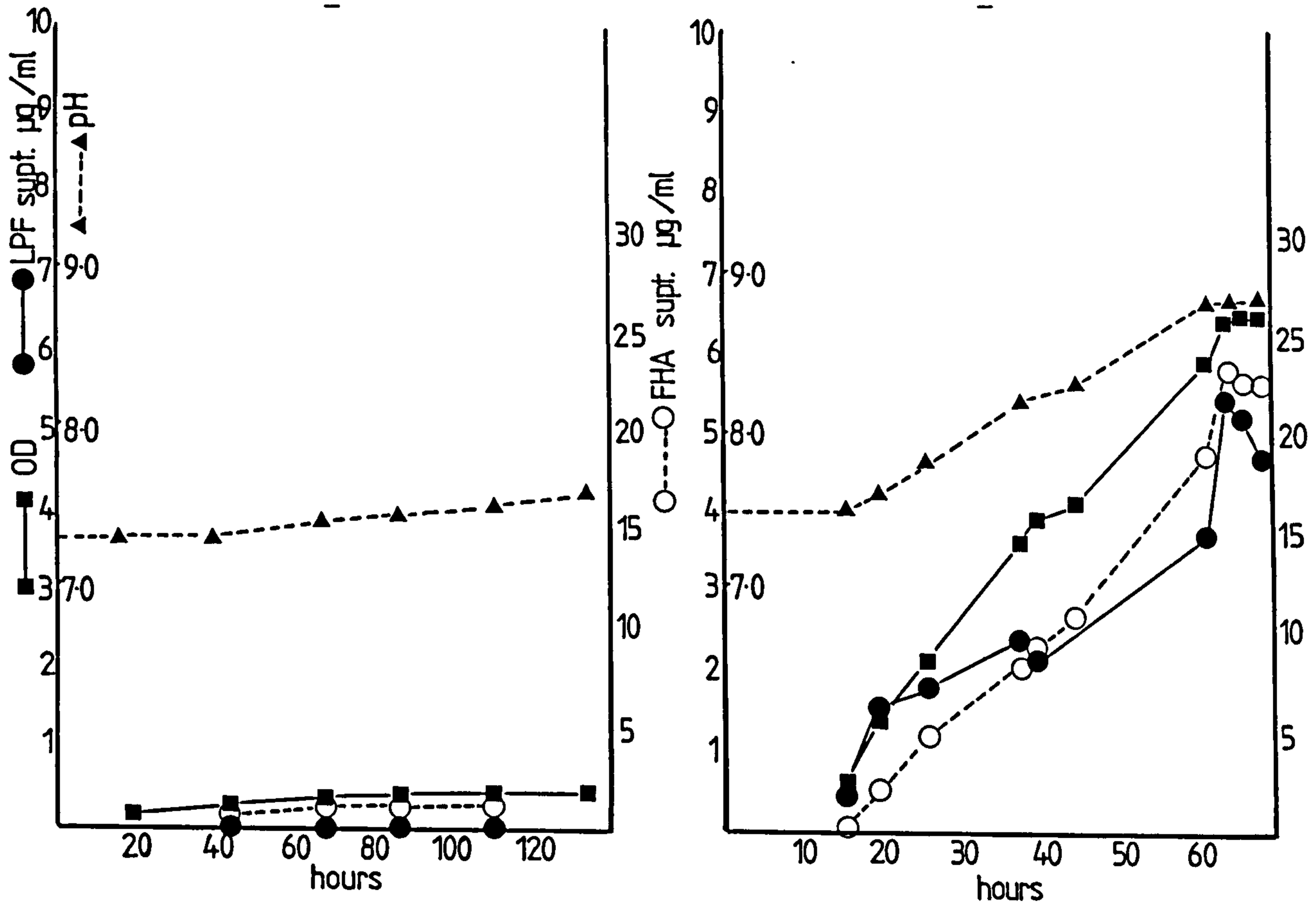
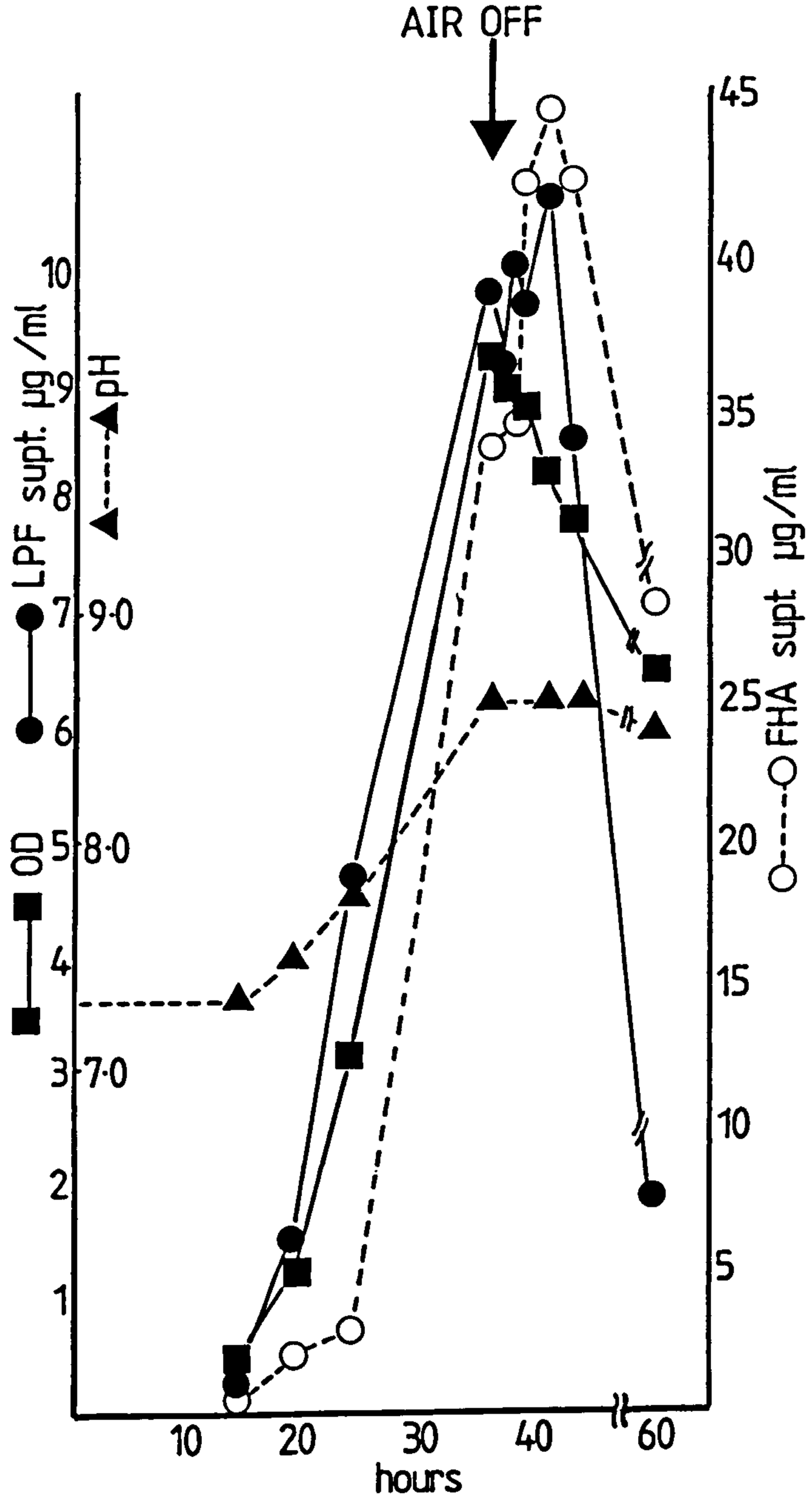


Fig. 13. Growth of B. pertussis in LH fermenter. The O_2 set point was 20% for the first 36 h when the air was turned off and the stirrer speed reduced.



can be seen that CD is necessary to obtain high yields of cells, LPF and FHA and that the optimum concentration of CD is 1 g/l. The O_2 set point was not important for growth but expression of LPF was enhanced by the lower set point used and FHA by the middle value. Growth without sparged aeration was poor as insufficient O_2 was transferred to the culture for optimum growth. The use of a constant, low stirrer speed did not produce maximum growth but these conditions favoured the production of FHA. Low stirrer speed and low O_2 concentrations at the end of growth also favoured FHA production. It can be concluded that CD and sparged aeration are required for maximum growth but that expression of FHA is enhanced by low aeration and mixing during the stationary phase of the culture.

4.3 Culture harvesting using the Millipore Pellicon.

Having grown a large batch of B. pertussis in a fermenter or Thompson bottles, a method of removing the bacteria from the culture is required so that the supernatant and cells can be processed to purify the LPF, FHA and agglutinogens for an acellular vaccine. The culture supernatant must be passed though a 0.2µm filter to remove all bacteria before purification of LPF and FHA. A very clear supernatant is therefore necessary if the filter is not to become blocked. A Padburg continuous flow centrifuge was used to remove cells from the culture but it did not prove effective with approximately 20% of the cells remaining in the supernatant. It was therefore attempted to concentrate the culture using the Pellicon tangential flow ultrafiltration system so that the resulting small volumes of culture could be more easily handled in a laboratory centrifuge. Also, small volumes of concentrated supernatant would be an advantage for the column chromatography purification steps.

4.3.1 Use for concentration of whole culture.

A typical example of the use of the Pellicon for concentration of whole culture is given in table 11A. The

Table 11A. Pellicon concentration of whole culture.
Culture concentrated from 8.81 to 1.481 (x5.94).

		LPF		FHA	
	Vol.	supt.	Total	supt.	Total
	l	µg/ml	mg	µg/ml	mg
Final pool, CL+CD culture	8.8	4.6	40.5	24.2	213
Pellicon concentrate	1.48	34.3	50.7	45.2	67
Pellicon filtrate	-	0		0	

Loss of LPF on concentration = 0
Loss of FHA on concentration = ~70%

Table 11B. The effect of coating the Pellicon membrane with bovine serum albumin.
Culture concentrated 9.01 to 1.61 (x5.62)

		LPF		FHA	
	Vol	supt.	Total	supt.	Total
	l	µg/ml	mg	µg/ml	mg
Final CL+CD culture pool	9.0	4.1	36.9	119.0	1071
Pellicon concentrate	1.6	23.5	37.6	60.0	96
Pellicon filtrate	-	0		0	

Table 12. Concentration of culture supernatant.
Supernatant concentrated from 18 l to 3.6 l (x5).

		LPF		FHA	
	Vol	supt.	Total	supt.	Total
	l	µg/ml	mg	µg/ml	mg
Pool culture supernatant	18	4.3	77.4	21.4	385
Pellicon concentrate	3.6	16.0	57.6	98.5	355

culture (48 h Thompson bottle growth) was concentrated from 8.8l to 1.48l (x 5.94). From the ELISA determinations of LPF and FHA concentrations it can be seen that there was no loss of LPF on concentration whereas the loss of FHA is approximately 70%. The LPF and FHA were not lost by passing through the membrane as no LPF or FHA was detected in the filtrate by ELISA.

The membrane in the Pellicon was precoated with BSA to determine if this would reduce the loss of FHA (table 11B). The culture was concentrated from 9l to 1.6 l (x5.62). The FHA concentration in the supernatant was high initially and approximately 90% was lost on Pellicon concentration. There was no loss of LPF and again no LPF or FHA was detected in the Pellicon filtrate. This indicates that the FHA was not lost by non-specific binding to the polysulfone membrane.

4.3.2 Use for concentration of culture supernatant.

FHA was lost from the concentrated culture supernatant when whole culture was concentrated. It was thought that the FHA may be removed from the supernatant by it binding to the cells in the concentrated cell slurry and then being removed with the cells on centrifugation. If this was occurring then the loss of FHA would be reduced if the culture was centrifuged to remove the bacteria and the supernatant then concentrated.

A 48 h Thompson bottle culture was centrifuged at 7280 xg for 60 min (Sorvall RC3B centrifuge, 6x11 swing-out rotor) and the supernatant then concentrated from 18 l to 3.6 l (table 12). Using the Pellicon in this way there was virtually no loss of LPF or FHA from the supernatant on concentration. This shows that the FHA was probably lost from the supernatant to the cell pellet when the concentrated culture was centrifuged.

This method did not replace the time consuming initial centrifugation of the culture but the reduction in supernatant volume was a significant advantage for subsequent processing. Therefore, this was the method

adopted for the CAMR vaccine manufacturing process.

4.4 Summary

For fermenter growth with sparged aeration it was necessary to use an antifoam but these proved to be toxic to B. pertussis. Silicone RD and M30 emulsions could be used sparingly. Unlike the 500ml fermenter results (3.1.4), only poor growth could be obtained without CD. Increasing the CD concentration from 1 to 2 g/l reduced the length of the lag phase of the culture and the maximum LPF concentration was produced earlier in the culture. As seen for Thompson bottle cultures, the concentration of CD in the supernatant remained constant throughout the culture and was not metabolised by B. pertussis, even at the high culture densities achieved in the 8l fermenter.

The effect on B. pertussis cultures of varying the aeration was determined. Controlling the oxygen saturation to 5, 20 and 50% produced equal growth as none of these levels were limiting for growth. Cultures grown at 5% oxygen saturation produced the highest levels of LPF and those grown at 20% oxygen the highest levels of FHA. Top aeration alone or sparged aeration with a constant slow stirrer speed did not supply sufficient oxygen for maximum growth. However, the use of a constant slow stirrer speed resulted in a high concentration of FHA in the supernatant relative to the culture density. Switching off the sparged aeration and reducing the mixing at the onset of the stationary phase of growth also increased the FHA concentration measured. These results show that B. pertussis will not achieve maximum growth unless there is sufficient oxygen transfer to the culture. However, once maximum culture density has been reached little oxygen and gentle mixing provide the most favourable conditions for production of FHA.

The Pellicon ultrafiltration system proved useful for downstream processing of B. pertussis cultures but only for concentration of culture supernatants, as FHA was lost when whole culture was concentrated. The FHA was probably

lost to the cell pellet when concentrated cell slurry was centrifuged.

The growth of B. pertussis in stainless steel fermenters does provide a viable alternative to the use of many small cultures for vaccine production. Equivalent or greater yields of LPF and FHA were obtained but only when medium containing CD was used.

Chapter 5.

The effects of antigenic modulation on B.pertussis.

The effects of conditions of culture on the growth and expression of virulence determinants of B. pertussis have been discussed in chapters 3 and 4. The growth environment can also affect the expression of virulence determinants in a process known as antigenic modulation (section 1.3.3). Antigenic modulation occurs when the NaCl in the medium is replaced with an equivalent amount of $MgSO_4$ (Lacey, 1960) or when the nicotinic acid acid concentration is increased ten-fold (Pusztai & Joo, 1967). This transition from virulent X-mode organisms to avirulent C-mode organisms has been studied using solid medium and using liquid batch cultures. Antigenic modulation was studied here to gain a further insight into the conditions that regulate expression of virulence determinants in B. pertussis.

The aims of this study were to observe antigenic modulation in the defined environment provided by a continuous culture system. This would allow X- and C-mode cultures with constant, equal growth rates to be compared. Growth in continuous culture also allows the rate of change from X- to C-mode to be studied in a population with a constant growth rate and whether different X-mode characteristics are lost at different rates could be determined. The conditions that trigger the switch from X-mode to C-mode were also defined in both batch and continuous culture. In chapters 3 and 4 it was found that CD enhanced expression of LPF and FHA. Therefore its effect on expression of X-mode characteristics during antigenic modulation was determined.

5.1 Effects of antigenic modulation on B. pertussis.

B. pertussis (strain Wellcome 28) cultures were grown in SS X-mode, SS $MgSO_4$ or SS NA C-mode media in continuous culture ($D=0.05h^{-1}$) to characterise the X- and C-mode variants produced. A comparison of the properties of the

X- and C-mode bacteria is given in table 13. LPF and FHA were detected by ELISA only in supernatants from X-mode cultures. Fimbriae were observed using the electron microscope on negatively stained organisms from X-mode cultures but not on organisms from C-mode culture. Likewise, SDS-PAGE analysis of whole cells (fig. 14) showed that certain outer membrane proteins, with molecular weights previously determined as 90 000, 86 000, 80 000, 33 000, 31 000 and 30 000 (Robinson & Hawkins, 1983) were only present in X-mode organisms. cAMP was detected in X-mode supernatants but not in those from C-mode cultures and adenylate cyclase activity in C-mode cells was less than 5% of the X-mode value. Thus B. pertussis lost all these virulence-associated components when grown in the C-mode in continuous culture and also there was no difference in the characteristics determined between C-mode organisms produced in the presence of $MgSO_4$ or increased NA.

5.2 Effect of antigenic modulation on adhesion of B. pertussis to Vero cells.

It has been demonstrated that virulent B. pertussis adhere to Vero cells cultured on glass cover slips (Sato et al., 1981). Therefore this assay was used to determine if the ability to adhere in this system was lost on transition from X- to C-mode.

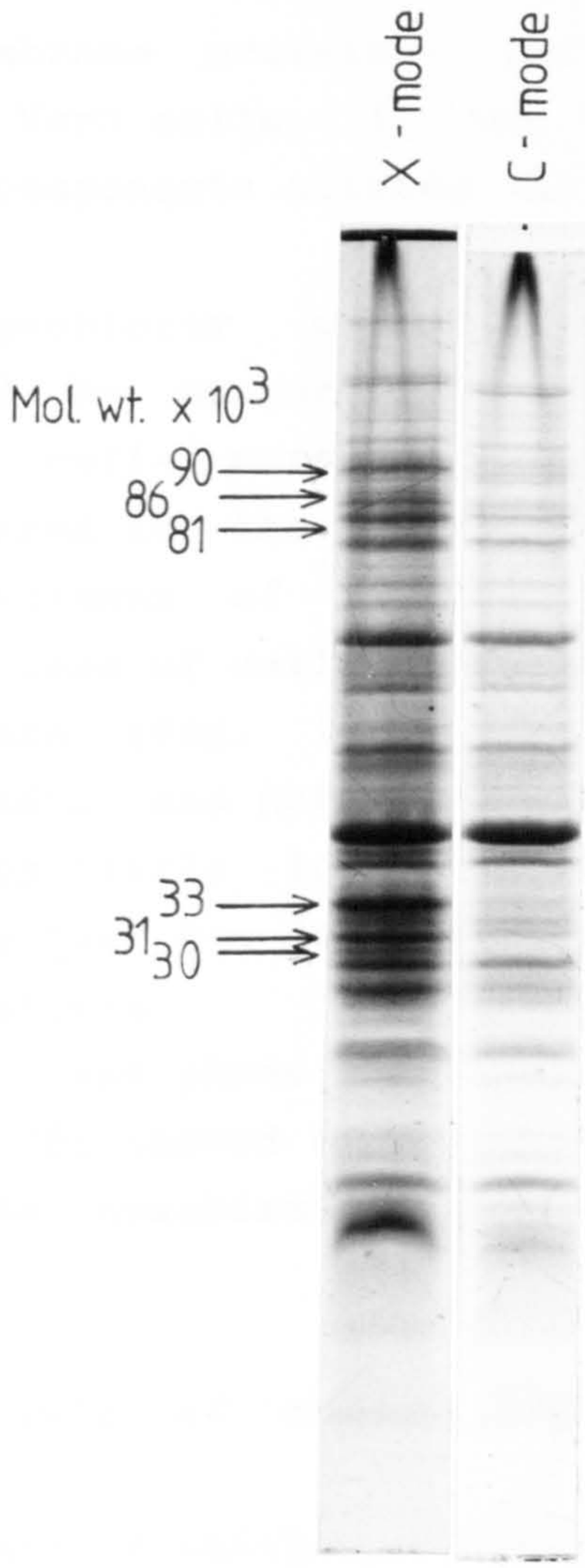
The adherence of X- and C-mode organisms to Vero cells was compared using B. pertussis grown in continuous culture at a dilution rate of $0.1h^{-1}$ in the X-mode and in the NA-induced C-mode. The mean number of X-mode organisms adhering per Vero cell was 10.5 (S.D.=5.1), whereas the mean number of C-mode organisms adhering per Vero cell was 2.9 (S.D.=2.8). Means were significantly different as determined by the Students' t test ($p>0.01$). Thus the antigenically deficient C-mode organisms have a greatly reduced ability to adhere to this cell line, indicating that the surface properties necessary for adhesion are amongst those lost on antigenic modulation.

Table 13. Comparison of X- and C-mode *B. pertussis* grown in continuous culture D = 0.05 h^{-1}.

	X-mode	$MgSO_4$ C-mode	NA C-mode
LPF in culture supt. µg/ml	1.7	0	0
FHA in culture supt. µg/ml	0.1	0	0
Fimbriae	present	absent	absent
X-mode OMPs	present	absent	absent
cAMP supt. pmol ml^{-1}	1760	ND	0
Adenylate cyclase nmol cAMP ml^{-1} mg^{-1} protein	45.1	ND	1.9

ND = Not determined.

Fig. 14. SDS-PAGE of B. pertussis cells grown in continuous culture, D = 0·05 h^{-1} with SS X-mode and $MgSO_4$ C-mode medium.



5.3 Effect of antigenic modulation on cell surface hydrophobicity.

B. pertussis grown in C-mode medium does not express several surface components (FHA, fimbriae, LPF and certain outer membrane proteins) and has a reduced ability to adhere to Vero cells. It was determined whether the loss of these components altered the hydrophobicity of the cell surface.

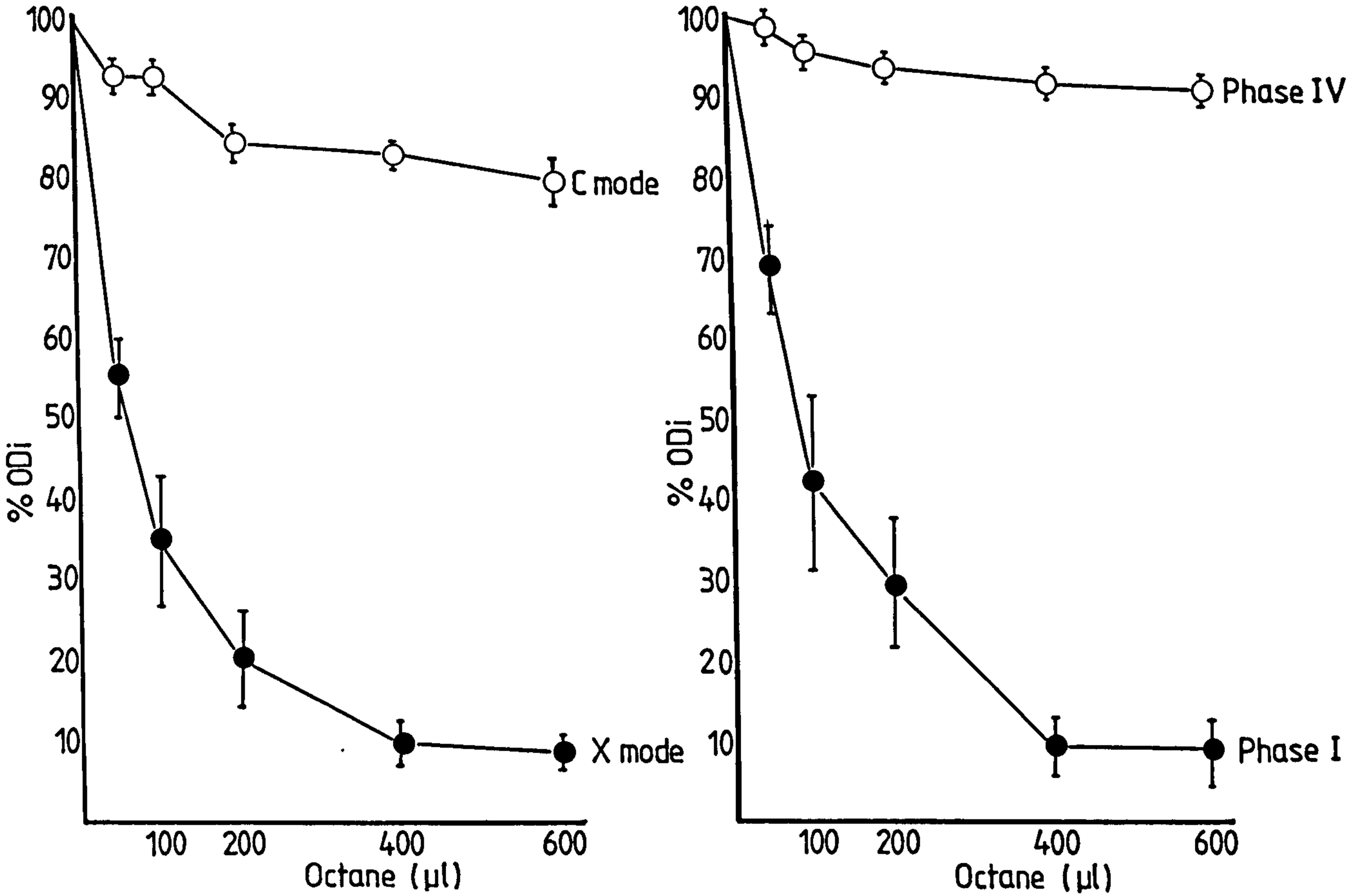
Hydrophobicity of X- and C-mode cultures was determined by measuring adhesion to octane. When B. pertussis cells were vortex-mixed with octane, X-mode cells adhered to the octane droplets formed and with larger volumes of octane (400µl), there was an almost complete loss of cells from the aqueous phase to the octane phase (fig. 15A). B. pertussis cells grown in C-mode media did not adhere to the octane droplets and showed very little loss of OD in the aqueous phase, indicating that C-mode organisms are more hydrophilic than X-mode bacteria.

Phase I and phase IV organisms of the same strain (Wellcome 28) showed similar hydrophobicity profiles as X- and C-mode organisms as determined by adhesion to octane (fig. 15B).

5.4 The rate of antigenic modulation in continuous culture.

The rate of antigenic modulation has been determined in batch cultures by Idigbe et al. (1981). They report that the rate of antigenic modulation was rapid, with X-mode components lost within 10 h. However, the results are difficult to assess as the rate cannot be related directly to growth rate and doubling times of the organism. The aims of this study were to determine whether reversible antigenic modulation of B. pertussis could be achieved in continuous culture and to investigate whether the rate of modulation could be varied by altering the dilution rate. It would also be possible to determine if

Fig. 15. Cell surface hydrophobicity of B. pertussis determined by adhesion to octane.



X-mode components were lost at equal or different rates.

5.4.1 $MgSO_4$-induced modulation.

B. pertussis was grown in continuous culture at a dilution rate of 0.1 h^{-1} in SS X-mode until steady state growth was achieved. The hydrophobicity of cells was determined by adhesion to octane, cellular LPF content of cells was measured by in vivo assay in mice and the SDS-PAGE profiles of bacteria were determined. The content of the 33 000 molecular weight X-mode specific outer membrane protein was measured by scanning SDS-PAGE gels and comparing the relative peak heights.

When the medium was changed to $MgSO_4$ C-mode medium there was a rapid loss of X-mode characteristics. There was a reduction in cell surface hydrophobicity and a decline in cell-associated LPF content. The loss of these characteristics, expressed as a percentage of their X-mode value is shown in fig. 16 together with the theoretical washout rate of the culture for this dilution rate. As the medium in the growth vessel was changed to C-mode, there was also a decrease in several cellular proteins previously identified to have molecular weights of 90 000, 86 000, 80 000, 33 000, 31 000 and 30 000 (fig. 17). The rate of decrease of the M_r 33 000 protein is shown in fig. 16. After 50 h, the chemostat medium was changed back to X-mode. There was a rapid increase in cell surface hydrohobicity, X-mode specific proteins and LPF content as the culture reverted to X-mode (figs. 16 and 17). Thus it can be seen that $MgSO_4$ antigenic modulation was completely reversible in continuous culture.

The rates of loss of LPF and of hydrophobicity at dilution rates of 0.15 and 0.05 (corresponding with doubling times of 5.25 and 14 h) are shown in fig. 18A and B. The rate of loss of X-mode characteristics for the three dilution rates studied was dependent on the dilution rate. The loss of LPF was equal to or greater than the theoretical washout rate. Of the X-mode characteristics measured it is suggested (figs. 16 and 18) that LPF was

Fig. 16. $MgSO_4$-induced antigenic modulation of B. pertussis in continuous culture, D = 0.1 h^{-1}. At 0h the medium was changed to C-mode and back to X-mode at 50 h. X-mode cells are considered to have 100% of each characteristic and C-mode cells 0%. ▲ , LPF; ● 33,000 molecular weight outer membrane protein; ○ , hydrophobicity; dashed line is theoretical washout rate.

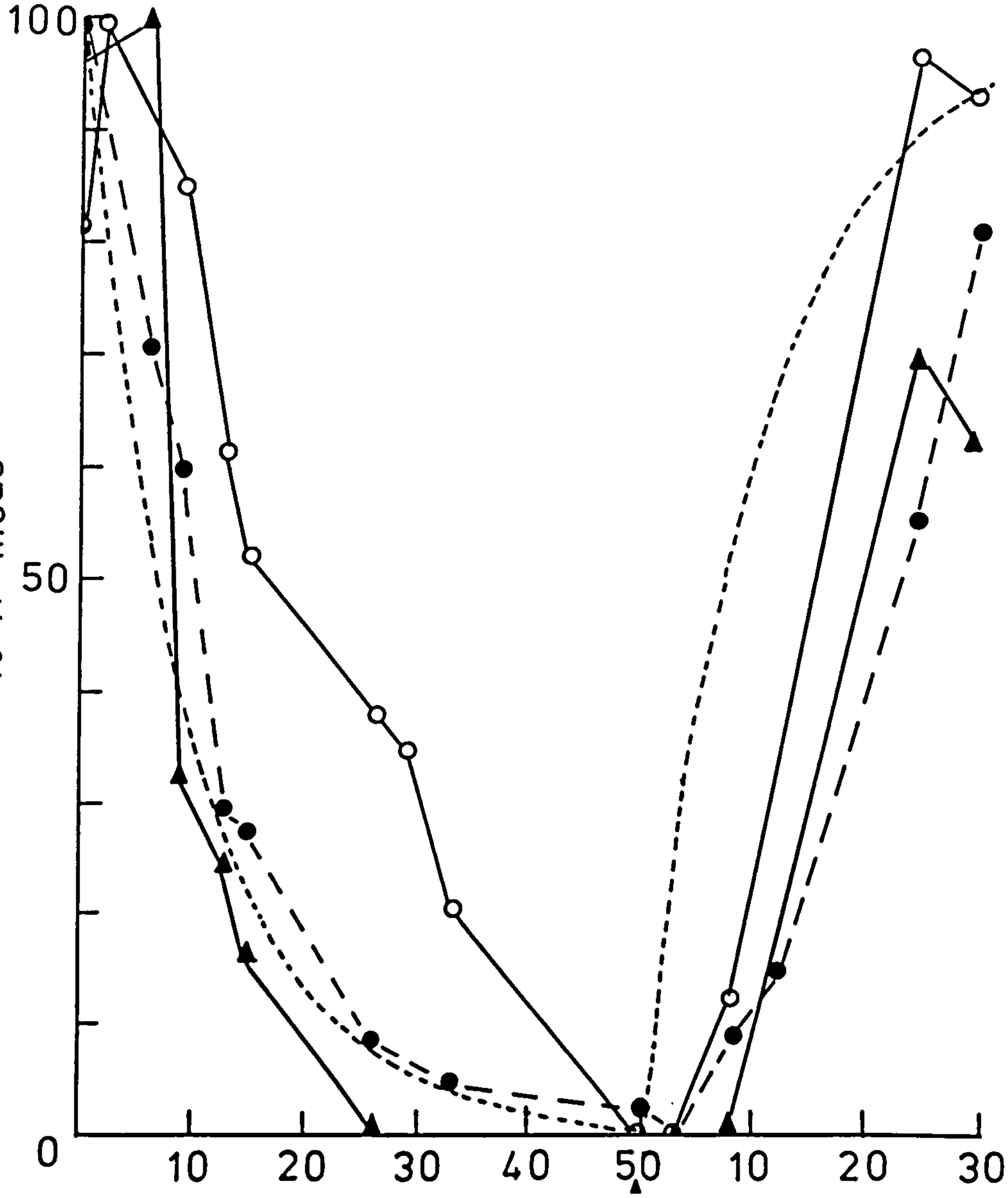


Fig. 17. SDS-PAGE of B. pertussis cells during $MgSO_4$-induced antigenic modulation in continuous culture, D = 0.1 h^{-1}.

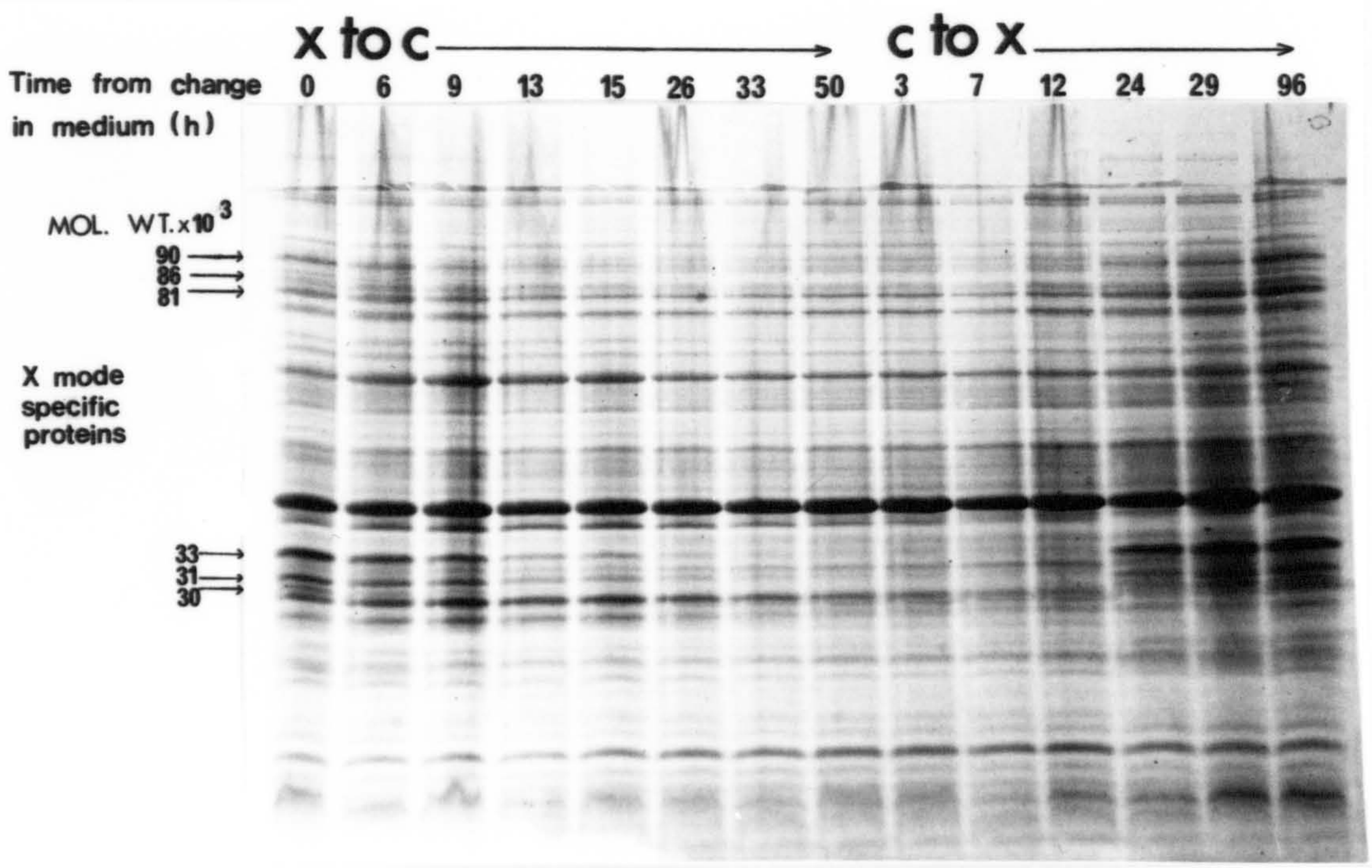
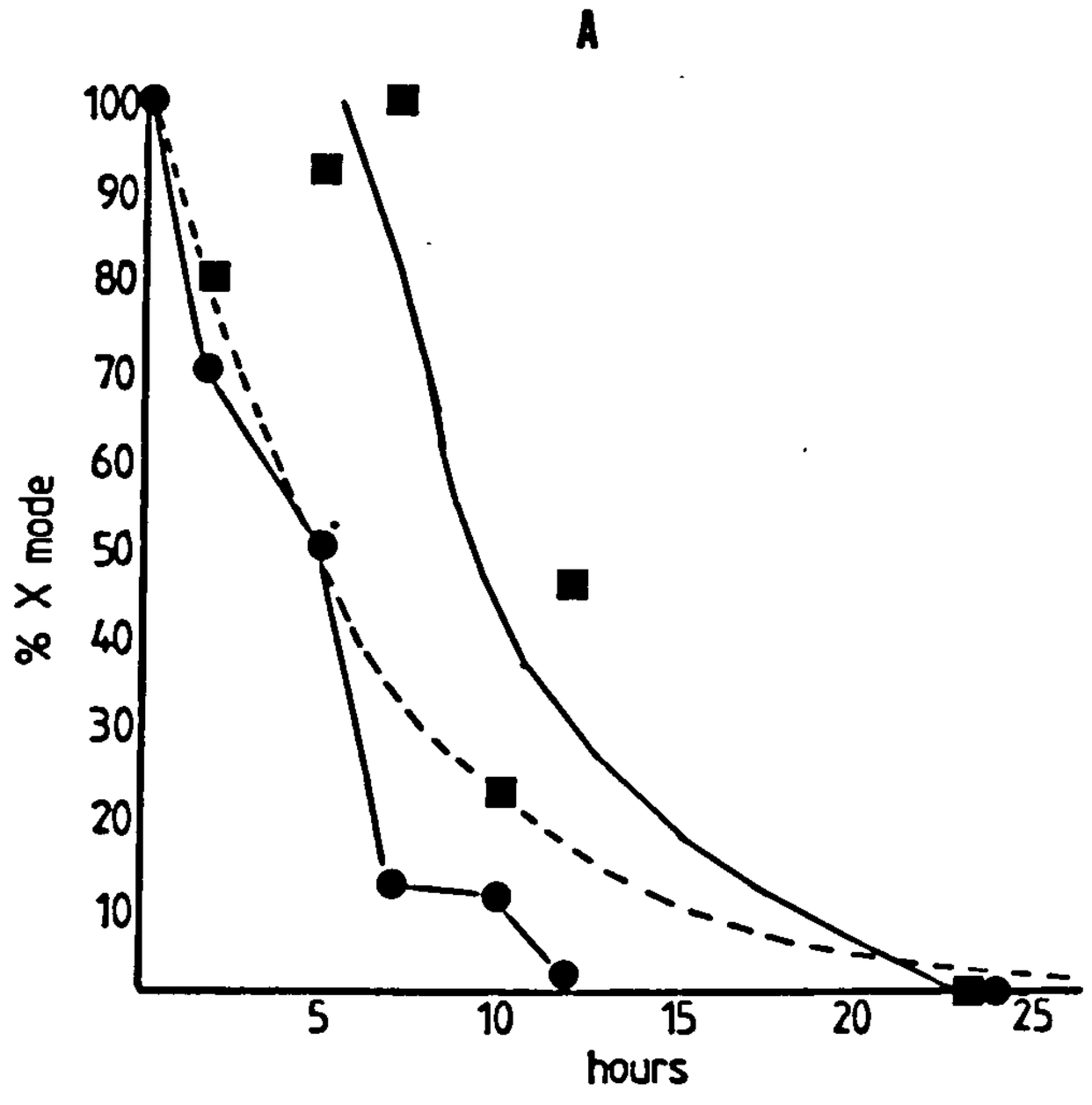


Fig. 18. $MgSO_4$-induced antigenic modulation of B. pertussis in continuous culture at A, D = 0.15 h^{-1} and B, D = 0.05 h^{-1}. ■, hydrophobicity; ●, LPF; dashed line is theoretical washout rate.



the first to be lost and hydrophobicity the last. The X-mode components were also regained when X-mode medium was reconnected at these dilution rates.

The decline of cellular LPF at the three different dilution rates (D=0.05, 0.1, 0.15 h^{-1}) is shown in fig. 19. In all cases the rate of loss of LPF was equal to or faster than the theoretical washout rate. This demonstrates that the transition from X- to C-mode is dependent on the growth rate of B. pertussis. The loss of LPF at a similar rate to the theoretical washout rate indicates that it depends on the replacement of X-mode with C-mode B. pertussis in the population.

5.4.2 The rate of nicotinic acid-induced antigenic modulation.

The rate of loss of X-mode components was also determined for NA-induced modulation in continuous culture. Modulation was induced by increasing the concentration of NA in the medium in both the medium reservoir and the culture vessel. Thus the medium was changed from X- to C-mode at 0 h and did not change gradually as occurred for $MgSO_4$ modulation (5.4.1). The hydrophobicity of cells was measured by adhesion to octane, cAMP in the supernatant was determined and LPF in the supernatant and cell-associated FHA were measured by ELISA.

When the concentration of NA was increased in the growth medium there was a similar loss of envelope proteins (fig. 20) to that shown in fig. 17. At a dilution rate of 0.1 h^{-1} (fig. 21A) LPF, cell-bound FHA and hydrophobicity were lost faster than the theoretical washout rate. These properties were lost sooner than with the $MgSO_4$-induced modulation at the same dilution rate. It can also be seen that cAMP in the supernatant was lost during antigenic modulation. For NA-induced modulation at this dilution rate (0.1 h^{-1}), LPF, cell-bound FHA and hydrophobicity were all lost at approximately the same rate. There was a lag of around 5 h before cAMP was lost

Fig. 19. Decline of cellular LPF during $MgSO_4$-induced antigenic modulation at ▲, D = 0.05 h^{-1}; ●, D = 0.1 h^{-1}; ○, D = 0.15 h^{-1}; dashed lines are theoretical washout rates.

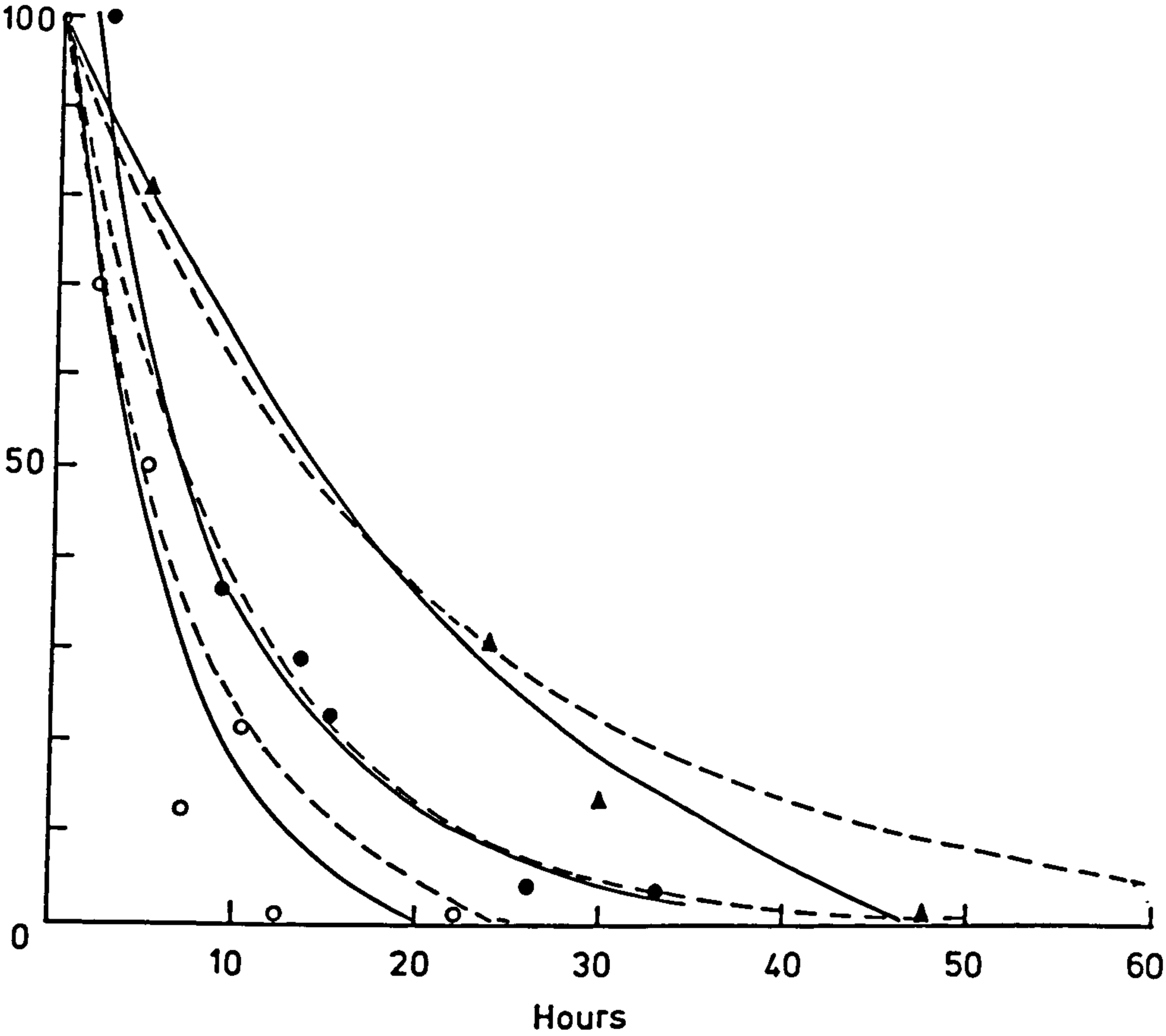


Fig. 20. SDS-PAGE of B. pertussis cells during NA-induced antigenic modulation in continuous culture, D = 0.1 h^{-1}.

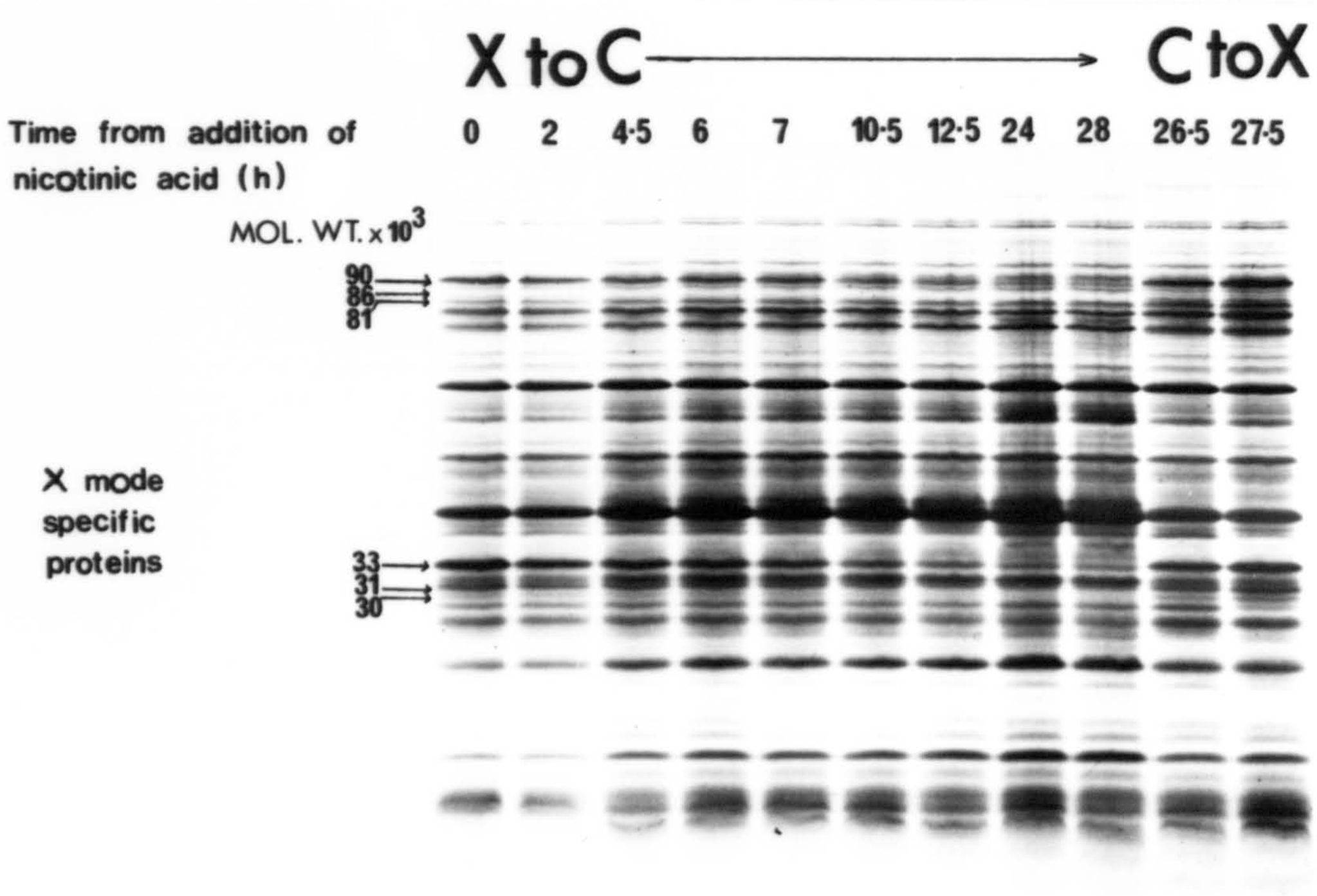
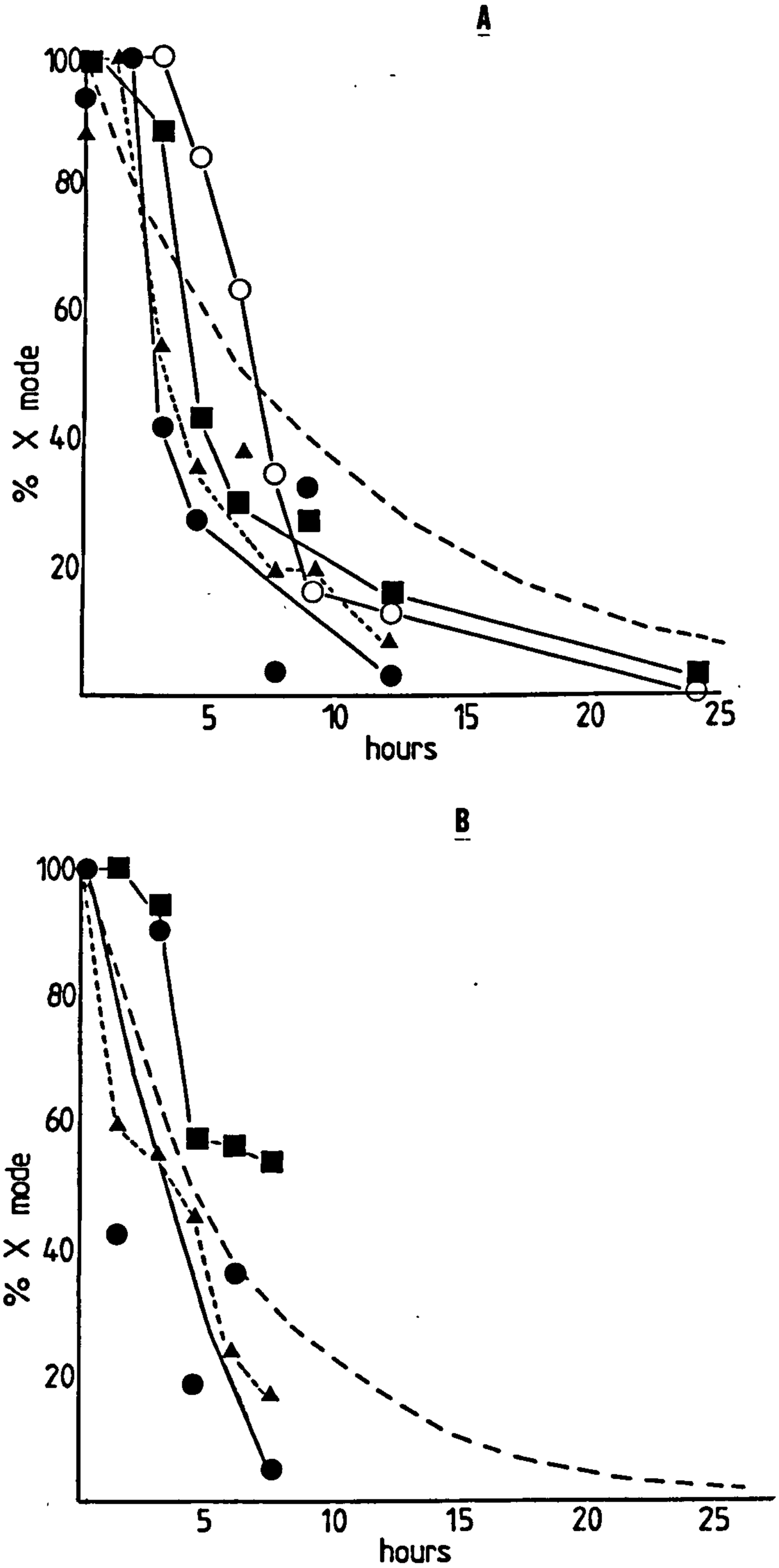


Fig. 21. NA-induced antigenic modulation of B. pertussis. A at D = 0.1 h^{-1}, B at D = 0.15 h^{-1}. At Oh the medium is changed to C-mode. X-mode cells are considered to have 100% of each characteristic. ■, hydrophobicity; ●, LPF; ▲, FHA on cells; ○, cAMP in supt.; dashed lines are theoretical washout rates.



from the supernatant. The NA modulation presumably occurs faster than observed for $MgSO_4$ because NA was added to the growth vessel, as well as to the medium reservoir, causing a more rapid changeover in medium from X- to C-mode. However, as the loss of X-mode components occurred faster than the washout rate it must mean that these X-mode components are lost from existing bacteria. The loss was not just caused by dilution of X-mode bacteria with newly produced C-mode organisms.

At a dilution rate of 0.15 h^{-1} (fig. 21B) LPF and cell-bound FHA were again lost faster than the theoretical washout rate. However, hydrophobicity was lost slower than the theoretical washout rate, with cells possessing 50% of X-mode hydrophobicity when C-mode levels of LPF were attained. This is in aggreement with $MgSO_4$ modulation where hydrophobicity was the last characteristic to be lost.

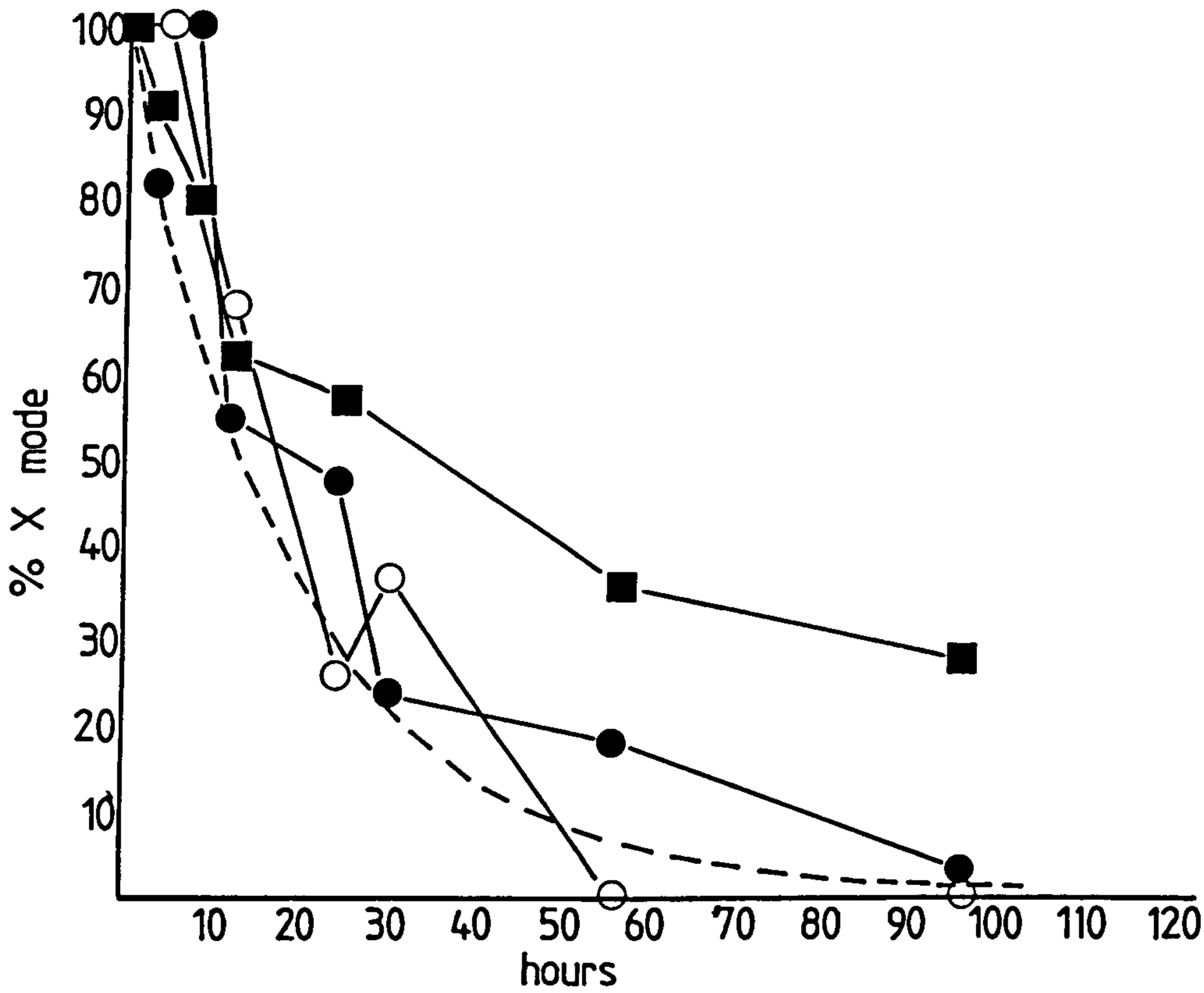
The rate of NA-induced modulation at a dilution rate of 0.05 h^{-1} followed the theoretical washout rate very closely (fig. 22). The loss of LPF and cAMP from the supernatant was approximately equal to the theoretical washout rate, not faster as for dilution rates 0.1 and 0.15 h^{-1}. Hydrophobicity was again the last of the X-mode characteristics to be lost, as it was at D = 0.15 h^{-1}.

The rate of antigenic modulation in continuous was dependent on the dilution rate for both NA and $MgSO_4$ modulation. The different X-mode components were lost at an approximately equal rate, with no component completely retained in the absence of the others. This equal rate of loss suggests that the expression of all the components is regulated by the same mechanism and not individual control of expression of the components.

5.4.3 Low temperature-induced antigenic modulation.

Antigenic modulation was also induced in continuous culture by reducing the temperature of the culture from 35°C to 25°C. B. pertussis was grown in continuous culture with SS X-mode medium at D=0.1 h^{-1}. When steady state

Fig. 22. NA-induced antigenic modulation of B. pertussis at $D=0.05h^{-1}$. The medium is changed to C-mode at 0h. X-mode cells are considered to have 100% of each characteristic. ■, hydrophobicity, ●, LPF; ○, cAMP in supernatant; dashed line is the theoretical washout rate.



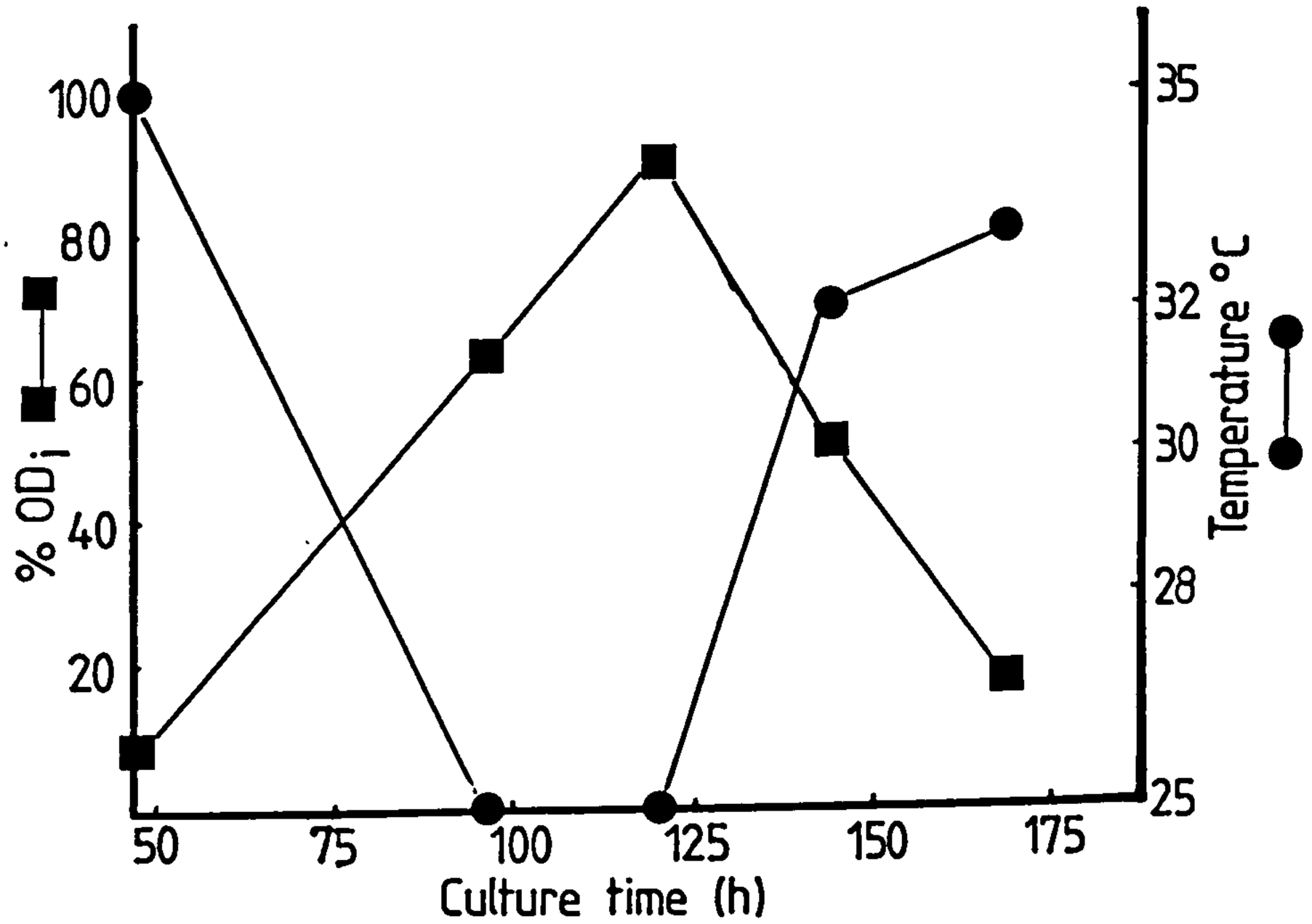
growth was achieved the temperature was reduced and the hydrophobicity of cells was measured. When the temperature was reduced the bacteria became more hydrophilic and when the temperature was returned to 35°C the bacteria regained X-mode hydrophobicity (fig. 23). Thus it is possible to induce reversible temperature antigenic modulation in continuous culture.

5.4.4 The effect of a gradual, stepwise change in medium composition from X- to C-mode in continuous culture.

In order to determine the $MgSO_4$/NaCl ratio that triggers the transition from X- to C-mode growth, medium was connected to the chemostat containing different ratios of $MgSO_4$ to NaCl, starting with X-mode SS medium (no $MgSO_4$) and finishing with a ratio (12 mM $MgSO_4$: 15 mM NaCl) that has been reported to produce C-mode growth (Brownlie et al.,1985b). B. pertussis was grown in continuous culture at a dilution rate of 0.1 h^{-1} and X-mode growth was determined by measuring cell surface hydrophobicity by adhesion to octane. The effect on cell surface hydrophobicity can be seen in fig. 24. No change in hydrophobicity was caused by medium containing 5 mM $MgSO_4$: 28 mM NaCl but there was a sharp decrease in hydrophobicity when medium containing 10 mM $MgSO_4$: 22 mM NaCl was connected. The hydrophobicity then remained at approximately one third of the X-mode value until medium containing 12 mM $MgSO_4$: 15 mM NaCl was connected. The hydrophobicity increased slightly the following day but then dropped to a fully C-mode value.

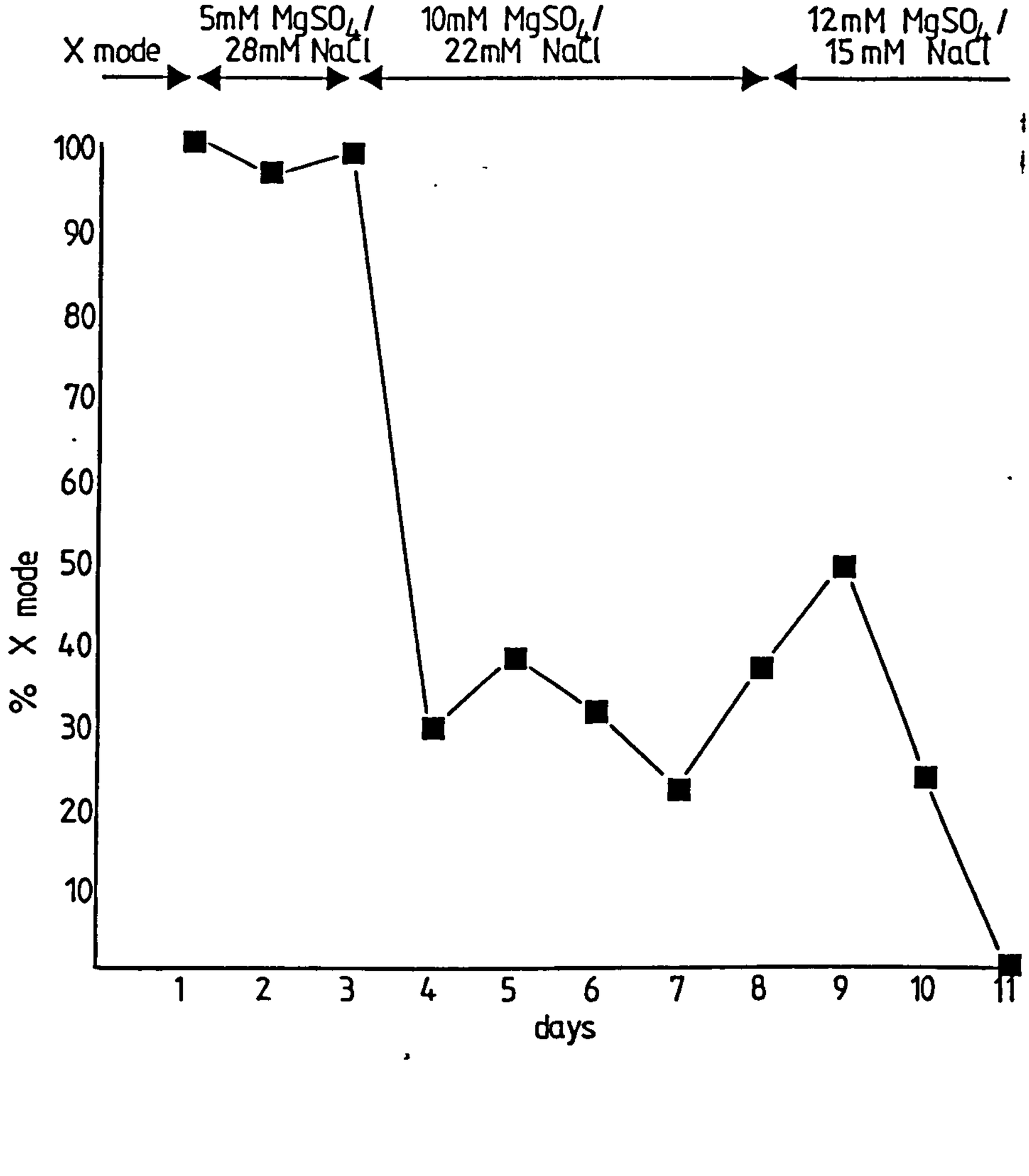
It appears that in continuous culture antigenic modulation is partially triggered by medium containing 10 mM $MgSO_4$. This concentration of $MgSO_4$ produced organisms with reduced hydrophobicity or a population of cells containing a proportion of C-mode cells. A fully C-mode population was achieved with medium containing 12 mM $MgSO_4$ after 72h, the same concentration reported by Brownlie et al. (1985b) for batch cultures.

Fig. 23. Hydrophobicity of B. pertussis during low temperature antigenic modulation in continuous culture.



$$\% \ OD_i = \frac{OD \ \underline{B.pertussis} \text{ suspension after mixing with octane}}{\text{Initial OD } \underline{B.pertussis} \text{ suspension}} \times 100$$

Fig. 24. Hydrophobicity of B. pertussis during growth in continuous culture with a stepwise change from X- to C- mode medium.



5.5 Effects of CD on antigenic modulation.

From chapters 3 and 4 it can be seen that CD enhanced the expression of LPF and FHA by B. pertussis. As these are components lost during antigenic modulation CD might have an effect on expression of X-mode components during antigenic modulation. CD was also seen to modify the growth requirements of B. pertussis, enabling the organism to grow on medium without glutathione (3.1.2c). The ability of CD to modify the C-mode growth produced in NA and $MgSO_4$ C-mode medium was determined. The experiments were performed using batch cultures to determine if CD had any effect on B. pertussis in different phases of growth, as CD enhanced expression of FHA particularly in stationary phase cultures (3.1.4, 4.2.1).

5.5.1 Effects of CD on NA-induced modulation in shake flask cultures.

B. pertussis was grown in shake flask cultures using SS and SS NA C-mode medium with and without 1g/l CD. The shake flasks were sampled at 48 and 72 h. The OD and HA titre were measured and LPF and FHA concentrations determined by ELISA (table 14). Both X- and C-mode cultures showed a greatly increased OD in the presence of CD, with the highest OD occurring in the C-mode cultures. The HA titres of X-mode culture supernatants were also greatly enhanced by the presence of CD. C-mode supernatants had no haemagglutinating activity at 48 or 72h. The production of LPF in the supernatant was increased in X-mode cultures by CD, the highest concentration (6.8 µg/ml) observed at 48 h. C-mode supernatants had no detectable LPF. The greatest effect of CD was seen in the amounts of FHA detected by ELISA in the supernatants and on cells. At 48h there was a great increase in expression of FHA in X-mode cultures. Again at 72 h X-mode + CD cultures showed a large increase in FHA whilst none was detected in cultures grown in C-mode medium. However, trace amounts of FHA were detected in both the supernatant and on the cells in 72 h C-mode

Table 14. The effect of CD on nicotinic acid antigenic modulation of B. pertussis.

48h	OD	HA titre	LPF supt. µg/ml	FHA supt. µg/ml	FHA cells µg/ml
SS	0.14(.11)	0.6	0	0	0.4(.7)
SS+CD	3.85(.32)	1.5	6.8(.58)	26.4(9.1)	26.4(9.1)
C mode	0.67(.58)	0	0	0	0
C mode+CD	1.90(.98)	0	0	0	0
72h					
SS	1.25(1.59)	4.6	0.7(1.2)	0.4(.7)	0.4(.4)
SS+CD	4.25 (.62)	12	1.9 (.7)	36.7(12.3)	4.9(.7)
C mode	2.85(2.05)	0	0	0	0
C mode+CD	4.77(1.12)	0	0	0.1(.1)	0.01(.025)

cultures containing CD. Thus the CD had, to a small degree, overcome the repression of FHA production caused by C-mode growth.

The hydrophobicity of cells grown in X- and C-mode medium with and without CD was determined after 48 h growth by adhesion to increasing volumes of octane (fig. 25). The hydrophobicity of X-mode cells was reduced by growth in CD-containing medium. The hydrophobicity of X- and C-mode cells was also reduced if the assay was performed using PBS containing 1mg/ml CD for cells grown in medium without CD (data not shown). So the presence of CD reduces the ability of the bacteria to adhere to the octane droplets. In contrast to this the hydrophobicity of the C-mode cells appeared increased by growth in the presence of CD, showing that the CD had partially overcome the repression of production of X-mode hydrophobic surface components caused by C-mode growth.

The results show that CD modified the C-mode bacteria produced in NA C-mode medium. The repression of X-mode characteristics was partially removed, with FHA detected and an increased hydrophobicity of the cells.

5.5.2 The effect of CD on $MgSO_4$-induced modulation.

The effect of CD on $MgSO_4$-induced modulation was studied to determine whether the C-mode organisms produced were altered by the presence of CD as seen for NA C-mode (5.5.1). The effect of CD on the ratio of $MgSO_4$/NaCl required to trigger antigenic modulation was also determined.

<u>B. pertussis</u> was grown in a series of shake flasks, in the presence and absence of CD, with a range of $MgSO_4$: NaCl ratios from 0 mM $MgSO_4$: 43 mM NaCl to 20 mM $MgSO_4$: 0 mM NaCl. The HA titres and FHA and LPF ELISA results are shown in table 15. Without CD, X-mode values of HA titre and LPF and FHA concentrations in the supernatant were still present in medium containing 5 mM $MgSO_4$: 28 mM NaCl. These properties were absent in the presence of higher concentrations of $MgSO_4$. In medium containing CD,

Fig. 25. Hydrophobicity of B. pertussis grown in X-mode and NA C-mode medium with and without CD. □ , X-mode; ■, X-mode+CD; ● , C-mode; ○ , C-mode+CD.

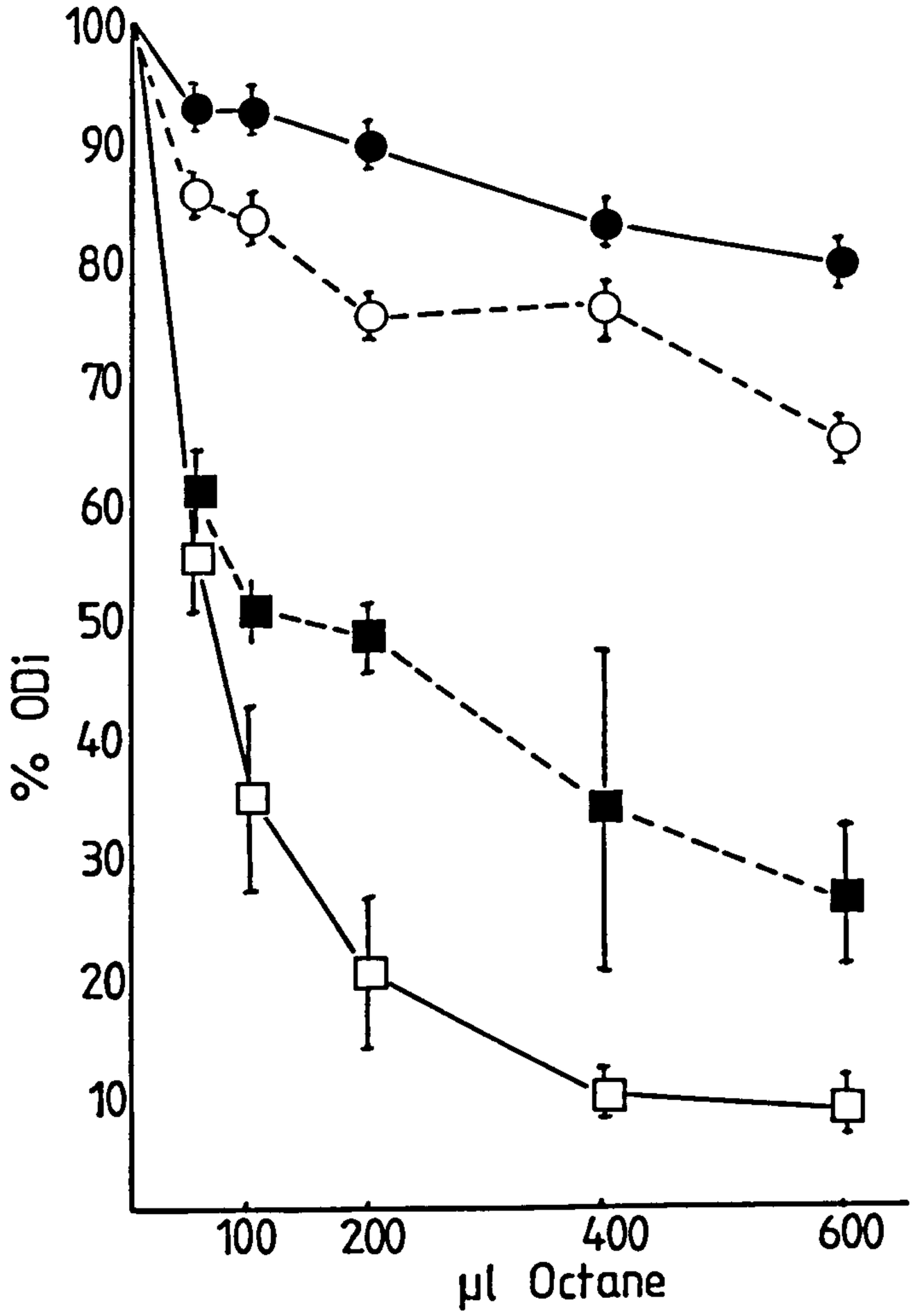


Table 15. Effect of CD on $MgSO_4$ modulation in a series of flasks with a gradient from X- to C-mode medium.

X MODE					C MODE	
$MgSO_4$ mM	0	5	10	12	15	20
NaCl mM	43	28	22	17	14	0
<u>48h No CD</u>						
HA titre	2.0	2.0	0	0	0	0
LPF supt. µg/ml	0.3	0.2	0	0	0	0
FHA supt. µg/ml	0.3	0.2	0	0	0	0
<u>48h +1g/l CD</u>						
HA titre	7	7	6	4	0	0
LPF supt. µg/ml	0.9	1.1	2.5	trace	0	0
FHA supt. µg/ml	9.3	8.6	5.1	1.2	0	0

Results are the mean of two determinations

the HA titres and concentrations of LPF and FHA in the supernatant were higher and remained at X-mode levels, even in medium containing 10 mM $MgSO_4$: 22 mM NaCl. LPF, FHA and HA activity were still detected in CD medium containing 12 mM $MgSO_4$: 17 mM NaCl. These X-mode characteristics were not found in CD medium containing 15 mM $MgSO_4$: 14 mM NaCl.

Cells from this experiment were analysed by SDS-PAGE (fig. 26). It can be seen that X-mode proteins are retained by the organism in higher $MgSO_4$ concentrations in the presence of CD than in its absence.

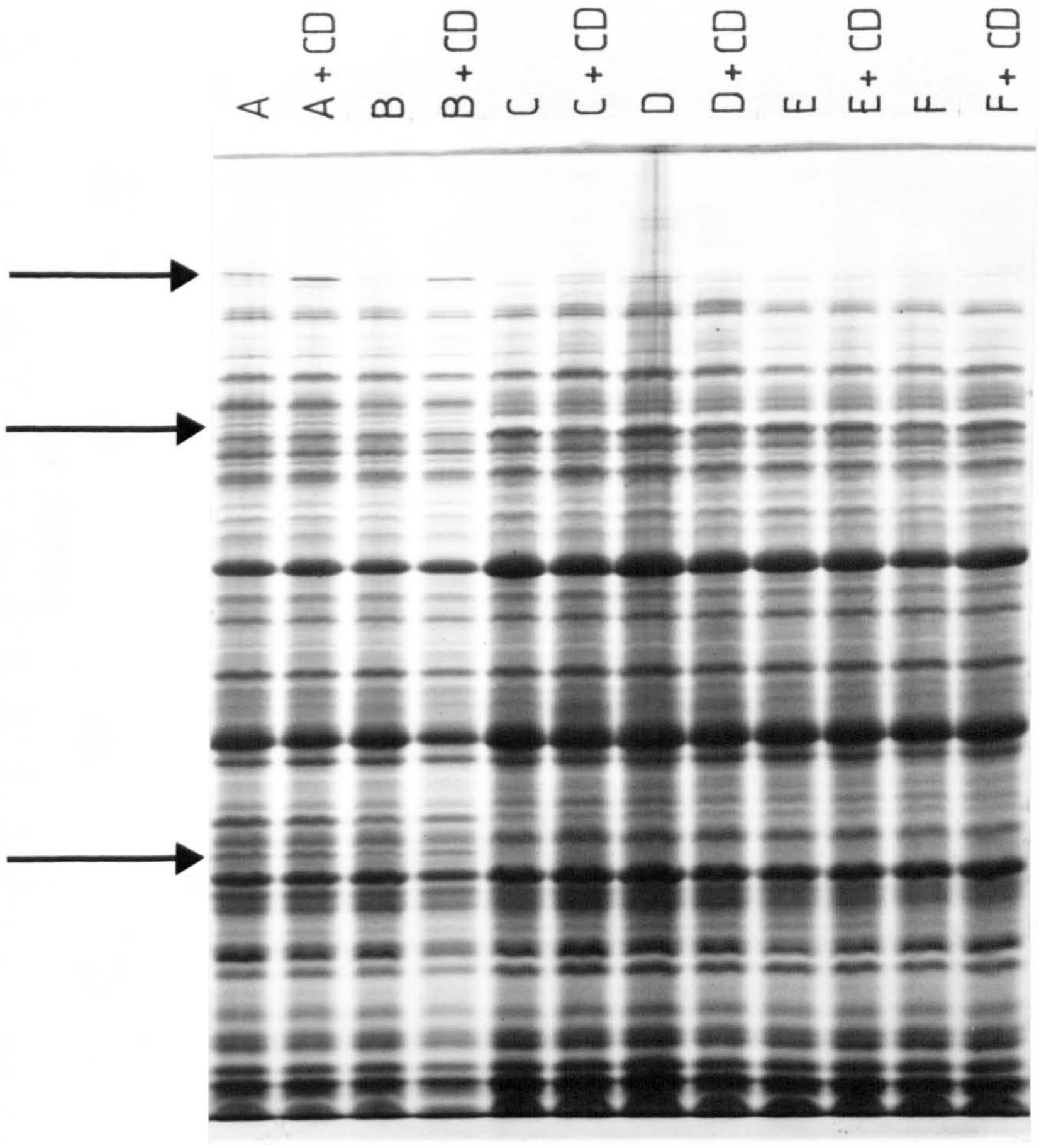
Cells were also assayed for their hydrophobicity by adhesion to octane (fig. 27A and B). For cells grown in the absence of CD (fig. 27A), typical X-mode hydrophobicity was exhibited only by cultures grown in medium containing 0 mM $MgSO_4$: 43 mM NaCl. Typical C-mode hydrophobicity was shown by cultures grown in medium containing from 12 to 20 mM $MgSO_4$. Intermediate hydrophobicities were shown by cells from 5 mM $MgSO_4$ and 10 mM $MgSO_4$ media. For cells grown in the presence of CD (fig. 27B), the X-mode (0 mM $MgSO_4$) hydrophobicity was slightly reduced, this is due to the effect of residual CD on the assay (see section 5.5.1). However, cells grown in medium containing up to, and including, 12 mM $MgSO_4$ showed similar hydrophobicty. Typical C-mode hydrophobicicty was only shown by cells grown in medium containing 15 and 20 mM $MgSO_4$

CD acts to increase the threshold concentration of $MgSO_4$ required to trigger C-mode growth. In the absence of CD, X-mode characteristics were only fully exhibited by cells grown in medium containing no $MgSO_4$, whilst with medium containing CD, LPF, FHA, X-mode OMP and hydrophobicity were enhanced at up to 10-12 mM $MgSO_4$.

5.6 Summary.

Antigenic modulation in continuous culture produced variants of B. pertussis which lacked LPF, FHA, fimbriae, X-mode specific outer membrane proteins and adenylate

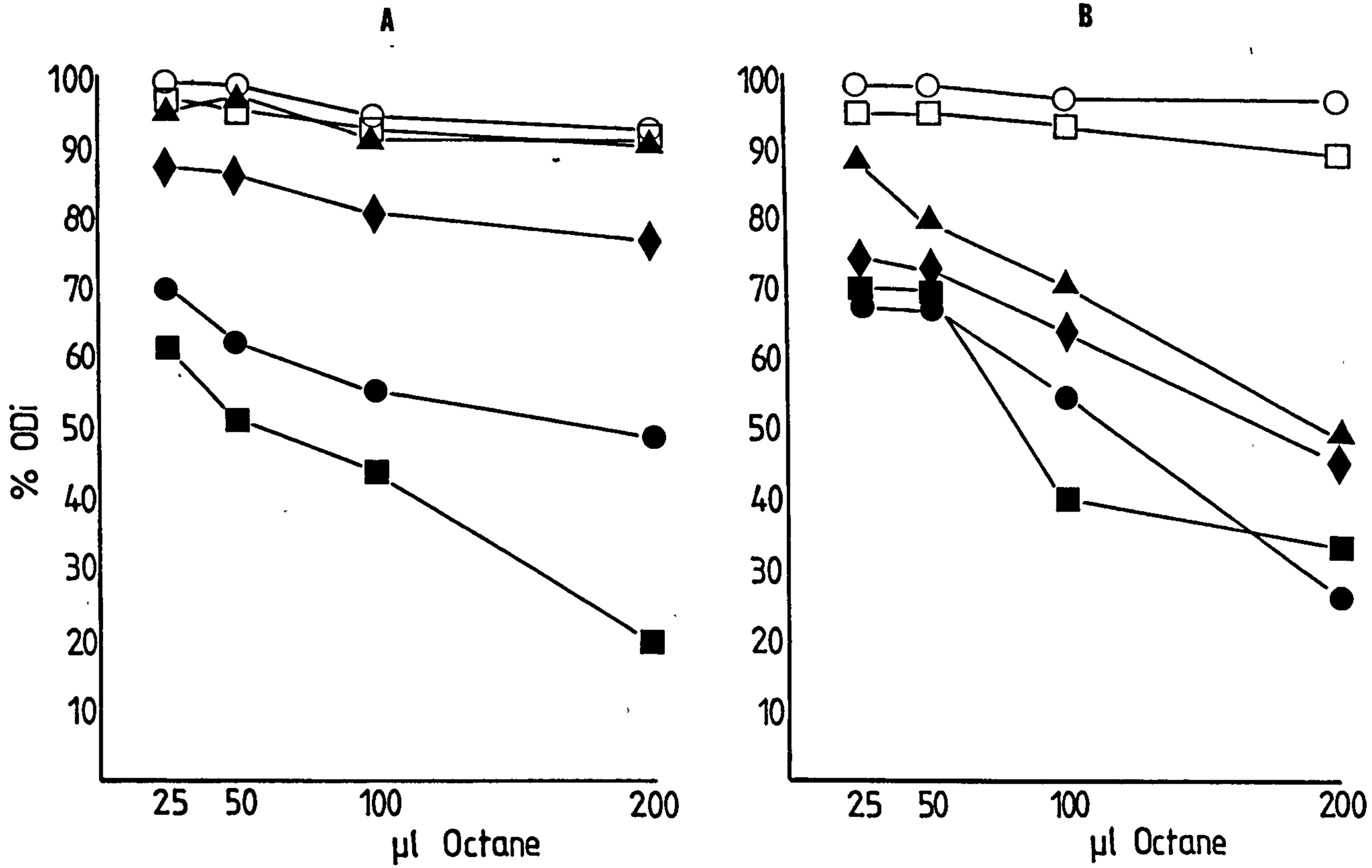
Fig. 26. SDS-PAGE of B. pertussis cells grown in shaken flask cultures with different $MgSO_4$:NaCl ratios, with and without 1 g/l CD.



X mode ⟶ C mode

		A	B	C	D	E	F
$MgSO_4$	mM	0	5	10	12	15	20
NaCl	mM	43	28	22	17	14	0

Fig. 27. Hydrophobicity of B. pertussis grown in media with a gradient of $MgSO_4$ from X to C mode A without CD, B with CD. Concentrations of $MgSO_4$ mM: ■, 0 ; ●, 5; ♦, 10; ▲, 12; □, 15; ○, 20.



cyclase. C-mode cells also had a reduced ability to adhere to Vero cells and reduced surface hydrophobicity. Previous workers have also observed a loss of many attributes of B. pertussis during antigenic modulation (table 1). The loss of FHA and surface hydrophobicity have not been previously reported. The change in hydrophobicity during antigenic modulation was the same as observed between phase I and phase IV B. pertussis.

Antigenic modulation was found to be completely reversible in continuous culture with X-mode components regained at approximately the same rate that they were lost. The rate of loss of X-mode components was followed in continuous culture at dilution rates 0.05, 0.1 and 0.15 h^{-1} (mean generation times of 14, 7 and 4.6 h). For $MgSO_4$ modulation all X-mode components measured were lost at approximately the same rate, although it appeared that hydrophobicity was lost after LPF had been lost. The loss of the X-mode components was approximately equal to the theoretical washout rate of the culture, indicating that the C-mode organisms were replaced by C-mode orgamisms upon cell division.

For NA-induced antigenic modulation in continuous culture, C-mode conditions were instantaneously achieved by adding NA to the growth vessel. In this case the loss of LPF and hydrophobicity was quicker than the theoretical washout rate at D=0.1 and 0.15 h^{-1}. cAMP was lost from the supernatant after a lag of approximately 5 h and as found for $MgSO_4$ modulation, hydrophobicity was lost after LPF. At D=0.05 h^{-1} the loss of LPF and hydrophobicity was approximately equal to the theoretical washout rate. The loss of X-mode characteristics at D=0.1 and 0.15 h^{-1} faster than the theoretical washout rate is an interesting phenomenon as it would require positive cellular inactivation or destruction of the X-mode characteristics.

In addition to increased $MgSO_4$ and NA concentrations, reversible antigenic modulation was also induced by lowering the temperature of the culture from 35°C to 25°C.

The ratio of $MgSO_4$ to NaCl that triggers antigenic

modulation in continuous culture was investigated. Fully C-mode hydrophobicity of bacteria was produced using medium containing 12mM $MgSO_4$: 15mM NaCl, as reported by Brownlie et al. (1985b) for batch cultures. However, medium containing 10mM $MgSO_4$: 22mM NaCl produced a population with approximately one third of X-mode hydrophobicity. So either the individual organisms all have a reduced hydrophobicity or the population contains a proportion of C-mode bacteria.

The effect of CD on antigenic modulation induced by $MgSO_4$ and NA was studied in batch culture. X-mode cultures showed increased expression of LPF and FHA. CD altered NA-induced C-mode organisms in that FHA could be detected on cells and in supernatants of stationary phase cultures and the hydrophobicity of C-mode organisms was increased. CD also altered the ratio of $MgSO_4$ at which antigenic modulation occurred. Cultures retained X-mode characteristics in higher concentrations of $MgSO_4$ in the presence of CD. The hydrophobicity of $MgSO_4$ C-mode cultures was also increased in the presence of CD. It appeared that CD had a slight effect in overcoming the repression of X-mode characteristics that occurred in C-mode conditions.

Chapter 6.

The effects of CD on the stability of filamentous haemagglutinin (FHA).

FHA can be purified from static liquid culture supernatants but it has not been found in shaken cultures (Arai & Munoz, 1979). However, the addition of CD to the growth medium enables shaken culture supernatants to be used as a source for the purification of FHA (Imaizumi et al., 1984). These findings were confirmed in chapters 3 and 4, where it was seen that the major effect of CD on B. pertussis was to enhance the yield of FHA. As the effect of CD was greatest with shaken cultures, the effect of CD on the stability of purified FHA in shaken solutions was therefore studied.

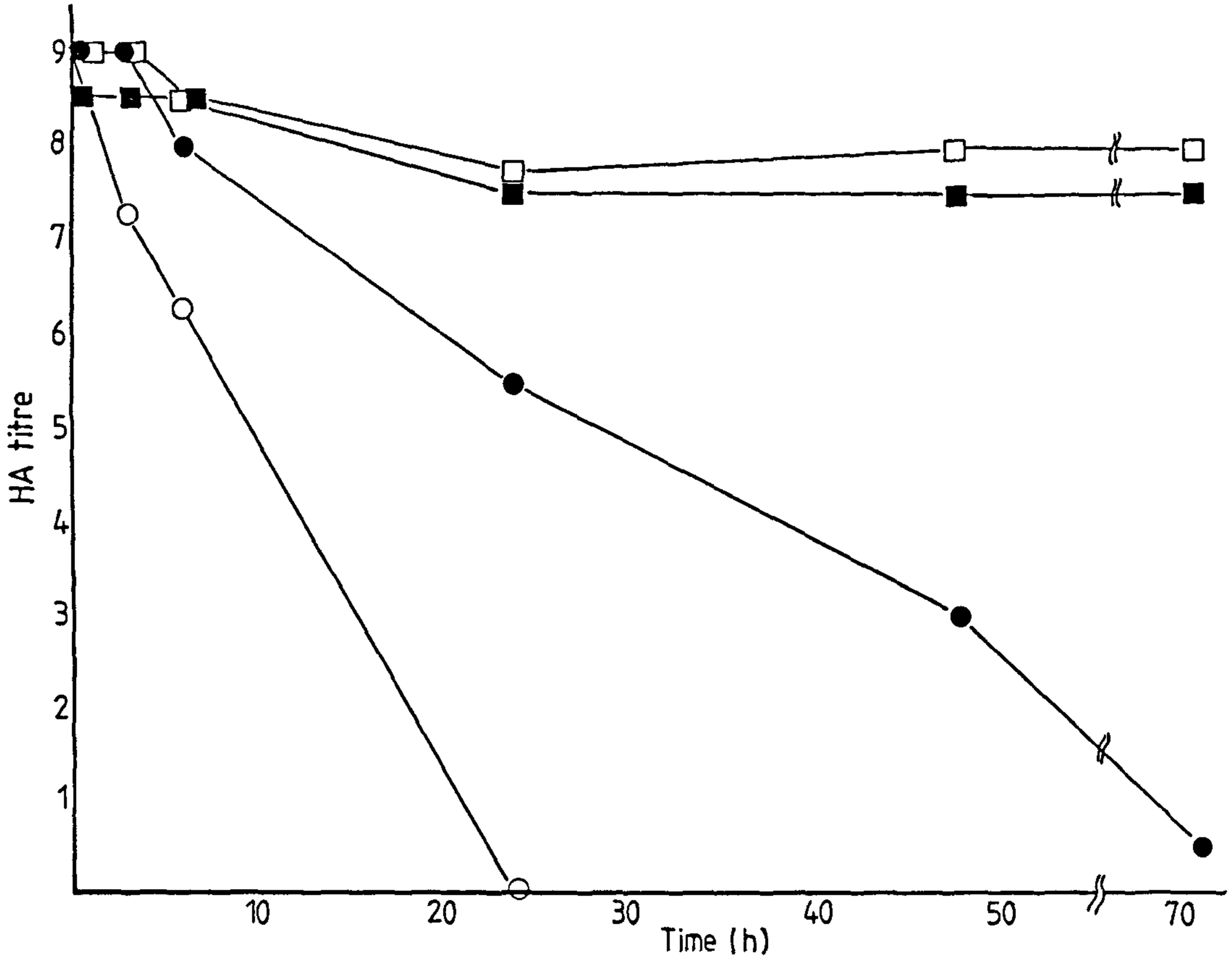
The FHA used for these studies was purified (by the method of Sato et al. (1983)) from Thompson bottle cultures using CL+CD medium incubated with shaking for 48h at 35°C (obtained from Dr. L. Irons, CAMR). No CD was detected in the purified protein by gas chromatography-mass spectrometry (assay performed by R. Waite, CAMR).

6.1 The effects of CD on the stability of FHA.

6.1.1 The effect of CD on the stability of FHA incubated with and without shaking.

Aliquots (20 ml) of a solution of FHA (50µg/ml in PBS) were incubated either statically or on an orbital shaker (180 rpm) at 35°C in 50ml conical flasks. Fig. 28 shows that the HA titre of FHA incubated without shaking remained almost constant for up to 70h, whilst FHA incubated with shaking showed a rapid decline in HA titre. However, in the presence of 1 g/l CD HA activity could still be detected after 50h shaking. Similar results were obtained when the FHA concentration was determined by ELISA (data not shown). As FHA is stable on static incubation but not when incubated with shaking, this would explain why FHA is only detected in very small amounts in cultures grown in shaken vessels or stirred fermenters.

Fig. 28. HA titres of 50 ug/ml solutions of FHA in PBS. Solutions incubated statically at 35° either without CD (□) or with 1 g/l CD (■). Solutions incubated with shaking at 35° either without CD (○) or with 1 g/l CD (●).



FHA activity was preserved in solutions containing CD so it may be that CD enhanced yields of FHA in shaken cultures by exerting a stabilising effect on the FHA produced. However, this stabilising effect appears to be weak and HA activity is still lost in the presence of CD.

6.1.2 The effect of CD concentration on the stability of FHA incubated with shaking.

Aliquots (20ml) of FHA solution (50µg/ml in PBS) were incubated with shaking as above but containing 0, 1.0, 2.0, and 4.0 g/l CD. The HA titres of three replicates of each CD concentration were determined periodically (fig. 29). It can be seen that the preservative effect of CD on HA activity was dependent on concentration. However, even with 4.0 g/l CD the stabilising effect of CD was not complete and a reduction in HA activity of 91% is obtained between 3 and 72h.

6.1.3 The effect of pH on the stability of FHA incubated with shaking.

In chapter 3 it was observed that the concentration of FHA in a stirred fermenter supernatant was greater in the high pH produced by the B. pertussis culture than in cultures where the pH was controlled to 7.6. The stability of purified FHA (50 µg/ml) on shaking at 35°C was examined in Tris/NaCl 0.5M buffer at pH 7.0, 8.0, 9.0 and 10.0 to determine if high pH increased FHA stability or the stabilising effect of CD (fig. 30). The HA titre of FHA incubated with shaking at pH 7.0 and 8.0 declined at approximately the same rate and FHA solutions at pH 9.0 and 10.0 declined at a faster rate. With the addition of 1 g/l CD, FHA solutions lost HA activity at approximately the same rate at all pH values tested. Thus it seems that FHA was not more stable at high pH but that CD was able to have the same stabilising effect on FHA at the higher pH values.

6.1.4 The stability of FHA incubated with shaking at 4°C.

Fig. 29. HA titres of 50 ug/ml solutions of FHA in PBS containing zero (○); 1 (▲), 2 (■) and 4 (●) g/l CD. The solutions were incubated at 35° with shaking (180 rpm) on an orbital shaker.

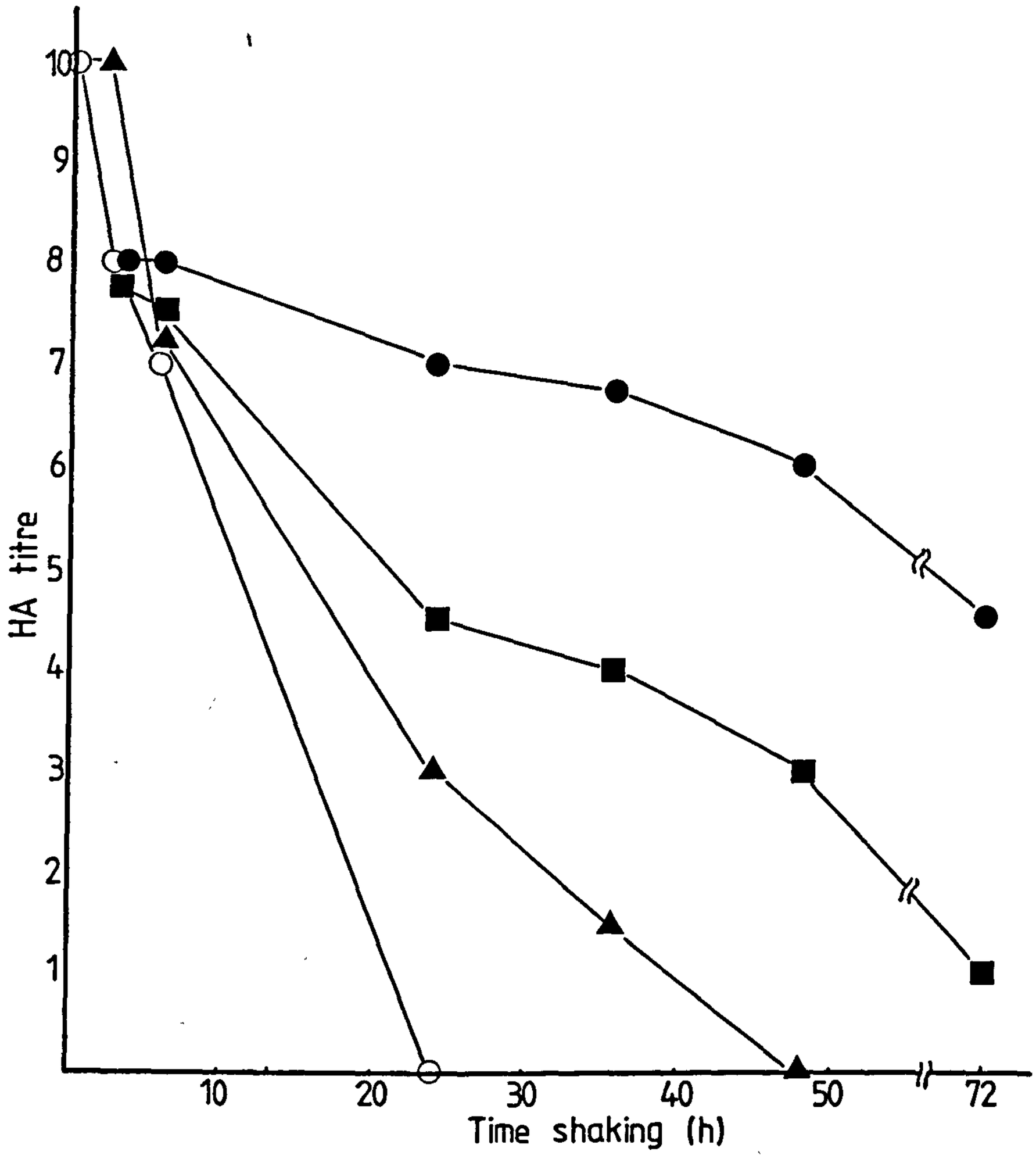
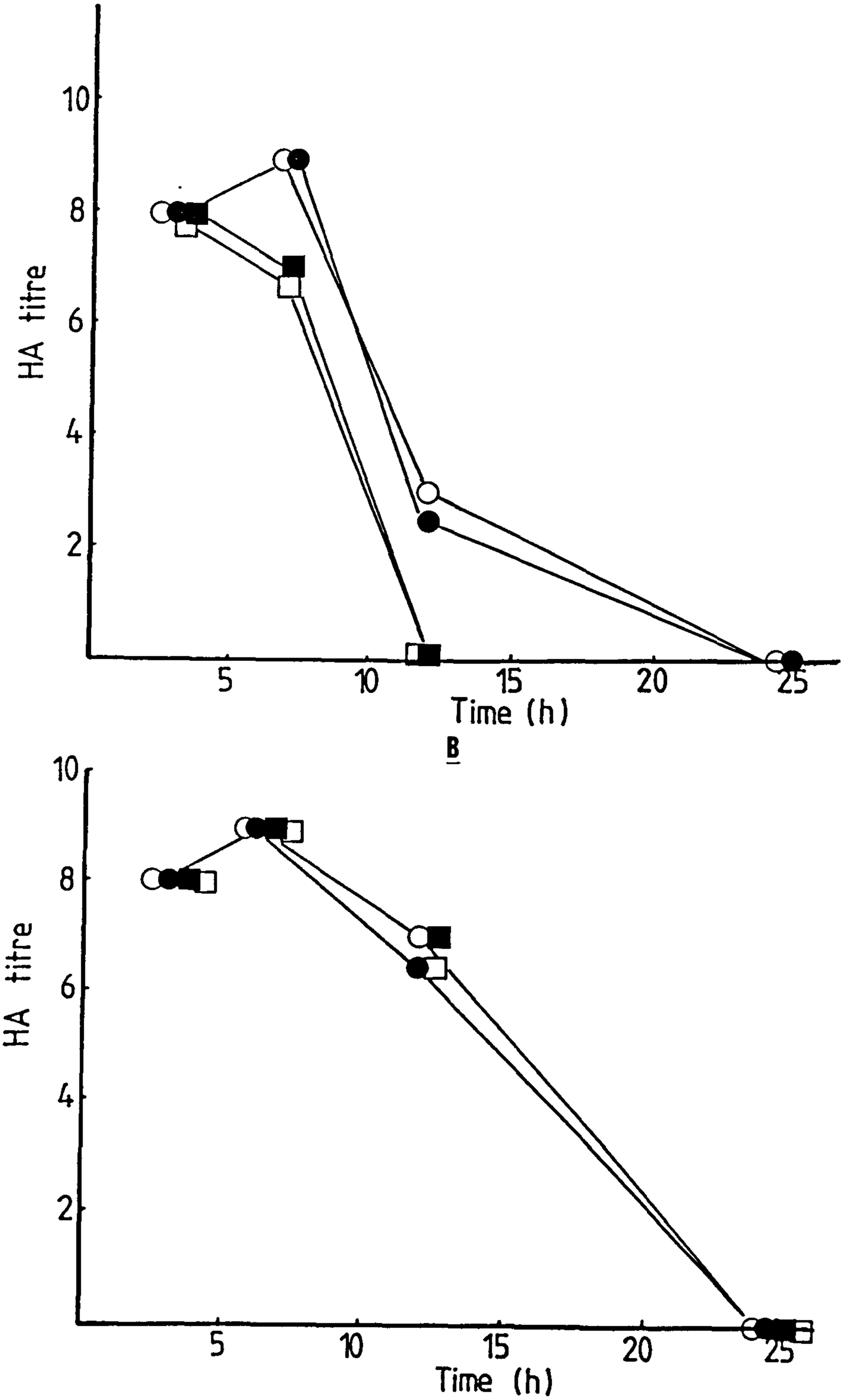


Fig. 30. HA titres of 50 ug/ml solutions of FHA in Tris/NaCl 0.5M buffer at ●, pH 7.0; ○, pH 8.0; ■, pH 9.0; □, pH 10.0. Solutions incubated with shaking. A, with CD. B, with 1 g/l CD.



A solution of FHA (50 µg/ml in PBS, 20 ml in 50 ml conical flasks) was incubated with shaking as above but at 4°C. It was found (fig. 31) that the HA activity of the FHA solution shaken at 4°C was retained for longer than at 35°C (see 6.1.1) and there was little loss of HA activity at 48h for flasks containing 1 g/l CD. Thus, the loss of HA activity from solutions of FHA on shaking is greatly reduced by lowering the temperature.

6.1.5 The effect of area of the air-liquid interface on the stability of FHA incubated with shaking.

It was thought that FHA may undergo surface aggregation as a result of forces generated by shaking, as coincident with the loss of HA activity, strands of aggregated protein appeared on the surface of the liquid. Aliquots (20 ml) of a 50 µg/ml solution of FHA in PBS were incubated with shaking at 35°C in 25 ml, 50 ml, 100 ml and 250 ml conical flasks. Mean HA titres of the solutions from each flask are shown in fig. 32. The loss of HA activity for the solutions in the 100ml and 250 ml flasks was very rapid and the rate of loss was dependent on the size of the flask used, with the greatest loss in the solution in the largest flask.

A solution of FHA (50 µg/ml in 20 ml PBS) was incubated statically in 50 ml conical flasks with and without sparged aeration. The FHA solution with air sparged through sintered glass (200 cm^3/min) possessed no HA activity after 3h incubation, whilst the HA activity of the statically incubated flask without sparged air remained constant. This suggests that the area of the air-liquid interface is important in the loss of HA activity from shaken solutions of FHA and that the loss of HA activity from FHA solutions shaken in 100ml and 250ml conical flasks is not simply due to absorption onto a large glass surface.

6.1.6 FHA analysed by SDS-PAGE during loss on incubation with shaking.

Fig. 31. HA titres of 50 μg/ml solutions of FHA in PBS incubated with shaking at 4°C. Without CD, ● and with CD, ○.

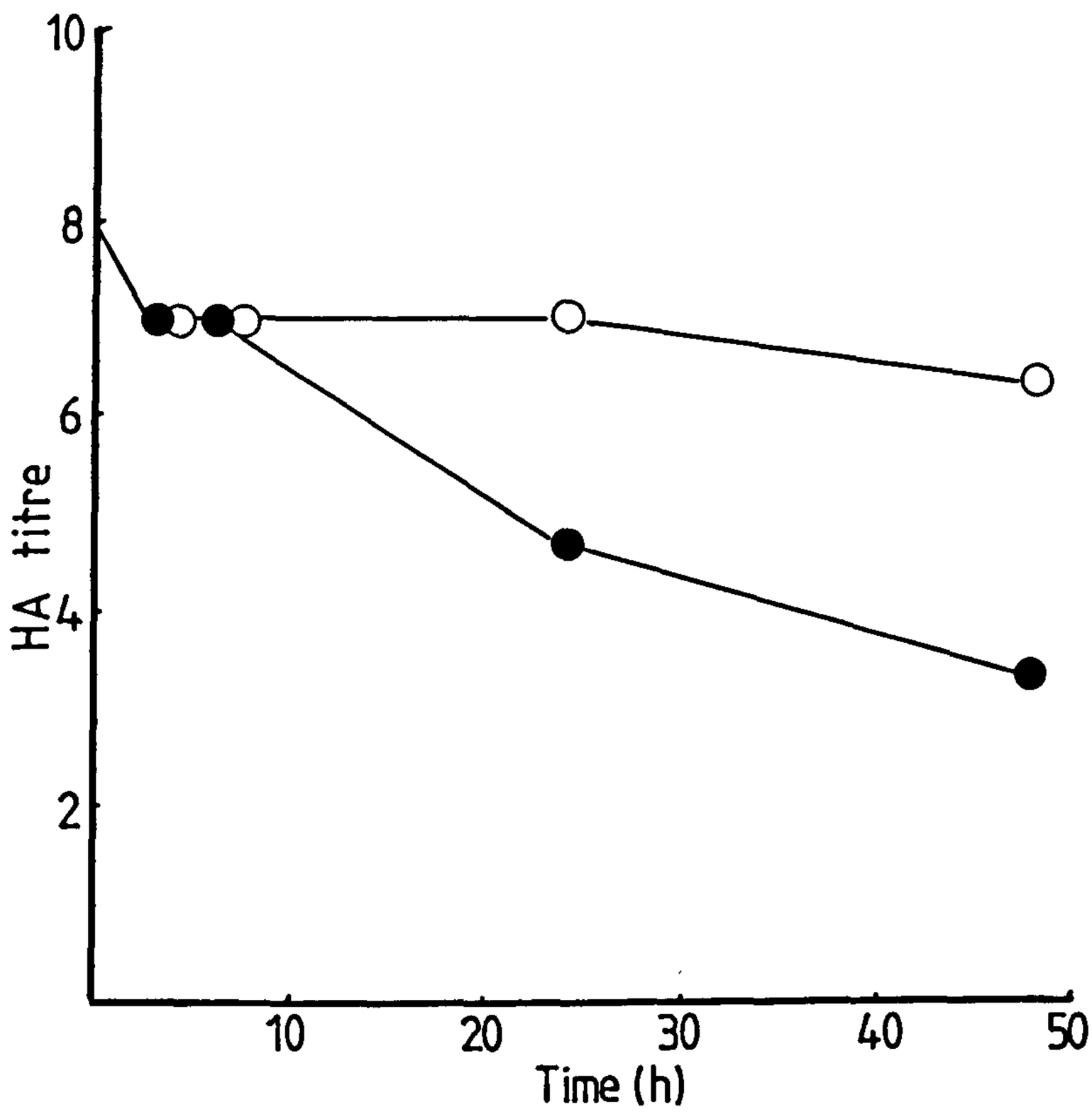
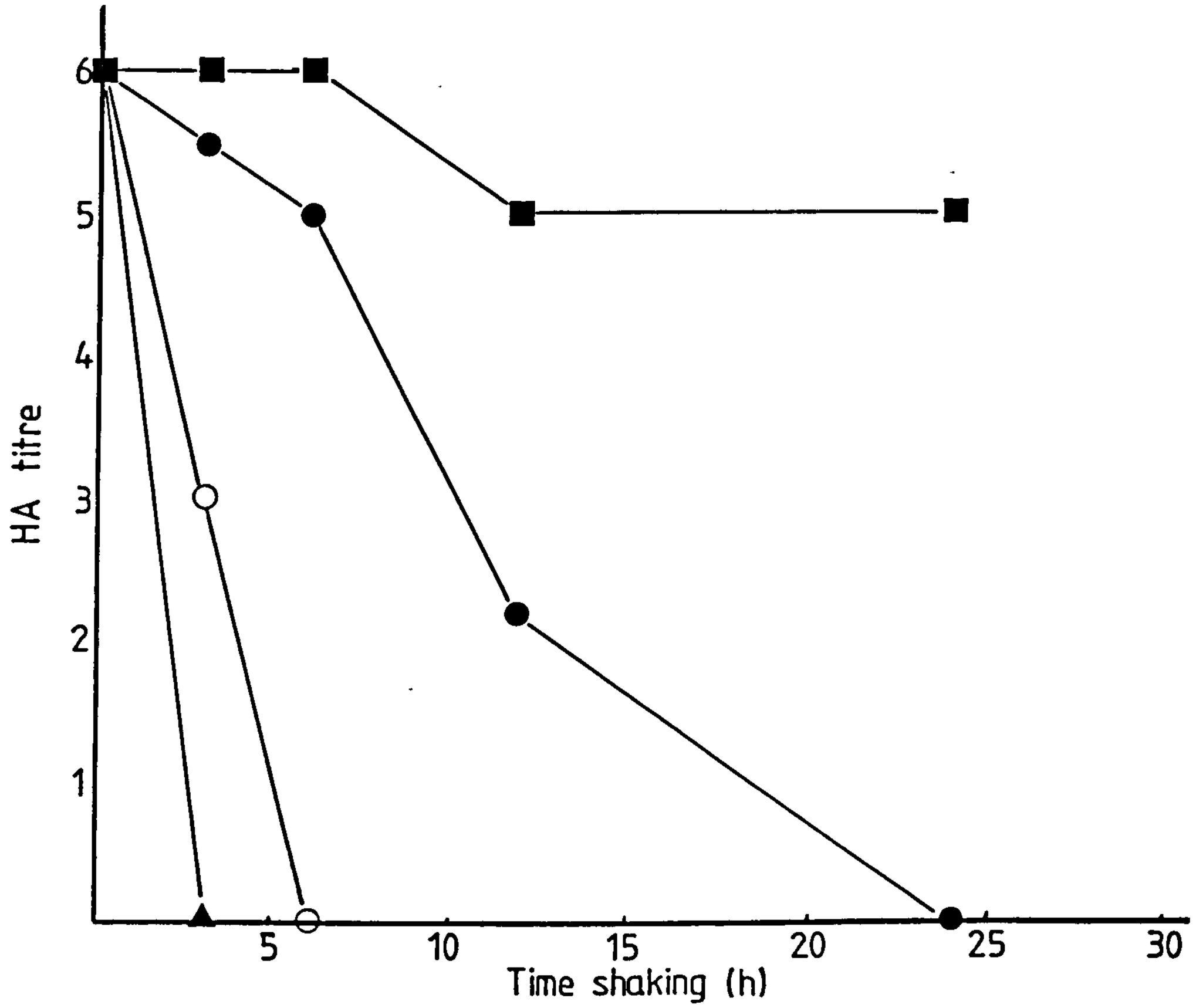


Fig. 32. HA titres of 50 ug/ml solutions of FHA in PBS incubated with shaking at 35° in 25 ml (■), 50 ml (●), 100 ml (○) and 250 ml (▲) conical flasks. Each flask contained 20 ml of FHA solution.



The loss of HA activity on shaking may have been caused by degradation of FHA to inactive lower molecular weight components. To determine if this had occurred an FHA solution (600 µg/ml) was incubated with shaking (20 ml in a 50 ml conical flask) at 35°C. Samples were taken at various times, centrifuged (11 600 xg for 5 min) to remove any large aggregates and the protein remaining in solution determined by an automated Lowry assay and analysed by SDS-PAGE (fig. 33). There was no obvious loss of protein bands or degradation to low molecular weight components from the FHA remaining in solution, so it seems that the loss of HA activity of FHA solutions incubated with shaking could be due to the loss of protein (shown in fig. 33) from the solution by surface aggregation at the air-liquid interface.

Comparison of HA activity of initial solutions of FHA (50 µg/ml) given in figures 28-32 shows a variability between experiments, with a particularly low HA titre obtained in fig. 32. The reasons for this may be the variability of HA titre obtained from different batches of FHA. Purified FHA is known to be heterogeneous from batch to batch (Irons et al., 1983) with variability in the proportions of components observed by SDS-PAGE analysis. Also, the goose red blood cells used for determination of HA activity may vary in sensitivity between batches. FHA concentrations were determined by ELISA for a number of the above experiments (data not shown) and these confirmed the results obtained from measurements of HA activity.

6.2 Binding of CD to FHA.

It has been observed that CD has a stabilising effect on purified FHA when incubated with shaking and it seems that this may be the mechanism by which greater yields of FHA are obtained from shaken cultures containing CD. Therefore an investigation into the possible binding of CD with FHA either in a purified form or expressed on the bacterial surface was attempted.

Fig. 33 SDS-PAGE of 600 µg/ml solutions of FHA in PBS incubated with shaking at 35°C in the presence and absence of 1 g/l CD.

		Protein conc. µg/ml
A	0 h	660
B	0 h + CD	640
C	6 h	600
D	6 h + CD	700
E	24 h	340
F	24 h + CD	340
G	48 h	140
H	48 h + CD	280
I	72 h	0
J	72 h + CD	320

6.2.1 Binding of CD to *B. pertussis* cells.

B. pertussis, strain Wellcome 28 phase I, a phase IV variant and a transposon Tn5-induced mutant that does not express FHA were grown in SS medium in Thompson bottles with static incubation for 5 days. Cells were harvested by centrifugation, washed in PBS and resuspended (10% w/v) in PBS containing 0.1 mg/ml CD. An even suspension was obtained using a hand homogeniser. Bacterial suspensions were then centrifuged and the supernatant analysed for CD using the TNS fluorescence assay and any loss of CD from the supernatant was calculated (table 16). Bacteria (Wellcome 28, phase I) from a 5 day static culture were also extracted with Tris/NaCl 0.5M pH8.0 buffer (1.0g in 10ml) to remove FHA from the cells before suspending in PBS containing CD. The concentration of FHA in culture supernatants and in the Tris/NaCl extract was determined by ELISA.

It can be seen from table 16 that some CD is lost from the suspensions of bacteria in PBS+CD when the bacteria are removed by centrifugation.This loss was approximately the same for Wellcome 28 bacteria before and after extraction with Tris/NaCl buffer to remove FHA. The % removal of CD from the supernatant was also no different when FHA$^-$ bacteria were used. A slightly smaller removal of CD from the supernatant was seen for the phase IV bacteria. FHA was detected by ELISA in the Wellcome 28 phase I culture supernatant (8.7 µg/ml) and also in the Tris/NaCl extract (29.9 µg/ml), whilst only a trace amount (0.05 µg/ml)was detected in the supernatant of the FHA$^-$ mutant culture. The similar % removal of CD from the suspensions of bacteria in PBS+CD on centrifugation suggests that the CD does associate with *B. pertussis* cells but that this is not related to the presence or absence of FHA.

6.2.2 Effect of CD on antibody binding to FHA in ELISA and immunoblotting.

Table 16. Binding of CD to B. pertussis cells grown in Thompson bottles with static incubation.

	% removal of CD from supt.	FHA in culture supt. (µg/ml)
0.1 mg/ml CD + 10% (w/v) phase I cells.	25.0 (3.0)	8.7
0.1 mg/ml CD + 10% (w/v) phase I cells extracted with tris/NaCl buffer).	27.1 (1.2)	29.9 (in extract)
0.1 mg/ml CD + 10% (w/v) FHA- cells.	27.1 (1.6)	0.05
0.1 mg/ml CD + 10% (w/v) phase IV cells.	20.7 (1.2)	0

Results are the mean of 3 determinations.
Figures in parenthesis = standard deviation.

The potential interaction of FHA and CD was investigated by determining if CD would inhibit or block antibody binding to FHA. This was performed using (i) antigen bound to a plastic surface (Nunc Immunoplate 1) and (ii) antigen electrophoretically transferred to nitrocellulose paper from an SDS-PAGE gel. If blocking of antibody binding was observed using the electrophoretic transfer technique, this would show the component to which the CD bound.

To determine if CD inhibited antibody binding to FHA in an ELISA system, FHA (2 µg/ml) was coated onto plates overnight in coating buffer (see 2.3). Plates were then washed with wash buffer (PBS containing 0.1% Tween 20) and incubated at room temperature for 2h with 100µl wash buffer per well. Doubling dilutions of CD from 10% (W/V) were made in a separate plate in either wash buffer or PBS and these were transferred to the washed, coated plates. After a further 2 h incubation, plates were washed and either FHA McAb F3 or F8 conjugated to horse radish peroxidase, was added and the assay continued as for other ELISA methods.

None of the ELISA plates tested, using either McAb conjugate or PBS with or without Tween 20, showed any evidence that CD had any effect on the binding of antibody to FHA. Whether this was because CD does not interact with the FHA in the structure that was presented on the ELISA plate is not known.

Subsequently, it was determined if CD could inhibit antibody binding to FHA components when separated by SDS-PAGE and transferred to nitrocellulose. FHA was electrophoresed on an 11.7% SDS-PAGE gel and then transferred electrophoretically for 12 h onto nitrocellulose paper in 0.2M glycine, 0.02M tris, 20% methanol buffer with a current of 40mA. After incubation of the nitrocellulose strips in wash buffer for 2 h, they were incubated in 0.1-10.0% (w/v) CD for 2 h, washed and then incubated with FHA McAb F2 overnight at 4°C. Strips were washed and incubated with rabbit anti-mouse conjugate

(diluted 1 in 1000) and visualised using 4-chloro, 1-naphthol as a substrate. The effect of incubation with 0.1, 1.0, and 10 mg/ml CD can be seen in fig. 34 and comparison with the amido black stained strip shows no evidence of the CD inhibiting binding of antibody to SDS-treated FHA components.

6.2.3 Separation of a mixture of FHA+CD by Sepharose G15 chromatography.

Gel filtration has been used to study the binding of small molecules to proteins (Wood & Cooper, 1970) with altered elution profiles of individual components indicating an association between them. Sephadex G15 (Pharmacia Ltd.) has a fractionation range of up to 1500 molecular weight with molecules greater than this excluded from the gel. CD (molecular weight=1331) is able to enter the gel matrix and will be eluted after larger molecules.

Conditions that allow the separation of FHA from CD in PO_4/NaCl 0.5M pH 7.2 were determined and a 1.5cm x 1m column packed with Sephadex G15 was used with a flow rate of 8ml h^{-1} at 4°C. FHA (0.1ml of 1.2 mg/ml solution) and CD (0.25ml of 1mM CD solution) in PO_4/NaCl buffer were applied to the column and 2ml fractions were collected. These were assayed for FHA by haemagglutination and for CD by the TNS fluorescence assay. Elution profiles of CD alone and a mixture of FHA + CD are shown in fig. 35. Binding of CD to FHA would be shown by detection of CD in the FHA peak, that occurred at the void volume of the column. However no additional fluorescence was determined in the FHA peak in excess of the low level of fluorescence detected with FHA alone. Also, the profile of the CD peak was little changed whether CD was applied alone or in a mixture with FHA.

These results suggest that if any binding of CD to FHA occurs, it is weak and insufficient to alter the elution profiles obtained. However, it is not known if the gel filtration conditions used may have caused a perturbation of the binding equilibrium.

Fig. 34. Use of FHA immunoblots to determine if binding of FHA McAb to SDS-treated FHA components is inhibited by preincubation with CD.

A: No CD.

B: 0.1 mg/ml CD.

C: 1.0 mg/ml CD.

D: 1.0 mg/ml CD (1.0 mg/ml CD also in McAb solution).

E: 10.0 mg/ml CD.

F: No CD, amido black stained.

G: Coomassie blue stained SDS-PAGE strip before transfer.

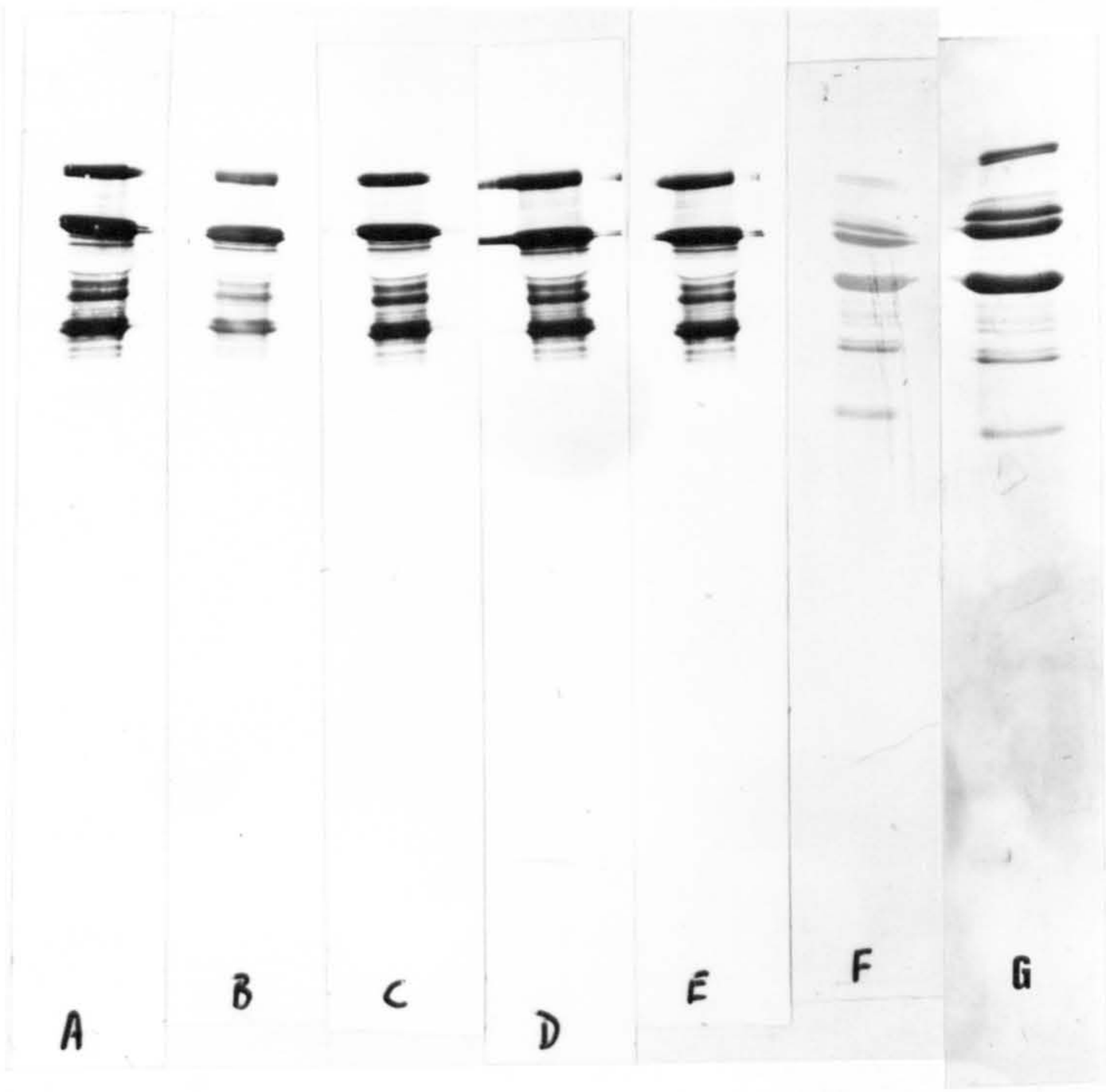
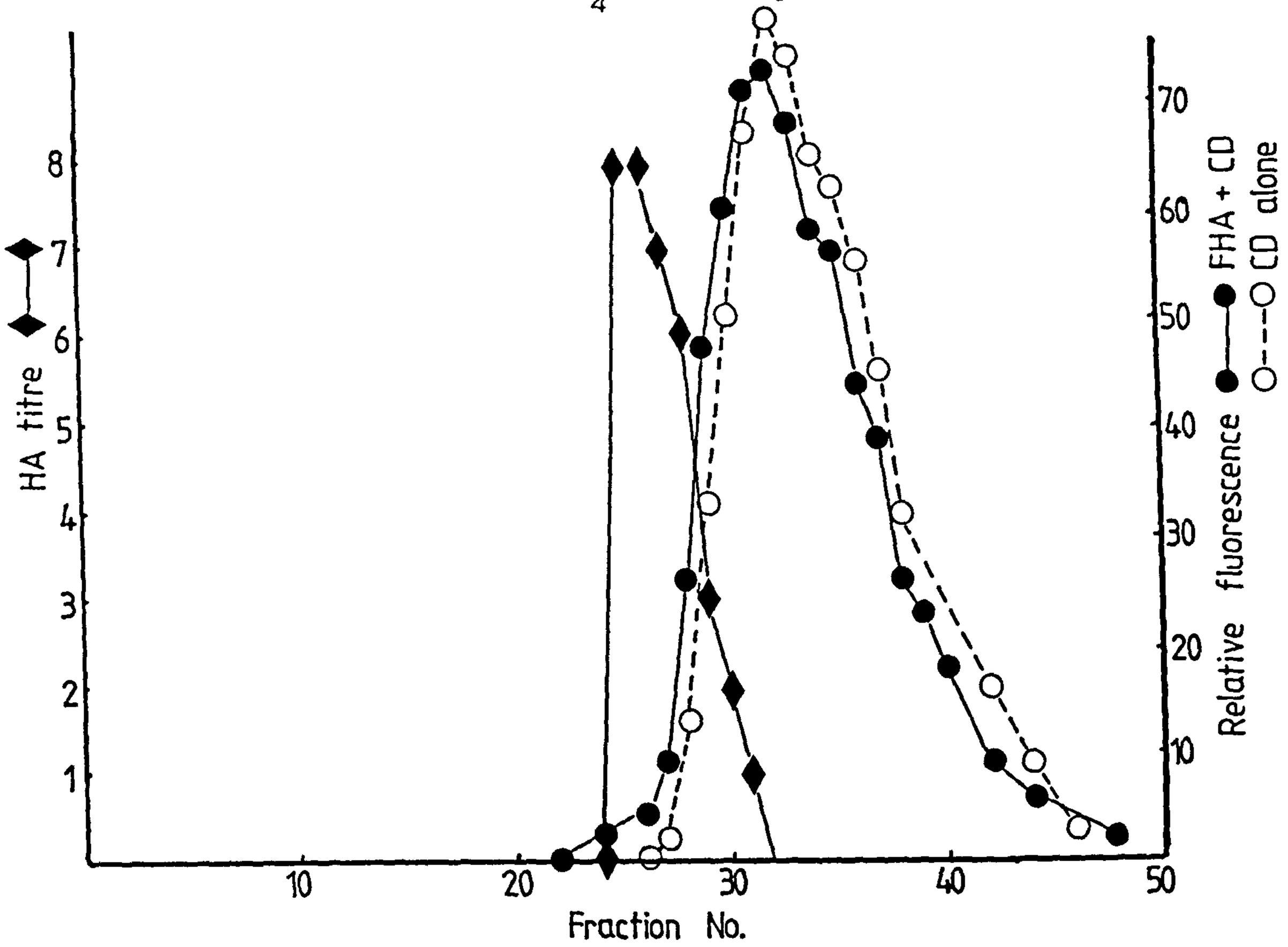


Fig. 35. Chromatography of CD and FHA+CD on Sephadex G15. 1.5cmx 1m column; flow rate 8 ml/h; 2 ml fractions; loading 250 µl of 1mM CD and 100 µl 1.2 mg/ml FHA; buffer PO_4/NaCl 0.5M pH 7.2



6.2.4 Binding of CD to FHA determined using the Amicon Centrifree micropartition system.

Equilibrium dialysis was also used to determine if FHA was able to bind CD. However, a dialysis bag system proved unsuccessful as standard dialysis membranes did not allow free diffusion of CD, even with membranes with molecular weight cut-off points of 6000-8000 and 12000-14000 (Spectropor). Also long dialysis times were needed to approach equilibrium.

Lin & Yang (1987) used Centriflo membrane cones (Amicon) to study the effect of cyclodextrins on the binding of warfarin to human albumin. A similar ultrafiltration micropartition system (Centrifree filters, Amicon) for separating free from protein-bound microsolute was used to study any interaction between CD and FHA. Separation of free from protein-bound ligand is achieved without the dilution of samples that occurs in equilibrium dialysis which may perturb the equilibrium. It has been demonstrated theoretically and experimentally (Sophianopoulos et al., 1978) that if one component of a reaction at equilibrium is freely diffusible through a semi-permiable membrane, and if an aliquot of this component is removed through the membrane at its equilibrium concentration, then the concentration of this component in the reaction mixture remains unchanged. Thus the concentration of free CD will be the same in the filtrate and the retentate of the system. Therefore any binding of CD to FHA will be seen as an increase in the total CD in the retentate and a decrease in CD concentration in the filtrate compared with a CD only control.

Samples (1 ml) of FHA, CD and FHA + CD in PBS were filtered through a 30 000 molecular weight cut-off membrane with the driving force provided by centrifugation at 1500 xg in a fixed angle rotor for 10 min. Ultrafiltrate containing free CD was separated from a mixture of FHA + CD and the concentration of CD in the

filtrate was determined using the TNS fluorescence assay.

CD determined in filtrates from Centrifree filters containing an initial CD concentration of 0.1 mg/ml and FHA concentrations of 0, 10, 100 and 1000 µg/ml are given in table 17. CD in the filtrates from samples containing FHA shows a reduction in concentration compared with CD only controls (14.0% for 1 mg/ml FHA) indicating an interaction between FHA and CD. However, for FHA concentrations of 10 and 100 µg/ml the reduction in CD concentration in the filtrate is very small, indicating that there is only a weak association between FHA and CD.

6.2.5 Binding of CD to B. pertussis cells determined using Centrifree filters.

Weak binding of CD to purified FHA was shown by ultrafiltration using Centrifree filters. Therefore this system was used to determine if CD binds to whole cells and if this was related to the presence of FHA on the surface of the cells. Phase I, phase IV and FHA⁻ B. pertussis were grown in static culture in SS medium as in section 6.2.1. Bacteria were harvested by centrifugation, washed in PBS and resuspended (1% and 10% w/v) in either PBS or PBS + 0.1 mg/ml CD. Even suspensions were obtained using a hand homogeniser. Aliquots (1 ml) of CD alone and each bacterial suspension were added to the Centrifree filters and centrifuged at 1500xg for 20 min. The concentration of CD in each filtrate was determined and results expressed as a % of the CD only control concentration (table 18). A similar % CD was bound to all the 10% (w/v) B. pertussis suspensions tested and only slightly greater binding was detected for Wellcome 28 cells at 1% compared with phase IV and FHA⁻ strains. These results confirm those given in section 6.2.1 where binding of CD to B. pertussis cells was not found to be related to the presence or absence of FHA.

6.3 Summary.

It was demonstrated that purified FHA was stable on

Table 17. Binding of CD to purified FHA determined using Centrifree filters.

	CD (mg/ml) in filtrate	% CD bound to FHA
0.1 mg/ml CD	0.098 (.004)	-
0.1 mg/ml CD + 10 µg/ml FHA	0.090 (.002)	8.2 (2.0)
0.1 mg/ml CD + 100 µg/ml FHA	0.093 (.004)	3.0 (3.7)
0.1 mg/ml CD + 1 mg/ml FHA	0.084 (.004)	14.0 (3.9)

Results are the mean of 3 replicates.
Figures in parenthesis = Standard deviation.

Table 18. Binding of CD to *B. pertussis* cells determined using Centrifree filters.

	% CD filtrate control	% CD bound to cells
Phase I cells 10% (w/v) + 0.1 mg/ml CD	89.5 (4.7)	10.5 (4.7)
FHA- cells 10% (w/v) + 0.1 mg/ml CD.	88.1 (7.2)	11.9 (7.2)
Phase IV cells 10% (w/v) + 0.1 mg/ml CD.	86.4 (2.4)	13.6 (2.4)
Phase I cells 1% (w/v) + 0.1 mg/ml CD.	95.3 (2.1)	4.7 (2.1)
FHA- cells 1% (w/v) in PBS + 0.1 mg/ml CD.	99.9 (1.4)	0.5 (3.1)
Phase IV cells 1% (w/v) + 0.1 mg/ml CD	96.4 (3.1)	3.0 (3.1)

B. pertussis cells grown for 5 days in Thompson bottles (SS medium) with static incubation.

Results are the mean of 3 determinations.

Figures in parenthesis = standard deviation.

static incubation but was unstable and quickly lost HA activity when incubated with shaking. CD was found to have a concentration-dependent stabilising effect on FHA incubated with shaking. This suggested that the ability of CD to enhance yields of FHA in shaken cultures could be wholly or partly due to a stabilising effect of CD on FHA. The loss of FHA activity on shaking was pH dependent and was slightly greater at high pH (9.0 and 10.0) than at neutral pH (7.0 and 8.0) but the stabilising effect of CD on FHA was independent of pH. The loss of FHA activity on shaking was markedly reduced by lowering the incubation temperature and was increased by increasing the area of the air-liquid interface. When FHA was incubated with shaking, protein was lost from the solution and strands of aggregated protein were seen at the surface of the liquid. However, the FHA remaining in solution did not show degradation when examined by SDS-PAGE.

It was proposed that CD may exert a stabilising effect by binding to FHA and interfering with its aggregation. CD was seen to associate with B. pertussis cells but this was not related to the presence or absence of FHA. Binding between CD and FHA was not demonstrated by blocking of antibody binding or gel filtration. However, micropartition by ultrafiltration showed weak binding between CD and purified FHA.

It is not known whether the increase in yield of FHA in shaken B. pertussis cultures is due solely to CD increasing the stability of FHA or if production and/or release of the protein are also stimulated by the presence of CD.

Chapter 7. Discussion.

7.1 Growth of B. pertussis.

The literature on the growth of B. pertussis in vitro has stressed the universal problem that the organism is difficult to isolate, and that cultures can be unreliable and variable. It is hoped that this problem can be addressed and some answers provided. It might be expected that B. pertussis is nutritionally demanding but this is not the case. B. pertussis is able to grow in the defined medium of Stainer & Scholte (1971) which consists of only 3 amino acids, salts, nicotinic acid, ascorbic acid, glutathione and Tris buffer. The ability of the organism to grow in this medium containing either cystine or cysteine (Parker, 1976; Novotny & Brookes, 1975) was confirmed in this study, with no difference to the growth produced. Modifications have been made to the concentration of Tris used in SS medium (Hewlett & Wolff, 1976) but these changes had little effect on final yield of cells. However, cultures with a reduced amount of Tris had a higher final pH due to the reduced buffering capacity of the medium.

It can be seen that B. pertussis is not nutritionally demanding but it is fastidious, especially in that it is sensitive to inhibitory compounds. The question thus arises, is the difficulty in obtaining reliable B. pertussis cultures due to its metabolism, the conditions of culture used or to its genetic instability or a combination of these? It is interesting to note that B. pertussis shows a high degree of DNA homology to B. parapertussis and B. bronchiseptica (Kloos et al., 1981) and these grow well in culture, implying that the problems associated with B. pertussis culture must be due to changes in only a few genes.

The use of CD as a medium additive by Imaizumi et al. (1983a & b, 1984) was investigated and it appears to have many important effects on B. pertussis. A marked effect of CD on growth of B. pertussis observed in this study was to

reduce the lag phase in shaken flask, Thompson bottle and fermenter cultures. This effect was noted by Imaizumi et al. (1983b) but the reduction in the lag phase was less marked. However, Perera et al. (1986) reported no change in the rate of growth or the length of the lag phase in the presence of CD. CD was also seen to increase the final optical density of shake flask and Thompson bottle cultures grown with SS medium. There was also an increase in final optical density with CL medium Thompson bottle cultures. In stirred fermenter cultures the maximum culture density was not increased by CD but a higher final culture density than seen in shaken cultures was achieved, even in the absence of CD. This may be because the fermenter cultures were not oxygen limited, as may be the case in shaken culture vessels, where greater growth may be facilitated by the presence of CD, even in oxygen limited conditions. Imaizumi et al. (1983b, 1984) and Perera et al. (1986) found little or no alteration in the final culture density by the addition of CD.

The effect of CD to promote growth in shaken cultures, where oxygen may become a limiting nutrient may be a similar effect to that observed where the addition of CD enabled B. pertussis to grow in SS medium in the absence of glutathione. Here, it appears that CD is altering the nutritional requirements of the organism. Vajdic et al. (1966) observed that glutathione was the primary source of organic sulphur for B. pertussis in a defined medium, its use corresponding with the start of exponential growth. They also observed that the concentration of cysteine did not decrease until the onset of the stationary phase of growth. It must be that in the presence of CD, B. pertussis can obtain all its organic sulphur from cystine and also that the reducing power of glutathione is no longer required.

The variable nature of B. pertussis cultures was observed when larger 8-10l cultures were studied. Novotny & Cownley (1979) state that certain types of stainless steel are toxic to B. pertussis, possibly due to the

manganese content of the steel. This statement was considered in the choice of 500ml fermenter for studying B. pertussis growth in batch and continuous culture. Consequently the steel head plate of the glass vessel was replaced with one made from teflon and all projections into the vessel were glass. Using this fermenter very reliable growth was obtained, even in the absence of CD, with cultures achieving a maximum optical density in excess of 8.0 (approximately 3.75 mg/ml dry weight). However, growth in the LH 2000 Series fermenter, which has a stainless steel vessel, proved unreliable when CD was not included in the medium. The maximum culture density achieved was half that seen in the presence of CD and this occurred at 60 h instead of approximately 40 h with CD. It is not known whether this poor growth resulted from an inhibitory effect of stainless steel or possibly from trace amounts of substances known to be toxic to B. pertussis, such as fatty acids (Rowatt, 1957b) remaining in the vessel.

Cameron et al. (1985) give an account of their own experiences and those of others in growing several different strains of B. pertussis in different fermenters made from different grades of stainless steel. In most cases good yields of B. pertussis (1.4-2.5 mg/ml dry weight) were obtained and cultures were used to make vaccines of acceptable potency and safety. Results from this study show that reliable growth of B. pertussis can be achieved using stainless steel fermenters but only when CD is used as a medium additive.

A major problem encountered with larger fermenter cultures of B. pertussis was foaming. Foam produced by these sparged cultures resulted in blockage of the air exhaust filter and a deposition of bacteria on the lid and walls of the fermenter, reducing the density of the culture. Initial attempts to grow B. pertussis with polypropylene glycol (PPG) antifoam resulted in death of the culture when the antifoam was first added. Novotny & Cownley (1979) observed that PPG antifoam inhibited growth

of B. pertussis and that MS silicone antifoam A was ineffective in controlling foaming during long fermenter runs. The toxicity of PPG antifoam was confirmed in shaken flask cultures and silicone RD and M30 emulsions were also tested. Both silicone emulsions could be used sparingly to control foaming in sparged fermenters but the M30 emulsion, Dow Corning's highest grade silicone antifoam, was the least inhibitory to B. pertussis growth. Control of foaming in fermenter cultures was achieved by timed-dose addition of antifoam over the last 24h of the culture. Csizer et al. (1977) also report the use of a silicone oil antifoam in 50l fermenter cultures of B. pertussis. The sensitivity of B. pertussis to toxic inhibitors necessitates the testing of any medium additive, such as antifoam, in small shaken flask cultures before use on a larger scale.

During growth in the LH 8l fermenter the dissolved oxygen concentration was maintained at several set point values with sparged aeration and automatic adjustment of stirrer speed. Controlling the oxygen concentration to 5, 20 and 50% of saturation produced equal growth as none of these levels was limiting for growth. Top aeration alone did not supply sufficient oxygen for maximum growth with a highest optical density of only 0.4. Growth with sparged aeration but a constant slow stirrer speed produced a reduced growth rate compared with cultures with $\%O_2$ controlled by stirrer speed. A maximum culture density of 6.5 was achieved at a culture time of 60 h. However antifoam was not required by top aerated cultures. Cameron et al. (1985) and Novotny & Cownley (1979) do not mention the method of aeration used during fermenter cultures but Csizer et al. (1977) also used sparged air in their 50l fermenter. From this study it appears that for maximum yields of bacterial mass from B. pertussis cultures the oxygen concentration of the medium must not become limiting and in larger cultures this can only be achieved by the use of sparged air.

It is interesting to attempt to compare the maximum

culture densities of B. pertussis fermenter cultures achieved in different laboratories. This comparison is obviously complicated by the use of different strains of the organism and different media. Also different means of expressing culture density have been used therefore the constant obtained by Csizer et al. (1977) which relates International Opacity Units to dry weight (mg/ml) has been used. Csizer et al. (1977) obtained cultures containing 0.62-0.94 mg/ml dry weight of B. pertussis using a 50l steel fermenter with the medium of Cohen & Wheeler (1946) modified to contain 0.5% Amberlite ion exchange resin. Novotny & Cownley (1979) present data comparing growth in several fermenters using SS medium. The best growth was obtained in a 70l glass vessel yielding a maximum of approximately 2.4 mg/ml dry weght of B. pertussis. Cameron et al. (1985) describe growth of B. pertussis in a 50l glass fermenter producing a maximum culture density of 2.5 mg/ml dry weight using the medium of Cohen and Wheeler (1946). They also used a 140l stainless steel fermenter and SS medium which in a number of cultures yielded 1.4 - 1.7 mg/ml dry weight of B. pertussis cells. All these cultures were performed without the addition of CD to the medium and in a variety of conditions of culture so the most direct comparison of results obtained in this study is with the work of Imaizumi et al. (1983b & 1984).

Using CL medium and 1.0 g/l CD Imaizumi et al. (1983b & 1984) produced shaken flask cultures with maximum cell concentrations of up to 115 International Opacity Units, equivalent to 1.8 mg/ml dry weight. This compares with maximum dry weights of B. pertussis obtained here using the same medium; using shaken flasks 3.0 mg/ml, shaken Thompson bottles 2.8 mg/ml, 500ml glass fermenter 3.9 mg/ml and 8l stainless steel fermenter 3.75-4.2 mg/ml. Thus it can be seen that conditions developed in this study resulted in cultures of greater density than have been previously reported, especially using the conditions described for fermenter cultures. However, it must be remembered that the comparison is not using the same

strain of the organism.

In comparison with fermentation processes for other bacteria the yields of cells obtained for B. pertussis are very low as yields well in excess of 25 mg/ml have been obtained with fermentations using E. coli (Zabriskie & Arcuri, 1986). The physiology of B. pertussis is less well understood but several approaches could be made to try to improve the fermentation of the organism. Addition of silicone antifoam can be deleterious to growth and expression of components required for vaccine production so it would be advantageous to use a mechanical foam breaker, perhaps in combination with a greatly reduced concentration of antifoam. This would also have great benefits for downstream processing for production of an acellular vaccine as antifoam in the culture supernatant can cause clogging of filters.

Greater yields of B. pertussis cells could possibly be obtained if the medium constituents that become depleted at the end of the exponential phase of growth are added to the culture, in a feeding policy that maintains growth beyond that normally achieved. Work is in progress to determine the nutrients that are depleted from CL+CD medium during growth. As FHA production appears to be favoured by a reduced stirrer speed in the fermenter this might be achieved whilst maintaining the controlled oxygen concentration by sparging with oxygen-enriched air.

An increased yield of B. pertussis haemagglutinins has been claimed when polyvinyl alcohol (PVA, MW = 10000) is added to the growth medium (G. Greenspan, US patent no. 4551429, 1985). However, results in the patent are only obtained from haemagglutination assays and a preliminary study of the effects of PVA has shown that even trace amounts of PVA interfere with HA measurements by preventing the red blood cells from settling. This may account for the high HA titres claimed.

Continuous culture has been used to study the growth of B. pertussis to assess whether this method was

applicable for vaccine production. Previous studies have shown it to be unsuitable (Van Hemert, 1974) or have not described expression of defined antigens (Novotny & Cownley, 1979; Jagicza et al., 1985) (see 1.3.5.1).

In this study it was demonstrated that growth of phase I B. pertussis could be maintained in continuous culture for extended periods (in excess of 1000 h) and at dilution rates of 0.05, 0.1 and 0.15 h^{-1}, which correspond to doubling times of 14, 7 and 5.25 h. Using a different strain Novotny and Cownley found the maximum dilution rate was 0.13 h^{-1}. Above this value the culture not only washed out but completely lost viability and proved impossible to recover by reducing the dilution rate. The same phenomenon was observed in this study when it was attempted to use a dilution rate in excess of 0.15 h^{-1} (data not shown).

Novotny & Cownley (1979) state that their continuous culture growth was limited by the concentration of glutamate and proline with medium containing 57 µM glutamate and 2 µM proline. It was attempted to achieve growth limited by cystine in this study. After an initial reduction in the culture density with reduced cystine, the culture density greatly increased and the culture appeared to have degraded to a phase IV variant which was able to grow more successfully under these conditions.

A similar effect was seen when it was attempted to achieve iron-limted growth in the presence of the iron chelator desferal as the culture appeared to degrade to phase IV organisms when analysed by SDS-PAGE. There was no reduction in culture density when B. pertussis was grown in medium with no added iron, indicating that the iron contaminating other medium constituents was sufficient for growth. B. pertussis was also able to grow in the presence of 0.1 g/l desferal and a reduction in the culture density was only caused by increasing the desferal concentration to 0.2g/l. This concentration produced phase IV variants but this population was unable to grow in these iron-depleted conditions and the culture washed out, in contrast to the superior growth seen for phase IV

organisms under reduced cystine conditions. No additional bands were observed on SDS-PAGE analysis of whole cells, indicating that B. pertussis does not produce iron-regulated outer membrane proteins in a similar manner to E. coli and Salmonella typhimurium (Griffiths, 1983). In the only other study of iron-limited growth of B. pertussis, Redhead et al. (1987) confirm that the organism does not appear to produce siderophores in batch cultures, with iron-limited conditions produced by the presence of lactoferrin. They report very poor growth of B. pertussis in SS medium with no added iron. This is in contrast to results presented here and may be due to higher levels of contaminating iron in medium and glassware in this study. Added iron has been included in B. pertussis culture medium since Cohen & Wheeler (1946). Whether added iron is required as a nutrient or if it serves to detoxify peroxides was questioned by Rowatt (1957b).

The change to phase IV variants when B. pertussis cultures are placed in an unfavourable nutrient environment may be because these organisms are less fastidious and are less sensitive to toxic inhibitors such as fatty acids (Field & Parker, 1979) and certain antibiotics (Bannatyne & Cheung, 1984).

The addition of CD to the growth medium for continuous culture greatly increased the culture density and as CD is not metabolised by B. pertussis, this suggests a nutrient limitation was not previously operating but that growth was inhibited. This relieving of a growth inhibition by CD supports the suggestion by Imaizumi et al. (1983b) that a major function of CD in B. pertussis cultures is to bind toxic fatty acids. It appears that in the presence of CD B. pertussis can fully use the available nutrients as when the concentration of cystine in the medium was reduced by 50% then the culture density also reduced by a similar degree.

The concentration of LPF in the culture supernatant was increased in excess of the increase in the culture density by the addition of CD but the FHA released into

the supernatant remained at a low concentration, even in the presence of CD. This may be because the results obtained using batch cultures show that FHA in supernatants occurs at the highest concentration during early stationary phase. In a continuous culture system bacteria are maintained in mid-exponential phase of growth by the continuous medium supply with stationary phase never permitted. For this reason continuous culture may not be a suitable system for growth of B. pertussis for the manufacture of an acellular vaccine as it is advantageous to have conditions allowing maximum yield of the required antigens. The suitability of continuous culture for vaccine manufacture would also be limited by the instability of virulent phase B. pertussis, with its tendency to degrade to avirulent phase IV organisms when the culture is subjected to any stress.

7.2 Antigenic modulation in B. pertussis.

During antigenic modulation induced by $MgSO_4$ and an increased concentration of NA, several components of B. pertussis were lost (table 14). The loss of some of these components has been observed by other workers (table 1). Also, antigenic modulation induced by $MgSO_4$ or NA resulted in a change in the hydrophobicity of B. pertussis cells as determined by adhesion to octane. X-mode cells were very hydrophobic and adhered to the octane droplets whereas C-mode cells were hydrophilic and did not adhere to octane. This method has been shown to correlate with attachment of bacteria to other surfaces including non-wettable plastics, epithelial cells and teeth, reviewed by Rosenberg (1984). Furthermore, C-mode cells adhered poorly to Vero cells compared with X-mode cells. C-mode B. pertussis also adhere poorly to mouse lungs (Burns & Freer, 1982) and to HeLa cells (Redhead, 1985). Therefore the loss of X-mode characteristics reduces the ability of B. pertussis to adhere in these assays. Addition work not included in this thesis using the Vero cell adhesion model has shown that monoclonal antibodies

to agglutinogen 3, FHA, LPF and to an X-mode specific polypeptide partially inhibited adhesion of X-mode B. pertussis. Also, monoclonal antibody to agglutinogen 2 inhibited adhesion of B. pertussis strains bearing homologous antigen (Gorringe et al., 1985).

Fish et al. (1987) studied the hydrophobicity of B. pertussis by adherence of bacteria to a polystyrene surface. They found that hydrophobic adherence of avirulent (phase III) strains was about 20% of that shown by virulent (phase I) strains. By studying a series of mutants lacking certain virulence factors they noted that hydrophobic adherence was maintained by mutants possessing FHA, even in the absence of LPF and haemolysin. From this they claim that FHA is the cell surface moiety responsible for hydrophobic binding. Whether it is the case that loss of a single component causes the loss of hydrophobicity seen on change from X- to C-mode B. pertussis could be determined using transposon Tn5-induced mutants (derived by I. Livey, CAMR).

The rate of transition from X- to C-mode has been studied in batch cultures. Lacey (1960) observed that the change from X- to C-mode occurred after 21-36 h on Bordet Gengou agar with a high $MgSO_4$ concentration and required 7-12 cell divisions. Idigbe et al. (1981) reported that strain 18334 grown in modified Hornibrook medium containing $MgSO_4$ underwent a more rapid loss of LPF-associated activities, HLT and X-mode polypeptides than could be accounted for by a simple growth-dilution effect. They suggested that selective destruction of X-mode components occurred during modulation. In these experiments there was a slow growth rate with a mean generation time of 6-7 h. The loss of adenylate cyclase activity during antigenic modulation was studied by Hall et al. (1982) and found to follow a curve indistinguishable from that expected if complete repression of adenylate cyclase synthesis occurred on the change from X- to C-mode medium. Brownlie et al. (1985b) found that the loss of adenylate cyclase, histamine

sensitising activity and X-mode polypeptides occurred in a synchronous manner and correlated closely with the theoretical loss (assuming no synthesis) with no inactivation or destruction. They found that the X- to C-mode transition took 8-10 h with a mean generation time of 3.2 h.

In this study continuous culture techniques were used to determine the rate of antigenic modulation. This provided a system with a constant growth rate, controlled by the dilution rate. Thus, the loss of X-mode components could be directly compared with the mean generation time and theoretical washout rate of the culture. It was found that all the X-mode characteristics assayed were lost and regained at approximately the same rate. The loss of LPF during $MgSO_4$-induced antigenic modulation, when X-mode medium in the chemostat was gradually changed to C-mode medium, was approximately equal to the washout rate at dilution rates of 0.05, 0.1 and 0.15 h^{-1}. The general consensus of opinion with the exception of Idigbe et al. (1981), is that the rate of modulation induced by change to a high $MgSO_4$ medium is equivalent to the theoretical value obtained if X-mode organisms are replaced by C-mode organisms upon cell division. The need for protein synthesis for antigenic modulation to occur was demonstrated by Brownlie et al. (1985b) as there was no loss of X-mode components in C-mode medium containing chloramphenicol, an inhibitor of protein synthesis. Chloramphenicol also prevented regain of X-mode properties when C-mode cells were transferred to X-mode medium.

For NA-induced modulation in continuous culture, C-mode-inducing conditions were instantaneously achieved by adding NA to the growth vessel. In this case loss of LPF and hydrophobicity (D=0.1 h^{-1}) was quicker than the theoretical washout rate, C-mode values of LPF content being reached after 8 h (1.14 generations) and of hydrophobicity after 12 h (1.71 generations). At a dilution rate of 0.15 h^{-1} LPF and FHA were also lost faster than the theoretical washout rate. However,

hydrophobicity was lost slower than the washout rate with cells possessing 50% of X-mode hydrophobicity when C-mode levels of LPF were obtained. At a slower dilution rate (0.05 h^{-1}) X-mode characteristics were lost at a rate approximately equal to the washout rate. At all dilution rates examined hydrophobicity was the last X-mode characteristic to be lost, this may because it is a property of a combination of surface components, some of which may be lost more slowly than those determined. The loss of X-mode components faster than theoretical washout rate is an interesting phenomenon since it requires positive cellular inactivation or destruction of the X-mode characteristics. Whether this phenomenon can also occur for $MgSO_4$-induced modulation remains to be elucidated as does the mechanism of active loss of antigens. The loss of X-mode components at a similar rate gives support to the proposed trans-acting positive effector, encoded by the virulent phase inducer gene, vir (Weiss & Falkow, 1984). Loss of components at different rates would indicate different control of expression of the different components.

In this study antigenic modulation was induced in continuous culture by reducing the temperature of the culture from 35°C to 25°C. At the reduced temperature C-mode hydrophobicity was observed and this returned to X-mode hydrophobicity when the temperature was returned to 35°C. Lacey (1960) also observed that C-mode growth was obtained when cultures of B. pertussis on Bordet-Gengou agar were incubated at 25°C. It may be that the enzymes responsible for production of X-mode components or the proposed positive effector protein are not able to function at this temperature.

Brownlie et al. (1985b) determined the ratio of $MgSO_4$:NaCl that caused C-mode growth of B. pertussis in batch cultures. In continuous culture antigenic modulation is partially triggered by medium containing 10 mM $MgSO_4$, producing organisms with reduced hydrophobicity or a population of cells containing a proportion of C-mode

cells. A fully C-mode population was achieved with medium containing 12 mM $MgSO_4$, the same concentration reported by Brownlie et al. (1985b).

The effects of CD on antigenic modulation were investigated. In NA C-mode cultures of B. pertussis containing CD, trace amounts of FHA were detected in both the supernatant and on the cells in 72 h cultures. Thus the CD had overcome the complete repression of FHA production that normally occurs in C-mode cultures. The hydrophobicity of cells from NA C-mode cultures was also increased by the presence of CD. In addition, it was found that the presence of CD increased the threshold concentration of $MgSO_4$ required to cause C-mode growth, with X-mode characteristics still expressed at concentrations of $MgSO_4$ that produced C-mode organisms without CD.

It has been suggested that the adenylate cyclase produced by B. pertussis may play a role in the control of expression of virulence factors and may be involved in the regulation of antigenic modulation (Hewlett et al., 1979; Parton & Durham, 1978; Wardlaw & Parton, 1979). This suggestion was made as cAMP is involved in regulation of expression of proteins in prokaryotes (Botsford,1981), including some virulence-associated proteins in E. coli (Eisenstein et al., 1981; Martinez-Cadena et al., 1981). Brownlie et al. (1985a) observed that loss of adenylate cyclase occurred concomitantly with loss of λ (histamine sensitizing activity) and X-mode specific polypeptides during modulation induced by different inorganic and inorganic salts. In continuous culture the rate of loss of cAMP was found to be approximately equal to the theoretical washout rate with NA-induced modulation. This, together with the findings of Brownlie et al. (1985b) indicate that adenylate cyclase does not have a causal role in the loss of virulence-related factors during modulation. The evidence for this is that (i) the loss of adenylate cyclase activity during modulation could be accounted for by complete repression of adenylate cyclase synthesis, and

there was no evidence for the inhibition of adenylate cyclase activity prior to modulation. (ii) Loss of adenylate cyclase in intact cells during modulation did not precede, but paralleled, the loss of X-mode characteristics. (iii) The addition of exogenous cAMP to the growth medium had no observable effect in counteracting modulation.

Further evidence that adenylate cyclase does not act in a regulatory role comes from the observation that adenylate cyclase negative mutants of B. pertussis produce normal levels of LPF, FHA and agglutinogens (Robinson et al., 1986b). If low levels of cAMP are the trigger for antigenic modulation then these strains might be expected to be permanently in the C-mode.

It is not known whether antigenic modulation plays a role in the pathogenesis of B. pertussis infections or is just an artifact of in vitro growth conditions. That B. pertussis grows in the X-mode in vivo can be seen from measurements of serum antibody responses of whooping cough patients to X-mode components e.g. LPF and FHA. In other bacteria antigenic changes have been considered to be a means of evading the host's immune response (Smyth, 1986; Heckels, 1986). However, antigenic modulation (and phase variation) appears too drastic a method just to evade the host immune response as it involves loss of all virulence characteristics. B. pertussis may also adapt to the host's immune response by less severe antigenic changes restricted to loss or gain of agglutinogen 2 or 3 (Preston et al., 1980; Stanbridge & Preston, 1974b; Robinson and Gorringe, unpublished observation). The fimbrial nature of these antigens makes them candidates for mediating attachment of B. pertussis to the ciliated cells of the upper respiratory tract. Although further studies are required to confirm the role of agglutinogens in adhesion, preliminary studies not included in this thesis have shown that monoclonal antibodies to agglutinogens 2 and 3 inhibit binding of B. pertussis to Vero cells in a serotype specific manner (Gorringe et al., 1985). It would

be interesting to determine any serotype changes in B. pertussis recovered from an intranasal infection in mice pre-immunised with either agglutinogen 2 or 3.

A possible benefit of antigenic modulation to B. pertussis may be to facilitate transmission of the disease. It is thought that virulence components, e.g. FHA, LPF and agglutinogens, are required for attachment in the respiratory tract of the child and to resist clearance mechanisms such as mucociliary flow and coughing. It is interesting to speculate that as disease progresses, local tissue damage and the enzymic action of LPF may cause local environmental conditions that favour the formation of C-mode organisms. As C-mode organisms adhere less well to mammalian cells than X-mode organisms, demonstrated in this study for Vero cells, this may cause the modulated cells to be expelled by the child and available for spread of the infection. This hypothesis depends on C-mode-inducing conditions being produced in the infected tissue. As antigenic modulation is not triggered by a single environmental condition but can be caused by a wide range of salts (Lacey, 1960), increased NA concentration and by the presence of certain pyridines (Schneider & Parker, 1982), then this increases the possibility that suitable conditions for antigenic modulation may occur. In addition, for C-mode organisms to have a role in the spread of disease, they must be capable of initiating disease and reverting to the X-mode form in vivo. This could be determined experimentally by seeing if C-mode grown B. pertussis inoculated into mice via the intranasal route could maintain an infection as seen for X-mode organisms (Robinson et al., 1986a). Reversion to X-mode growth could be detected by measuring serum antibody responses to X-mode components.

The observation that several phase I or X-mode characteristics are all lost during phase variation and antigenic modulation might suggest that the loss of genetic information results from the curing of a plasmid

or prophage coding for the virulence factors. However, a correlation between plasmid content and virulence in B. pertussis has not been shown. Furthermore, phase variation has been shown to be reversible (Weiss & Falkow, 1984) which also indicates an alternative mechanism of control of virulence determinants. A model has been considered in which the virulence-associated genes are arranged in one operon with their expression being under the control of a single promoter. This hypothesis is not now accepted as it is not consistent with the results of studies using transposon mutagenesis with Tn5. Insertion of Tn5 into a gene on such a polycistronic operon would cause not only loss of the virulence factor coded by the target gene but also those functions encoded downstream from the insertion site, as Tn5 has been shown to cause polar mutations (Berg et al., 1980). In fact, most virulence mutants obtained by Tn5 mutagenesis lack only a single virulence factor and mutants lacking only haemolysin, LPF, FHA or agglutinogen 3 have been obtained (Weiss et al., 1983; Robinson et al., 1986b). These Tn5 insertions producing haemolysin, LPF and FHA negative mutants have been mapped within different restriction fragments in the B. pertussis chromosome, indicating that they are not closely associated (Weiss et al., 1983). Haemolysin and adenylate cyclase are the only attributes that have been lost together indicating that these genes are on a single operon (Weiss et al., 1983).

A model of the molecular basis of phase variation was proposed by Weiss & Falkow (1984). This is based on the premise that there is a gene, termed vir, that codes for a trans-acting gene product which acts as a positive inducer for the virulence-associated genes. Support for this model comes from the observation that that a single insertion of Tn5 into one particular region of the B. pertussis chromosome can result in the loss of many, if not all, of the virulence factors. This model would allow for co-ordinate expression of the virulence-associated genes even if they are widely spaced on the chromosome with avirulent organisms resulting from non-production of the

proposed trans-acting gene product. This model would also explain the failure to obtain expression of B. pertussis virulence factors when fragments of B. pertussis DNA are cloned into E. coli (Weiss & Falkow, 1983; Robinson et al., 1986b) as the positive effector gene would be required in addition to the structural gene.

As well as providing an explanation for the mechanism of phase variation, the model proposed by Weiss & Falkow (1984) is also a model for antigenic modulation. Thus it may be that environmental signals, e.g. replacing NaCl with $MgSO_4$, can modulate the expression of the postulated trans-acting gene product and therefore effect the expression of the virulence factors.

7.3 Expression of LPF and FHA by B. pertussis.

In addition to the unreliable growth produced by B. pertussis with different workers and in different laboratories, cultures have often been variable as to their content of virulence determinants (Parker, 1979). Before the identification of these virulence determinants and the development of assays for their detection, cultures were assessed for their agglutination titre with serotyping antisera and also for their mouse protective activity in the Kendrick test, see for example, Novotny & Cownley (1979). Therefore only very recent reports discuss expression of characterised B. pertussis antigens.

LPF is found in culture supernatants after growth of the organism. However, lymphocytosis-promoting activity can be detected in both supernatants and cell pellets of B. pertussis cultures (Morse & Bray, 1969; Sato et al., 1974). Using a gold labelled monoclonal antibody and electron microscopy, Ashworth et al. (1986) found that LPF appeared to be distributed over the cell surface.

Little is known about the mechanism of release of LPF into the supernatant. Sato et al. (1974) followed specific LPF activity in cells and supernatants of statically incubated cultures and they found that activity was higher in the cells until the fourth day of culture, after

which the activity in the supernatant superseded cell-bound activity. Protein determinations with cells and supernatants demonstrated no apparent autolysis of the cells, suggesting that LPF was released from the cells by some other means. Perera et al. (1986) also found that LPF release was not associated with cell lysis by measuring the cytoplasmic marker malate dehydrogenase in supernatants. They subsequently demonstrated the accumulation of a precursor of the S1 subunit of the toxin PT (pS1) in cell envelopes when B. pertussis was treated with the membrane perturbing agent phenethyl alcohol (Perera & Freer, 1987). Recent molecular cloning studies (Locht & Keith, 1986; Nicosia et al., 1986) have shown that the amino terminus of each LPF subunit is preceded by a typical bacterial signal peptide. This indicates that the subunits are translated as precursors and secreted individually into the periplasmic space. This is similar to the mechanism proposed for the export of heat-labile enterotoxin of Escherichia coli in which the A and B subunits are synthesised as precursors in the cytoplasm. These are then translocated and processed to mature A and B subunits in the periplasmic space where they assemble into holotoxin (Hirst, 1986).

Several workers have measured the concentration of LPF in B. pertussis culture supernatants during growth. It appears that the maximum concentration in the supernatant corresponds with the end of the exponential and the beginning of the stationary phase of growth (Sato et al., 1974; Sekura et al., 1983; Bellalou & Relyveld, 1984). Bellalou & Relyveld (1984) also noted that LPF activity declined rapidly after reaching a peak at the end of the exponential growth phase. This reduction in LPF activity may be due to the elevated pH seen in the stationary phase, as a high culture pH has been shown to reduce the toxicity of B. pertussis cultures (Lane, 1968).

Imaizumi et al. (1983b) studied the effect of CD on growth of B. pertussis and production of LPF in liquid media. CD was found to stimulate growth, reduce the lag

time of the cultures and enhance by about 100 fold the production of LPF in SS medium compared with CD-free medium. A modification of SS medium was also described in which the concentration of glutathione and ascorbic acid was increased and 10 g/l casamino acids was added to the medium. This further enhanced LPF production and in the presence of CD, a maximum of 50 mg per litre of culture was estimated to be present by *in vivo* and *in vitro* assays.

The effect of CD on LPF production has been studied by Perera *et al*. (1986) who found that growth rate and maximum culture density were not affected by the presence of CD, although the LPF was produced somewhat earlier in cultures containing CD. Their maximum yield of LPF was 4.3 mg/l at 48 h in the presence of CD and 1.8 mg/l at 32 h in the absence of CD. For cultures grown without CD, the LPF as measured by ELISA was distributed in approximately equal amounts between cells and supernatants. The addition of CD to the medium caused an approximately five fold increase in LPF detected in the supernatant.

In this study the effect of CD on LPF production by *B. pertussis* (strain Wellcome 28) in Thompson bottle cultures in five different media was determined. Supernatants from these cultures were assayed for LPF by ELISA and the maximum concentration was found at the end of the exponential phase of growth. The addition of CD increased the concentration of LPF in SS supernatants from 3.5 to 8.7 µg/ml and that in CL medium from 6.7 to 11.0 µg/ml. Very little LPF was detected in cultures grown in the medium of Sato *et al*. (1974). Growth and production of LPF was also studied in 500ml stirred fermenters using CL medium containing increasing amounts of CD at a controlled pH of 7.6 and at 20% dissolved oxygen. The production of LPF is detected earlier in the cultures as the concentration of CD increases, which reflects the shorter lag phase of these cultures. The maximum concentration of LPF found in CL medium was 3.2 µg/ml at 72 h, whereas for cultures containing 2 g/l CD it was 9.8

ug/ml.

Larger scale (8l) fermenter cultures of B. pertussis produced approximately equivalent concentrations of LPF in the supernatant. Although without CD, yields were very low reflecting the poor growth obtained. Control of O_2 in the culture to a low concentration (5%) favoured expression of LPF whilst a high level (50%) reduced the LPF determined in the supernatant. The reduction of toxicity of B. pertussis cultures grown with vigorous aeration was observed by Lane (1968), who also noted that cultures were detoxified by the high pH occuring in stationary phase cultures. Results from this study show a reduction in the LPF concentration as the culture pH approaches 9.0. Fermenter cultures where the pH is controlled to 8.1 do not show this reduction in LPF concentration (data not shown). Therefore for fermenter cultures for vaccine production pH control to 8.1 was adopted as a compromise between maximum yields of LPF and FHA.

These studies, together with those of Perera et al. (1986) have shown that CD causes a definite enhancement of LPF production by B. pertussis but the levels of LPF reported by Imaizumi et al. (1983b) have not been achieved. This cannot be accounted for by differences of strain used as Perera et al. (1986) found little difference in LPF production by four strains of B. pertussis including the Tohama strain used by the Japanese workers. However, Sekura et al. (1983) found that Tohama produced only 10-20% of the amount of LPF observed for strain 165. Further work is required to determine the reasons for these differences and to elucidate the effects of CD on expression of LPF by B. pertussis.

The observation that FHA is detected in statically incubated cultures and not in shaken cultures (Arai & Munoz, 1979) was confirmed in Thompson bottle cultures. However, the addition of CD to the medium increased FHA measured in shaken cultures, reported by Imaizumi et al. (1984). However it is difficult to compare yields of

Imaizumi et al. (1984) with results obtained in this study as they express ELISA results in arbitary units related to a reference preparation of FHA, the concentration of which is not stated. However, they describe that CD caused an increase in FHA production per organism and that the effect was due not only to stimulation of cell growth but also by some other mechanism.

The effect of CD in increasing FHA in culture supernatants is clearly illustrated with 500 ml fermenter cultures (fig.7). Without CD very little FHA is measured in the supernatant, even though the final density of the culture is equivalent to cultures containing CD. For the culture containing 2.0 g/l CD with the pH controlled to 7.6, there was a peak of FHA concentration in the supernatant (30 μg/ml) at 35 h, after which the concentration dropped to less than 10 μg/ml at 50 h.

The culture pH was also found to be important for optimal expression of FHA. In a culture containing 2.0 g/l CD but with no pH control the FHA concentration did not peak at 35 h and then reduce but it carried on rising to a maximum value of 70 μg/ml at 55 h. Cultures without both pH control and CD did not show this effect. It appears then, that expression of FHA is favoured by the high pH produced in stationary phase cultures as well as by the presence of CD. Thus subsequent fermenter cultures were performed without pH control.

Yields of FHA obtained in the LH 8l fermenter were approximately 50% of those seen in the 500 ml fermenter. Reasons for this may have been the use of antifoam in the larger fermenter and the different stirring and aeration systems in the two vessels. The $\%O_2$ set point did not greatly affect the FHA determined in the 8l fermenter supernatant but a set point of 20% proved most favourable. Top aeration only, as used in the 500ml fermenter, supplied insufficient O_2 to the culture, producing poor growth and poor expression of FHA. Aeration with sparged air and a constant slow stirrer speed produced a greater concentration of FHA for the culture density, compared

with cultures with O_2 control by stirrer speed.

As gentle mixing seemed to favour expression of FHA but did not produce as great a yield of cells, cultures were grown with the % O_2 controlled to 20% until the end of exponential growth. The sparged aeration was then stopped and the stirrer speed reduced for the final period of the culture. This produced a further increase in the FHA measured in the supernatant and a slight increase in the LPF measured. However, it is not known whether the expression of FHA was favoured by the more gentle mixing of the culture or by the reduced O_2 saturation of the medium that is a consequence of the reduced aeration and mixing.

As FHA production in B. pertussis cultures was favoured by static incubation in Thompson bottles and by gentle mixing in fermenter cultures, the stability of purified FHA was investigated (chapter 6). It was demonstrated that FHA was stable on static incubation but quickly lost HA activity when incubated with shaking. However, CD had a concentration dependent stabilising effect on FHA incubated with shaking. This suggested that the ability of CD to enhance yields of FHA in shaken cultures could be wholly or partly due to this stabilising effect of CD on FHA. It was observed that the expression of FHA in 500 ml fermenter cultures was enhanced in cultures containing CD without pH control (3.1.4b). The loss of HA activity from solutions of purified FHA was slightly greater at the higher pH that occurred in the cultures without pH control. However, the effect of CD to stabilise purified FHA was found to be pH independent. Therefore it may be that expression of FHA is favoured by the high pH occurring in stationary phase B. pertussis cultures but without CD the FHA produced is more rapidly degraded, giving lower yields. The loss of HA activity was also markedly increased by increasing the area of the air-liquid interface.

The loss of FHA activity from solutions of purified FHA was thought to occur by aggregation as aggregated

strands of protein appeared on the surface of the liquid and no degradation of the protein remaining in solution was seen when examined by SDS-PAGE. Recent light scattering studies have shown that FHA undergoes a reversible temperature dependent association in solution (L. Irons, unpublished work). Thus it seems likely that on shaking at 35°C this process becomes irreversible, leading to the formation of large aggregates and loss of FHA from solution. The stabilising effect of CD on FHA is presumably due to the CD interfering with this aggregation process.

The mechanism by which CD increased the stability of FHA remains unknown. It was proposed that CD may bind to FHA, thereby preventing its aggregation. However, binding of CD to FHA was not conclusively demonstrated, with binding of CD to B. pertussis cells not related to the presence or absence of FHA on the cells. Also, binding between CD and FHA was not demonstrated by blocking of antibody binding or gel filtration but micropartition by ultrafiltration showed weak binding between CD and FHA with concentrated solutions of FHA (1 mg/ml).

The interaction between CD and FHA requires further study as CD clearly has a major effect on the yields of FHA that can be obtained from agitated cultures of B. pertussis. Results presented here suggest that it is unlikely that the stabilising effect is caused solely by binding of CD to FHA as even if more sensitive analytical techniques could detect a very small amount of CD bound to FHA, this amount would be much less than that required in solution to have any effect on FHA in B. pertussis cultures.

The effects of CD on growth and expression of LPF and FHA by B. pertussis have already been seen. However, the mechanisms by which these effects are achieved are poorly understood. Imaizumi et al. (1983b) suggested some possible mechanisms for the great increase in LPF production that they observed. They suggested:

(i) CD absorbs some growth inhibitors, either derived from medium constituents or from secondary metabolites of B. pertussis. The addition of CD was previously shown to overcome the toxic effects of palmitic and oleic acids added to solid medium, allowing normal growth (Imaizumi et al., 1983a). Imaizumi et al. (1983b) recovered CD from culture supernatants and found that some fatty acids could be detected in the CD. However, these were not identified. The removal of growth inhibitors by CD was demonstrated in this study by growth in continuous culture. When CD was added to a steady state culture of B. pertussis in SS medium there was an approximate doubling of the culture density, indicating that a nutrient limitation was not operating but that the culture was under a growth inhibition. This was relieved by the addition of CD and B. pertussis was then able to maximally utilise the available nutrients. From the results obtained in this study it is unlikely that the major effect of CD is to absorb toxic secondary metabolites as CD was seen to reduce the length of the lag phase in batch cultures, presumably by encouraging growth when secondary metabolites would not have accumulated in the medium.

(ii) CD acts as a stabiliser to assist glutathione, according to Imaizumi et al. (1983b) an essential medium component for LPF production. CD could incorporate monomeric reduced glutathione in its cavity (Imaizumi et al., 1983a) and so may enhance uptake of glutathione into B. pertussis cells. This is not confirmed by results presented here as B. pertussis was found to grow and produce LPF without glutathione if CD was present.

(iii) CD acts as a stabiliser of LPF and the actual enhancement was due to protection against degradation of the toxin. It is possible that CD does afford some protection to LPF as this was seen to be the case for FHA in this study. CD is known for its ability to stabilise certain substances. The light sensitivity of the insecticide pyrethrin and vitamin D_3 (cholecalciferol) have been greatly reduced by complexation with

cyclodextrin. Cyclodextrins have been used commercially to stabilise prostaglandin E_1 and can be used to stabilise benzocaine, atropine, aspirin, phenylbutazone and many other drugs. The aromatic substances contained in onions, garlic, horseradish and mustard can also be stabilised in powdered inclusion complexes with cyclodextrin (Saenger, 1980). CD can also alter the physicochemical properties of complexed molecules, particularly to increase the solubility of poorly soluble drugs. With the addition of CD clear, stable aqueous solutions of some steroids and fat soluble vitamins can be prepared (Szejtli, 1983). Thus it is possible that CD may also enhance the stability of LPF and FHA, increasing the final yields obtained.

(iv) CD could act to enhance or stimulate the uptake of components essential for growth. It is possible that nutrients trapped within the CD cavity could be more easily taken up by B. pertussis as CD has been seen to associate with B. pertussis cells (6.2.1). Cyclodextrins are known to catalyse a number of reactions and have thus been studied as enzyme models (Bender & Komiyama, 1978). It could be speculated that the uptake of some nutrients is enhanced by their presentation to the cell membrane as guest molecules in a CD complex with the CD remaining free in the medium.

Another possible mechanism by which CD could increase the final yields of LPF and FHA in B. pertussis cultures is by its action to maintain B. pertussis in the X-mode, even in C-mode conditions. This was observed in this study when B. pertussis retained X-mode characteristics in medium that, in the absence of CD, produced C-mode growth. It may be that the conditions produced by a dense, late exponential or stationary phase culture favour C-mode growth and CD acts to maintain organisms in the X-mode. This would be difficult to determine experimentally as no marker, exclusive to C-mode cells, is known that could be assayed.

Recent genetic studies of LPF and FHA have increased

the understanding of the expression of these antigens. A DNA fragment that contains the genes for the 5 LPF subunits has been cloned and sequenced in two laboratories (Nicosia et al., 1986; Locht & Keith, 1986) with close agreement between their findings. It is thought that the genes are organised as a single operon which is transcribed as a single polycistronic mRNA. Both groups note that the amino terminus of each mature protein subunit is preceded by a peptide of variable length which is typical of bacterial signal peptides. This indicates that the five subunits are translated as precursors and secreted individually into the periplasm. Nicosia et al. (1986) state that the putative promoter sequence for the LPF gene would be inefficient in E. coli and was also likely to be inefficient in causing expression of LPF in B. pertussis. They suggest that this implies that a different regulatory mechanism must work and this role would be fulfilled by the positive regulatory elememt vir, proposed by Weiss & Falkow (1984).

Nicosia & Rappouli (1987) reported further studies on the promoter of the LPF operon which was shown to be weak in E. coli due to a suboptimal spacing of the promoter sequence. When the promoter sequence spacing was optimised for E. coli in a promoterless plasmid then expression of plasmid product was enhanced 1000-fold. They suggest that if the reqirements for a good promoter are the same in B. pertussis as in E. coli then the LPF promoter would also be rather inefficient in B. pertussis, requiring an additional regulatory mechanism, namely the vir gene product. Nicosia & Rappouli (1987) also show that the transcription of the LPF operon in B. pertussis is constant throughout the phases of growth until stationary phase, when LPF mRNA is almost absent. This agrees with the findings of this study and others that LPF in culture supernatants reaches a peak towards the end of the exponential phase of growth and does not increase during stationary phase.

Less work has focussed on the genetic control of the

expression of FHA. However, Brown & Parker (1987) have cloned the FHA gene and studied its expression in E. coli. B. pertussis DNA was cloned into a plasmid containing a kanamycin resistance gene and an E. coli clone was obtained that expressed FHA. However this protein was larger than that expressed in B. pertussis, perhaps due to difference in processing of this protein in the two bacteria. Brown & Parker (1987) found evidence that the kanamycin resistance gene provided the promoter function for FHA expression in E. coli but production was less than that seen in B. pertussis. Also, conjugative transfer of the plasmid containing the FHA and kanamycin resistance genes to a transposon Tn5-induced FHA$^-$ mutant increased the expression of FHA in excess of that seen with wild-type B. pertussis. This suggested that the FHA promoter was on the plasmid but this was not active in E. coli in the absence of the B. pertussis vir gene.

Work describing expression of B. pertussis proteins in other hosts e.g. E. coli begs the question whether this will provide a more suitable system for production of components for an acellular vaccine than the growth of B. pertussis? Work is obviously hindered by the inefficient promoters that precede the B. pertussis genes which require another level of control of expression via the Vir gene product. If this obstacle can be overcome by the construction of a plasmid with a suitable promoter(s) to regulate expression of the B. pertussis gene then expression in another host might become an alternative to growth of B. pertussis. However, it is unlikely that proteins such as FHA and LPF would be secreted into the supernatant as in the majority of cases recombinant proteins are expressed intracellularly and in an insoluble form (Marston, 1986). This would cause problems in the isolation and purification of these proteins as the host cells would have to be disrupted releasing many other intracellular components into the mixture for purification. Also, it may not be possible to obtain direct expression of B. pertussis components because the

protein would be recognised as foreign and degraded. Thus it may be necessary to link the B. pertussis gene with a host gene to produce a fusion protein (Marston, 1986). Fusion proteins may prove to produce protective antibodies against B. pertussis but should they require separation this would add to the difficulty of any production process for a vaccine. Also, for a three component vaccine of the type developed at CAMR either a recombinant organism would have to produce three products or three separate fermentations and purifications would be required.

Therefore it is my view that the most practical direction in development of a process for production of components for acellular vaccines against B. pertussis is to improve the existing fermentation of the organism. Also, to try to derive a B. pertussis clone with improved promoter efficiency for the existing virulence factor genes. It would also be advantageous to induce a modification in the LPF gene so that LPF produced remains immunogenic but has reduced or abolished toxicity.

In summary, CD has been observed to have several marked effects on B. pertussis by Imaizumi et al. (1983a&b; 1984), Perera et al. (1986) and in this study. It overcomes the fastidious nature of B. pertussis allowing reproducible fermenter cultures to be produced which are suitable for use in vaccine manufacture. It is possible that many properties of CD, particularly its ability to form inclusion complexes, are responsible for its different effects on B. pertussis. These effects include reducing the length of the lag phase of cultures, allowing growth in previously unfavourable conditions (e.g. in stainless steel fermenters or without glutathione in the medium), removing toxic inhibitors of growth (demonstrated in continuous culture), increasing the stability of FHA and maintaining growth in the X-mode in otherwise C-mode conditions. Thus it has been demonstrated that CD has a variety of effects on growth, expression and stability of virulence determinants of B. pertussis.

References.

ANDERSON,E.K. (1953). Serological studies on Haemophilus pertussis, H. parapertussis and H. bronchisepticus. Acta Pathologica et Microbiologica Scandinavica 33, 202-224.

AOYAMA, T., MURASE, Y., IWATA, T., IMAIZUMI, A., SUZUKI,Y. & SATO, Y. (1986). Comparison of blood-free medium (cyclodextrin solid medium) with Bordet-Gengou medium for clinical isolation of Bordetella pertussis. Journal of Clinical Microbiology 23, 1046-1048.

ARAI, H. & MUNOZ, J.J. (1979). Purification and crystalisation of fimbrial hemagglutinin from Bordetella pertussis. Infection and Immunity 25, 460-462.

ARAI, H. & MUNOZ, J.J. (1981). Crystallisation of pertussigen from Bordetella pertussis. Infection and Immumity 31, 495-499.

ARAI, H. & SATO, Y. (1976). Separation and characterisation of two distinct haemagglutinins contained in purified leukocytosis-promoting factor from Bordetella pertussis. Biochimica et Biophysica Acta 444, 765-782.

ASHWORTH, L.A.E., IRONS, L.I., & DOWSETT, A.B. (1982a). The antigenic relationship between serotype-specific agglutinogen and fimbriae of Bordetella pertussis. Infection and Immunity 37, 1278-1281.

ASHWORTH, L.A.E., FITZGEORGE, R.B., IRONS, L.I., MORGAN,C.P. & ROBINSON, A. (1982b). Rabbit nasopharyngeal colonisation by Bordetella pertussis: the effects of immunisation on clearance and on serum and nasal antibody levels. Journal of Hygiene, Cambridge 88, 475-486.

ASHWORTH, L.A.E., DOWSETT, A.B., IRONS, L.I. & ROBINSON, A. (1986). The location of surface antigens of Bordetella

pertussis by immuno-electron microscopy. Developments in Biological Standardisation 61, 143-151.

ASKELOF, P., GRANSTROM, M., GILLENIUS, P. & LINDBERG, A.A. (1982). Purification and characterisation of a fimbrial haemagglutinin from Bordetella pertussis for use in an enzyme-linked immunosorbent assay. Journal of Medical Microbiology 15, 73-83.

AYME, G., CAROFF, M., CHABY,R., HAEFFER-CAVAILLON, N., LE DUR, A., MUREAU, M., MUSET, M., MYNARD, M-C., ROUMIANTZEFF, M., SCHULTZ, D. & SZABO, L. (1980). Biological properties of fragments derived from Bordetella pertussis endotoxin: isolation of a non-toxic, Schwartzman-negative lipid A possessing high adjuvant properties. Infection and Immunity 27, 739-745.

BANNATYNE, R.M. & CHEUNG, (1984). Antibiotic resistance of degraded strains of Bordetella pertussis. Antimicrobial Agents and Chemotherapy 25, 537-538.

BASSILI, W.R. & STEWART, G.T. (1976). Epidemiological evaluation of immunisation and other factors in the control of whooping cough. Lancet (i), 471-473.

BELLALOU, J. & RELYVELD, E.H. (1984). Studies on culture conditions of Bordetella pertussis and relationship to immunogenicity of vaccines. Annals de Microbiologie (Institute Pasteur) 135B, 101-110.

BELLMAN, M.H., ROSS, E.M. & MILLER, D.L. (1983). Infantile spasms and pertussis immunisation. Lancet (i), 1031-1034.

BENDER, M.L. & KOMIYAMA, M. (1978). Cyclodextrin Chemistry. Berlin: Springer-Verlag.

BERG, D.E., WEISS, A.A. & CROSSLAND, L. (1980). Polarity of Tn5 insertation mutations in Escherichia coli. Journal

of Bacteriology 142, 439-446.

BLOM, J., HANSEN, G.A. & POULSEN, F.M. (1983). Morphology of cells and haemagglutinins of Bordetella species: Resolution of substructural units in fimbriae of Bordetella pertussis. Infection and Immunity 42, 308-317.

BORDET, J. & GENGOU, O. (1906). Le microbe de la coqueluche. Annals de l'Institute Pasteur 20, 731-741.

BOTSFORD, J.L. (1981). Cyclic nucleotides in procaryotes. Microbiological Reviews 145, 620-642.

BRONNE-SHANDBURY, C.J. & DOLBY, J.M. (1976). The stability of the serotypes of Bordetella pertussis with particular reference to serotype 1,2,3,4. Journal of Hygiene, Cambridge 76, 227-286.

BROOKES, R. & SIKYTA, B. (1967). Influence of pH on the growth characteristics of Neisseria gonorrhoeae in continuous culture. Applied Microbiology 15, 224-227.

BROOME, C.V., PREBLUD, S.R., BRUNER, B., McGOWAN, J.E., HAYES, P.S., HARRIS, P.P., ELSEA, W. & FRASER, D.W. (1981). Epidemiology of pertussis, Atlanta, 1977. Journal of Paediatrics 98, 362-367.

BROWN, D.R. & PARKER, C.D. (1987). Cloning of the filamentous haemagglutinin of Bordetella pertussis and its expression in Escherichia coli. Infection and Immunity 55, 154-161.

BROWN, M.W.R., ANWAR, H. & LAMBERT, P.A. (1984). Evidence that mucoid Pseudomonas aeruginosa in the cystic fibrosis lung grows under iron-restricted conditions. FEMS Microbiology Letters 21, 113-117.

BROWN, M.W.R. & WILLIAMS, P. (1985).The influence of

environment on envelope properties affecting survival of bacteria in infections. Annual Review of Microbiology 39, 527-556.

BROWNLIE, R.M., PARTON, R. & COOTE, J.G. (1985a). The effect of growth conditions on adenylate cyclase activity and virulence related properties of Bordetella pertussis. Journal of General Microbiology 131, 17-25.

BROWNLIE, R.M., COOTE, J.G. & PARTON, R. (1985b). Adenylate cyclase activity during phenotypic variation of Bordetella pertussis. Journal of General Microbiology 131, 27-38.

BULLEN, J.J. (1981). The significance of iron in infection. Reviews of Infectious Diseases 3, 1127-1138.

BURNS, K.A. & FREER, J.H. (1982). Mouse lung adhesion assay for Bordetella pertussis. FEMS Microbiology Letters 13, 271-274.

BWIBO, N.O. (1971). Whooping cough in Uganda. Scandinavian Journal of Infectious Diseases 3, 41-43.

CAMERON, J. (1967). Variation in Bordetella pertussis. Journal of Pathology and Bacteriology 94, 367-374.

CAMERON, J., ROUSSEAU, P. & TREPANIER, G. (1985). Bordetella pertussis: growth in stainless steel. Journal of Biological Standardisation 13, 97-100.

CASU, B., REGGIANI,M. & SANDERSON, G.R. (1979). Methylated cycloamyloses (cyclodextrins) and their inclusion properties. Carbohydrate Research 76, 59-66.

CLARKSON, J.A. & FINE, P.E. (1985). The efficiency of measles and pertussis notification in England and Wales. International Journal of Epidemiology 14, 153-168.

COHEN, S.M. & WHEELER, M.W. (1946). Pertussis vaccine prepared with phase I cultures grown in fluid medium. American Journal of Public Health 36, 371-376.

COLLIER, A.M., PETERSON, L.P. & BASEMAN, J.B. (1977). Pathogenesis of infection with Bordetella pertussis in hamster tracheal organ culture. Journal of Infectious Diseases 136, S196-S203.

COMMITTEE ON SAFETY OF MEDICINES AND THE JOINT COMMITTEE ON VACCINATION AND IMMUNISATION. (1981). In Department of Health and Social Security. Whooping cough: reports from the Committee on Safety of Medicines and the Joint Committee on Vaccination and Immunisation. London, HMSO.

CONFER, D.L. & EATON, J.W. (1982). Phagocyte impotence caused by an invasive bacterial adenylate cyclase. Science 217, 948-950.

COOK, R. (1979). Pertussis in developing countries: problems and possibilities of control through immunisation. In International Symposium on Pertussis, pp. 283-286. Edited by C.R. Manclark and J.C. Hill. Washington D.C.: United States Department of Health, Education and Welfare Publication No. (NIH) 79-1830.

COWELL, J.L., HEWLETT,E.L. & MANCLARK, C.R. (1979). Intracellular localisation of the dermonecrotic toxin of Bordetella pertussis. Infection and Immunity 25, 896-901.

COWELL, J.L., SATO, Y., SATO, H., AN DER LAN, B. & MANCLARK,C.R. (1981). Separation, purification and properties of the filamentous hemagglutinin and leukocytosis promoting factor-hemagglutinin from Bordetella pertussis. Seminars in Infectious Diseases 4, 371-379.

CSIZER, Z., SZAMMET, A., NEIDERMAYER, N., ZSIDAI, J., AUBER, E. & JOO, I. (1977). Estimation of bacterial mass of pertussis vaccine by opacity and dry weight determinations. Journal of Biological Standardisation 5, 289-295.

DAWSON, B., FARNWORTH, E.H. MACLEOD, J.W. & NICHOLSON, D.E. (1951). Observations on the value of the Bordet-Gengou medium for the cultivation of Haemophilus pertussis. Journal of General Microbiology 5, 408-415.

DHSS JOINT COMMITTEE ON VACCINATION AND IMMUNISATION. (1977). Whooping cough vaccination. Review of the evidence of whooping cough vaccination. London: DHSS.

DHSS (1981). Whooping cough. Reports from the Committee on Safety of Medicines and the Joint Committee on Vaccination and Immunisation. (1981). London: HMSO.

DICK, G. (1975). Whooping cough vaccine. British Medical Journal 4, 161.

DOBROGOSZ, W.J., EZZELL, J.W., KLOOS, W.E. & MANCLARK, C.R. (1979). Physiology of Bordetella pertussis. In International Symposium on Pertussis, pp. 86-93. Washington D.C.: United States Department of Health, Education and Welfare. Publication No. (NIH) 79-1830.

DOULL, J.A., SHIBLEY, G.S. & McCLELLAND, J.E. (1936). Active immunisation against whooping cough. Interim report of the Cleveland experience. American Journal of Public Health 26, 1097-1105.

EISENSTEIN, B.I., BEACHEY, E.H. & SOLOMON, S.S. (1981). Divergent effects of cyclic adenosine 3', 5'-monophosphate on formation of type I fimbriae in different K-12 strains of Escherichia coli. Journal of Bacteriology 145, 620-623.

ELDERING, G., HORNBECK, C. & BAKER, J. (1957). Serological study of Bordetella pertussis and related species. Journal of Bacteriology 74, 133-136.

ELDERING, G. & KENDRICK, P.L. (1938). Bacillus parapertussis: a species resembling both Bacillus pertussis and Bacillus bronchisepticus but identical with neither. Journal of Bacteriology 35, 561-572.

ELLWOOD, D.C. & ROBINSON, A. (1981). Bacterial envelope structure and macromolecular composition. In Continuous Culture of Cells, pp.39-68. Edited by P.H. Calcott. Florida: CRC Press.

EVANS, C.G.T., HERBERT, D. & TEMPEST, D.W. (1970). The continuous cultivation of micro-organisms. In Methods in Microbiology, Vol. 2, pp. 278-327. Edited by J.R. Norris and D.W. Ribbons. London: Academic Press.

EZZELL, J.W., DOBROGOSZ, W.J., KLOOS, W.E. & MANCLARK, C.R. (1981a). Phase shift markers in Bordetella: alterations in envelope proteins. Journal of Infectious Diseases 143, 562-569.

EZZELL, J.W., DOBROGOSZ, W.J., KLOOS, W.E. & MANCLARK, C.R. (1981b). Phase shift markers in the genus Bordetella: loss of cytochrome d629 in phase IV varants. Microbios, 31, 171-182.

FIELD, L.H. & PARKER, C.D. (1979). Effects of fatty acids on growth of Bordetella pertussis in defined medium. Journal of Clinical Microbiology 9, 651-653.

FINE, P.E. & CLARKSON, J.A. (1982). The recurrence of whooping cough: possible implications for assessment of vaccine efficacy. Lancet 1, 666-669.

FINE, P.E. & CLARKSON, J.A. (1984). Distribution of

immunity to pertussis in the population of England and Wales. Journal of Hygiene, Cambridge 92, 21-36.

FISH, F., NAVON, Y. & GOLDMAN, S. (1987). Hydrophobic adherence and variation in Bordetella pertussis. Medical Microbiology and Immunology 176, 37-46.

FLOSDORF, E.W., DOZOIS, T.F. & KIMBALL, A.C. (1941). Studies with Haemophilus pertussis V. Agglutinogenic relationships of the phases. Journal of Bacteriology 41, 457-471.

FREDRICKSEN, J.H., FROHOLM, L.O. & PAULSEN,B.S. (1986). Purification of agglutinogen 3 from Bordetella pertussis. Developments in Biological Standardisation 61, 187-196.

GOLDMAN, W.E. & HERWALDT, L.A. (1986). Bordetella pertussis tracheal cytotoxin. Developments in Biological Standardisation 61, 103-111.

GOLDMAN, W.E., KLAPPER, D.G. & BASEMAN, J.B. (1982). Detection, isolation and analysis of a Bordetella pertussis product toxic to cultured tracheal cells. Infection and Immunity 36, 782-794.

GOLDMAN,S., HANSKI, E. & FISH, F. (1984). Spontaneous phase variation in Bordetella pertussis is a multistep non-random process. European Molecular Biology Organisation Journal 3, 1353-1356.

GOLDNER, M., JAKUS, C.M., RHODES, H.K. & WILSON, R.J. (1966). The amino acid utilisation by phase I Bordetella pertussis in a chemically defined medium. Journal of General Microbiology 44, 439-444.

GOODNOW, R.A. (1980). Biology of Bordetella bronchiseptica. Microbiological Reviews 44, 722-738.

GORRINGE, A.R., ASHWORTH, L.A.E., IRONS, L.I. & ROBINSON, A. (1985). Effect of monoclonal antibodies on the adherence of Bordetella pertussis to Vero cells. FEMS Microbiology Letters 26, 5-9.

GRIFFITH, A.H. (1978). Reactions after pertussis vaccine: a manufacturer's experiences and difficulties since 1964. British Medical Journal 1, 809-815.

GRIFFITH, A.H. & FREESTONE, D.S. (1981). Pertussis vaccine. British Medical Journal 282, 2051.

GRIFFITHS, E. (1983). Bacterial adaptation to a low iron environment. In Microbiology-1983 pp.329-333. Washington D.C.: American Society for Microbiology.

GRIFFITHS, E., STEVENSON, P. & JOYCE, P. (1983). Pathogenic Escherichia coli express new outer membrane proteins when growing in vivo. FEMS Microbiology Letters 16, 95-99.

HALL, G.W., DOBROGOSZ, W.J., EZZELL, J.W., KLOOS, W.E. & MANCLARK, C.R. (1982). Repression of adenylate cyclase in the genus Bordetella. Microbios 33, 45-52.

HANSKI, E. & FARFEL, Z. (1985). Bordetella pertussis invasive adenylate cyclase. Partial purification and properties of its cellular penetration. Journal of Biological Chemistry 290, 5526-5532.

HARDER, W. & DIJKHUIZEN, L. (1983). Physiological responses to nutrient limitation. Annual Review of Microbiology 37, 1-23.

HAUSCHILD, A.H.W. & PIVNICK, H. (1961). Continuous culture of Brucella abortus S19. Canadian Journal of Microbiology 7, 491-505.

HECKELS, J.E. (1986). Gonococcal antigenic variation and pathogenesis. In Antigenic Variation in Infectious Diseases, Special Publication of the Society for General Microbiology Vol. 19, pp.77-94. Edited by T.H. Birkbeck and C.W. Penn. Oxford: IRL Press.

HELTING, T.B. & BLACKKOLB, F. (1981). Extracted protective antigen of Bordetella pertussis. I. Preparation and properties of the solubilized surface of components. Acta Pathologica et Microbiologica Scandinavica (B) 89, 93-101.

HEWLETT, E.L., UNDERHILL, L.H., VARGO, S.A., WOLFF, J. & MANCLARK, C.R. (1979). Bordetella pertussis adenylate cyclase: regulation of activity and its loss in degraded strains. In International Symposium on Pertussis pp.81-85. Edited by C.R. Manclark and J.C. Hill. Washinton D.C.: United States Department of Health, Education and Welfare. Publication No. (NIH) 79-1830.

HEWLETT, E.L., WEISS, A.A., CRANE, J.K., PEARSON, R.D., ANDERSON, H.J., MYERS, G.A., EVANS, W.S., HANTSKE, L.L., KAY, H.D. & CRONIN, M.J. (1986). Bordetella extracytoplasmic adenylate cyclase: actions as a bacterial toxin. Developments in Biological Standardisation 61, 21-26.

HEWLETT,E.L. & WOLFF,J. (1976). Soluble adenylate cyclase from the culture medium of Bordetella pertussis: purification and characterisation. Journal of Bacteriology 127, 890-898.

HIRST, T.R. (1986). Mechanism of enterotoxin secretion from Escherichia coli and Vibrio cholerae. In Protein Carbohydrate Interactions in Biological Systems: The Molecular Biology of Microbial Pathogenicity, FEMS Symposium No. 31 pp.415-422. London: Academic Press.

HORNIBROOK, J.W. (1939). Cultivation of phase I

Haemophilus pertussis in a semi-synthetic liquid medium. Public Health Reports, Washington D.C. 54, 1847-1851.

HORNIBROOK, J.W. (1940). Nicotinic acid as a growth factor for Haemophilus pertussis. Proceedings of the Society for Experimental Biology, New York 45, 598-599.

IDIGBE, E.O., PARTON, R. & WARDLAW, (1981). Rapidity of antigenic modulation of Bordetella pertussis in modified Hornibrook medium. Journal of Medical Microbiology 14, 409-418.

IMAIZUMI, A., SUZUKI, Y., ONO, S., SATO, H. & SATO, Y. (1983a). Heptakis (2,6-O-dimethyl)β cyclodextrin: a novel growth stimulant for Bordetella pertussis phase I. Journal of Clinical Microbiology 17, 781-786.

IMAIZUMI, A., SUZUKI, Y., ONO, S., SATO, H. & SATO, Y. (1983 b). Effect of heptakis (2,6-O-dimethyl) beta-cyclodextrin on the production of pertussis toxin produced by Bordetella pertussis. Infection and Immunity 41, 1138-1143.

IMAIZUMI,A., SUZUKI, Y., GINNAGA,Y. SAKUMA, S. & SATO, Y. (1984). A new culturing method for the production of filamentous haemagglutinin of Bordetella pertussis. Journal of Microbiological Methods 2, 339-347.

IRONS, L.I., ASHWORTH, L.A.E. & WILTON-SMITH, P. (1983). Heterogeneity of the filamentous haemagglutinin of Bordetella pertussis studied with monoclonal antibodies. Journal of General Microbiology 129, 2769-2778.

IRONS, L.I. & MACLENNAN, A.P. (1979). Substrate specificity and the purification by affinity combination methods of the two Bordetella pertussis haemagglutinins. In International Symposium on Pertussis, pp. 338-349. Edited by C.R. Manclark and J.C. Hill. Washington D.C.:

United States Department of Health, Education and Welfare. Publication No. (NIH) 79-1830.

JAGICZA, A., BALLA, P. LENDVAY, N., ZSIDAI, J. & ERDEI, S. (1985). Production of active vaccine by large scale continuous fermentation of Bordetella pertussis. Annals of Immunology of Hungary 25, 17-22.

JEBB, W.H.H. & TOMLINSON, A.H. (1955). The nutritional requirements of Haemophilus pertussis Journal of General Microbiology 13, 1-8.

JEBB, W.H.H. & TOMLINSON, A.H. (1957). The minimal amino acid requirements of Haemophilus pertussis. Journal of General Microbiology 17, 59-63.

JONES, G.W. & ISAACSON, R.E. (1983). Proteinaceous bacterial adhesins and their receptors. CRC Critical Reviews in Microbiology 10, 229-260.

KASUGA, T., NAKASE, Y., UKISHIMA, K. & TAKATSU, K. (1953). Studies on Haemophilus pertussis. I. Antigen structure of H. pertussis and its phases. Kitasato Archives of Experimental Medicine 26, 121-134.

KASUGA, T., NAKASE, Y., UKISHIMA, K. & TAKATSU, K. (1954). Studies on Haemophilus pertussis. III. Some properties of each phase of H. pertussis. Kitasato Archives of Experimental Medicine 27, 37-48.

KATADA, T., AMANO, T. & UI, M. (1982). Modulation by islet-activating protein of adenylate cyclase activity in C6 glioma cells. Journal of Biological Chemistry 257, 3739-3746.

KATADA, T., TAMURA, M. & UI, M. (1983). The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalising ADP-ribosylation of a membrane protein.

Archives of Biochemistry and Biophysics 224, 290-298.

KATADA, T. & UI, M. (1982). ADP-ribosylation of the specific membrane protein of C6 cells by the islet-activating protein associated with modification of adenylate cyclase activity. Journal of Biological Chemistry 257, 7210-7216.

KENDRICK, P.L. & ELDERING, G. (1936). Progress report on pertussis immunisation. American Journal of Public Health 26, 8-12.

KENDRICK, P.L., ELDERING, G., DIXON, M.K. & MISNER, J. (1947). Mouse protection tests in the study of pertussis vaccines. American Journal of Public Health 37, 803-810.

KESSIN, R.H. & FRANKE, J. (1986). Secreted adenylate cyclase of Bordetella pertussis: calmodulin requirements and partial purification of two forms. Journal of Bacteriology 166, 290-296.

KILHOFFER, M-C., HOPE COOK, G. & WOLFF, J. (1983). Calcium-independent activation of adenylate cyclase by calmodulin. European Journal of Biochemistry 133, 11-15.

KIMURA, M. & HIKINO, N. (1986). Results with a new DTP in Japan. Developments in Biological Standardisation 61, 545-561.

KLOOS, W.E., MOHAPATRA, N., DOBROGOSZ, W.J., EZZELL, J.W. & MANCLARK, C.R. (1981). Deoxyribonucleotide sequence relationships among Bordetella species. International Journal of Systematic Bacteriology 31, 173-176.

KONDO, H. NAKATANI, H. & HIROMI, K. (1976). Analysis of mixtures of α and β cyclodextrins using fluorescent dyes. Carbohydrate Research 52, 1-10.

LACEY, B.W. (1960). Antigenic modulation of Bordetella pertussis. Journal of Hygiene, Cambridge 58, 57-93.

LANE, A.G. (1968). Detoxification of liquid cultures of Bordetella pertussis by forced aeration at high pH. Applied Microbiology 16, 1211-1214.

LAPIN, J.H. (1943). Whooping cough. Springfield, Illinois: Charles C. Thomas.

LAWSON, G. McL. (1939). Immunity studies in pertussis. American Journal of Hygiene 29 (Section B), 119-131.

LE DUR, A., CHABY, R. & SZABO, L. (1980). Isolation of two protein-free and chemically different lipopolysaccharides from Bordetella pertussis phenol-extracted endotoxin. Journal of Bacteriology 143, 78-88.

LEE, S.W., WAY, A.W. & OSEN, E.G. (1986). Purification and subunit heterogeneity of pili of Bordetella bronchiseptica. Infection and Imm unity 51, 586-593.

LESLIE, P.H. & GARDNER, A.D. (1931). The phases of Haemophilus pertussis. Journal of Hygiene, Cambridge 31, 423-434.

LIN, S-Y. & YANG, J-C. (1987). The effect of cyclodextrin inclusion complexation on the in vitro protein binding and in vivo prothrombin time of warfarin. Drug Development and Industrial Pharmacy 13, 329-343.

LIVEY, I., PARTON, R. & WARDLAW, A.C. (1978). Loss of heat-labile toxin from Bordetella pertussis grown in modified Hornibrook medium. FEMS Microbiology Letters 3, 203-205.

LIVEY, I. & WARDLAW, A.C. (1984). Production and properties of Bordetella pertussis heat-labile toxin.

Journal of Medical Microbiology 17, 91-103.

LOCHT, C. & KEITH, J.M. (1986). Pertussis toxin gene: Nucleotide sequence and genetic organisation. Science 232, 1258-1264.

LOEB, M.R. & SMITH, D.H. (1982). Human antibody response to individual outer membrane proteins of Haemophilus influenzae type B. Infection and Immunity 37, 1032-1036.

MACDONALD, H. & MACDONALD, E.J. (1933). Experimental pertussis. Journal of Infectious Diseases 53, 328-330.

MADSEN, T. (1933). Vaccination against whooping cough. Journal of the American Medical Association 101, 187-188.

MALLORY, F.B. & HORNOR, A.A. (1912). Pertussis: the histological lesion in the respiratory tract. Journal of Medical Research 27, 115-123.

MANCHEE, R.J., EVANS, C.G.T. & ROBINSON, A. (1980). Strain variation in continuous culture. In The Stability of Industrial Organisms pp.53-57. Edited by B.E. Kirsop. Kew: Commonwealth Mycological Institute.

MANCLARK, C.R. & COWELL, J.L. (1984). Pertussis. In Bacterial Vaccines pp. 96-106. Edited by R. Germanier. New York: Academic Press.

MARSTON, A.O. (1986). The purification of eukaryotic polypeptides synthesised in Escherichia coli. Biochemical Journal 240, 1-12.

MARTINEZ-CADENA, M.G., GUZMAN-VERDUZCO, L.M., STIEGLITZ, H. & KUPERSZTOCH-PORTNOY, Y.M. (1981). Catabolite repression of Escherichia coli heat-stable enterotoxin activity. Journal of Bacteriology 145, 722-728.

MATSUYAMA, T. (1977). Resistance of Bordetella pertussis phase I to mucociliary clearance by rabbit tracheal mucus membrane. Journal of Infectious Diseases 136, 609-616.

MAZLOUM, H.A. & ROWLEY, D. (1955). growth requirements of Bordetella pertussis on solid media. Journal of Pathology and Bacteriology 70, 439-446.

McPHEAT, W.L., WARDLAW, A.C. & NOVOTNY, P. (1983). Modulation of Bordetella pertussis by nicotinic acid. Infection and Immunity 41, 516-522.

MEDICAL RESEARCH COUNCIL (1951). The prevention of whooping cough by vaccination. British Medical Journal 1, 1463-1471.

MEDICAL RESEARCH COUNCIL (1956). Vaccination against whooping cough: relation between protection in children and results of laboratory tests. British Medical Journal 2, 454-462.

MILES, A.A. & MISRA, S.S. (1938). The estimation of the bactericidal power of blood. Journal of Hygiene, Cambridge 38, 732-748.

MILLER, C.L. & FLETCHER, W.B. (1976). Severity of whooping cough. British Medical Journal 1, 117-119.

MILLER, D.L., ALDERSLADE, R. & ROSS, E.M. (1982). Whooping cough and whooping cough vaccine: The risks and benefits debate. Epidemiological Reviews 5, 1-24.

MONOD, J. (1950). La technique de culture continue: theorie et applications. Annals de l'Institute Pasteur, Paris 79, 390-410.

MORSE, S.I. & BRAY, K.K. (1969). The occurrence and properties of leukocytosis and lymphocytosis stimulating

material in the supernatant fluids of Bordetella pertussis cultures. Journal of Experimental Medicine 129, 523-550.

MORSE, S.I. & MORSE, J.H. (1976). Isolation and properties of the leukocytosis and lymphocytosis promoting factor of Bordetella pertussis. Journal of Experimental Medicine 143, 1483-1501.

MULLER, A.S & LEEUWENBURG, J. (1985). Pertussis - epidemiology and control. World Health Organisation Expanded Programme on Immunisation Report.

MULLER, A.S., LEEUWENBURG, J. & VOORHOEVE, A.M. (1984). Pertussis in a rural area of Kenya. Epidemiology and results of a vaccine trial. Bulletin of the World Health Organisation 62, 899-908.

MUNOZ, J.J. (1985). Biological activity of pertussigen (pertussis toxin). In Pertussis Toxin pp.1-18. Edited by R. Sekura. London: Academic Press.

MUNOZ, J.J., ARAI, H. & COLE, R.L. (1981a). Mouse-protecting and histamine sensitising activities of pertussigen and fimbrial haemagglutinin from Bordetella pertussis. Infection and Immunity 32, 243-250.

MUNOZ, J.J., ARAI, H., BERGMAN, R.K. & SADOWSKI, P.L. (1981b). Biological activities of crystalline pertussigen from Bordetella pertussis. Infection and Immunity 33, 820-826.

MUNOZ, J.J & BERGMAN,R.K. (1977). Bordetella pertussis. Immunological and other biological activities. New York: Marcel Dekker Inc..

MUSE, K.E. COLLIER, A.M. & BASEMAN, J.B. (1977). Scanning electron microscope study of hamster tracheal organ cultures infected with Bordetella pertussis. Journal of

Infectious Diseases 136, 768-777.

MUSSER, J.M., HEWLETT, E.L., PEPPLER, M.S. & SELANDER, R.K. (1986). Genetic diversity and relationships in populations of Bordetella species. Journal of Bacteriology 166, 230-237.

NAKASE, Y. & ENDOH, M. (1986). Bordetella heat-labile toxin: further purification, characterisation and mode of action. Developments in Biological Standardisation 61, 93-102.

NAKASE, Y., TAKATSU, K., TATEISHI, M., SEKIYA, K. & KASUGA, T. (1969). Heat-labile toxin of Bordetella pertussis purified by preparative acrylamide gel electrophoresis. Japanese Journal of Microbiology 13, 359-366.

NICOSIA, A., PERUGINI, M., FRANZINI, C., CASAGLI, M.C., BORRI, M.G., ANTONI, G., ALMONI, M., NERI, P., RATTI, G. & RAPPUOLI, R. (1986). Cloning and sequencing of the pertussis toxin genes: Operon structure and gene duplication. Proceedings of the National Academy of Sciences of the United States of America 83, 4631-4635.

NICOSIA, A. & RAPPUOLI, R. (1987). Promoter of the pertussis toxin operon and production of pertussis toxin. Journal of Bacteriology, 169, 2843-2846.

NOBLE, G.R., BERNIER, R.H., ESBER, E.C., HARDEGREE, M.C., HINMAN, A.R., KLEIN, D. & SAAH, A.J. (1987). Acellular and whole-cell pertussis vaccines in Japan. Journal of the American Medical Association 257, 1351-1356.

NOGIMORI, K., ITO, K., TAMURA, M., SATOH, S., ISHII, S. & UI, M. (1984). Chemical modification of islets-activating protein, pertussis toxin. Essential role of free amino groups in its lympocytosis-promoting

activity. Biochimica et Biophysica Acta 801, 220-231.

NOGIMORI, K., TAMURA, M., NAKAMURA, T., YAJIMA, M., ITO, K. & UI, M. (1986a). Dual mechanisms involved in the diverse biological activities of islets-activating protein (pertussis toxin), as revealed by chemical modification of the toxin molecule. Developments in Biological Standardisation 61, 51-61.

NOGIMORI, K., TAMURA, M., YAJIMA, M., HASHIMURA, N., ISHII, S. & UI, M. (1986b). Structure function relationships of islets-activating protein, pertussis toxin: biological activities of hybrid toxins reconstsiuted from native and methylated subunits. Biochemistry 25, 1355-1363.

NOVICK,A. & SZILARD, L. (1950). Experiments with the chemostat on spontaneous mutation of bacteria. Proceedings of the National Academy of Sciences of the United States of America 36, 708-719.

NOVOTNY, P. & BROOKES, J.E. (1975). The use of Bordetella pertussis preserved in liquid nitrogen as a challenge suspension in the Kendrick mouse protection test. Journal of Biological Standardisation 3, 11-29.

NOVOTNY, P., CHUBB, A.P., COWNLEY, K., MONTARAZ,J.A. & BEESLEY, J.E. (1986). Bordetella adenylate cyclase: a genus specific protective antigen and virulence factor. Developments in Biological Standardisation 61, 27-41.

NOVOTNY, P. & COWNLEY, K. (1979). Effect of growth conditions on the composition and stability of the outer membrane of Bordetella pertussis. In International Symposium on Pertussis pp.99-123. Edited by C.R.Manclark and J.C. Hill. Washington D.C.: United States Department of Health, education and Welfare. Pubication No. (NIH) 79-1830.

OLIN, P. (1986). Clinical results obtained to date in the Swedish trials. In Workshop on Acellular Pertussis Vaccines, September 22-24 1986 pp.106-110. Transcript by the United States Department of Health and Human Services.

OLSON. L.C. (1975). Pertussis. Medicine 54, 427-468.

OPREMCAK, L.B. & RHEINS, M.S. (1983). Scanning electron microscope study of mouse ciliated oviduct and tracheal epithelium infected in vitro with Bordetella pertussis. Canadian Journal of Microbiology 29, 415-420.

PARISH, H.J. (1965). A History of Immunisation. Edinburgh and London: E. and S. Livingstone Ltd..

PARKER, C.D. (1976). The role of the genetics and physiology of Bordetella pertussis in the study of host-parasite relationships in pertussis. Advances in Applied Microbiology 20, 27-42.

PARKER, C.D. (1979). The genetics and physiology of Bordetella pertussis. In International Symposium on Pertussis pp. 65-69. Edited by C.R. Manclark and J.C. Hill. Washington D.C.: United States Department of Health Education and Welfare. Publication No. (NIH) 79-1830.

PARKER, C.D., BRANES, L.V., ARMSTRONG, S.K., FRANK, D.W. & COLE, A. (1986). Cell surface antigens of Bordetella pertussis. Developments in Biological Standardisation 61, 123-136.

PARTON, R. (1986). The effect of anti-inflammatory agents on the haemorrhagic response of mouse skin to Bordetella pertussis heat-labile toxin. Journal of Medical Microbiology 21, 265-270.

PARTON, R. & DURHAM, J.P. (1978). Loss of adenylate

cyclase activity in variants of Bordetella pertussis. FEMS Microbiology Letters 4, 287-289.

PARTON, R. & WARDLAW, A.C. (1975). Cell-envelope proteins of Bordetella pertussis. Journal of Medical Microbiology 8, 47-57.

PEPPLER, M.S. (1982). Isolation and characterisation of isogenic pairs of domed hemolytic and flat non-hemolytic colony types of Bordetella pertussis. Infection and Immunity 35, 840-851.

PEPPLER, M.S. (1984). Two physically and serologically distinct lipopolysaccharide profiles in strains of Bordetella pertussis and their phenotype variants. Infection and Immunity 43, 224-232.

PEPPLER, M.S. & SCHRUMPF, M.E. (1984). Isolation and characterisation of Bordetella pertussis phenotype variants capable of growing on nutrient agar: comparison of phases III and IV. Infection and Immunity 43, 217-223.

PERERA, V.Y. & FREER, J.H. (1987). Accumulation of a precursor to Subunit S1 of pertussis toxin in cell envelopes of Bordetella pertussis in response to the membrane perturbant phenethyl alcohol. Journal of Medical Microbiology 23, 269-274.

PERERA, V.Y., WARDLAW, A.C. & FREER, J.H. (1986). Pertussis toxin : its distribution and form in growing cultures. In Bacterial Protein Toxins. Proceedings of the Second European Conference on Bacterial Protein Toxins, Wepion pp.383-388. Edited by P. Falmagne. Berlin: Fischer Verlag.

PIRT, S.J., THACKERAY, E.J. & HARRIS-SMITH, R. (1961). The influence of environment on antigen production by Pasteurella pestis studied by means of the continuous flow

culture technique. Journal of General Microbiology 25, 119-130.

PITTMAN, M. (1974). Genus Bordetella Moreno-Lopez. In Bergey's Manual of Determinative Bacteriology, 8th edition pp 282-283. Edited by R.E. Buchanan and N.E. Gibbons. Baltimore: Williams and Williams.

PITTMAN, M. (1979). Pertussis toxin: the cause of the harmful effects and prolonged immunity of whooping cough. A hypothesis. Reviews of Infectious Diseases 1, 401-412.

POLLOCK, M.R. (1947). The growth of Haemophilus pertussis on media without blood. British Journal of Experimental Pathology 28, 295-307.

POLLOCK, T.M., MILLER, E. & LOBB, J. (1984). Severity of whooping cough in England before and after the decline of pertussis immunisation. Archives of Disease in Childhood 59, 162-165.

POLLOCK, T.M. & MORRIS, J.A. (1983). A seven year survey of disorders attributed to vaccination in North West Thames region. Lancet 1, 753-757.

PRESTON, N.W. (1986). Essential immunogens in human pertussis: the role of fimbriae. Developments in Biological Standardisation 61, 137-141.

PRESTON, N.W. & STANDBRIDGE, T.N. (1972). Efficacy of pertussis vaccines: a brighter horizon. British Medical Journal 3, 448-451.

PRESTON, N.W., TIMEWELL, R.M. & CARTER, E.J. (1980). Experimental pertussis infection in the rabbit: similarities with infection in primates. Journal of Infection 2, 227-235.

PROOM, H. (1955). The minimal nutritional requirements of organisms of the genus Bordetella Lopez. Journal of General Microbiology 12, 63-75.

PUBLIC HEALTH LABORATORY SERVICE (1982). Report from the PHLS Epidemiological Research Laboratory and 21 Area Health Authorities. Efficacy of pertussis vaccination in England. British Medical Journal 285, 357-359.

PUSZTAI, Z. & JOO, I. (1967). Influence of nicotinic acid on the antigenic structuire of Bordetella pertussis. Annals of Immunology of Hungary 10, 63-67.

REDHEAD, K. (1983). Variability in the surface exposure of the outer membrane proteins of Bordetella pertussis. FEMS Microbiology Letters 17, 35-39.

REDHEAD, K. (1985). An assay of Bordetella pertussis adhesion to tissue-culture cells. Journal of Medical Microbiology 19, 99-108.

REDHEAD, K., HILL, T. & CHART, H. (1987). Interaction of lactoferrin and transferrins with the outer membrane of Bordetella pertussis. Journal of General Microbiology 133, 891-898.

ROBINSON, A., HAWKINS, D.C. & IRONS, L.I. (1981). The preparation of distinct protective antigens from Bordetella pertussis. FEMS Microbiology Letters 10, 241-244.

ROBINSON, A., ASHWORTH, L.A.E., GORRINGE, A.R. & IRONS, L.I. (1985a). Progress in the development of acellular pertussis vaccines. In Bacterial Protein Toxins. Proceedings of Second Symposium on Bacterial Toxins, Wepion, Belgium pp.389-396. Edited by P. Falmagne. Berlin: Fischer Verlag.

ROBINSON, A., IRONS, L.I. & ASHWORTH, L.A.E. (1985b) Pertussis vaccine: present status and future prospects. Vaccine 3, 11-22.

ROBINSON, A., ASHWORTH, L.A.E., BASKERVILLE, A. & IRONS, L.I. (1986a). Protection against intranasal infection of mice with Bordetella pertussis. Developments in Biological Standardisation 61, 165-172.

ROBINSON, A., DUGGLEBY, C.J., GORRINGE, A.R. & LIVEY, I. (1986b). Antigenic variation in Bordetella pertussis. In Antigenic Variation in Infectious Diseases, Special Publication of the Society for General Microbiology Vol. 19 pp.147-161. Edited by T.H. Birkbeck and C.W. Penn. Oxford: IRL Press.

ROBINSON, A. & HAWKINS, D.C. (1983). Structure and biological properties of solubilised envelope proteins of Bordetella pertussis. Infection and Immunity 39, 590-598.

ROBINSON, A. & IRONS, L.I. (1983). Synergistic effect of Bordetella pertussis lympocytosis promoting factor on protective activities of isolated Bordetella antigens in mice. Infection and Immunity 40, 523-528.

ROBINSON, A. & MANCHEE, R.J. (1979). Solubilisation of the protective antigens of Bordetella pertussis. FEMS Microbiology Letters 5, 131-134.

ROSENBERG, M. (1984). Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiology Letters 22, 289-295.

ROSENBERG, M., GUTNICK, D. & ROSENBERG, E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiology Letters 9, 29-33.

ROSS, R.F. & MUNOZ, J. (1971). Antigens of Bordetella pertussis. V. Separation of agglutinogen 1 and mouse-protective antigen. Infection and Immunity 3, 243-248.

ROWATT, E. (1955). Amino acid metabolism in the genus Bordetella. Journal of General Microbiology 13, 552-560.

ROWATT, E. (1957a). Some factors affecting the growth of Bordetella pertussis. Journal of General Microbiology 17, 279-296.

ROWATT, E. (1957b). The growth of Bordetella pertussis, a review. Journal of General Microbiology 17, 297-326.

RUTTER, D.A., ASHWORTH, L.A.E., DAY, A., FUNNELL, S., LOVELL, F. & ROBINSON, A. (1988). Trial of a new acellular pertussis vaccine in healthy adult volunteers. Vaccine 6, 29-32.

SAENGER, W. (1980). Cyclodextrin inclusion compounds in research and industry. Angewandte Chemie International Edition in English 19, 344-362.

SATO, Y., ARAI,H. & SUZUKI, K. (1974). Leucocytosis promoting factor of Bordetella pertussis. III. Its identity with protective antigen. Infection and Immunity 9, 801-810.

SATO, Y., IZUMIYA, K., SATO, H., COWELL, J.L. & MANCLARK, C.R. (1981). Role of antibody to leucocytosis-promoting factor haemagglutinin in immunity to pertussis. Infection and Immunity 31, 1223-1231.

SATO, Y., COWELL, J.L., SATO, H., BURSTYN, D.G. & MANCLARK, C.R. (1983). Separation and purification of haemagglutinins from Bordetella pertussis. Infection and

Immunity 41, 313-320.

SATO, Y., KIMURA, M. & FUKUMI, H. (1984). Development of a pertussis component vaccine in Japan. Lancet 1, 122-126.

SCHNEIDER, D.R. & PARKER, C.D. (1982). Effect of pyridines on phenotypic properties of Bordetella pertussis. Infection and Immunity 38, 548-553.

SEKIYA, K. (1983). Effects of Bordetella pertussis components on IgE and IgG1 responses. Microbiology and Immunology 27, 905-915.

SEKIYA, K., MUNOZ, J.J. & NAKASE, Y. (1982). Effect of dermonecrotic toxin of Bordetella pertussis on the spleen of CFW and C57BL/10SCN mice. Microbiology and Immunology 26, 971-977.

SEKURA, R.D., FISH, F., MANCLARK. C.R., MEADE, B. & ZHANG, Y. (1983). Pertussis toxin: Affinity purification of a new ADP-ribosyltransferase. Journal of Biological Chemistry 258, 14648-14651.

SHATTUCK, R.L. & STORM, D.R. (1985). Calmodulin inhibits entry of Bordetella pertussis adenylate cyclase into animal cells. Biochemistry 24, 6323-6328.

SILVERTHORNE, N. & CAMERON,C. (1942). Whooping cough. IV. Beef liver extract blood agar media for the growth of Bordetella pertussis. Journal of Paediatrics 20, 13-17.

SLUNGAARD, A., CONFER, D.L., JACOB, H.S. & EATON, J.W. (1983). Antineoplastic effects of a bacterial adenylate cyclase. Clinical Research 31, 547A.

SMITH, H. (1977). Microbial surfaces in relation to pathogenicity. Bacteriological Reviews 41, 475-500.

SMYTH, C.J. (1986). Fimbrial variation in Escherichia coli. In Antigenic Variation in Infectious Diseases, Special Publication of the Society for General Microbiology Vol. 19 pp. 95-125. Edited by T.H. Birkbeck and C.W. Penn. Oxford: IRL Press.

SOPHIANOPOULOS, J.A., DURHAM, S.J., SOPHIANOPOULOS, A.J., RAGSDALE, H.L. & CROPPER, W.P. (1978). Ultrafiltration is theoretically equivalent to equilibrium dialysis but much simpler to carry out. Archives of Biochemistry and Biophysics 187, 132-137.

STAINER, D.W. & SCHOLTE, M.J. (1971). A simple chemically defined medium for the production of phase I Bordetella pertussis. Journal of General Microbiology 63, 211-220.

STANBRIDGE, T.M. & PRESTON, N.W. (1974a). Experimental pertussis infection in the marmoset: type specificity of active immunity. Journal of Hygiene, Cambridge 72, 213-228.

STANBRIDGE, T.N. & PRESTON, N.W. (1974b). Variation of serotype in strains of Bordetella pertussis. Journal of Hygiene, Cambridge 73, 305-310.

STANDFAST, A.F.B. (1951). The phase I of Haemophilus pertussis. Journal of General Microbiology 5, 531-545.

STEWART. G.T. (1977). Vaccination against whooping cough. Efficacy versus risks. Lancet 1, 234-237.

STEWART, G.T. (1979). Toxicity of pertussis vaccine: frequency and probability of reactions. Journal of Epidemiology and Community Health 33, 150-156.

SUTHERLAND, I.W. & WILKINSON, J.F. (1961). A new growth medium for virulent Bordetella pertussis. Journal of Pathology and Bacteriology 82, 431-438.

SZEJTLI, J. (1983). Dimethyl-β-cyclodextrin as a parenteral drug carrier. Journal of Inclusion Phenomena 1, 135-150.

TAMURA, M., NOGIMORI, K., YAJIMA, M., ITO, K., KATADA, T., UI, M. & ISHII, S. (1982). Subunit structure of islet-actvating protein, pertussis toxin, in conformity with the A-B model. Biochemistry 21, 5516-5522.

TAMURA, M., NOGIMORI, K., YAJIMA, M., ASE,K. & UI, M. (1983). A role of the B-oligomer moiety of the islet activating protein, pertussis toxin, in the development of the biological effects on intact cells. Journal of Biological Chemistry 258, 6756-6761.

TEMPEST, D.W. (1970). The continuous cultivation of micro-organisms. In Methods in Microbiology Volume 3 pp. 259-276. London: Academic Press.

TUOMANEN, E.I. & HENDLEY, J.O. (1983). Adherence of Bordetella pertussis to human respiratory cells. Journal of Infectious Diseases 148, 125-130.

TUOMANEN, E.I., NEDELMAN, J., HENDLEY, J.O. & HEWLETT, E.L. (1983). Species specificity of Bordetella adherence to ciliated respiratory cells. Infection and Immunity 42, 696-695.

TUOMANEN, E., WEISS, A., RICH, R., ZAK,F. & Zak, O. (1986). Filamentous haemagglutinin and pertussis toxin promote adherence of Bordetella pertussis to cilia. Developments in Biological Standardisation 61, 197-204.

TURNOWSKY, F., BROWN, M.W.R., ANWAR, H. & LAMBERT, P.A. (1983). Effect of iron limitation and growth rate on the binding of penicillin G to the penicillin binding proteins of mucoid and non-mucoid strains of Pseudomonas aeruginosa. FEMS Microbiology Letters 17, 243-245.

aeruginosa. FEMS Microbiology Letters 17, 243-245.

UI, M., NOGIMORI, K. & TAMURA, M. (1985). Islets-activating protein, pertussis toxin: subunit structure and mechanism for its multiple biological actions. In Pertussis Toxin pp. 19-44. Edited by R.D. Sekura J. Moss and M. Vaughan. New York: Academic Press.

UNGAR, J. JAMES, A.M., MUGGLETON, P.W., PEGLER, H.F. & TOMICH, E.G. (1950). The cultivation of Haemophilus pertussis in partially defined medium. Journal of General Microbiology 4, 345-359.

URISU, A., COWELL, J.L. & MANCLARK, C.R. (1986). Filamentous haemagglutinin has a major role in mediating adherence of Bordetella pertussis to human Widr cells. Infection and Immunity 52, 695-701.

VAJDIC, A.H., GOLDNER, M. & WILSON, R.J. (1966). The amino acid use in cultures of phase I Bordetella pertussis during growth in chemically defined media. Journal of General Microbiology 44, 445-449.

VAN HEMERT, P. (1974). Vaccine production as a unit process. In Progress in Industrial Microbiology, Volume 13 pp. 151-272. Edited by D.J.D. Hockenhull. London: Churchill Livingstone.

VERWAY, W.F., THIELE,T.H., SAGE, D.N. & SCHUCHHARDT, L.F. (1949). A simplified liquid culture medium for the growth of Haemophilus pertussis. Journal of Bacteriology 58, 127-134.

WALKER, E., PINKERTON, I.W., LOVE, W.C., CHAUDHURI, A.K.R. & DATTA, J.B. (1981). Whooping cough in Glasgow 1969-1980. The Journal of Infection 3, 150-158.

WARDLAW, A.C. & PARTON, R. (1979). Changes in envelope

proteins and correlation with biological activities in Bordetella pertussis. In International Symposium on Pertussis pp.94-98. Edited by C.R. Manclark and J.C. Hill. Washinton D.C.: United States Department of Health, Education and Welfare. Publication No. (NIH) 79-1830.

WARDLAW, A.C. & PARTON, R. (1983a). Bordetella pertussis toxins. Pharmacology and Therapeutics 19, 1-53.

WARDLAW, A.C. & PARTON, R. (1983b). Pertussis vaccine. In Medical Microbiology, Volume 2 pp. 207-253. Edited by C.S.F. Easmon ad J. Jeljaszewicz. London and New York: Academic Press.

WARDLAW, A.C., PARTON, R. & HOOKER, M.J. (1976). Loss of protective antigen, histamine-sensitising factor and envelope polypeptides in cultural variants of Bordetella pertussis. Journal of Medical Microbiology 9, 89-100.

WEIHL, C., RILEY, H.D. & LAPIN, J.H. (1963). Extracted pertussis antigen. A clinical appraisal. American Journal of Diseases of Childhood 106, 210-215.

WEISS, A.A. & FALKOW, S. (1984). Genetic analysis of phase change in Bordetella pertussis. Infection and Immunity 43, 263-269.

WEISS, A.A., HEWLETT, E.L., MYERS, G.A. & FALKOW, S. (1983). Tn5-induced mutations affecting virulence factors of Bordetella pertussis. Infection and Immunity 42, 33-41.

WEISS, A.A., HEWLETT, E.L., MYERS, G.A. & FALKOW, S. (1984). Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of Bordetella pertussis. Journal of Infectious Diseases 150, 219-222.

WILLIAMS, A.W. (1914). The etiology of pertussis. Archives of Paediatrics 31, 567-572.

WILSON, R.J. (1963). Cultivation of Bordetella pertussis phase I in a chemically defined medium. Canadian Journal of Public Health 54, 518-523.

WOLFF, J., COOK, G.H., GOLDHAMMER, A.R. & BERKOWITZ, S.A. (1980). Calmodulin activates prokaryotic adenylate cyclase. Proceedings of the National Academy of Sciences of the United States of America 77, 3841-3844.

WOOD, G.C. & COOPER, P.F. (1970). The application of gel filtration to the study of protein-binding of small molecules. Chromatography Reviews 12, 88-107.

WORLD HEALTH ORGANISATION (1985). Developments in pertussis vaccines: Memorandum from a WHO meeting. Bulletin of the World Health Organisation 63, 241-248.

ZABRISKIE, D.W. & ARCURI, E.J. (1986). Factors influencing productivity of fermentations employing recombinant micro-organisms. Enzyme and Microbial Technology 8, 706-717.

ZAK, K., DIAZ, J-L., JACKSON, D. & HECKELS, J.E. (1984). Antigenic variation during infection with Neisseria gonorrhoeae: detection of antibodies to surface proteins in sera of patients with gonorrhoeae. Journal of Infectious Diseases 149, 166-174.

ZHANG, J.M., COWELL, J.L., STEVEN, A.C. & MANCLARK, A.C. (1986). Purification of srotype 2 fimbriae of Bordetella pertussis and their identification as a mouse protective antigen. Developments in Biological Standardisation 61, 173-185.