The Effect of Haem Limitation and Iron Restriction on Outer Membrane Proteins and on Respiratory Systems of Non Typable *Haemophilus influenzae*.

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Doctor of Philosophy.

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Summary The effects of haem limitation and iron restriction on cells of non typable *Haemophilus influenzae* were investigated. Haem limitation was achieved by adding concentrations of haem to growth media which resulted in substantial decreases in final cell yields. Iron restriction was achieved by substituting protoporphyrin IX (PPIX) for haem in the growth medium and adding an iron chelator to the system. The effect of these nutrient limitations on a) outer membrane composition, and b) respiratory systems of non typable *H.influenzae* was investigated. Several of the strains examined produced new PPIX-specific outer membrane proteins when cultured utilising PPIX as a porphyrin source. The immune response of patients with bronchiectasis to outer membrane antigens of *H.influenzae* cultured under iron-restricted conditions was analysed by ELISA and immunoblotting techniques. ELISA analysis revealed that individuals with severe bronchiectasis had high titres of antibodies directed against *H.influenzae* OMs in both serum and sputum. Immunoblotting with homologous serum showed that where PPIX-specific OMPs were produced they were antigenic and were recognised by patients serum. This suggested that these *H.influenzae* OMPs may be expressed *in vivo*. Additionally, the development of the immune response to non typable *H.influenzae* outer membrane antigens was investigated using a rat lung model. Bacteria encased in agar beads were inoculated intratracheally into rat lungs, infection was established, and the immune response monitored for 6 weeks. The animals developed antibodies to PPIX-specific OMPs during the course of infection, providing further evidence that *H.influenzae* express these novel OMP antigens when growing *in vivo*. Studies *in vitro* on respiratory systems of phenotypically altered *H.influenzae* showed that bacteria grown utilising PPIX as a porphyrin source, or under conditions of iron-restriction produced ten fold fewer cytochromes than cells grown in nutrient excess, while haem limited *H.influenzae* produced no detectable cytochromes. Respiration of various substrates was depressed in haem limited and in PPIX-grown cultures as compared with cells grown in nutrient excess.

Key Words: *Haemophilus influenzae* pulmonary infection; Outer membrane proteins; Haemin; Protoporphyrin IX; Phenotypic change.

TO MY

FATHER AND MOTHER

The heavens declare the Glory of God,
The earth displays His handiwork
Day after day they pour forth speech
Night after night they display knowledge
There is no speech or language
Where their voice is not heard.

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ABBREVIATIONS.

Abbreviations used throughout this thesis are listed below.

BHI	=	Brain Heart Infusion
ELISA	=	Enzyme-Linked Immunosorbent Assay
LPS	=	Lipopolysaccharide
OM	=	Outer Membrane
OMP	=	Outer Membrane Protein
PBS	=	Phosphate buffered saline
PPIX	=	Protoporphyrin IX
SDS-PAGE	=	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Tris	=	Tris (hydroxymethyl) aminoethane

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1. INTRODUCTION.

1. INTRODUCTION

1.1 Historical Background.

Taxonomically placed in the family *Pasteurellaceae*, the genus *Haemophilus* is closely related to *Pasteurella* and *Actinobacillus*. Haemophili are typically short Gram-negative rods or coccobacilli 0.5-0.8 x 1-2µ in size. They are nutritionally fastidious, having an absolute requirement for an exogenous source of haemin or certain other porphyrins (X factor) and / or NAD or some other definable coenzyme -like substance (V factor) (Kilian and Biberstein, 1984).

The first recorded observation of bacteria belonging to this genus occurred in 1883 when Robert Koch described small rod shaped bacteria present in purulent exudate from the eye of a patient suffering from conjunctivitis. Nine years later in 1892, Pfeiffer - while investigating the cause of an influenza epidemic - noted that a small rod shaped bacterium, fastidious in its growth requirements (only culturable *in vitro* if blood was added to the medium) was present in large numbers in purulent sputum samples from every case of influenzae examined. On the basis of this evidence Pfeiffer implicated this organism as the cause of influenza.

The specific name *Haemophilus influenzae* (*Haemophilus* = blood loving) given to this organism in 1917 by the Society of American Bacteriologists (Winslow *et al*, 1917) reflects

the attitudes which prevailed at the time regarding the pathogenicity of the species. However over the 2-3 decades following its first isolation there was considerable controversy as to whether *H. influenzae* was in fact the aetiological agent of influenza. Some workers, having failed to culture it from sputum samples from patients in subsequent influenza epidemics, were of the opinion that it was merely a secondary invader (as reviewed by Turk and May, 1967). The discovery of influenza virus in 1934 confirmed that this was indeed the case.

Haemophili form a significant proportion of the normal flora of the upper respiratory tract and, more rarely, may also colonise other mucosal surfaces such as the genital tract and the surface of the eye (Moxon 1985). Of all *Haemophilus* species colonising the respiratory tract *H. influenzae* is undoubtedly the most important both from a numerical standpoint and also with regard to pathogenic potential.

In a study involving 137 children under 8 years of age Kilian *et al* (1972) reported that of all *Haemophilus* species isolated from the nasopharynx, 49% were *H.influenzae* (Table 1.1.1a). *Haemophilus influenzae* is a strictly human parasite, there is no evidence of any animal reservoir for this species (Turk, 1981). It is spread from individual to individual in droplet spray or by contact with secretions from a colonised host (Glode *et al*, 1980).

TABLE 1.1.1a.

Distribution of species of 137 *Haemophilus* strains isolated from the nasopharynx of children aged less than eight years.

H. influenzae.	49%
H. parainfluenzae.	25%
H. haemolyticus.	13%
H. parahaemolyticus /	11%
V factor independent species	3%

from Kilian *et al* (1972).

TABLE 1.1.1b. Common nasopharyngeal carriage rates for *H. influenzae.*

H. influenzae	Carriage Rate.
Capsulated type b	2-4%
Other capsular types	1-2%
Non capsulated	50-80%

from Turk (1981).

Probably every individual will encounter *H.influenzae* at some point during his/her lifetime. Kilian *et al* (1972) isolated this species from the nasopharynx of several infants within days of birth and Sell *et al* (1973) suggest that all children have been colonised with *H.influenzae* by the time they are four years of age. Over the years there have been numerous studies of carriage rates in the upper respiratory tract of normal individuals (Dawson and Zinneman, 1952; Masters *et al*, 1958; Turk, 1963; Kilian *et al*, 1972; Spinola *et al*, 1986). The results of the earlier studies have been summarized by Turk (1981) and have indicated 28 - 80% carriage at any given time.

The species *H.influenzae* can be subdivided into several different serotypes. Pittman (1931) described six biochemically distinct capsular serotypes, designated types a - f. All six have high molecular weight, negatively charged surface polysaccharides consisting of disaccharide repeat units (Egan *et al*, 1982). Of the six serotypes, type b is the most pathogenic (Zwahlen *et al*, 1983). Greater than 95% of strains responsible for systemic infections in man belong to type b (Turk and May, 1967). A further group within the species do not posess any capsule (Gyorkey *et al* 1984). These non typable strains constitute the vast majority of the *H.influenzae* species carried in the upper respiratory tract of a given population at any one particular time (Table 1.1.1b).

Although the initial ideas and observations regarding the pathogenicity of *H.influenzae* were wrong, the species does have a wide range of pathological implications (Table 1.1.1c). In general, infections caused by *H.influenzae* may be divided into two broad groups: a) Acute infections arising as a result of bloodstream invasion, the vast majority of which (>95%) are caused by type b strains (Turk and May, 1967; Musher, 1983), and b) Chronic infections caused by spread of non typable *H.influenzae* within the respiratory tract. Capsular types other than type b very rarely cause problems in infection.

1.1.2 Pathogenicity of Non Typable *H.influenzae*.

Non typable *H.influenzae* strains are being increasingly reported as important bacterial pathogens. They account for up to 20% of all cases of otitis media (Howie *et al* 1970) being second only to *Streptococcus pneumoniae* as a cause of this disease in young children (Klein 1981). Non typable strains have also been shown to cause bacteraemia and arthritis in neonates (Friesen and Cho 1986, Granoff and Nankervis 1975) and have, on rare occasions been reported to cause urinary tract infections, the latter usually only in patients with predisposing factors such as a stone or some other abnormality of the urinary tract (Turk 1981). In recent years it has been recognised as a cause of sinusitis in children and adults (Wald *et al* 1981, Evans *et al* 1975) and also as a cause of pneumonia in children, particularly

TABLE 1.1.1c. Infections Caused by *H.influenzae*.

1. *H.influenzae* type b.	
a) Common.	meningitis epiglottitis pneumonia septicaemia arthritis
b) Uncommon.	cellulitis peritonitis cerebral abcess pericarditis
2. Non Typable *H.influenzae*.	
a) Common.	Acute exacerbations of bronchitis and bronchiectasis, sinusitis conjunctivitis otitis media pneumonia
b) Uncommon	bacteraemia without focus neonatal sepsis urinary tract infection

Adapted from Turk (1981) and Lacey (1986).

in underdeveloped countries, (Shann *et al* 1984) and in the elderly (Berk *et al* 1982, Brabender *et al* 1984). Non typable *H.influenzae* have also been implicated as a cause of invasive disease in adults (Simon *et al* 1980, Wallace *et al* 1981, Wallace *et al* 1983).

Chiefly however, non typable *H.influenzae* is a pathogen of the respiratory tract. Classically, non typable *H.influenzae* has been recognised as an infective agent which causes disease in individuals who have underlying respiratory tract damage, for example children with cystic fibrosis (May *et al* 1972, Schiotz and Høiby 1979, Corey *et al* 1984) and in individuals suffering from chronic bronchitis and bronchiectasis (Turk and May 1967, Turk 1981, Wilson and Cole 1986). In these patients infection with *H.influenzae* is facilitated by underlying pulmonary dysfunction.

It has been demonstrated in bronchiectatic patients that there is an association between infection with *H.influenzae* and sputum purulence (Roberts, 1984), furthermore eradication of *H.influenzae* by antimicrobial chemotherapy leads to a marked clinical improvement (Cole *et al*, 1983; Cole, 1984). However, a characteristic of chronic bronchitis and bronchiectasis is the tendency for infective episodes to recurr frequently. In such instances clinical relapse has been clearly associated with the re-emergence of *H.influenzae* in the sputum (May 1953, May and Delves, 1964;

Roberts, 1984). At the time when the earlier studies were performed no typing schemes for serologically non typable *H.influenzae* were available so it was not possible to determine whether relapse was associated with re-emergence of the previous infecting strain or whether it was the result of infection with a novel strain.

1.1.3. Typing of *H.influenzae* Isolates.

/

Epidemiologically, it is important to identify differences between both type b and non typable *H.influenzae*, strains and thus ascertain possible sources of infection. Additionally it is of interest to determine whether strains causing a particular type of infection (e.g. of the respiratory tract) have any characteristics which distinguish them from the majority of strains normally found colonising the nasopharynx. In situations such as bronchiectasis where recurrent infection is the norm it is also useful to determine whether the same or new strains are involved in successive infective episodes. Several typing schemes have been proposed for *H.influenzae* based on various biochemical characteristics, outer membrane protein profiles and lipopolysaccharide types. Each of these has been used with varying degrees of success in epidemiological studies of the species.

1.1.3a Biotyping.

A biotyping scheme has been devised for *H.influenzae* based on enzymatic and biochemical properties (Kilian 1976). More specifically 3 biochemical properties are employed to distinguish between isolates namely; indole production, urea hydrolysis, and production of ornithine decarboxylase. Eight biotypes have been identified based on this scheme and these are listed in table 1.1.3.

Several studies have indicated that the majority of capsulate strains belong to biotype I, accordingly biotype I strains are commonly associated with acute infections (Kilian 1976, Wallace *et al* 1981, van Alphen *et al* 1987). In general, most non typable strains isolated from the respiratory tract have been found to belong to biotypes II and III (Albritton *et al* 1978, Kamme 1980, Brabender *et al* 1984). In a study of invasive disease caused by non typable strains, Wallace *et al* (1981) confirm that the majority of non typable isolates fall into these two biotypes. However, the same authors report that most non typable *H.influenzae* causing obstetrical infections are of biotype IV. Kilian (1976) and Barenkamp *et al* (1982) also suggest that biotype IV strains may predominate in the female genital tract. This evidence suggests that non typable *H.influenzae* colonising

TABLE 1.1.3:

Haemophilus influenzae Biotypes.

BIOTYPE	INDOLE	UREASE	ORNITHINE DECARBOXYLASE
I	+	+	+
II	+	+	–
III	–	+	–
IV	–	+	+
V	+	–	+
VI	–	–	+
VII	+	–	–
VIII	–	–	–

this particular niche may constitute a population which is biochemically distinct from that of the upper respiratory tract.

Differences in biotype have proved useful in determining that the small proportion of non typable strains which cause invasive disease are not unencapsulated mutants of capsulate strains. Wallace *et al* (1981) demonstrated that invasive non typable *H.influenzae* constitute a population which is different in biotype distribution from the majority of capsulate strains. Non typable organisms colonising the nasopharynx appear to share the same range of biotypes as those isolated from sites of active infection. The fact that nasopharyngeal and lower respiratory tract populations do not differ significantly in biotype suggests that bacteria colonising the lungs may have originated as part of the normal pharyngeal flora. In situations where there is underlying respiratory damage it seems likely that this may indeed be the case.

There has been a suggestion that biotype I may be indicative of other virulence factors apart from capsule production (Kilian *et al* 1979). The identity and significance of such factors has yet to be ascertained and, although the majority of type b strains do belong to biotype I, it is not unusual for type b strains of other biotypes to cause acute invasive disease (Prober *et al* 1982, Albritton *et al* 1978).

In a study of *H.influenzae* colonisation of CF patients over a two year period, Watson *et al* (1988) have demonstrated on

the basis of biotype that strains carried by these individuals vary frequently with time. A single biotype is rarely carried for more than two months and is often replaced with a new biotype within one month of the initial isolation. These findings corroborate previous ideas that colonisation with *H.influenzae* is a dynamic process involving frequent aquisition and loss of strains (Turk and May 1967, Spinola *et al*, 1986). Additionally, CF patients have been found to be colonised with several different biotypes at a particular time (Watson *et al* 1988), this is in stark contrast to the findings of the same authors for other respiratory infections where more than one biotype was rarely detected. The question of whether recurrent infections in individuals with chronic pulmonary disease are caused by survival of a single strain or the advent of a new strain remains to be resolved.

Biotyping has proved useful in monitoring colonisation of individuals and in identifying certain <u>populations</u> within the species. The majority of *H.influenzae* strains fall within a small range of biotypes - type b strains are predominantly of biotype I and most non typable strains are of biotypes II and III - this scheme is therefore of limited use in pinpointing individual strains with certainty.

<u>1.1.3b OMP Subtyping of *H.influenzae*</u>.

Several subtyping systems based on outer membrane protein (OMP) profiles of *H.influenzae* have been proposed.

H. influenzae OMPs are stable on subculture (Loeb and Smith 1980, Barenkamp *et al*, 1982) and on transmission from host to host (Barenkamp *et al*, 1981a) and thus are excellent candidates to form the framework of a typing system. In general OMPs of *H. influenzae* type b are less variable than those of non typable strains and consequently are more amenable to typing systems (Loeb and Smith 1982).

OMP subtyping systems for *H. influenzae* type b have been devised and employed usefully in epidemiological studies. These systems are based on variations in the electrophoretic mobilities of several major outer membrane proteins on SDS-PAGE (see 2.4.1). Loeb and Smith (1980) describe six such proteins designated a-f and use variabilities in molecular weight to define eight different subtypes. Barenkamp *et al* (1981a and b) proposed a similar system utilising variability of the same group of proteins and also note an additional protein present in all type b strains which is heat modifiable and may be present in heavy (H), or light (L) form. Using these parameters these authors defined 21 subtypes but note that 92% of all type b isolates fall into only 5 of these groups. This typing system concentrates on the major OMPs and ignores any variations in minor proteins which may be present.

In contrast to *H. influenzae* type b, individual strains of non typable *H. influenzae* show a great deal of variation in major as well as minor OMPs. Several authors have reported that OMPs of 16K and 30K are common to all strains of

H. influenzae both type b and non typable (Loeb and Smith 1980, Barenkamp *et al* 1982, Murphy *et al* 1983, van Alphen *et al* 1983). Spinola *et al* (1986) report an additional 64K band present in all non typable strains, no other protein has been consistently found in all clinical isolates of non typable *H. influenzae*.

Murphy *et al* (1983) devised a subtyping scheme for non typable strains. They observed that each non typable isolate had two major OMPs which were typically of molecular weight between 32 and 42K. On the basis of differing patterns of these two principal OMPs they were able to classify 48 clinical isolates into 8 distinct subtypes. Other authors however, have failed to note any trends or patterns in the OMP profiles of non typable *H. influenzae* which could form the basis of a typing system (Barenkamp *et al* 1982, Spinola *et al* 1986, van Alphen *et al* 1987).

A typing system based on only 2 OMPs, and ignoring differences in all others is of limited usefulness, Murphy and Apicella (1985) have proposed a serotyping system for non typable strains which takes account of multiple antigenic determinants. Reactivity patterns with each of 3 different antisera raised against 3 non typable isolates enable 6 distinct serogroups to be defined. As yet however, no typing systems based on non typable *H. influenzae* OMPs have been shown to be universally applicable.

OMP subtyping schemes have proved useful in epidemiological studies of type b disease. OMP profiles have been examined

during epidemics in order to determine whether a single source or multiple strains were responsible (Loeb and Smith 1980) and have been used to confirm epidemiologically related cases and contacts (Barenkamp *et al*, 1981a).

Although not amenable to classification into subtyping groups like type b, OMP profiles of non typable isolates are useful in defining individual strains. Analysis of OMP patterns has proved effective in determining whether recurrent otitis media infections are due to identical or novel non typable strains (Barenkamp *et al*, 1984) and have also been useful strain markers in longitudinal studies of nasopharyngeal colonisation both by type b and by non typable organisms (Spinola *et al* 1986). Interestingly, a particular subset of non typable strains causing unrelated episodes of neonatal sepsis have been shown to be identical in OMP profile (Barenkamp *et al*, 1982). In this instance OMP typing lends support to the idea, generated by studies of *H.influenzae* biotypes, that non typable *H.influenzae* causing obstetric and neonatal infections may constitute a unique population within the species (Wallace *et al* 1983).

Epidemiologically related pairs of isolates identical in biotype, can be distinguished on the basis of OMP profile in many instances. However, isolates identical in OMP profile are usually identical in biotype (Spinola *et al* 1986). Previous authors, when subtyping *H.influenzae* into groups on the basis of a few OMPs noted no correlation between subgroups and biotypes (Murphy *et al* 1983). Typing strains

on the basis of OMP profiles relies on the assumption that proteins which have identical mobilities on SDS-PAGE are identical. However, it has been reported that on rare occasions isolates which are identical in OMP profile may be immunologically distinct (Spinola *et al* 1986), additionally a limited degree of antigenic diversity in the major 39K OMP of *H.influenzae* type b strains has been reported (Gulig *et al*, 1983), so this system may not be accurate in 100% of cases. Nevertheless, it is the most effective method available to date for identifying and describing individual strains of *H.influenzae*, particularly when used in conjunction with biotyping.

1.1.3c LPS-Based Subtyping Systems.

Subtyping systems based on interstrain differences in the LPS component of the outer membrane have been devised (Inzana 1983) and applied in epidemiological studies of *H.influenzae* type b disease (Inzana and Pichichero 1984, Losonsky *et al* 1984). However SDS-PAGE patterns of LPS from non typable strains demonstrate little diversity and have proved too homogeneous in gel profiles for the development of this kind of typing system (Inzana 1983).

Inzana (1983) described 11 subtypes of *H.influenzae* type b using this approach and was able to demonstrate that isolates identical in OMP profile and biotype could be distinguished on the basis of electrophoretic mobility of LPS. Subsequently, several authors (Tolan *et al* 1986,

Kimura and Hansen 1986, and Kimura *et al* 1987) have demonstrated that *H.influenzae* type b LPS is not a stable epidemiological marker - having the tendency to variation on laboratory and on *in vivo* passage at high frequency.

Non typable isolates demonstrate antigenic heterogeneity in both lipid A (Apicella *et al* 1985) and oligosaccharide components (Campagnari *et al* 1987, Patrick *et al* 1987) of LPS. Patrick *et al* (1987) were able to segregate 69 non typable isolates into 14 serogroups on the basis of reactivity with a series of oligosaccharide specific monoclonal antibodies. The same authors noted extensive cross reactivity between non typable and type b LPS and found that, like that of *H.influenzae* type b, LPS from non typable *H.influenzae* was subject to variation on *in vitro* or *in vivo* passage and was therefore not a suitable epidemiological marker.

In conclusion, of all *H.influenzae* typing systems described to date, OMP analysis appears to be the most accurate and useful in distinguishing between individual strains. *Haemophilus influenzae* type b is more amenable to subgrouping on the basis of OMP profile than non typable *H.influenzae*, which displays more heterogeneity in OMP composition. Biotyping systems are useful in conjunction with OMP analysis for defining individual strains and are effective in describing populations within the species as a whole. LPS typing is not reliable due to the variability of this character *in vivo* and *in vitro*.

Non typable *H.influenzae*, having lain in the shadow of type b strains and having been dismissed as a contaminating commensal in many situations in the past has, in recent years, begun to be recognised as an important pathogen in its own right. As a cause of suppurative infection in patients with chronic bronchitis and bronchiectasis *H.influenzae* strains are of great economic importance. Such individuals are commonly in need of prolonged courses of antimicrobial chemotherapy, may need repeated hospitalisation and are frequently unfit for work (Turk 1981). In the light of these facts and the emergence of non typable *H.influenzae* as a pathogen in other situations (Table 1.1.1c) the importance of gaining a better understanding of possible virulence factors and mechanisms of pathogenicity of this species in the respiratory tract - and elsewhere - is apparent.

1.2 Non Typable *H.influenzae* in Chronic Obstructive Pulmonary Disease.

Being in direct contact with the environment via inhaled air, the lungs are frequently challenged with particulate matter and microorganisms. The fact that such inhaled material only rarely leads to respiratory problems or infection demonstrates the efficiency of pulmonary defence mechanisms.

A complex array of mechanical and immunological barriers maintain the sterility of the lower respiratory tract (Table 1.2.1). These include specific and non specific, and both local and systemic components (for review see Reynolds 1983, Cole and Wilson 1986).

Mechanical barriers to pulmonary infection include the cough and sneeze reflexes and the integrity of the mucosal epithelium lining the respiratory tract. Cilia on the surface of epithelial cells are bathed in periciliary fluid above which is a layer of mucus. Foreign particulate matter entering the respiratory tract may become trapped in this layer. Cilia, beating in synchrony propell the mucus upwards towards the nasopharynx where it is expelled by coughing or swallowing. This 'mucociliary escalator' provides an efficient relatively non specific mechanical barrier to invading microorganisms (Wilson and Cole 1986). Additionally, periciliary fluid and mucus contain

Table 1.2.1. Specific and Non Specific Immune Defences in the Lung.

	NON SPECIFIC	SPECIFIC
LOCAL	Cough reflex	Local immunoglobulins
	Mucosal epithelium	Pulmonary macrophages
	Mucociliary escalator	
	Lysozyme	
	Lactoferrin	
SYSTEMIC	Complement components	Serum immunoglobulins
	PMN Leucocytes	Cellular immune response.
	Transferrin	

Adapted from Reynolds (1983) and Cole and Wilson (1986).

antibacterial substances such as lysozyme and secretory IgA - creating a hostile environment for any potential pathogens (Reynolds 1983).

Fragments of bacteria, whole cells or inert particles which reach the terminal airways have to contend with specific and non specific immune mechanisms such as opsonizing antibodies (mainly IgG), complement components and alveolar macrophages (Toews 1983). It has been estimated that on >95% of all occasions foreign material entering the lungs is cleared imperceptibly by the above mechanisms (Cole and Wilson 1986). Occasionally, in the case of a particularly virulent organism or if there is a particularly large inoculum, local immune defences may be overwhelmed. In such circumstances a controlled inflammatory response takes place, involving recruitment of PMN leucocytes and serum components. Thus elements of systemic immunity are recruited to augment local mechanisms and regain control over infection (Reynolds 1983). When the normal immune response fails to eliminate an infecting organism the inflammatory response may be further amplified and a state of chronic infection can become established.

Bronchiectasis is a condition which is characterized by chronic pulmonary infection often with bacteria which are normally considered to be of low pathogenic potential. Individuals with this condition have an irreversible dilation of the bronchii, the exact cause of which is unknown. Clinical symptoms vary widely between patients, in

some cases lung function may be near normal with intermittent infective episodes, while in more severe cases abnormal lung function is associated with persistent production of purulent sputum, chronic ill health and destruction of lung tissue (Thurlbeck, 1976; Cole and Wilson 1986). The disease is more common in women than men and the mean age of sufferers is 41 years (Cole, 1984). Thus this condition affects a relatively young population in comparison with, for example, chronic bronchitis and in contrast to the latter condition patients with bronchiectasis largely constitute a non smoking population. They do not appear to be immunodeficient, having a sufficient antibody response, however it has been suggested that specific antibodies to certain bacterial antigens may be lacking (Lane and Maclennan 1986) and that certain IgG subclasses may be deficient. Recent evidence suggests that deficiency of IgG subclasses is in fact rare in such patients (Veale *et al* 1988).

Due to underlying disease therefore, but for specific reasons which remain to be elucidated the immune response fails to eliminate bacteria entering the lungs of individuals with bronchiectasis. The continued presence of bacteria then leads to further amplification of the inflammatory response and this exaggerated, poorly controlled immune response may cause damage to normal lung tissue, thus facilitating further infection. A 'viscious

circle' of infection, immune response, and tissue damage is thus created (Fig.1.2.1, Cole 1984).

Damage created by a poorly controlled host response such as this may include destruction of the elastin component of bronchial walls (Cole and Wilson 1986) and also may have a role to play in destruction of ciliated epithelial cells. Polymorphonuclear leukocytes, entering the lung during the inflammatory response can cause damage to 'bystander' lung tissue by leakage of proteolytic enzymes and oxygen radicals which are normally involved in bacterial killing. There are antiprotease and antioxidant enzymes present in the lung and these normally inactivate any PMN released enzymes thus preventing damage to lung tissue. Individuals with deficiencies in antiproteases, for example α_1 antitrypsin, are known to be susceptible to pulmonary deterioration (Eriksson, 1965). It has been suggested that the resident host antiproteases in the bronchiectatic lung are unable to cope with the continuous neutrophil traffic caused by a persistent microbial load (Cole, 1984; Stockley, 1983). Thus a protease - antiprotease imbalance is thought to exist, facilitating pulmonary damage by PMN - derived protease enzymes. Impairment of pulmonary clearance in bronchiectasis (Laurenco *et al*, 1972; Currie *et al*, 1987) may in part be the result of reduction in ciliary beat frequency by neutrophil elastase (Smallman *et al* 1984) although microbial factors are also known to have a deleterious effect on ciliated epithelium (Sykes *et al* 1987).

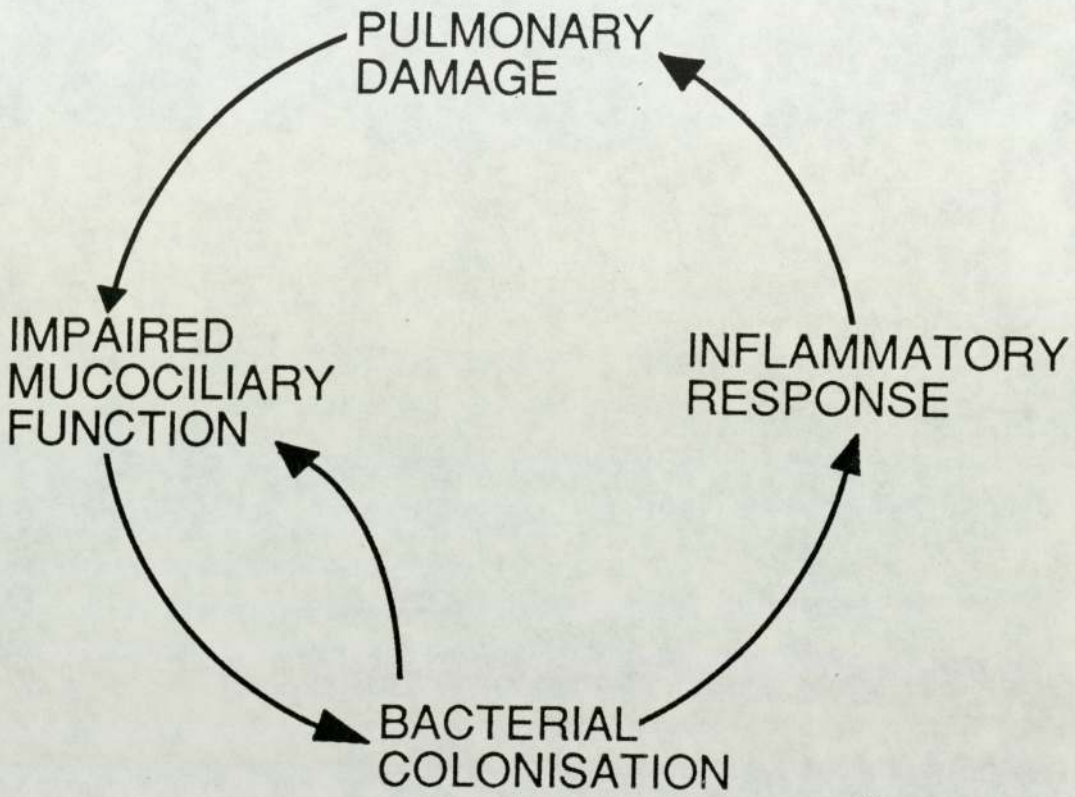


FIG. 1.2.1
The 'viscious circle' hypothesis of pulmonary damage in bronchiectasis

(from Cole, 1984)

Structural damage to the bronchial tree in bronchiectasis can therefore result in chronic lung infection with bacteria which normally form part of the commensal flora. Of these *Streptococcus pneumoniae* and non typable *H.influenzae* are the two species most commonly associated with exacerbations of infection and clinical deterioration of patients (Smith 1983). There are no outstanding virulence factors recognised in non typable *H.influenzae* strains which would explain their propensity for colonising the lower respiratory tract of such individuals. Several factors have been described which may facilitate survival of non typable *H.influenzae* in the respiratory tract. These are discussed below.

1.2.2 ADHERANCE.

Bacterial adherance to host epithelial cells is considered to be a critical first step in the pathogenesis of many infections (Beachey 1981). In several species, for example *Neisseria gonorrhoeae* (McGee *et al* 1981) and *Escherichia coli* (Thorne *et al* 1979) ability to adhere to epithelial cells is correlated with virulence and is known to be mediated by proteinaceous, non flagellar surface appendages known as pili or fimbriae.

Several authors have reported the isolation of fimbriae from both non typable and type b strains of *H.influenzae* (Scott and Old 1981, Pichichero *et al* 1982, Bakaletz *et al* 1988). *Haemophilus influenzae* fimbriae are described as flexible rods of varying length and of diameter 2-6nm (Guerina *et al*

1985, Bakaletz *et al* 1988). They are composed of repeating protein subunits of molecular weight 23-25Kdal (Guerina *et al* 1985, van Alphen *et al* 1988), degree of fimbriation may vary markedly between cells of a given isolate and they may be located in polar positions or peritrichiously on cells (Bakaletz *et al* 1988).

The presence of fimbriae on *H.influenzae* is known to correlate with ability to cause haemagglutination of human erythrocytes and ability to adhere to human oropharyngeal cells *in vitro* (van Alphen *et al* 1988). Nasopharyngeal isolates of *H.influenzae* type b have been demonstrated to consist of a mixture of fimbriated and non fimbriated forms and bloodstream isolates have been shown to be uniformly non fimbriated (Kaplan *et al* 1983, Mason *et al* 1985). In contrast, the majority of nasopharyngeal isolates of non typable *H.influenzae* are fimbriated. Lampe *et al* (1982) report that 90% of non typable isolates were fimbriated compared with a value of 5% for type b. Another study (Bakaletz *et al* 1988) showed 100% fimbriation of non typable strains studied. However, degree of fimbriation varied from 10-100% between individual bacteria. Apicella *et al* (1984) reported only three of fifteen sputum isolates to be fimbriated. Whether this result reflects differences in assay procedure between the above studies or a genuine difference between nasopharyngeal and sputum isolates remains to be investigated.

Fimbriae of type b and non typable *H.influenzae* share some common epitopes (Guerina *et al* 1985, van Alphen *et al* 1988). Binding to epithelial cells and haemagglutination is mannose resistant (Scott and Old 1981) and the Anton blood group antigen has been demonstrated to be the receptor site for haemagglutination of human erythrocytes (van Alphen *et al* 1986). The binding site is conserved between non typable and type b strains although they display variation in other regions (van Alphen *et al* 1988). The receptor on erythrocytes and epithelial cells is not identical. The two are different molecules containing particular common epitopes (van Alphen *et al* 1987). Definitive evidence of the role of *H.influenzae* fimbriae in adherance to human oropharyngeal cells showing that purified fimbriae bind to epithelial cells and red blood cells and cause haemagglutination has recently been published (van Alphen *et al* 1988).

The exact contribution of adhesive organelles in the pathogenesis of non typable *H.influenzae* infection in patients with chronic obstructive pulmonary disease is not well understood. Fainstein and Musher (1979) report increased adherance of non typable strains to oropharyngeal cells of smokers compared to those of non smokers. This may be relevant in cases of chronic bronchitis but the majority of bronchiectasis patients do not smoke. Additionally viral infection has been shown to facilitate attachment of *H.influenzae* to mucosal epithelium (Fainstein *et al* 1980).

Whether the above evidence reflects genuine increased adhesion to damaged epithelial cells or whether it reflects decreased clearance mechanisms as a result of damage to host cells remains to be clarified.

1.2.3 CILIOTOXINS.

Mucociliary clearance is a first line pulmonary defence mechanism, functioning to protect the host from inhaled foreign particles including bacteria by transporting them upwards towards the pharynx where they are either swallowed or expelled by coughing (Reynolds 1983).

In healthy individuals ciliated epithelial cells are present lining the airways from the nasal passages to the terminal airways. Cilia function within a 2 layered fluid system - a watery layer, the periciliary fluid, lies beneath a mucus layer which covers the surface of the epithelium. Upward movement of mucus is achieved by the coordinated beating of cilia within the periciliary fluid. Each beat consists of a stiff downstroke, during which hooks at the tip of each cilium engage the mucus and propell it foreward, and a paddle-like reverse stroke during which the cilia sweep back to their starting point by moving through the periciliary fluid at right angles to the epithelial cell surface (Wilson and Cole 1986). To achieve efficient movement of mucus forewards each cilium must beat in a coordinated fashion with all others on the same and adjacent cells.

Individuals with rare inherited conditions which affect ciliary motility are known to have increased risk of respiratory tract infection (Eliasson *et al* 1977). Mucociliary clearance is also known to be decreased in bronchial sepsis of other aetiology. In the bronchiectatic lung there is evidence of decreased mucociliary clearance and epithelial cell damage (Lourenco *et al* 1972). Loss of ciliated epithelium may be observed at sites of purulent infection (Wilson and Cole, 1986), and purulent sputum sols from bronchiectasis patients have been demonstrated to have deleterious effects on human ciliated epithelium *in vitro* - both host derived and microbial factors have been implicated in this process (Smallman *et al* 1984, Sykes *et al* 1987). Neutrophil elastase present in purulent sputum has been shown to be responsible for decreases in ciliary beat frequency (CBF) observed using human nasal epithelium *in vitro* (Smallman *et al* 1984). Similarly *Pseudomonas aeruginosa*, *S. pneumoniae* and *H. influenzae* have been shown to produce factors which interfere with ciliary function and cause epithelial cell damage (Wilson and Cole 1986, Sykes *et al* 1987).

Cultures and culture filtrates of non typable *H.influenzae* can cause decreases in CBF, disorganization of beating patterns (ciliary dyskinesia) and epithelial cell extrusion in *in vitro* preparations of human ciliated epithelium (Wilson *et al* 1985; Mylotte *et al*, 1985; Wilson *et al*, 1986a). Culture filtrates may cause disruption of beating

patterns of individual cilia, causing some to merely oscillate to and fro, others to stop beating altogether (ciliostasis), and others to beat in opposite directions (Wilson *et al* 1985).

Denny (1974) described the isolation of a heat stable, non trypsin digestible, non dialysable toxin from *H.influenzae* which caused ciliostasis and epithelial cell damage and implicated LPS as the cause. Subsequent workers confirmed that purified *H.influenzae* LPS caused extrusion of ciliated and non ciliated cells from human respiratory epithelium *in vitro* and further suggested that the lipid A moiety was responsible (Johnson and Inzana 1986, Wilson *et al* 1986b). Purified LPS did not however cause decreases in CBF (Wilson *et al* 1986b). Therefore it seems that in addition to LPS *H.influenzae* produce a second ciliotoxic factor. This has been reported to be a non proteinaceous low molecular weight molecule (Wilson *et al* 1986a) but has not been more accurately defined to date.

The onset of ciliotoxic activity by culture filtrates of non typable strains is rapid *in vitro* (Wilson *et al* 1985). This may be an important factor in the early stages of infection, possibly assisting in the perpetuation and spread of organisms within the lung. If, as in bronchiectasis, the immune response fails to clear bacteria and chronic infection becomes established then increasing levels of elastase may cause further damage to ciliated epithelial cells. Thus the 'viscious circle' described earlier would

become established (Cole 1984, Sykes *et al* 1987). Antibiotic treatment has been shown to decrease elastase levels in sputum even in clinically stable patients (Stockley *et al* 1984). Therefore bacteria, specifically *H.influenzae* may cause damage to the respiratory epithelium in patients with chronic obstructive pulmonary disease either directly by production of ciliotoxic factors and/or indirectly by stimulation of the immune response, causing concentrations of elastase in the lungs to increase.

1.2.4 IgA PROTEASES.

IgA is the principal mediator of immunity at mucosal surfaces. Secretions bathing the mucosal sufaces contain IgA which is synthesised by plasma cells below the epithelium. This secretory IgA has specificity for bacterial surface antigens (Abrahams and Beachey 1985), toxins (Mansa and Kilian 1986) and viruses (Plaut 1983).

In serum IgA is mainly in monomeric form. In secretions however it exists as a dimer held together by a polypeptide chain known as secretory component. There are 2 IgA subclasses, IgA_1 and IgA_2, these differ in the amino acid sequence of the hinge region of the α chain (review by Kilian *et al* 1988). IgA_1 is the predominant class of secretory IgA found on respiratory mucosal surfaces (Mestecky *et al*, 1986; Kilian *et al*, 1988).

Several bacterial species are capable of producing IgA proteases - these are extracellular, highly specific

proteolytic enzymes which hydrolyse human IgA$_1$ by cleaving specific peptide bonds between amino acids in the hinge region, yielding Fab and Fc fragments (Plaut *et al*, 1978; Mulks, 1985). The biological significance of the IgA$_1$ proteases is strongly suggested by their association with bacteria which cause diseases which are either localized at or originate from mucosal surfaces (Kilian and Reinholdt 1986). IgA protease producers include *Streptococcus sanguis*, *S. mitior*, certain *Bacteroides* species, *Neisseria meningitidis*, *N. gonorrhoeae*, *S. pneumoniae* and *H. influenzae* (Mulks *et al* 1982, Plaut 1983).

Among *Haemophilus sp.*, only *H. influenzae* produce IgA proteases (Kilian *et al* 1979, Male 1979). Several different types of IgA$_1$ protease are produced by this species, Mulks *et al* (1982) have described three different types of enzyme based on cleavage patterns seen on SDS-PAGE; these different types cleave IgA$_1$ at different positions within the hinge region. The type of enzyme produced correlates closely with serotype but not biotype (Mulks *et al* 1982). Kilian and Thomsen (1983) were able to classify *H. influenzae* IgA$_1$ proteases into each of 15 'inhibition types' based on antigenic differences. In this way enzymes having identical cleavage patterns were shown to be antigenically different. Non typable isolates are extremely variable, IgA proteases in these strains being distributed over 12 different inhibition types.

IgA proteases are vulnerable to host produced neutralizing antibodies (Gilbert *et al* 1983). It has been suggested that the diversity of IgA protease types throughout the species may be a means of avoiding the action of these (Kilian and Thomsen 1983). The antigenic diversity of proteases of non typable strains may give these an advantage in the avoidance of neutralizing antibodies raised against previous non typable colonisers giving newly infecting strains the opportunity for protease production, thus increasing survival time in the respiratory tract (Kilian *et al* 1988). The role of IgA_1 proteases in the pathogenesis of respiratory infection in bronchiectasis is not known. IgA_2 is also present on mucosal surfaces, although IgA_1 predominates, and IgA_2 is insusceptible to these proteases (Kilian and Reinholdt 1986). It is possible that Fab fragments generated by IgA_1 proteases may stick to the bacterial cell surface and block the action of neutralising antibodies. This has been demonstrated for intact IgA present in bronchopulmonary secretions (Musher *et al* 1984). It is easy to speculate on a role for IgA_1 proteases in respiratory infection and the propensity of producer species for colonising the respiratory tract gives some grounds for this. IgA_1 protease production is possibly only one of many factors, both host and microbial, which determine pathogenicity. Conclusive proof of the role of IgA_1 proteases in *H.influenzae* colonisation of the bronchiectatic lung remains to be demonstrated. The establishment of a

relevant animal model system of chronic pulmonary infection by non typable *H.influenzae* would greatly facilitate studies in this area and would render analysis of *in vivo* production of IgA proteases possible.

1.2.5 THE HOST IMMUNE RESPONSE.

In bronchiectasis stagnation of secretions in the lower air passages, brought about by underlying pulmonary dysfunction, provides favourable conditions for bacterial colonisation and infection. There is however, a marked degree of variation in the clinical prominence of infection among individuals with this condition. Bronchiectasis patients form a heterogeneous group ranging from those who usually produce mucoid sputum with occasional infective exacerbations to those who persistently produce purulent sputum even in an apparently stable clinical state (Hill *et al*, 1986). The factors which determine susceptibility to infection remain undefined.

Clearing of purulent sputum can be achieved with antibiotic therapy (Cole *et al*, 1983; Hill *et al*, 1986), but individuals who persistently produce purulent sputum may require more prolonged courses and higher dosages than those whose sputum is normally mucoid. On cessation of therapy however, relapse is rapid in patients whose sputum is persistently purulent (Hill *et al* 1986). *Haemophilus influenzae* is isolated much more frequently from purulent than from mucoid sputum (May and Delves, 1964; Roberts,

1984) and relapse after treatment is often associated with the reappearance of *H.influenzae* in the sputum (May 1953). The isolation of *H.influenzae* from mucoid sputum is presumed to reflect a simple carrier state (Turk and May 1967), although it has been suggested that continuous low grade infection without obvious purulent sputum could eventually lead to inflammation and damage to bronchial walls (Davies, 1984). The precise reasons why some individuals with bronchiectasis are chronically colonised with bacteria while others remain relatively free from infection remain to be elucidated.

A few studies have been performed which compare the immune response to *H.influenzae* of subjects producing purulent with that of those producing mucoid secretions with a view to identifying factors present in serum from individuals with mucoid sputum which may protect against *H.influenzae* infection (May 1965, Morgan and Wood 1965, Clarke 1979). Morgan and Wood (1965) using a type a *H.influenzae* strain demonstrated increases in serum antibody titres as sputum changed from mucoid to purulent and also demonstrated that patients with chronic obstructive pulmonary disease had higher anti-*H.influenzae* titres than normal controls. The *H.influenzae* antigens against which this response was directed were not identified. Burns and May (1967) identified two specific *H.influenzae* directed precipitins in the sera of chronic bronchitis and bronchiectasis patients. These precipitins were designated H_1 and H_2. H_1 precipitins

were found to be present in the sera of 69% of individuals producing purulent but only in 25% of those with mucoid sputum and in only 6% of normal controls (Burns and May 1967, 1968). The strongest reactions were observed in those who had a long history of purulent sputum production. May *et al* (1973) showed that there was a positive correlation between the presence of H_1 precipitins and purulent sputum production, and also cigarette smoking, in chronic bronchitics.

The presence of H_1 antibodies obviously does not protect against *H.influenzae* infection. These antibodies are generally not acquired by normal nasopharyngeal colonisation and their presence is correlated with repeated infective episodes. The functions of these antibodies and the precise nature of the antigens for which they are specific have not been described although they are known to be heat labile and cytoplasmic in origin. Clarke (1979) reported that bronchiectasis patients also have elevated levels of precipitins directed against *H.influenzae* LPS compared with normal controls.

Several studies have shown that non typable *H.influenzae* OMPs are a major target of bactericidal antibodies present in normal human serum and in convalescent serum from patients with pneumonia and otitis media caused by this species (Gnehm *et al* 1985, Karasic *et al* 1985, Barenkamp 1986). Normal human serum displays variable amounts of bactericidal activity against different non typable strains

(Musher *et al* 1983b) but levels of antibody specifically directed against non typable OMPs in sera from bronchiectasis patients have yet to be determined.

Immunoglobulins directed against OMPs and LPS have been shown to enhance pulmonary clearance of non typable *H.influenzae* in mice (Hansen *et al* 1988) but in chronic infections, bacteria are known to persist for long periods of time even in the presence of opsonising and bactericidal antibody directed against surface antigens (Musher *et al* 1983a, Yamaguchi *et al* 1986).

No antibody has been demonstrated exclusively in convalescent sera from patients recovering from non typable *H.influenzae* pneumonia (Hansen *et al* 1985). Antibodies present in convalescent sera seem to be as diverse as the OMP antigens of the species. As there is no protective antigen recognised exclusively by convalescent sera the above authors suggested that antibodies in the sera of individuals recovering from infection have a greater affinity for OMPs than do those present in acute phase serum.

It is possible that antigenic diversity among OMPs of non typable *H.influenzae* gives the species capacity for avoidance of the host immune response. Musher *et al* (1983b) demonstrated that absorption of normal human serum with one non typable strain did not remove all bactericidal activity against other isolates. Spinola *et al* (1986) however showed that IgG levels do not alter greatly on loss and acquisition

of different *H.influenzae* strains and do not appear to play a major role in terminating colonisation. The latter study involved nasopharyngeal colonisation only. Whether the situation is different in the bronchiectatic lung has yet to be investigated.

If antibody responses to particular antigens are restricted to particular subclasses of IgG, then low levels of a given subclass would predispose to a particular type of recurrent infection. It has been proposed that selective deficiency of certain IgG subclasses may be a predisposing factor to recurrent infection in bronchiectasis. Lane and Maclennan (1986) suggested that the ability to make IgG_2 to specific bacterial antigens may be deficient in individuals suffering from recurrent upper respiratory tract infections, even where total IgG_2 levels appear normal. Additionally Fick *et al* (1986) reported decreased levels of IgG_1 in CF patients compared with normal controls. A recent report however, detected no such immunodeficiencies in bronchiectasis patients (Veale *et al* 1988).

Local IgA deficiency has been implicated in the past as a factor influencing mortality in chronic obstructive pulmonary disease (Soutar 1977), but more recent studies have demonstrated that local IgA levels do actually rise during episodes of acute infection (Stockley *et al* 1980). Musher *et al* (1984) provided evidence that IgA present in bronchopulmonary secretions from patients with non typable *H.influenzae* pneumonia blocked the effect of bactericidal

and opsonising antibodies present in the patients serum. Such blocking antibodies may permit colonisation of individuals who already have adequate antibody levels against infecting *H. influenzae* strains.

1.2.6 Sputum Culture.

The significance of isolation of *H. influenzae* in expectorated sputum samples has been a subject of debate over the years. Inevitably sputum becomes contaminated with saliva during expectoration and consequently will contain a number of bacterial species representative of the oropharyngeal region. Interpretation of the isolation of *H. influenzae* from such a sample is complicated by their usual presence as part of the pharyngeal flora of the normal healthy individual. This difficulty has led to the recommendation that routine sputum cultures be abandoned in the initial assessment of patients with community aquired pneumonia (La Force, 1985).

Cytologic screening of sputum, and the rejection of specimens containing many squamous epithelial cells, has been suggested as a partial solution to this problem (Klein *et al*, 1986). Bartlett and Finegold (1978), recommend the washing of sputum with either physiological saline or water as a method for the removal of saliva and any surface adherant contaminants. Allibone *et al* (1956) suggest that if a large piece of sputum is cultured the bulk of surface

contaminants will be small in comparison with the total mass.

Ideally, this problem is overcome by obtaining samples directly from the lung using such techniques as bronchoscopy or trans-tracheal aspiration. In many instances however such procedures are impractical. Recently, in cysic fibrosis patients, it has been demonstrated that there is a good correlation between the bacterial flora of endobronchial specimens and that of washed sputum samples (Gilljam *et al*, 1986; Thomassen *et al*, 1984).

Another difficulty which arises when culturing sputum is the question of how accurately a single loopful of material represents the flora of the lower respiratory tract. Turk and May (1967) state that the flora of a single portion of sputum may vary with respect to the sample as a whole and recommend that several inocula be taken from each sample in order to overcome this problem. Homogenisation or digestion of the sputum before culture is another way in which error of this type may be minimised. Pancreatin (Rawlins, 1953) or dithiothreitol / Sputolysine (Hammerschlag *et al*, 1980) may be used for this purpose. May and Delves (1964) observed variations in flora between different sputum samples from the same patient over short periods of time and therefore recommended that several sputum samples be obtained from

each individual under study in order to ensure an accurate diagnosis. This is not always a practical possibility.

In many instances in individuals with chronic pulmonary disease sputum culture reveals the presence of a large number of species. *Haemophilus influenzae*, being fastidious in their growth requirements, are liable to overgrowth by other organisms which grow profusely on the rich media required for *Haemophilus* isolation. Occasions have been reported in bronchiectasis and cystic fibrosis patients where clinical improvement after antimicrobial chemotherapy did not correlate with any marked changes in sputum flora (Beaudry *et al*, 1980; Roberts and Cole, 1980; Bauernfeind *et al*, 1987). It has been suggested that in such individuals *H.influenzae* may be the underlying pathogen but that it may have been overgrown in culture by other organisms present in the sputum (Bauernfeind *et al*, 1987).

Kilian *et al* (1972) showed that incorporation of bacitracin into chocolate agar plates resulted in substantially increased yields of haemophili. Roberts and Cole (1980) report an improvement in isolation rates of *H.influenzae* from 5 to 80% from sputum samples containing large numbers of *Pseudomonas aeruginosa*. They created a selective medium by incorporating bacitracin into blood agar plates supplemented with NAD which were then incubated anaerobically. Bauernfeind *et al* (1987) describe a selective

medium containing pyocins which facilitates the isolation of *Haemophilus* from sputa containing large numbers of *P. aeruginosa.*

It is evident from the above that *H. influenzae* growing *in vivo* within the respiratory tract of individuals with chronic obstructive pulmonary disease are in an environment which is vastly different to that of *H. influenzae* cultured *in vitro* in standard laboratory media.

The *in vivo* environment is 'hostile' to the invading microorganism, containing many elements of the host immune response inhibitory to bacterial growth and may also contain other bacterial species which compete for vital nutrients and whose products may be lethal to *H. influenzae*. In contrast, bacteria cultured *in vitro* are often grown in media designed to encourage maximal growth where all nutrients required for survival are present in excess.

The cell envelopes of Gram-negative bacteria represent the interface between the bacterium and its environment and are therefore immunologically important - being accessible to host defence mechanisms. Envelope components may be involved in avoidance of the host immune response, or may facilitate attachment to host cells. Others have a vital role to play in the transport of essential nutrients into the cell. Many bacteria are known to display altered cell surface

composition *in vivo* compared with cell surface composition *in vitro* under ideal conditions of temperature, pH and nutrient availability (Brown and Williams, 1985; Brown *et al*, 1984; Sciortino and Finkelstein, 1983).

An understanding of how conditions which may prevail *in vivo* affect the cell surface composition of non typable *H. influenzae* may be useful in determining how this species survives within the respiratory tract and also may prove useful in designing effective strategies against *H. influenzae* in chronic obstructive pulmonary disease.

1.3.1 The Gram-Negative Cell Envelope.

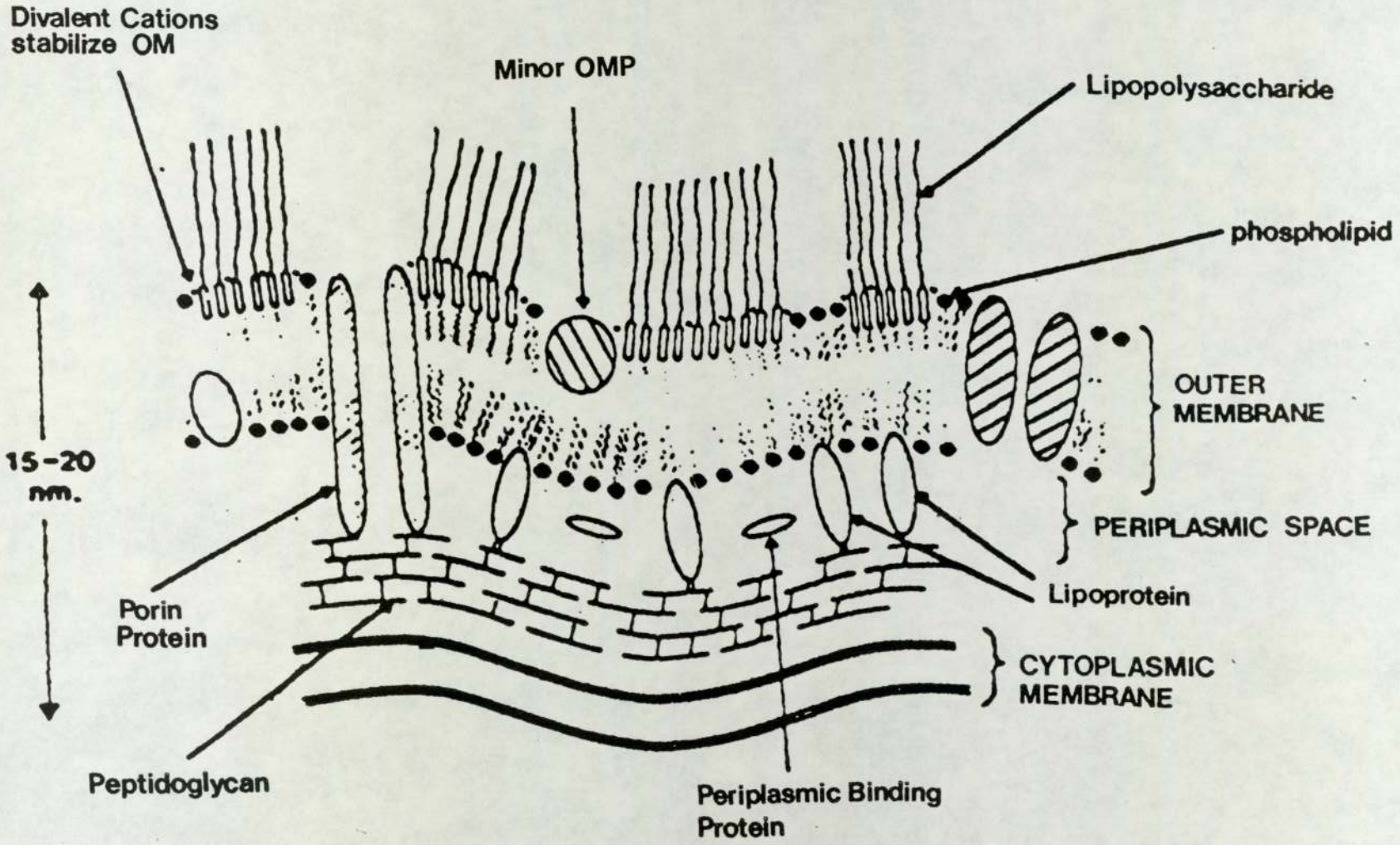
A generalised structure of the Gram negative cell envelope is shown in Fig.1.3.1. Briefly, the envelope is composed of three structurally and functionally distinct layers - the cytoplasmic membrane, the peptidoglycan layer and the outer membrane. The area between the cytoplasmic membrane and the outer membrane is termed the periplasmic space. Some bacteria also have an additional polysaccharide (Sutherland 1977) or protein (Sleytr 1978) layer external to the outer membrane.

Gram-negative envelope composition has been extensively reviewed (Inouye, 1979; Nikaido and Nakae, 1979; Lugtenberg and van Alphen, 1983; Hammond *et al*, 1984; Nikaido and Vaara, 1985; Nakae, 1986) and will not be discussed in detail here. The majority of studies have been performed with *Escherichia coli* and *Salmonella typhimurium* and in comparison with the amount of information gained about these two systems, knowledge of envelope composition in other species is limited. Few such studies have investigated cell envelope structure and function in *H.influenzae* and most of those which have have involved type b rather than non typable strains.

The Gram-Negative Outer Membrane.

The outer membrane (OM) constitutes the outermost structure of the cell in many Gram-negative bacteria which do not

Figure 1.3.1 Diagrammatic Representation of the Outer Membrane of Gram - Negative Bacteria. (from Hammond *et al* 1984).



posess surface polysaccharide or protein layers and as such is in direct contact with the environment (Smit and Nikaido, 1978). Consequently outer membrane components of bacteria growing within a human host are immunologically important, being accessible to elements of the host immune response. The OM is composed of phospholipid, protein and lipopolysaccharide. It renders the cell impermeable to hydrophobic and most high molecular weight hydrophilic substances and also protects the cell from the action of various hydrolytic enzymes, bile salts and detergents (Rest *et al*, 1977; Nikaido and Nakae, 1979; Nikaido and Vaara, 1985). If the integrity of the OM is disrupted by treatment with EDTA or certain antimicrobial agents the cell envelope undergoes a non specific increase in permeability and becomes susceptible to the action of various harmful substances such as lysozyme (Lugtenberg and van Alphen, 1983; Nikaido, 1985). In addition to providing a non specific permeability barrier the components of the outer membrane may be involved in avoidance of the immune response (Makela *et al*, 1980) and also play an essential role in the uptake of vital nutrients from the environment (Nikaido and Vaara, 1985; Nakae, 1986).

1.3.2 Outer Membrane Phospholipids.

In *E.coli* and *S.typhimurium* the phospholipid component of the OM consists of phosphatidylethanolamine, phosphatidylglycerol and phosphatidyldiglycerol but the

relative proportion of phosphatidylethanolamine is increased relative to that of the CM (Cronan and Gelman, 1975; Lugtenberg and Peters, 1976). Gram negative bacteria are usually resistant to the action of phospholipase enzymes and certain detergents such as SDS to which phospholipid bilayers are notoriously sensitive. This has led to the theory that the OM is not a phospholipid bilayer but is composed of an inner phospholipid leaflet and an outer leaflet composed of LPS. Measurement of the actual amount of phospholipid present in the OM of *E.coli* and the use of labelled antibodies to locate LPS have shown that this may indeed be the case (Smit *et al*, 1975; Funahara and Nikaido, 1980). However Nixdorff *et al* (1978) note that rough mutants of LPS are more sensitive to phospholipases than smooth strains and have proposed that the outer leaflet LPS may protect small areas of phospholipid bilayer from the action of phospholipase enzymes. *Haemophilus influenzae* and *Neisseria gonorrhoeae* both have rough LPS and both are very sensitive to certain hydrophobic antibiotics. It has been proposed that these species may have more areas of phospholipid bilayer in the OM than other Gram negatives (review by Lugtenberg and van Alphen, 1983) and this has been shown to be the case for *N.gonorrhoeae* (Lysko and Morse 1981).

1.3.3. Lipopolysaccharide./

1.3.3 Lipopolysaccharide.

Lipopolysaccharide is an amphiphilic molecule, located exclusively in the outer leaflet of the OM of Gram negative bacteria (Funahara and Nikaido 1980, Muhlradt and Golecki 1975). It is composed of three biologically and structurally distinct regions: 1) lipid A - a hydrophobic portion anchored in the OM consisting of fatty acids linked to glucosamine; 2) a hydrophilic core region extending outwards from lipid A and consisting of an inner region containing the unique sugar 3-deoxy-D-manno-2-octulosonic acid (KDO) and heptose and an outer, short oligosaccharide chain; and 3) O-Antigen, a branched polymer of repeating sugar units extending outwards from the core (Westphal *et al* 1983, Hammond *et al* 1984).

LPS molecules are negatively charged and are tightly held together within the outer leaflet by divalent cations, usually Ca^{2+} and Mg^{2+} (Costerton *et al* 1974). Treatment of cells with reagents which remove these cations leads to a non specific increase in cell permeability and facilitates the entry of potentially harmful substrates. In addition to providing a permeability barrier to hydrophobic substances, the hydrophilic side chains of LPS are thought to be important in avoidance of phagocytosis and of the humoral immune response (Nikaido and Vaara, 1985).

H. influenzae LPS is 'rough' consisting of lipid A and core oligosaccharide but having no detectable O-antigen (Flesher

and Insel, 1978; Inzana, 1983). LPS of type b strains has been well characterized (Flesher and Insel, 1978; Parr and Bryan, 1984; Inzana *et al*, 1985; Apicella *et al*, 1985) and has been shown to be antigenically cross reactive with LPS from rough isolates of *E.coli* and *Salmonella* (Marks *et al* 1982, Flesher and Insel 1978), however *H.influenzae* type b LPS is not identical in chemical composition to enterobacterial LPS. The lipid A region of LPS in typable strains differs in fatty acid composition to that of the enterobacteriaceae (Zamze and Moxon 1987) and the oligosaccharide portion contains much less KDO than has been reported for either *Salmonella* or *E.coli* (Zoon and Scocca 1975, Flesher and Insel 1978, Inzana *et al* 1985). Although LPS oligosaccharides from types a-f are similar in sugar composition they vary in terms of the quantity of each carbohydrate residue present (Zamze and Moxon 1987). Significant antigenic differences in LPS may exist between different serotypes (Flesher and Insel 1978) or within individual serotypes (Apicella *et al* 1985). Generally *H.influenzae* LPS yields 1-4 bands when subjected to SDS-PAGE. Using this method Inzana (1983) observed a greater degree of variation in LPS profiles of type b compared with those of non typable isolates. However Patrick *et al* (1987) were able to identify 14 distinct antigenic groups among non typable *H.influenzae* based on reactivity with a series of monoclonal antibodies directed against the oligosaccharide portion of the LPS. Using a similar approach Apicella *et al*

(1985) demonstrated that antigenic heterogeneity also exists among lipid A regions of both type b and non typable LPS. Concomitantly, significant commonality between type b and non typable LPS was demonstrated (Apicella *et al*, 1985; Patrick *et al*, 1987).

Biological Activity of *H.influenzae* LPS.

The lipid A region of *H.influenzae* LPS exhibits endotoxic activity (Flesher and Insel, 1978). Additionally, acquisition of resistance to serum bactericidal activity is known to be associated with a change in LPS phenotype and with increased virulence in type b strains (Anderson *et al*, 1980; Zwahlen *et al*, 1985; Kimura *et al*, 1987).

LPS from non typable *H.influenzae* has been implicated as a cause of epithelial cell damage in chronic obstructive pulmonary disease (see section 1.2) and recently it has been demonstrated that antibodies directed against non typable *H.influenzae* LPS aid pulmonary clearance of bacteria in a mouse model system (Hansen *et al*, 1988). Studies of non typable *H.influenzae* LPS are still incomplete and its role in the host-pathogen interaction has yet to be clearly elucidated.

1.3.4 Outer Membrane Proteins.

The outer membrane is poor in enzymatic activity and contains substantially fewer protein species than the cytoplasmic membrane (Lugtenberg and van Alphen, 1983;

Hammond *et al*, 1984). Outer membranes of *H.influenzae* are typical of those of other Gram negative bacteria in that they contain 20-40 different protein species but 4-6 major proteins account for most of their protein content (Loeb and Smith, 1980). The outer membrane proteins (OMPs) of most Gram negative bacteria can generally be divided into 3 broad groups i.e. lipoproteins, porin proteins and 'minor' proteins.

1.3.4a Lipoprotein.

Lipoprotein is the most abundant protein species in the *E.coli* cell envelope, representing about 6% of the total cell protein (Hammond *et al*, 1984). The best characterized lipoprotein is murein lipoprotein, or Brauns' lipoprotein (Braun and Rehn, 1969), this represents about 1/3 of the lipoprotein in *E.coli* cells and is found covalently attached to peptidoglycan (Hantke and Braun, 1973). It is thought that one function of peptidoglycan linked lipoprotein is to anchor the outer membrane to the cell wall, mutants lacking lipoprotein have been isolated and have been shown to be more prone to release outer membrane vesicles or blebs than wild type cells (Lugtenberg and van Alphen, 1983) suggesting instability of the outer membrane.

A second group of lipoproteins, termed peptidoglycan associated lipoproteins (PAL), are closely but not covalently linked to peptidoglycan and a third group have been identified free in the outer membrane (Ichihara *et al*,

1981). Recently 12 lipoproteins have been described in *H.influenzae* type b ranging in molecular weight from 14-67Kdal (Weinberg *et al*, 1988). One of these, P6 - a 16Kdal protein, has been shown to be covalently attached to peptidoglycan (Green *et al*, 1987, Weinberg *et al*, 1988). This protein has been the focus of much attention as a possible vaccine component (Murphy *et al*, 1986) and passive immunisation with antisera raised against P6 has been shown to confer protection from *H.influenzae* type b disease (Munson and Granoff, 1985; Green *et al*, 1987). P6 is also present in all non typable strains and is known to be a target for bactericidal antibodies against this pathogen (Murphy *et al*, 1986).

On the available evidence it would seem that lipoprotein P6 spans the outer membrane as certain epitopes of this protein are known to be surface exposed (Munson and Granoff, 1985; Murphy *et al*, 1986), this is in contrast to the situation in the *E.coli* outer membrane where murein lipoprotein is inaccessible to specific antibodies and is thought to be deeply buried in the cell envelope (review by Hammond *et al* 1984).

1.3.4b Porin Proteins.

The porin proteins of Gram negative bacteria form non specific diffusion channels for the entry of hydrophilic molecules into the cell. They are non covalently attached to peptidoglycan and may be complexed with LPS (Schindler and

Rosenbusch, 1978). In enterobacteriaceae porins are organised into trimers with subunit masses ranging from 34-42Kdal (Nakae *et al*, 1979; Nikaido and Vaara 1985) and form aqueous channels across the outer membrane (Decad and Nikaido 1976). These porins are involved in the translocation of small hydrophilic molecules such as sugars and amino acids into the cell, large molecules are unable to enter - the exclusion limit for enterobacterial pores being 600-700dal (Nakae and Nikaido 1975).

The OM of *H.influenzae* appears more permeable than that of the enterobacteriaceae, as demonstrated by increased sensitivity to β-lactam antibiotics, (Vachon *et al*, 1985). P2, a 39K major OMP of *H.influenzae* type b has been demonstrated to be extensively complexed with LPS (Gulig *et al* 1985). This OMP has also been identified in non typable *H.influenzae*, in these strains it is reported to vary in molecular weight from 36-41K (Murphy and Bartos, 1988). P2 has been shown to function as a porin in type b and in certain non typable strains (Vachon *et al*, 1985; Burns *et al*, 1985; Burns and Smith, 1987). This protein, when incorporated into liposomes, was demonstrated to render them permeable to oligosaccharides of molecular weight up to 1,400 dal (Vachon *et al*, 1985). This is double the exclusion limit reported for enterobacterial pores (Nakae and Nikaido, 1975). Mutants of non typable *H.influenzae* lacking this protein exhibit slower growth rate and have decreased antibiotic sensitivity compared with their wild type parents

(Burns and Smith, 1987). However, such mutants remain viable, therefore these authors suggest the presence of other porins in non typable strains, either constitutively expressed or inducible.

1.3.4c Minor OMPs. The Gram negative cell envelope is not of invariable composition and may undergo major changes in response to environmental stimuli (Ellwood and Tempest, 1972; Williams *et al*, 1984; Brown and Williams, 1985). Many minor OMPs represent inducible or derepressible proteins which, under appropriate environmental conditions, may reach levels comparable to those of the major proteins. Several proteins may be produced in increased quantities if certain nutrients are lacking in the environment. Examples of such proteins include the Pho E and lam B porins of *E.coli*. These are involved in uptake of phosphate and maltose respectively. They may allow passage of other solutes through the membrane but have specific binding sites for phosphate and maltose, thus selectively increasing uptake of these nutrients (Nikaido 1985)

Perhaps the most well studied group of minor OMPs are those involved in uptake of iron. There is much evidence which suggests that bacteria grown under iron restricted conditions have altered cell envelope properties compared with those grown in conventional laboratory conditions (Anwar *et al*, 1984; Griffiths *et al*, 1983; Black *et al*, 1986). Despite the abundance of iron present in animal hosts

it is relatively unavailable to invading microorganisms. Inorganic ferric iron is highly insoluble at neutral pH and most iron in humans is found intracellularly, bound to ferritin, haemosiderin, myoglobin and haemoglobin (Finch and Hubers, 1982; Griffiths, 1987a) any extracellular free iron is bound by the host iron binding proteins lactoferrin, present on mucosal surfaces and in secretions and transferrin, present in serum (Masson *et al*, 1966; Morgan, 1981; Bezkorovainy, 1987). Both transferrin and lactoferrin have association constants for iron of the order of 10^{36} (Griffiths, 1987a) hence the amount of free iron in the host is extremely low, of the order of 10^{-18}M (review by Finkelstein *et al* 1983). Microorganisms are thought to require between 0.4 and 4μM iron for growth (Bullen *et al*, 1978; Bullen, 1981)) therefore any successful pathogen must be able to compete effectively for iron with the host iron binding proteins.

Gram negative bacteria typically respond to iron restriction by production of their own iron uptake systems (Griffiths, 1987b). Many produce powerful iron chelators, or siderophores, (Lankford, 1973; Neilands, 1981) which are released from the bacterial cell when levels of free iron in the environment are low. These siderophores have an extremely high affinity for ferric iron, for example enterobactin, an iron chelator produced by *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella typhimurium* and some *Shigella* species has an association constant for iron

of 10^{52} (Harris *et al*, 1979). Microbial iron chelators can therefore compete effectively for iron with host iron binding proteins, they chelate iron which is transported back into the cell via specific outer membrane protein receptors (Griffiths, 1987b).

Sciortino and Finkelstein (1983) showed that *Vibrio cholerae* grown *in vivo* in rabbit intestines express iron regulated membrane proteins (IRMPs) and Brown *et al* (1984) have demonstrated the presence of IRMPs in outer membranes of *Pseudomonas aeruginosa* isolated without subculture from the lungs of cystic fibrosis patients. Similarly *Klebsiella pneumoniae* and *Proteus mirabilis* exhibit IRMPs, indicative of iron restricted growth, when harvested directly from infected urine (Shand *et al* 1985). Interestingly IRMPs also appear to be antigenic (Anwar *et al* 1984, Black *et al* 1986), and passive immunization with antibodies raised against IRMPs of *E.coli* has been shown to be protective in an animal model system (Bolin and Jensen 1987).

1.3.5 Iron Restriction of *H.influenzae*.

Haemophilus influenzae has an absolute requirement for haem or certain haem precursors and iron (White and Granick 1963). In the human host there is little free haem available to the invading microorganism, as in addition to the sequestering of free iron by lactoferrin and transferrin there are a variety of host proteins which bind free haem. Any free haemoglobin is readily complexed with haptoglobin and removed from the circulation (Eaton *et al*, 1982),

similarly free haem is complexed with the serum protein hemopexin (Muller-Eberhard, 1970).

Haemophilus influenzae can aquire haem from haemoglobin, haptoglobin and hemopexin (Stull, 1987; Pidcock *et al*, 1988). While these proteins may be utilised by bacteria growing in the blood their availability in sputum remains undetermined. *Haemophilus influenzae* also has the capacity to sequester iron from transferrin via cell surface expressed receptors but few strains have been reported capable of removing iron from lactoferrin (Herrington and Sparling, 1985; Pidcock *et al*, 1988; Schryvers, 1988). Therefore the source of haem or iron for non typable *H.influenzae* growing on mucosal surfaces remains to be determined.

OMP profile changes have been shown to occur when *H.influenzae* type b are subject to haem (Coulton and Pang, 1983; Stull, 1987) or iron restriction (Herrington and Sparling, 1985; Williams and Brown, 1986; Pidcock *et al*, 1987). Different strains appear to vary in their responses to haem limitation. Coulton and Pang (1983) report increases in a 43Kdal protein while Stull (1987) reports a haem repressible 38Kdal protein in a different strain. Iron restriction of *H.influenzae* type b results in the induction of a group of proteins of molecular weight 90-95Kdal in both BHI broth and in defined medium (Herrington and Sparling 1985, Williams and Brown 1986, Pidcock *et al* 1988). However, there appears to be some variation in the nature of

these IRMPs depending on the amount of iron available in the medium. As levels of iron increase up to 1mM additional OMPs appear of molecular weight 77, 65, 24, 83, and 74Kdal (Pidcock *et al* 1988). These authors also report production of hydroxamate type siderophores in both type b and non typable strains which peaks as iron concentration increases. Siderophores appeared to be produced when concentrations of iron present in the media were between 10 and 100μM, at iron concentrations below 10μM siderophores were undetectable and at iron concentrations above 100μM siderophore production fell sharply.

No evidence has been presented regarding expression of *H.influenzae* IRMPs *in vivo*, similarly their antigenicity and potential as immunogens remain to be investigated.

On the available evidence it can be seen that Gram-negative bacteria grown under conventional laboratory conditions (at ideal temperature, aeration and nutrient concentrations) may in fact bear little resemblance to bacteria actually growing in an *in vivo* environment. Adaptability of cell surface components to suit environmental conditions is one means by which bacteria can maximise their chances of survival within a given host tissue.

Non typable *H.influenzae* are successful colonisers of the upper respiratory tract and are a persistant cause of infection in patients with underlying pulmonary disease - surviving despite the presence of an inflammatory response. It is known that bacteria can alter their metabolism and

cell envelope composition in response to changing environmental conditions (Griffiths, 1987b). Analysis of the envelope composition of non typable *H.influenzae* grown *in vitro* under conditions which may approximate to those occurring *in vivo* may help in understanding how this organism survives in and persistently colonises the lungs of individuals with chronic obstructive pulmonary disease.

As *H.influenzae* have an absolute requirement for haem, an iron containing compound, and given that haem and iron are relatively unavailable to bacteria growing *in vivo* (Koskelo and Muller-Eberhard, 1977; Seery and Muller-Eberhard, 1973; Griffiths, 1987a) any attempt to grow *H.influenzae in vitro* in such a way as to mimic conditions which may prevail *in vivo* must involve haem limitation and iron restriction.

1.4 THE HAEMIN REQUIREMENT OF *HAEMOPHILUS INFLUENZAE*.

The main criterion for the inclusion of Gram negative, facultatively anaerobic bacteria in the genus *Haemophilus* is the demonstration of a requirement for either or both of two nutritional factors - haemin (X factor) and nicotinamide adenine dinucleotide - NAD (V factor) or certain precursors of these. Bacteria belonging to the species *H.influenzae* require an exogenous source of both of the above nutrients (Granick and Gilder 1946, Gilder and Granick 1947). Laboratory media designed for cultivation of this species must therefore incorporate both of these factors.

1.4.1 Culture Media.

Blood is a rich source of haemin and NAD and several blood based media provide excellent substrates for growth of *H.influenzae*. Blood agar itself however, is not a satisfactory medium because although haemin is readily available, NAD is relatively unavailable due to the action of cell-associated NAD nucleosidase enzymes which convert NAD to nicotinamide, a substrate which cannot be utilised by haemophili (O'Reilly and Niven, 1986). Colonies formed by *H.influenzae* on blood agar are consequently small and easily overlooked. This problem can be overcome by inoculating blood agar plates with a streak of *Staphylococcus aureus*. This bacterium secretes V factor into the surrounding medium and haemophili, utilising this, display enhanced growth

around *S.aureus* colonies. This 'satellitism' is a useful means of identifying *Haemophilus sp.* on blood agar plates (Turk, 1981; Kilian and Biberstein, 1984).

Alternatively, blood agar may be subjected to heat (80°C for several minutes). Heating causes lysis of red blood cells thereby releasing intracellular haemin and NAD and also inactivates NAD nucleosidase enzymes. The brown coloured media thus formed is termed 'chocolate' agar. Other suitable media may be prepared by the addition of peptic digest of blood (Fildes enrichment) or of boiled filtered blood (Levinthals medium) to nutrient agar or broth. These are all excellent media for the *in vitro* cultivation of *H.influenzae* (Turk 1981, Turk and May, 1967).

The majority of *H.influenzae* will also grow on rich complex media which has been supplemented with pure preparations of haemin and NAD. An advantage of using this method is that the precise amounts of X and V factors available in the medium are known and can be varied, thus facilitating studies of the precise requirements of individual strains for each factor (White and Granick 1963, Coulton and Pang 1983). Discs impregnated with X factor, V factor, or both are commercially available and many labs use these for identification purposes. On unsupplemented brain heart infusion agar, growth of *H.influenzae* occurs only in the zone surrounding discs containing both X and V factors placed on the surface of the plate (Fig. 1.4.1).

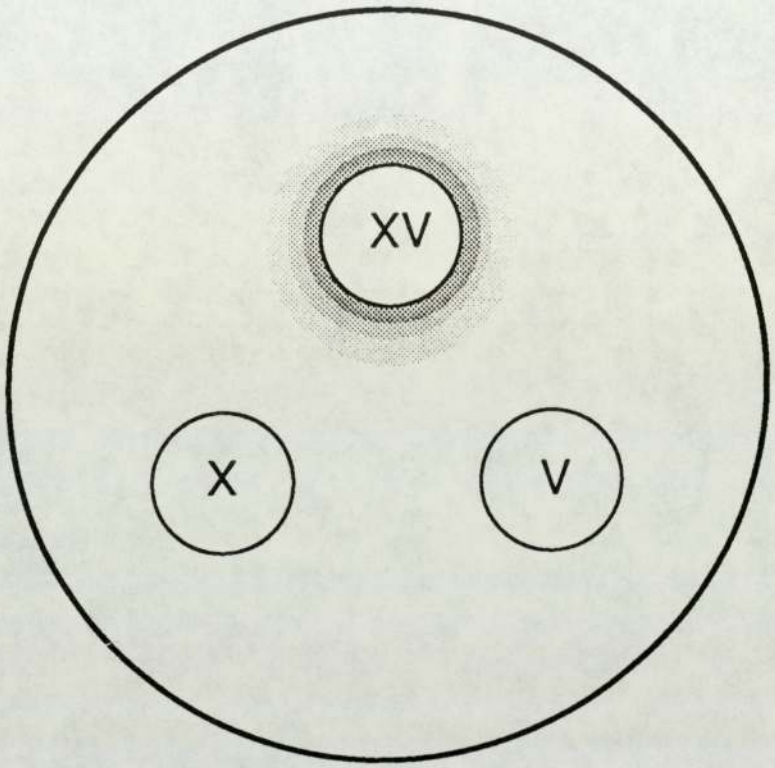


Figure 1.4.1.

Growth of H. influenzae on brain heart infusion agar supplemented with X and V factor impregnated discs

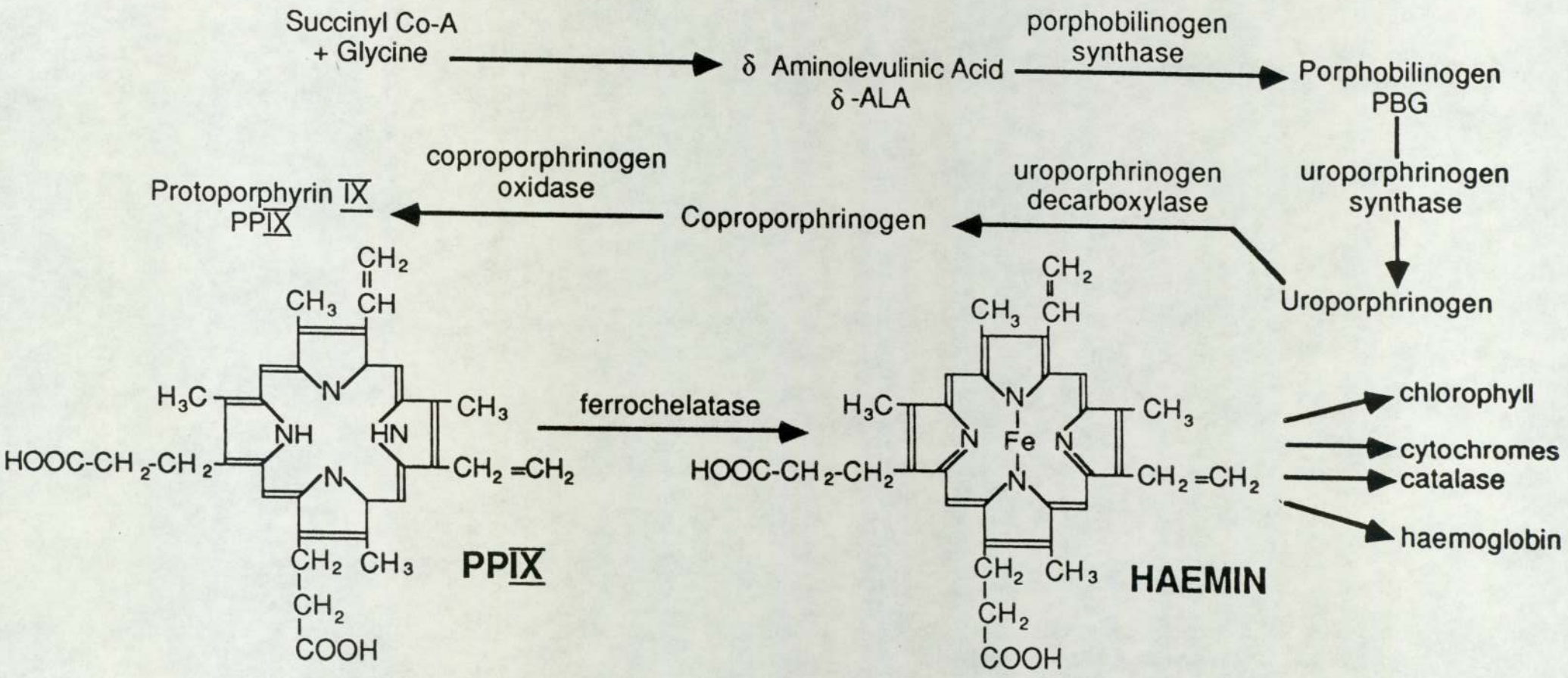
(from Turk, 1981)

1.4.2. *H.influenzae* Haemin Requirements.

The haemin biosynthetic pathway is illustrated diagrammatically in Fig.1.4.2. The enzymes and intermediates involved in the synthesis of protoporphyrin IX from δ-aminolevulinic acid are the same in plants, animals and micro-organisms regardless of whether the end product is cytochrome-haemin, haemoglobin, or chlorophyll. In this respect the pathway is unique (White and Granick 1963). Species which lack the ability to synthesise haem are found exclusively among micro-organisms (Lascelles 1961) and of these *H.influenzae* has been the most extensively studied. Among *Haemophilus* species there is a perfect correlation between haemin requirement and the inability to convert δ-aminolevulinic acid (δ-ALA) to porphyrins (Biberstein *et al* 1963). White and Granick (1963) showed that neither porphobilinogen, uroporphrinogen, or coproporphrinogen could be utilised as haem precursors by *H.influenzae*. The enzymes catalysing the conversion of these substrates to protoporphyrin IX (PPIX) were found to be missing or inactive in all strains examined. However, the same authors report that *H.influenzae* can utilise PPIX as a porphyrin source. All strains therefore possess the enzyme ferrochelatase (Porra and Jones 1963) which catalyses the insertion of iron into the centre of the porphyrin ring of PPIX, thus forming haem. Haem, once formed may be incorporated into catalase or modified into various

Figure 1.4.2 The Haemin Biosynthetic Pathway

(White and Granick, 1963, Kilian 1974)

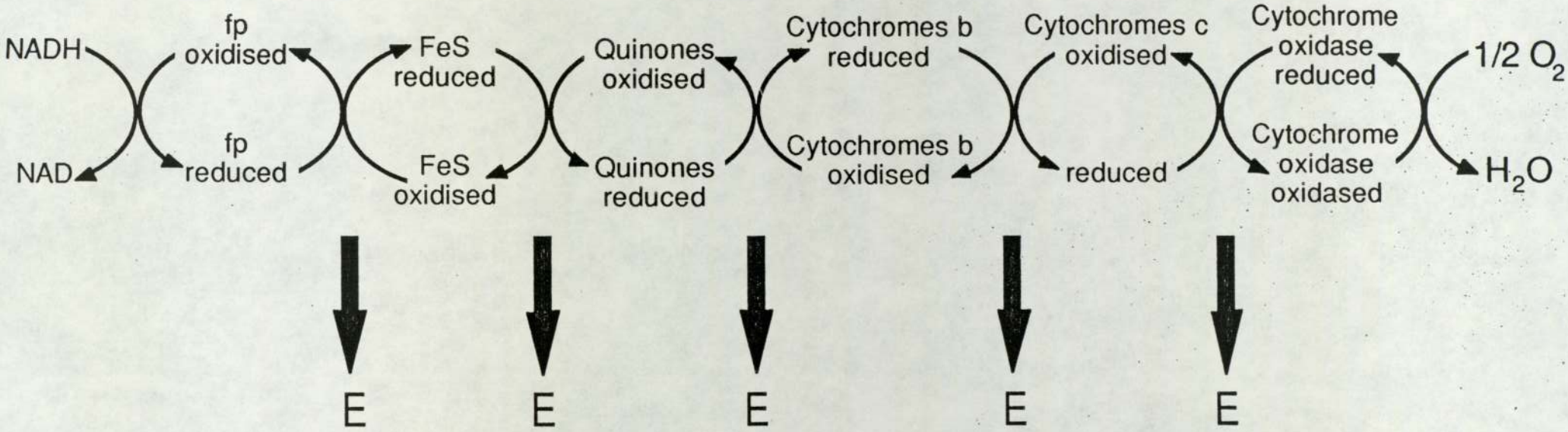


cytochrome haemins (White and Granick 1963). The means of uptake of exogenously supplied haemin or PPIX by *Haemophilus influenzae* remains to be elucidated.

1.4.3. Cellular Utilisation of Haem.

Haem is an important component of both catalase and cytochromes, hence a supply of haem is essential to aerobically growing cells. Aerobic metabolism exposes the cell to the deleterious effects of endogenously derived oxygen free radicals. In response to this bacteria have several antioxidant enzymes of which catalase is one. Cytochromes are haemoproteins which function as electron carriers in the respiratory chains of oxidative phosphorylation (for review see Jones and Poole, 1985). During the process of oxidative phosphorylation energy is generated as electrons, or reducing equivalents, are transferred from organic donors such as NADH or succinate to a final electron acceptor (O_2 in aerobic systems). This occurs via a series of electron carriers which may include flavoproteins, iron-sulphur compounds, quinones and cytochromes. Reducing equivalents are transferred down the chain through a series of steps involving exergonic redox reactions from substrates with a tendency to donate electrons to those with a tendency to accept them, i.e. in the direction of increasing redox potential (Fig.1.4.3a). The amount of energy generated at each step is determined by differences in redox potential between donor and acceptor

FIGURE 1.4.3a.



The electron transport chain of oxidative phosphorylation

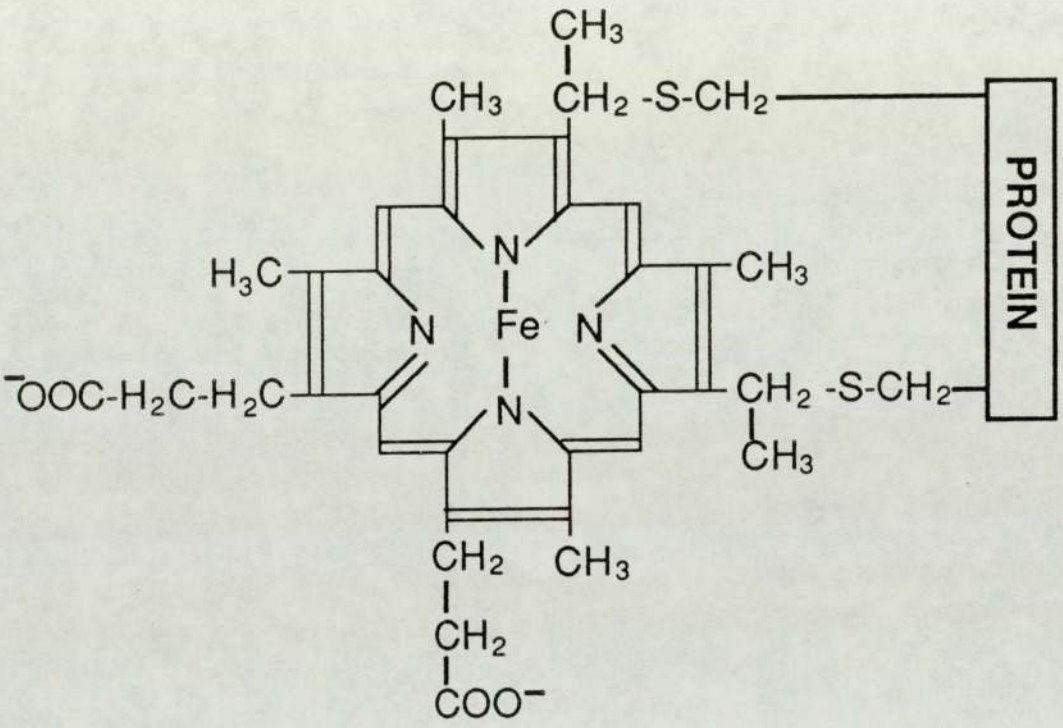
couples. Respiratory assemblies are located in the cytoplasmic membrane of bacterial cells and energy generated may be used to produce a membrane associated charge gradient which in turn can be used to drive the synthesis of ATP by membrane bound ATP phosphohydrolase enzymes (Jones 1982).

Cytochromes are proteins which contain a haem prosthetic group (Fig.1.4.3b). They function as electron carriers by alternating between reduced (Fe^{2+}) and oxidised (Fe^{3+}) forms. There are 4 classes of cytochromes generally recognised. These are designated types a,b,c, and d. They have distinctive structures and properties, differing from one another in the way in which haem groups are linked to protein components and also in the types of side chains present on haem prosthetic groups (Jones and Poole 1985). All, with the exception of certain c type cytochromes (White 1962, 1963, Niven 1984) are tightly membrane bound. All a and d type cytochromes and certain b types are termed cytochrome oxidases. These are auto-oxidisable cytochromes which can react directly with molecular oxygen and therefore are the terminal members of respiratory chains (Jones and Poole 1985).

1.4.4a. The Analysis of Cytochromes.

Cytochromes undergo spectral changes concomitant with oxidation and reduction. These differences can be exploited to gain qualitative and quantitative information about the cytochrome complement of any particular bacterial species.

FIGURE 1.4.3b.



Cytochrome c.

The structural integrity of the cytoplasmic membrane must be preserved for the functionality of bacterial respiratory systems (White and Smith 1962) and therefore analysis of cytochromes must be performed on membrane preparations. Such preparations are optically unfavourable for conventional spectrophotometry as changes in absorbance due to reduction of cytochromes represent very small spectral changes against a highly light scattering background. To overcome this problem analysis of cytochromes is carried out in spectrophotometers which are capable of analysing two samples simultaneously (one oxidised, the other reduced) and recording only the difference between them - i.e. reduced-oxidised difference spectra. Split beam spectrophotometers, where a single beam is shared between two samples via an oscillating mirror, or computer-assisted spectrophotometers with the ability to store scans in memory and subtract subsequent scans from each other are ideal for the analysis of cytochrome difference spectra.

Cytochromes of different types give characteristic absorbance peaks on reduced-oxidised difference spectra. If oxidation and reduction of all cytochromes is assured by chemical means then the area under each absorbance peak is proportional to cytochrome concentration. Spectrophotometry thus facilitates both qualitative and quantitative analyses (review - Jones and Poole 1985).

1.4.4b. Respiratory Systems in *Haemophilus influenzae*.

When grown under aerobic conditions in media containing a rich supply of haem *H. influenzae* produce a respiratory chain consisting of 2 flavoproteins and 6 cytochromes - b, c and 4 cytochrome oxidases - (White, 1963) see Fig.1.4.4. The relative amounts of each cytochrome species produced vary according to phase of growth and levels of aeration. In general cytochrome oxidases become more numerous relative to other cytochromes as stationary phase progresses and as levels of available oxygen decrease (White 1963).

Final levels of growth achieved by *H. influenzae* cultures are directly proportional to the amount of haem available in the growth medium (White and Granick, 1963). Additionally, as haemin concentration increases rates of oxygen uptake also increase (Biberstein and Spencer 1962). This would reflect progressively increasing concentrations of cytochromes. This was confirmed by White (1963) who demonstrated that the amount of cytochrome produced by *H. influenzae* decreases with falling haemin concentration. At very low haemin concentration no respiratory pigments are detectable in aerobically growing *H. influenzae* yet under such conditions growth is still haemin dependent. Catalase production in this species is also directly proportional to the amount of haemin present in the growth medium (Biberstein and Gills 1961).

This ability to limit cytochrome production (a haem sparing mechanism) in unfavourable environmental conditions e.g. low

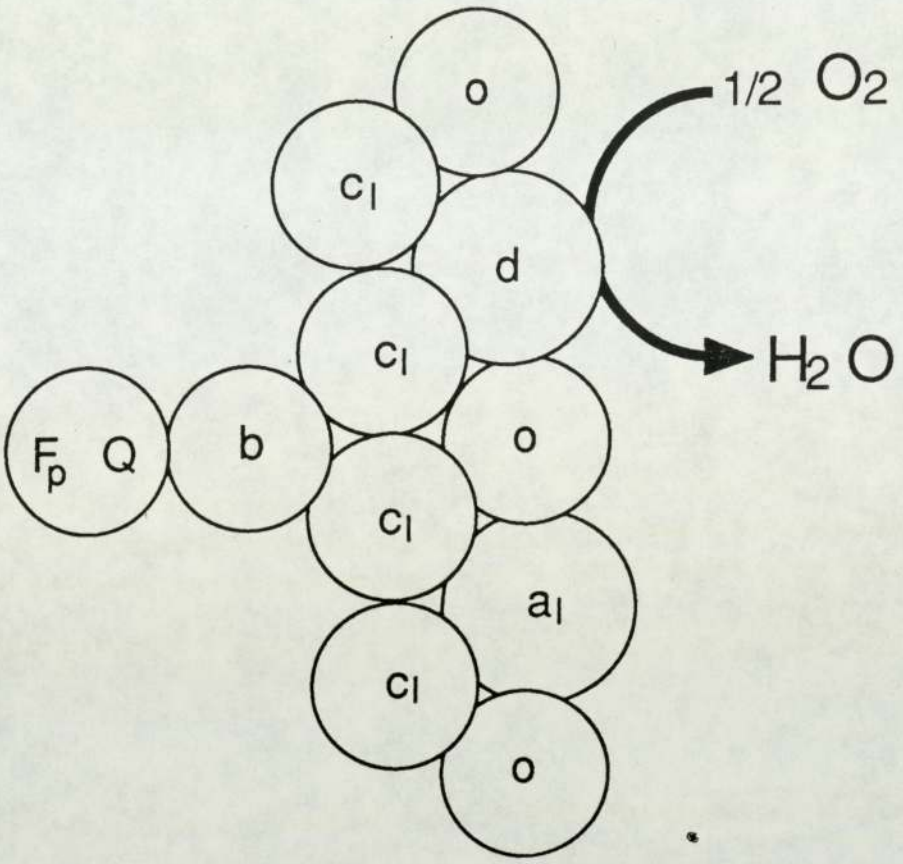


FIGURE 1.4.4.

Representation of the electron transport system formed by H. influenzae

(Adapted from White, 1963)

aeration or limited haem availability is peculiar to haem requirers among *Haemophilus* species. Haemin independent *Haemophilus* species such as *H.parainfluenzae* do not have this flexibility. Paradoxically therefore, haem requiring species are able to survive in certain conditions which may inhibit the growth of haem independent Haemophili (White 1963).

1.5. AIMS.

Surface structures play a crucial role in interaction with host defences in infection (Costerton *et al*, 1981; Smith, 1977). If expressed *in vivo*, *H.influenzae* iron regulated membrane proteins (IRMPS) may provide the basis for development of effective vaccines against non typable strains in chronic obtructive pulmonary disease and in other situations.

Individuals with bronchiectasis form a heterogeneous group, severity of disease varies from those who normally produce mucoid sputum to those whose sputum is persistently purulent having a heavy microbial load (Hill *et al*, 1986). An analysis of the immune response to outer membrane antigens of non typable *H.influenzae* in these patients may provide clues as to why *H.influenzae* proliferates in the lungs of people who have purulent sputum and not in the lungs of

those with mucoid sputum. It has been suggested that individuals whose sputum is normally mucoid may have antibodies to key antigens, which are absent in those individuals who persistently produce purulent secretions (Turk and May, 1967).

Bacterial cells are extremely versatile. Their physiology and structure at any one time being determined by a number of factors, both microbial and environmental (Brown and Williams, 1985; Griffiths, 1987b). In order to maximise the probability of effectively combatting bacterial infection in man, it is important to gain as much information as possible about environmental factors which may be in operation at sites of infection and to ascertain how bacteria respond to these in terms of altered metabolism, cell envelope composition and antigenicity. Growth media can then be designed so that bacteria cultured *in vitro* reflect as accurately as possible their *in vivo* counterparts.

Iron availability is one factor which has been shown to have a profound effect on the envelope composition of several bacterial species, resulting in the expression of new outer membrane protein antigens (Black *et al*, 1986; Anwar *et al*, 1984; Ward *et al*, 1988). The effect of haem limitation on *H.influenzae* type b has been described (Coulton and Pang, 1983; Stull, 1987), and inducible IRMPs have been demonstrated in *H.influenzae* type b grown *in vitro* under

iron restricted conditions (Herrington and Sparling, 1985; Pidcock *et al*, 1988). However there have been no studies performed to determine whether *H.influenzae* IRMPs are antigenic, or whether they are expressed by bacteria growing *in vivo*.

The aims of this study were to investigate the effects of haem limitation and iron restriction on the OM composition and on respiratory systems of non typable *H.influenzae*. It was proposed to investigate the immune response of individuals with bronchiectasis of varying degrees of severity to haem limited and iron restricted outer membranes of their homologous isolates with a view to determining a) whether non typable *H.influenzae* growing *in vivo* in the human lung express a haem and iron restricted phenotype and b) whether there are significant differences in immune response to *H.influenzae* OM antigens between individuals producing mucoid and those producing purulent sputum.

It was proposed to further define the cell surface and physiological characteristics of *in vivo* grown *H.influenzae* using a rat lung model of chronic pulmonary infection (Slater, Abstracts of the American Society for Microbiology, 1986 Abstract B195), and to directly observe the *in vivo* composition of the OM of non typable *H.influenzae* using bacteria harvested directly, without subculture, from infected lungs. By investigation of antibodies present in

patients serum and sputum and in serum and lung lavage fluid obtained from animal infections it was proposed to gain qualitative and quantitative information about OM antigens expressed by non typable *H. influenzae* growing *in vivo* in a pulmonary environment. Additionally it was proposed to apply information gained *in vitro* about respiratory systems of *H. influenzae* grown under conditions of haem and iron restriction to *in vivo* material and thus gain information about levels of haem available to *H. influenzae* growing in the lungs.

Thus it was hoped to determine whether *H. influenzae* grown *in vitro* under conditions of haem and iron restriction reflect cells growing *in vivo* in pulmonary infections more accurately than non typable *H. influenzae* grown under conventional laboratory conditions.

2. MATERIALS AND METHODS.

2.1. MATERIALS.

2.1.1. Equipment.

Anaerobic Cabinet : Germfree Labs. Inc., Miami, Fla. USA. with modification by Don Whitley Scientific Ltd., Shipley, W. Yorkshire.

Balances : Macro - Oertling MC22, Oertling Ltd., Orpington, Kent.
Micro - Sartorius 1702, Sartorius Instruments Ltd., Belmont, Surrey.

Candle Extinction Jar : Gaspak Systems, Oxoid UK.

Centrifuges : 1. MSE Super Minor bench top centrifuge. Measuring and Scientific Equipment, Crawley, W. Sussex.
2. Eppendorf centrifuge - MSE Microcentaur. Crawley, W. Sussex.
3. Beckman J2-21 high speed centrifuge, Beckman RIIC Ltd., High Wycombe, Bucks.
4. Ultracentrifuge - Model L8-M, class H, 70Ti rotor, Beckman RIIC Ltd.

Chart Recorders : 1. Model 555, Linear Instruments Corp., Irvine, CA. USA.
2. Hewlett Packard HP7010B X-Y recorder.

ELISA Plate Washer: Model 120, Flow Laboratories Ltd., Irvine, Scotland.

ELISA Plate Reader: Titertek® Multiscan Model 310C, Flow Laboratories Ltd.

ELISA Plates	:	Dynatech Microtiter, Flow Laboratories Ltd.
Freeze Dryer	:	Edwards Modylo Freeze Dryer, Edwards High Vacuum Ltd, Crawley, W. Sussex.
French Press	:	Amicon Corp., Gloucester, UK.
Electrophoresis Units.	:	1. Large Units - made in house by Aston Services. 2. Minigel Apparatus - Model II Mini - Protean 125 BR vertical slab gel, Bio-Rad Laboratories Ltd., Watford, Herts.
Immunoblotting Equipment	:	Transblot Cell. Bio-Rad Laboratories Ltd.
Incubator	:	IG10 Gyratory Shaker, New Brunswick Scientific Co. Inc. Edison, NJ. USA.
Membrane Filters	:	Gelman Acrodisc (0.2μ), Gelman Sciences, Brackmills, Northampton.
Oxygen Electrode	:	Rank Brothers., Bottisham, Cambs. UK.
pH Meter	:	PTI-15, Fisons Scientific Apparatus, Loughborough, Leics. UK.
Pipettes	:	Gilson Pipetman P200 and P1000, Anachem, Luton, Beds. UK. Titertek multichannel pipette - Flow Laboratories Ltd.
Power Supplies (D.C)	:	Model 200/2.0 and 250/2.5 power supplies, Bio-Rad Laboratories Ltd.

Power Supplies (D.C)	:	Model SAE 2761, Shandon Southern Products Ltd., Astmoor Ind. Est., Runcorn, Cheshire.
Rotary Evaporator	:	Buchi Rotavapor R., Fisons Scientific Apparatus.
Sonicator	:	Sonoprep 150, MSE., Crawley, W. Sussex.
Spectrophotometers:		1. LKB Ultrospec 4050, Pharmacia House, Milton Keynes, Bucks. 2. Two wavelength double beam spectrophotometer Model 356, Perkin-Elmer Corp., Norwalk, CT. USA. 3. Johnson Foundation DBS-3 dual wavelength scanning spectrophotometer as described by Williams and Poole (1987).
Syringes and Needles	:	1ml, 5ml and 10ml sterile syringes supplied by Beckton, Dickinson & Co. Ltd. York House, Wembley, Middx. UK. Single use sterile hypodermic needles supplied by Terumo Corp., 3030 Leuven, Belgium.
Water Bath	:	Grant Instruments Ltd., Barrington, Bucks. UK.

2.1.2. Chemicals and Reagents.

All chemicals and reagents used were supplied by Sigma Chemical Company, Poole, Dorset. or by British Drug Houses

(BDH), Poole, Dorset., except where otherwise stated. Glass distilled water was used throughout.

2.1.3. Antibody-Conjugates used in Immunological Assays.

Horseradish peroxidase (HRP) conjugated protein-A, HRP conjugated goat anti human IgA and IgM, and HRP conjugated goat anti rat IgG were supplied by Sigma Chemical Company. HRP conjugated goat anti human IgG was supplied by Dako Ltd. High Wycombe Bucks.

Absorption of Conjugates

All conjugates, with the exception of protein-A-peroxidase, had to be absorbed with outer membranes (OMs) of *H. influenzae* (see section 2.3.1.) before use in order to remove any antibodies to OM components which were present. OMs were added to each conjugate to give a final OM protein concentration of 2mg/ml. The mixture was incubated for 2 hours at 37°C. Insoluble material was then removed by centrifugation (48,000 x g, 4°C, 40 min). The process was repeated until conjugates gave A_{450} readings below 0.2 in an OM ELISA system (see section 2.4.4). Conjugates were stored at 4°C until used.

2.1.4. Blood and Sputum Samples.

Fresh blood was allowed to clot for 2 hours at 37°C, the serum was then removed by centrifugation (1,000 x g, 18°C, 10 min). Serum was stored in 200µl aliquots at -20°C.

Each sputum sample was collected over a 4 hour period after waking by each patient involved in the study. Sol phase

sputum was prepared by ultracentrifugation of whole sputum at 90 000 x g for 1 hour. The supernatant (sol phase) was removed and stored at -20°C in 200µl aliquots until used.

2.1.5. Culture Media.

Brain heart infusion (BHI) agar and BHI broth were supplied by Lab M Ltd., Bury, Lancs.

Chocolate agar was prepared by adding 2.5% v/v defibrinated horse blood (Lab M) to molten BHI agar which had been allowed to cool to 60°C. The agar-blood mixture was then incubated at 80°C until a brown colour was observed. Plates were then poured.

Agar plates containing BHI agar alone, BHI supplemented with 10µg/ml NAD, and BHI supplemented with 10µg/ml NAD and 10µg/ml haemin (bovine type I) were used to identify bacteria as *H. influenzae*.

2.1.6. Media Supplements

Stock solutions of media supplements were prepared as follows.

a) Haemin. 100mg of haemin (bovine type I) was dissolved using 1N NaOH, adjusted to pH 7.0, and made up to 100ml using distilled water.

b) Protoporphyrin IX. Protoporphyrin IX (PPIX) was dissolved in distilled water to give a final concentration of 1mg/ml.

Haemin and PPIX stock solutions were heat sterilised, and stored at 4°C prior to use.

c) NAD. NAD was dissolved in distilled water and sterilised by filtration using a 0.2μ membrane filter. NAD stock solutions were stored at -20°C.

d) Desferal. Desferal (desferrioxamine mesylate - Ciba Geigy Ltd., Horsham, Surrey.) was made up in distilled water to a final concentration of 30mM, filter sterilised and stored at -20°C until use.

2.2 CULTURE AND IDENTIFICATION OF *H. INFLUENZAE.*

2.2.1. Bacterial Strains.

Non typable *H. influenzae* strains HM1-HM29 were all isolated from sputum samples which were kindly provided by Dr. R.A. Stockley of The General Hospital, Birmingham, UK. Bacteria were stored at -70°C in haemin and NAD supplemented BHI broth containing 15% glycerol until use.

2.2.2. Culture of *H. influenzae* from Sputum.

Before culture for *H. influenzae* sputum samples were washed with 10ml of 0.85% NaCl in order to remove any saliva present (Bartlett and Finegold, 1978). After washing, sputum was collected by centrifugation (1,000 x g, 18°C, 5 min).

Sputum samples were streaked out onto chocolate agar plates which were then incubated overnight at 37°C in a candle extinction jar. Any colonies having a grey, translucent

appearance on chocolate agar were re-inoculated onto each of the following: (i) BHI agar

(ii) BHI agar + 10µg/ml NAD

(iii) BHI agar + 10µg/ml NAD + 10µg/ml Haemin

Bacteria which grew only on (iii) were identified as *H. influenzae.*

Digestion of Sputum with Pancreatin

After washing with saline, pancreatin enzyme was added to some sputum samples to give a final concentration of 1mg/ml. The reaction was allowed to proceed for 1 hour at 37°C. Sputum was then cultured for *H. influenzae* as above.

2.2.3. Serotype and Biotype Analysis of *H. influenzae* Isolates.

a) Serotyping *H. influenzae* strains were serotyped using capsule specific antisera (types a-f, Difco Laboratories Ltd. Detroit, Michigan. USA). Strains were identified as non typable by failure to aggutinate in the presence of type specific antisera.

b) Biotyping. Biotyping of *H. influenzae* strains was performed using API 20E strips (API Systems, La Balme Les Grottes, 38390 Montalieu-Vercieu, France). Each strain was inoculated into 20ml of BHI broth supplemented with 10µg/ml haemin +10µg/ml NAD and allowed to grow to early stationary phase. Cells were harvested by centrifugation (17,000 x g, 4°C, 10 mins) in sterile centrifuge vessels, washed once with sterile

0.85% NaCl and finally resuspended in 0.85% NaCl supplemented with 10μg/ml haemin and 10μg/ml NAD to an OD_{470} of 1.0.
This bacterial suspension was used to inoculate API 20E strips, which were then incubated for 24 hours at 37°C. API strip results were developed and interpreted according to the manufacturers instructions.
H.influenzae isolates were biotyped on the basis of ornithine decaboxylase, urease, and indole production according to the scheme devised by Kilian (1976).

2.2.4. Batch Culture of *H.influenzae*.

Bacteria were cultured in BHI broth supplemented with 10μg/ml NAD and with excess or growth yield limiting concentrations of either haemin or PPIX. Iron restricted conditions were achieved by substituting PPIX for haemin in the growth medium and adding the iron chelator desferal at a concentration of 30μM. Several colonies from chocolate agar plates were used to inoculate 20ml of BHI broth contained in 100ml erlenmeyer flasks fitted with polyurethane foam closures. After 15 hours cells from these cultures were used to inoculate 1L flasks containing 200ml of supplemented BHI broth, or 5L flasks containing 2L of supplemented BHI broth. An inoculum size of 0.1% was used. All cultures were incubated at 37°C in an orbital shaking incubator set at 180rpm in an environment not enriched for CO_2. Growth was monitored by measurement of optical density at 470nm, a wavelength at which there was

minimal background absorbance due to media components or bacterial products.

2.2.5. Anaerobic Culture.

H. influenzae were grown in an anaerobic cabinet in closed 500ml bottles containing 400ml BHI broth + 10µg/ml NAD and further supplemented as follows:

a) unsupplemented

b) 5µg/ml haemin

c) 0.25µg/ml haemin

e) 5µg/ml PPIX

f) 0.25µg/ml PPIX

The media was incubated in an anaerobic environment for 48 hours prior to inoculation, to ensure that it became completely reduced.

2.3. PREPARATIVE TECHNIQUES.

2.3.1. Preparation of Bacterial Membrane Fractions.

a) Outer Membranes. Outer membranes (OMs) were prepared by sarkosyl extraction according to the method of Filip *et al* (1973). Stationary phase cells were harvested by centrifugation (17,000 x g, 4°C, 10 min), washed with an equal volume of 0.85% w/v NaCl and finally resuspended with distilled water. Bacteria were disrupted by sonication (10 x 20s, 18µ amplitude) and the detergent sodium - N - lauroyl sarcosine was added to 2% w/v final concentration. After 30

min incubation at room temperature unbroken cells were removed by centrifugation (10,000 x g, 4°C, 10 min). Outer membranes were then harvested by ultracentrifugation (150,000 x g, 4°C, 40 min). After washing with distilled water, OMs were finally resuspended in a small volume of distilled water and stored at -20°C.

2.3.2. Preparation of Cell Fractions for Spectrophotometric Analysis of Cytochromes.

Cells of *H.influenzae* from stationary phase (4 x 200ml) and from log phase cultures (8 x 200ml) were harvested by centrifugation (17,000 x g, 4°C, 10 min), washed with an equal volume of phosphate buffered salts solution (PBS) containing 25mM Na_2HPO_4, 100mM NaCl, 10mM KCl, 5mM $MgSO_4$, and HCl to pH 7.4. Cells were resuspended in 20ml PBS and disrupted by sonication (10 x 20s, 18u amplitude). Samples were contained in a 50ml beaker on ice during sonication and were allowed to cool for one minute between bursts. Unbroken cells were removed by centrifugation (10,000 x g, 4°C, 10 min) and the top 80% of the supernatant fraction was carefully removed. This is referred to as the cell free extract (CFE). Ten ml of the CFE was stored on ice, the remainder was subjected to high speed centrifugation (180,000 x g, 4°C, 3 hours). The supernatant, or soluble fraction (S180) was carefully removed and the pellet (P180), which consisted of bacterial membranes, was rinsed with PBS and

resuspended in 5ml cold PBS using a glass homogeniser. All cell fractions were stored on ice until used.

2.3.3. Isolation of Lipopolysaccharide (LPS)

a) Proteinase K Digestion (Hitchcock and Brown 1983). Stationary phase cells were harvested by centrifugation (17,000 x g, 4°C, 10 min), washed with an equal volume of 0.85% w/v NaCl, and finally resuspended with distilled water to a final OD_{470} of 1.0. Proteinase K, dissolved in SDS-PAGE sample buffer (see table 2.4), was added to a final concentration of 2.5mg/ml. The cells and proteinase K mixture was then incubated for 1 hour at 60°C. Samples were then heated to 100°C for 10 minutes before being loaded onto SDS-PAGE gels (see 2.4.1).

b) Purification of LPS for use in Immunological Assays. LPS was prepared from *H. influenzae* according to the method of Galanos *et al* (1969). Stationary phase cells were harvested from 2x2L of culture and washed once using distilled water. The bacteria were treated successively with ethanol, acetone and twice with distilled water and were dried *in vacuo*. 200ml of extraction mixture consisting of phenol, chloroform and petroleum ether (b.p. 40-60°) in a volume ratio of 2:5:8 was added to the dried bacteria with mixing, so that the cells formed a fine suspension. The bacterial cells were then removed by centrifugation (10,000 x g, 4°C, 10 min) and the supernatant was filtered through filter paper into a round

bottomed flask. The cell pellet was retained and the extraction procedure repeated once more.

Petroleum ether and chloroform were removed completely from the pooled supernatant solutions using a rotary evaporator. The solution was then transferred to a glass test tube and distilled water added dropwise until the LPS formed a precipitate. The precipitate was collected by centrifugation (1,000 x g, 10 min) and was then washed 3 times with 80% phenol and finally with ether before being dried *in vacuo*. After drying the LPS was taken up in distilled water, heated to 45°C and a vacuum was applied to remove air. The solution was shaken for a few minutes until it became viscous. LPS was harvested by ultracentrifugation (150,000 x g, 4°C, 4 hours), redissolved in distilled water and freeze dried.

2.4. ANALYTICAL PROCEDURES.

2.4.1. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

a) Outer Membrane Proteins (OMPs) were separated by SDS-PAGE using 10% gels (5cm in length, Bio-Rad Mini Protean II system) essentially according to the method of Lugtenberg *et al* (1975). The exact composition of the gels and buffer solutions is shown in table 2.4.1. Outer membranes, suspended in sample buffer containing 0.1% bromophenol blue dye were

TABLE 2.4.1.

COMPOSITION OF GELS AND BUFFERS FOR SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS.

INGREDIENT	RUNNING GEL		STACKING GEL	SAMPLE BUFFER
	10%	14%		
Stock I :	2.5ml	18.75ml		
Stock II :			5.0ml	
10% SDS :	0.3ml	1.50ml	0.3ml	5.0ml
1.5M tris-HCl pH 8.0:	3.75ml	18.75ml		
0.5M tris-HCl pH 6.8:			7.5ml	2.5ml
Distilled water :	3.50ml	18.50ml	16.0ml	5.0ml
10% APS :	0.04ml	0.20ml	0.1ml	
TEMED :	0.028ml	0.14ml	0.08ml	
Glycerol :				2.5ml
2-mercapto-ethanol :				0.5ml
5% bromo-phenol blue:				0.2ml

Stock I = 44g acrylamide, 0.8g N,N' methylene bisacrylamide, in 100ml distilled water.

Stock II = 30g acrylamide, 0.8g N,N' methylene bisacrylamide in 100ml distilled water.

APS = Ammonium persulphate (prepared freshly immediately before use).

TEMED = N,N,N',N' tetramethylene diamine.

Electrode buffer = 28.8g glycine, 6g tris, 20ml 10% SDS in 2L of distilled water to pH 8.0.

Tris = tris (hydroxymethyl) aminoethane.

maintained at 100°C for 10 minutes before being applied to gels. Ten µg of protein was loaded onto each lane of the gel and electrophoresis was carried out at 200V for 35 minutes.
After electrophoresis, separated proteins (or peptides) were stained overnight using 0.1% w/v coomassie blue R250 in 5% methanol - 10% acetic acid. Gels were then destained for 3-4 hours in 5% methanol - 10% acetic acid solution until proteins were visible as dark blue bands against a clear background.
Molecular weights of proteins separated by SDS-PAGE were estimated from a calibration curve of $\log_{10}$ molecular weight of protein standards against Rf values.

$$Rf = \frac{\text{Distance moved by standard protein through the running gel}}{\text{Distance moved by the bromophenol blue dye front.}}$$

Standard marker proteins used included lysozyme (14.3K), β lactoglobulin (18.4K), trypsinogen (24K), carbonic anhydrase (29K), ovalbumen (45K), bovine serum albumen (66K), and β amylase (200K).

b) Lipopolysaccharide. Separation of LPS by SDS-PAGE was achieved using 14% gels prepared as shown in table 2.4.1, essentially according to the method of Lugtenberg *et al* (1975). Large gel systems measuring 200mm x 250mm x 2mm were

used. Electrophoresis was carried out at a constant current of 30mA until the bromophenol blue dye front had reached the bottom of the running gel.

2.4.2. Silver Stain of LPS.

LPS, separated by SDS-PAGE, was stained using silver nitrate according to the method of Tsai and Frasch (1982). LPS was fixed in the polyacrylamide gel by overnight immersion in a solution containing 40% ethanol - 5% acetic acid. This solution was then replaced with fresh fixing solution containing 0.7% periodic acid and oxidation was allowed to proceed for 45 minutes. Gels were then washed for 90 minutes using large volumes of double distilled water and applying fresh water every 15 minutes. After washing gels were stained, with shaking, for 45 minutes. Staining solution was prepared by adding 5ml of 20% w/v silver nitrate solution to a mixture containing 28ml 0.1M NaOH and 2ml 35% ammonia solution. The volume of the staining reagent was then made up to 150ml with double distilled water. The gels were washed for a further 45 minutes after staining. Finally the stain was developed with 1L of double distilled water containing 50mg of citric acid and 0.5ml of 37% formaldehyde. After LPS in the gel became visible as dark brown bands, the reaction was terminated by replacing the developer with fixing solution.

2.4.3. Immunoblotting. /

2.4.3. Immunoblotting.

OMPs, previously separated by SDS-PAGE, were electrophoretically transferred onto nitrocellulose paper (Bio-Rad Laboratories Ltd.) and antigenic sites visualised according to the method of Towbin *et al* (1979). Briefly, gels were placed on top of nitrocellulose sheets, sandwiched between sheets of blotting paper surrounded by scotch-brite pads and placed in a trans-blot cell filled with tris-glycine-methanol buffer (125mM tris, 192mM glycine, 20% v/v methanol pH 8.3) cooled to 0°C. Electrophoretic transfer was carried out at 80V for 1 hour followed by 50V for 15 hours. The protein-impregnated nitrocellulose paper was first incubated for 1 hour at room temperature in tris-buffered saline (TBS pH 7.4) containing 0.3% v/v tween 20 (TBS-tween) and then incubated (4 hours) with serum diluted 1/25 with TBS-tween. After washing with TBS the paper was incubated (2 hours) with HRP-conjugated protein-A, anti human or anti rat IgG as required, diluted 1/1000 in TBS-tween. After further washing with TBS, antigenic sites were visualised with a 25µg/ml solution of 4-chloro-1-naphthol in TBS containing 0.01% v/v H_2O_2.

Complete transfer of proteins from gel to nitrocellulose paper was confirmed by staining a sample strip of nitrocellulose paper with 1% amido black in 5% methanol-10% acetic acid and also by staining the gel, after transfer, with coomassie blue (see 2.4.1) to determine the extent of transfer to the nitrocellulose paper.

2.4.4. Enzyme Linked Immunosorbent Assay (ELISA).

ELISA assays were performed essentially according to the method of Gnehm *et al*, 1985.

a) Outer Membranes. ELISA microtiter plate wells were coated with 250µl of a 10µg/ml (protein) suspension of *H.influenzae* OMs in 0.05M carbonate buffer pH 9.6. Plates were incubated for 18 hours at 4°C to allow adsorption of OMs onto the plate. After washing with phosphate buffered saline (PBS: 0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4. pH7.4) containing 0.5% tween 20 (PBS-tween), non specific protein binding sites were blocked with a solution of PBS-tween containing 1% w/v bovine serum albumen. Plates were incubated at room temperature for 1 hour with 250µl of blocking solution in each well. After washing with PBS-tween 100µl of serially diluted serum or sol phase sputum was applied to each test well and plates were incubated for 2 hours at room temperature. After further washing 200µl of HRP-conjugated anti human IgG, IgA, IgM, or anti rat IgG diluted 1/1000 in PBS-tween was added to each well. After 2 hours incubation with conjugate the plates were again washed and 200µl of substrate solution applied to each well. Substrate solution was prepared by dissolving 10mg of 3,3',5,5' tetramethylbenzidine in 100ml of 0.1M sodium acetate / citrate buffer pH 6.0 and, immediately before use, 8µl of H_2O_2 was added and the solution mixed thoroughly. The plates were incubated for 10 minutes at room temperature before the reaction was stopped by the addition of 50µl of 2N

H_2SO_4 to each well, causing the development of a yellow colouration.

The absorbance of each well at 450nm was recorded immediately. Control wells onto which no OMs had been adsorbed or which were coated with OMs but recieved no serum (or sol phase sputum) consistently gave readings below 0.2. The mean absorbance of the controls was subtracted from the mean absorbance of the test wells. Corrected absorbance readings >0.1 were considered positive. Results are expressed as the reciprocal of the serum dilution giving a corrected absorbance of 0.1.

b) Lipopolysaccharide. ELISA to detect antibodies to purified LPS of *H.influenzae* present in serum or sol phase sputum was carried out exactly as described above except that the washing solutions consisted of PBS alone. At no stage in the procedure was tween-20 used. Ten µg/ml (dry weight) LPS was used in the antigen suspension.

2.4.5. Analysis of Cytochrome Difference Spectra.

Cytochrome difference spectra were measured at room temperature in a two wavelength double beam spectrophotometer in split beam mode. The slit width was 1mm and the light path 1cm. Each sample was diluted as necessary with PBS and divided equally between two glass cuvettes. Reduced minus oxidised difference spectra were obtained by reducing the contents of one cuvette with a few grains of sodium dithionite ($Na_2S_2O_4$), and oxidising the contents of the other

by vigorous aeration or by adding a few grains of sodium ferricyanide ($Na_3Fe[CN]_6$). Samples were scanned across a range of wavelengths from 380-780nm and reduced - oxidised difference spectra were plotted on a chart recorder coupled to the spectrophotometer.

Cytochrome quantitation was performed with sodium dithionite reduced minus ferricyanide oxidised spectra using peak (or shoulder) - trough wavelength pairs and the extinction coefficients given by Jones and Redfearn (1966).

For CO spectra, CO gas was allowed to bubble through a reduced sample in a cuvette for 5 minutes. The CO-reduced sample was kept in the dark prior to spectral analysis. CO-reduced minus dithionite reduced spectra were recorded and plotted.

2.4.6. Determination of Respiratory Rates.

Respiration was measured at 30°C using an oxygen electrode coupled to a chart recorder. The incubation mixture consisted of 200µl of either whole cells or cell fractions of *H.influenzae* in PBS to a total volume of 2.97ml. Endogenous respiratory activity was recorded for 5 minutes prior to the addition of substrate in order to give a base respiratory rate. The reaction was initiated by the addition of 0.03ml of substrate in PBS to give a final concentration of 1mM. Respiratory activity induced by each of the following substrates was measured; NADH, NADPH, Glucose, L-lactate, D-lactate, succinate, pyruvate and formate. Respiration rates

were calculated as ng atoms oxygen consumed / minute / 100mg protein using the oxygen concentrations / ml at 30°C specified by Chappell (1964).

2.4.7. Production of Acids from Glucose.

Cells from 20ml of broth cultures in late log phase were harvested by centrifugation (17,000 x g, 4°C, 10 mins) and washed once with 0.85% w/v NaCl. Cells were then resuspended in 1ml PBS, this cell suspension was used to inoculate 10ml 10mM glucose in PBS contained in 50ml erlenmeyer flasks fitted with polyurethane foam bungs. Flasks were incubated for 2 hours at 37°C (with shaking). Cells were then harvested by centrifugation (17,000 x g, 4°C, 10 min), supernatants were retained for analysis by gas liquid chromatography (GLC).

GLC was performed by P.G. Martin of Macdonald College of McGill University, Montreal, Canada. His contribution to this work is gratefully acknowledged.

2.5. ANIMAL MODEL STUDIES.

2.5.1. Experimental Procedure

A rat lung model of chronic infection with non typable *H. influenzae* was developed using a modification of the method described by Slater (1986). Bacteria were introduced into the lungs encased in agar beads which were prepared as follows: Bacteria were harvested during late log phase from 200ml of

BHI broth containing excess haemin (2.5μg/ml) and NAD (10μg/ml). The cells were resuspended in 10ml of casamino acids buffer (200mM NaCl, 20mM KCl, 20mM Na_2HPO_4, 4% casamino acids, 20mM glucose pH 7.4). Five ml of bacterial cell suspension, warmed to 45°C, was then added to 5ml of 4% w/v molten agar held at 45°C. Five ml of the agar-bacterial suspension was taken up into a syringe fitted with a 23 guage needle and immediately added, with stirring, to 45ml of ice cold buffer (100mM NaCl, 10mM KCl, 10mM Na_2HPO_4, 2% w/v casamino acids, 10mM glucose, pH 7.4). As the agar enters the cold buffer it solidifies, forming small beads of diameter 20-30μm.

Infection Procedure (Figure 2.5.1 a-f).

Six week old female Sprague-Dawley rats 180-280g in weight (specific pathogen free, Harlan Olac Ltd., Shaws Farm, Bicester, Oxon) were sedated with fentanyl citrate (0.015mg), and fluanisone (0.495mg) (Hypnorm, Roche) and then anaesthatized with 1mg midazolam hydrochloride (Hypnovel, Janssen). Just prior to surgical exposure of the trachea rats were given 0.02mg of naxolone hydrochloride (Narcan, Dupont) to promote respiration. After exposure of the trachea each rat was then inoculated intratracheally with 0.2ml agar bead slurry containing 10^6 c.f.u of non typable *H.influenzae* strain HM1 using a 1ml syringe fitted with a 23 gauge needle. The animal was held in an upright position for a few seconds, then placed ventral side down until breathing returned to normal. The wound was then closed with 2 Michel clips (12mm

FIGURE 2.5.1.

Rat Model of Chronic *H.influenzae* Lung Infection: Infection Procedure.

a) Rats were individually anaesthatized using 1mg midazolam hydrochloride. Just prior to surgery each rat was given 0.02mg naxolone hydrochloride to promote respiration.

b) An incision 1.5-2cm in length was made, exposing the muscles above the trachea.

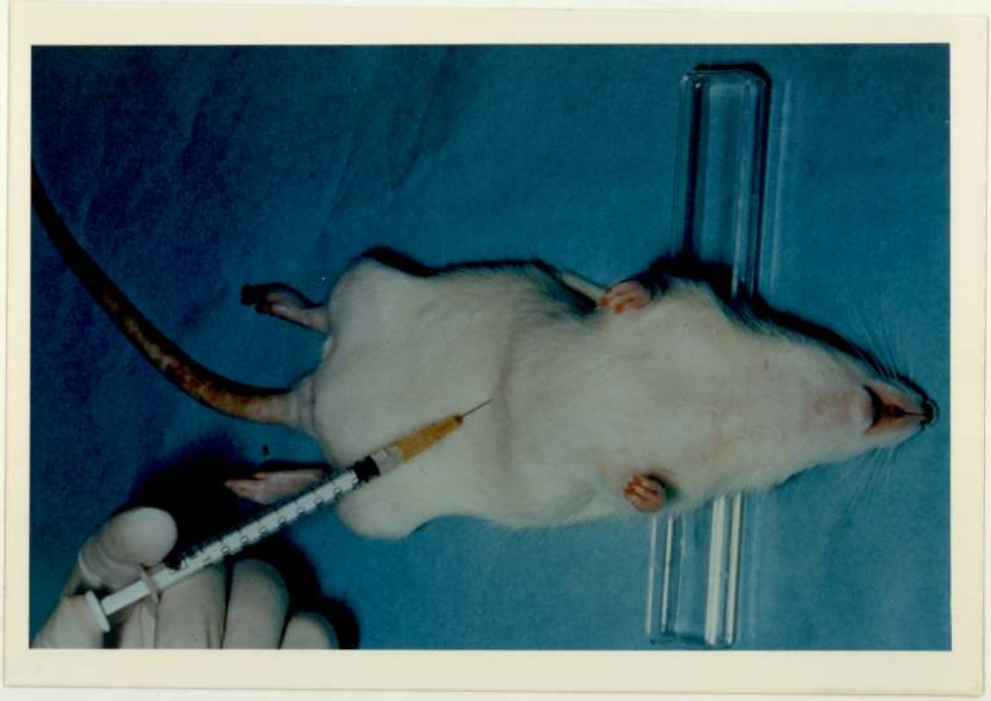
c) Surgical exposure of the trachea.

d) 0.2ml of agar bead slurry containing 10^6, or 10^8 c.f.u of non typable *H.influenzae* was inoculated into the lungs using a 1ml syringe fitted with a 23 gauge needle.

e) The wound was closed using two 12mm michele clips.

f) The animals were allowed to recover from anaesthesia.

a



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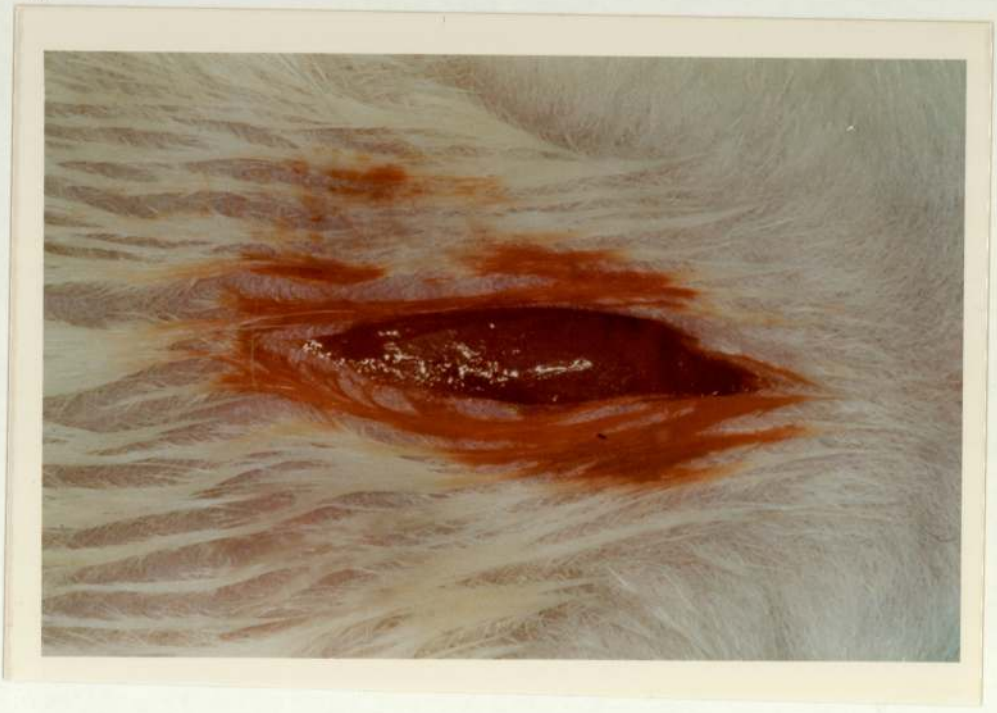

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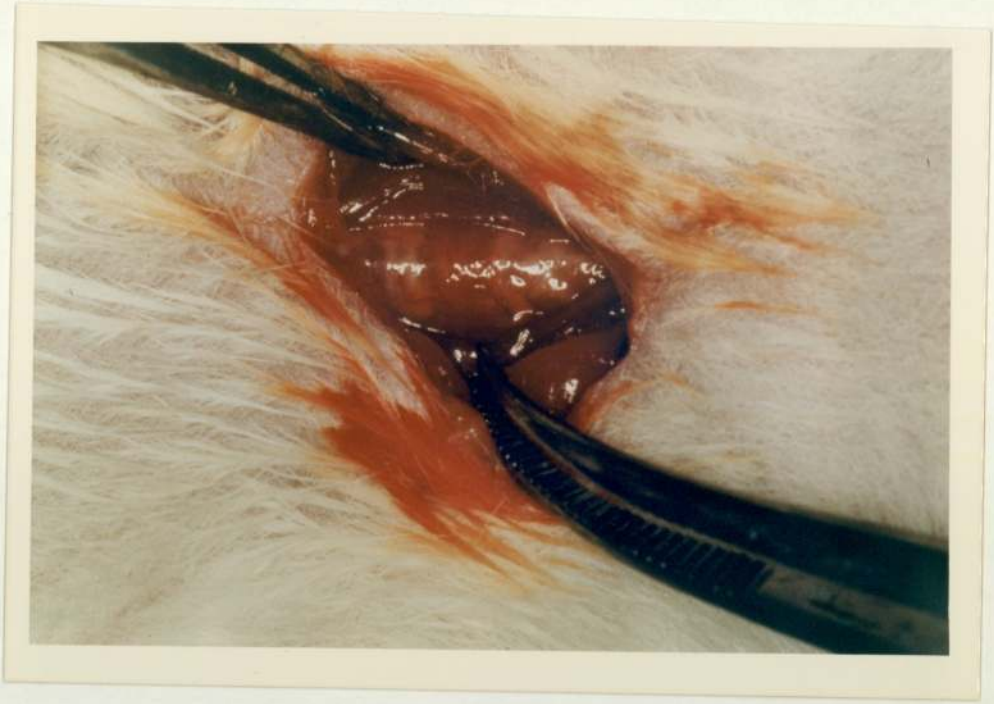
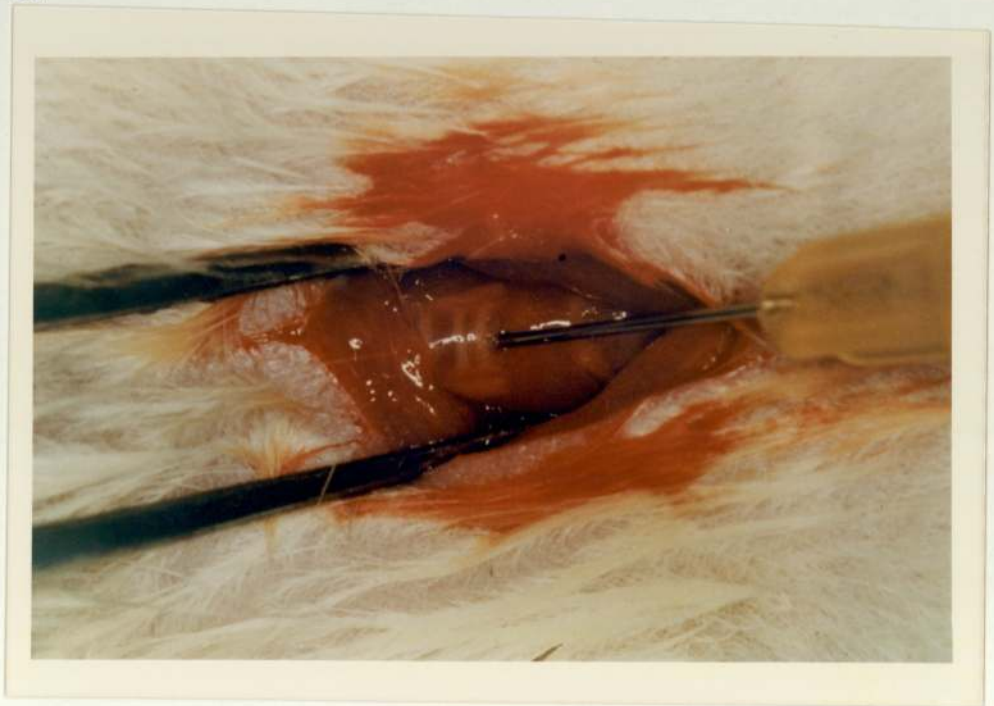
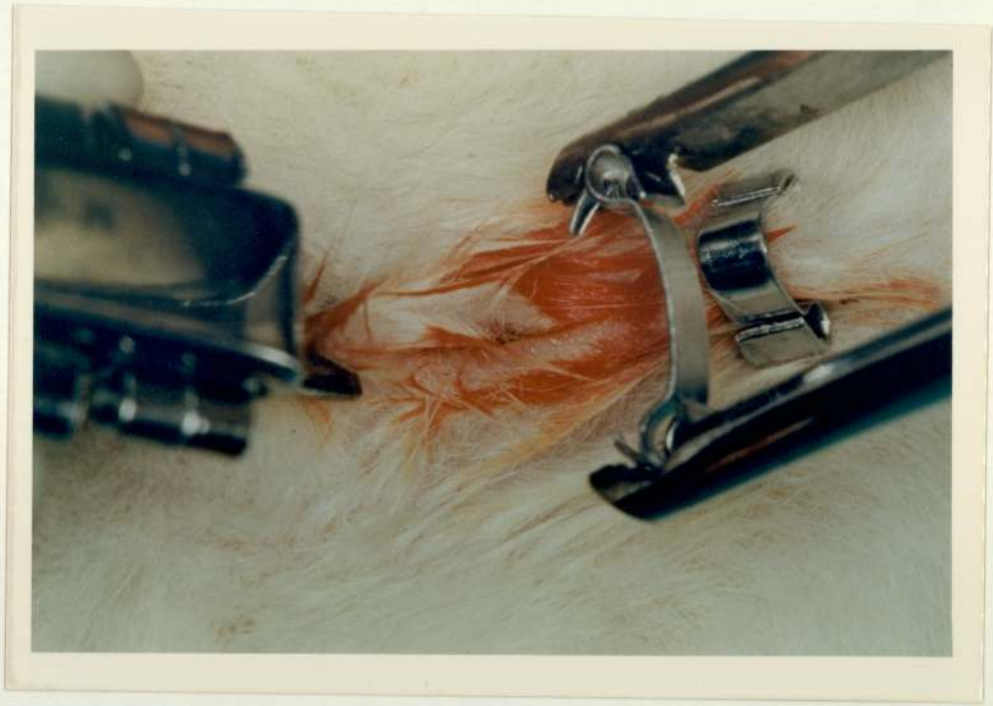


d



e



f

in length, Mercian Surgical Supplies, Wolverhampton, UK.) and the animals allowed to recover from anaesthesia.

Four to five animals were sacrificed at 0, 3, 7, 14, 21, 28, 35, and 42 days post infection. Blood was collected by cardiac puncture and serum separated as described previously (2.1.4). Lungs from each animal were weighed in sterile petri dishes and homogenised in ice cold 0.85% NaCl using a glass homogeniser fitted to a Black and Decker drill (Model BD163V). Samples of lung homogenate were serially diluted in sterile BHI broth and viable counts performed using chocolate agar and BHI agar. The numbers of *H.influenzae* present in each lung were estimated, results were expressed as c.f.u./g of lung tissue.

The above procedure was repeated using an increased inoculum size. Agar bead slurry containing 10^8 c.f.u. of non typable *H.influenzae* strain HM1 was inoculated into each rat.

Experimental Controls /

Five unmanipulated control animals were sacrificed on day 0 and on day 42. A second set of controls were inoculated with sterile agar beads and five animals from this set of controls were sacrificed at 0, 21, and 42 days post-operatively. Blood was collected by cardiac puncture and serum separated as described previously.

2.5.2. Lung Washes. /

2.5.2. Lung Washes.

Lung lavage fluid was collected by infusion of 5ml NaCl into the lung through a catheter secured in the trachea (Fig. 2.5.2 a-b). NaCl could be inserted and removed from the lungs several times using this apparatus, 25 - 50% of the volume was recovered. Lavage fluid was centrifuged for 2 minutes (13,000 x g, Eppendorf). The supernatant was removed and stored at -20°C.

2.5.3. Augmentin Therapy of *H.influenzae* Infections.

Forty rats were infected with 10^6 c.f.u. of non typable *H.influenzae* strain HM1 as described in section 2.5.1. Fourteen days post infection 20 of the animals were given an i/m dose of 30mg/kg augmentin (amoxycillin + clavulanic acid). The remaining 20 animals received an injection consisting of pyrogen free water and served as controls. Four animals were sacrificed at 0, 1, 5, 12, and 19 days post infection. The lungs were homogenised as previously described and viable counts performed.

2.5.4. Recovery of Bacterial Outer Membranes from *in vivo*.

Lungs from 50 rats collected 42 days post infection, were homogenised vigorously. Large debris was removed by course filtration through glass wool and the resulting filtrate was divided in two. Half was passed twice through a french pressure cell (14,000 psi), the remainder was subjected to differential centrifugation using 3 rounds of alternate slow

FIGURE 2.5.2.

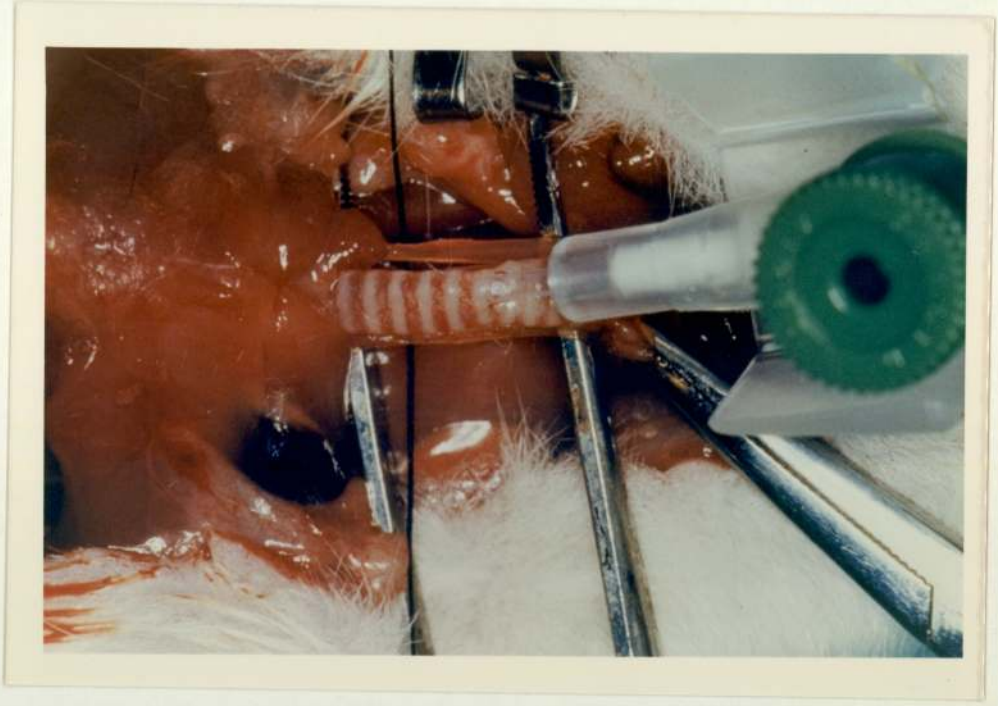
Rat Model of Chronic *H.influenzae* Lung Infection.

Procedure for the Collection of Lung Lavage Fluid:

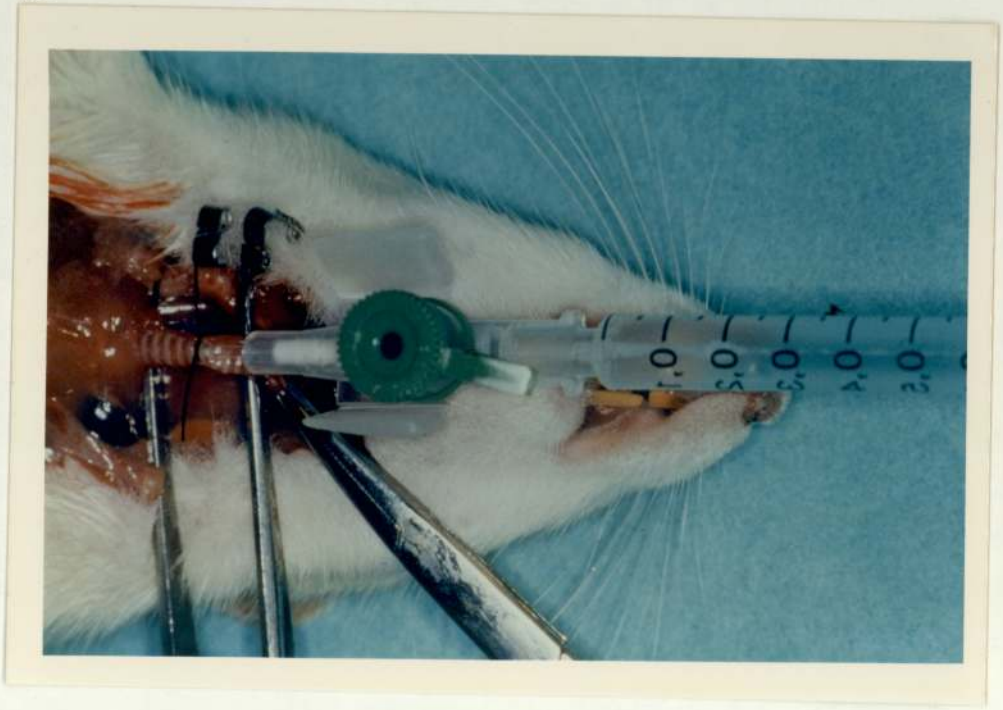
a) A catheter was secured in the trachea using a piece of thread.

b) Five ml of 0.85% w/v NaCl was passed into the lungs using a syringe. Lung lavage fluid was obtained by drawing the NaCl solution back up into the syringe and repeating the procedure. 25-50% of the original volume was recoverable.

a



b



(1 000 x g, 10 min) and fast (15,000 x g, 20min) spins. The supernatant remaining after differential centrifugation was then passed twice through a french pressure cell (14,000 psi).

After french pressing, sarkosyl (Sodium - N - lauroyl sarcosinate) was added to each fraction to a final concentration of 2% w/v. After 30 minutes incubation at room temperature unbroken cells were removed by centrifugation (10,000 x g, 4°C, 10 min). Outer membranes were then harvested by ultracentrifugation (150,000 x g, 4°C, 40 min) and washed with distilled water. OMs were finally resuspended in a small volume of distilled water and stored at -20°C.

2.6. QUANTITATIVE ANALYSES.

2.6.1. Protein Assays

The concentration of protein present in each bacterial cell fraction was estimated using the method of Lowry *et al* (1953). A standard curve was obtained using solutions of bovine serum albumen of known concentration (0-200µg/ml), prepared freshly each time the assay was performed. Sample protein determinations were performed in triplicate.

2.6.2. Formic Acid Assay.

Formic acid in bacterial culture supernatants and in homogenised infected and control (uninfected) rat lungs was

measured using a commercially available test kit (Boehringer Mannheim GMBH).

Formic acid is oxidised in the presence of formate dehydrogenase (FDH) and NAD, yielding NADH and CO_2.

$$\text{Formate} + \text{NAD} + H_2O \xrightarrow{\text{FDH}} HCO_3^- + \text{NADH} + H^+.$$

The amount of NADH formed is stoichiometric with the amount of formic acid present. The increase in NADH is measured spectrophotometrically by absorbance at 340_{nm}.

The test kit was used according to the manufacturers instructions. Formic acid present in culture supernatants could be assayed directly. Rat lung homogenates (see 2.5.1.) were treated as follows before analysis; 4ml of 1N perchloric acid was added to 1g of homogenised tissue. The mixture was adjusted to pH 9.0 with 2N KOH and was made up to 20ml with distilled water. After refrigeration for 20 min to allow precipitation of potassium perchlorate, the mixture was filtered through course filter paper. The resulting filtrate was assayed for formic acid content.

2.6.3. Statistical Analysis.

Statistical differences were determined using the Wilcoxon matched pairs signed rank test.

3. RESULTS AND DISCUSSION.

3.1 Non Typable *H.influenzae* Infection in Bronchiectasis.

Bronchiectasis patients can be subdivided into three broad groups based on the usual nature of their bronchial secretions, these are; 1) MUCOID (M group) consisting of individuals producing mucoid sputum which occasionally becomes purulent; 2) MUCOPURULENT (MP group) consisting of patients normally producing mucopurulent sputum which occasionally becomes purulent; and 3) PURULENT (P group) consisting of individuals whose secretions are persistently of a purulent nature (Hill *et al*, 1986).

3.1.1 Isolation of Non Typable *H.influenzae* from Sputum.

Sputum samples, collected over 4 hours on waking, were obtained from 20-30 bronchiectasis patients in each of the three groups described above. After washing with 0.85% NaCl to remove surface contaminants of oropharyngeal origin, sputa were cultured for *H.influenzae* as described previously (see section 2.2.2). Bacteria identified as *H.influenzae* were serotyped (2.2.3) using polyclonal anti-capsular antisera specific for *H.influenzae* capsular types a-f.

Table 3.1.1 shows the percentage of sputa examined which contained serologically non typable *H.influenzae* for each of the three patient groups. This species was isolated more frequently from mucopurulent (65.2%) and purulent (53.3%) than from mucoid sputum samples (38.8%).

TABLE 3.1.1.

Frequency of Isolation of Non Typable *H.influenzae* from the Sputum of Patients with Bronchiectasis.

SPUTUM CHARACTER	% POSITIVE CULTURES
Mucoid	38.8%
Mucopurulent	65.2%
Purulent	53.3%

Six patients with mucoid sputum, six with mucopurulent sputum and five producing purulent sputum, all of whom had positive non typable *H.influenzae* cultures at the time of initial sampling, were selected for further investigation. Sputum and serum samples were collected from each of these individuals over an eight month period and colonisation with non typable *H.influenzae* was monitored. Three to four samples were obtained from each individual over the study period. At the beginning of the study none of the patients were receiving antibiotic therapy.

Figure 3.1.1 a-c lists patients involved in the study by group and number together with sampling dates and the nature of sputum produced on each occasion (M = mucoid, MP = mucopurulent, P = purulent). Non typable *H.influenzae* isolates are listed and their biotypes given. Biotyping was performed on the basis of ornithine decarboxylase, urease and indole production according to the scheme devised by Kilian (1976) (sections 1.1.3a and 2.2.3b). The proportion of the sputum flora represented by Haemophili was estimated on each occasion as follows: + = a few isolated *Haemophilus* colonies, mixed flora; ++ = 50% of the sputum flora represented by Haemophili; +++ = sputum flora predominantly Haemophili. It should be emphasised that these scores are estimates and that they represent the proportion of the aerobic flora of the piece of sputum cultured consisting of

FIGURE 3.1.1a.

Non Typable *H.influenzae* Isolated From Mucoid Sputum.

Patient	Date	Sputum Character*	*H.influenzae* Isolate.	Score	Biotype
M1	24.09.87	M	HM5	+	III
	20.10.87	non productive			
	21.03.87	M/MP	HM6	++	II
	28.04.88	M/MP	no isolate		
M2	26.08.87	M	HM7	+	III
	10.12.87	M	no isolate		
	21.03.88	M	no isolate		
M3	03.12.87	M	HM8	++	III
	11.02.88	M	no isolate		
	12.03.88	M	no isolate		
M4	10.12.87	M	HM9	+	II
	21.03.88	M	no isolate		
	28.04.88	M	no isolate		
M5	25.02.88	M	HM10	+	III
	21.03.88	M	no isolate		
	28.04.88	M	no isolate		
M6	24.09.87	M	HM11	+	IV
	26.11.88	M	no isolate		
	21.03.88	M	no isolate		

* M = Mucoid, MP = Mucopurulent.

FIGURE 3.1.1b.

Non Typable *H. influenzae* Isolated From Mucopurulent Sputum.

Patient	Date	Sputum Character*	*H. influenzae* Isolate.	Score	Biotype
MP1	19.09.86	P	HM2	+++	III
	26.08.87	P	HM12	++	III
	07.01.88	M	no isolate		
	23.03.88	MP	no isolate		
MP2	06.11.86	MP	HM3	++	III
	10.09.87	MP	no isolate		
	22.10.87	MP	HM13	++	I
	11.02.88	MP	no isolate		
	23.03.88	MP	HM14	++	III
MP3	10.09.87	MP	HM15	+	II
	14.10.87	MP/P	HM16	++	I
	07.01.88	MP	no isolate		
	23.03.88	MP/P	HM17	++	IV
MP4	02.09.87	M/MP	HM18	+	
	28.10.87	M/MP	no isolate		
	03.03.88	MP	HM19	++	I
	25.03.88	MP/P	HM19	++	I
MP5	10.09.87	MP/P	HM20	+++	III
	12.11.87	P	no isolate		
	07.01.88	MP/P	HM21	+++	I
	23.03.88	MP	HM22	+++	III
MP6	14.10.87	MP	HM4	+++	V
	23.03.88	MP	HM23	+++	II
	28.04.88	MP	no isolate		

* M = Mucoid, MP = Mucopurulent, P = Purulent.

FIGURE 3.1.1c.

Non Typable *H.influenzae* Isolated From Purulent Sputum.

Patient	Date	Sputum Character*	*H.influenzae* Isolate.	Score	Biotype
P1	14.10.87	P	HM24	+++	V
	05.11.87	P	no isolate		
	07.01.88	P	no isolate		
	25.02.88	MP	no isolate		
P2	14.10.87	P	HM25	++	I
	10.12.87	P	HM25	++	I
	07.04.88	MP/P	no isolate		
P3	14.10.87	P	HM26	++	III
	12.11.87	P	no isolate		
	10.12.87	P	HM26	+++	III
	11.02.88	P	no isolate		
P4	24.09.87	P	HM27	+	II
	11.02.88	P	HM28	+++	III
	25.02.88	P	no isolate		
P5	19.09.86	MP	HM1	++	III
	10.09.87	MP/P	HM29	+++	III
	10.12.87	MP/P	no isolate		
	25.03.88	MP/P	no isolate		

* M = Mucoid, MP = Mucopurulent, P = Purulent.

Note that strains HM1, HM2 and HM3 were collected outside the main study period which began in August 1987 and ended in April 1988.

Haemophilus as no culture for anaerobes was performed and whole sputum samples were not cultured.

Purulent and mucopurulent sputum were heavily colonised with bacteria, while mucoid samples contained relatively few bacteria. Non typable *H.influenzae* strains were present in larger numbers and formed a greater proportion of the flora in mucopurulent and purulent than in mucoid sputum. In mucoid samples non typable *H.influenzae* formed up to 50% of the sputum flora only on two occasions (HM6 and HM8, Fig. 3.1.1a) but on all five other occasions only a few isolated colonies were found. In mucopurulent and purulent samples however, *H.influenzae* were commonly found to be present in large numbers (Fig. 3.1.1 b and c). On one occasion a change in sputum character from mucoid to mucopurulent appeared to correlate with an increase in numbers of *H.influenzae* (HM6, Fig. 3.1.1a) however, on another occasion (HM8, Fig. 3.1.1a) the patients sputum remained mucoid despite the presence of relatively large numbers of *H.influenzae*.

Although the majority of purulent and mucopurulent samples contained *H.influenzae*, usually in large numbers, many patients continued to produce purulent secretions when *H.influenzae* could no longer be detected. It seems likely therefore that organisms other than *H.influenzae* also contribute to sputum purulence in these instances.

3.1.2 *H.influenzae* Biotype Distribution.

The biotype distribution of 28 of the 29 non typable *H.influenzae* isolated from bronchiectasis patients is shown in Figure 3.1.2. The remaining isolate, HM18, could not be biotyped as it would not grow in supplemented BHI broth and when colonies were washed off chocolate agar plates and used to inoculate API strips no reaction took place with any substrate and no viable bacteria could be recovered.

Eighty-six percent of the remaining 28 strains were distributed over biotypes I, II and III. The majority of strains (50%) were of biotype III, 18% were of biotype II and 18% were biotype I. Two isolates (7%) were of biotype IV and a further 7% were biotype V. No strains of biotypes VI-VIII were identified.

Non typable *H.influenzae* isolated from mucoid sputum were of biotypes II, III and IV. Those cultured from mucopurulent samples were of biotypes I to V and *H.influenzae* recovered from purulent sputum were distributed over biotypes I, II, III and V (Fig. 3.1.2).

3.1.3 OMP Profile Analysis of *H.influenzae* Isolates.

The OMP profiles of 23 non typable *H.influenzae* strains isolated from individuals with bronchiectasis are shown in Fig. 3.1.3 A and B. Bacteria were grown in BHI broth supplemented with excess haemin and NAD (each at 5µg/ml). OMs were prepared and outer membrane proteins separated by SDS-PAGE as described previously (2.3.1 and 2.4.1).

Figure 3.1.2. Biotype Distribution of *H.influenzae* Isolates.

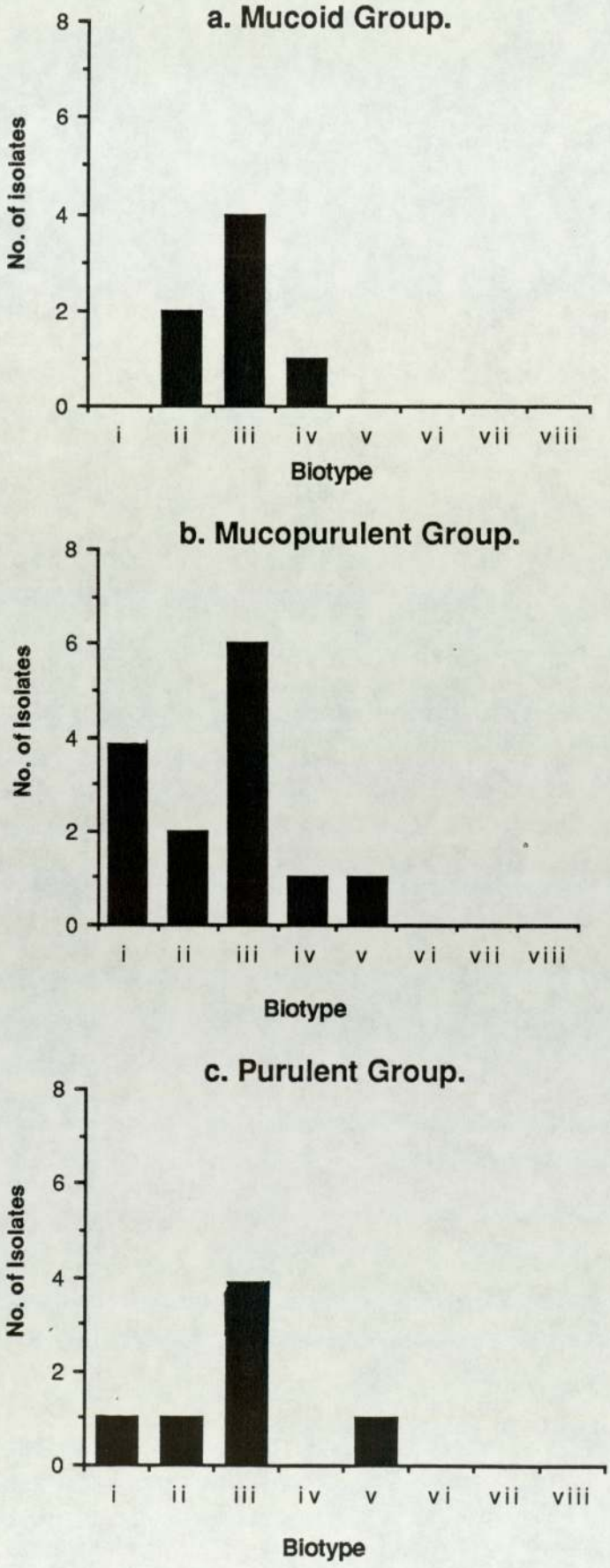


FIGURE 3.1.3.

Outer Membrane Protein Profiles of Non Typable *H.influenzae* Isolated from the Sputum of Patients with Bronchiectasis.

Bacteria were cultured in BHI broth supplemented with excess haemin and NAD (5µg/ml). OMs were prepared by sarkosyl extraction according to the method of Filip *et al* (1973) (2.3.1) and OMPs separated by SDS-PAGE (2.4.1) using 10% gels, according to the method of Lugtenberg *et al* (1975). Separated proteins or peptides were stained using coomassie blue. Numbers in the margins refer to molecular weight in kilodaltons, or to *H.influenzae* OMPs by standard nomenclature.

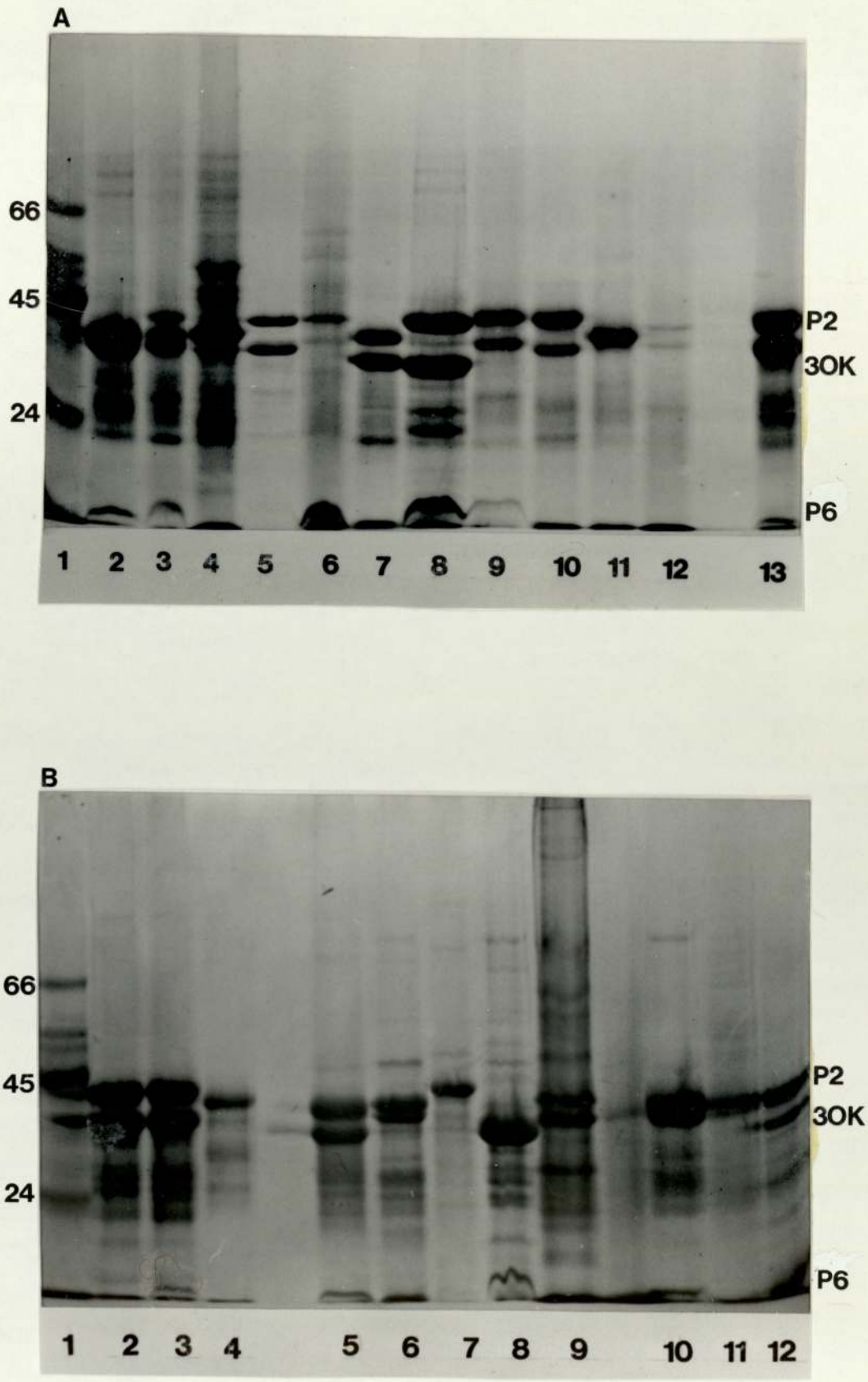
Haemophilus influenzae strains represented are listed below:

A + B. Lane 1: molecular weight standards

A.
Lane 2: HM1
Lane 3: HM29
Lane 4: HM2
Lane 5: HM12
Lane 6: HM3
Lane 7: HM13
Lane 8: HM19
Lane 9: HM15
Lane 10: HM16
Lane 11: HM17
Lane 12: HM23
Lane 13: HM26

B.
Lane 2: HM25 (14.10.87)
Lane 3: HM25 (10.12.87)
Lane 4: HM20
Lane 5: HM27
Lane 6: HM28
Lane 7: HM22
Lane 8: HM10
Lane 9: HM11
Lane 10: HM7
Lane 11: HM9
Lane 12: HM8

FIGURE 3.1.3.


A
66
45
24
P2
30K
P6
1 2 3 4 5 6 7 8 9 10 11 12 13
B
66
45
24
P2
30K
P6
1 2 3 4 5 6 7 8 9 10 11 12

Studies of biotype and OMP profiles showed that individual *H.influenzae* strains colonised the sputum of patients with bronchiectasis for relatively short periods of time. All but two of the individuals under study were infected with *H.influenzae* strains of different OMP type and biotype each time their sputum was cultured. For example patient no. MP3 (Fig. 3.1.1b) produced mucopurulent sputum on 10.09.87 which contained a biotype II strain, HM15, the OMP profile of which is shown in Fig. 3.1.3A lane 9. One month later (14.10.87) the same individual produced mucopurulent sputum with a profuse growth of a biotype I strain, HM16, (Fig. 3.1.3A lane 10). The molecular weights of the major OMPs of these two strains are similar, but they differ in some minor OMPs; while they may be confused on SDS-PAGE, the biotypes of these isolates confirm that they are in fact distinct. Two months later the same patient produced mucopurulent sputum from which no *H.influenzae* were isolated, after a further two months a biotype IV strain predominated, the OMP profile of which is shown in Fig. 3.1.3A, lane 11.

Similarly, patient MP5 (Fig. 3.1.1b) produced sputum which was mucopurulent to purulent in character between the dates of 10.09.87 and 23.03.88. On 10.09.87 a biotype III strain, HM20, was cultured (Fig. 3.1.3B lane 4), on 07.01.88 a new biotype I strain, HM21, was isolated in virtual pure culture. By 23.03.88 however, a new biotype III strain, HM22, (Fig. 3.1.3B lane 7) predominated in the sputum.

Non typable *H.influenzae* HM25 (Fig. 3.1.3B lanes 2 and 3), a biotype I isolate, and a biotype III strain, HM26 (Fig. 3.1.3A lane 13) persisted in purulent sputum samples from patients P2 and P3 (Fig. 3.1.1c) for two to three months. HM25 was first isolated on 14.10.87 and was still present in the sputum when the patient next attended clinic two months later. Similarly HM26 was cultured from purulent sputum samples from patient P3 on 14.10.87 and again on 10.12.87. Culture of purulent sputum in the intervening month however, failed to yield *H.influenzae*. Although this may represent a failure to isolate *H.influenzae* on one occasion, given the large numbers of *H.influenzae* HM26 cultured from this particular individual during October and December, it seems likely that there was a decrease in the numbers of *H.influenzae* present in the sputum between October and November, followed by an increase during November and December.

The above are the only two instances where a single strain persisted in an individual over a two month period between visits to the clinic. In all other instances *H.influenzae* isolates were replaced by new strains or could no longer be cultured each time the patient involved attended the hospital (Fig. 3.1.1a-c).

Each non typable *H.influenzae* isolate was unique in OMP profile. Only two proteins were found to be present in all strains, these were the lipoprotein P6 (16K) (Weinberg *et al*, 1988), seen as a diffuse, weakly staining band in many

strains, and a protein of molecular weight 30K, both of which have been previously shown to be universally distributed amongst non typable *H.influenzae* (Barenkamp *et al*, 1982; Murphy *et al*, 1983). However, a 64K protein present in all non typable *H.influenzae* isolated in a study of nasopharyngeal carriage in children (Spinola *et al*, 1986) was not produced by all strains isolated during the present study (Fig. 3.1.3).

The OMP profiles shown in figure 3.1.3 reveal that each isolate produced one to three major outer membrane proteins, the majority of strains producing two. These were of molecular weights between 30K and 45K and displayed significant strain to strain variation. The higher molecular weight OMP in these bronchiectasis isolates varied in molecular weight between 34K and 45K. This protein, designated P2 in both type b and non typable *H.influenzae* (Munson *et al*, 1983; Murphy and Apicella, 1987) is thought to function as the major porin protein in this species (Vachon *et al*, 1985; Burns and Smith, 1987).

No patterns in OMP profile were discerned which could be utilised to group these bronchiectasis isolates and form the basis of a typing system. Similarly no correlation between biotype and OMP type was noted, strains belonging to each biotype being heterogeneous in OMP composition.

3.1.4 Sputum Culture.

All sputum specimens received were washed with 0.85% NaCl

prior to the removal of a large sample for culture (2.2.2). It has been suggested that the flora of a single loopful of sputum may vary with respect to the sample as a whole and may therefore not accurately reflect the sputum flora (Turk and May, 1967). Additionally it is known that two sputum specimens, collected within minutes of each other from a single patient may vary in their bacterial load and content (May and Delves, 1964; Roberts, 1984). This problem was overcome in the present study as each sputum specimen received represented secretions expectorated over a 4 hour period and was therefore presumed to reflect as accurately as possible the sputum flora of the individual concerned.

Homogenisation of sputum with pancreatin (Rawlins, 1953) yielded *H.influenzae* identical in biotype and OMP profile to those cultured from large loopfuls of washed, undigested samples (Fig. 3.1.4A). On no occasion was an *H.influenzae* strain isolated from digested sputum which was not also isolated from undigested samples. It was therefore concluded that culture of a large inoculum of washed, untreated sputum was a sufficient indicator of the *Haemophilus* content of the specimen as a whole.

Furthermore, on no occasion was more than one strain of non typable *H.influenzae* isolated from a single sputum sample. All 28 isolates represented the only strain cultured from sputum at the time of isolation. Several colonies, picked from different areas of culture plates were identical in biotype and OMP profile. Figure 3.1.4B shows three such

FIGURE 3.1.4.

A. OMP Profiles of *H.influenzae* Isolated from Pancreatin-Digested Sputum Samples.

Sputum samples were washed with 0.85% NaCl. A large piece of washed sputum was then removed and cultured for *H.influenzae* as described in section 2.2.2. Pancreatin extract (1mg/ml) was added to the remainder. After incubation for 1 hour at 37°C a loopful of pancreatin digested material was cultured for *H.influenzae*.

Lane 1: *H.influenzae* HM5, (biotype III) isolated from untreated mucoid sputum.

Lane 2: *H.influenzae* HM5 isolated from mucoid sputum after digestion with pancreatin.

Lane 3: *H.influenzae* HM21 (biotype I), isolated from untreated purulent sputum.

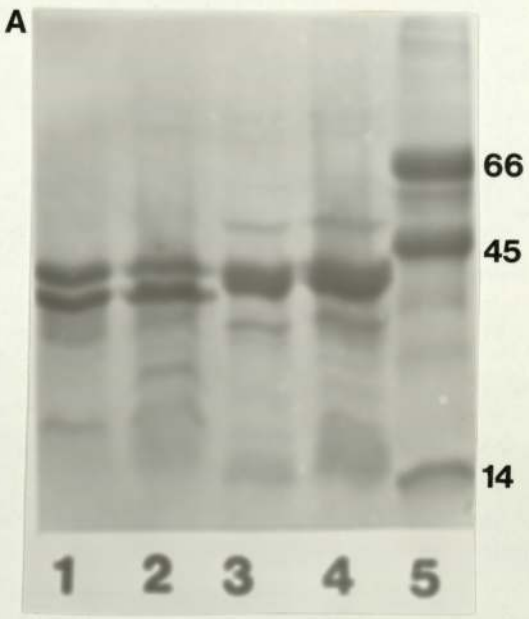
Lane 4: *H.influenzae* HM21 isolated from purulent sputum after digestion with pancreatin.

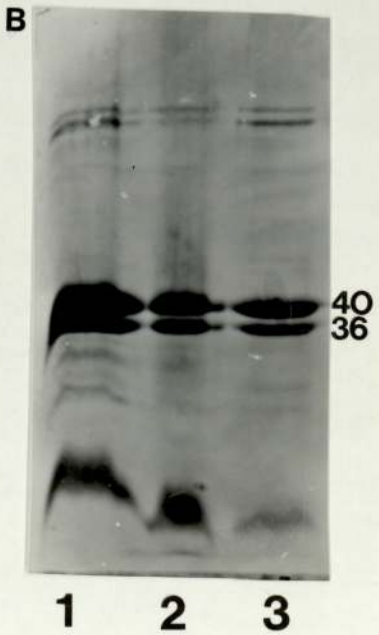
Lane 5: molecular weight standards.

B. The OMP profiles of 3 *H.influenzae* isolates from 3 distinct areas of a single purulent sputum sample.

Numbers in the right hand margins refer to molecular weight in kilodaltons.

FIGURE 3.1.4.





isolates cultured from a single specimen of purulent sputum. All were identical in biotype and OMP profile and represented a single strain, HM4. These results suggest that only one *H.influenzae* strain predominated in the sputum of the patients under study at any one particular time. It can not however, be concluded with certainty that other *H.influenzae* strains were absent as they may have been present in the sputum in very much smaller numbers than the dominant strain and hence have escaped detection.

3.1.5 DISCUSSION.

In chronic obstructive pulmonary disease an initial lung-damaging event, the exact nature of which remains ill-defined, provides an opportunity for microbial colonisation of the previously sterile pulmonary environment. Bacterial colonisation may persist as a relatively stable parasitic state or may progress and result in increased lung damage, either directly by production of toxic factors or indirectly by continuous stimulation of an excessive inflammatory response (Cole, 1984; Wilson and Cole, 1986). In bronchiectasis, individuals with relatively mild disease characteristically produce mucoid sputum and only occasionally experience purulent exacerbations. These may represent the former category (stable parasitic state) while individuals with more extensive lung damage who continually expectorate purulent sputum may represent the latter.

As the bronchial tree is normally sterile (Roberts 1984) any organism isolated from sputum could be regarded as a potential pathogen. Sputum from patients with bronchiectasis commonly contains large numbers of bacterial species (Turk and May, 1967; Roberts, 1984). *Haemophilus influenzae* and *Streptococcus pneumoniae* are the two organisms most commonly associated with purulent exacerbations in such individuals (Smith *et al*, 1983) and the eradication of *H.influenzae* from the lungs of such individuals is known to be correlated with clinical improvement (May and Delves, 1964; Cole *et al*, 1983). The results presented here would tend to confirm the association of culturable *H.influenzae* with purulent sputum. Non typable *H.influenzae* were frequently isolated in large numbers from purulent and mucopurulent sputum, while culture of mucoid sputum yielded this species less frequently and in greatly reduced quantities.

Analysis of OMP profiles and biotypes has proved useful in identifying subsets among non typable *H.influenzae* populations which are commonly associated with bacteraemic and neonatal infections (Barenkamp *et al*, 1982; Wallace *et al*, 1983). However, despite efforts to classify non typable strains into distinct subtypes on the basis of mobility of major OMPs on SDS-PAGE (Murphy *et al*, 1983) no associations between particular OMP types or biotypes and nasopharyngeal, pulmonary, or otitis media isolates have been reported (Barenkamp *et al*, 1982; Spinola *et al*, 1986). Similarly OMP profiles of non typable *H.influenzae* isolated from

bronchiectasis patients as part of the present study, demonstrated heterogeneous OMP profiles and no correlation between particular OMP profiles and biotypes was noted.

The majority of non typable *H.influenzae* isolated during the present study were of biotypes I, II and III. Previous studies of biotype distribution among non typable strains isolated from individuals with chronic bronchitis (Kilbourn *et al*, 1982), from elderly individuals with pneumonia (Brabender *et al*, 1984), and nasopharyngeal isolates from normal individuals (Kamme, 1980) report a predominance of biotypes II and III, but low levels (3.5%, 3.8% and 4% respectively) of biotype I strains. The results described here resemble these in that a vast predominance of biotype III (50%) was observed but differ in that 18% of non typable *H.influenzae* were of biotype I, all of which were isolated from purulent or mucopurulent sputum. The study of Kilbourn *et al* (1982) involved a similar number of isolates to the present study (29) and noted only 3.5% biotype I in chronic bronchitis.

Individuals with bronchiectasis who continually produce mucopurulent or purulent sputum may therefore be more likely to be colonised with biotype I strains than bronchiectasis patients with mucoid sputum or individuals with other forms of pulmonary disease such as chronic bronchitis or pneumonia. Similarly Musher *et al* (1983) suggest that biotype I may predominate among non typable *H.influenzae* isolated from patients with acute exacerbations of chronic

obstructive pulmonary disease and a predominance of biotype I strains has also been reported for *H. influenzae* infection in cystic fibrosis (Hoiby and Kilian, 1976). However, in contrast to the above results Rhind *et al* (1987) in a study of biotype distribution among *H. influenzae* isolated from patients suffering from a range of pulmonary diseases including chronic bronchitis, bronchial carcinoma, bronchiectasis and pneumonia found no differences in biotype distribution between these groups or between patients in a stable condition and those experiencing infective exacerbations.

It has been suggested that for *H. influenzae* type b biotype I may be indicative of virulence factors other than capsule production (Kilian *et al*, 1979) but the exact nature of such virulence factors remains undefined as does their association with non typable strains. No definate conclusions about the pathogenicity of biotype I strains in bronchiectasis can be drawn from the present study in view of the small number of samples involved. Further investigation involving much greater numbers of patients is required.

The present study indicates that individual non typable *H. influenzae* strains colonise the lungs of bronchiectasis patients for relatively short periods of time, no strain apparently persisting for more than two to three months. Similarly in cystic fibrosis (Watson *et al*, 1988) and otitis media (Barenkamp *et al*, 1984) individual strains of

H. influenzae rarely persist for more than one or two months before being replaced by new strains. The host and microbial factors which regulate initiation and termination of colonisation by individual *H. influenzae* strains remain to be elucidated.

In contrast to the situation in cystic fibrosis where several distinct strains are present simultaneously in the lungs (Watson *et al*, 1988), more than one strain was never found in a single bronchiectasis sputum sample, suggesting that only one strain predominated in the sputum at any one time. The presence of other strains in small numbers cannot be completely ruled out as their presence may have been obscured by large numbers of the predominant strain or by other bacteria.

Haemophilus influenzae are persistent colonisers of purulent sputum in bronchiectasis. Often sputum cleared on antibiotic therapy becomes purulent again very quickly on cessation of therapy (Hill *et al*, 1986). This purulence is on many occasions associated with the re-emergence of *H. influenzae* (Cole *et al*, 1983). If doses of β-lactam antibiotic administered are insufficient to achieve bactericidal concentrations in the sputum a 'reservoir' of *H. influenzae* defective in cell wall composition and consequently of aberrant shape (termed spheroplasts) are able to survive and revert to normal forms on withdrawal of the antibiotic. In certain instances purulent sputum, yielding no apparent pathogens on culture has been demonstrated to contain

spheroplasts (Roberts *et al*, 1984). These authors were able to recover *H.influenzae* from such sputa by incorporation of the cell wall constituent N-acetyl-glucosamine into culture media. It has been demonstrated on several occasions that high doses of oral antibiotics (3g amoxycillin) are required in order to achieve sputum concentrations which are bactericidal for *H.influenzae* (Cole, 1984; Hill *et al*, 1986).

The present study demonstrates that in the absence of antimicrobial chemotherapy, colonisation of the bronchiectatic lung with non typable *H.influenzae* is a dynamic process involving frequent acquisition and loss of strains. This would suggest that sphaeroplasts are not always the source of subsequent infection but rather that new strains are acquired from exogenous sources. A previous study of *H.influenzae* and *S.pneumoniae* infection in chronic bronchitics also concluded that exacerbation in these individuals was due to acquisition of novel strains from exogenous rather than endogenous sources (Ellis *et al*, 1978).

A longer term study is necessary in order to ascertain whether strains no longer culturable from sputum were actually eradicated or whether they can survive in the lung in dormant form or in small numbers 'masked' by heavier loads of other strains only to re-emerge at a later date causing further exacerbations. The factors controlling proliferation and influencing loss and acquisition of

H. influenzae within the pulmonary environment remain to be elucidated. Analysis of surface antigens expressed by *H. influenzae* growing *in vivo* and of the immune response of bronchiectasis patients to these antigens may lead to a better understanding of how non typable *H. influenzae* survive within the lungs, and why they are prevalent in some bronchiectasis patients and, in comparison, rarely isolated from others.

3.2 The Effects of Haem-Limitation and Iron-Restriction on the Outer Membrane of Non typable *Haemophilus influenzae*.

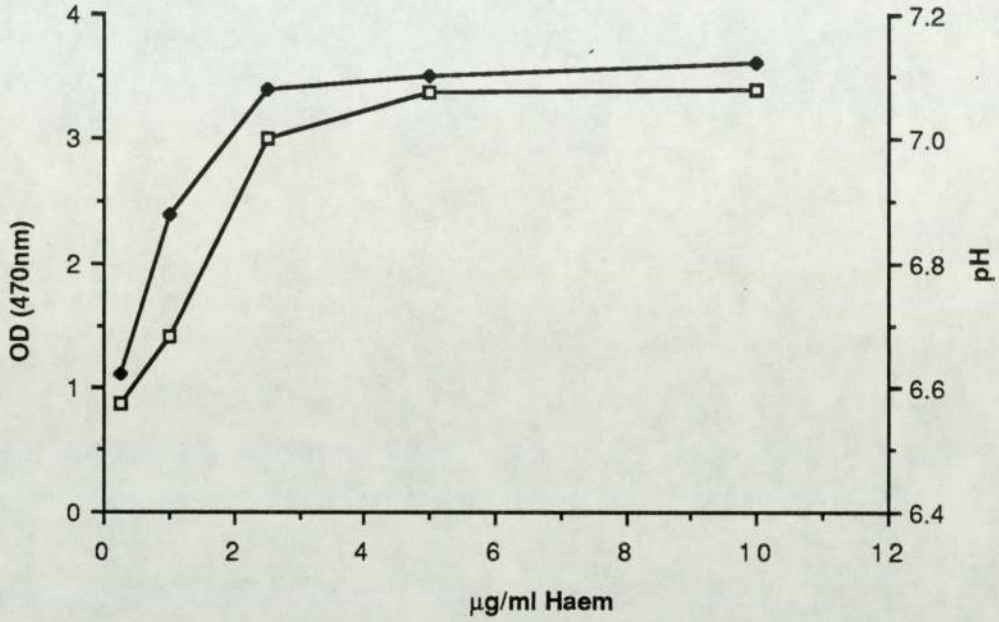
3.2.1. Culture Characteristics of Haem-Limited and Iron-Restricted *H.influenzae*.

The final OD_{470} and pH values for 18 hour cultures of non typable *H.influenzae* HM1 and HM3 grown in NAD supplemented BHI broth containing varying concentrations of haem or PPIX are shown in Figures 3.2.1.1 and 3.2.1.2. Concentrations of exogenously supplied haem or PPIX of 2µg/ml or greater resulted in maximal growth yields. However, at haem or PPIX concentrations below 2µg/ml the cell yield (as measured by OD_{470}) drops steadily. Reductions in final OD of 65% and 61% respectively were recorded for non typable *H.influenzae* isolates HM1 and HM3 when the external haem concentration was decreased from 5 to 0.25µg/ml. Similarly reductions in final culture OD of 64% and 55%, respectively, were recorded for the same two strains when initial PPIX concentrations were reduced from 5µg/ml to 0.25µg/ml.

There was little difference in final cell yield between haem and PPIX grown cells, indicating that PPIX was an adequate substitute for haem, and implying the production of ferrochelatase enzyme (Porra and Jones, 1963; see Fig. 1.4.2) by these isolates.

Figure 3.2.1.1.

a. Final Cell Yield and pH of Cultures of *H.influenzae* HM1 Grown in Varying Concentrations of Haem.



b. Final Cell Yield and pH of Cultures of *H.influenzae* HM1 Grown in Varying Concentrations of PPIX.

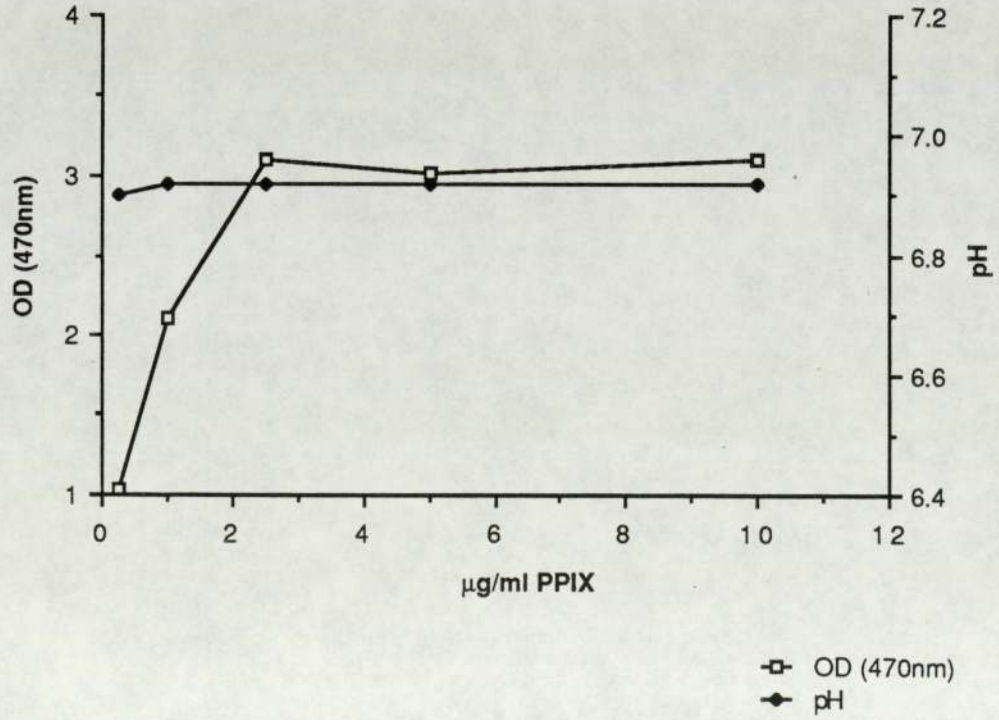
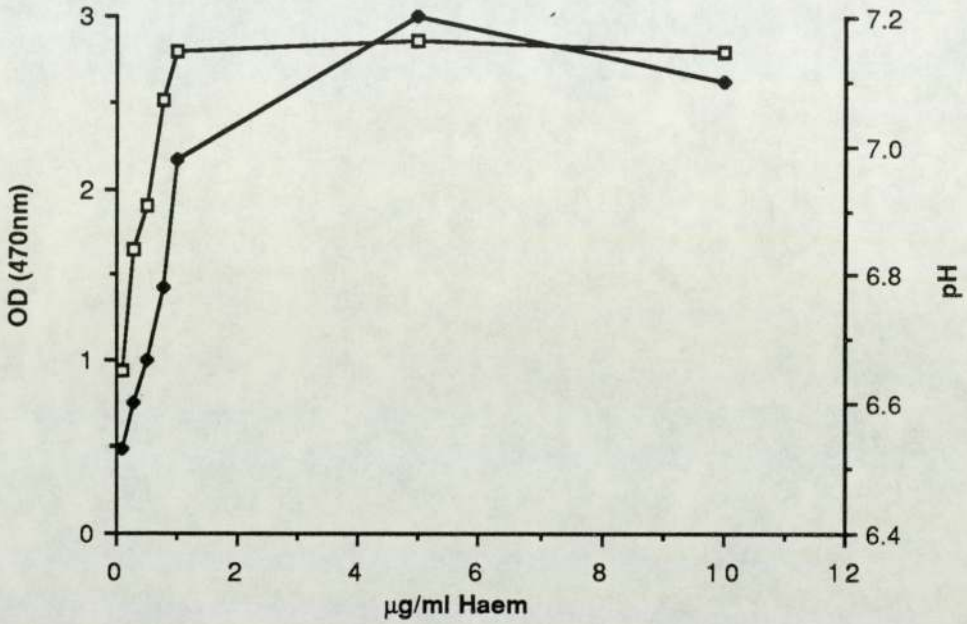
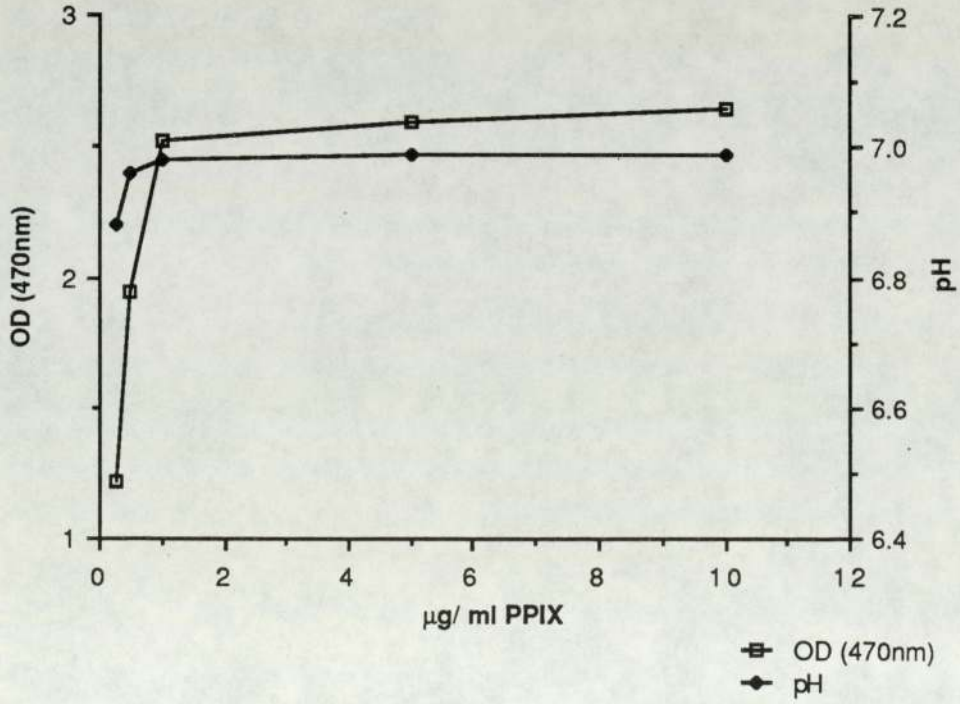


Figure 3.2.1.2.

a. Final Cell Yield and pH of Cultures of *H.influenzae* HM3 Grown in Varying Concentrations of Haem.



b. Final Cell Yield and pH of Cultures of *H.influenzae* HM3 Grown in Varying Concentrations of PPIX.



Although final cell yields are analogous regardless of haem source, there are marked differences in the final pH of non typable *H.influenzae* cultured in growth yield limiting concentrations of haem (Fig. 3.2.1.1a and 3.2.1.2a) and those cultured in growth yield limiting concentrations of PPIX (Fig. 3.2.1.1b and 3.2.1.2b). The final pH of cultures of *H.influenzae* HM1 grown in haem excess was 7.12 ± 0.02, however, when the initial haem concentration was reduced to growth yield limiting amounts (0.25µg/ml) the final culture pH was reduced to 6.62 ± 0.01 in spite of the fact of a 65% decrease in biomass. Similarly the final pH of cultures of *H.influenzae* HM3 was reduced from 7.1 ± 0.18 to 6.53 ± 0.06 when the amount of available haemin was reduced from excess to growth yield limiting concentrations.

When PPIX is used as a haem source, these pH changes do not occur. Reduction in the initial PPIX concentration from 5µg/ml to 0.25µg/ml was accompanied by only a small drop in final culture pH from 6.94 ± 0.02 to 6.92 ± 0.02 for HM1 and from 6.98 ± 0.01 to 6.90 ± 0.01 for *H.influenzae* HM3. These results seem to indicate altered metabolic responses by non typable *H.influenzae* depending on whether the porphyrin source is haem or PPIX and would also suggest alterations in metabolism between *H.influenzae* cultured in an environment where haem is limiting and *H.influenzae* grown in conditions of haem excess, the products of which lead to increased acidification of the growth medium.

Growth curves of four non typable *H.influenzae* bronchiectasis isolates, designated HM1-HM4 are shown in Figs. 3.2.1.3 - 3.2.1.6. Bacteria were grown in NAD supplemented BHI broth under conditions of decreasing haem (panel A) and PPIX (panel B) availability.

Reduction of initial amounts of haem source from 5µg/ml to 0.25µg/ml resulted in a decrease in growth yield but did not appear to greatly affect growth rate for any of the strains examined. Doubling times for cells of *H.influenzae* HM1 were 37 minutes (haem excess), 40 minutes (haem-limited), 36 minutes (PPIX excess), and 38 minutes (PPIX-limited). Similarly strains HM2 - HM4 did not show alterations in growth rate when concentrations of exogenously supplied haem or PPIX were reduced to growth yield-limiting levels. In all instances growth rates, lag times and final biomass yields were similar regardless of whether the porphyrin source was haem or PPIX.

Iron-restriction of *H.influenzae* in complex media can be achieved by substitution of PPIX for haem and addition of an iron chelator to the system (Williams and Brown, 1986; Herrington and Sparling, 1985). Any growth occurring is then dependent on the amount of free iron available in the medium. The effect of addition of the iron chelator Desferal (desferrioxamine mesylate, CIBA GEIGY Limited) on the growth curve of non typable *H.influenzae* cultured under conditions where PPIX was present in limiting concentrations (0.25µg/ml) is shown in Fig. 3.2.1.7. Culture in PPIX

supplemented BHI broth containing Desferal at a concentration of 30μM resulted in a 20% reduction in growth yield compared with cells grown under PPIX-limitation alone. Further reductions in growth yield could be achieved using higher concentrations of Desferal but also resulted in increased lag time and decreased growth rate. Desferal was therefore used at a concentration of 30μM in order to achieve iron-restricted conditions in all future experiments.

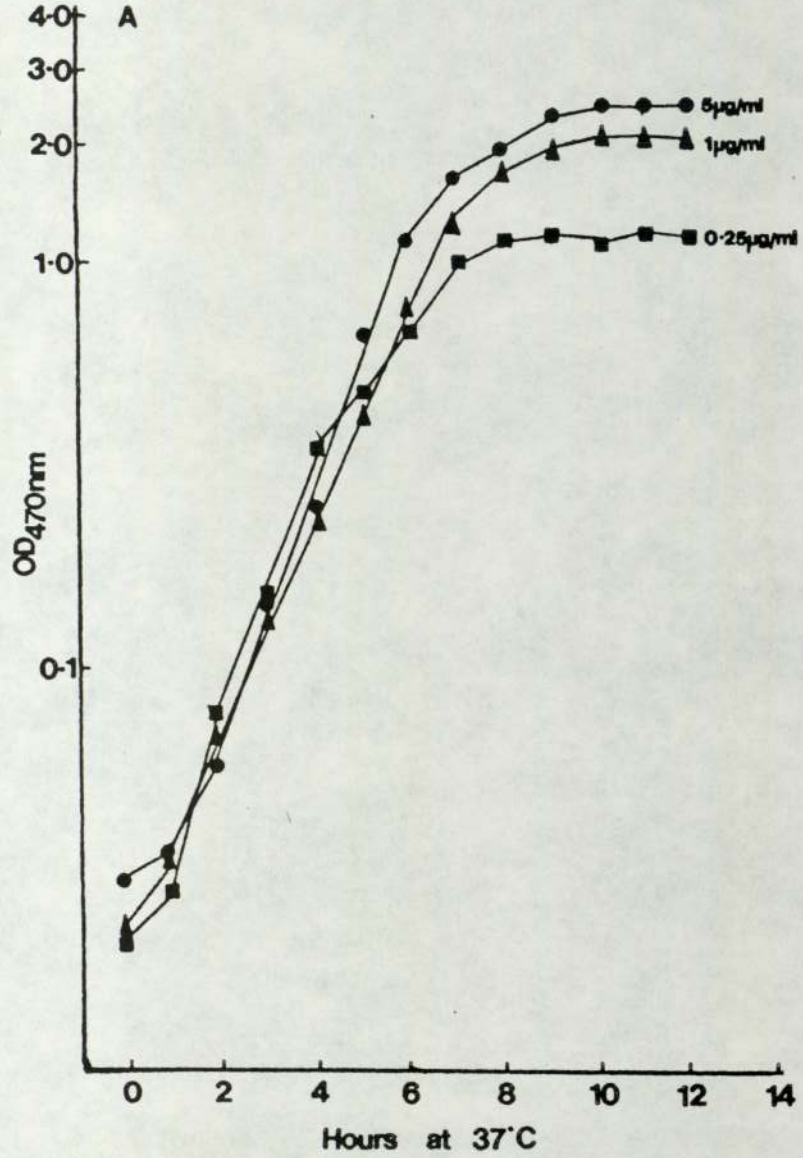
Iron-restriction of cultures of *H.influenzae* isolates HM1-HM4 (Fig. 3.2.1.8 a-d) using this method resulted in similar decreases in final cell yield in each case and did not cause major changes in growth rate in any instance.

Figure 3.2.1.3

Panel A Effect of Initial Haem Concentration on the Growth Curve of *H.influenzae* HM1

Panel B Effect of Initial PPIX Concentration on the Growth Curve of *H.influenzae* HM1

Bacterial growth in BHI broth supplemented with excess NAD (5μg/ml) and containing decreasing concentrations of haem or PPIX was monitored turbidimetrically.


A
4·0
3·0
2·0
1·0
0·1
OD470nm
5µg/ml
1µg/ml
0·25µg/ml
0
2
4
6
8
10
12
14
Hours at 37°C

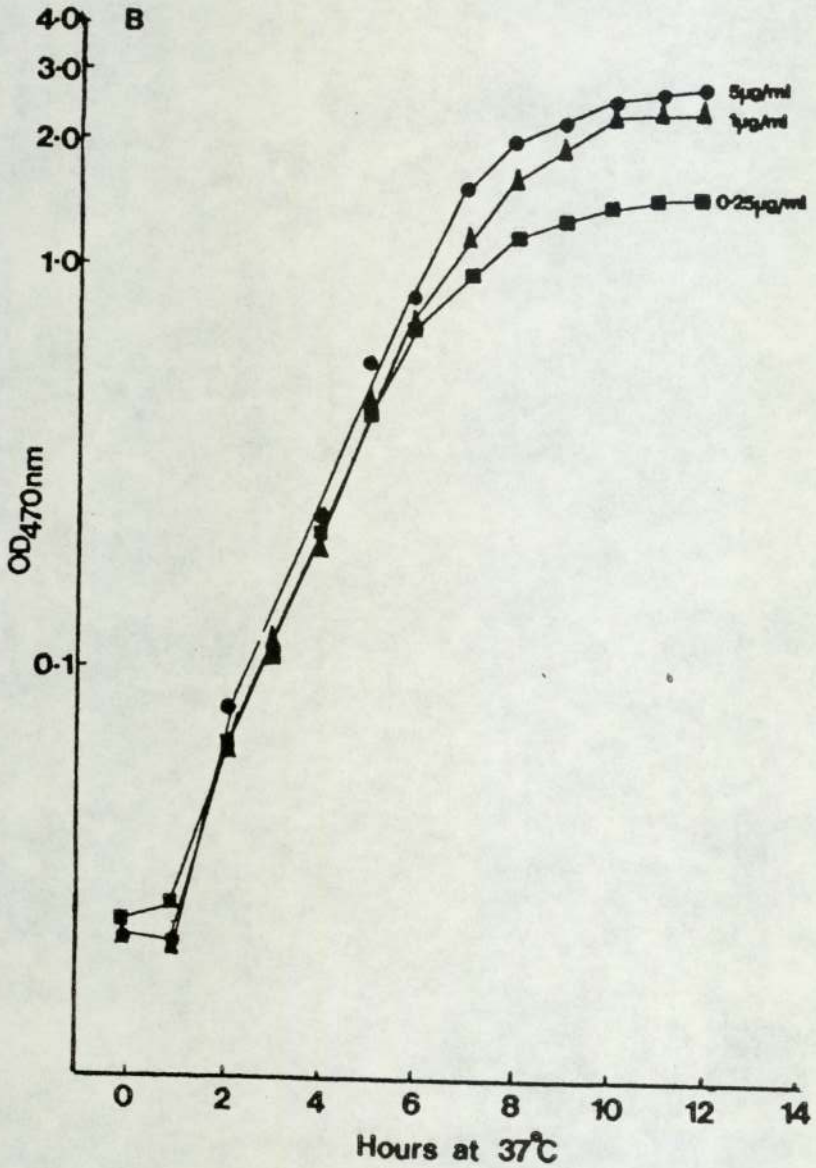
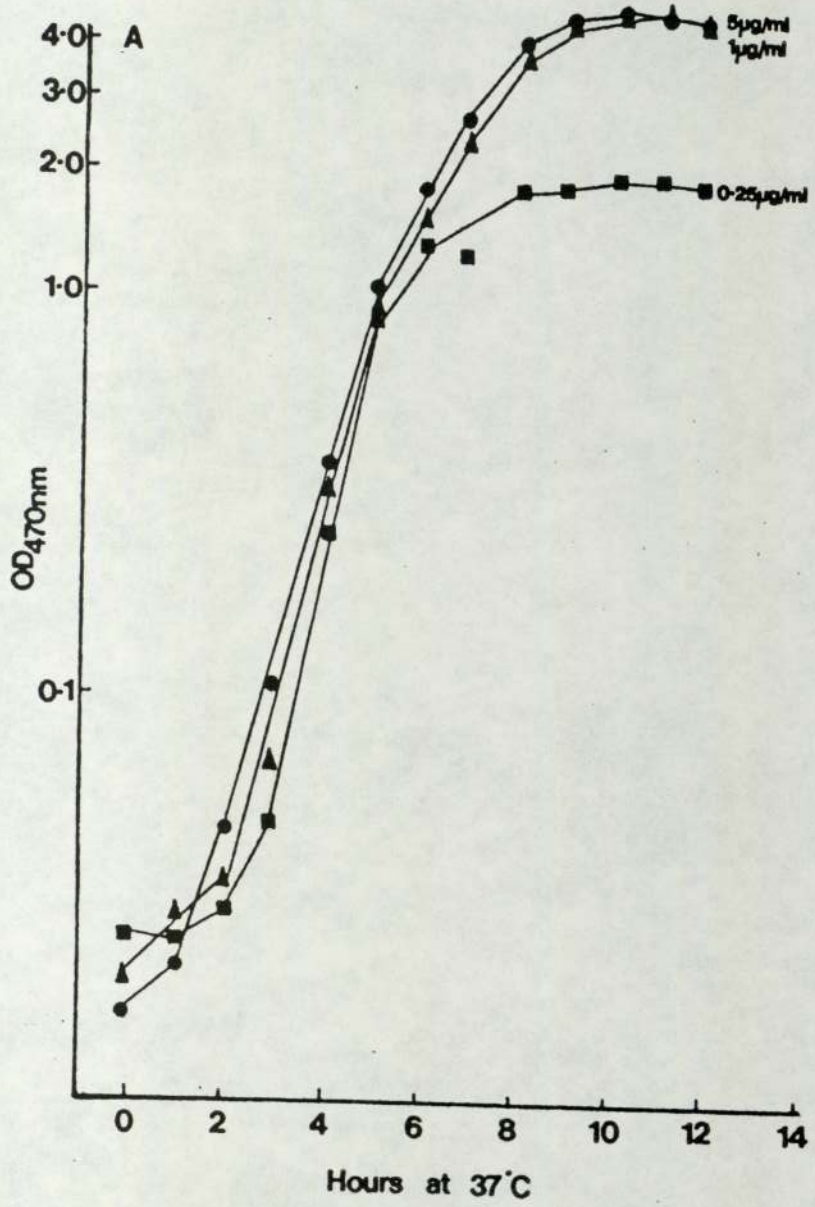

B
4·0
3·0
2·0
1·0
0·1
OD470nm
5µg/ml
1µg/ml
0·25µg/ml
0
2
4
6
8
10
12
14
Hours at 37°C

Figure 3.2.1.4

Panel A Effect of Initial Haem Concentration on the Growth Curve of *H.influenzae* HM2

Panel B Effect of Initial PPIX Concentration on the Growth Curve of *H.influenzae* HM2

Bacterial growth in BHI broth supplemented with excess NAD (5μg/ml) and containing decreasing concentrations of haem or PPIX was monitored turbidimetrically.


A
4·0
3·0
2·0
1·0
0·1
OD470nm
5µg/ml
1µg/ml
0·25µg/ml
0
2
4
6
8
10
12
14
Hours at 37°C

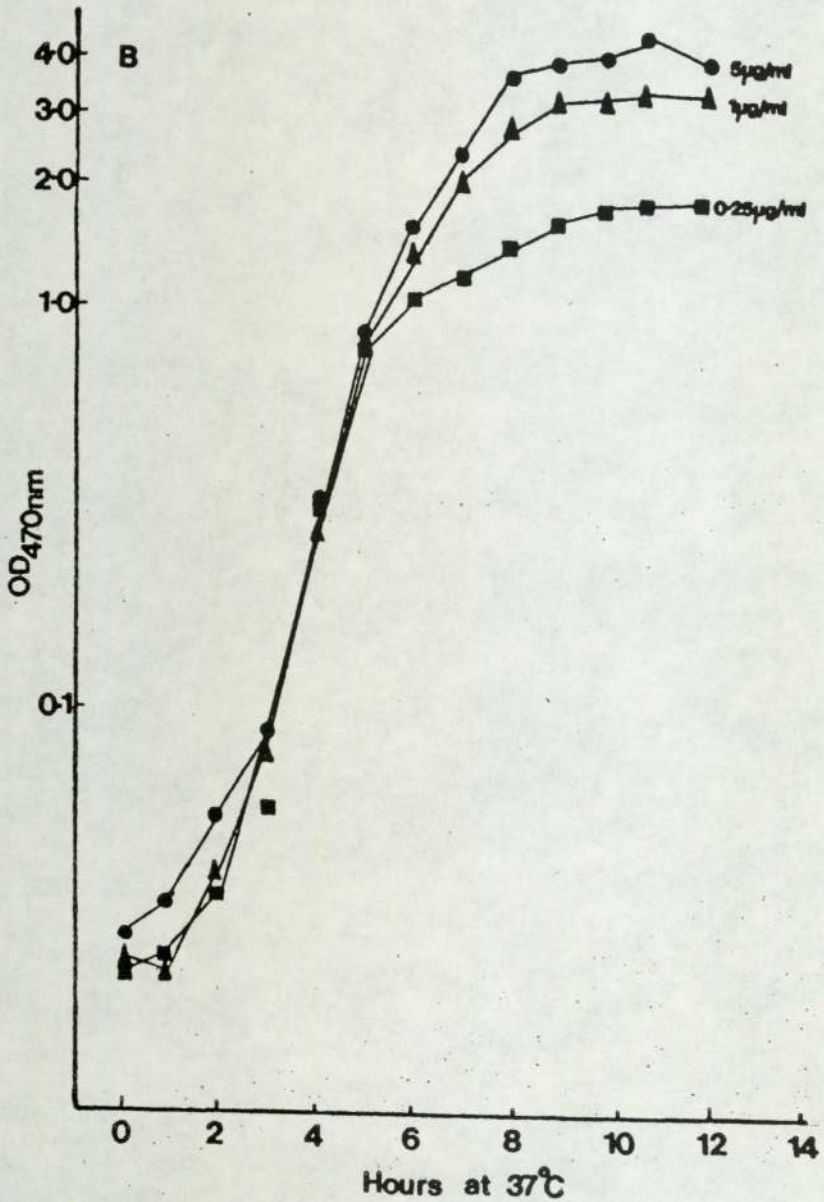
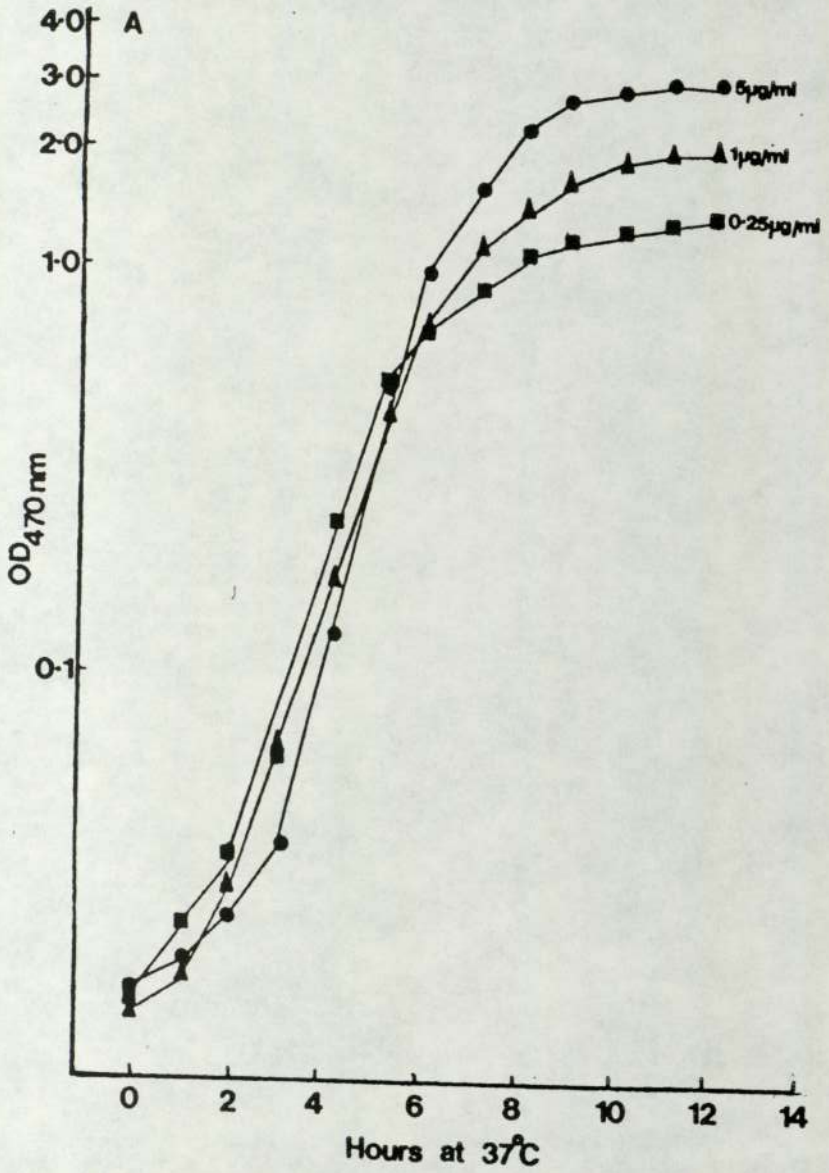

B
4·0
3·0
2·0
1·0
0·1
OD470nm
5µg/ml
1µg/ml
0·25µg/ml
0
2
4
6
8
10
12
14
Hours at 37°C

Figure 3.2.1.5

Panel A Effect of Initial Haem Concentration on the Growth Curve of *H.influenzae* HM3

Panel B Effect of Initial PPIX Concentration on the Growth Curve of *H.influenzae* HM3

Bacterial growth in BHI broth supplemented with excess NAD (5μg/ml) and containing decreasing concentrations of haem or PPIX was monitored turbidimetrically.


A
4·0
3·0
2·0
1·0
0·1
OD470nm
5µg/ml
1µg/ml
0·25µg/ml
0
2
4
6
8
10
12
14
Hours at 37°C

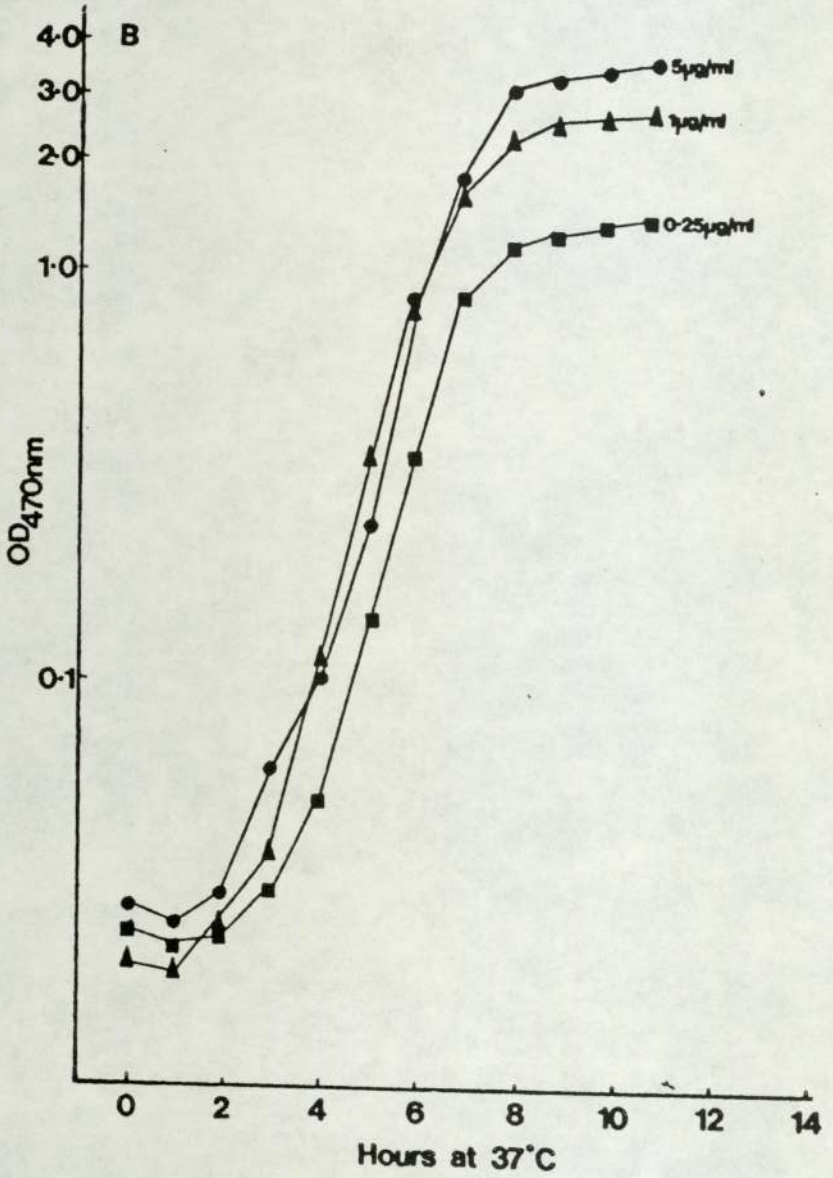
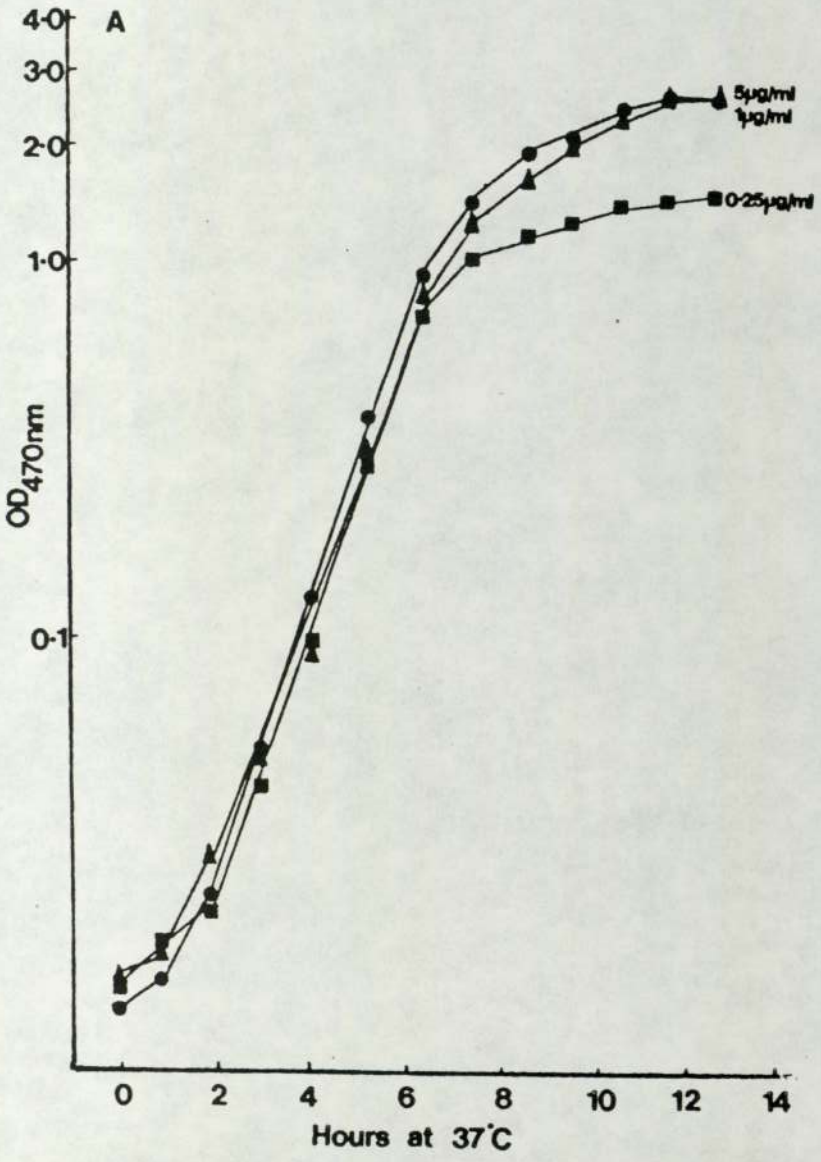

B
4·0
3·0
2·0
1·0
0·1
OD470nm
5µg/ml
1µg/ml
0·25µg/ml
0
2
4
6
8
10
12
14
Hours at 37°C

Figure 3.2.1.6

Panel A Effect of Initial Haem Concentration on the Growth Curve of *H.influenzae* HM4

Panel B Effect of Initial PPIX Concentration on the Growth Curve of *H.influenzae* HM4

Bacterial growth in BHI broth supplemented with excess NAD (5μg/ml) and containing decreasing concentrations of haem or PPIX was monitored turbidimetrically.


A
4·0
3·0
2·0
1·0
0·1
OD470nm
5µg/ml
1µg/ml
0·25µg/ml
0
2
4
6
8
10
12
14
Hours at 37°C

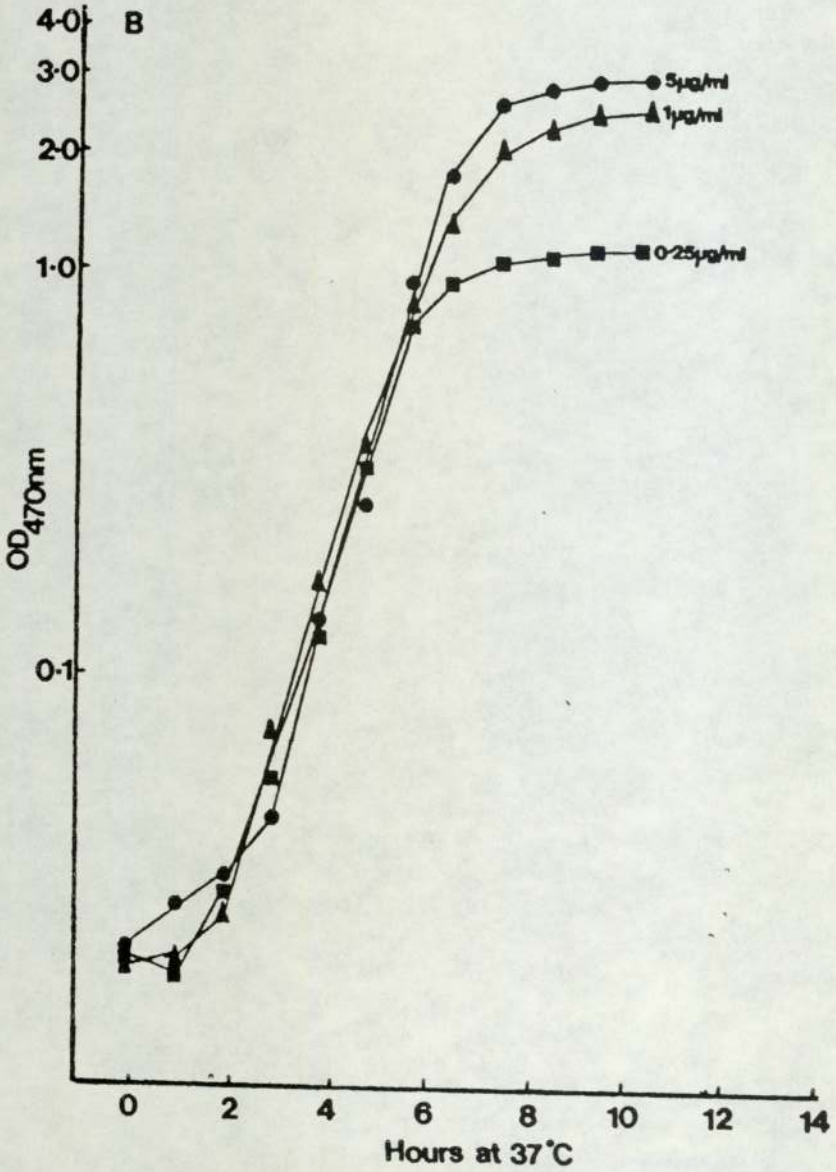

B
4·0
3·0
2·0
1·0
0·1
OD470nm
5µg/ml
1µg/ml
0·25µg/ml
0
2
4
6
8
10
12
14
Hours at 37°C

Figure 3.2.1.7

Effect of Increasing Concentrations of the Iron Chelator Desferal on the Growth Curve of *H.influenzae* HM1.

Bacterial growth in BHI broth supplemented with excess NAD, 0.25μg/ml PPIX and 0 (■), 30 (◇), 45 (○), or 60 (▼) μM Desferal was monitored turbidimetrically.

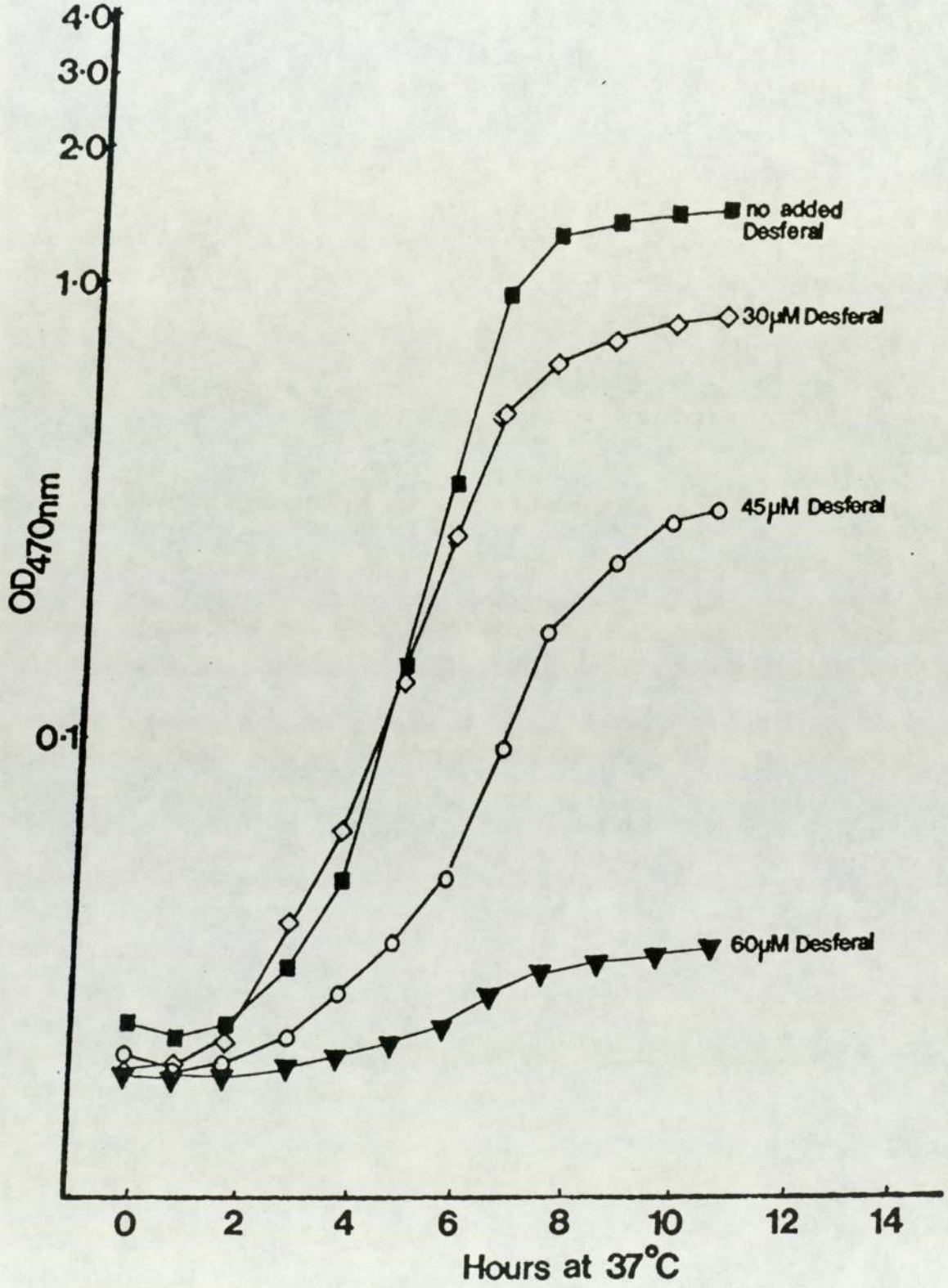

4·0
3·0
2·0
1·0
0·1
OD_{470nm}
no added Desferal
30μM Desferal
45μM Desferal
60μM Desferal
0
2
4
6
8
10
12
14
Hours at 37°C

Figure 3.2.1.8

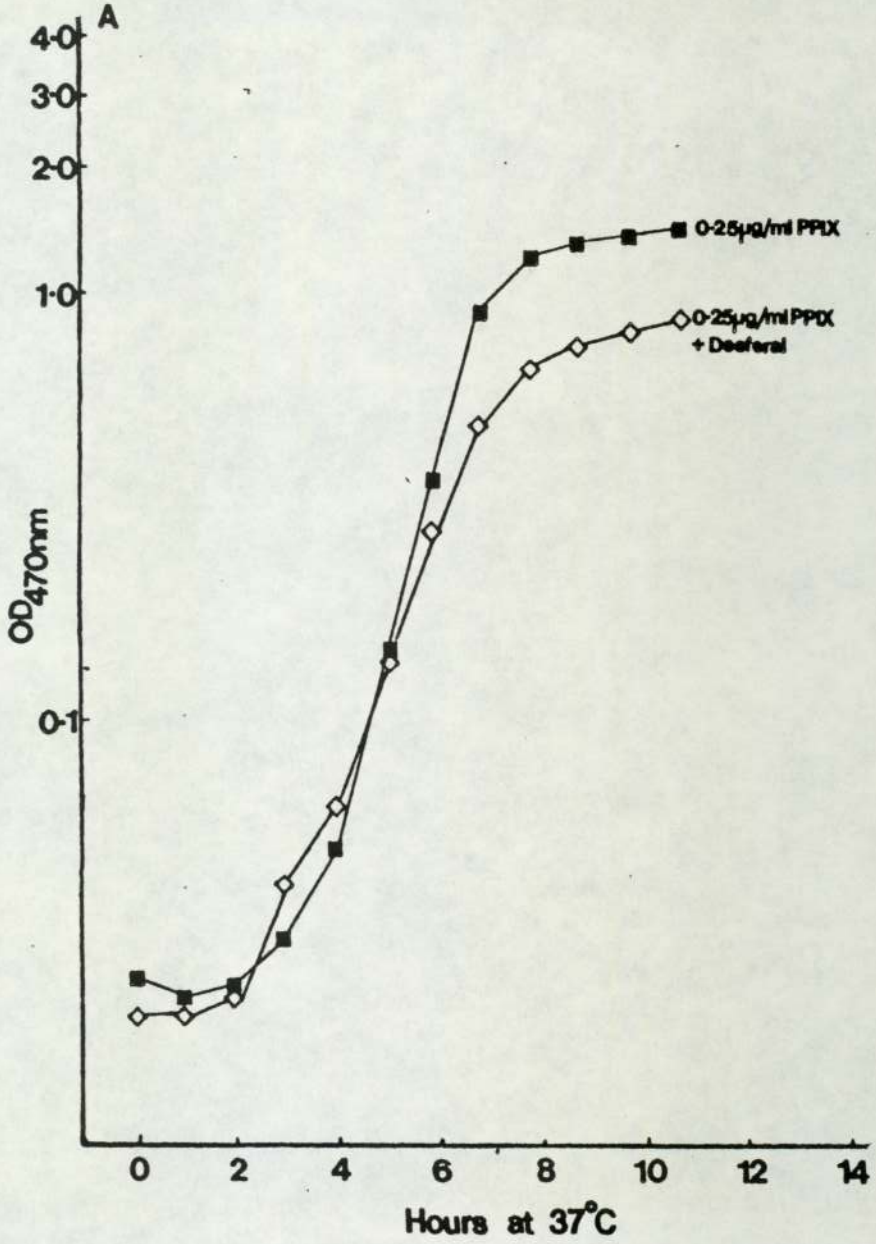
Panel A Effect of Iron Restriction on the Growth Curve of *H.influenzae* HM1.

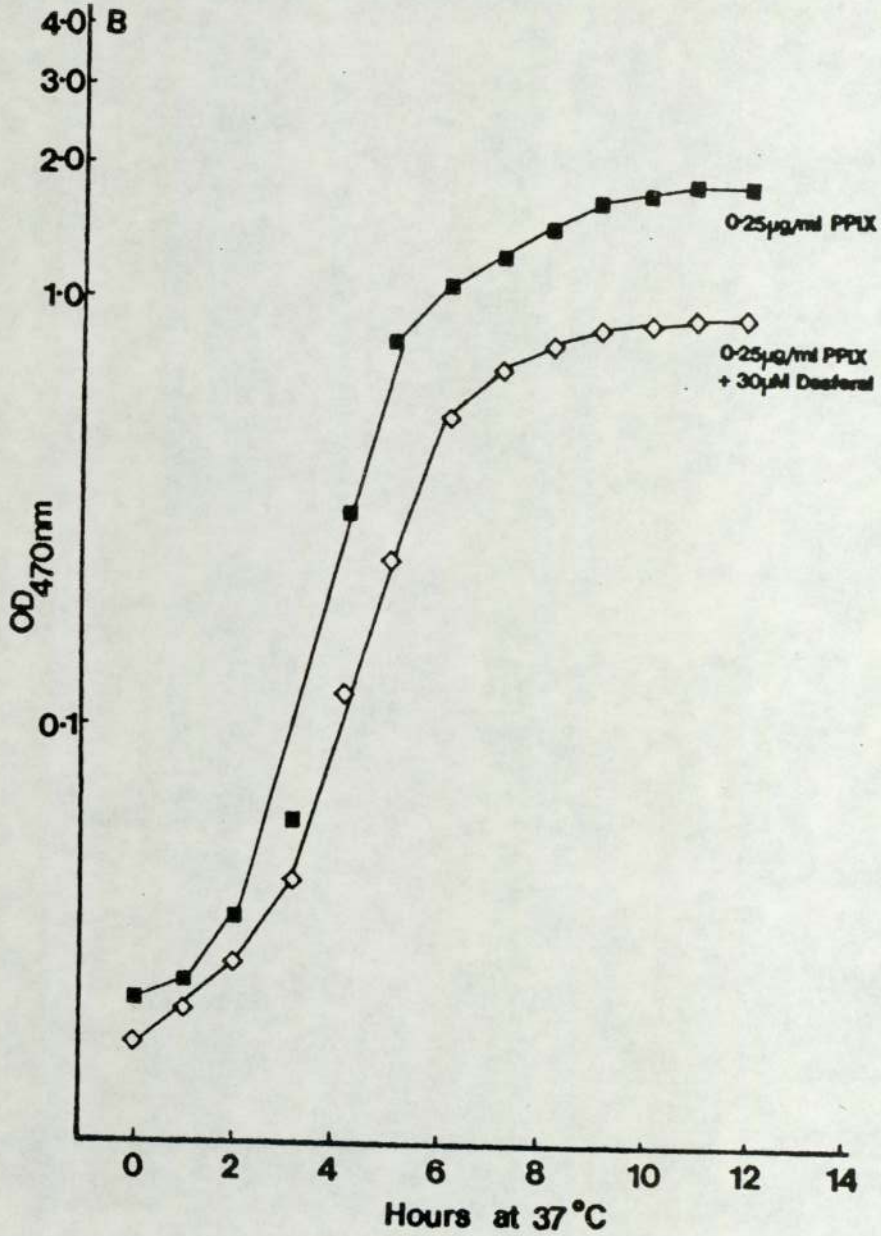
Panel B Effect of Iron Restriction on the Growth Curve of *H.influenzae* HM2.

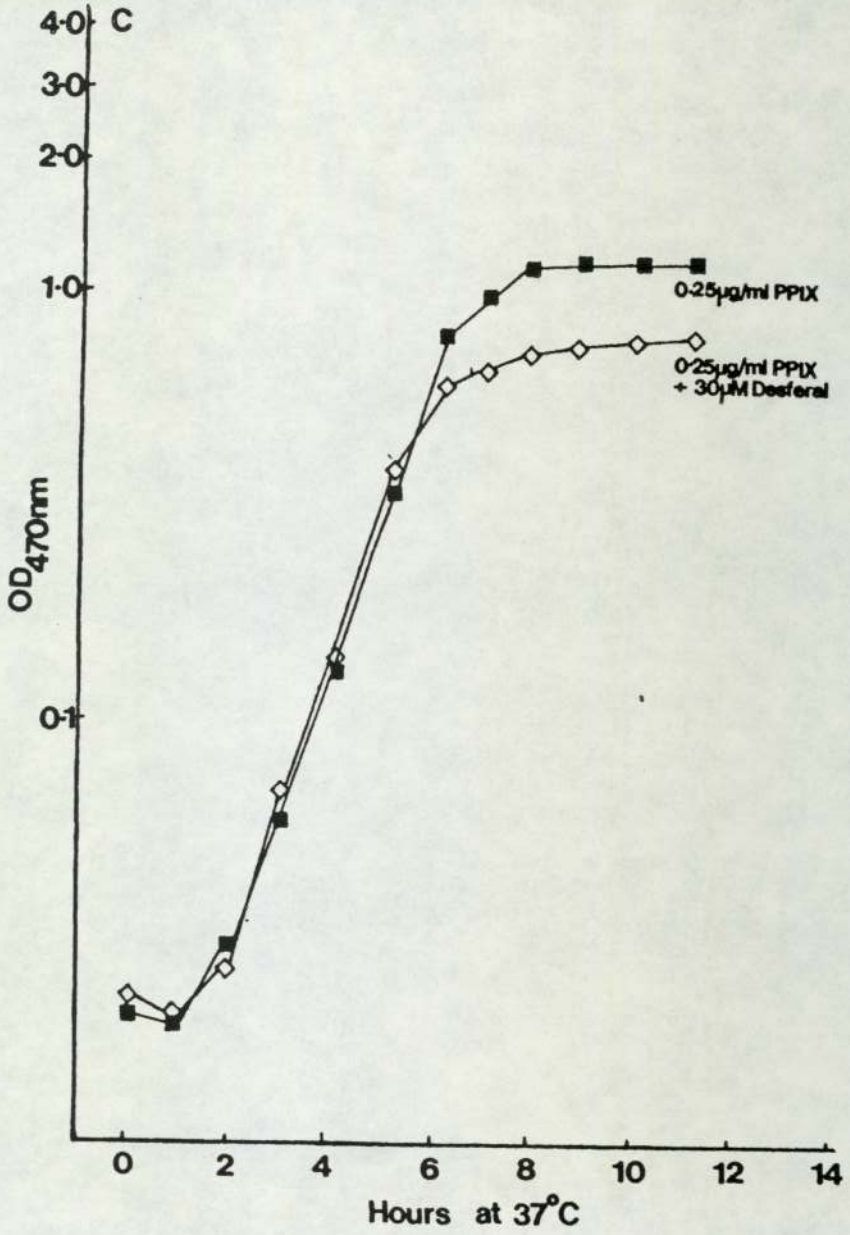
Panel C Effect of Iron Restriction on the Growth Curve of *H.influenzae* HM3.

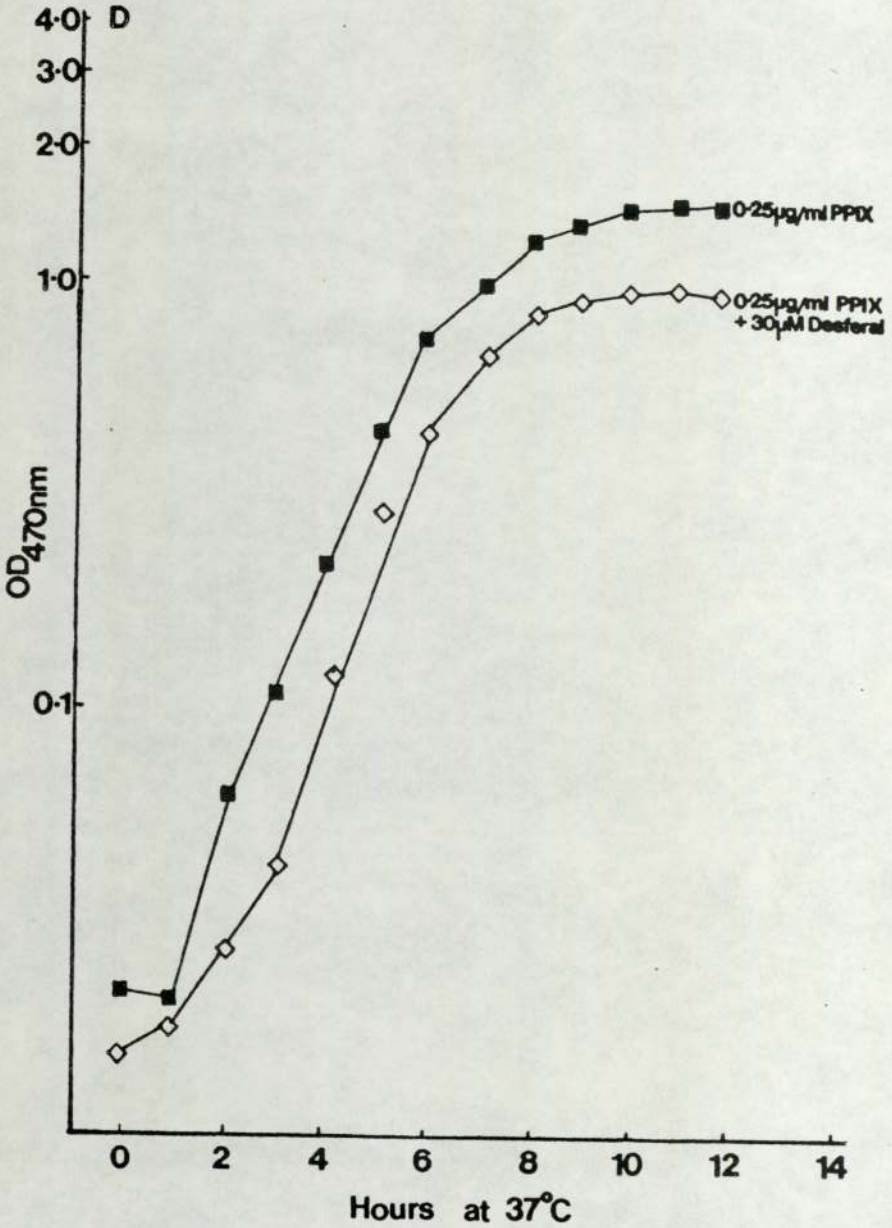
Panel D Effect of Iron Restriction on the Growth Curve of *H.influenzae* HM4.

Bacterial growth in BHI broth supplemented with excess NAD, 0.25μg/ml PPIX, and with either 0 (■) or 30 (◇) μM Desferal was monitored turbidimetrically.


A
4·0
3·0
2·0
1·0
0·1
OD470nm
0·25µg/ml PPIX
0·25µg/ml PPIX
+ Desferal
0
2
4
6
8
10
12
14
Hours at 37°C


B
4·0
3·0
2·0
1·0
0·1
OD470nm
0·25µg/ml PPIX
0·25µg/ml PPIX
+ 30µM Desferal
0
2
4
6
8
10
12
14
Hours at 37°C


C
4·0
3·0
2·0
1·0
0·1
OD_{470nm}
0·25µg/ml PPIX
0·25µg/ml PPIX
+ 30µM Desferal
0
2
4
6
8
10
12
14
Hours at 37°C


D
4·0
3·0
2·0
1·0
0·1
OD_{470nm}
0·25µg/ml PPIX
0·25µg/ml PPIX
+ 30µM Desferal
0
2
4
6
8
10
12
14
Hours at 37°C

3.2.2. The Effect of Haem-Limitation and Iron-restriction on the Outer Membrane of Non Typable *H.influenzae*.

The effect of growth under iron-restricted conditions on the OMP profile of non typable *H.influenzae* is illustrated in Figure 3.2.2.1 a-e. In total 12 out of 28 strains examined (43%) displayed alterations in OMP profile on iron-restriction (lane 2, Fig. 3.2.2.1 a-e) compared with growth under conditions of haem excess (lane 1, Fig. 3.2.2.1 a-e). All of the *H.influenzae* isolates responding to iron-restriction in terms of altered OMP profile produced increased amounts of a protein of molecular weight 84K. Seventy-five percent (9/12) of responders also produced small amounts of a novel 150K OMP and 25% (3/12) also produced a new 120K OMP under conditions of iron-restriction. The OMP profiles shown in Fig. 3.2.2.1 a-e typify the range of responses to iron restriction observed.

Analysis of OMP profiles of *H.influenzae* cultured under conditions of PPIX excess, PPIX-limitation, and iron restriction (Fig. 3.2.2.2) showed that the OMP changes described above occur in all conditions where PPIX is used as a porphyrin source regardless of whether or not Desferal is included in the culture medium, this would suggest that these OMPs are PPIX-specific rather than iron-regulated proteins. However, the OMP profile of *H.influenzae* grown under conditions of haem-limitation and iron-restriction (0.25μg/ml haem + 30μM Desferal) also shows increased production of an 84K protein (lane 4, Fig. 3.2.2.2) compared

FIGURE 3.2.2.1a-e.

The Effect of Iron-Restriction on OMP Profiles of Non Typable *H.influenzae* Isolated From Bronchiectasis Patients.

Bacteria were cultured in BHI broth containing excess NAD (5µg/ml) and were subject to conditions of either haem excess or iron-restriction. OMs were prepared as described previously (2.3.1); OMPs were separated by SDS-PAGE (2.4.1) and stained using coomassie blue. Numbers and letters in the right hand margin refer to proteins of *H.influenzae*, either by molecular weight or by standard nomenclature (Munson *et al*, 1983; Murphy and Bartos, 1988).

(a) *H.influenzae* HM17

Lane 1: molecular weight standards.

Lane 2: Haem excess

Lane 3: Iron-restricted (PPIX-limited + 30µM Desferal).

(b) *H.influenzae* HM1.

Lane 1: Haem excess.

Lane 2: Iron-restriced (PPIX-limited + 30µM Desferal).

(c) *H.influenzae* HM11. Legend as for (b).

(d) *H.influenzae* HM2. Legend as for (b).

(e) *H.influenzae* HM10. Legend as for (b).

FIGURE 3.2. 2.1

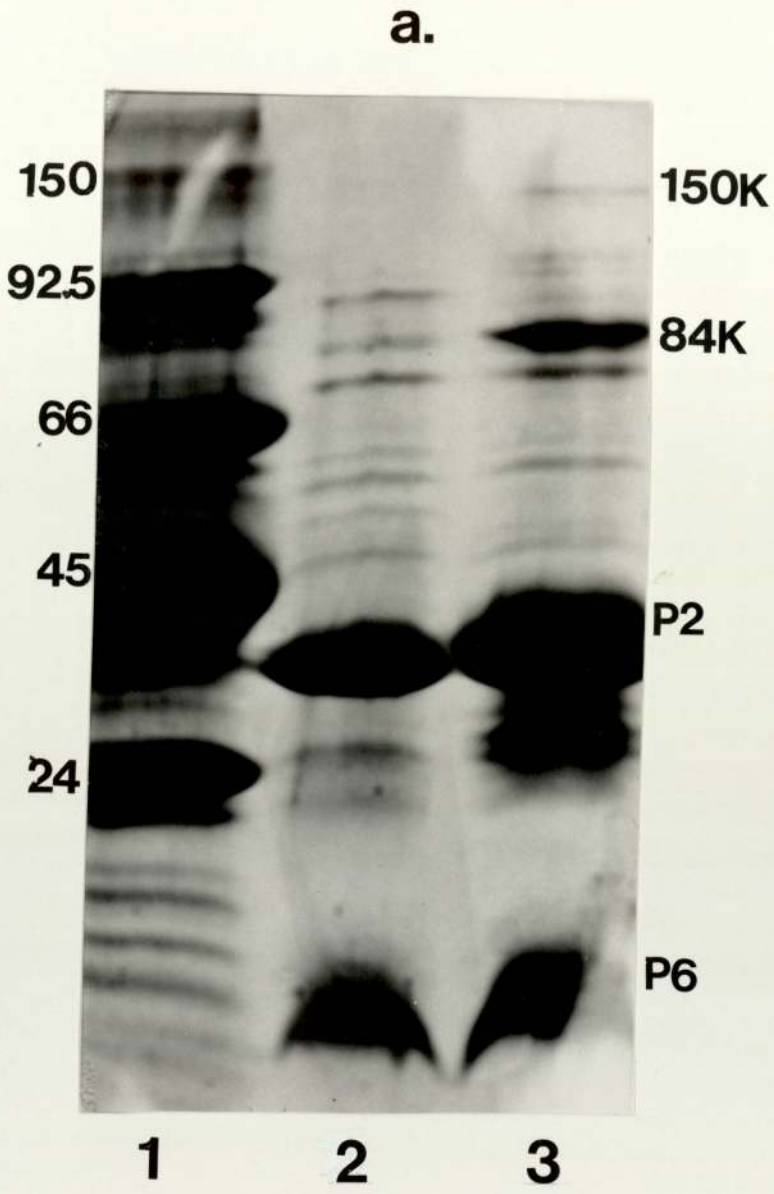


FIGURE 3.2.2.1

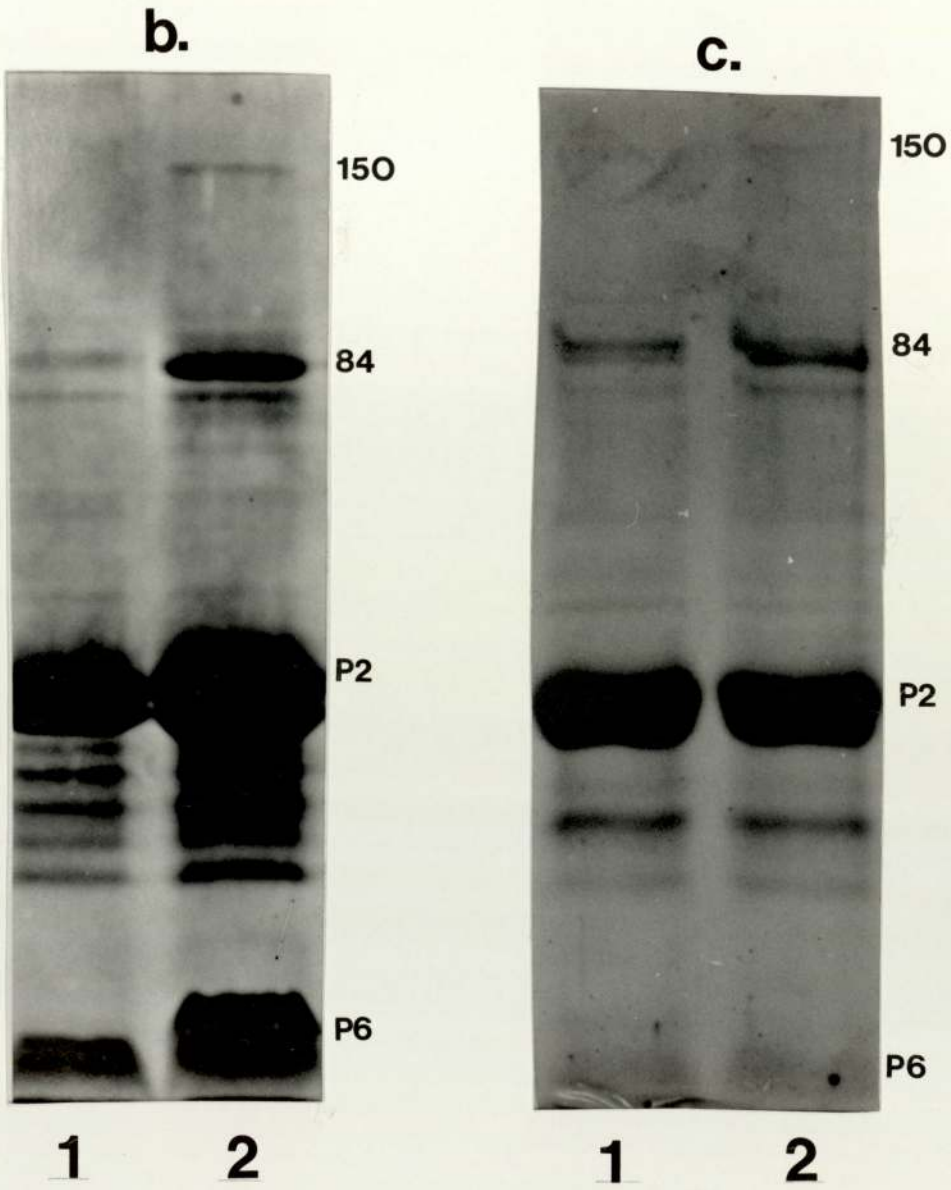


FIGURE 3.2. 2.1

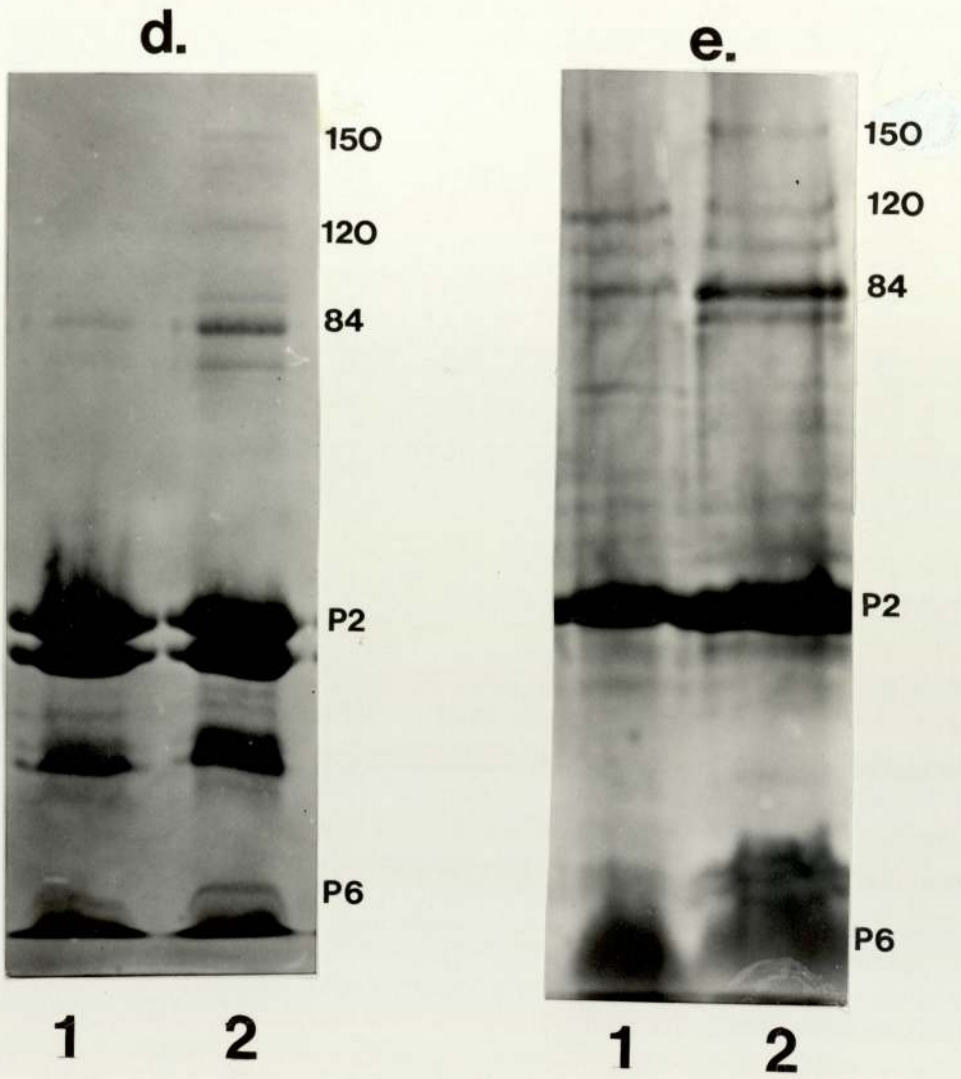


FIGURE 3.2.2.2

Outer Membrane Protein Profiles of Non Typable *H.influenzae* HM1: The Effect of Haem or PPIX Limitation and Iron-Restriction.

Bacteria were grown in BHI broth containing excess NAD (5µg/ml) and supplemented with haem, PPIX and Desferal as indicated below. OMs were prepared by sarkosyl extraction as described previously (2.3.1). OMPs were separated by SDS-PAGE (2.4.1) and stained using coomassie blue. Figures given in the margins refer to molecular weight in kilodaltons, molecular weight standards are shown in lane 1.

Lane 2: Haem excess (5µg/ml).

Lane 3: Haem-limited (0.25µg/ml).

Lane 4: Haem-limited + 30µM Desferal.

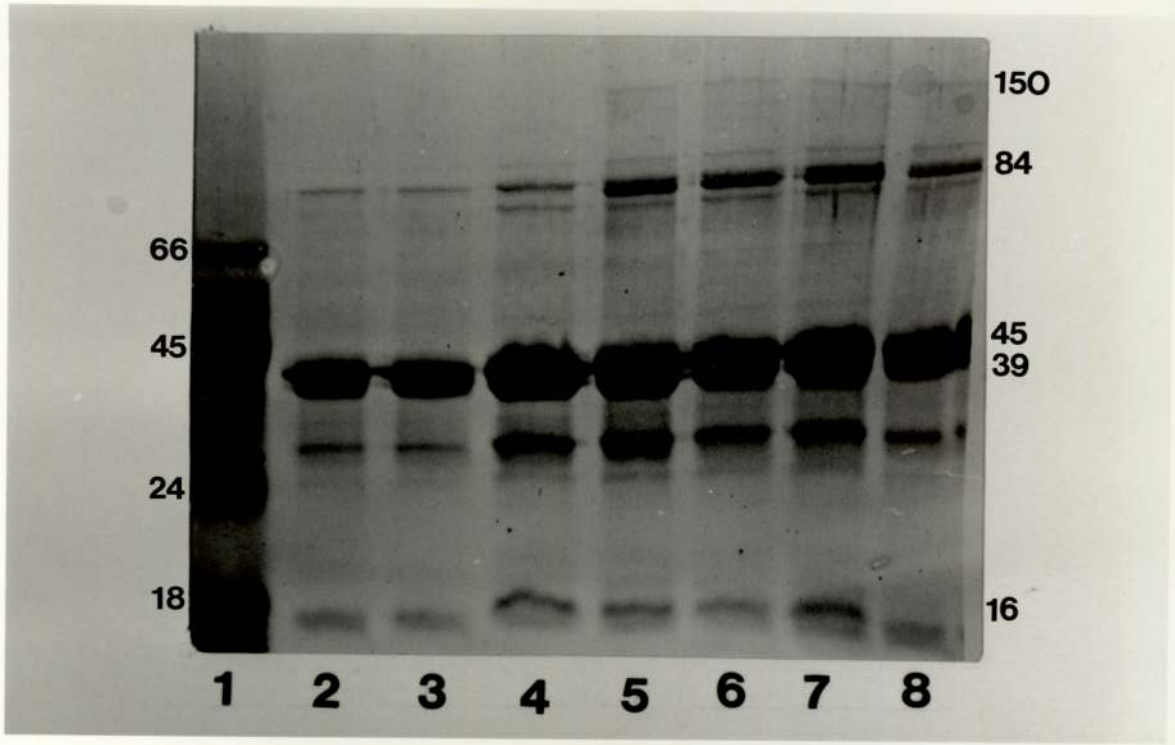
Lane 5: PPIX excess (5µg/ml).

Lane 6: PPIX excess + 30µM Desferal.

Lane 7: PPIX-limited (0.25µg/ml).

Lane 8: Iron restricted (0.25µg/ml PPIX + 30µM Desferal).

FIGURE 3.2.2.2.



to cells grown under conditions of haem excess (lane 2) or haem-limitation (lane 3), these cells also weakly expressed a 150K protein but this was very faint and did not show up on photographs. These results suggest that iron availability does play a role in the induction of these OMP changes.

The remaining 57% of *H.influenzae* isolated during the course of this study did not show any OMP changes on haem-limitation, on substitution of PPIX for haem, or on iron-restriction. This is illustrated by *H.influenzae* HM3, shown in Fig. 3.2.2.3. The OMP profile of this isolate remained constant regardless of porphyrin source or iron availability.

Figure 3.2.2.4 shows the OMP profiles of *H.influenzae* HM1 grown under anaerobic conditions in the absence of haem (lane 1) and in conditions of haem excess (lane 2), haem-limitation (lane 3), PPIX excess (lane 4) and PPIX-limitation (lane 5). *Haemophilus influenzae* do not require haem in order to grow anaerobically (White, 1963). The absence of high molecular weight material, including inducible PPIX-specific proteins in these cells suggests a role for these OMPs in aerobic metabolism, or a requirement of oxygen for their production. Cells cultured under iron-restricted conditions (0.25μg/ml PPIX + 30μM Desferal) would not grow under anaerobic conditions.

LPS profiles of non typable *H.influenzae* isolates were analysed by SDS-PAGE (2.4.1) after proteinase K digestion of

FIGURE 3.2.2.3.

Outer Membrane Protein Profiles of Non Typable *H.influenzae* HM3: The Effect of Haem or PPIX Limitation and Iron-Restriction.

Bacteria were grown in BHI broth containing excess NAD (5µg/ml) supplemented with haem or PPIX and Desferal as shown below. OMs were prepared by sarkosyl extraction as described previously (2.3.1), OMPs were separated by SDS-PAGE (2.4.1) and stained using coomassie blue. Numbers in the right hand margin refer to *H.influenzae* proteins by molecular weight or standard nomenclature.

Lane 1: Haem excess (5µg/ml).

Lane 2: Haem-limited (0.25µg/ml).

Lane 3: PPIX excess (5µg/ml).

Lane 4: Iron-restricted (0.25µg/ml PPIX + 30µM Desferal).

FIGURE 3.2.2.3

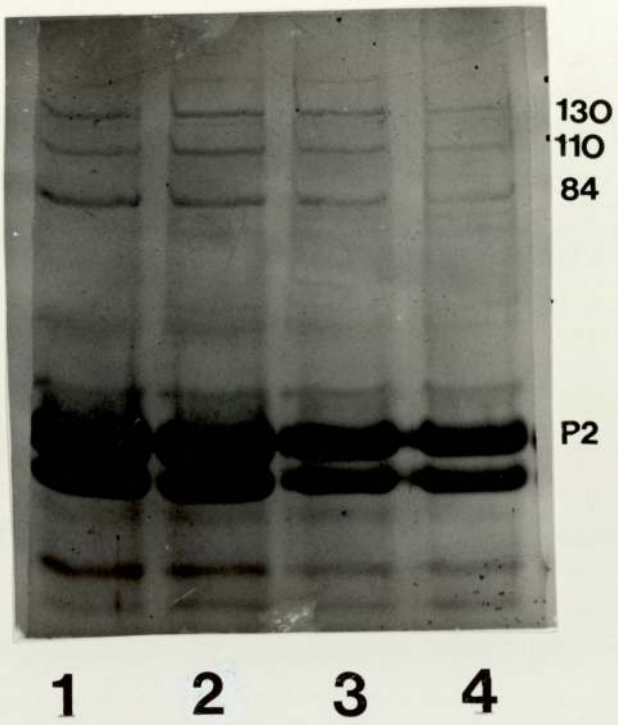


FIGURE 3.2.2.4.

The Effect of Growth Under Anaerobic Conditions on the OMP Profile of Non Typable *H.influenzae* HM1.

Bacteria were cultured under anaerobic conditions (see 2.2.5) in BHI broth containing excess (5µg/ml) NAD and supplemented with haem or PPIX as shown below. OMs were prepared by sarkosyl extraction (2.3.1), OMPs were separated by SDS-PAGE (2.4.1) and stained using coomassie blue.

Lane 1: No haem added.

Lane 2: Haem excess (5µg/ml).

Lane 3: Haem-limited (0.25µg/ml).

Lane 4: PPIX excess (5µg/ml).

Lane 5: PPIX-limited (0.25µg/ml).

FIGURE 3.2.2.4

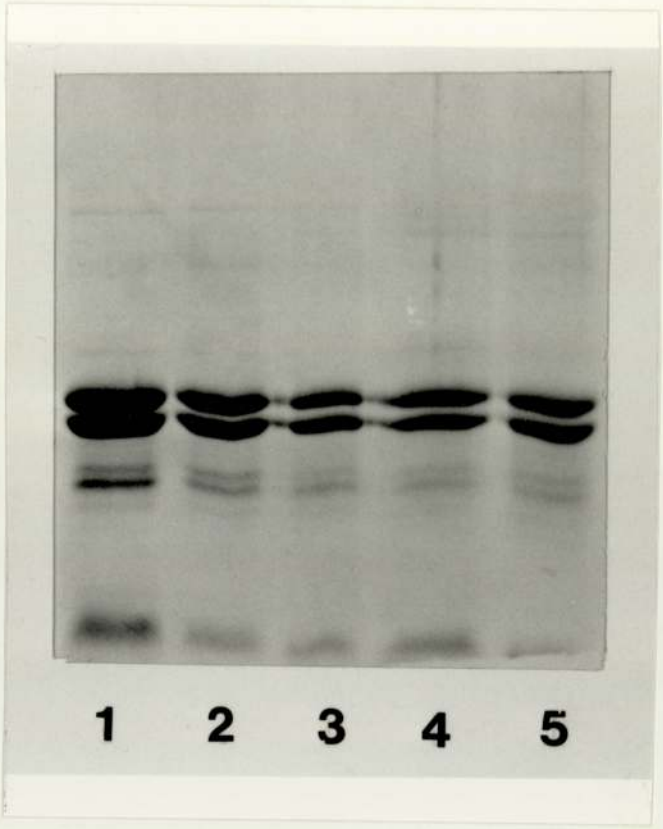


FIGURE 3.2.2.5.

LPS Profiles of *H.influenzae*: The Effect of Haem or PPIX Limitation and Iron-Restriction.

Bacteria were grown in BHI broth containing excess NAD (5µg/ml) and supplemented with either 5µg/ml haem or 0.25µg/ml PPIX + 30µM Desferal. LPS was prepared by proteinase K digestion of whole cells as described in section 2.3.3. After separation by SDS-PAGE (2.4.1b) LPS was stained using silver nitrate according to the method of Tsai and Frasch (1982) (2.4.2).

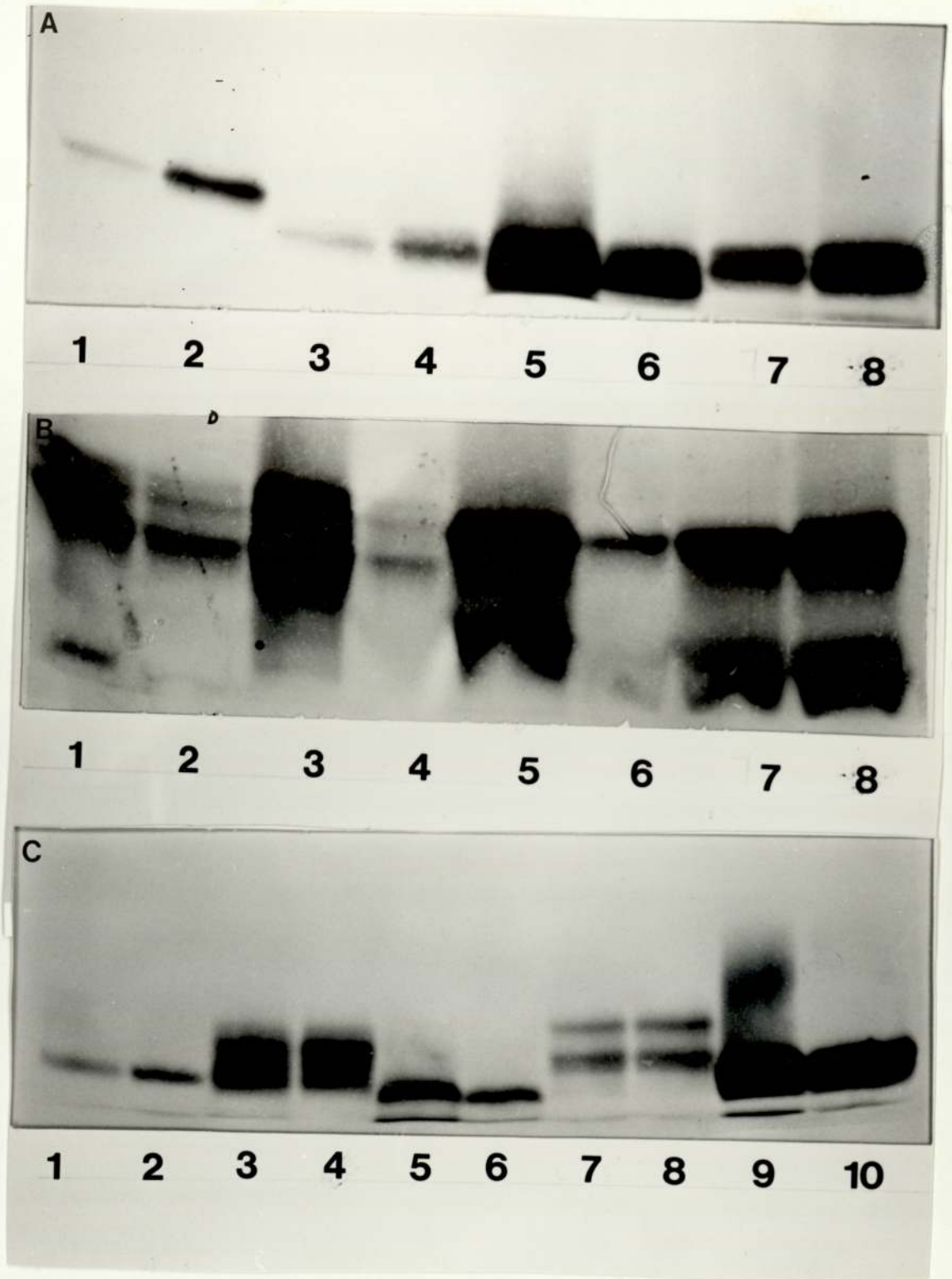
H. influenzae strains represented are as follows:

(a)
1. HM24; haem excess.
2. HM24; iron-restricted
3. HM11; haem excess
4. HM11; iron-restricted
5. HM3; haem excess
6. HM3; iron-restricted
7. HM2; haem excess
8. HM2; iron-restricted

(b)
1. HM25; haem excess
2. HM25; iron-restricted
3. HM25; haem excess
4. HM25; iron-restricted
5. HM26; haem excess
6. HM26; iron-restricted
7. HM26; haem excess
8. HM26; iron-restricted

(c)
1. HM27; haem excess
2. HM27; iron-restricted
3. HM20; haem excess
4. HM20; iron-restricted
5. HM21; haem excess
6. HM21; iron-restricted
7. HM10; haem excess
8. HM10; iron-restricted
9. HM17; haem excess
10. HM17; iron-restricted

FIGURE 3.2.2.5



whole cells (2.3.3), and apparently remained constant regardless of changing porphyrin ring source or iron availability. LPS profiles of 13 isolates grown under haem excess and iron-restricted conditions are shown in Fig. 3.2.2.5. On no occasion did alterations in porphyrin ring source or iron availability result in qualitative changes in LPS profiles as seen on SDS-PAGE.

3.2.3. The Immune Response of Patients with Bronchiectasis to OM Antigens of their Homologous *H.influenzae* Isolates.

OMPs from non typable *H.influenzae* grown utilising either haem (1) or PPIX (2) as a porphyrin source were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose paper and probed with homologous patients serum. After washing and incubation with HRP conjugated protein-A antigenic sites were visualised using a solution of 25µg/ml 4-chloro-1-naphthol containing 0.01% H_2O_2 (see 2.4.3).

Figure 3.2.3.1 represents SDS-PAGE OMP profiles (a and b) and corresponding immunoblots (c and d) of *H.influenzae* HM1 and HM2 which were isolated from bronchiectasis patients producing purulent and mucopurulent sputum respectively. Fig. 3.2.3.2 a and b represent immunoblots performed using serum from individuals producing mucoid sputum, and their homologous *H.influenzae* isolates HM11 and HM10. SDS-PAGE profiles of the latter two isolates are shown in Fig. 3.2.2.1 c and e.

Individuals producing purulent or mucopurulent sputum appear to have a much stronger immune response to their homologous *H.influenzae* OM antigens than those who produce mucoid secretions, as evidenced by greater intensity of antigenic bands seen on immunoblots (Fig. 3.2.3.1 c and d compared with Fig. 3.2.3.2 a and b). Where PPIX-specific OMPs were produced they were recognised by homologous serum, strongly in cases where purulent sputum was produced and very weakly in cases where secretions were mucoid in nature. These observations, based on intensity of banding patterns on immunoblots, were confirmed by quantitative analysis using an ELISA system (see section 3.2.4).

Figure 3.2.3.1c shows strong recognition of PPIX-specific proteins by homologous serum from an individual producing purulent sputum. In addition to the 150K and 84K proteins, OMPs of 120K and 98K which were barely visible on the corresponding SDS-PAGE gel (Fig. 3.2.3.1.a) proved to be strong antigens. Proteins above 45K in molecular weight were the most strongly recognised by this individual, those of lower molecular weight including the LPS/P6 region gave rise to antigenic bands of less intensity, despite being present in larger quantities (3.2.3.1a) than the high molecular weight material.

Immunoblotting of *H.influenzae* HM2 OM antigens (Fig. 3.2.3.1d) with homologous serum demonstrates strong recognition of the 84K OMP in PPIX grown cells but in this case the 120K and 150K OMPs induced by PPIX utilisation do

FIGURE 3.2.3.1.

The Immune Response of Bronchiectasis Patients Producing Purulent or Mucopurulent Sputum to OMP Antigens of their Homologous Non Typable *H.influenzae* Isolates.

(a) and (b)

OMP profiles of *H.influenzae* HM1 (a) and HM2 (b) grown under conditions of haem excess (lane 1) or under iron-restricted conditions (lane 2).

(c) and (d)

Immunoblots of OMPs of *H.influenzae* HM1 and HM2 cultured under conditions of haem excess (lane 1) or under iron-restricted conditions (lane 2) and probed with homologous patients serum.

Bacteria were grown under conditions of haem excess (5µg/ml) or iron-restriction (0.25µg/ml PPIX + 30µM Desferal). OMs were prepared by sarkosyl extraction (2.3.1) and separated by SDS-PAGE (2.4.1) (a and b). OMPs were then then electrophoretically transferred to nitrocellulose paper and incubated with a 1/25 dilution of homologous serum. After washing with TBS the protein impregnated paper was then incubated with HRP conjugated protein - A. Immune complexes were then visualised using 4-chloro-1-naphthol and H_2O_2.

FIGURE 3.2.3.1.

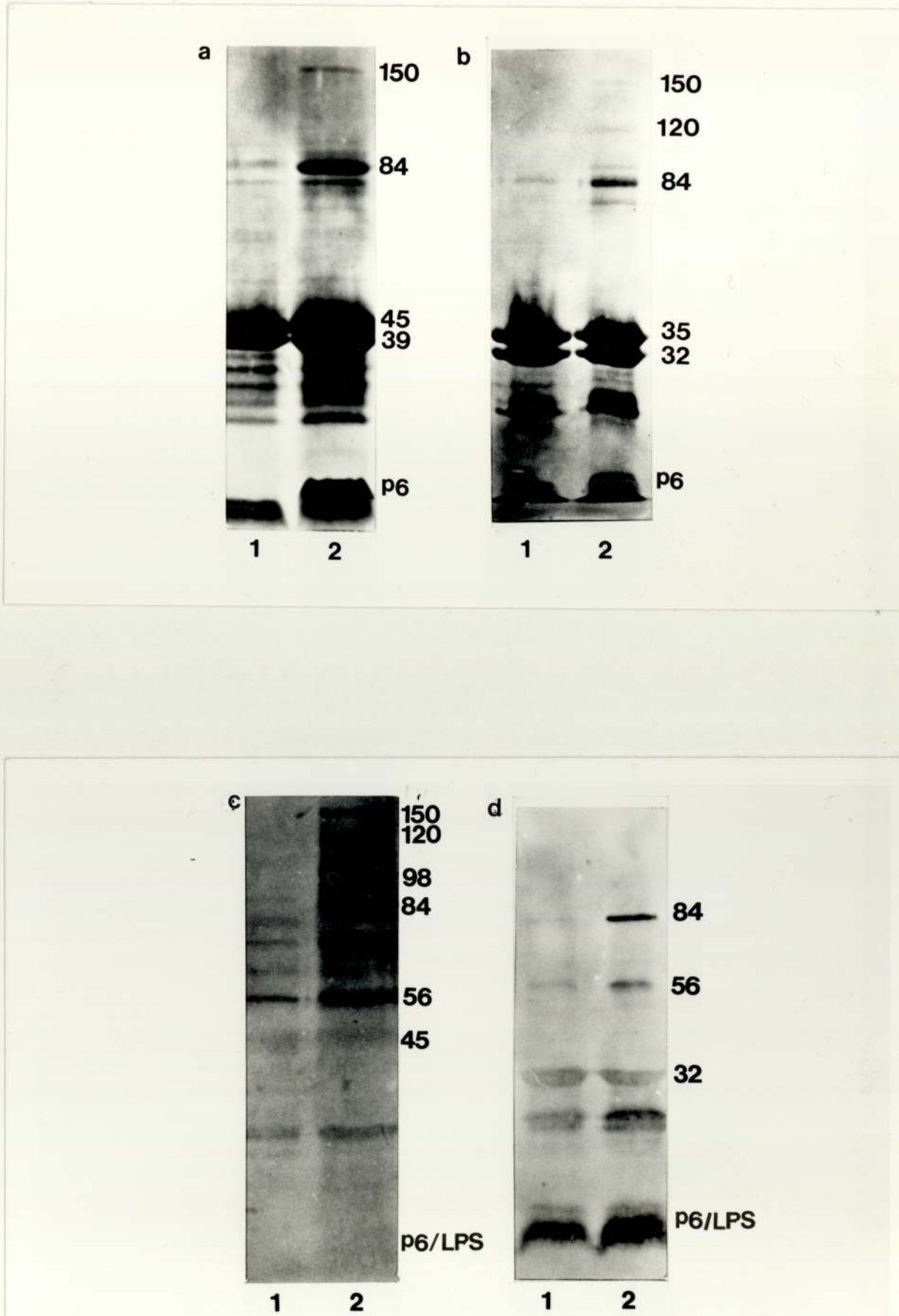

a
150
84
45
39
p6
1
2
b
150
120
84
35
32
p6
1
2
c
150
120
98
84
56
45
p6/LPS
1
2
d
84
56
32
p6/LPS
1
2

FIGURE 3.2.3.2.

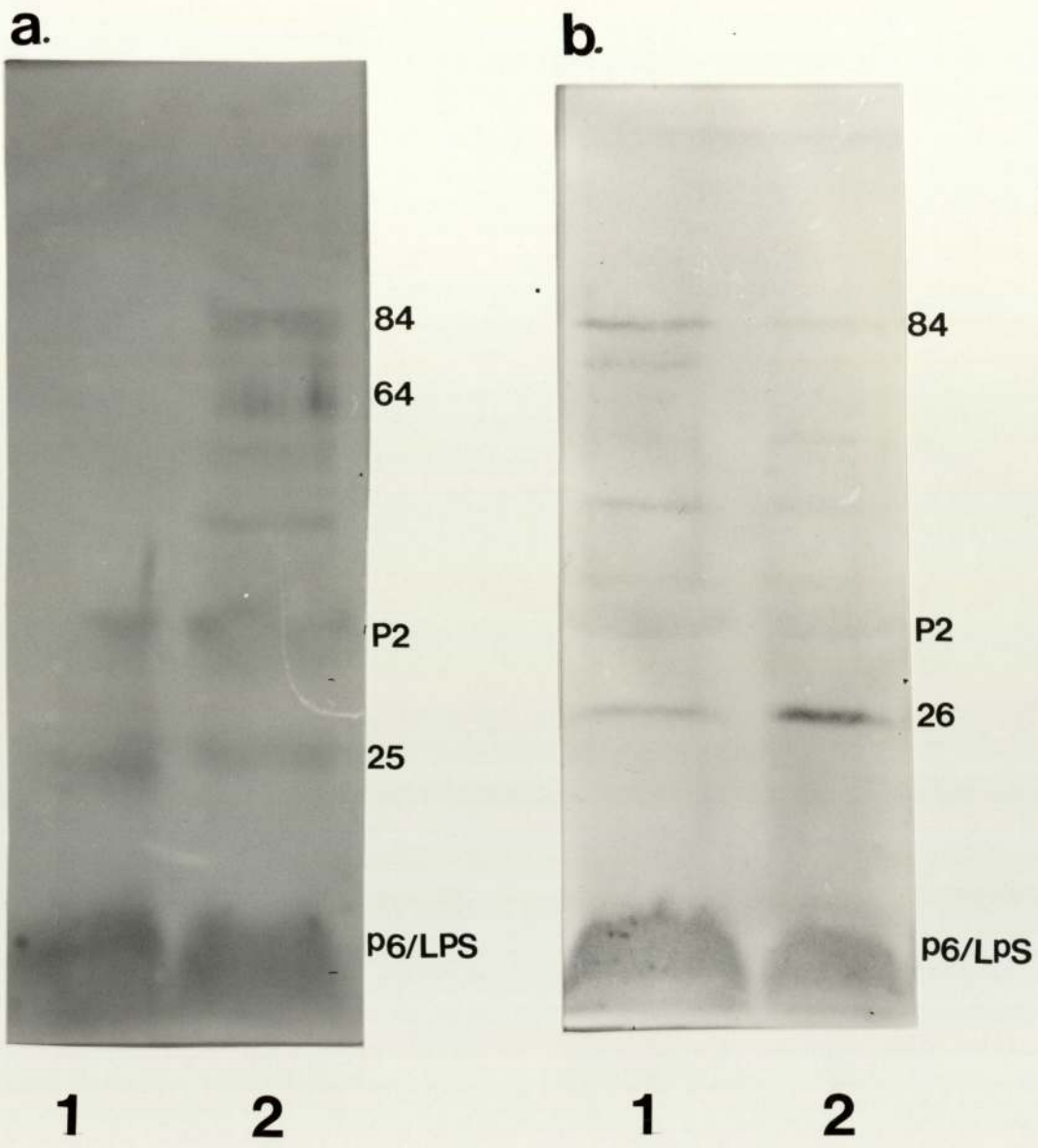
The Immune Response of Bronchiectasis Patients Producing Mucoid Sputum to OMP Antigens of their Homologous Non Typable *H.influenzae* Isolates.

Haemophilus influenzae strains HM11 (a) and HM10 (b) isolated from mucoid sputum samples were grown *in vitro* under conditions of haem excess (lane 1) or iron-restriction (lane 2). The OMP profiles of these isolates, cultured under each of the above conditions are shown in Figures 3.2.2.1 c and e respectively.

Immunoblots were prepared as described in the legend to Figure 3.2.3.1.

Numbers in the right hand margin refer to molecular weight in kilodaltons.

FIGURE 3.2.3.2



not appear to be antigenic. The major antigens recognised by this patients serum appear to be the 84K, 32K and 27K OMPs and the P6/LPS region.

Immunoblots of OMPs from *H.influenzae* isolated from mucoid sputum probed with homologous serum (Fig. 3.2.3.2 a and b) revealed several antigenic bands of much less intensity than those produced on immunoblotting with serum from individuals with characteristically purulent or mucopurulent secretions. Where 84K and 150K PPIX-specific OMPs were expressed they were recognised by patients serum but with low intensity. Antigenic bands of molecular weight 150K were visible to the naked eye in both HM11 and HM10 immunoblots but were too faint to show up on photographs. There were no OMP bands consistently recognised by serum from patients producing mucoid sputum which were not also apparent on immunoblotting with serum from individuals in the purulent group.

Haemophilus influenzae HM3, isolated from an individual producing mucopurulent sputum, did not show any apparent OMP changes on substitution of PPIX for haem (Fig. 3.2.2.3). However, immunoblotting with homologous serum (Fig. 3.2.3.3) revealed enhanced recognition of proteins in PPIX grown cells (lanes 3 and 4) compared with those cultured under conditions of haem excess or haem-limitation (lanes 1 and 2), indicating that variations in avidity of antibody binding may be brought about by changing culture conditions even although mobility of proteins on SDS-PAGE remain unchanged. Figure 3.2.3.4 shows immunoblots of a single

FIGURE 3.2.3.3.

Immunoblot of OMPs of *H.influenzae* HM3: The Effect of Varying Haem, PPIX and Iron Availability.

Bacteria were cultured in BHI broth containing excess NAD (5µg/ml) and supplemented with haem, PPIX and Desferal as shown in Fig. 3.2.2.3. SDS-PAGE separated OMPs were then electrophoretically transferred to nitrocellulose paper. Antigenic sites were visualised after incubation with homologous serum followed by protein-A peroxidase as described in the legend to Fig.3.2.3.1.
Numbers in the right hand margin refer to molecular weight in kilodaltons.

FIGURE 3.2.3.3

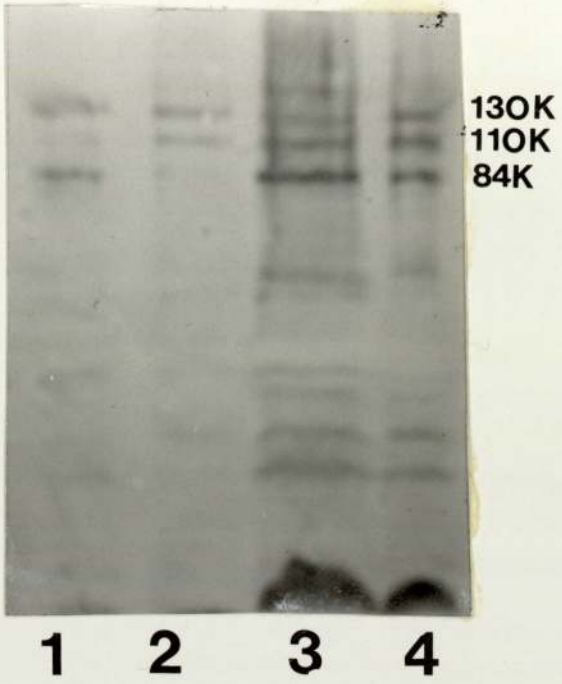
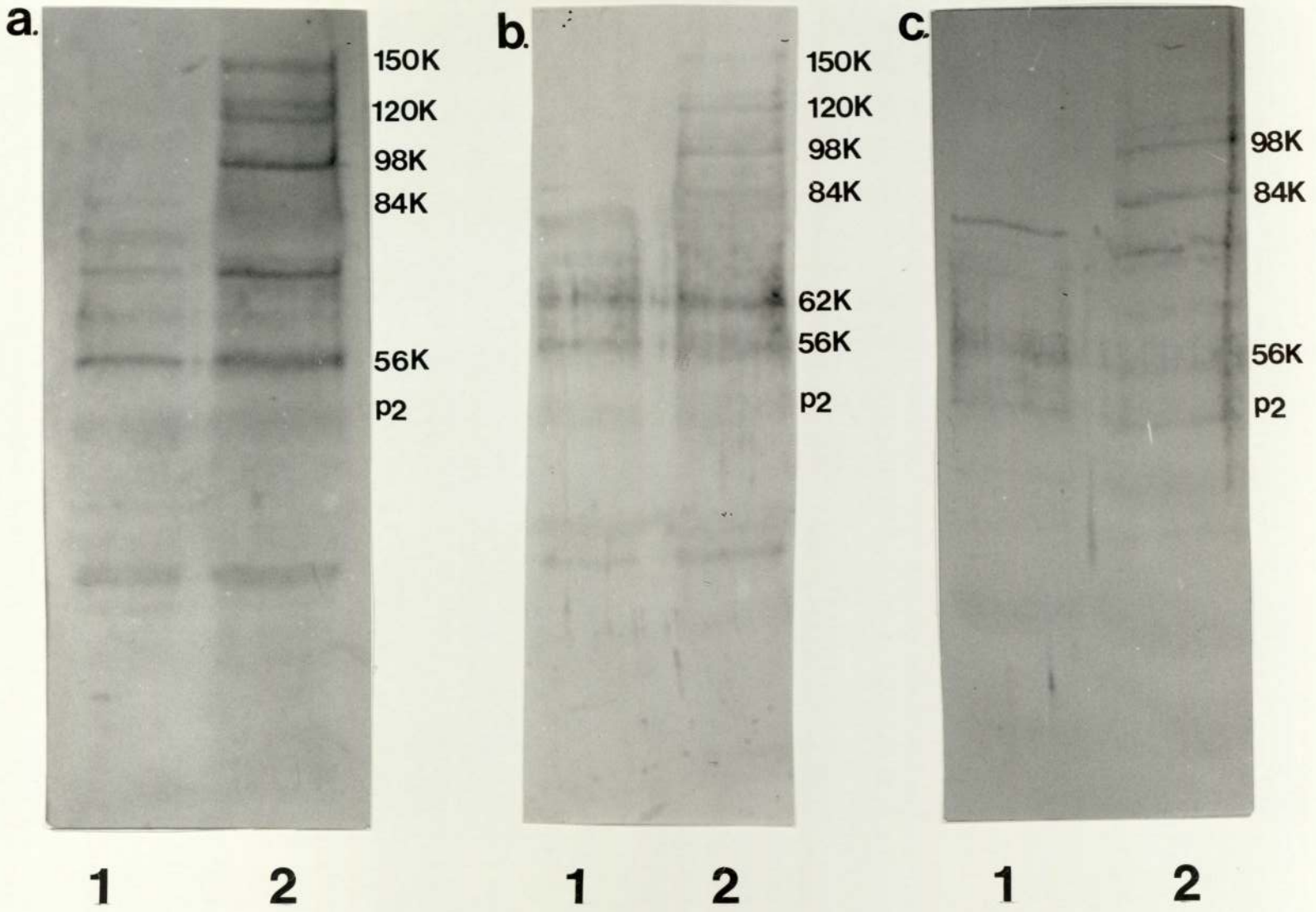


FIGURE 3.2.3.4.

Immunoblots of *H.influenzae* HM1 grown under conditions of haem excess (1) and iron-restriction (2), probed with serum from patients producing (a) purulent and (b) mucoid sputum, and with (c) normal human serum.

OMs were purified (2.3.1) and separated by SDS-PAGE (2.4.1) before electropheretic transfer to nitrocellulose paper. Immunoblotting was carried out as described in the legend to Figure 3.2.3.1 and in section 2.4.3. Numbers in the right hand margin refer to molecular weight in kilodaltons.

FIGURE 3.2.3.4


a.
150K
120K
98K
84K
56K
p2
1
2
b.
150K
120K
98K
84K
62K
56K
p2
1
2
c.
98K
84K
56K
p2
1
2

isolate of *H.influenzae*, HM1, cultured using haem (1) or PPIX (2) as a porphyrin source. Immunoblots were probed with (a) homologous serum from a patient with purulent sputum, (b) heterologous serum from a patient producing mucoid sputum and (c) with normal human serum. In each case antibodies to PPIX-specific OMPs were present. Antigenic bands were identical regardless of the serum used in immunoblotting but were of greatly reduced intensity when control serum or serum from patients with mucoid sputum was used, apparently indicating quantitative differences but no qualitative differences in antibody response to OMPs of non typable *H.influenzae* between normal individuals, those suffering from mild and those with severe bronchiectasis.

OMPs from *H.influenzae* strains HM13 and HM24, isolated from mucopurulent and purulent sputum samples respectively, were separated by SDS-PAGE and transferred to nitrocellulose paper as described previously (2.4.3). Each protein-impregnated nitrocellulose sheet was then probed with homologous sol phase sputum (2.4.1) diluted 1/10 with TBS-tween. Antigenic sites were then visualised using protein-A-peroxidase as described previously (2.4.3). Figure 3.2.3.5A shows that IgG antibodies present in homologous sol phase sputum were directed against the LPS/P6 region of the OM of both HM13 and HM24. Similar results were obtained with all other *H.influenzae* strains isolated from purulent and

mucopurulent sputum. Mucoid sputum yielded no apparent anti-*H.influenzae* antibodies on immunoblotting.

Attempts to identify which OMP antigens of *H.influenzae* sol phase sputum IgA was directed against were frustrated by the fact that commercially purchased anti-human IgA HRP conjugates contained antibodies which reacted directly with *H.influenzae* OMPs (Fig. 3.2.3.5B). Absorption of conjugates with *H.influenzae* OMs was performed until readings below 0.2 were obtained on ELISA (2.1.3) however, even use of such absorbed conjugates resulted in banding patterns on immunoblotting controls. Furthermore, when immunoblots which had been incubated with the sol phase of purulent or mucopurulent sputum were probed with absorbed anti-human IgA conjugate no antigenic bands could be seen (Fig. 3.2.3.5B) despite the presence of such bands on controls which had not been treated with sputum but had been incubated with conjugate alone. This phenomenon was observed with all sputum samples tested. It appeared therefore that some component of the sputum prevented the attachment of anti-human IgA, but not protein-A peroxidase conjugate to the nitrocellulose paper. No such inhibition of conjugate binding was observed in ELISA systems.

3.2.4. Quantitative Analysis of the Systemic and Local Humoral Immune Response to Non Typable *H.influenzae* Outer Membranes in Patients with Bronchiectasis./

FIGURE 3.2.3.5A and B.

The Local Immune Response of Patients with Bronchiectasis Producing Mucopurulent and Purulent Sputum to OM Antigens of their Homologous *H.influenzae* Isolates.

(A) IgG

OMs of *H.influenzae* HM13 (a) and HM24 (b) cultured under iron-restricted conditions were prepared as described previously (2.3.1) and separated by SDS-PAGE (2.4.1). After electrophoretic transfer to nitrocellulose paper OMs were probed with homologous sol phase sputum (1/10 dilution). After washing with TBS each nitrocellulose sheet was incubated with HRP conjugated protein - A, antigenic sites were then visualised using a solution of 25µg/ml 4-chloro-1-naphthol containing 0.01% H_2O_2 (2.4.3).

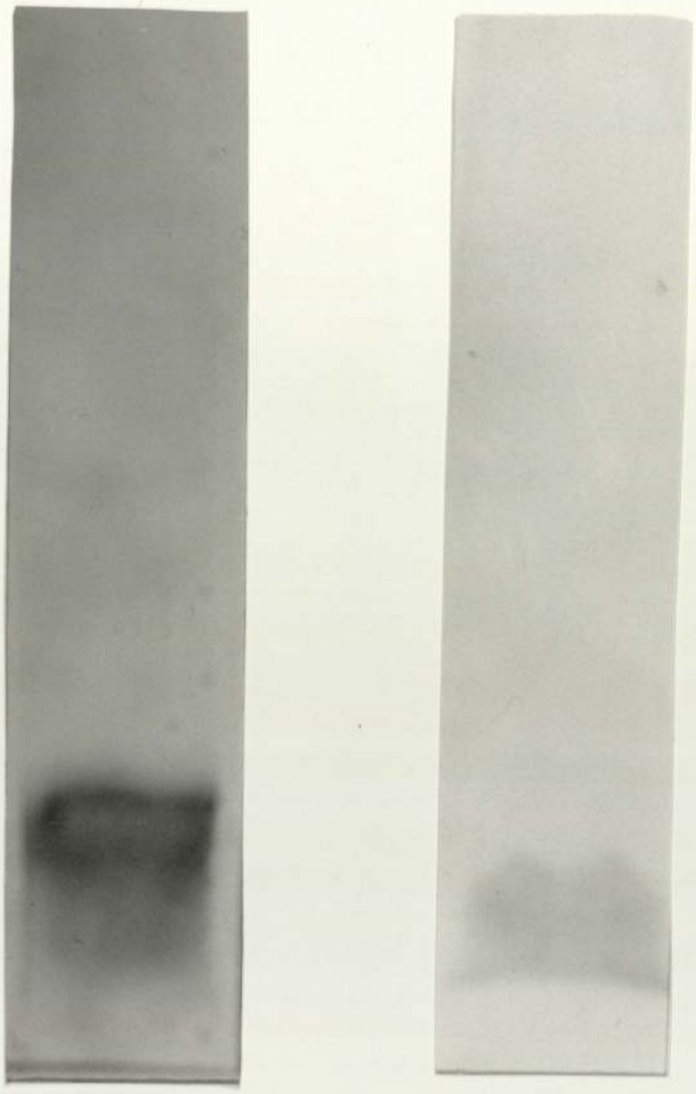
(B) IgA

Details as in the legend to (A) above except that antigenic sites were visualised using HRP conjugated anti-human IgA.

Lane 1: Control immunoblot, OMs from HM13 probed with HRP conjugated anti-human IgA alone.

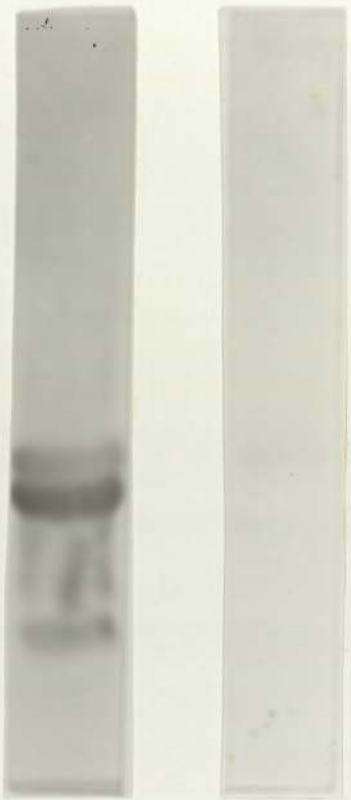
Lane 2: Immunoblot of OMs from HM13 probed with sol phase sputum followed by anti-human IgA conjugate.

FIGURE 3.2.3.5A



a b

FIGURE 3 2 3 5B



3.2.4. Quantitative Analysis of the Systemic and Local Humoral Immune Response to Non Typable *H.influenzae* Outer Membranes in Patients with Bronchiectasis.

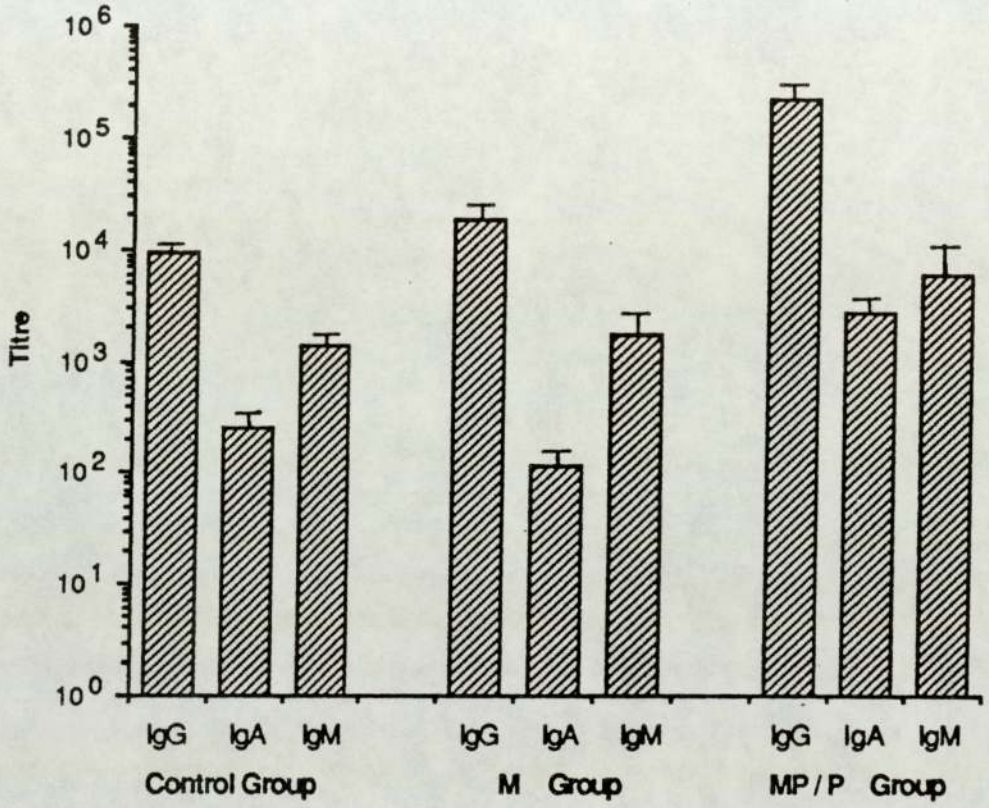
Titres of IgG, IgA and IgM directed against OMs of non typable *H.influenzae* cultured under iron-restricted conditions were quantified using an ELISA system (2.4.4a). Serum and sol phase sputum were obtained from each individual involved in the study and quantities of IgG, IgA and IgM directed against OMs of their homologous *H.influenzae* isolates were determined. Mean serum titres for individuals with mucoid sputum, purulent or mucopurulent sputum, and for normal human serum are shown in Figure 3.2.4.1.

Anti-*H.influenzae* serum titres of bronchiectasis patients producing purulent and those characteristically producing mucopurulent sputum were not significantly different from each other and so were treated as a single group (MP/P group). Mean serum IgG and IgA titres were reduced ten fold in patients who continually expectorated mucoid sputum (mean IgG 18,666 ± 5006, IgA 116 ± 58) compared with mean serum IgG and IgA levels in patients with persistently purulent sputum (IgG 220,000 ± 100,000, IgA 2681 ± 1000).

Serum anti-*H.influenzae* IgG titres in mucoid group patients were significantly higher than those of the normal human serum ($p<0.05$) but there were no significant differences between serum IgA and IgM titres of these two groups.

Figure 3.2.4.1.

Serum Antibody Titres Against Outer Membranes of Non Typable *Haemophilus influenzae*.

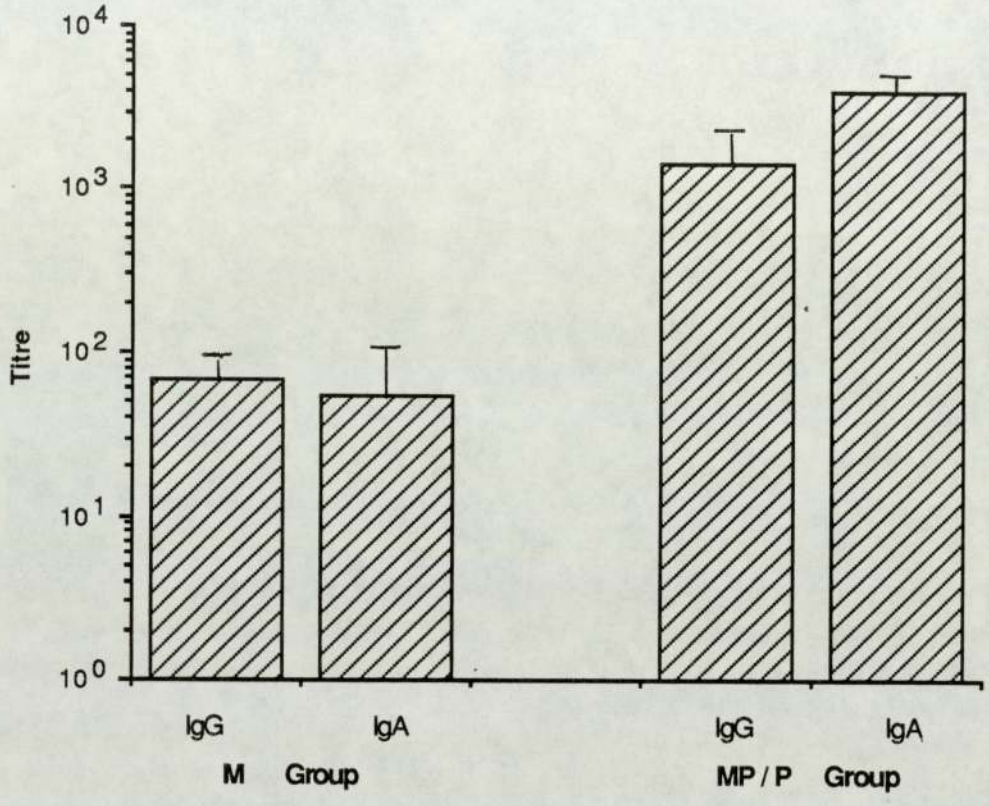


Test Groups were composed as follows:

1. Control Group (n = 6) consisted of individuals with no history of pulmonary disease.
2. M Group (n = 6) consisted of bronchiectasis patients who usually produced mucoid sputum.
3. MP/P Group (n = 11) consisted of bronchiectasis patients who usually produced mucopurulent or purulent sputum.

Values presented are mean serum titres for each group. SEM values are represented by vertical bars.

Figure 3.2.4.2.
Sol Phase Sputum Antibody Titres Against Outer Membranes of Non Typable *Haemophilus influenzae*.



Test groups were composed as follows:

1. M Group (n = 6) consisted of bronchiectasis patients who usually produced mucoid sputum.

2. MP/P Group (n = 8) consisted of bronchiectasis patients who usually produced mucopurulent or purulent sputum.

Values presented are mean sol phase sputum titres for each group. SEM values are represented by vertical bars.

However, as titres of anti-*H.influenzae* antibodies in control sera were determined using a single strain while homologous isolates were used in M group and MP/P group assays this result requires further clarification using homologous nasopharyngeal isolates from normal individuals. Titres of IgG and IgA directed against non typable *H.influenzae* OMs present in sol phase sputum from bronchiectasis patients are shown in Fig. 3.2.4.2. Both IgG and IgA titres were significantly higher in purulent and mucopurulent than in mucoid sputum ($p<0.01$). Purulent sputum contained more IgA than IgG antibodies and displayed IgA titres which were significantly higher than IgA titres present in the corresponding serum samples ($p<0.01$), demonstrating involvement of the local immune response. This was not true for mucoid sputum samples; in these IgG levels were slightly higher than those of IgA. IgA titres in mucoid sputum covered a large range of values from 10 to 180 (mean 55) in comparison with a mean sputum IgA titre of 4025 ± 1407 for purulent secretions.

3.2.5. DISCUSSION.

Many bacteria respond to iron-restriction by the induction of high affinity iron uptake systems (Griffiths, 1987b) the most well studied of which involve the synthesis and release of iron solubilising compounds, or siderophores, which compete with host iron binding proteins for essential iron.

Iron-siderophore complexes are recognised by specific OMP receptors, production of which is repressed by iron and derepressed by iron starvation (Braun, 1985; Griffiths, 1987b). There is much evidence to suggest that these iron regulated membrane proteins (IRMPs) are expressed by bacteria growing *in vivo* (Sciortino and Finkelstein, 1983; Brown *et al*, 1984; Shand *et al*, 1985; Cochrane *et al*, 1988) and that in some situations IRMPs may have potential as vaccine candidates (Bolin and Jensen, 1987).

Production of IRMPs (Herrington and Sparling, 1985; Williams and Brown, 1986) and of hydroxamate type siderophores (Pidcock *et al*, 1988) has been reported for *H.influenzae* type b. The latter authors report production of a number of novel OMPs in type b strains expression of which is controlled by levels of iron available in the growth medium. Certain bacterial species are known to express IRMPs but do not produce siderophores. These include obligate human parasites such as *Neisseria spp.*, *Bordetella pertussis* and *Leigonella pneumophila* (Dyer *et al*, 1985). *Neisseria spp.*, *Bordetella pertussis* and *H.influenzae* are all able to sequester iron from iron-saturated transferrin (Mickelsen and Sparling, 1981; Redhead *et al*, 1987; Herrington and Sparling, 1985) and it has been suggested that specific IRMPs induced in these bacteria may function as transferrin receptors. *Neisseria meningitidis* growing *in vivo* expresses IRMPs one of which appears to function in this manner (Black *et al*, 1986; Tsai *et al*, 1988). Transferrin binding activity

is known to be regulated by iron availability in *H.influenzae* as the addition of an iron chelator to BHI broth containing PPIX as a porphyrin source results in a 300 fold increase in transferrin binding activity of *H.influenzae* cultures (Schryvers, 1988). It is possible therefore that any of the IRMPs described in *H.influenzae* may function in this capacity rather than as siderophore receptors. However, as all *H.influenzae* have been demonstrated to be able to acquire iron from transferrin (Herrington and Sparling, 1985; Stull, 1987; Schryvers, 1988) and only 43% of the non typable *H.influenzae* strains examined as part of the present study responded to iron restriction in terms of altered OMP profiles, it seems unlikely that any of the novel OMPs described in this instance function in this manner.

Despite their proven ability to sequester iron from transferrin, none of the non typable *H.influenzae* isolates examined by Herrington and Sparling (1985) or Stull (1987), and only one out of fifteen non typable strains investigated by Schryvers (1988) were able to sequester iron from lactoferrin. The mechanisms of iron acquisition operative in non typable strains colonising mucosal surfaces therefore remain undetermined.

Haem binding proteins of 43K and 38K have been identified in two different strains of *H.influenzae* type b (Coulton and Pang, 1983; Stull, 1987). No such OMP changes were observed in non typable isolates grown under conditions of haem-

limitation in the present study. This may reflect genuine differences between responses of type b and non typable strains to haem-limitation *in vitro*, however it may also reflect differences in experimental conditions between studies. The former studies involved much lower culture haem concentrations, which resulted in greatly decreased growth rate in comparison with the present study where growth yield limiting concentrations of haem were such that initial growth rates remained largely unchanged.

Haemophilus influenzae can use haem as a sole source of required iron (Stull, 1987; Pidcock *et al*, 1988). Consequently any attempt to grow this species aerobically under iron-restricted conditions must involve substitution of the haem precursor PPIX (Fig. 1.4.1) as a porphyrin source. The results presented here indicate that culture of *H. influenzae* using PPIX alone causes OMP changes regardless of the presence or absence of an iron chelator. Similar OMP changes occur to a more limited extent when *H. influenzae* are cultured under conditions of haem-limitation in the presence of an iron chelator and this would suggest that these OMPs, expressed strongly in the presence of PPIX, may have a role in iron acquisition. It is conceivable that although haem alone can satisfy cellular iron requirements (Stull, 1987; Pidcock *et al*, 1988) bacteria limited for haem in the presence of an iron chelator may experience a degree of iron stress, resulting in the induction of IRMPs; further experiments reducing haem concentrations further in the

presence of an iron chelator may serve to clarify this point.

PPIX is a strong inducer of new OMPs, although the OMP changes observed on growth of non typable *H.influenzae* in PPIX are relatively minor in comparison with the inducible IRMPs of other species (Griffiths *et al*, 1983; Shand *et al*, 1985; Ward *et al*, 1988). It seems unlikely that PPIX is available to bacteria growing *in vivo* as synthesis of haem from PPIX and iron is an intracellular event, taking place within mitochondria (Israels *et al*, 1975). *In vitro* growth utilising PPIX as a porphyrin source may act as a trigger for induction of an iron uptake system as *H.influenzae* cultured in the presence of PPIX require iron for insertion into the porphyrin ring and the formation of haem. Alternatively it may be that growth *in vitro* utilising PPIX non specifically induces OMP changes in non typable strains, resulting in an OMP profile which is more like that of *H.influenzae* grown *in vivo* than the OMP profile of cells grown under conditions where haem is freely available.

Immunoblotting with homologous patients serum reveals a strong antibody response to PPIX-specific OMPs, particularly in serum from individuals producing purulent sputum even although quantitatively they represented very minor components of the OM and were barely visible on SDS-PAGE. These results suggest that proteins induced *in vitro* by PPIX utilisation are expressed by non typable *H.influenzae* growing *in vivo* within the pulmonary environment. Similarly

it has been demonstrated that serum from individuals with cystic fibrosis contains antibodies reactive with IRMPs of *Pseudomonas aeruginosa* (Anwar *et al*, 1984). The presence of large amounts of antibody to these PPIX-specific proteins of *H.influenzae* in individuals who continually expectorated purulent sputum reveals that in bronchiectasis at least, antibodies raised against these OMPs are not protective.

On the basis of the results presented here it would seem that in addition to heterogeneity of OMP profiles (Barenkamp *et al*, 1982; Loeb and Smith, 1980) non typable *H.influenzae* display heterogeneity in terms of their response to PPIX substitution and iron-restriction. Forty-three percent of bronchiectasis isolates produced new OMPs on substitution of PPIX for haem, the remaining 57% did not. Williams and Brown (1986) also report non-responsiveness to iron-restriction among non typable strains. Strains judged to be non responders in the present study did however display altered avidity of antibody binding to OMPs from bacteria grown under PPIX and iron-restricted conditions compared with OMPs from the identical strain cultured under conditions of haem excess.

Previous studies have generated a degree of controversy regarding the significance of non typable *H.influenzae* as a pathogen in chronic obstructive pulmonary diseases such as chronic bronchitis and bronchiectasis (review by Murphy and Apicella, 1987). Studies seeking to demonstrate an immune response to antigens of non typable strains in chronic

bronchitic and bronchiectatic patients, thus demonstrating the existence of infection with *H.influenzae* have yielded conflicting results (Reichek *et al*, 1970; Gump *et al*, 1976; Smith *et al*, 1976; McHardy *et al*, 1980). Murphy and Apicella (1987) suggest that a reason for the confusion existing about antibody levels to *H.influenzae* in chronic obstructive pulmonary disease may be that previous studies have failed to take antigenic differences between individual strains of *H.influenzae* into account, using a single isolate as the source of antigen in all assays.

In the present study, each patients serum was assayed for antibodies to *H.influenzae* OMs using his/her own isolate as antigen. No antibodies reactive with OM antigens of non typable *H.influenzae* were found to be universally present in normal human serum and in serum from individuals producing mucoid sputum, but absent from the serum of individuals with purulent sputum. Additionally anti-OM titres were ten fold greater in purulent than in mucoid group patients and purulent sputum was shown to have high titres of locally produced IgA. It seems unlikely therefore that humoral immune mechanisms operative in patients with mucoid sputum are absent or defective in individuals who continually expectorate purulent sputum and are chronically colonised with non typable *H.influenzae*.

These results clearly implicate non typable *H.influenzae* as a cause of infection in patients with severe bronchiectasis. It has also been demonstrated that in individuals with

chronic obstructive pulmonary disease and acute tracheobronchitis convalescent serum has increased opsonising activity for the patients own *H.influenzae* isolate following periods of illness (Musher *et al*, 1983). The capacity of antibody and complement to kill invading microorganisms or for opsonisation plays a major role in protection of the host from pulmonary infection (Reynolds and Merrill, 1981). OMPs of non typable *H.influenzae* have been shown to be targets for the majority of bactericidal antibody present in normal human serum and in serum from individuals with otitis media (Gnehm *et al*, 1985) and OMPs P2 (Murphy and Bartos, 1988) and P6 (Murphy *et al*, 1986) have been identified as specific targets for bactericidal antibody. Antibodies directed against LPS of non typable *H.influenzae* are not thought to contribute significantly to bactericidal activity of normal human serum (Gnehm *et al*, 1985).

It has been demonstrated in patients with non typable *H.influenzae* pneumonia that IgA present in bronchopulmonary secretions blocks the bactericidal and opsonising effects of antibody present in homologous and in normal human serum (Musher *et al*, 1984). In addition to bronchiectasis patients as reported in the present study, high titres of locally produced IgA reactive with whole cell digests and LPS of non typable strains have been reported to be present in purulent secretions from individuals with a history of recurrent otitis media (Faden *et al*, 1988) and from people

experiencing recurrent episodes of sinusitis (Harada and Sakakura, 1988) caused by non typable *H.influenzae*.

If local IgA antibodies in these instances are acting to block the binding of bactericidal antibodies to the bacterial cell surface it may explain why *H.influenzae* persistently colonises the lungs of individuals with bronchiectasis who continue to produce purulent sputum despite the presence of high local and systemic anti-*H.influenzae* titres.

Pulmonary damage due to bacterial infection in bronchiectasis appears to be exacerbated rather than relieved by the host immune response and there is some evidence to suggest that high levels of local antibody directed against *H.influenzae* may actually assist in the persistence of this species within the lung (Musher *et al*, 1984). Vaccination procedures against non typable *H.influenzae* are unlikely to be of benefit to patients with severe bronchiectasis characterized by persistent purulent sputum production and may in fact have deleterious effects as they have very high titres of anti-*H.influenzae* antibody already.

One way in which the 'viscious circle' of infection and pulmonary damage in bronchiectasis may be broken is by the removal of colonising microoranisms by antimicrobial chemotherapy. Antibiotic treatment of patients with purulent sputum is effective in the short term if sufficiently high doses of appropriate antibiotics are administered (Cole *et*

al, 1983) but sputum may rapidly become infected again after cessation of therapy (Hill *et al*, 1986). In addition to reduction of the microbial load it has been suggested that reduction of the host inflammatory response by treatment with anti-inflammatory agents may prove beneficial to patients (Cole, 1984). As yet no studies using this approach to therapy have been published.

3.3 Rat Lung Model of Chronic Non Typable *H.influenzae* Infection.

Haemophilus influenzae strain HM1, cultured in BHI broth containing haem concentrations which did not result in alterations in OMP profile (2.5µg/ml) were incorporated into agar beads according to the procedure described in section 2.5.1. Following intratracheal inoculation of agar bead encased bacteria rat lungs became chronically colonised and this colonisation persisted for at least 42 days (Fig. 3.3.1B and Fig. 3.3.2B). Inocula of 10^6 (○) or 10^8 (□) c.f.u were used.

Despite the use of different densities of bacterial suspensions to prepare the beads animals appeared to contain the same initial number of c.f.u./g of lung tissue. In view of this finding and as lung homogenisation as performed in this instance is unlikely to disrupt agar beads, it appears that use of this protocolresults in the production of beads containing different numbers of bacteria. When the lower inocula was used an initial slow decline in pulmonary bacterial load was observed which increased again following 21 days of infection (Fig. 3.3.1B (○)), when the higher inoculum was used (□) a rapid but limited decline in numbers of bacteria /g of lung tissue was observed, numbers increasing again after 3 days post infection.

Figure 3.3.1

Chronic *H.influenzae* Lung Infection: Antibody Response To Outer Membranes.

Panel A shows the ELISA titre of IgG directed against OM components in serum from unmanipulated control animals (△), animals injected with sterile agar beads (▲), or with agar beads containing 10^6 (●) or 10^8 (■) CFU of *H.influenzae* HM1.

Panel B shows the pulmonary *H.influenzae* population following injection of 10^6 (○) or 10^8 (□) CFU.

Values presented are means (n = 4 - 5) and the SEM is either encompassed by the size of the symbol or represented by vertical bars.
Titres of infected animals are different from controls at $P < 0.025$ *, $P < 0.01$ **, $P < 0.005$ ***.

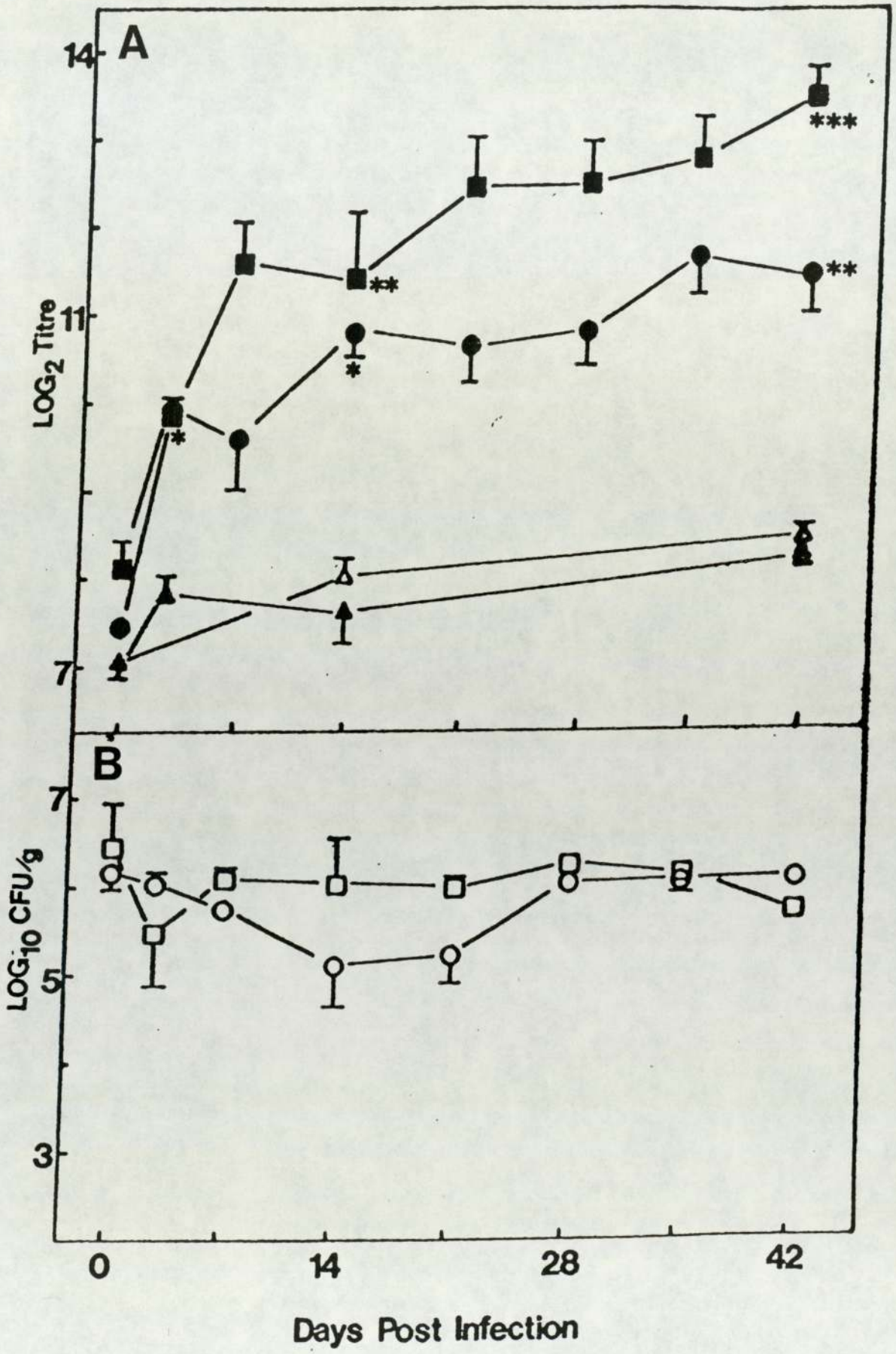

A
B
14
11
7
LOG_2 Titre
*
**
***
7
5
3
LOG_{10} CFU/g
0
14
28
42
Days Post Infection

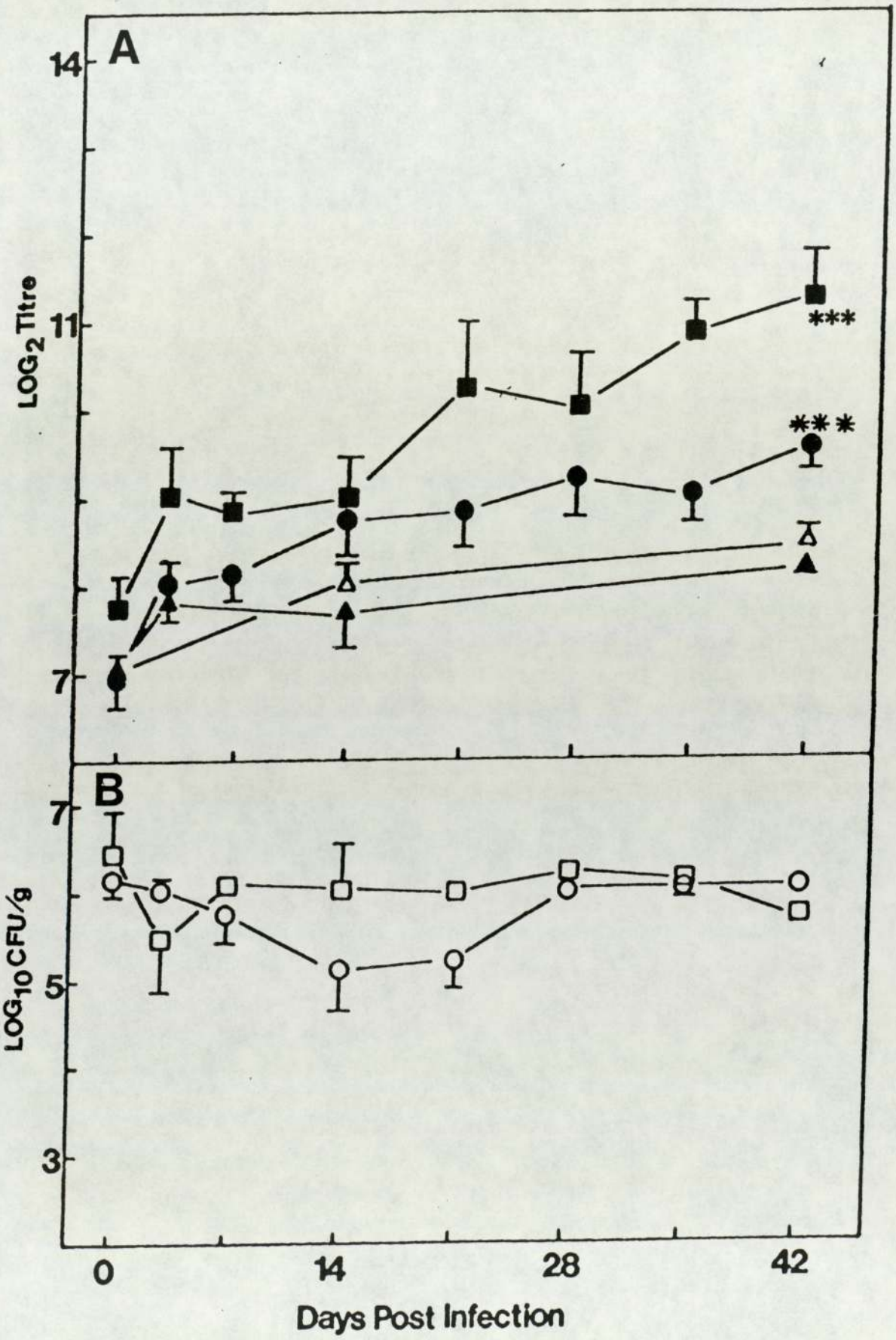
Figure 3.3.2

Chronic *H.influenzae* Lung Infection: Antibody Response to LPS.

Panel A shows the ELISA titre of IgG directed against LPS in serum from unmanipulated control animals (△), animals injected with sterile agar beads (▲), or with agar beads containing 10^6 (●) or 10^8 (■) CFU of *H.influenzae* HM1.

Panel B shows the pulmonary *H.influenzae* population following injection of 10^6 (○) or 10^8 (□) CFU.

Values presented are means (n = 4-5) and the SEM is either encompassed by the size of the symbol or represented by vertical bars. Titres of infected animals are different from controls at $P < 0.025$ *, $P < 0.01$**, $P < 0.005$***.


A
14
11
7
LOG2 Titre
***
***
B
7
5
3
LOG10 CFU/g
0
14
28
42
Days Post Infection

Contamination of the lungs by non-*Haemophilus* bacteria occurred with increasing frequency as infection progressed. The proportion of contaminating bacteria increased from 1% at 3 days to 7% at day 21 and 31% by 42 days post-infection when an inoculum size of 10^8 c.f.u was used. When 10^6 c.f.u of *H.influenzae* HM1 was used contaminants increased from <1% to 18% at 42 days post-infection.

Persistant non typable *H.influenzae* infection was accompanied by increasing titres of serum IgG antibodies directed against OMs from the infecting strain (Fig. 3.3.1A). A rapid rise in antibodies occurred from initial titres of naturally occurring antibodies (mean 318 ± 194), with animals receiving higher initial inocula (■) demonstrating a higher OM titre by day 42 (13,400 ± 6794) than those receiving lower inocula (●) (3400 ± 1935). Animals injected with sterile agar beads (▲) demonstrated titres not significantly different from unmanipulated controls (△) (302 ± 87) although both groups demonstrated increasing titres over the six week study period.

Serum IgG directed against LPS of non typable *H.influenzae* HM1 (Fig. 3.3.2A) also increased from initial titres indicative of naturally occurring antibodies (278 ± 131) to become significantly higher than controls at 42 days post-infection (4100 ± 2388 (■)). Unmanipulated controls (△) and control animals inoculated with sterile agar beads (▲) demonstrate anti-LPS titres not significantly different from each other at 42 days post-infection.

Lavage fluid (see 2.5.2) collected from rat lungs 42 days post-infection with 10^8 c.f.u of *H.influenzae* HM1 contained high titres of IgG directed against OMs in comparison with uninfected controls. The majority of these antibodies appeared to be directed against the LPS component of the OM (Table 3.3.1).

Figure 3.3.3 shows the OMP profile of *H.influenzae* HM1 cultured using haem (lane 1) or PPIX (lane 2) as a porphyrin source. A new OMP of 150K is expressed and production of an existing 84K OMP is increased only in the OM of bacteria cultured in the presence of PPIX. Bacteria used to inoculate rat lungs were grown *in vitro* in BHI broth containing 2.5μg/ml haem and so did not express any of these PPIX-induced proteins at the time of introduction into the rat lungs.

Immunoblots of OMPs from *H.influenzae* HM1 cultured *in vitro* using PPIX as a porphyrin source were probed with serum from infected rats (10^8 inoculum) collected at 0, 3, 7, 14, 21, 28, 35, and 42 days post-infection (Fig. 3.3.4). Increased intensity of antibody binding to several OMPs was observed throughout the course of infection. A weak response to the 150K PPIX induced OMP was apparent at 3 days post-infection, the intensity of this band did not increase further however, until 35 days post-infection. Antibodies to 84K and P2 proteins were apparent by 3 days post-infection; antibodies to the porin protein, P2, were also weakly visible in control sera. An IgG reponse to the LPS/P6 region on

TABLE 3.3.1

IgG Titres Against OMs and LPS of *H.influenzae* HM1 in PBS Lavage of Rat Lungs.

	LOG_2 ANTIBODY TITRE		
ANTIGEN	CONTROLS	INFECTED	P VALUE
OMs	1.47 ± 0.9	8.82 ± 1.4	< 0.025
LPS	2.86 ± 0.9	7.82 ± 0.9	< 0.025

Test groups were composed as follows:

1. Control Group (n = 4) consisted of uninfected animals.
2. Infected Group (n = 4) consisted of animals infected with 10^8 CFU of *H.influenzae* HM1 encased in agar beads. Lung washes were performed at 42 days post infection.

Values presented are means ± SEM.

FIGURE 3.3.3.

OMP Profiles of Non Typable *H.influenzae* HM1: The Effect of Iron-Restriction.

Bacteria were cultured in BHI broth containing excess NAD (5μg/ml) and supplemented with (1) haem excess (5μg/ml) or (2) 0.25μg/ml PPIX + 30μM Desferal. OMs were prepared as described previously (2.3.1), separated by SDS-PAGE (2.4.1) and stained using coomassie blue.

FIGURE 3. 3. 3

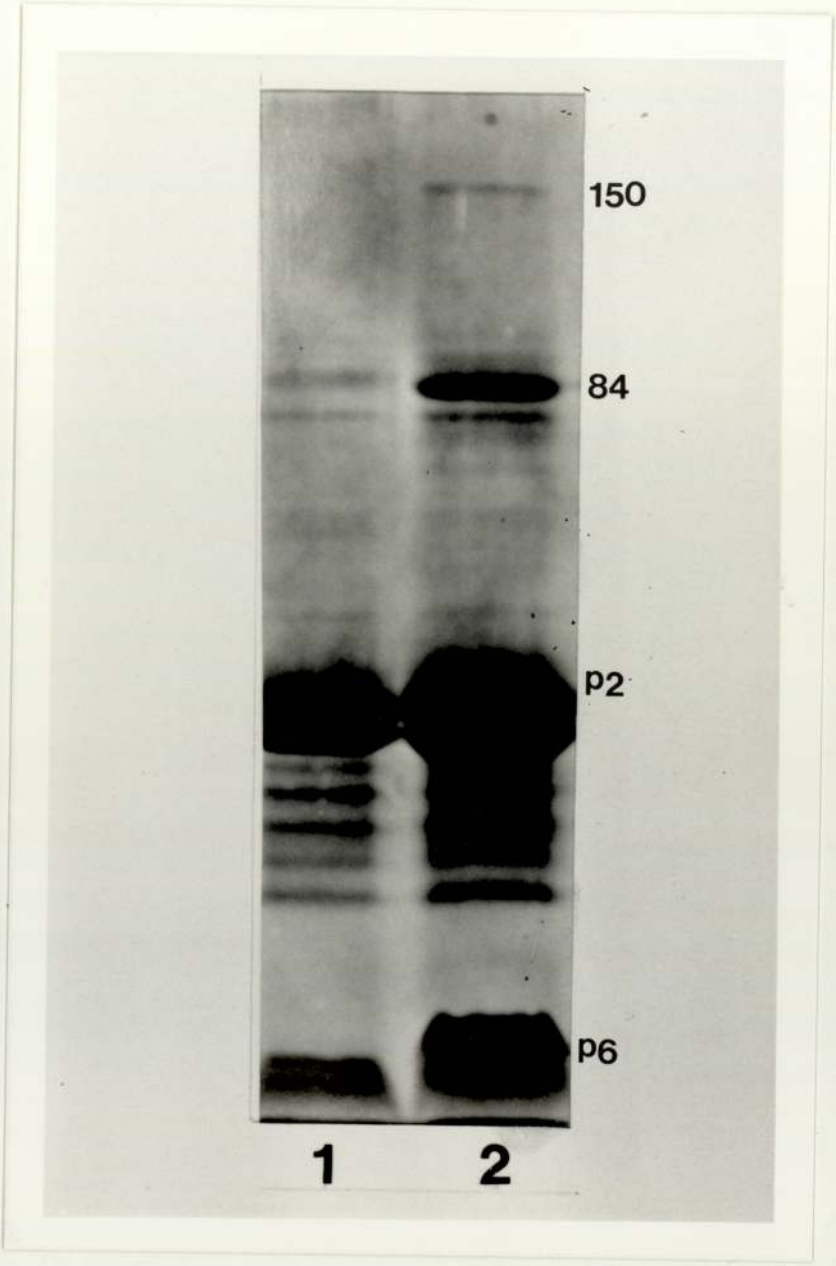


FIGURE 3.3.4

Rat Model of Chronic Non Typable *H.influenzae* Lung Infection.

The Development of the Immune Response to Iron-Restricted OM Antigens of *H.influenzae* HM1 After Intratracheal Inoculation of 10^8 c.f.u. of Bacteria Encased in Agar Beads.

OMPs from *H.influenzae* HM1, cultured under iron-restricted conditions were separated by SDS-PAGE (2.4.1) and electrophoretically transferred to nitrocellulose paper (2.4.3). The protein impregnated paper was cut into strips each of which was probed with pooled sera from experimentally infected rats.

Four to five animals were sacrificed at 0, 3, 7, 14, 21, 28, 35, and 42 days post-infection. Blood was collected by cardiac puncture and serum separated as described previously (2.1.4). Immunoblotting was carried out as described previously (2.4.3) using 1/50 dilutions of pooled serum (n=4-5). Antigenic sites were visualised using HRP conjugated anti-rat IgG and 25µg/ml 4-chloro-1-naphthol containing 0.01% H_2O_2.

Numbers along the horizontal axis refer to days post-infection. C = control sera collected from unmanipulated animals; Co = Paper probed with conjugate alone.

FIGURE 3.3.4

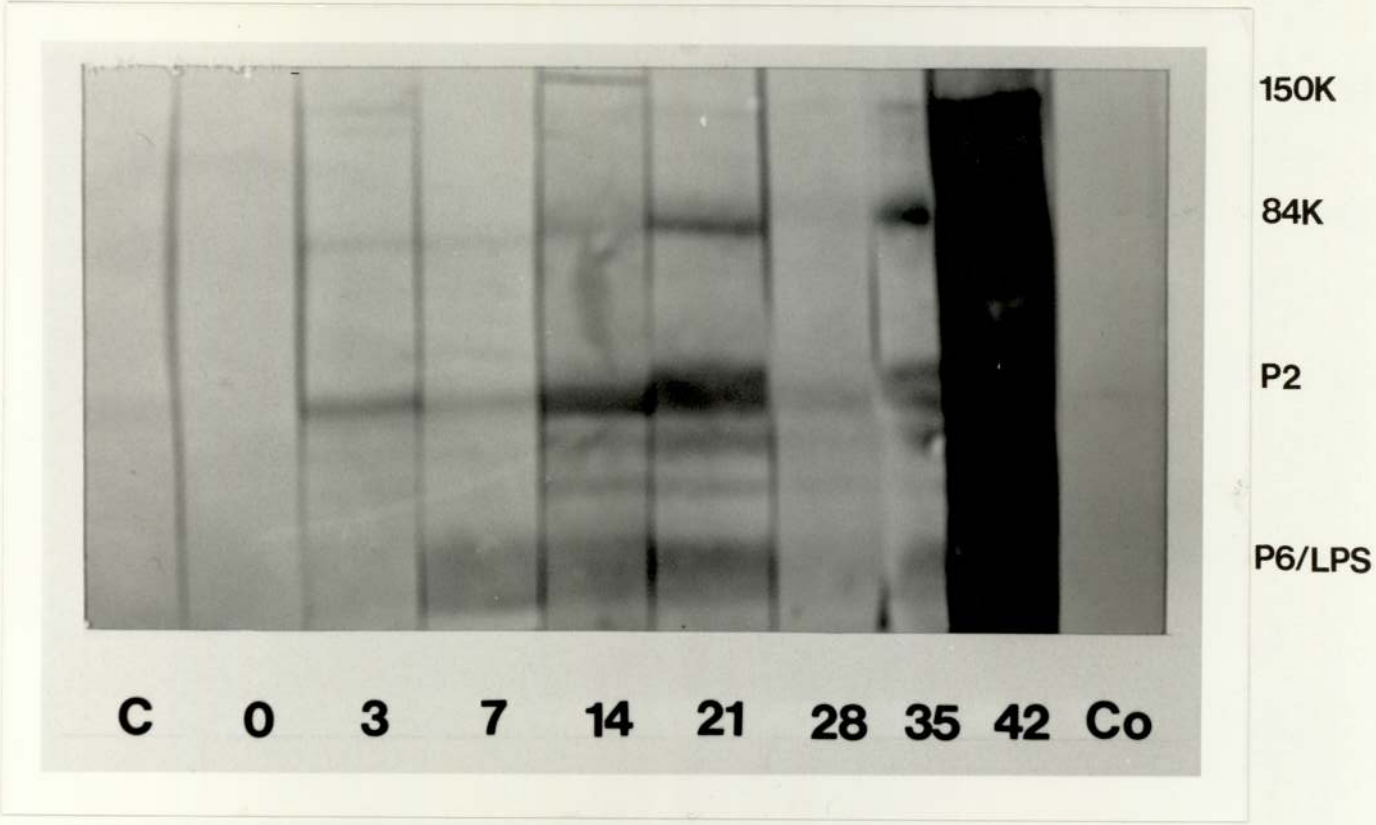


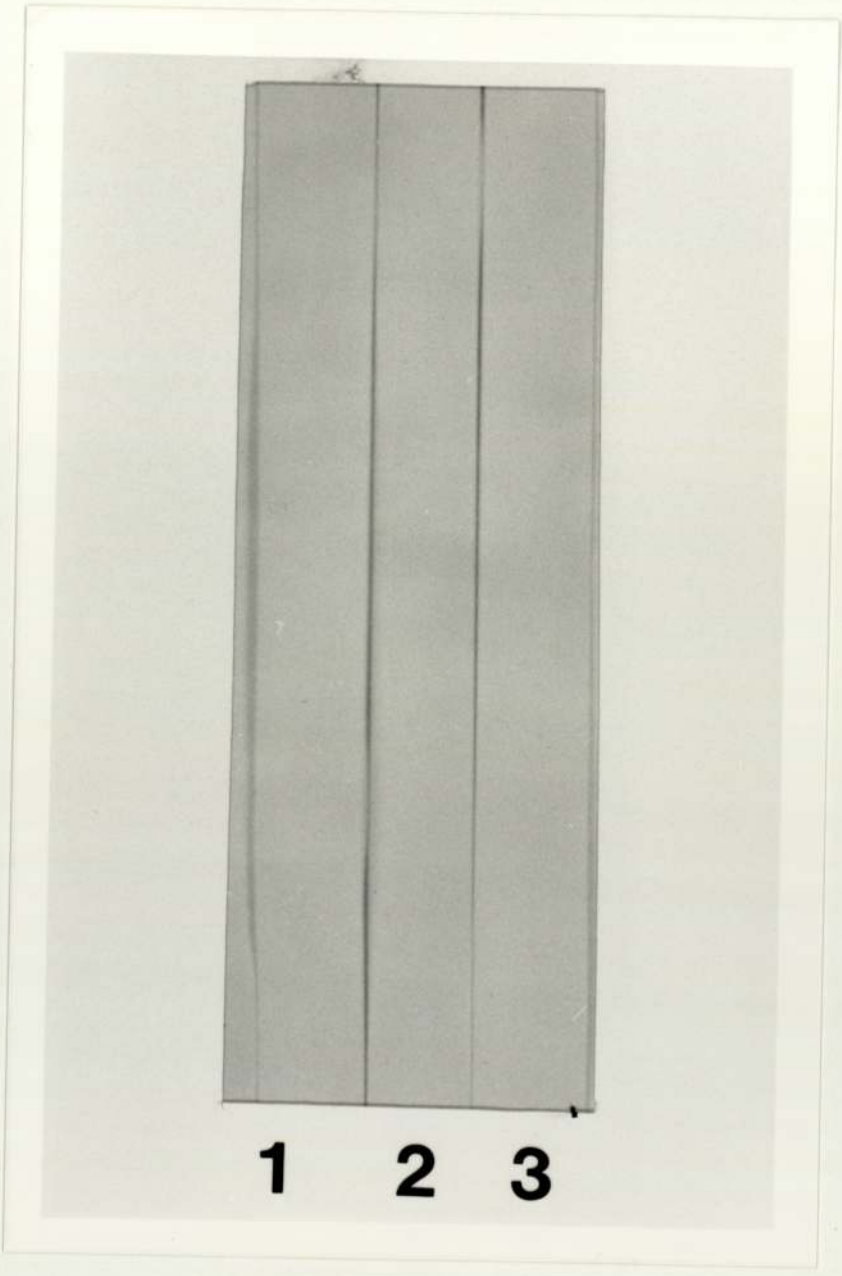
FIGURE 3.3.5.

The Immune Response of Control Animals to Iron-Restricted OM Antigens of Non Typable *H.influenzae* HM1.

Details as in the legend to figure 3.3.4.

Immunoblots were probed with sera collected at 3 (lane 1), 14 (lane 2) and 42 (lane 3) days post-operatively from animals inoculated intratracheally with sterile agar beads.

FIGURE 3.3.5



immunoblots was weakly visible on day 3, becoming stronger by day 7. Additional bands of molecular weight between 16K and 39K were apparent at 14 days post-infection. By day 35 the 150K, 84K, P2 and the LPS/P6 region appeared to be the major OM antigens recognised by serum from animals infected with *H.influenzae* HM1. At 42 days post-infection antibodies to almost every OMP of the infecting strain appeared to be produced. Control immunoblots are shown in Figure. 3.3.5. OMPs from *H.influenzae* HM1 shown here were probed with sera collected at day 3 (lane 1), day 14 (lane 2) and day 42 post inoculation from control animals which had been injected intratracheally with sterile agar beads.

These results suggest that non typable *H.influenzae* HM1, within the pulmonary environment are phenotypically altered in comparison with cells grown *in vitro* under conditions of haem excess, in that they appear to express a 150K OMP when growing *in vivo* which is only apparent *in vitro* when this strain is cultured using PPIX as a porphyrin source.

Several attempts were made to harvest bacterial OMs directly from *in vivo* material. Homogenised rat lungs collected 42 days post-infection with 10^{8} c.f.u of non typable *H.influenzae* HM1 were subjected to differential centrifugation followed by sarkosyl extraction as described previously (2.5.4). Coomassie blue stained SDS-PAGE gels of material extracted in the above manner, together with OMPs prepared by sarkosyl extraction of bacteria grown *in vitro* in BHI broth supplemented with haem or PPIX are shown in

Figs. 3.3.6 a and b. It is evident that complete OMP profiles of *in vivo* grown non typable *H.influenzae* could not be obtained from infected rat lungs using this procedure. OMPs P2, P6 and a 56K protein were the only *H.influenzae* proteins visible in *in vivo* extracts and no conclusions as to the protein composition of the OM of non typable *H.influenzae* HM1 growing *in vivo* could be drawn from this experiment.

OMPs from five non typable *H.influenzae* strains which expressed PPIX-specific 150K and 84K proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper. The protein-impregnated nitrocellulose was then incubated with a 1/50 dilution of antisera raised against *H.influenzae* HM1 obtained from rats 42 days post-infection with 10^8 c.f.u. Antigenic sites were visualised using protein-A-peroxidase conjugate and a solution of 25µg/ml 4-chloro-1-naphthol containing 0.01% H_2O_2. The results are shown in Fig. 3.3.7 and indicate substantial cross reactivity between OMPs of these non typable isolates. IgG antibodies raised against HM1 reacted strongly with OMP P2 and the LPS/P6 region of all five strains. No other protein was consistently antigenic in all isolates. Antibodies raised against the 84K protein of *H.influenzae* HM1 reacted with the 84K OMP expressed by three out of the five isolates tested, but antibodies to the PPIX-specific 150K OMP of HM1 were not cross reactive with the 150K protein produced by any of the other isolates, apparently

FIGURE 3.3.6. Recovery of Bacterial Outer Membranes from *In Vivo* Material.

Lungs from 50 rats, collected 42 days post-infection with *H.influenzae* HM1, were homogenised vigorously and large debris removed by course filtration. The resulting filtrate was divided in two. Half was passed twice through a french pressure cell and OMs recovered by sarkosyl extraction (fraction 1). The remaining 50% was subjected to three rounds of differential centrifugation before passage through a french press and sarkosyl extraction (fraction 2). A second sarkosyl extraction of fraction 2 was performed, and the resultant material designated fraction 3 (see 2.5.4).

Proteins present in each of the above fractions were separated by SDS-PAGE (2.4.1) and stained using coomassie blue.

(A) Lane 1: molecular weight standards

Lane 2: OMPs of *H.influenzae* HM1 cultured *in vitro* under conditions of haem excess.

Lane 3: OMPs of *H.influenzae* HM1 cultured under iron-restricted conditions.

Lane 4: fraction 1.

Lane 5: fraction 2.

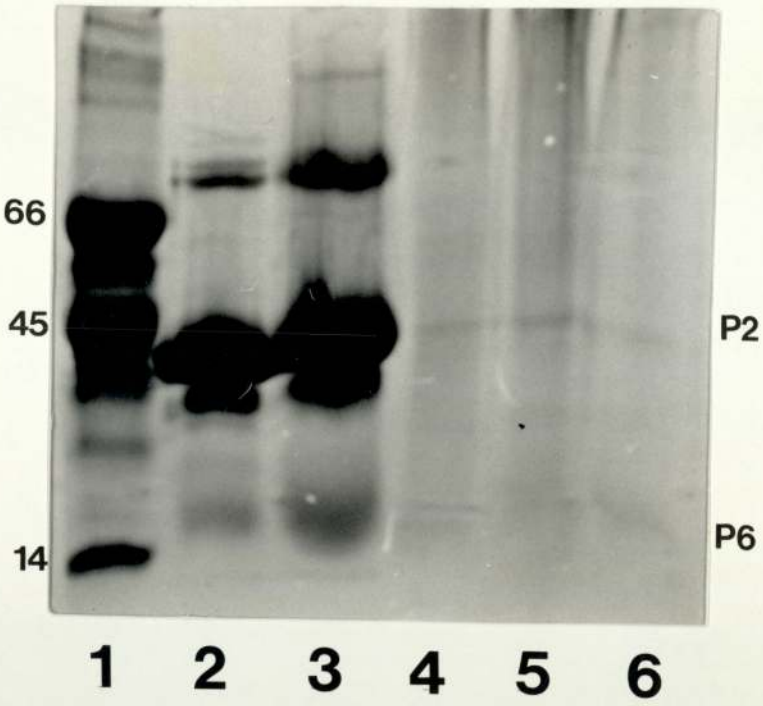
Lane 6: fraction 3.

(B) Lane 1: OMPs of *H.influenzae* HM1 cultured *in vitro* under iron-restricted conditions.

Lane 2: fraction 3, gel loading 100% greater than that shown in (A) lane 6.

FIGURE 3. 3. 6.

(A)



(B)

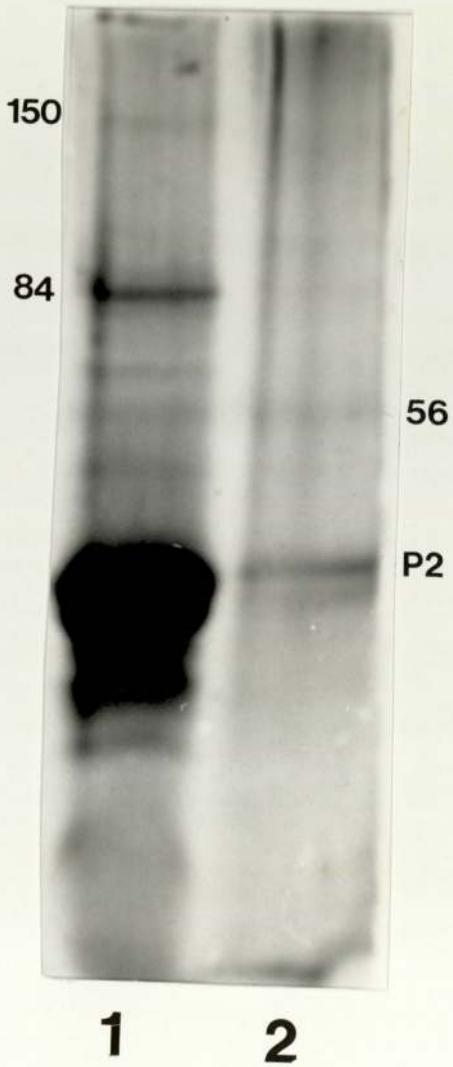


FIGURE 3.3.7.

Cross-Reactivity of OM Antigens Derived from Non Typable *H.influenzae* Cultured Under Iron-Restricted Conditions.

OMs from 5 strains of non typable *H.influenzae*, all of which expressed 84K and 150K PPIX-specific OMPs, were purified as previously described (2.3.1) and separated by SDS-PAGE (2.4.1). Separated OMPs were electrophoretically transferred to nitrocellulose paper and immunoblotting carried out as described in section 2.4.3. A 1/50 dilution of serum collected from rats 42 days post-infection with *H.influenzae* HM1 was incubated with the protein-impregnated nitrocellulose paper for 4 hours. After washing with TBS, antigenic sites were visualised using HRP conjugated anti-rat IgG and 25µg/ml 4-chloro-1-naphthol containing 0.01% H_2O_2.

Numbers in the margins refer to *H.influenzae* proteins by molecular weight (kilodaltons) or by standard nomenclature.

Lane 1: *H.influenzae* HM2.

Lane 2: *H.influenzae* HM10.

Lane 3: *H.influenzae* HM25.

Lane 4: *H.influenzae* HM11.

Lane 5: *H.influenzae* HM24.

FIGURE 3.3.7

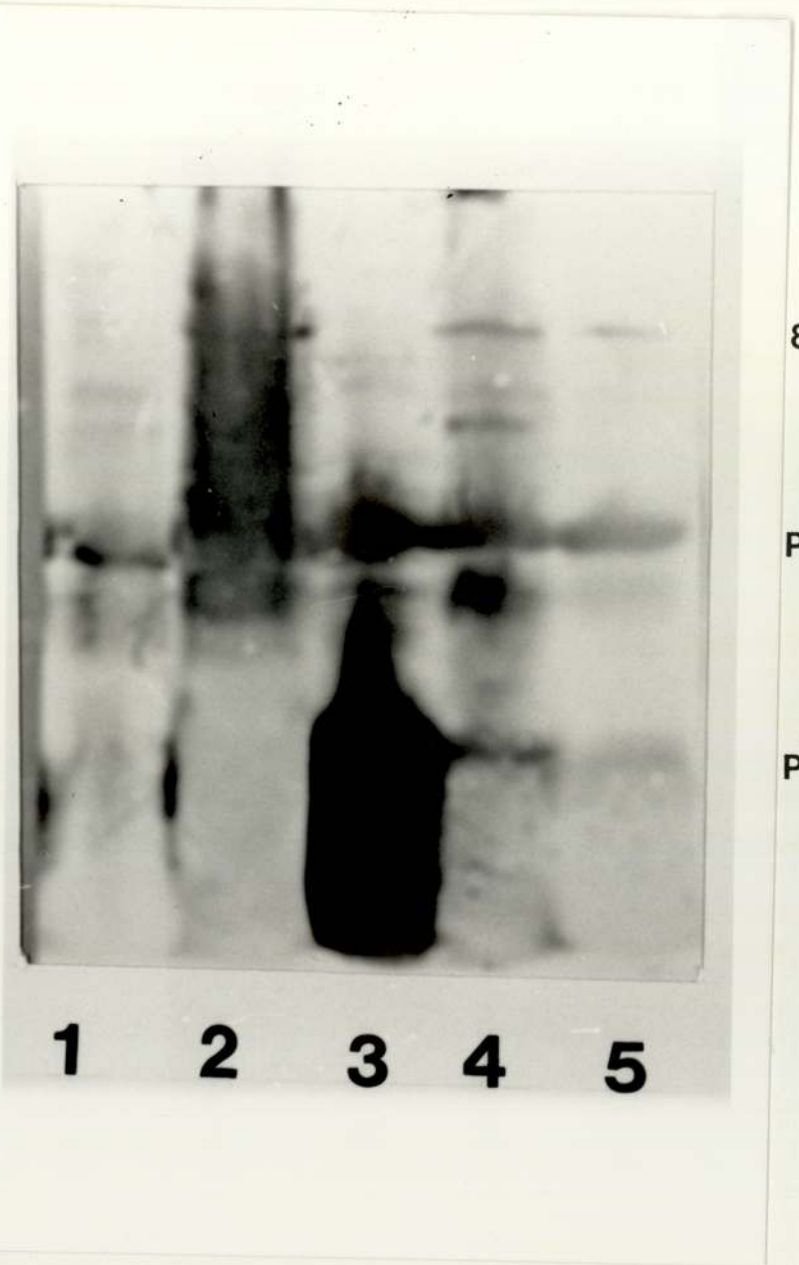
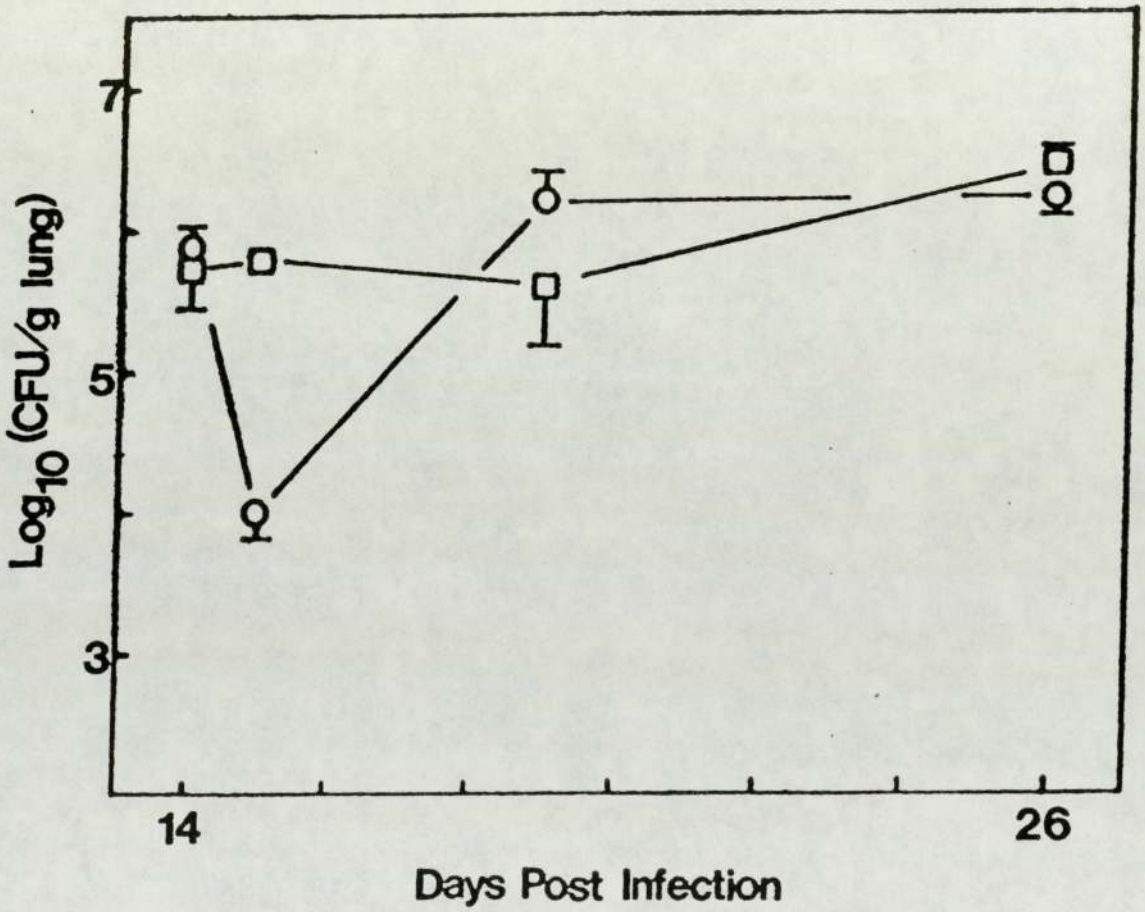


Figure 3.3.8

Amoxycillin Induced Reduction of Pulmonary *H.influenzae.*

A single dose of Augmentin (30mg / Kg) was administered to animals which had been infected with 10^8 CFU of *H.influenzae* HM1 14 days previously (○); controls received sterile water (□).

Values presented are means (n = 4) and the SEM is either encompassed by the size of the symbol or represented by vertical bars. Bacterial load on day 15 was decreased as compared to controls.


7
5
3
Log10 (CFU/g lung)
14
26
Days Post Infection

indicating antigenic heterogeneity among 150K OMPs produced by different strains of non typable *H.influenzae*.

Despite encasement with agar beads *H.influenzae* remains susceptible to amoxycillin *in vivo* (Fig. 3.3.8). In the present study a single dose of augmentin (30mg/kg) resulted in a 98% reduction in pulmonary load in rats inoculated intratracheally with 10^6 c.f.u of *H.influenzae*. As only one, subtherapeutic dose was administered bacteria were not completely eradicated and the pulmonary population subsequently increased to pre-treatment levels.

3.3.2. DISCUSSION.

The present study has demonstrated persistent colonisation of rat lungs by a single isolate of non typable *H.influenzae*. Encasement of *H.influenzae* into agar beads appears to facilitate resistance to normal cellular lung defence mechanisms. Infection persists despite the presence of naturally occurring antibodies which appear to be largely directed against the LPS component of the OM, in both serum and lung lavage fluid.

Previous agar bead models of pulmonary infection using *Pseudomonas aeruginosa* have demonstrated differences between strains with respect to pathogenesis and pulmonary persistence (Woods *et al*, 1982; Cash *et al*, 1983). Strains of *P.aeruginosa* producing exotoxin A and elastase appear to persist in the lung for longer periods of time than isogenic mutants lacking the ability to produce one or both of these

factors and are consequently associated with increased lung damage (Woods *et al*, 1982). Although *H.influenzae* does not appear to produce exotoxins, liberated LPS from this bacterium has been demonstrated to have toxic effects which include ciliostasis and destruction of ciliated epithelium (Denny, 1974; Johnson and Inzana, 1986) and so may be an important precipitating factor of pulmonary damage. Complexation of LPS from certain bacterial species (e.g. *E.coli*, *K.pneumoniae* and *P.aeruginosa*) and lung surfactant is reported to increase LPS toxicity in an animal model (Brogden *et al*, 1986), but whether this applies to *H.influenzae* LPS in the bronchiectatic lung remains to be demonstrated. The present model would be amenable to studies comparing virulence of various *H.influenzae* isolates, for example strains with differing LPS moieties (Apicella *et al*, 1985).

Serum and lavage IgG titres increased 7 to 10 fold during the course of infection and are apparently correlated to inoculum size. In this respect they resemble *H.influenzae* antibody titres in bronchiectasis as described in section 3.2, in cystic fibrosis (Burns and May, 1968) and chronic bronchitis (Murphy and Apicella, 1987) where individuals almost uniformly present increased serum anti-*H.influenzae* titres compared with normal subjects.

In the present study a rapid rise in serum IgG titres was observed when OMs were used as antigen, which was faster than the rise in anti-LPS titre alone. As immunoblotting

demonstrates a rapid immune response towards OMPs, these may be potent inducers of the immune system, perhaps more so than LPS. Use of purified OMP antigens in assays would clarify this.

Immune recognition of the 150K PPIX-specific OMP by infected rat sera suggests production of this antigen by *H.influenzae* HM1 growing *in vivo* in rat lungs. As antiserum produced by the bronchiectasis patient from whose sputum this strain was isolated also contained IgG reactive with this protein (3.2.3.1c) it would seem that the *in vivo* environment afforded by this model may be in some respects similar to the environment of bacteria in human pulmonary infection. Similarly, cystic fibrosis patients colonised with *P.aeruginosa* demonstrate antibody to *Pseudomonas* IRMPs (Anwar *et al*, 1984) which can be detected in the OMs of bacteria harvested directly, without subculture, from infected sputum (Brown *et al*, 1984). Attempts to harvest bacteria without subculture from infected lungs in the present study were unsuccessful.

The majority of IgG present in lung lavage fluid from infected rats appears to be directed against LPS. Similarly most of the IgG present in purulent sputum from patients with severe bronchiectasis also appears to be directed against the LPS/P6 region of the *H.influenzae* OM on immunoblots (Fig. 3.2.3.5A). Sputa from chronic bronchitics also contain specific anti-*H.influenzae* IgG and IgA antibodies (Gump *et al*, 1973). Titres of IgA in lung lavage

fluid were not determined in the present study and the antigens against which IgA antibodies in purulent sputum were targetted also remain to be elucidated. Antibodies detected in lung lavage may represent locally produced IgG but this must be interpreted with caution due to the lavage technique employed. Additionally, increases in serum proteins present in sputum have been reported during the course of exacerbations of chronic pulmonary disease (Stockley and Burnett, 1980).

Antibodies reactive with OMPs and LPS of non typable *H.influenzae* have also been observed in serum and bronchoalveolar lavage fluid from intraperitoneally vaccinated mice, and these have been shown to be effective in enhancing pulmonary clearance (Hansen *et al*, 1988). In the present study, despite increases in serum and lung lavage antibodies there was no decrease in net bacterial load. Resistance to clearance may in part be due to inaccessibility of antibodies afforded by the agar bead vehicle, poor opsonic activity of antibodies induced by intratracheal administration of antigen (McGeehee *et al*, 1987), induction of blocking IgA antibodies (Musher *et al*, 1984) phenotypic shift to increased resistance to anti-LPS antibodies as occurs in *H.influenzae* type b (Inzana and Anderson, 1985) or low complement activity (Reynolds and Newball, 1974). However, the fact that enhanced pulmonary clearance of non typable strains in complement depleted mice vaccinated with OMPs and LPS has been demonstrated (Hansen

et al, 1988) would suggest that of the possibilities listed above, lack of complement activity alone is not a factor which would prevent clearance.

Gnehm *et al*, (1985) report that in human serum most anti-non typable *H.influenzae* bactericidal activity is directed against OMPs rather than LPS. In a situation where most locally produced IgG is directed against LPS one would therefore expect poor bactericidal activity. Determination of IgA levels in lung lavage fluid and elucidation of the antigens against which they are directed may help to identify key antigens and antibodies involved in non typable *H.influenzae* pathogenesis within the respiratory tract.

This experimental model is amenable to antibiotic therapy indicating retention of susceptibility by the bacteria and sufficient penetration of antibiotic into the agar bead to attain bactericidal concentrations. The effects of various antimicrobial regimens and of sub-MIC concentrations of antibiotic (in sputum) on *H.influenzae* persistence and antigen presentation could be further investigated using this type of animal model.

Levels of contamination by other bacteria during the course of *Haemophilus* infection in this model increased from <1% to 31% by day 42, control lungs inoculated with sterile agar beads did not become contaminated in this manner. Colonisation by other bacteria may have been facilitated as time progressed by increasing levels of pulmonary damage due to persistent *H.influenzae* infection. This theory remains to

be confirmed by histological analysis of infected lung tissue.

Problems with this model include the fact that there was no net increase in bacterial load from day 1 to day 42. After an initial decrease in numbers the number of bacteria present in the lung increased again but did not reach levels above that of the initial inoculum. Despite this however, bacteria growing in the lungs did show evidence of phenotypic change (as evidenced by apparent alterations in OMP profile) *in vivo* compared with cells used as the initial inoculum.

This model of chronic non typable *H.influenzae* pulmonary infection presents characteristics similar to such infections in individuals with chronic obstructive pulmonary disease and may therefore be useful in future for experimental examination of immunological, pathological, and therapeutic aspects of these infections.

3.4. Respiratory Systems of Non Typable *H.influenzae* Cultured Under Haem-Limited or Iron-Restricted Conditions.

The final pH of cultures of *H.influenzae* HM1 and HM3 grown *in vitro* under conditions of haem excess, PPIX excess or limitation, and haem-limitation differed markedly from one another (see Fig. 3.2.1.1), suggesting that changes in the quantity of exogenously supplied haem source resulted in alterations in metabolism. *Haemophilus influenzae* cultured utilising PPIX as a porphyrin source showed similar final pH values regardless of whether excess or growth yield limiting amounts of PPIX were available. In contrast however, reduction in available haem from excess to growth yield limiting concentrations resulted in a significant drop in final culture pH in spite of a 60-65% decrease in biomass. Figures 3.4.1 a and b show pH changes throughout the growth curve for two *H.influenzae* isolates (HM1 and HM3) and show that after a drop in pH during log phase re-alkalinisation of the medium occurs in haem-sufficient cultures as stationary phase progresses. In haem-limited cultures however, the culture pH continues to drop throughout stationary phase. Figure 3.4.2 confirms that the buffering capacity of BHI broth remains constant across the range of pH values covered, hence the effect observed must be due to

Figure 3.4.1a

H.influenzae HM3 Culture: Optical Density and pH Changes with Time.

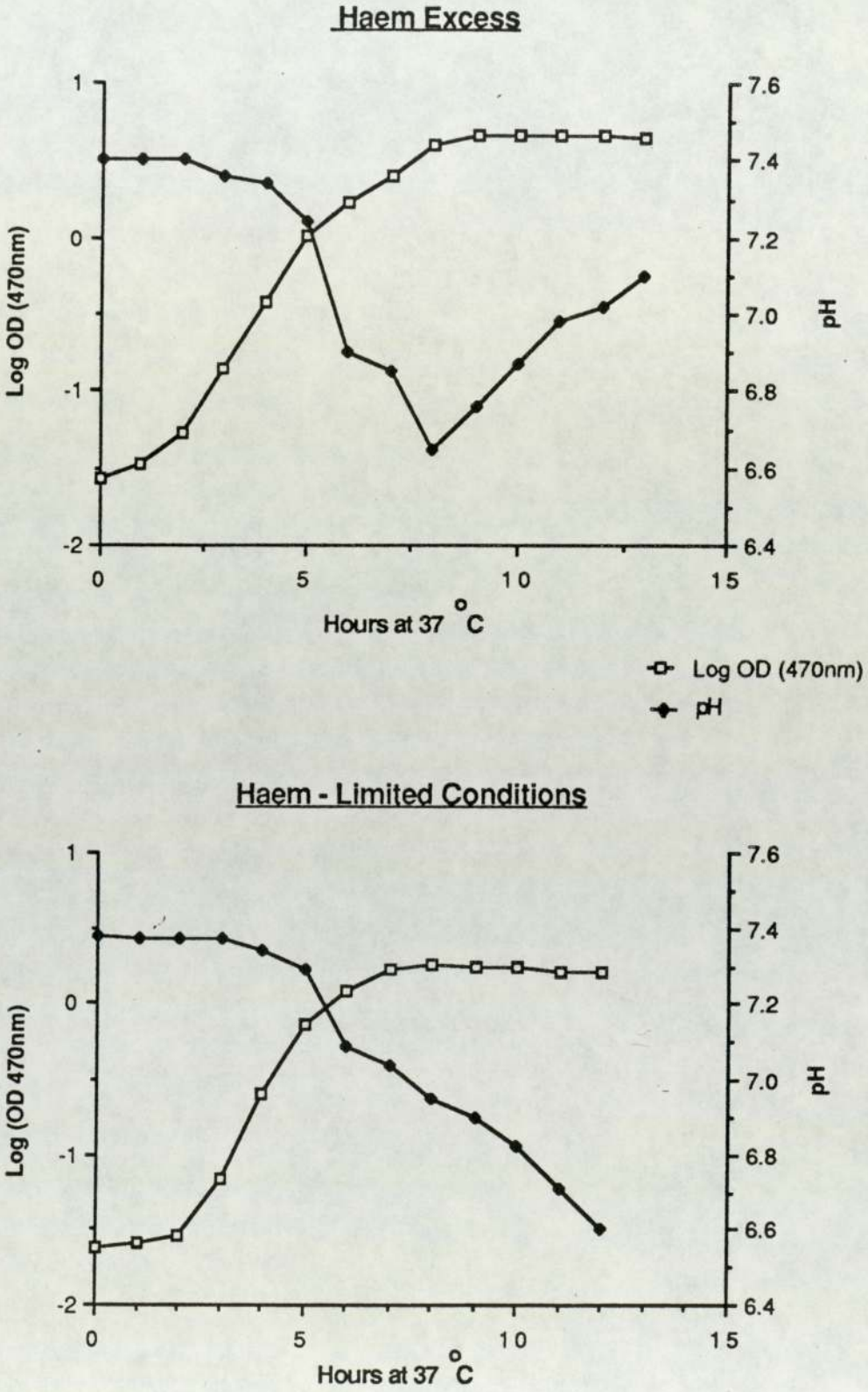
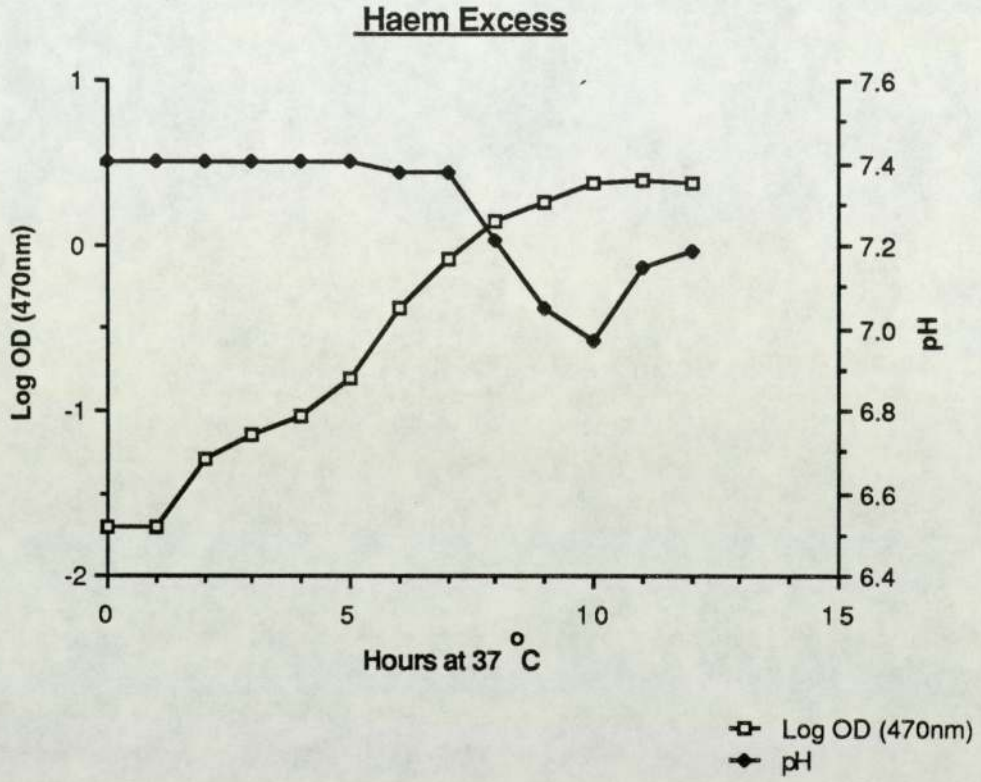


Figure 3.4.1b

H.influenzae HM1 Culture: Optical Density and pH Changes with Time.



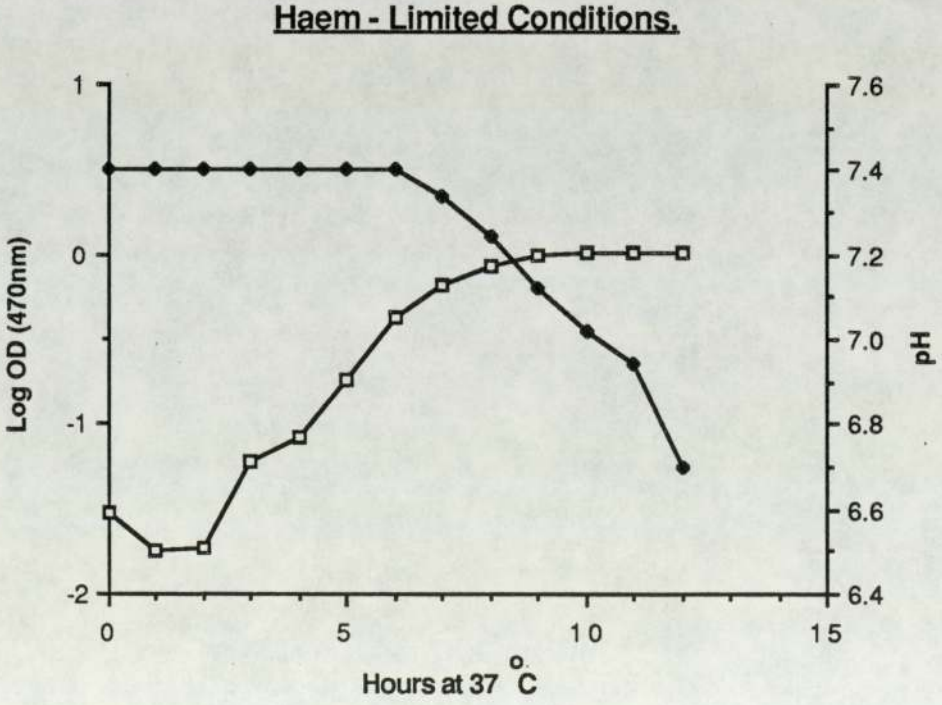
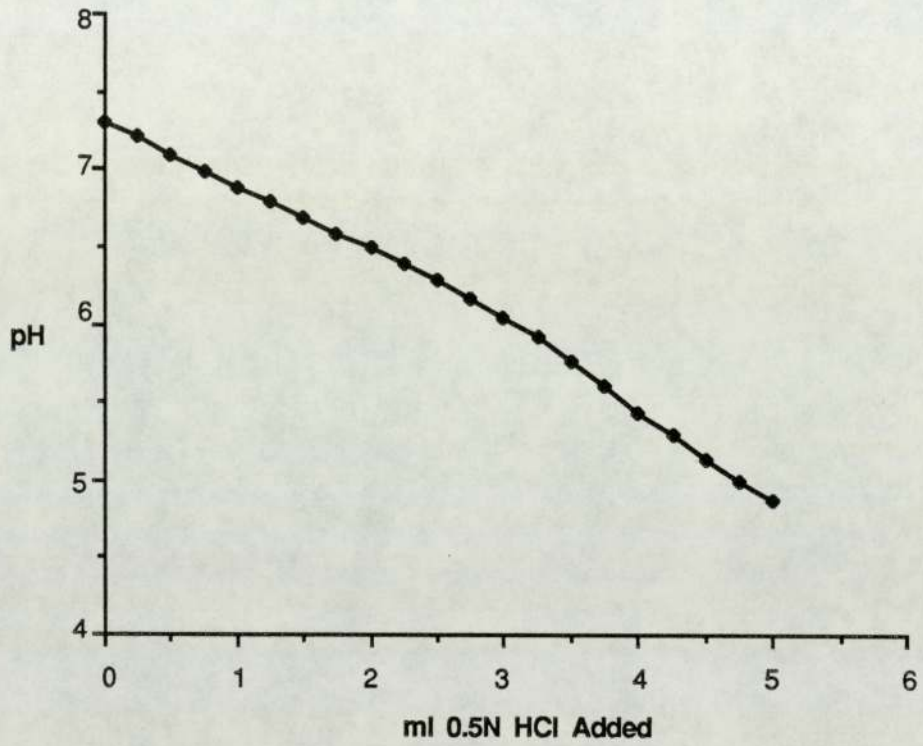


Figure 3.4.2

Titration of BHI Broth With 0.5N HCl.



increased acid production of haem-limited compared with haem-sufficient or PPIX grown cultures.

The final OD_{470} values of haem-sufficient and haem-limited *H.influenzae* HM3 cultured in glucose-free BHI broth supplemented with increasing glucose concentrations are shown in Figure 3.4.3, analogous results were obtained for strain HM1. These results indicate more efficient utilisation of glucose by haem-sufficient compared with haem-limited cultures as evidenced by greater accumulation of biomass in haem-sufficient cultures as available glucose increases.

Decreased efficiency of glucose utilisation and reduction in final pH of haem-limited cultures is correlated with increased organic acid production (Table 3.4.1). Haem-limited *H.influenzae* uniquely produce lactic acid and also produce an increased ratio of succinic:acetic acid in comparison with cells grown under conditions of haem excess and cells utilising PPIX as a porphyrin source. Culture supernatants from *H.influenzae* grown under all of the conditions of haem and iron availability desribed in table 3.4.1 were also assayed for the presence of formic acid and gave uniformly negative results.

Figure 3.4.3.

Final Cell Yields of Haem -Sufficient and Haem - Limited Cultures of *H.influenzae* HM3 Grown in Varying Concentrations of Glucose.

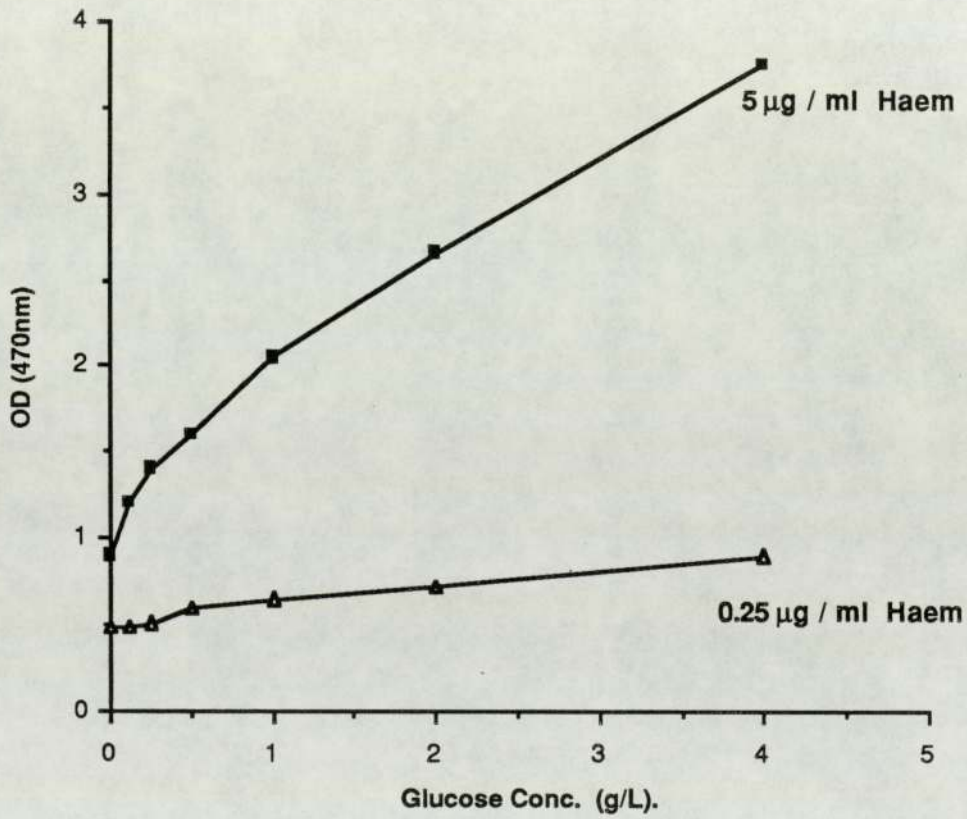


TABLE 3.4.1

Production of Acids from Glucose by Non Typable *H.influenzae* - Effect of Varying Haem and Iron Availability.

Culture Conditions	Acetate	Lactate	Succinate
Haem Excess	2.15	ND	0.63
Haem Limited	0.63	7.4	1.9
PPIX Excess	1.43	ND	1.28
PPIX Limited	2.53	ND	1.12
Iron Restricted	2.64	ND	1.16
NAD Limited	1.66	ND	0.73

Values represent mM Acids in Stationary Phase Culture Supernatants.

ND = Not Detectable

The Influence of Haem-Limitation, PPIX Substitution, and Iron-Restriction on the Cytochrome Complement of Non Typable *H.influenzae*.

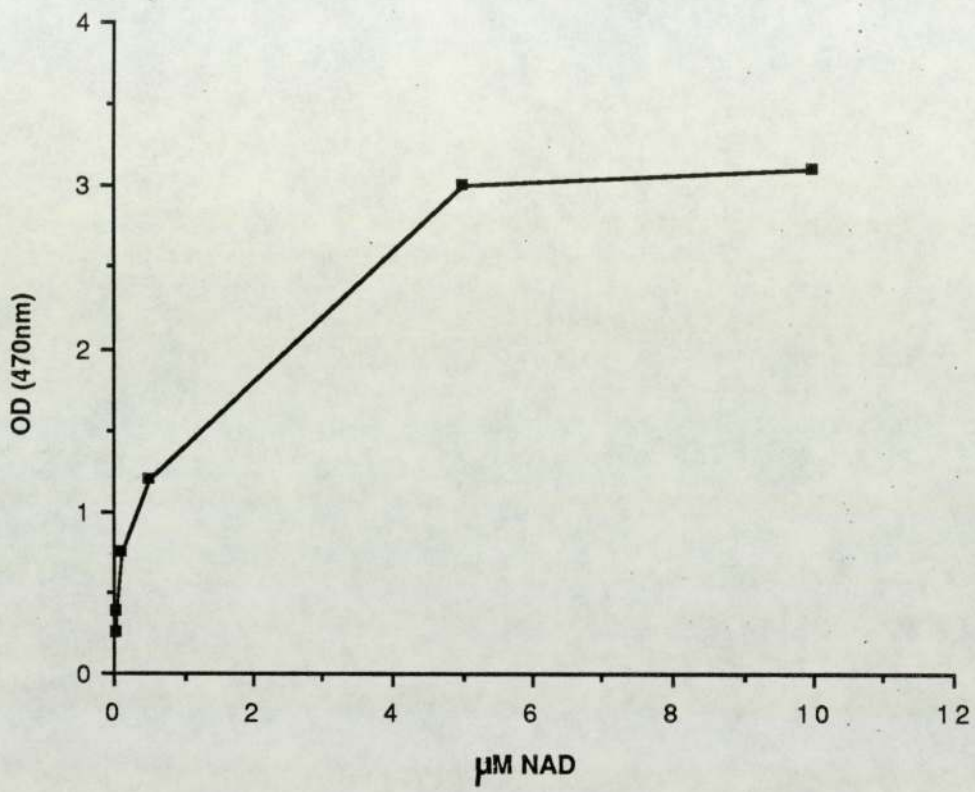
During analysis of the influence of porphyrin ring source and iron availability on the cytochrome complement of *H.influenzae*, bacteria grown under conditions of NAD-limitation were used as controls in order to ensure that any changes observed were due to test limitations and not due to oxygen limitation at high biomass levels, which is known to affect cytochrome concentrations (White, 1963; Jones and Poole, 1985). Figure 3.4.4 shows the final OD_{470} of *H.influenzae* HM3 cultured in BHI broth containing excess haem and varying concentrations of NAD. On the basis of these results a concentration of 0.1μM NAD was used to achieve NAD-limited bacteria.

Reduced minus oxidised difference spectra (see 1.4.4a) for cell free extracts (CFE), soluble cell fractions (S180) and membrane fractions (P180) (see 2.3.2) of non typable *H.influenzae* grown under various conditions of haem, PPIX and iron availability are shown in Figures 3.4.5 to 3.4.8. Reduced minus oxidised cytochrome spectra of *H.influenzae* cultured under conditions of NAD-limitation are shown in Fig. 3.4.9.

In general, the reduced minus oxidised difference spectrum of a cytochrome exhibits 3 major absorption bands, the α band (in the range 545-650nm); the β band (range 520-530nm) and the γ, or soret band (range 410-450nm). Exceptions to

Figure 3.4.4

Final Cell Yields of Cultures of *H.influenzae* HM3 Grown in Varying Concentrations of NAD.



Bacterial growth in BHI broth supplemented with excess haem and varying concentrations of NAD was monitored turbidimetrically.

this rule include d-type cytochromes, which have only weak soret bands, and a-type cytochromes, which do not have appreciable β bands (Jones and Poole, 1985). Figs. 3.4.5 - 3.4.9 show that *H.influenzae* HM3 grown in haem-sufficient (3.4.5), in PPIX-limited (3.4.7), iron-restricted (3.4.8) and NAD-limited (3.4.9) conditions produce a cytochrome complement consisting of d, b and c-type cytochromes. These appear to be produced in greatly reduced quantities in cells grown in PPIX-limited and in iron-restricted conditions compared with those grown under conditions of haem-excess.

The α peaks of d, b and c-type cytochromes of *H.influenzae* HM3 appear at 630, 560, and 550nm respectively. A trough indicative of flavoprotein was observed at 438-440nm under each of the growth conditions. a-type cytochromes were not apparent on these spectra but their production cannot be excluded, as a small peak between 570 and 580nm which would be characteristic of these may be present but masked by the large α peak exhibited by b and c-type cytochromes.

The membrane bound nature of cytochromes is illustrated by the relatively flat traces obtained on analysis of soluble (S180) fractions. *Haemophilus influenzae* cultured in haem-sufficient conditions (Fig.3.4.5 A and B) produce a soluble c-type cytochrome which is not apparent in cells grown under any other conditions. Analysis of dithionite reduced minus dithionite + CO reduced spectra (Fig.3.4.5C) of the S180 fraction showed CO binding capacity and indicated that this

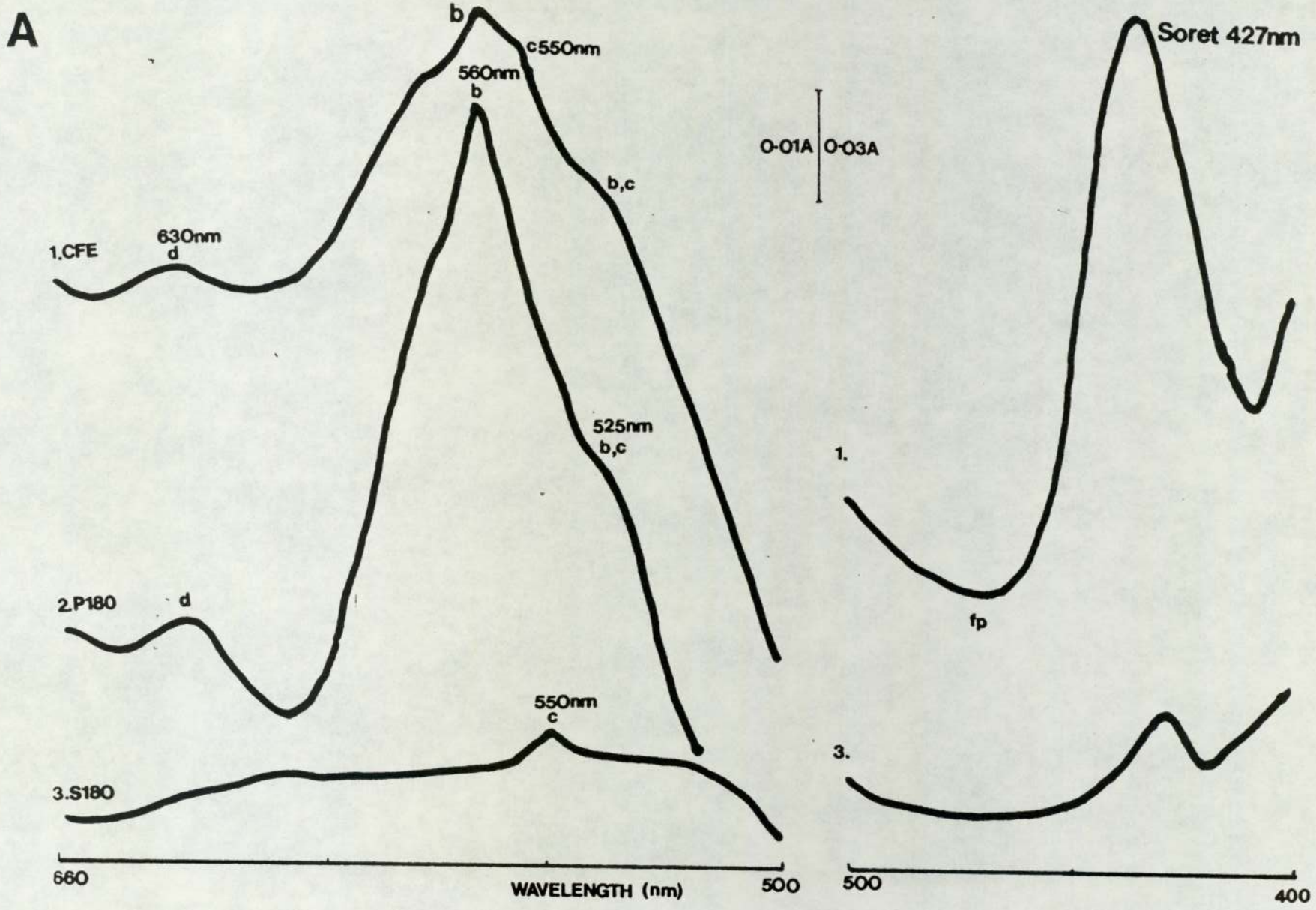
Figure 3.4.5 A and B.

Dithionite Reduced Minus Air Oxidised Difference Spectra of Non Typable *H.influenzae* Cultured Under Haem - Sufficient Conditions.

Dithionite reduced minus air oxidised difference spectra of 1. Cell free extracts (CFE), 2. Particulate fractions (P180), and 3. Soluble fractions (S180) derived from (A) stationary phase and (B) log phase cells of *H.influenzae* cultured under conditions of haem excess (5μg/ml).

Absorbance peaks characteristic of b, c, and d - type cytochromes are indicated by letter and wavelength. fp = flavoprotein.

The bar represents 0.01 A between 660 and 500nm and 0.03A between 500 and 400nm.


A
b
c550nm
560nm
b
b,c
630nm
d
1.CFE
525nm
b,c
2.P180
d
550nm
c
3.S180
660
WAVELENGTH (nm)
500
0·01A
0·03A
Soret 427nm
1.
fp
3.
500
400

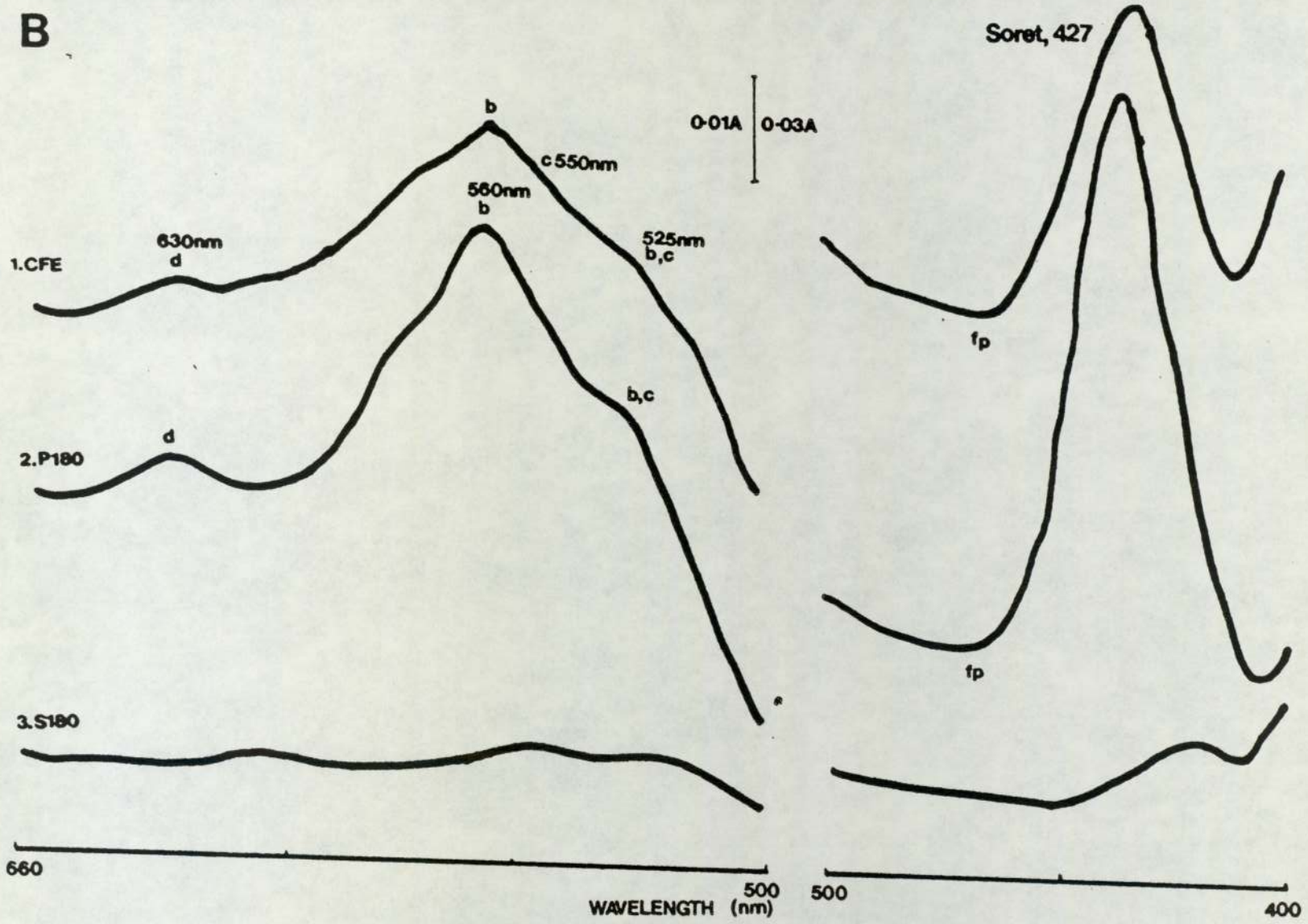

B
Soret, 427
0·01A
0·03A
b
c 550nm
560nm
b
630nm
d
525nm
b,c
1.CFE
b,c
d
2.P180
fp
fp
3.S180
660
500
WAVELENGTH (nm)
500
400

Figure 3.4.5C

Dithionite Reduced Minus Air Oxidised , and Dithionite Reduced + CO Minus Dithionite Reduced Difference Spectra of Soluble Fractions (S180) Derived From Stationary Phase Cells of Non Typable *H.influenzae* Cultured Under Haem - Sufficient Conditions.

A. Dithionite reduced minus air oxidised difference spectra

B. Dithionite reduced plus CO, minus dithionite reduced difference spectra.

Absorbance peaks and troughs characteristic of b and c - type cytochromes are indicated by letter and wavelength. fp = flavoprotein

The bar represents 0.02 Absorbance units between 660 and 500nm and 0.04A between 500 and 380nm.

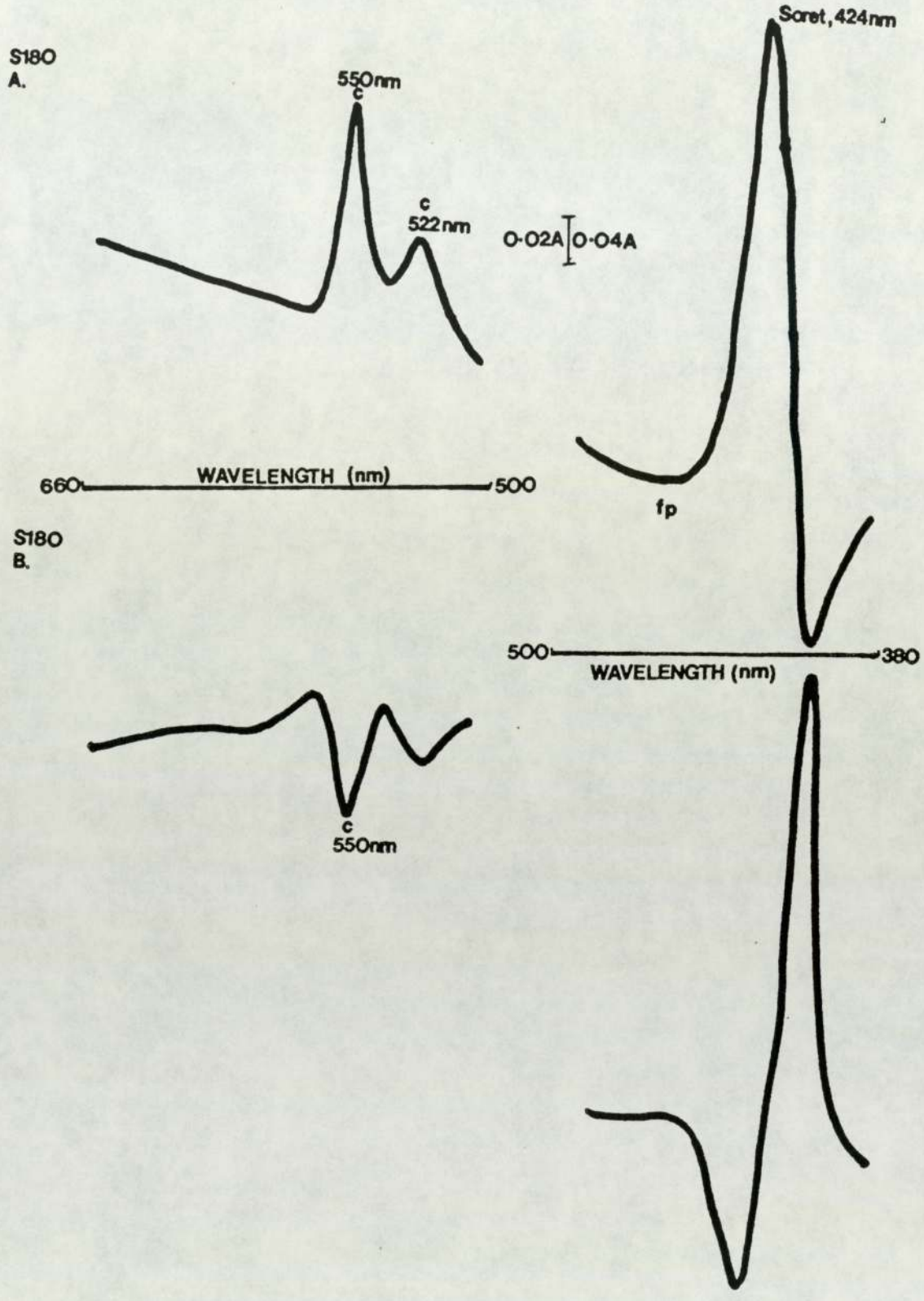
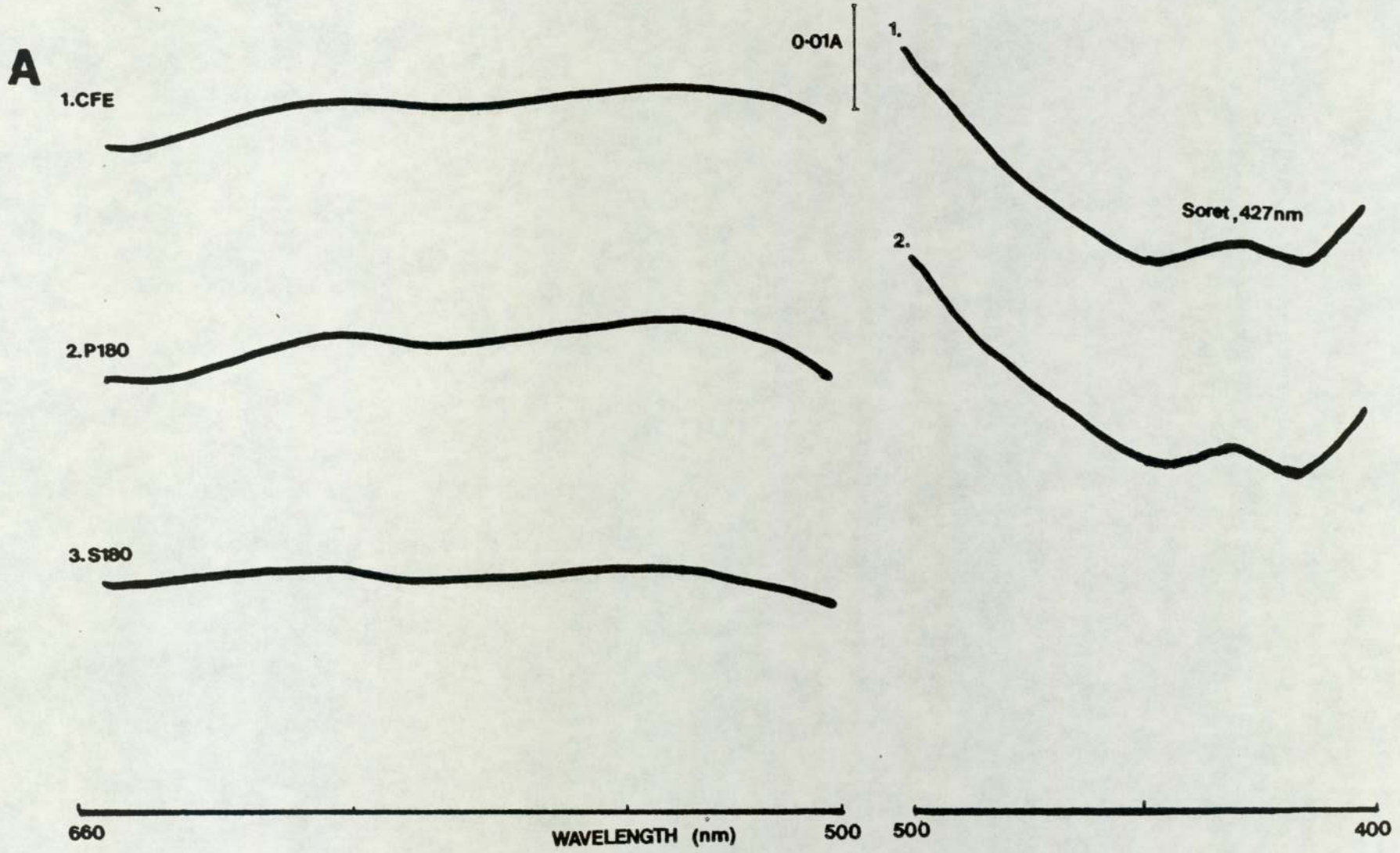

S180
A.
550nm
c
c
522nm
0·02A
0·04A
Soret, 424nm
660
WAVELENGTH (nm)
500
fp
S180
B.
500
380
WAVELENGTH (nm)
c
550nm

Figure 3.4.6 A and B.

Dithionite Reduced Minus Air Oxidised Difference Spectra of Non Typable *H.influenzae* Cultured Under Conditions of Haem - limitation.

Dithionite reduced minus air oxidised difference spectra of 1. Cell free extracts (CFE), 2. Particulate fractions (P180), and 3. Soluble fractions (S180) derived from A) stationary phase and B) log phase cells of *H.influenzae* cultured under haem - limited conditions.

The bar represents 0.01Absorbance units.


A
0·01A
1.CFE
2.P180
3.S180
660
WAVELENGTH (nm)
500
1.
Soret, 427nm
2.
500
400

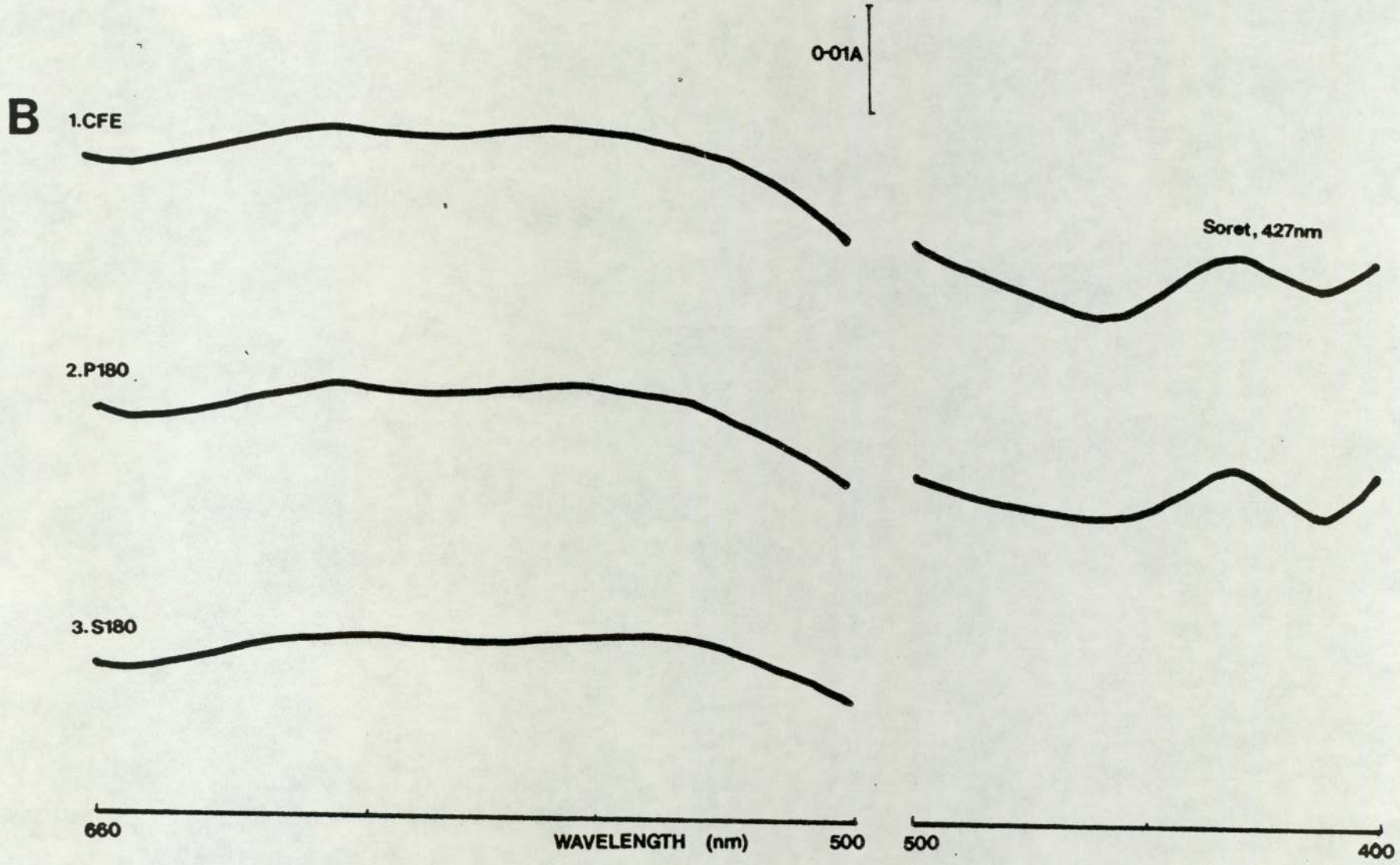

B
1.CFE
2.P180
3.S180
0·01A
Soret, 427nm
660
WAVELENGTH (nm)
500
500
400

Figure 3.4.7

Dithionite Reduced Minus Air Oxidised Difference Spectra of Non Typable *H.influenzae* Cultured Under PPIX - Limited Conditions.

Dithionite reduced minus air oxidised difference spectra of 1. Cell free extracts (CFE), 2. Particulate fractions (P180), and 3. Soluble fractions (S180) derived from stationary phase cells of *H.influenzae* cultured under conditions where PPIX was limiting (0.25μg/ml).

Absorbance peaks characteristic of b, c, and d type cytochromes are indicated by letter and wavelength. fp = flavoprotein.

The bar represents 0.01 Absorbance units between 660 and 500 nm and 0.03 A between 500 and 400nm.

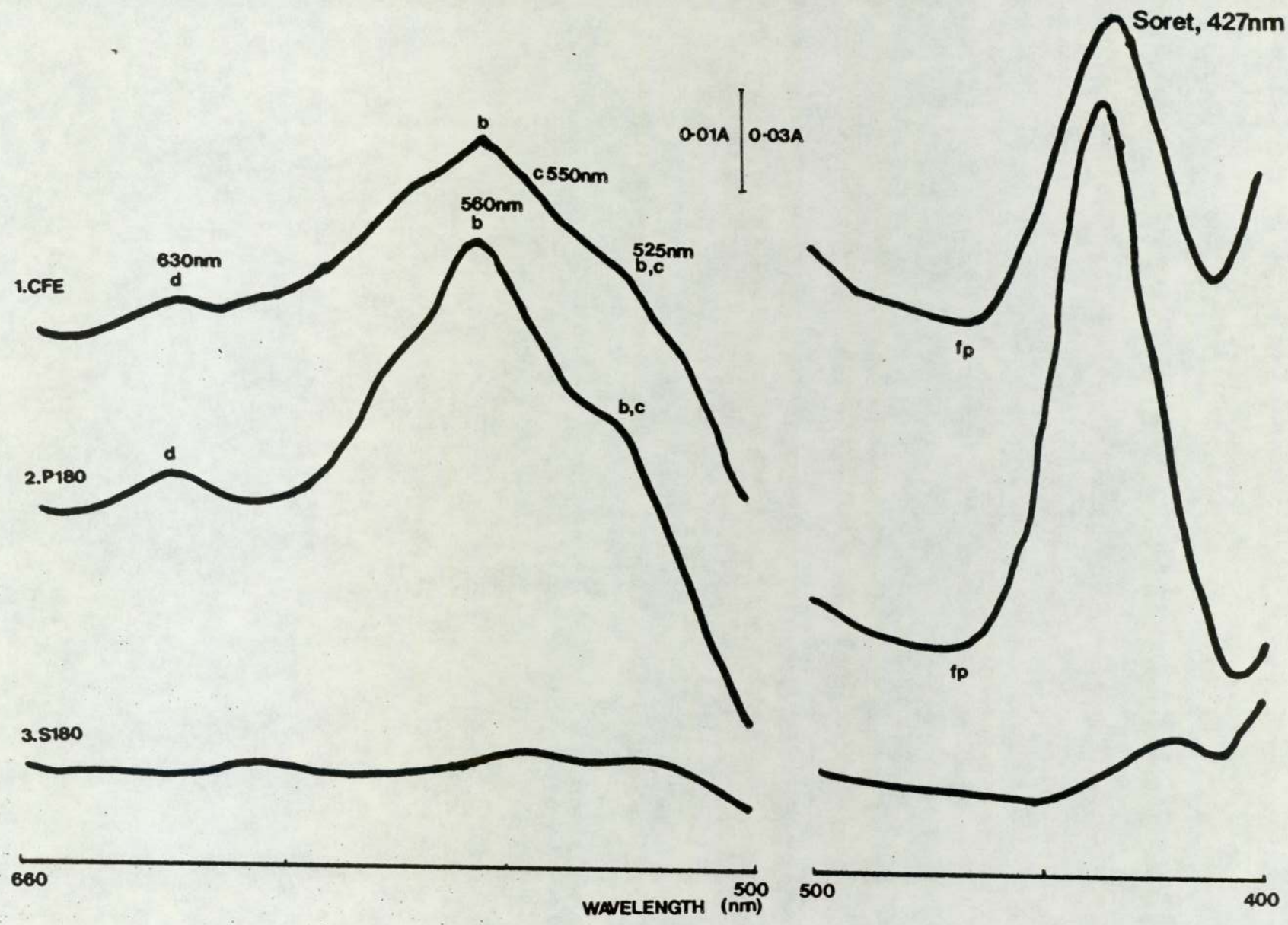

Soret, 427nm
0·01A
0·03A
b
c 550nm
560nm
b
525nm
b,c
630nm
d
1.CFE
b,c
d
2.P180
3.S180
fp
fp
660
500
WAVELENGTH (nm)
500
400

Figure 3.4.8

Dithionite Reduced Minus Air Oxidised Difference Spectra of Non Typable *H.influenzae* Cultured Under Iron Restricted Conditions.

Dithionite reduced minus air oxidised difference spectra of 1. Cell free extracts (CFE), 2. Particulate fractions (P180), and 3. Soluble fractions (S180) derived from stationary phase cells of *H.influenzae* cultured under iron - restricted conditions (0.25μg/ml PPIX + 30μM Desferal).

Absorbance peaks characteristic of b, c, and d - type cytochromes are indicated by letter and wavelength. fp = flavoprotein.

The bar represents 0.01 Absorbance units between 660 and 500nm and 0.03A between 500 and 400nm.

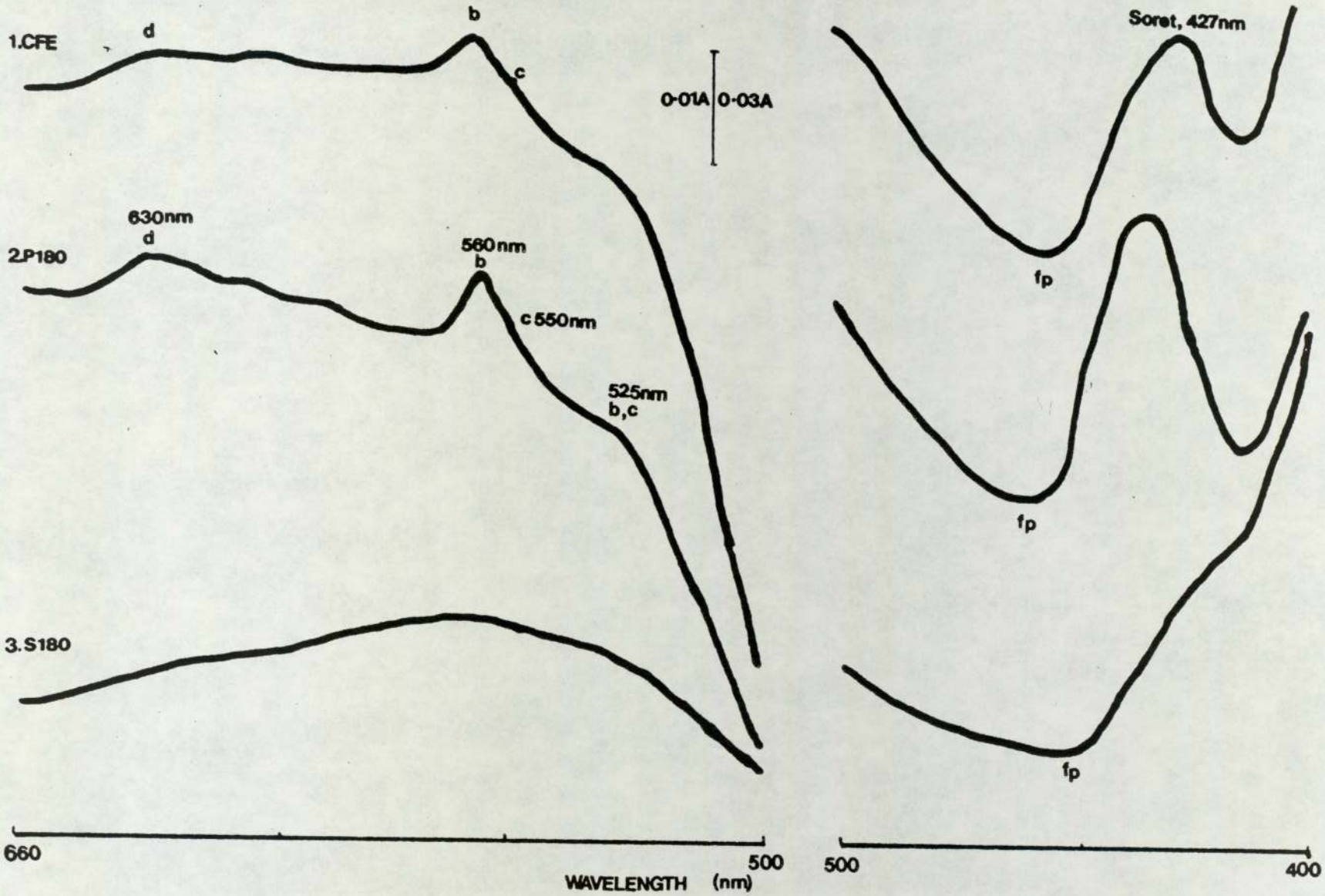

1.CFE
d
b
c
0·01A
0·03A
630nm
d
2.P180
560nm
b
c 550nm
525nm
b,c
3.S180
660
500
WAVELENGTH (nm)
Soret, 427nm
fp
fp
fp
500
400

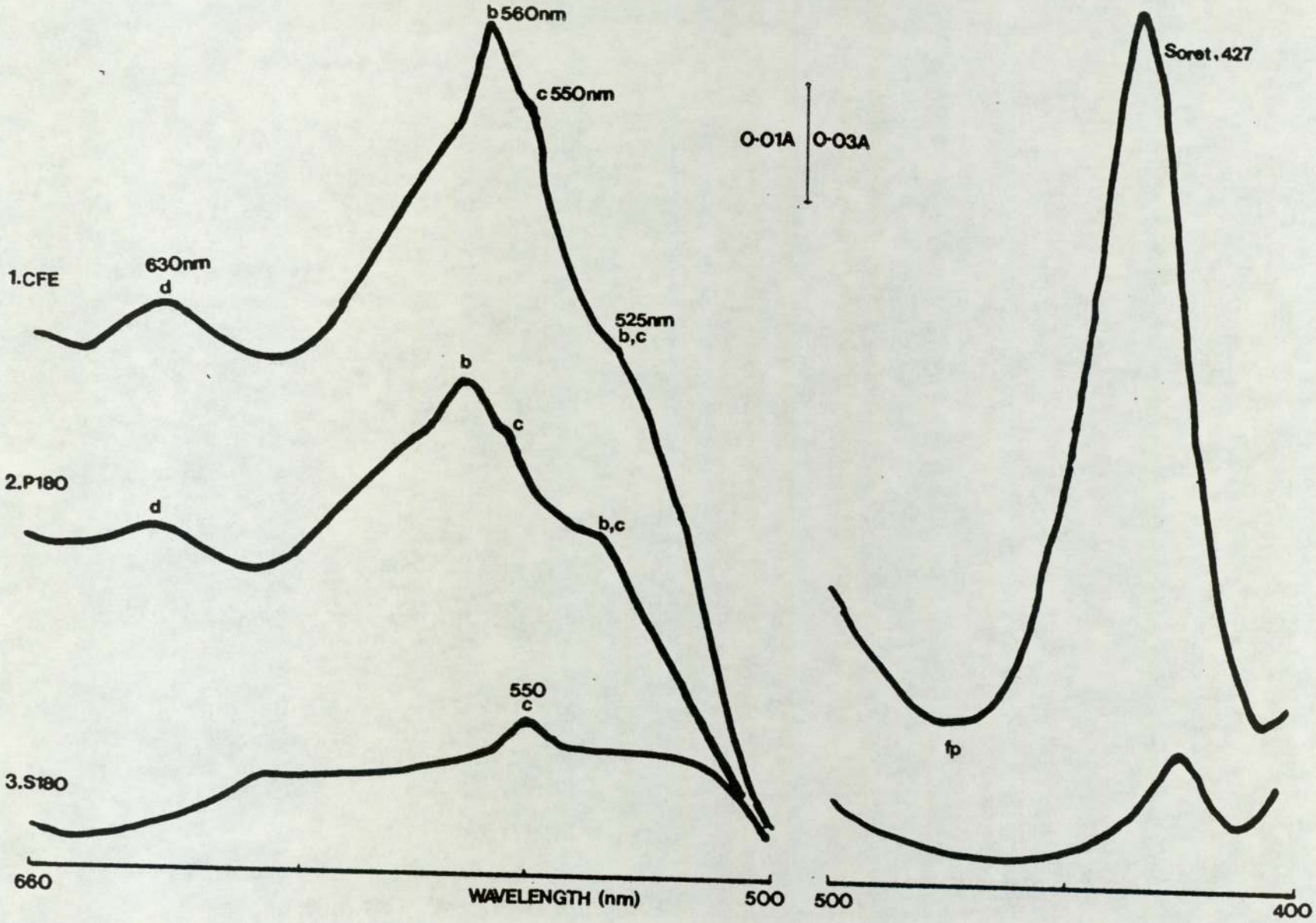
Figure 3.4.9

Dithionite Reduced Minus Air Oxidised Difference Spectra of Non Typable *H.influenzae* Cultured Under Conditions of NAD - Limitation.

Dithionite reduced minus air oxidised difference spectra of 1. Cell free extracts (CFE), 2. Particulate fractions (P180), and 3. Soluble fractions (S180) derived from stationary phase cells of *H.influenzae* cultured under NAD - limited conditions (0.025 μM NAD, 5μg/ml haem).

Absorbance peaks characteristic of b, c, and d - type cytochromes are indicated by letter and wavelength. fp = flavoprotein.

The bar represents 0.01 Absorbance units between 660 and 500nm and 0.03 A between 500 and 400nm.


b 560nm
c 550nm
0·01A
0·03A
Soret, 427
1.CFE
630nm
d
525nm
b,c
b
c
2.P180
d
b,c
550
c
3.S180
fp
660
WAVELENGTH (nm)
500
500
400

soluble c-type cytochrome could function as a cytochrome oxidase.

In contrast to all other culture conditions, bacteria grown under conditions of haem-limitation show no peaks indicative of the presence of cytochromes in the range 500-660nm (Figs. 3.4.6 A and B), but exhibit a small soret peak at 427nm and a shallow trough at 438nm indicative of flavoprotein production. Haem-limited bacteria therefore do not appear to produce appreciable amounts of cytochrome either in stationary or in log phase. Spectra from *H.influenzae* grown under haem-limited conditions in fact resemble those obtained from anaerobically grown cells (Fig. 3.4.10).

The amount of each cytochrome produced under each of the culture conditions under investigation was quantified from dithionite reduced minus ferricyanide oxidised difference spectra which ensured complete oxidation and reduction of all cytochromes. Changes in absorbance between peak-trough wavelength pairs were calculated for each cytochrome and concentrations of each type present were obtained using the following equation $A = \epsilon c \lambda$ where A indicates peak height, λ indicates wavelength and ϵ refers to the extinction coefficient for each cytochrome type as given by Jones and Redfearn (1965).

Concentrations of cytochromes b, c and d present in CFE, S180 and P180 cell fractions derived from *H.influenzae* cultured under conditions of varying haem, PPIX and iron availability are shown in Table 3.4.2. These indicate

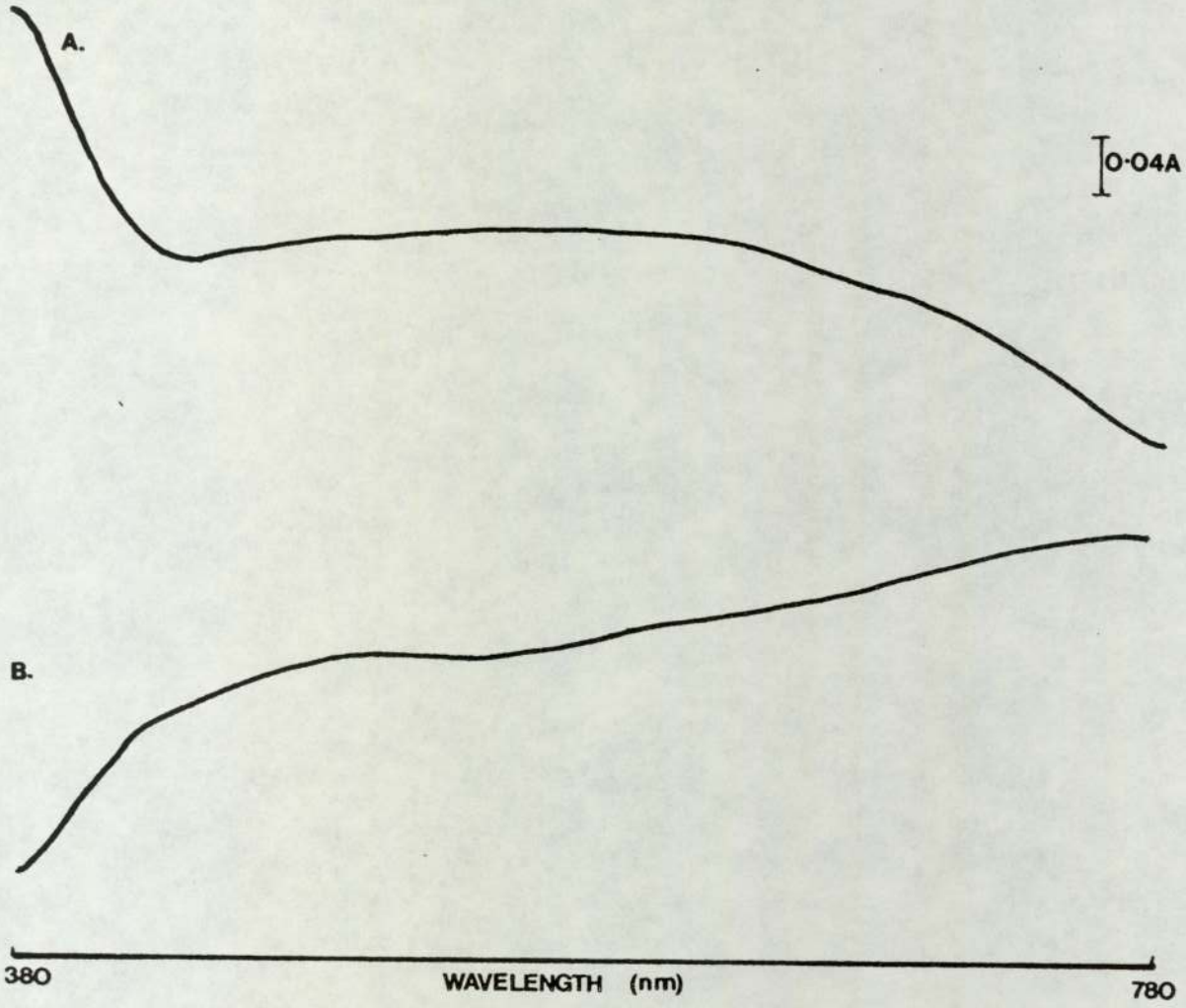
Figure 3.4.10

Dithionite Reduced Minus Air Oxidised Difference Spectra of Non Typable *H.influenzae* Cultured Under Anaerobic Conditions.

Dithionite reduced minus air oxidised difference spectra of cell free extracts (CFE) derived from stationary phase cells of *H.influenzae* cultured under anaerobic conditions and supplemented with excess NAD and varying concentrations of haem or PPIX as follows;

A. No added haem or PPIX
B. Haem excess (5μg/ml)
C. Growth yield limiting haem (0.25μg/ml).
D. PPIX excess (5μg/ml)
E. Growth yield limiting PPIX (0.25μg/ml)

The bar represents 0.04 Absorbance units.


A.
0·04A
B.
380
WAVELENGTH (nm)
780

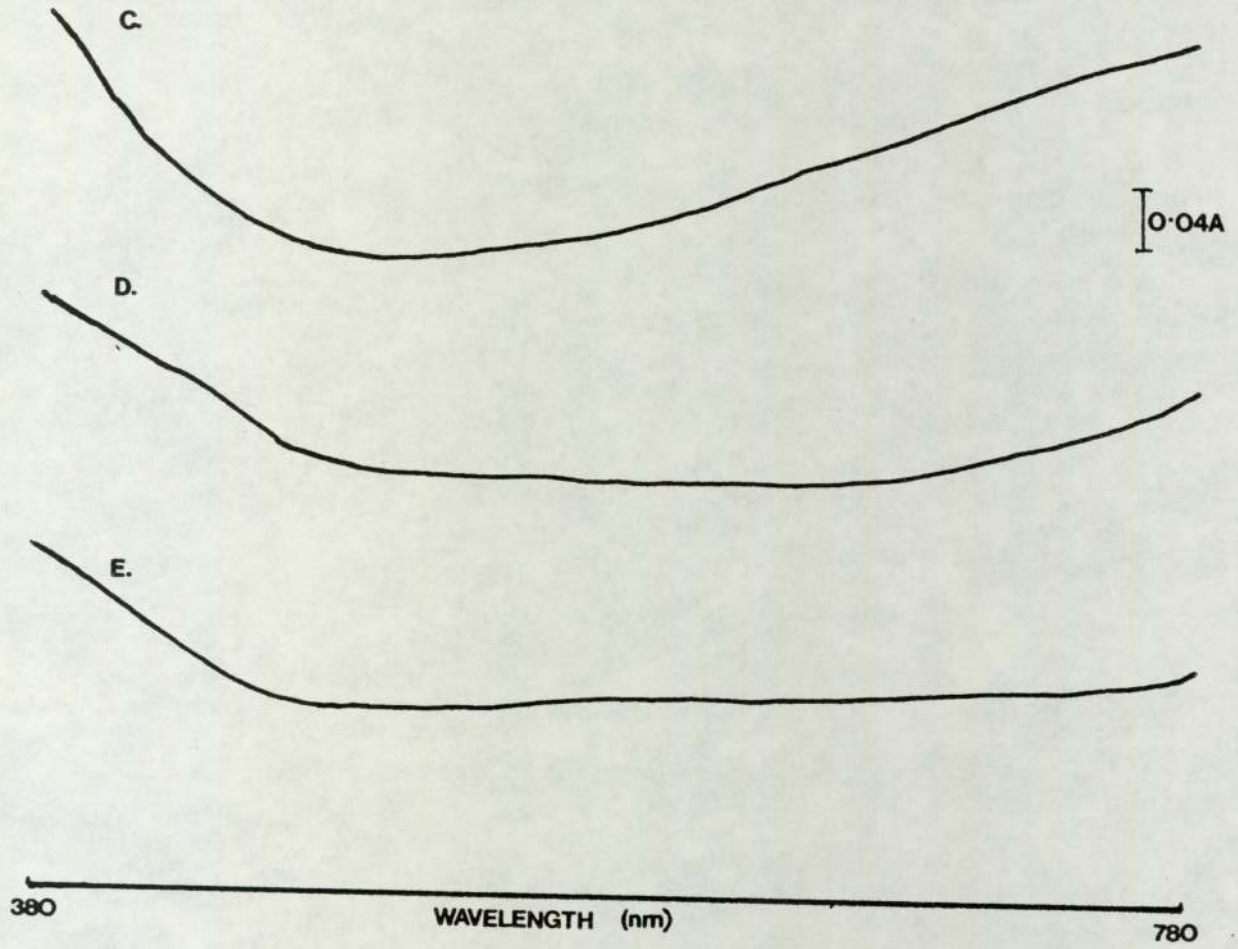

C.
0·04A
D.
E.
380
WAVELENGTH (nm)
780

TABLE 3.4.2. Cytochrome Concentrations in Cell Fractions of *H.influenzae* HM3 (pmol/mg protein) - Effect of Varying Haem and Iron Availability.

Culture Conditions	Growth Phase	CFE			S180			P180		
		cyt b	cyt c	cyt d	cyt b	cyt c	cyt d	cyt b	cyt c	cyt d
Haem Excess	Stationary	270	256	39.5	ND	58.7	ND	717	600	93
	Log	341	276	93	ND	46	ND	425	347	70
Haem Limited	Stationary	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Log	ND	ND	ND	ND	ND	ND	ND	ND	ND
PPIX Limited	Stationary	24.5	18	Tr	ND	ND	ND	42	21	Tr
Iron Restricted	Stationary	51	38	Tr	ND	ND	ND	80.7	37	22
NAD Limited	Stationary	303	229	75	ND	69.6	ND	796	605	51

ND = Not Detectable
Tr = Trace

equivalent, maximal production of all cytochromes in haem-sufficient and NAD-limited *H.influenzae* including production of small amounts of a soluble c-type cytochrome (58-69 pmol/mg protein). Culture in growth yield limiting concentrations of PPIX, regardless of the presence or absence of an iron chelator resulted in a ten fold decrease in production of all cytochromes, d-type cytochrome peaks although visible in these spectra were so small as to prevent accurate measurement and so are marked trace (Tr) in table 3.4.2. Haem-limited *H.influenzae* in contrast to PPIX-limited and haem-sufficient bacteria yielded flat spectra in which no measurable cytochrome peaks could be detected.

Differences in amounts of cytochrome detectable in cell fractions derived from *H.influenzae* cultured under various conditions of haem, PPIX and iron availability were reflected by differences in respiration of various potential energy sources. Figure 3.4.11 a-c shows oxygen uptake curves for each of haem excess (a), haem-limited (b) and PPIX-limited (c) whole cells of *H.influenzae* HM3 and demonstrates the effect of addition of formate or l-lactate on oxygen uptake. *Haemophilus influenzae* grown under each of these conditions appear to show levels of respiratory activity which correspond with quantities of cytochrome apparent on spectrophotometric analysis of cell fractions.

FIGURE 3.4.11.

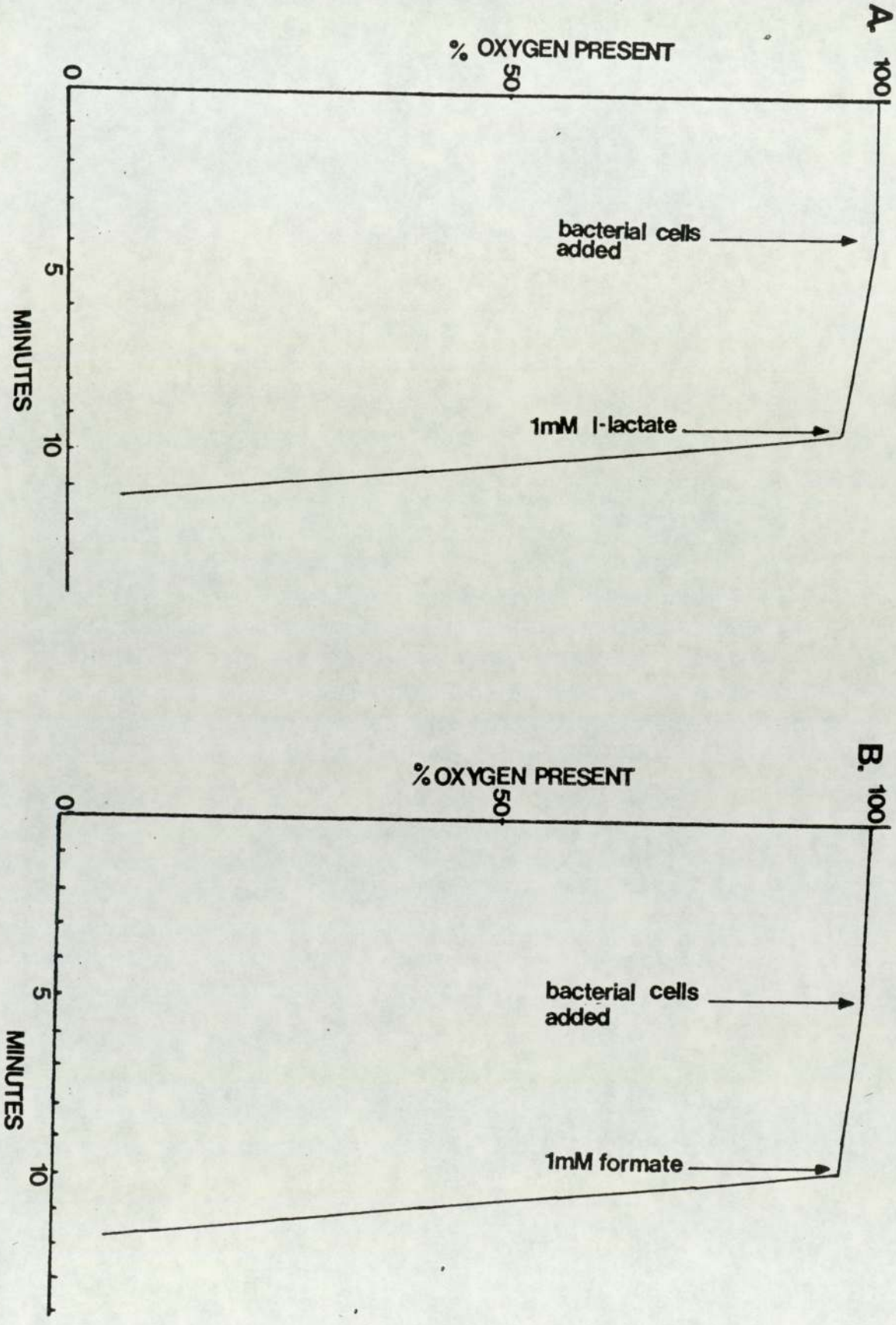
OXYGEN UPTAKE OF WHOLE CELLS OF NON TYPABLE *H. INFLUENZAE* IN PBS (pH 7.4) AT 30°C IN THE PRESENCE OF FORMATE OR 1-LACTATE: THE EFFECT OF VARYING HAEM AVAILABILITY.

Figure 3.4.11 (a). *Haemophilus influenzae* cells grown in conditions of haem excess (5µg/ml). Panel A shows respiratory activity on the addition of 1mM l-lactate; Panel B shows respiratory activity on the addition of 1mM formate.

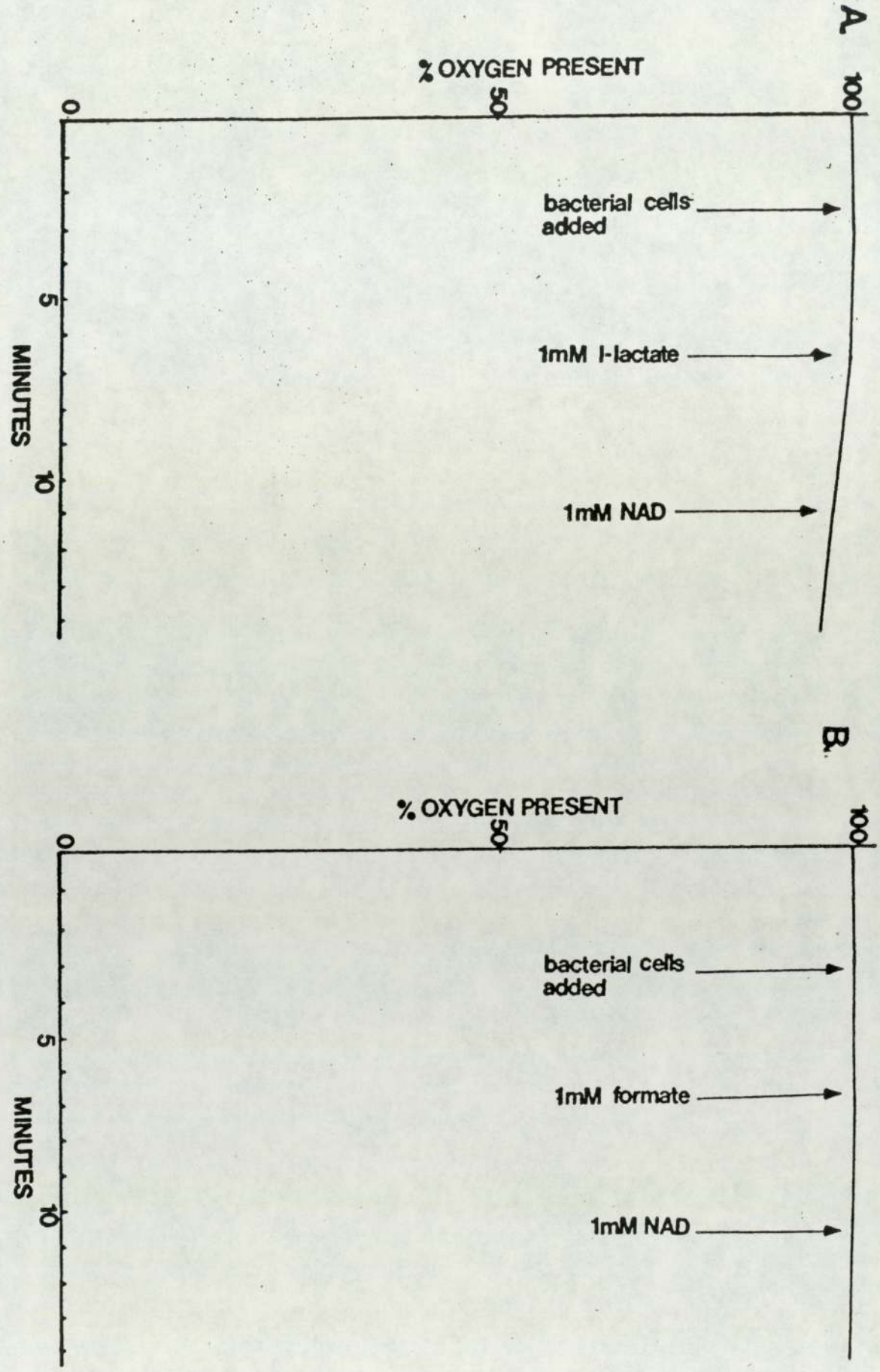
Figure 3.4.11 (b). *Haemophilus influenzae* cells grown under haem-limited conditions (0.25µg/ml). Panel A shows respiratory activity on the addition of 1mM l-lactate; Panel B shows respiratory activity on the addition of 1mM formate.

Figure 3.4.11 (c). *Haemophilus influenzae* cells grown under PPIX-limited conditions (0.25µg/ml). Panel A shows respiratory activity on the addition of 1mM l-lactate; Panel B shows respiratory activity on the addition of 1mM formate.

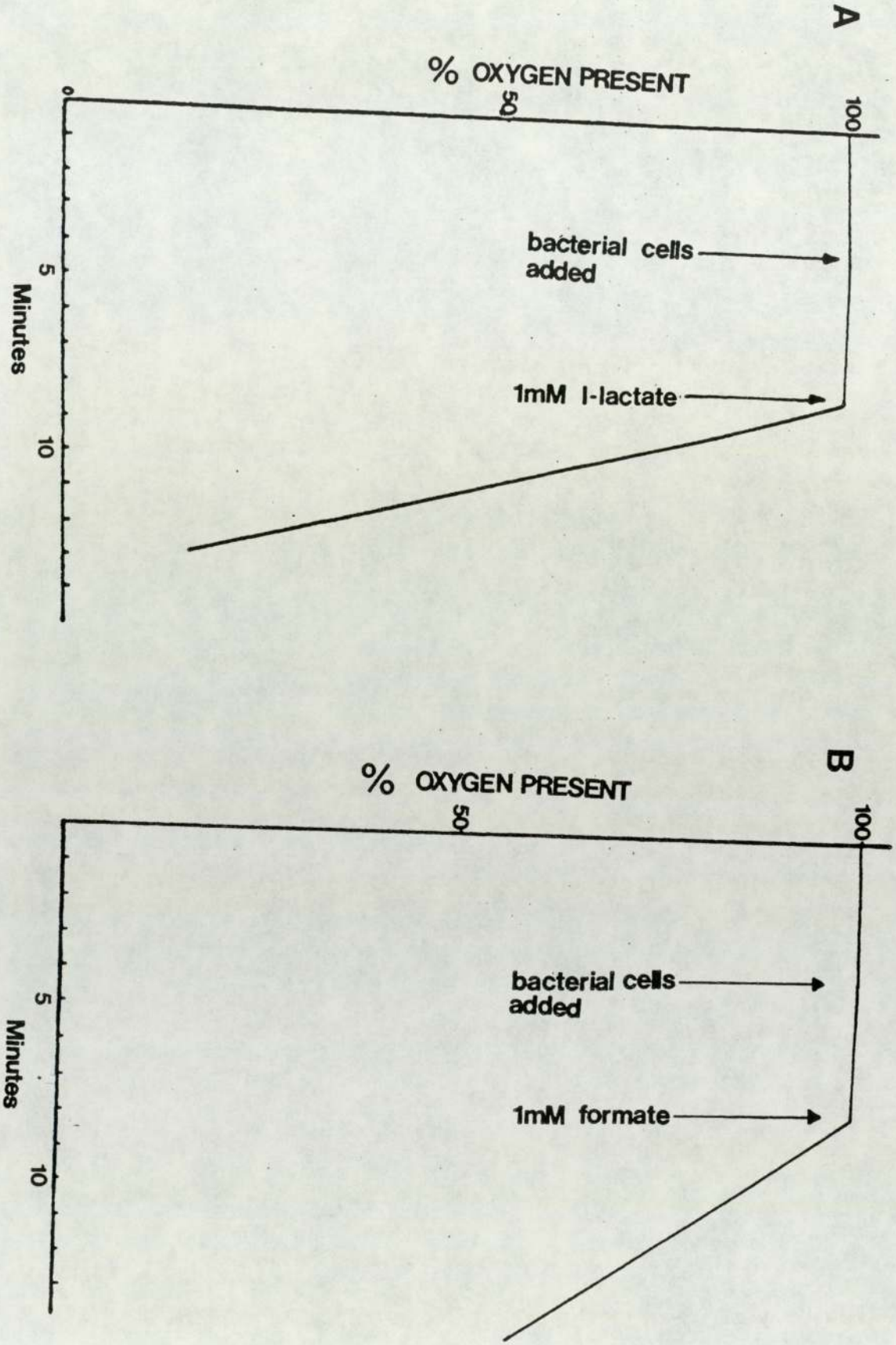
(a)


A.
% OXYGEN PRESENT
0
50
100
bacterial cells added
1mM l-lactate
5
10
MINUTES
B.
% OXYGEN PRESENT
0
50
100
bacterial cells added
1mM formate
5
10
MINUTES

(b)


A.
% OXYGEN PRESENT
0
50
100
bacterial cells added
1mM l-lactate
1mM NAD
5
10
MINUTES
B.
% OXYGEN PRESENT
0
50
100
bacterial cells added
1mM formate
1mM NAD
5
10
MINUTES

(c)


A
% OXYGEN PRESENT
0
50
100
5
10
Minutes
bacterial cells added
1mM l-lactate
B
% OXYGEN PRESENT
50
100
5
10
Minutes
bacterial cells added
1mM formate

Respiration rates of these and various other potential energy sources by whole cells and by membrane fractions are shown in Tables 3.4.3 and 3.4.4.

Haemophilus influenzae cultured using PPIX as a porphyrin source show reduced respiratory rates compared with bacteria grown in haem excess. Haem-limited stationary phase cells respire glucose, pyruvate, l-lactate d-lactate and NADH although at rates at least ten fold lower than respiratory rates exhibited by cells grown under conditions of haem-sufficiency. However, log phase haem-limited *H.influenzae* respire glucose, pyruvate, l-lactate and d-lactate at rates comparable to those of log phase haem-sufficient cells, indicating that the apparent cytochromeless state is not accompanied by a complete absence of respiratory metabolism. Cell membrane fractions (Table 3.4.4) derived from log phase haem-limited cells respire NADH at rates ten times lower than those of haem-sufficient bacteria, suggesting that functional electron transport chains are present in these cells but in much reduced quantities compared with haem excess or PPIX grown cells in spite of the fact that cytochromes are undetectable by spectrophotometry.

Additionally there appears to be a selective loss in the capacity to oxidise formate and succinate in haem-limited *H.influenzae* compared with those grown under the other culture conditions investigated (Table 3.4.3). Membrane fractions from *H.influenzae* grown under conditions of haem-limitation also fail to display any activity of membrane

TABLE 3.4.3 The Effect of Varying Haem and Iron Availability on Respiration of Various Substrates by Whole Cell Preparations of Non Typable *H.influenzae*

CULTURE CONDITIONS	GROWTH PHASE	GLUCOSE	PYRUVATE	FORMATE	L-LACTATE	D-LACTATE	SUCCINATE
Haem Excess	Stationary	38.49	49.02	96.62	111.80	30.16	14.25
	Log	18.02	20.00	40.00	40.00	5.01	1.32
Haem Limited	Stationary	2.80	3.70	0.00	3.70	3.87	0.00
	Log	16.16	7.60	0.00	30.30	10.10	0.00
PPIX Excess	Stationary	6.29	13.90	4.89	40.58	9.09	6.04
PPIX Limited	Stationary	5.54	8.77	7.39	27.70	8.31	1.73
Iron Restricted	Stationary	13.19	15.70	3.01	60.00	14.20	
NAD Limtied	Stationary	10.69	7.56	59.23	52.20	11.76	

All figures represent ng atoms O consumed/minute/100mg protein.

TABLE 3.4.4 The Effect of Varying Haem Availability on Respiration of Various Substrates by Cell Membrane Fractions (P180) of Non Typable *H.influenzae*.

CULTURE CONDITIONS	GROWTH PHASE	NADH	SUCCINATE	NADPH (+ NAD)	FORMATE
Haem Excess	Stationary	89.89	4.49	2.24 (17.97)	13.97
	Log	72.00	1.35	2.17 (10.10)	8.32
Haem Limited	Stationary	0.61	ND	0.43 (0.43)	ND
	Log	8.61	ND	0.80 (0.80)	ND

All figures represent ng atoms O consumed / minute / 100mg protein.

ND = not detectable.

bound pyridine nucleotide transhydrogenase enzyme as indicated by stimulation of NADPH oxidation in the presence of NAD (Table 3.4.4). This enzyme is produced when bacteria are grown under conditions of haem excess.

Non typable *H.influenzae* cultured in the presence of PPIX are similar in terms of respiratory activity, regardless of whether the porphyrin source is present in excess or growth yield limiting concentrations. These exhibit oxidation rates of glucose, pyruvate, d-lactate, succinate and NADH which are three to four times greater than those of haem-limited cells but which are greatly reduced compared with cells grown under conditions of haem excess.

Non typable *H.influenzae* cultures whose growth was limited by exogenous NAD concentration also displayed reduced ability to oxidise certain substrates compared with haem excess-NAD excess cultures (Table 3.4.3). This probably indicates rate limiting concentrations of intracellular NAD, as has been suggested previously (Lwoff and Lwoff, 1937).

Haemophilus influenzae grown in the presence of PPIX have much reduced capacity to oxidise formate compared with bacteria grown in haem excess, while haem-limited cells lack this capacity altogether. Uninfected rat lungs were also tested for the ability to oxidise formate and were found to lack this capacity. These differences in formate oxidising capacity between haem-sufficient and haem-limited *H.influenzae* could provide a means of indirectly determining the amounts of haem available to non typable strains growing

in vivo within the pulmonary environment. If formate oxidising activity could be demonstrated in infected rat lungs it would indirectly indicate that *H.influenzae* growing in the lungs are subject to environmental conditions more akin to those of bacteria growing *in vitro* under conditions of haem excess than haem limitation.

DISCUSSION.

Culture of *H.influenzae* in graded amounts of haem can result in corresponding decreases in final biomass (White, 1963). Additionally, growth in progressively decreasing concentrations of PPIX produces a similar effect. However, reduction in exogenous haem from excess to growth yield limiting amounts is accompanied by a significant decrease in final culture pH which is not mimicked in *H.influenzae* cultured in growth yield limiting concentrations of PPIX. these observations suggest major differences in metabolism between *H.influenzae* cultured using haem and those using PPIX as a porphyrin source.

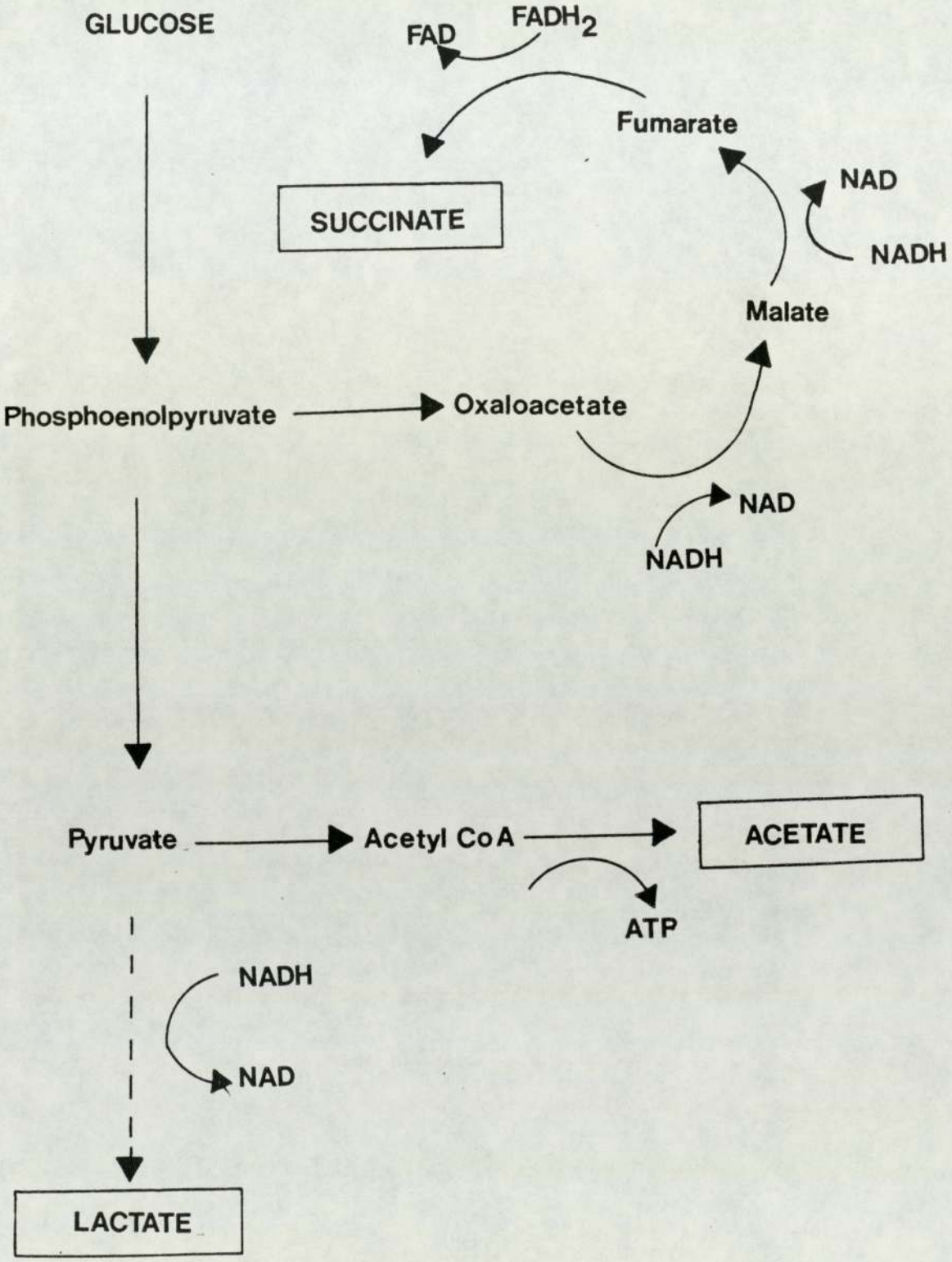
Haem-limited *H.influenzae* also exhibit decreased efficiency of glucose utilisation and in this respect resemble anaerobically grown cells (Hollander, 1976). The acid end products of glucose metabolism in haem-limited cells differ from those produced by bacteria grown under any of the other culture conditions investigated as part of the present study in that they uniquely produce lactic acid. Production of this acid has not previously been reported in this species.

Haemophilus influenzae cultured under conditions of haem excess, NAD limitation or in the presence of PPIX produce succinic and acetic acid, these have been previously reported to be the principal acids produced from glucose by members of this species (Tuyau *et al*, 1984). Reduction in the concentration of available haem therefore results in alterations in the pathway of glucose metabolism employed by *H. influenzae*.

Following analysis of the enzymes of the TCA cycle produced by haemophili, Tuyau *et al* (1984) have demonstrated that only PEP carboxylase, malate dehydrogenase, fumarase and succinic dehydrogenase are present. Consequently these authors suggest that haemophili posess only a partial TCA cycle and propose that succinate and acetate are produced as shown in Figure 3.4.12. These authors used only one phenotype of *H. influenzae* (Haem excess) and therefore did not detect formation of lactic acid.

Haem-limited *H. influenzae* appear to acquire a more fermentative type of metabolism in which lactic acid is the principal end product of glucose catabolism. Presumably lactate is produced from pyruvate, consuming NADH not oxidised by an electron transport chain (broken line Fig. 3.4.12). Similarly a mutant of *E. coli* K12 has been described which is unable to synthesize haem and which is restricted to a fermentative type of metabolism the principal end product of which is also lactic acid (Schellhorn and Hassan, 1988). Haem deficient mutants of

FIGURE 3.4.12


GLUCOSE
FAD
FADH2
Fumarate
SUCCINATE
NAD
NADH
Malate
Phosphoenolpyruvate
Oxaloacetate
NAD
NADH
Pyruvate
Acetyl CoA
ACETATE
ATP
NADH
NAD
LACTATE

this species are also deficient in catalase and cytochromes and in this respect also resemble haem-limited *H. influenzae* (Biberstein and Gills, 1961; White, 1963).

Haemophilus influenzae cultured in graded amounts of haem have been shown to produce successively decreasing concentrations of cytochromes, White (1963) described b, c, and d-type cytochromes produced by *H. influenzae* but did not attempt to localize these and so did not detect a soluble c-type cytochrome in S180 fractions. In the present study, PPIX grown *H. influenzae* appeared to produce intermediate amounts of cytochrome compared with cells grown under conditions of haem excess and those cultured under conditions of haem limitation. Stationary phase cultures of NAD-limited cells were similar in cytochrome complement to haem-sufficient *H. influenzae*, demonstrating that a simple decrease in biomass, and probable resultant change in culture oxygen tension, does not result in cytochrome loss and that effects observed in other culture conditions are due to haemin and PPIX limitation.

In general, graded differences in cytochrome production are correlated to differences in substrate oxidation rates by *H. influenzae* grown under various conditions of haem, PPIX and iron availability. However, exponential phase bacteria from haem-limited cultures show oxidation rates for certain substrates which are quantitatively similar to those exhibited by exponential phase, haem-sufficient cells despite an apparent lack of cytochromes in the former.

Unless flavoprotein alone can account for these changes in rates of substrate oxidation by haem-limited *H.influenzae*, this observation suggests that undetectable amounts of cytochrome are produced and that these can sustain oxidation rates similar to those observed in bacteria grown under conditions where a full complement of cytochromes are produced.

These results suggest that if *H.influenzae* growing *in vivo* are subject to porphyrin ring source limitation they may posess a pathway of glucose metabolism which is different from that normally observed in *H.influenzae* cultured *in vitro* under conditions where haem is freely available.

4. CONCLUDING REMARKS.

In terms of alterations in OMP profile non typable *H.influenzae* appear heterogeneous in their response to PPIX substitution and iron-restriction. Some strains exhibit PPIX inducible OMPs while in others PPIX or iron uptake systems appear to be constitutively expressed. Outer membrane proteins induced *in vitro* by PPIX utilisation in certain strains appear to be expressed *in vivo* when these strains are growing in the respiratory tract. However it is unlikely that PPIX itself is available to bacteria growing on mucosal surfaces as, as an intermediate in haem synthesis it is confined to an intracellular location within mitochondria (Israels *et al*, 1975).

Neither haem nor iron are freely available to bacteria growing *in vivo*, each being tightly bound to host haem and iron binding proteins. Haem binding proteins such as hemopexin (Muller-Eberhard, 1970) and haptoglobin (Eaton *et al*, 1982) remove any free haem from the circulation and host iron binding proteins, transferrin and lactoferrin, bind any free iron in serum and on mucosal surfaces (Bezkorovainy, 1987; Masson and Heremans, 1966). *Haemophilus influenzae* can aquire iron from transferrin but not generally from lactoferrin (Herrington and Sparling, 1985; Schryvers, 1988) and can sequester haem from haemoglobin, haptoglobin and hemopexin (Stull, 1987). Availability of these serum proteins to bacteria on mucosal surfaces is

unknown and consequently the sources of haem and iron available to non typable *H.influenzae* within the pulmonary environment remain undefined.

Haemophilus influenzae cultured *in vitro* under conditions of haem-limitation in the presence of an iron chelator show outer membrane protein changes which resemble those produced on growth utilising PPIX as a porphyrin source, but to a lesser extent. It is possible therefore that growth utilising PPIX may be a strong inducer of proteins involved in iron uptake.

Haemophilus influenzae can grow anaerobically in the absence of haem (White, 1963). This would suggest that acquisition of haem *in vivo* is not essential for survival. However, the absence of high molecular weight OMPs in anaerobically grown cells and the apparent presence of high molecular weight OMP antigens in *H.influenzae* growing *in vivo* in bronchiectasis patients and in chronically infected rats would suggest that *H.influenzae* growing in the respiratory tract acquire enough haem to grow using a respiratory metabolism to generate usable energy. If haem and iron are available only in limiting concentrations, then non typable *H.influenzae* occupying this particular niche may utilise different metabolic pathways and express different surface antigens compared with bacteria growing *in vitro* under conditions of haem sufficiency. The presence of antibodies to phenotypically altered OMPs in serum from patients with

bronchiectasis and from experimentally infected rats suggests that this may indeed be the case.

Individuals with bronchiectasis and experimentally infected rats exhibited a strong antibody response to most OMPs of their infecting *H.influenzae* strain, including PPIX specific OMPs. As deficiencies in specific antibody subclasses are not considered to be a widespread problem in bronchiectasis (Cole, 1984; Veale *et al*, 1988) it would seem that persistance of *H.influenzae* infection in bronchiectasis is not due to lack of specific OM-directed antibodies but may in fact be due to inefficiency of antibodies produced or the production of blocking antibodies as described by Musher (1984). The fact that individuals with more severe disease and persistently purulent sputum have higher titres of anti-*H.influenzae* antibodies than those whose symptoms are less severe would tend to confirm the association of non typable *H.influenzae* with infection in bronchiectasis and also suggest that pulmonary infection in bronchiectasis is not curtailed but rather exacerbated by an overstimulated immune response.

Future Considerations.

Possibilities for the future progression of this research include the determination of IgA levels in lavage fluid from infected rat lungs and the identification of any *H.influenzae* antigens against which locally produced IgA may be directed. If blocking IgA antibodies are induced by

H. influenzae in bronchiectasis, it would be important to identify these and define the antigens for which they are specific. In this respect it would also be of interest to determine the respective abilities of mucoid and purulent sputum to inhibit the bactericidal activity of their own or normal human serum for their homologous *H. influenzae* isolates.

The present study identified non typable *H. influenzae* OMP antigens against which serum IgG is directed using immunoblotting techniques, and noted no qualitative differences between serum from patients with purulent and those producing mucoid sputum. The specific OMP antigens against which serum IgA and IgM are directed against remain to be identified and compared between the two test groups.

A longer term study of *H. influenzae* infection in bronchiectasis could be utilized to monitor any changes in serum and local antibody responses as changes occurr in the quantity and nature of *H. influenzae* cultured from sputum.

In view of the high anti-*H. influenzae* titres observed in bronchiectasis patients producing purulent sputum, it is possible that immune complex formation is a problem in these individuals. Future experimentation may therefore include determination of local and serum immune complex formation in individuals with severe bronchiectasis.

Purification of individual OMP antigens would allow the immune response of patients to individual OMPs to be assayed. Additionally, the vaccinogenic potential of

individual proteins could be assayed using the rat lung model of *H.influenzae* infection. While vaccines against non typable strains are unlikely to be of value in bronchiectasis, they remain a possibility for the prevention of other infections caused by these bacteria (Clancy *et al*, 1983; Hansen *et al*, 1988).

Determination of the haem content of purulent and mucoid sputum may yield further information about the availability of haem to *H.influenzae* growing on mucosal surfaces in infected and non-infected lung tissue. Furthermore, non typable *H.influenzae* isolated from patients with bronchiectasis of varying severity could be investigated for their ability to sequester iron from transferrin and lactoferrin. The influence of haem or PPIX limitation and iron-restriction on these parameters could also be investigated.

Production of siderophores by *H. influenzae* type b has been reported previously (Pidcock *et al*, 1988). The effect of growth under conditions of varying haem, PPIX, and iron availability on siderophore production by non typable *H.influenzae*, including strains which do not appear to respond to PPIX and iron-restriction in terms of alterations in OMP profile, is a further area into which the present study could be extended.

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