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MODULATION OF *BORDETELLA PERTUSSIS* BY NICOTINIC ACID

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Presented for the degree of Doctor of Philosophy

in the Faculty of Science, University of Glasgow

Department of Microbiology

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A man is judged by what he looks for .....

not by what he finds.

Le Carré.

SUMMARY

The characteristics of nicotinic acid (NA) and nicotinamide (ND) uptake by Bordetella pertussis were determined. This investigation was prompted by the previous work of Pusztai and Joó (Ann. Immunologiae Hungaricae, 10, 63-67, 1967) on NA modulation which suggested that certain cell envelope proteins having pathophysiological activities in experimental animals might also be involved in the uptake of NA, but not of ND.

On investigation, the rates of uptake of the two vitamins by B. pertussis were found to be almost identical and were linear for at least 30 min; uptake of both required a source of energy, satisfied by glutamate, and potassium ions; and was temperature-dependent. The uptake systems for both vitamins displayed saturation kinetics and Km and Vmax values for their uptake were estimated. The Km values were 1.0 and 0.04 μM for NA and ND respectively, whilst the Vmax values were 13.5 and 18.4 pmol mg dry weight^{-1}min^{-1} respectively.

The overall uptake process was defined as including both transport and metabolism of the vitamins. ND taken up by B. pertussis was found to be rapidly converted to NA by the activity of nicotinamide deamidase. Subsequent metabolism of NA was difficult to determine, but it appeared that it was metabolised first to NAD and then recycled to NA via ND.

NA and ND uptakes had the same pattern of inhibition by a variety of substituted pyridine compounds, although ND uptake was always inhibited slightly more than NA. Both vitamins were inhibited by 3-pyridine-carboxaldehyde (3PC), 3-pyridine-aldoxime (3PA) and 3-pyridyl-carbinol. No other analogues were inhibitory. However, whereas 3PC completely inhibited ND uptake at molar ratios of analogue

to vitamin of 100, 10 and 1, its inhibitory effect on NA uptake decreased as the molar ratio decreased; 3PC and 3PA were shown not to inhibit the oxidation of glutamate or the growth of B. pertussis. NA was found to inhibit ND uptake and did so to a greater extent than ND inhibited NA uptake when compared on an equimolar basis.

From these results, three possible models were suggested for the uptake of NA and ND by B. pertussis. The evidence available was in favour of a model (Model 3, Discussion, Section I) with separate periplasmic binding-proteins for NA and ND which interact with a common cytoplasmic membrane carrier protein for translocation into the cell. Transport of ND is then followed by its rapid metabolism to NA.

To investigate the possible regulatory role of adenylate cyclase (AC) in NA modulation, assays of AC were done on intact cells and cell lysates of cells grown under different conditions. Intact cell-associated AC was found in all three species of Bordetella. The AC activity in B. pertussis was confirmed to be greater in cells grown on solid media containing blood than in liquid media, due to activation of the AC by a protein activator in the blood. The cellular distribution of AC in B. pertussis was examined by fractionation of intact cells. The percentage distribution varied depending on whether or not the AC activity was assayed in the presence of this activator. This was because soluble AC (50000 g supernate) responded less to the activator than membrane-containing cell fractions. However, the majority of the AC was in that soluble fraction, with a small amount in the cell envelope and the outer membrane. Association of some of the AC with the outer membrane was confirmed by sucrose density gradient centrifugation analysis.

Wardlaw, Parton and Hooker (J.Med.Micro., 9, 89-100, 1976) had shown that whereas growth in high NA media (NA500) resulted in modulation, growth in ND500 media did not. In the present study, growth in NA500 media in batch cultures resulted in a marked reduction in the uptakes of both NA and ND and AC levels and a loss of agglutinogen factors 2 and 3. The titre of factor 1 was increased in these growth conditions. There also appeared to be a slight alteration in the cellular distribution of AC.

By contrast, growth in ND500 media had no effect on any of these properties, except for a possible slight reduction in ND uptake only. B. pertussis 18323 was atypical in its response to growth in ND500 media in that AC activity was stimulated 15-fold.

B. pertussis 2992 did not modulate when grown in NA500 media in semi-continuous culture, although it did modulate in batch culture. This strain modulated in semi-continuous culture, however, when grown at 28°C.

These results suggest that NA and ND interact with separate components for their transport and that high concentrations of NA also affect another factor in the cell envelope that regulates the expression of both virulence genes and AC.

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DECLARATION

This thesis is the original work of the author. Part of the results in Section I have been published in FEMS Microbiology Letters.

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* STRIKE OUT EITHER a. or b. BOTH IN SECTION 1 and SECTION 2.

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LIST OF ABBREVIATIONS

AC	Adenylate cyclase
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
ATP	Adenosine-5'-triphosphate
BG	Bordet-Gengou medium
BG-NA500	BG containing 500 $\mu g\ ml^{-1}$ nicotinic acid
CA	Charcoal agar
CA-NA500	CA containing 500 $\mu g\ ml^{-1}$ nicotinic acid
cAMP	Cyclic adenosine-3',5'-monophosphate
CD	Cell debris
Ci	Curie, 2.22×10^{12} disintegrations per minute
CW	Cell wall
CWBA	Cohen and Wheeler's horse blood agar
CWBA-NA500	CWBA containing 500 $\mu g\ ml^{-1}$ nicotinic acid
CWBA-ND500	CWBA containing 500 $\mu g\ ml^{-1}$ nicotinamide
Cys	Cysteine
EAE	Experimental autoallergic encephalomyelitis
F-HA	Fimbrial haemagglutinin
F-SN	Final supernate
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
HA	Haemagglutinin
His	Histidine

HLT	Heat-labile toxin
HSF	Histamine-sensitising factor
IAP	Islet-activating protein
Ile	Isoleucine
JCVI	Joint Committee on Vaccination and Immunisation
k	Thousand
Km	Michaelis-Menten constant
Leu	Leucine
LPF	Lymphocytosis-promoting factor
LPF-HA	LPF haemagglutinin
LPS	Lipopolysaccharide
Met	Methionine
MRC	Medical Research Council
NA	Nicotinic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NAMN	Nicotinic acid mononucleotide
NAPRTase	Nicotinate phosphoribosyltransferase
ND	Nicotinamide
NDMN	Nicotinamide mononucleotide
OM	Outer membrane
OMV	Outer membrane vesicles
ou	Opacity unit
PA	Protective antigen
3PA	3-pyridine-aldoxime

PBG	PBS containing 1% (w/v) glutamate
PBS	Phosphate-buffered saline
3PC	3-pyridine-carboxaldehyde
PEP:PTS	Phosphoenol pyruvate:phosphotransferase system
PHLS	Public Health Laboratory Service
PMF	Proton motive force
PNC	Pyridine nucleotide cycle
Pro	Proline
SCC	Semi-continuous culture
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine
SS	Stainer and Scholte's liquid medium
SS-NA5	SS containing 5 $\mu g\ ml^{-1}$ NA
SS-NA500	SS containing 500 $\mu g\ ml^{-1}$ NA
SS-ND500	SS containing 500 $\mu g\ ml^{-1}$ ND
Thr	Threonine
Tris	Tris-[hydroxymethyl]-aminomethane
Tyr	Tyrosine
Val	Valine
Vmax	Maximum velocity constant of Michaelis and Menten
WHO	World Health Organisation

INTRODUCTION

SECTION I : BORDETELLA PERTUSSIS

1. Taxonomy of the Genus Bordetella

The genus Bordetella is classified under "Gram-negative Aerobic Rods and Cocci : Genera of Uncertain Affiliation" in the latest (8th) edition of Bergey's Manual of Determinative Bacteriology (Pittman, 1974). The genus consists of three species: the non-motile B. pertussis and B. parapertussis and the motile B. bronchiseptica. The organisms have an optimum temperature of 35-37°C for growth and do not attack carbohydrates. They are chemoorganotrophs which oxidise amino acids and require for growth an organic sulphur source (e.g. Cys) and either nicotinic acid (NA) or nicotinamide (ND) (Pittman, 1974).

The three species are obligate mammalian parasites and pathogens of the respiratory tract and B. pertussis is the causative organism of whooping-cough. Genus-specific antigens include a heat-stable 'O' antigen, a heat-labile dermonecrotic toxin and the heat-labile agglutinogen No. 7. In addition, each species has its own species-specific agglutinogen. All species are haemolytic (Pittman, 1974; Wilson and Miles, 1975). Their differential characteristics are shown in Table 1.

B. pertussis, which was isolated as the causative organism of whooping-cough by Bordet and Gengou (1906), was originally included in the genus Haemophilus (Winslow et al, 1920) by its microscopical appearance and its apparent requirement for blood. Unlike H. influenza, however, there was no requirement for either the X- or V-factor (Fildes, 1923).

B. parapertussis was first isolated by Eldering and Kendrick

TABLE 1. Differential characteristics for species of the genus *Bordetella*

CHARACTERISTICS[1]	*B.pertussis*	*B.parapertussis*	*B.bronchiseptica*
Flagella	-	-	+
Reduction of nitrates	-	-	+
Citrate utilisation	-	+	+
Urease	-	+	+ (in 4 h)
Oxidase	+	-	+
Catalase	+ or - [2]	+	+
Growth on peptone agar:			
- growth	-	+	+
- browning	-	+	-
Sensitisation of mice to histamine	+	-	-
Species-specific heat-labile agglutinogens:			
- Factor 1	+	-	-
- Factor 12	-	-	+
- Factor 14	-	+	-
G + C Content, moles %	61	61	66

(1) Adapted from Lautrop (1960) and Pittman (1974)

(2) 70% of freshly isolated strains are positive.

(1937, 1938) from cases of whooping-cough, but from its cultural characteristics was distinct from B. pertussis. It also was originally placed in the genus Haemophilus. B. bronchiseptica was first isolated from the respiratory tract of dogs suffering from distemper (Ferry, 1911) and was first isolated from a case of whooping-cough by Brown in 1926 (Medical Research Council (MRC), 1951). This organism has been previously classified under Haemophilus and Brucella (Pittman, 1974).

López (1952) summarised the evidence for the properties shared by these three organisms and proposed they be grouped together in a new genus: Bordetella. Further metabolic and nutritional similarities among the three species were established by Proom (1955). Pittman (1955) demonstrated they shared a common heat-labile dermonecrotic toxin and other serological and pathogenic properties. As a result of these and other studies, the proposal of López was accepted and the new genus was first included in the 7th edition of Bergey's Manual (Pittman, 1957).

There have been some recent studies which have questioned the separate species status of these three organisms (Kumazawa and Yoshikawa, 1978; Kloos et al, 1979), particularly the separation of B. pertussis and B. parapertussis. Both groups of workers have converted B. pertussis into B. parapertussis by treatment with N-methyl-N'-nitro-nitrosoguanidine.

2. Pertussis - the Disease

Whooping-cough is a highly communicable, acute, localised infection of the human respiratory tract which, in its typical form, is characterised by paroxysmal coughing, lymphocytosis and hypoglycaemia and is sometimes accompanied by neurological disorders (Pittman, 1970).

Whooping-cough is a clinical syndrome which can be caused by any one of the three species of Bordetella and there is no cross-immunity among the species (Lorber, 1975; Olson, 1975). The majority of whooping-cough infections are due to B. pertussis. B. parapertussis accounts for about 5% of the cases and human infections with B. bronchiseptica are very rare. Lautrop (1960) proposed that the term PERTUSSIS be used solely for infections with B. pertussis, PARAPERTUSSIS for infections with B. parapertussis and WHOOPING-COUGH for the clinical syndrome caused by any one of three species.

Pertussis infections show three clinical stages following an incubation period of 7-14 days: catarrhal, paroxysmal and convalescent (Lorber, 1975). The catarrhal stage usually lasts 7-14 days and is accompanied by the symptoms common to many respiratory tract infections: cough, sneezing, general malaise and a mild fever. The victim is highly infectious during this stage, the disease being spread via respiratory droplets. Attack rates of 80-90% of unvaccinated children exposed at home to pertussis infection have been reported (MRC, 1951; Joint Committee on Vaccination and Immunisation (JCVI), 1977). The bouts of coughing become much more violent and frequent during the paroxysmal stage and are often accompanied by the characteristic "whoop". The patient is no longer infectious during this stage, which can last for up to four weeks, but neurological disorders and secondary infections may occur. The paroxysms begin to decrease during the convalescent stage, although full recovery from the infection can take up to several months (Lorber, 1975; Linneman, 1979).

Adenoviruses have also been reported to cause a paroxysmal cough and pertussis-like disease (Olson, 1975; Linneman, 1979). However, it is not known if they also cause the lymphocytosis that is

characteristic of B. pertussis infections (Linneman, 1979).

Pertussis remains widely distributed throughout the world, especially in highly populated and underdeveloped countries (Table 2). The infection can occur in any age group, but is most common, most severe and has the highest mortality rate in infants up to one year old (Bradford, 1969; Lorber, 1975). Epidemics of pertussis occur in cycles with peaks every 3-4 years (JCVI, 1977).

Little is known of the pathology of pertussis infection other than that the bacteria remain localised among the cilia of the epithelial cells of the respiratory tract and multiply there without invasion of the epithelium (Mallory and Horner, 1912; Muse et al, 1977). Necrosis of the epithelium layer occurs to a limited extent and there is a lymphocyte infiltration of the epithelial tissue (Pittman, 1970; Linneman, 1979).

The nature of the immunity to pertussis infection remains unknown. It has been proposed that a short-term, local immunity is provided by secretory antibodies preventing adhesion of the bacteria to the cilia (Pittman, 1979). Further, prolonged immunity may then be due to serum antibodies neutralising an exotoxin released by the bacteria attached to the cilia (Pittman, 1979). However, immunity to pertussis either from infection or from active immunisation lasts only for a few years (Pittman, 1970) and there appears to be no passive transfer of immunity from mother to foetus (Lorber, 1975).

3. Pertussis Vaccine and Vaccination

Many attempts have been made to immunise actively against pertussis since the causative organism was first isolated. Madsen

TABLE 2. Incidence of pertussis in selected countries

COUNTRY	NO. OF REPORTED CASES 1976(1)	1977(2)
ALGERIA	2862	666
ARGENTINA	44195	44875
CANADA	3009	1903
DENMARK	16385	11452
FIJI	28	15
FRANCE	353	189
ITALY	19246	8478
JAPAN	2471	5417
NEW HEBRIDES	30	10
THAILAND	1980	3010
UNITED KINGDOM	4369	18717
UNITED STATES OF AMERICA	927	1915
VENEZUELA	9204	10267
ZAIRE	18975	17114

(1) World Health Statistics Report (1977)

(2) World Health Statistics Quarterly (1978)

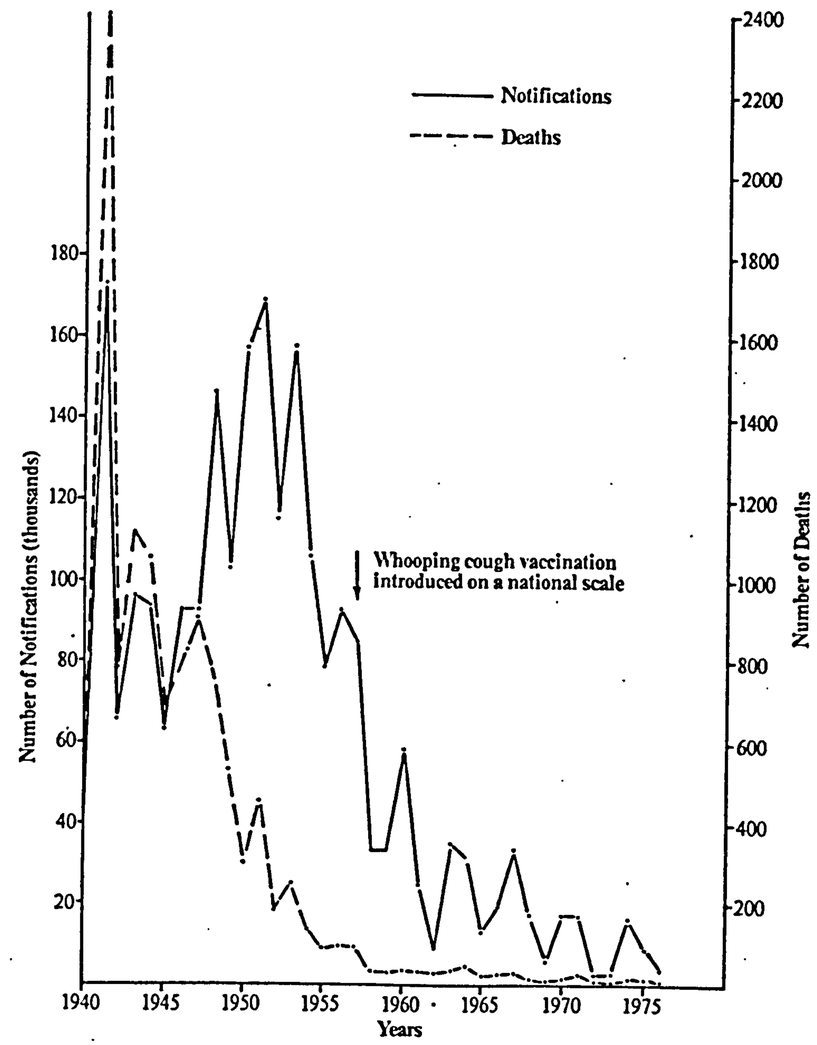
(1925, 1933) reported a limited amount of success in the first large scale trials of pertussis vaccine in that only 68% of vaccinated children became infected compared with 98% of unvaccinated children. Kendrick and Eldering (1939) reported the first well-controlled trials in the U.S.A. with an attack rate of 35% in vaccinated and 89% in unvaccinated children.

The Medical Research Council (MRC), in the United Kingdom, conducted the best organised series of pertussis vaccine field trials in the 1950's. A large number of vaccines were compared and found to have a wide range of protective potency, with an overall attack rate following exposure to pertussis at home of 18% in vaccinated and 87% in unvaccinated children (MRC, 1951, 1956, 1959). These trials established a correlation between protective potency of the vaccines and their performance in the intracerebral mouse protection test (Kendrick et al, 1947). The World Health Organisation (WHO) subsequently recommended this test as the standard assay of vaccine protective potency (WHO, 1964). Pertussis vaccine, combined with diphtheria, and later also with tetanus, toxoids was was introduced in the United Kingdom on a national scale in 1957.

The introduction of the vaccine appeared to be responsible for an accelerated rate of decline in the incidence of pertussis which continued, interrupted only in the 1960's, until 1976 (Figure 1). The efficiency of the pertussis vaccine was questioned when the Public Health Laboratory Service (PHLS) reported that in the pertussis epidemic of 1966-1967, 56% of fully vaccinated and 67% of unvaccinated children became infected (PHLS, 1969). Preston (1963, 1965) had already shown that the serotype of fresh isolates of B. pertussis had changed from types 1, 2 and 1,2,3 prevalent prior to 1958 to type 1,3, prevalent

Figure 1. Whooping-cough notifications and deaths in England and Wales from 1940-1976.

The data for 1940-1976 were taken from the report of the Joint Committee on Vaccination and Immunisation (1977).



from 1963 onwards. These findings were confirmed by the PHLS (1973) and the suggestion was made that the change in serotype may have occurred because the vaccines in use then did not contain antigen no. 3. The vaccines were therefore altered to contain high levels of antigens 1, 2 and 3. The efficacy of the new vaccines was analysed by Preston and Stanbridge (1972) for the period 1969-1971 and they were found to be superior to the earlier vaccines.

Adverse reactions to pertussis vaccine have been known since its earliest usage (Madsen, 1925). Many of these are local reactions at the site of injection, but a number, whose size is a subject of some debate, are more serious neurological complications such as convulsions or encephalitis (JCVI, 1977). Adverse publicity of these reactions reached a peak in the mid-1970's. Claims were made that changing socio-economic conditions were more important than vaccination as the cause of the decline in the incidence of pertussis and that the risk of serious neurological complications was too great to justify the national use of the vaccine (Bassili and Stewart, 1976; Stewart, 1977). As a result of this publicity, the acceptance rate for pertussis vaccine dropped from 75-80% for 1972-1974 to about 40% for 1977 (JCVI, 1977) and a committee was set up to review the efficacy of pertussis vaccines in the United Kingdom (JCVI, 1977). The committee reported that the incidence of neurological complications could not be measured with certainty as such disorders may arise spontaneously in unvaccinated children of a similar age. They also noted that the mortality rate from the disease itself had remained unaltered since 1958 at about 1 death per 1000 notifications. However, pertussis remains a very debilitating disease. Miller and Fletcher (1976) reported that of 8092 notified cases in 1974-1975, 10% required hospitalisation the majority of which were

children 0-1 year old. Pertussis vaccination has repeatedly been reported as reducing the severity and length of the disease in vaccinated children (Madsen, 1933; MRC, 1951; Miller and Fletcher, 1976). In view of this modifying effect and their belief that the vaccine was protective , JCVI recommended the continuation of pertussis vaccination on a national scale (JCVI, 1977).

The next pertussis epidemic in the UK occurred, as expected, in 1978 and thus followed the period with very low levels of acceptance of pertussis vaccine. In contrast to previous trends (Figure 1), the incidence of pertussis was the highest recorded since before 1960 (Stuart-Harris, 1979). However, the controversy over the efficacy of the vaccine continues (Stewart, 1979; Stuart-Harris, 1979).

4. Pathophysiological Activities and Antigens

The complex nature of the cell envelope of B. pertussis was shown by Parton and Wardlaw (1975) who resolved about thirty-five proteins, by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), of molecular weights ranging from 10,000 to well over 70,000. Infection with B. pertussis causes a variety of responses in both humans and laboratory animals. A partial list of these responses is given in Table 3. These responses have been extensively reviewed elsewhere (Morse, 1976; Munoz and Bergman, 1977) and only a brief account will be given.

4.1. Heat-Labile Dermonecrotic Toxin (HLT)

HLT is a protein responsible for acute death in experimental animals and dermonecrosis (Morse, 1976) after infection of B. pertussis

TABLE 3. Pathophysiologically-active components associated with *B. pertussis*[1]

1. Heat-labile dermonecrotic toxin (HLT)
2. Agglutinogens
3. Haemagglutinins (HA)
4. Histamine-sensitising factor (HSF)
5. Lymphocyte-promoting factor (LPF)
6. Islet-activating protein (IAP)
7. Polymorphonuclear leukocyte-inhibitory factor[2],[3]
8. Protective antigen (PA)
9. Adjuvanticity
10. Mitogenicity for lymphocytes
11. Endotoxin (or lipopolysaccharide)

(1) Details of all these properties, except 7, can be found in the reviews of Pittman (1970), Morse (1976) and Munoz and Bergman (1977).

(2) Utsumi *et al* (1978).

(3) Imagawa *et al* (1979).

cells. It is destroyed by heating at 56°C for 30 min and has been purified by Onoue et al (1963). The toxin was recently shown to be a cytoplasmic component (Cowell et al, 1979).

4.2. Haemagglutinins (HA)

Keogh et al (1947) first described the HA activity of B. pertussis. It has been shown recently that B. pertussis possesses two distinct HA's (Arai and Sato, 1976). There is a fimbrial HA (F-HA) which is identical with the filamentous fimbriae of B. pertussis and whose formation is inhibited in shake flask cultures (Arai and Munoz, 1979b). F-HA has been purified and crystallised (Arai and Munoz, 1979a). The second HA, which has a lower specific HA activity, is associated with the spherical, cell envelope component LPF (see 4.4). LPF-HA has been purified and analysed by Irons and MacLennan (1979a, 1979b).

4.3. Histamine-Sensitising Factor (HSF)

HSF is an envelope protein of B. pertussis which, on injection into mice, is responsible for the sensitisation of the mice to subsequent injection of histamine, serotonin or other stress stimuli (Morse, 1976). The effect was first reported by Parfentjev and Goodline (1948) and HSF has since been purified and analysed from intact cells (Lehrer et al, 1975; Lehrer, 1979) and from culture supernates (Morse and Morse, 1976). In common with other B. pertussis pathophysiologically-active components such as HA, LPF and IAP, HSF is found both in intact cells and culture supernates.

4.4. Lymphocyte-Promoting Factor (LPF)

LPF is a cell envelope protein of B. pertussis which causes lymphocytosis in both humans and mice due to a redistribution of T and B lymphocytes between blood and lymph (Morse, 1976, 1977b). Morse and Morse (1976) purified LPF from culture supernates of B. pertussis and showed that the component was a spherical molecule. The LPF has also been shown to be a potent mitogen for cultured murine T-lymphocytes (Morse, 1977a; Morse et al, 1979).

4.5. Islet-Activating Protein (IAP)

Sumi and Ui (1975) showed that previous injection of B. pertussis sensitised mice to produce a hyperinsulinaemia on subsequent injection of various insulin secretagogues. The component of B. pertussis responsible for this effect (IAP) has been isolated from culture supernates (Yajima et al, 1978a). The IAP was found to contain also both HSF and LPF activities (Yajima et al, 1978b; Ui et al, 1979).

4.6. Agglutinogens

The term "agglutinogen" refers to the bacterial substance(s) which react with their corresponding antibodies and cause agglutination of the bacteria (Munoz and Bergman, 1977). The "agglutinogen" was long thought to represent a single component until Andersen (1953) and Eldering et al (1957) described eight different agglutinogens, Factor 1 being common to all strains of B. pertussis. Whether the various agglutinogens are separate chemical entities or merely different determinants of a single molecule has not been resolved (Pittman, 1970; Munoz and Bergman, 1977). The relationship of the agglutinogens to protection in children is controversial.

5. Interrelationships of the Pathophysiological Activities and the Nature of the Protective Antigen

There is now good evidence that many of the pathophysiological activities of B. pertussis are the result of a common underlying mechanism of action affected by a single bacterial component (Morse, 1976). Levine and Pieroni (1966) first suggested such a Unitarian Hypothesis and concluded that the LPF, HSF, protective antigen (PA), reaginic antibody adjuvant and hyperacute experimental autoallergic encephalomyelitis (EAE) adjuvant activities were all caused by a single bacterial component of the cell envelope. Munoz (1976) isolated such a component and termed it PERTUSSIGEN. He, along with several other groups of workers, has shown that the above list of properties and also including IAP, epinephrine refractory hypoglycaemia and lymphocyte mitogenicity activities are all due to a single, cell envelope component of molecular weight 77,000 to 90,000 (Lehrer et al, 1975; Arai and Sato, 1976; Morse and Morse, 1976; Morse, 1977a; Munoz and Bergman, 1977; Yajima et al, 1978b; Irons and MacLennan, 1979a, 1979b; Lehrer, 1979; Munoz and Bergman, 1979). Pertussigen was not equivalent to the agglutinogens, HLT or endotoxin and did not contain HA activity (Munoz and Bergman, 1979). A lack of HA activity in an LPF preparation was also found by Morse and Morse (1976), but not by Arai and Sato (1976).

Wardlaw et al (1976), Wardlaw and Parton (1979) and Wardlaw et al (1979) noted that the presence or absence of PA, HSF, LPF, EAE adjuvant and reaginic antibody adjuvant activities closely correlated with the presence or absence of two main envelope proteins of molecular weight 28,000 (28k) and 30,000 (30k) seen in SDS-PAGE (Parton and Wardlaw, 1975). However, Irons and MacLennan (1979b) showed that

purified preparations of LPF-HA and F-HA were not equivalent to these two gel bands when run in SDS-PAGE.

Pittman (1979), having reviewed all the evidence on the pathophysiological activities of B. pertussis, concluded that the disease process depended on two factors in particular:

a) a factor responsible for the specific adhesion of B. pertussis cells to ciliated epithelium, and

b) the release of an exotoxin, which she termed PERTUSSIS TOXIN.

Her definition of pertussis toxin is virtually the same as for the pertussigen of Munoz, with the important exception that she does not include PA in the definition.

Contrary to the definition of pertussigen given above, two groups of workers have shown that highly purified preparations of LPF do not have PA activity in the mouse intracerebral test (Irons and MacLennan, 1979b; Sato et al, 1979). This may be due to the purified material being a poor immunogen or to PA being more unstable than LPF.

The term "protective antigen" is usually taken to mean the bacterial substance(s) responsible for protecting mice against intracerebral challenge with virulent B. pertussis, this protection having been shown to correlate with the protective potency of a vaccine in children (MRC, 1956, 1959). However, the exact nature of the PA remains unknown and it is quite possible that, as indicated above by Pittman (1979),there is more than one stage in the disease process at which protection could be effected.

The PA activity of purified pertussis toxin is controversial, as mentioned above. However, purified F-HA has been shown to contain PA activity (Sato et al, 1979; Irons and MacLennan, 1979b), although the level of PA is only a fraction of that produced by intact cells

(Irons and MacLennan, 1979b). Furthermore, cells grown in shaken cultures have little or no F-HA, yet still have high levels of PA.

On present evidence it seems that the pathophysiological activities and antigens of B. pertussis can be grouped into four cell envelope and one cytoplasmic component:

i) F-HA,
ii) Pertussis toxin,
iii) Agglutinogens,
iv) Endotoxin, and
v) HLT.

The combination of these components would be sufficient to cause the pathogenesis of B. pertussis and consequently, the immune response directed against them would afford protection against the disease.

6. Nutrition, Metabolism and Physiology

6.1. Nutrition

6.1.1. Culture Media

Bordet and Gengou isolated B. pertussis on a solid medium containing a glycerol-extract of potatoes and 50% (v/v) whole blood. Subsequent attempts to eliminate blood from the medium were unsuccessful (reviewed by Rowatt, 1957b) until Hornibrook (1939) described a liquid medium which contained a hydrolysate of casein, inorganic salts, Cys and starch. The addition of yeast dialysate to this medium stimulated growth. Hornibrook (1940) modified the medium to one containing a defined mixture of amino acids in place of the casein, inorganic salts, Cys, starch and NA (Table 4). Both the NA and Cys were essential for

TABLE 4. Composition of the liquid media of Hornibrook (1940)[1], Cohen and Wheeler (1946) and Stainer and Scholte (1971)

Component	mg l^{-1} Hornibrook (1940)	Cohen and Wheeler (1946)	Stainer and Scholte (1971)
Amino acids:			
Casamino acids	-	10,000[2]	-
Glu	900	-	10,720
Pro	200	-	240
Tyr	100	-	-
Gly	22.5	-	-
His	100	-	-
Cys [3]	5	25	40
Arg	100	-	-
Inorganic salts:			
NaCl	3,500	2,500	2,500
KH_2PO_4	125	500	500
KCl	100	-	200
$MgCl_2.6H_2O$	50	400	100
$CaCl_2$	100	10	20
$FeSO_4.7H_2O$	-	10	10
NH_4Cl	100	-	-
Na_2CO_3	25	-	-
$CuSO_4.5H_2O$	-	0.5	-
Other factors:			
Nicotinic acid[4]	0.5	-	4
Ascorbic acid	-	-	20

TABLE 4 (Contd)

Component	Hornibrook (1940)	Cohen and Wheeler (1946)	Stainer and Scholte (1971)
Glutathione	-	-	100
Tris base	-	-	1519
Yeast dialysate	-	50 ml l^{-1}	-
Starch	500	1,500	-

(1) The medium normally referred to as "Hornibrook's" is his 1939 formulation which has Casamino acids for the amino acid source and yeast dialysate in place of nicotinic acid.

(2) Casamino acids contain approximately 40 per cent NaCl, in addition to the added NaCl.

(3) Can be added as either cystine or cysteine.

(4) Can be replaced by nicotinamide.

growth, but Hornibrook noted that ND could replace NA and was equally efficient in supporting growth. However, this medium required a large inoculum and thus was not entirely satisfactory. Modifications such as those of Wilson (1945) and Farrell and Taylor (1945) were not much of an improvement (Rowatt, 1957b).

Cohen and Wheeler's (1946) modification of Hornibrook's medium contained Casamino acids, Cys, inorganic salts, yeast dialysate and starch (Table 4) and these workers were the first authors to determine the optimum salt concentrations experimentally. Although their medium also required a large inoculum, it yielded cells with high levels of PA and was recommended by WHO for the preparation of Pertussis vaccines (WHO, 1953).

The above media and others (Rowatt, 1957b) were formulated primarily for the production of effective vaccines. However, for physiological and metabolic studies, a chemically defined medium would be desirable. The first such medium was described by Wilson (1963) and was a modification of Cohen and Wheeler's medium with the addition of Tris buffer. Wilson (1963) noted that the salts NaCl, KCl, $MgCl_2$ and $CaCl_2$ were required to keep the cells in even suspension. However, the medium contains a large number of constituents (20 amino acids and 24 other factors) which belies the simple growth requirements of B. pertussis (see 6.1.2). Stainer and Scholte (1971) described the most recent and simplest chemically defined medium (Table 4). It consists of one or two amino acids, inorganic salts, Cys, NA, three other growth factors and Tris buffer. It gives high yields of cells with good levels of PA and has become the medium of choice for studies on B. pertussis. Stainer and Scholte's medium was solidified by Parker (1976) by the addition of agarose, and also modified by incor-

poration of an anionic exchange resin and sodium acetate. This medium supports the growth of small inocula and should be of great benefit to further physiological and metabolic studies on B. pertussis.

6.1.2. Growth Requirements

B. pertussis is a slow-growing organism with a mean generation time of about 2.5-2.8 h (Rowatt, 1957a) yet has very simple growth requirements. Thus, B. pertussis will grow in a liquid medium containing one or two amino acids, an organic source of sulphur, NA or ND and inorganic salts (Jebb and Tomlinson, 1955). The growth requirements were reviewed by Rowatt (1957b) and more recently by Parker (1976).

i) Amino acids:

Hornibrook (1940) was the first to demonstrate that B. pertussis can derive its carbon, nitrogen and energy from amino acids. Subsequent authors have examined which amino acids are utilised and differences in their results can be attributed to different analytical methods, culture media, strains and the size of inoculum. Ideally, small inocula (about 200 organisms ml^{-1}) should be used and in this respect, the works of Rowatt (1955), Jebb and Tomlinson (1955, 1957) and Lane (1970) are of particular importance.

The evidence of these workers suggests that in fact only a few amino acids are utilised: Glu, Pro, Ala, Ser, Asp, Gly and Gln. Cys is an essential amino acid and is discussed separately (see below). Other amino acids are either not utilised or used only in very small amounts. Proom (1955) is the only author whose results deviate substantially from those above. He reported that Pro, Leu and Ala were essential amino acids and could be replaced only by a complex mixture of amino acids. However, the composition of his medium was

very suspect as there was no added NaCl, but there was $MgSO_4$ (shown by Lacey (1960) to cause antigenic modulation, see 7) and $MnSO_4$ (manganous ions were suggested by Parker (1976) to be inhibitory, see 6.1.3).

All other workers agree that Glu is the principle source of carbon, nitrogen and energy for B. pertussis (Jebb and Tomlinson, 1951, 1955; Rowatt, 1955, 1957a; Goldner et al, 1966; Vajdic et al, 1966; Stainer and Scholte, 1971) and that the organism will grow in a medium containing Glu and Cys as the only amino acids (Jebb and Tomlinson, 1955, 1957; Stainer and Scholte, 1971). Several authors noted that addition of large amounts of Glu to the medium increased the final yield of cells (Dawson et al, 1951), although this was not found by Sutherland and Wilkinson (1961).

Certain amino acids have been suggested as capable of replacing Glu as the main amino acid. Thus, Pro and Cys as the only two amino acids were found to support growth of B. pertussis from large inocula (Jebb and Tomlinson, 1955; Stainer and Scholte, 1971), although not from small inocula (Jebb and Tomlinson, 1955). Furthermore, Goldner et al (1966) and Stainer and Scholte (1971) found that addition of large amounts of Pro increased the cell yield, although this was not found by Pusztai et al (1960). Jebb and Tomlinson (1955) found that Asp and Cys alone were capable of supporting growth of B. pertussis from small inocula, and Dawson et al (1951) found that addition of large amounts of Asp increased the cell yield. However, Goldner et al (1966) and Vajdic et al (1966) suggested that Asp was not a main amino acid and was only used in small amounts late in the growth cycle.

(ii) Organic sulphur source

Hornibrook (1939) found that B. pertussis had a requirement

for an organic source of sulphur which was satisfied only by Cys, cystine or glutathione (a tri-peptide of Glu-Cys-Gly) and not by Met. The requirement for one or other of these three compounds was confirmed by many other workers (Proom, 1955; Jebb and Tomlinson, 1955, 1957; Rowatt, 1957a; Stainer and Scholte, 1971; Parker, 1976). Most of these workers used large inocula. Jebb and Tomlinson (1957), using small inocula, tested a large number of organic and inorganic sulphur sources adjusted to contain the same concentration of sulphur. Only Cys, cystine or glutathione supported growth, but glutathione gave only about one eighth of the growth yielded by Cys. Vajdic et al (1966) found, with large inocula, that glutathione was the preferred sulphur source and was utilised before Cys.

In addition to satisfying the sulphur requirement of the cells, these compounds may also have a non-specific role in relation to the redox potential of the medium and to the neutralisation of certain inhibitors (see 6.1.3) (Goldner et al, 1966; Parker, 1976). However, Goldner et al (1966), Lane (1970) and Stainer and Scholte (1971) have shown that these compounds are completely removed from the medium during growth.

iii) NA, ND and other growth factors

Hornibrook (1940) showed that B. pertussis needed either NA or ND and that levels between 1 and 500 ng ml^{-1} of either vitamin supported maximum growth. The requirement for either NA or ND has been confirmed by others (Jebb and Tomlinson, 1955; Proom, 1955; Stainer and Scholte, 1971; Parker, 1976). Jebb and Tomlinson (1955) also titrated the level of NA or ND required for maximum growth and found that about 500 ng ml^{-1} of either vitamin was sufficient.

In Stainer and Scholte's (1971) liquid medium, ascorbic acid was required for growth, but was not needed for growth from small inocula when the medium was solidified (Parker, 1976). Whilst no other growth factors are essential for growth, yeast dialysate is often used as a source of other factors which stimulate growth. In an analysis of such non-essential factors, Jebb and Tomlinson (1955) found the addition of three purines (xanthine, hypoxanthine and guanine), biotin and haemin stimulated growth. Rowatt (1957b) reported that, distinct from those factors above, other unknown constituents of blood, casein hydrolysate and large, unwashed inocula could cause a rapid and profuse growth when added to the medium.

iv) Inorganic ions

Cohen and Wheeler (1946) were the first authors to determine experimentally the optimal inorganic ion composition for their medium. Their formula was based on that of Hornibrook (1939) except that they omitted KCl, increased the KH_2PO_4 concentration to increase the buffering capacity and added $FeSO_4$ and $CuSO_4$ as these salts stimulated growth. Their formulation was adopted with little modification by most other workers. Wilson (1963) modified their formula by the addition of KCl as he found that KCl, NaCl, $CaCl_2$ and $MgCl_2$ were necessary to maintain the cells in even suspension.

$FeSO_4$ has been used in all media since its introduction by Cohen and Wheeler (1946). As well as supplying ferrous ions, $FeSO_4$ probably functions as a neutraliser of certain inhibitors (see 6.1.3). Parker (1976) found that $FeSO_4$ was not required for growth from small inocula on solidified Stainer and Scholte (1971) liquid medium. Stainer and Scholte (1971) adopted Wilson's (1963) inorganic ion

formulation, but omitted $CuSO_4$ as, like Jebb and Tomlinson (1955), they found it unnecessary.

The importance of the ionic composition of the medium was shown by Lacey (1960) who tested a large number of salts for their effect on the cell envelope antigens of B. pertussis (see 7). He found that certain ions had a tendency to cause modulation of these antigens and that $MgSO_4$ was particularly potent in this respect. The modulation was freely reversible by then growing the cells in the presence of NaCl. Furthermore, certain ions (e.g. manganous ions) appear to be inhibitory to B. pertussis (Parker, 1976, see 6.1.3).

v) pH and buffers

A large amount of ammonia is formed during the growth of B. pertussis (see 6.2) and this causes a rise in the pH of the medium. Several authors have noted that growth stops when the pH reaches a value of about 8.2-8.3 (Rowatt, 1957a; Pusztai et al, 1960; Sutherland and Wilkinson, 1961; Wilson, 1963). Buffers, although not essential, allow a prolonged growth and increased cell yield. Tris buffer was first used by Sutherland and Wilkinson (1961) and subsequently by Wilson (1963) and Stainer and Scholte (1971).

Cohen and Wheeler (1946) increased the KH_2PO_4 concentration to improve the buffering of their medium, and amino acids may also act as buffers (Rowatt, 1957b). The increase in pH can also be compensated for by having a lower initial pH in the medium as several workers have shown that the initial rate of growth is the same over the pH range 6.9-7.6 (Rowatt, 1957b; Sutherland and Wilkinson, 1961; Wilson, 1963).

6.1.3. Growth Inhibitors

Jebb and Tomlinson (1955) concluded that the problems

encountered in the cultivation of B. pertussis were due to the sensitivity of the bacterium to inhibitors in the medium and that it was the need to neutralise these inhibitors that gave rise to unnecessarily complex media. Rowatt (1957a,b) described and reviewed inhibitors of the growth of B. pertussis. Some of the inhibitors are discussed below.

i) Fatty acids

Hornibrook (1939) and Cohen and Wheeler (1946) found that starch was essential for growth in their liquid medium and earlier workers had found blood to be essential for growth on solid media (reviewed by Rowatt, 1957b). Pollock (1947) showed that albumen was the active fraction of blood and could be replaced by charcoal or starch. Pollock (1947) demonstrated that these components protected B. pertussis from inhibition by unsaturated fatty acids by binding to them. Rowatt (1957a) concluded that fatty acids had a bacteriostatic effect on B. pertussis. More recently, Field and Parker (1979b) with fresh isolates of B. pertussis, have further confirmed the high sensitivity of B. pertussis to unsaturated fatty acids. Mono-unsaturated fatty acids were more inhibitory than multi-unsaturated fatty acids of the same chain length. Saturated fatty acids were less inhibitory and, depending on their concentration, could even be stimulatory.

As well as fatty acids being present in media constituents such as agar or peptones or released from cotton wool plugs on autoclaving (Pollock, 1948; Parker, 1976), there is some evidence that B. pertussis excretes fatty acids during growth. Thus, Pollock (1947) found that charcoal had to be present throughout growth and it was not sufficient to pretreat the medium with charcoal. Likewise, Kuwajima et al (1957) and Sutherland and Wilkinson (1961) found that the anion

exchange resins which they used in place of starch had to be present throughout growth. Furthermore, Pollock (1947) extracted a substance inhibitory to B. pertussis from the charcoal removed from the medium after growth and also found that more of this substance could be extracted from flasks inoculated with B. pertussis than from uninoculated ones (Pollock, 1949).

However, Rowatt (1957a) did not detect excretion of fatty acids by B. pertussis. Stainer and Scholte's (1971) liquid medium when solidified with agarose did not require an anion exchange resin for growth from small inocula (Parker, 1976). It may be that excretion of fatty acids is a strain-dependent property or is repressed in these media.

ii) Colloidal sulphur and sulphides

Proom (1955) demonstrated that growth of B. pertussis was inhibited by either colloidal sulphur or colloidal sulphides which are formed when Cys, cystine or other sulphur-containing media constituents (e.g. agar) are autoclaved in the medium. These sulphur inhibitors have a bacteriostatic effect on B. pertussis, the cells remaining viable for about 40 h before starting to die (Rowatt, 1957a). The inhibition is distinct from that caused by fatty acids, however, and the addition of albumen, activated charcoal, filtered Cys (or cystine) or large numbers of red blood cells gave protection from the inhibition (Proom, 1955; Rowatt, 1957a,b; Parker, 1976).

iii) "Organic peroxides"

Mazloum and Rowley (1955) found that B. pertussis would not grow on Cohen and Wheeler's (1946) liquid medium solidified with agar unless charcoal and/or catalase was also added, the best growth being

obtained when both were present. They suggested that the catalase decomposed hydrogen peroxide which inhibited growth of B. pertussis.

Rowatt (1957a) described an inhibitor of B. pertussis that was distinct from fatty acids or sulphur compounds as it rapidly killed the cells. Protection was afforded by low concentrations of red blood cells, haemin, filtered (but not autoclaved) $FeSO_4$ or some unknown factor present in sufficient quantity in 1% (w/v) casein hydrolysate (but not in 0.1%). She concluded that the inhibitor was probably "organic peroxides" formed on autoclaving the medium rather than hydrogen peroxide. However, the exact nature of this inhibitor remains unknown.

iv) Manganous ions

Cameron and Dunning (1970) showed that manganese was inhibitory to the growth of B. pertussis. Cameron and Stainer (1977) showed that the manganese content of various brands of agar correlated inversely with the ability of that brand to support growth of B. pertussis from small inocula. Parker (1976) observed that this inhibition was neutralised by the addition of red blood cells or haemin but could not discount the possibility that it was the same inhibition as Rowatt's (1957a) "organic peroxides".

v) Amino acid antagonisms

Dawson et al (1951) reported that Ser was inhibitory to B. pertussis. Jebb and Tomlinson (1955) found two sets of amino acids with antagonistic effects. In the first set, Ser, Pro and Met together had an inhibitory effect that was overcome by the presence of Gly. Separately, the amino acids were only slightly inhibitory. Secondly, the inhibitory effect of Val was overcome by the presence of either Leu or Ile, but most effectively by both together. The concentration

of Gly used was 3×10^{-4}M. Mason (1966) found that concentrations of Gly above 0.2M were inhibitory and concentrations slightly less than 0.2M caused the formation of sphaeroplasts.

The protective effect of Gly was not confirmed by Goldner et al (1966) who found no inhibition of growth if Gly was omitted from the defined medium of Wilson (1963).

6.2. Metabolism

The metabolism of B. pertussis is based on the oxidation of amino acids to provide the necessary energy and metabolites for growth, but knowledge of the details of this metabolism is slight and has been little studied since the 1950's.

Jebb and Tomlinson (1951) showed that a wide range of carbohydrates were not attacked. The means by which B. pertussis converts the amino acids into the carbohydrates found in ribonucleic acid, deoxyribonucleic acid and cellular polysaccharides is unknown. However, Fukumi et al (1953) demonstrated the presence of the enzymes aconitase and isocitrate dehydrogenase and Kobayashi and Fukumi (1954) showed the presence of aldolase. These studies suggest the presence of a functioning tricarboxylic acid cycle and Embden-Meyerhof pathway which would thus be a source of carbohydrates.

As mentioned in 6.1.2, most authors are agreed that Glu is the main source of cellular carbon, energy and nitrogen. Jebb and Tomlinson (1951) found that of the few amino acids oxidised by washed cell suspensions, all were completely oxidised except Glu. Rowatt (1955) concluded that Glu provided the majority of the cellular nitrogen, with only a small amount coming from other amino acids. Apart from Glu, the other amino acids utilised are Pro, Gln, Ala, Ser, Gly and Asp (Jebb and Tomlinson, 1951; Rowatt, 1955; Goldner et al, 1966; Lane, 1970).

Jebb and Tomlinson (1951) reported the production of both carbon dioxide and ammonia from amino acids which they concluded were metabolised by B. pertussis by oxidative deamination. They demonstrated that Glu was first deaminated to α ketoglutarate. They tested the ability of B. pertussis to oxidise various keto acids and found that succinate and lactate were oxidised as rapidly as Glu, α ketoglutarate at 50% and pyruvate at 15% the rate of Glu. Oxaloacetate, fumarate, malate and acetate were not oxidised (Jebb and Tomlinson, 1951).

Several authors have suggested that some form of control mechanism operates whereby the oxidation of other amino acids is repressed until the Glu is utilised (Jebb and Tomlinson, 1951; Rowatt, 1955; Vajdic et al, 1966). Pusztai et al (1960), however, reported that Pro was metabolised before the other amino acids. Lane (1970) detected no such repression mechanism and found that Glu, Pro, Ser, Gly, Ala and Asp were all completely used simultaneously. He suggested that the previous reports of repression were due to a combination of the use of a less sensitive paper chromatographic analysis and higher initial concentrations of Glu, which is the main amino acid found in hydrolysates of casein.

On the other hand, Jebb and Tomlinson (1951) reported that oxidation of Pro was 25% greater by cells grown in media with excess Pro added, implying some form of induction. However, no such increase was found in the oxidation of Gly, Ala or Asp when the respective amino acid was added in excess to the medium.

B. pertussis grows in the presence of only two amino acids: Cys plus one of Glu, Pro or Asp (Jebb and Tomlinson, 1955, 1957; Stainer and Scholte, 1971) and so must be able to synthesise all other amino acids from any one of the latter three (Cys satisfies the sulphur

requirement). Goldner et al (1966) suggested that Gln and Pro were converted first to Glu and only when these three amino acids were used up were other amino acids used (Vajdic et al, 1966). However, Stainer and Scholte (1971) found that when grown in Glu and Cys only in liquid medium, three amino acids were excreted: Thr, Val and Ala. There was no excretion of any amino acids when the cells were grown in the presence of only Pro and Cys, thus implying separate metabolism of Glu and Pro.

Excretion of Ala from cells grown in media enriched with Glu was tentatively suggested by Rowatt (1957a). Lane (1970) reported that B. pertussis excreted Ile.

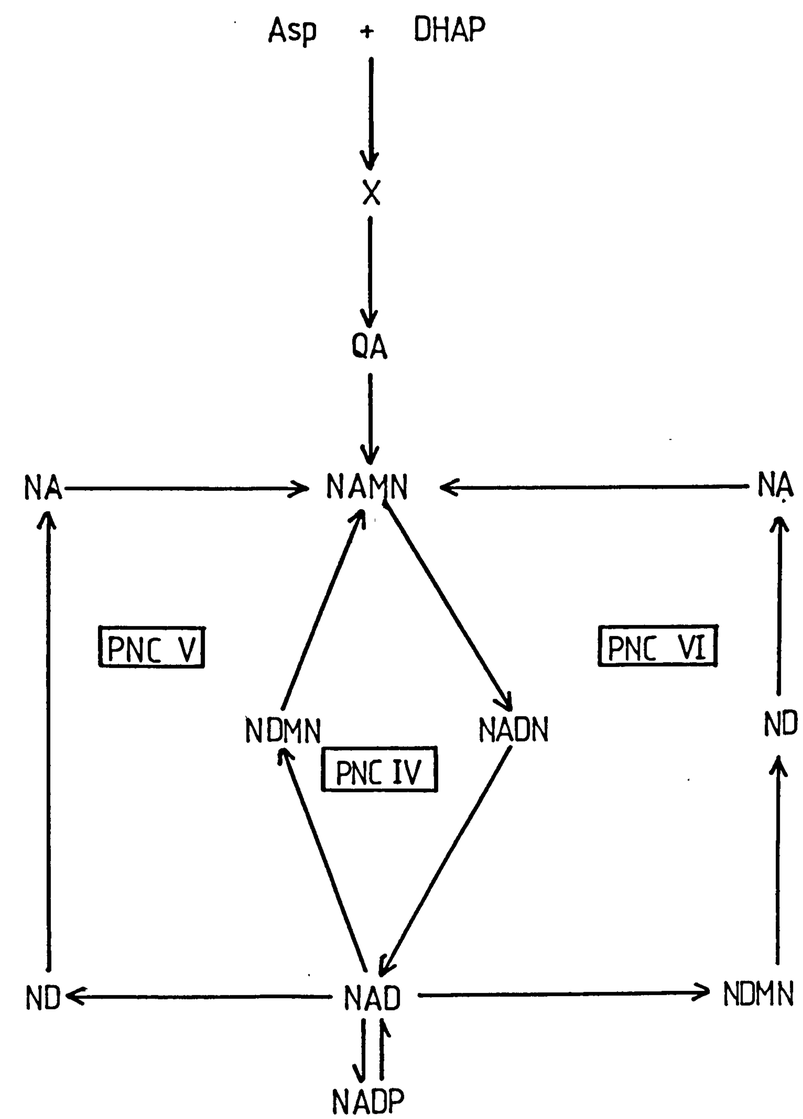
The metabolism of NA and ND by B. pertussis has not been studied, even although one or other vitamin is essential for growth (see 6.1.2) and high concentrations of either vitamin have quite different effects on cellular properties (see 7). NA and ND are the precursors of nicotinamide adenine dinucleotide (NAD) and NAD-phosphate (NADP), compounds of central metabolic importance which function in numerous anabolic and catabolic reactions and are widely distributed throughout biological systems. A comprehensive review of NAD metabolism has been published recently (Foster and Moat, 1980) and some of the more pertinent points are discussed below.

Most procaryotes synthesise NAD de novo from Asp and dihydroxyacetone phosphate via quinolinic acid (Figure 2) (Foster and Moat, 1980). However, bacteria such as B. pertussis and Lactobacillus species do not possess this ability and so have an absolute requirement for either NA or ND (Ohtsu et al, 1967). NAD is synthesised from these by a reaction sequence common to almost all species so far studied and known as the Preiss-Handler pathway (Figure 2) (Preiss and Handler, 1958a,b):

Figure 2. Pathways of NAD biosynthesis and PNC metabolism[1]

(1) Adapted from Foster and Moat (1980).

<u>Abbreviations</u> DHAP, dihydroxyacetone phosphate; QA, quinolinic acid; NAMN, nicotinic acid mononucleotide; NDMN, nicotinamide mononucleotide; NADN, nicotinic acid dinucleotide; PNC, pyridine nucleotide cycle.



$$NA \longrightarrow NAMN \longrightarrow NADN \longrightarrow NAD$$

NAD, although essential, is only required in very small amounts and the cells strive to maintain a constant NADP:NAD ratio and a constant level of NAD (Lundquist and Olivera, 1971; Foster et al, 1979b). This requires a strict control over the synthesis and breakdown of NAD. Recycling pathways of NAD degradation products, known as pyridine nucleotide cycles (PNC's), function in the majority of species studied (Figure 2) (Foster and Moat, 1980). The enzymatic steps for the recycling of ND, the final breakdown product of NAD, back to NAD are the same for all species capable of this conversion (Figure 2). However, the degradation of NAD to ND varies between species and gives rise to three PNC's (Figure 2).

PNC V uses the direct conversion of NAD to ND by NAD glycohydrolase, an enzyme which is common in eucaryotic species but relatively rare in procaryotes. The enzyme has been found in Mycobacterium species (Kasařov and Moat, 1972) and Vibrio cholerae (Fernandes et al, 1979), but not in Escherichia coli (Andreoli et al, 1972).

Most procaryotes degrade NAD to ND in two steps (Foster and Moat, 1980):

$$NAD \longrightarrow NDMN \longrightarrow ND$$

PNC VI uses both these steps, whereas in PNC IV the NDMN is deaminated to form NAMN, thus by-passing both ND and NA (Figure 2). There is now substantial evidence that both PNC IV and PNC VI function in vivo, with PNC IV being the predominant PNC (Manlapaz-Fernandez and Olivera, 1973; Foster and Baskowsy-Foster, 1980; Foster and Moat, 1980). PNC VI, however, is better suited to scavenging preformed pyridine compounds from the environment and to the "fine control" of NAD levels by

repression of NAPRTase levels (see below).

The enzymes involved in NAD biosynthesis and PNC metabolism are fully documented in the review by Foster and Moat (1980) and only two are mentioned here. ND deamidase converts ND to NA and is located in the cell cytoplasm (McLaren et al, 1973; Baecker et al, 1978). The enzyme is constitutive, though present in small amounts (Imsande and Pardee, 1962; Baecker et al, 1978; Foster et al, 1979b) and is not subject to feedback inhibition by NAD (Foster et al, 1979b).

NA phosphoribosyl transferase (NAPRTase) converts NA to NAMN and is the rate-limiting enzyme in PNC metabolism (Imsande and Pardee, 1962; McLaren et al, 1973; Cobb et al, 1977). In E. coli, most of the enzyme is in the periplasmic space (Baecker et al, 1978) and is subject to a modest degree of repression (Imsande and Pardee, 1962; Baecker et al, 1978) and likewise in Salmonella typhimurium (Foster et al, 1979b). It appears that NAD is the co-repressor molecule in these cases (Foster et al, 1979b; Foster and Moat, 1980). However, the NAPRTase enzymes of Bacillus subtilis, B. megatherium and Serratia marcescens are not controlled by repression (Imsande, 1964; Saxton et al, 1968). NAPRTase in all species is not subject to feedback inhibition by NAD (Imsande and Pardee, 1962; Imsande, 1964; Foster et al, 1979b). Furthermore, Mycobacterium tuberculosis appears to have all the enzymes of PNC VI with the sole exception of NAPRTase and so excretes NA (Kasařov and Moat, 1972).

6.3. Physiology

Some further aspects of the physiology of B. pertussis relevant to this thesis are discussed below.

6.3.1. Composition of the Cell Envelope

The structure and composition of the cell envelope of *B. pertussis* have not been well studied. There have, however, been some recent reports on the lipopolysaccharide and protein content.

i) Lipopolysaccharide (LPS)

LPS extracted from intact cells of *B. pertussis* was reported to contain both 2-keto-3-deoxyoctonate (KDO) and KDO-5-phosphate (Chaby and Szabó, 1975). However, the structure of the LPS is different from that of enterobacteria. It consists of two, low molecular weight polysaccharides linked to Lipid A of a composition similar to that in enterobacteria (Le Dur *et al*, 1978). A second lipid, Lipid X, is also present although its point of attachment is unknown (Le Dur *et al*, 1978; Chaby *et al*, 1979). The LPS has also been found to contain two unusual fatty acids (Chaby *et al*, 1979). Novotny and Cownley (1979) were unable to confirm the presence of KDO in *B. pertussis* LPS.

ii) Proteins

The proteins of the cell envelope were examined by SDS-PAGE and about 35 bands, mostly of molecular weight 10000-70000, were resolved (Parton and Wardlaw, 1975). Two main bands, whose molecular weights were subsequently corrected to 28k and 30k (Wardlaw *et al*, 1976), were heat-modifiable in that their position in the gel was dependent on the temperature of solubilisation of the envelopes in SDS. Heat-modifiable proteins have been described in *E. coli* (Schnaitman, 1973) and *Neisseria meningitidis* (Frasch and Mocca, 1978). Schnaitman (1973) suggested the phenomenon was due to a complete reaction of the proteins with SDS at high temperatures to form the "rigid-rod" conformation characteristic of protein-SDS complexes.

Dobrogosz et al (1979) studied the proteins in the outer membrane of B. pertussis by SDS-PAGE. They reported a total of about 10 bands of which 4-5 were major ones. This is similar to the protein composition of the outer membranes of many other gram-negative bacteria (Nikaido and Nakae, 1979).

Novotny and Cownley (1979) isolated outer membrane vesicles from B. pertussis by mechanical disruption of intact cells. Mild acid hydrolysis of the vesicles released about 10 proteins, all of molecular weight about 10000, which were separated by crossed immunoelectrophoresis.

6.3.2. Adenylate Cyclase

Adenylate cyclase (AC) catalyses the reaction:

ATP $\longrightarrow$ cyclic adenosine 3',5' monophosphate (cAMP) + inorganic phosphate.

cAMP is a compound of great importance in the regulation of bacterial metabolism (Pastan and Adhya, 1976; Peterkofsky, 1976) and is involved in the regulation of the composition of the cell envelope (Aono et al, 1978; Alderman et al, 1979; Mallick and Herrlich, 1979).

Commercial pertussis vaccine was found to contain a relatively stable, soluble AC (Wolff and Cook, 1973). The enzyme was subsequently purified from culture supernates of B. pertussis by Hewlett and Wolff (1976). They found the AC had a molecular weight of 70,000, was heat-labile, had an optimum temperature of 30°C and an optimum pH of 7-8. The AC had an absolute requirement for divalent cations, with Mg^{2+} giving maximal activity, but was inhibited by sodium fluoride. α Keto acids (e.g. pyruvate) and non-substrate nucleoside triphosphates had no effect on the AC (Hewlett and Wolff, 1976). Ide (1971) examined the lysates of 21 bacterial species for AC and divided them into two groups: i) soluble intracellularly (30,000 g supernate) and stimulated

by pyruvate (e.g. Brevibacterium liquefaciens) and ii) membrane-associated (30,000 g pellet) and not stimulated by pyruvate (e.g. E. coli Crooke). He also found 11 strains that had no detectable AC activity. The properties of B. pertussis AC are different from those of B. liquefaciens and E. coli (Table 5) and the organism appears unique in excreting AC into its culture supernate. Hewlett et al (1976) showed that B. pertussis excretes AC during exponential growth, with the culture supernate containing 10-20% of the total AC activity. Julina-Zlateva et al (1979) reported that the presence of an anionic resin in the culture medium stimulated excretion of the AC. Julina-Zlateva et al (1979) and Endoh et al (1980) have also found AC in the culture supernate of B. parapertussis and B. bronchiseptica.

Hewlett et al (1976) also demonstrated that B. pertussis AC could be assayed in untreated, intact cells. They concluded that about 90% of the AC associated with intact cells was extracytoplasmic and that the remaining 10% or so was intracellular. Cowell et al (1979) confirmed the extracytoplasmic location but concluded that rather than being free in the periplasm, the AC was loosely associated with some cell envelope component having an extracytoplasmic exposure.

Hewlett et al (1978) found the level of intact-cell AC was much greater in B. pertussis grown on a solid medium containing blood than in a chemically defined liquid medium. This was due to the red blood cells containing a protein activator of B. pertussis AC (Hewlett et al, 1978). The activator is present in the erythrocytes of human, sheep and rabbit blood and in commercial preparations of beef liver catalase and creatine phosphokinase (Hewlett et al, 1978, 1979b). The activator has been purified from rabbit erythrocytes (Hewlett et al,

TABLE 5. Comparison of the location and properties of the adenylate cyclases of *B. liquefaciens*, *E. coli* and *B. pertussis*[1]

PROPERTY	*E. coli*	*B. liquefaciens*	*B. pertussis*
Present in culture supernate	NO	NO	YES
Predominant location:	Intracellular, cell membrane (30,000g pellet)	Intracellular, soluble (30,000g supernate)	Extracytoplasmic and extracellular
Molecular weight:	110,000	94,000	70,000
Optimum pH	9.0 - 9.5	7-9 (without pyruvate) 9-10 (with pyruvate)	7-8
Effects of[2] :			
nucleotides	-	-	0
αketo acids	-	+	0
fluoride	-	0	-

(1) Adapted from Hewlett *et al* (1977)

(2) - : inhibition
+ : stimulation
0 : no effect

1979a) and from commercial beef liver catalase (Hewlett et al, 1978). The molecular weight of the activator from the erythrocytes was given as 105,000, but on rechromatography dissociated to give two fractions of molecular weights 75,000 and 55,000. After two cycles of gel chromatography, the activator from catalase was found to have a molecular weight of 40,000 and gave a single band in SDS-PAGE. The activator is heat-, acid- and base-stable, but is destroyed by trypsin, chymotrypsin and pronase (Hewlett et al, 1978, 1979a).

The activator stimulated the AC of B. pertussis grown in chemically defined liquid medium by about 100-fold, whereas it only gave a 2-fold stimulation of AC in cells grown on blood-containing media (Hewlett et al, 1978). The exact nature of the activator and its mechanism of action are unknown. However, the activation process requires about 3-4 min and this is not affected by lowering the temperature to 4°C. Furthermore, the AC remains activated even when the activator is removed by washing (Hewlett et al, 1978, 1979a).

The above studies with the activator were performed on intact cells, although cell lysates show the same effects. However, the AC from culture supernates is stimulated by the activator to a much lesser extent than intact-cell AC. Furthermore, response to the activator decreases with increasing purification of the AC from cell lysates and there is no response with the purified enzyme (Hewlett et al, 1978, 1979a). Thus it seems probable that some other, as yet unidentified, cell envelope component is also involved in the activation of B. pertussis AC.

7. Antigenic Variation

One of the most striking features of B. pertussis is its great propensity for variation both culturally and serologically. Two categories of variation in B. pertussis are recognised: i) Phase variation in which a series of successive mutations occurring over prolonged subculture on laboratory media results in the irreversible loss of a variety of cell properties; and ii) Phenotypic variation in which changes in the growth environment cause a rapid and freely reversible loss of certain cellular properties. Phenotypic variation does not involve mutational changes.

7.1. Phase Variation

Bordet and Sleeswyk (1910, cited by Aprile, 1972) observed that fresh isolates of B. pertussis grown on Bordet-Gengou medium (BG) differed serologically from old stock cultures grown on nutrient agar. Leslie and Gardner (1931) showed that with repeated subculture of B. pertussis, a series of antigenic changes occurred that gave rise to 4 serologically and culturally distinguishable forms: Phases I, II, III and IV. Thus, Phase I organisms were virulent fresh isolates, homogeneous with regard to agglutination, whereas Phase IV organisms were avirulent, degraded strains that grew on nutrient agar and were not agglutinated by Phase I antiserum. Phases II and III were intermediate.

Standfast (1951) examined the effect of repeated subculture on a number of properties such as growth requirements, agglutination, virulence, PA and HA. He found that these varied randomly and independently, although the tendency was for mutations to accumulate. Thus it was only possible to recognise the Phase I and IV states and a

large number of intermediate states were formed. Field and Parker (1979a) have observed a similar random loss of properties leading to degraded states.

Parker (1979) has suggested that the terms fresh isolate, intermediate strain and degraded strain be used in place of Phases I, II, III and IV. Thus, fresh isolates are strains isolated and maintained with a minimum number of subcultures on BG medium without peptone. There is no acceptable definition for degraded strains as yet, but they would be strains showing a large number of differences from the parent fresh isolate. Thus, degraded strains grow on solid media which do not support growth of fresh isolates and have lost pathophysiological activities such as PA, HSF, LPF, HLT, HA, agglutinogens and virulence. They have altered serological specificity in the LPS, have lost cytochrome d_{629} and gained resistance to certain antibiotics and fatty acids (Standfast, 1951; Aprile, 1972; Aprile and Wardlaw, 1973; Parker, 1976; Dobrogosz et al, 1979; Field and Parker, 1979a; Hewlett et al, 1979b; Wardlaw and Parton, 1979). Intermediate strains show various combinations of these properties, having accumulated them in a random order (Standfast, 1951; Field and Parker, 1979a).

Phase variation also produces changes in the cell envelope proteins and loss of the AC. Parton and Wardlaw (1975) reported that the SDS-PAGE profiles of Phase I, Phase IV and C-mode (see 7.2) cell envelopes differed only in that Phase IV's and C-modes lacked the two heat-modifiable bands: 28k and 30k (Wardlaw et al, 1976). Gel scans of C-mode cell envelopes also showed the loss of two higher molecular weight bands (Wardlaw et al, 1976). This would agree well with Dobrogosz et al (1979) who reported the loss of 4 outer membrane proteins of molecular weight 27.5k, 30k, 88k and 98k in Phase IV cells.

Parton and Durham (1978) reported that Phase IV strains of B. pertussis had much reduced AC levels in cell lysates and culture supernates compared with the parent Phase I strain. Endoh et al (1980) confirmed this loss. Hewlett et al (1979b) reported that the AC activity was reduced about 10^4-fold in Phase IV cells grown either on BG or in Stainer and Scholte's (1971) liquid medium (SS). They also found that the AC levels in Phase IV cells grown on BG were about 10-fold greater than those from SS-grown cells. However, the AC from Phase IV cells grown in SS no longer responded to the protein activator (see 6.3.2) and it is not possible to state whether the loss of AC in Phase IV cells is due to loss of AC or to loss of responsiveness to the activator (Hewlett et al, 1979b).

Parker (1976, 1979) has proposed a "genetic hypothesis" to explain the Phase Variation of B. pertussis. The hypothesis assumes that some abnormality in the metabolism of B. pertussis renders the organism unusually sensitive to inhibitors in the growth medium (Parker, 1979). There would then be strong selective pressure for mutations reducing this sensitivity. Parker (1979) notes that random mutations in a small number of genes (e.g. 4-10) could yield a large number of possible phenotypes (14-1022 for 4-10 genes). If these small numbers of genes code for the organisms growth and biological properties, accumulated random mutations in these would lead to degraded strains via a large number of possible intermediate strains. The effect of multiple mutations would be to make the process essentially irreversible, as observed.

7.2. Phenotypic Variation

Two distinct forms of phenotypic variation have been

described in B. pertussis:

i) Antigenic Modulation (Lacey, 1960), and

ii) Nicotinic Acid Modulation (Pusztai and Joó, 1967).

7.2.1. Antigenic Modulation

Lacey (1951) found that replacement of NaCl with $MgSO_4$ on a weight for weight basis in the growth medium caused a remarkable number and variety of phenotypic changes in B. pertussis. Further studies (Lacey, 1960) showed that these changes were demonstrable with all fresh isolates, were completely reversible within a single subculture on the original medium and were non-mutational and therefore quite different from the phase variation described by Leslie and Gardner (1931). Lacey (1960) termed this variation "antigenic modulation" and defined it as "a change of phenotype occurring in all, or almost all, members of a population as the expression of a reversible and continuously-environmentally-dependent change in metabolism".

Lacey (1960) described 3 antigenic modes: X, I and C, which were in fact dynamic equilibrium states in a continuous change from X- to C-mode with an infinite number of intermediate states. X stands for "xanthic" which describes the yellowish-ochre hue of confluent growth of B. pertussis on BG medium; C or "cyanic" describes the greyish-blue appearance when the strains were grown on BG with added $MgSO_4$. The I-mode is an intermediate mode formed only within a narrow range of conditions.

Lacey (1960) reported that the following factors could cause modulation:

a) the ionic composition of the medium, e.g. $MgSO_4$ in place of NaCl,

b) growth at low temperatures, e.g. 25°C in place of 37°C, and

c) miscellaneous factors such as fatty acids, tellurite or old blood. He further stated that all these factors acted by the same means to produce the same end result: C-mode cells. Lacey (1960) examined in more detail the effects of ions and temperature on agglutinability of B. pertussis and used differences in agglutination to define the 3 modes. He tested over a hundred salts by replacing the NaCl with an equivalent amount of another salt and concluded that cations and anions had independent potencies for modulation. The ratio of the ionic species and not their absolute concentrations determined the final mode of the cells. Furthermore, he studied the combined effects of temperature and salts and showed they were independent but that the sum of their individual potentials for modulation determined the final mode of the cells.

Lacey (1951) had observed that mere suspension of the cells in a salt solution did not produce modulation. There was a requirement for cell division and subsequent studies have shown that a particular mode becomes established after 4-7 cell divisions (Lacey, 1960). Furthermore, Lacey (1960) concluded that the 3 modes observed were the result of the growth environment in some unknown way influencing the relative rates of synthesis of the 3 main antigens: X, C and I. However, recent studies by Idigbe (1979) suggested that repression alone is not responsible for antigenic modulation as loss of HSF, LPF and 28k and 30k gel bands occurred within 10 h of transfer of cells to C-mode media. This 10 h period was of the same length as the lag phase and thus the modulation occurred without the need for cell division.

Lacey (1960) characterised the X-mode as being favoured by growth in certain salts (e.g. NaCl) or at high temperatures (e.g. 37°C), and as possessing the X-antigen. It was also known that X-mode cells formed

a serologically heterogeneous group (Andersen, 1953). The C-mode was characterised as being favoured by growth in certain other salts (e.g. $MgSO_4$) or at low temperatures (e.g. 25°C), as possessing the C-antigen in place of the X-antigen and as being a serologically homogeneous group.

Subsequent studies have shown that the C-mode closely resembles Leslie and Gardner's (1931) Phase IV in the variety of other cell properties lost compared with the X-mode. Those lost properties are the PA (Holt and Spasojević, 1968; Wardlaw et al, 1976), HSF (Wardlaw et al, 1976), LPF (Idigbe, 1979), HA (Lacey, 1951, 1960), adjuvant activity for reaginic antibody and for hyperacute EAE (Wardlaw et al, 1979), mouse virulence (Lacey, 1951), HLT (Livey et al, 1978), agglutinogens (Adams, 1970) and cytochrome d_{629} (Dobrogosz et al, 1979).

SDS-PAGE analysis of C-mode cell envelopes showed the loss of the 2 main bands at 28k and 30k (Wardlaw et al, 1976). Their gel scans appear also to show a loss of 2 higher molecular weight bands (see also 7.1). This would agree with Dobrogosz et al (1979) who found the loss of 4 outer membrane bands, in C-mode cells, of molecular weights 27.5k, 30k, 88k and 98k. Idigbe (1979) reported that C-mode cell envelopes differed from X-modes only in the loss of 3 bands at 28k, 30k and 100k. Furthermore, Parton and Wardlaw (1975) noted that Phase IV and C-mode cell envelope profiles were indistinguishable and that Phase IV cell envelope profiles did not alter when the cells were grown in C-mode medium.

Parton and Durham (1978) noted that C-mode cells resembled Phase IV cells in showing a reduction in AC activity of about 100-1000-fold in both cell lysates and culture supernates. Their results also showed that whilst the AC activities of the 6 X-mode strains tested varied from 19.3-360 nmol cAMP/mg/15 min, the AC activities of the

corresponding C-mode cultures varied only over the range 0.08-0.14 nmol cAMP/mg/15 min. However, they did not state whether, as in Phase IV's (see 7.1), the AC of C-modes was unresponsive to the protein activator of AC (see 6.3.2).

It should be noted that with the exception of the cytoplasmic HLT (Cowell et al, 1979) and the predominantly extracytoplasmic AC (Hewlett et al, 1976), all the other properties mentioned above are located in the cell envelope.

Lacey (1951, 1960) reported that all the fresh isolates of B. pertussis he tested underwent modulation. However, Adams (1970) found that strain 18323, a high virulence strain, did not lose PA or agglutinogens when grown either at 25°C or in the presence of high $MgSO_4$ levels. This is a laboratory passaged strain, however, and is most likely not equivalent to a fresh isolate. One of the differences may be the loss of the ability to modulate. Alternatively this particular strain may differ genetically from others such that it is incapable of modulation.

7.2.2. Nicotinic Acid Modulation

Media used for growing B. pertussis in Phase I or X-mode normally contain about 1 μg ml^{-1} of either NA or ND. Pusztai and Joó (1967) described the effect of increased NA concentrations (5, 50 or 500 μg ml^{-1}) in the growth medium on various properties of the cells. Increasing the NA concentration 500-fold to 500 μg ml^{-1} (NA500) had no effect on the germ count, pH, oxidation-reduction potential or bacterial morphology. However, certain of the pathophysiological properties were affected. Thus, all of the 7 strains tested showed a gradual loss of PA and HSF activities with increasing NA concentration and 5 of these

strains also became inagglutinable by Phase I specific immune serum. None of the 7 strains showed a loss of HLT over the range of NA concentrations tested.

The loss of PA and HSF from cells grown in NA500 has been confirmed by Ackers and Dolby (1972) and Wardlaw et al (1976). Ackers and Dolby (1972) also concluded that growth in NA500 had no effect on either the amount or antigenic specificity of the LPS.

Pusztai and Joó (1967) reported further that the NA500-grown cells had about half the amount of precipitating protein antigens of NA1-grown cells and that immunoelectrophoretic analysis of disintegrated cells showed a loss of 4 precipitin bands from the NA500-grown cells. SDS-PAGE analysis of cell envelopes of NA500-grown cells showed they were indistinguishable from those of Phase IV's and C-modes and lacked the 28k, 30k and some higher molecular weight gel bands (Wardlaw et al, 1976).

The changes in phenotype occurred within a single subculture and were likewise reversible by culture in NA1 medium (Pusztai and Joó, 1967). Thus, although this effect has not been studied in as much detail as antigenic modulation, the definition of modulation given by Lacey (1960) appears to be applicable in this instance also.

NA and antigenic modulations are similar in terms of loss of PA, HSF, agglutinogens and cell envelope profiles in SDS-PAGE (Pusztai and Joó, 1967; Wardlaw et al, 1976). Furthermore, both modulations occur in B. parapertussis as judged by loss of agglutinability (Pusztai and Joó, 1967; Dobrogosz et al, 1979). Pusztai and Joó (1967) concluded that NA modulation was the result of an altered rate of synthesis of cell envelope components such as PA, HSF and agglutinogens. This is very similar to the postulated effect of antigenic modulation by

Lacey (1960) (see also 7.2.1). However, Pusztai and Joó (1967) stated that despite certain similarities, NA and antigenic modulations are different phenomena as shown by two findings. First, there is no loss of HLT in NA-modulated B. pertussis, unlike C-modes (Pusztai and Joó, 1967; Livey et al, 1978). Second, hyperimmune sera raised against NA500-grown B. pertussis did not agglutinate these cells, but did give a low titre of agglutination against NA1-grown cells. Immune sera raised against NA1-grown cells gave much higher titres against these cells, but no agglutination of NA500-grown cells (Pusztai and Joó, 1967). Thus, cells grown in NA500 media had much reduced levels of the main agglutinogen and with no altered antigenic specificity. In antigenic modulation, however, the X-antigen is not only much reduced in C-mode cells but is replaced by another agglutinogen, the C-antigen, of a different specificity (Lacey, 1960). Thus, antisera raised against C-mode cells agglutinated C- but not X-mode cells.

Pusztai and Joó (1967) noted that 2 strains did not lose agglutinability when grown in NA500-media. One of these strains, however, was shown to lose PA and HSF activities in NA500-media and thus the agglutinogen must be under a separate form of control from the PA and HSF. The other strain, which was not tested for PA and HSF activities, was 18323. This was the strain shown by Adams (1970) not to undergo antigenic modulation (see also 7.2.1) and this strain may therefore lack the means to modulate and would therefore be worthy of more detailed study.

Whilst comparing the effects on B. pertussis of growth in NA1 and NA500 media, Wardlaw et al (1976) also examined the effects of growth in ND1 and ND500 media. Whereas growth in NA1 and ND1 had no effect on PA and HSF activities and the 28k/30k cell envelope bands,

growth in NA500 caused their loss (Table 6). Thus, of two very similar vitamins, either one of which is required for growth, only NA caused modulation when present in high concentration.

High concentrations of NA and ND have been found to cause a modest degree of repression of the enzyme NAPRTase in both E. coli and Sal. typhimurium (Baecker et al, 1978; Foster et al, 1979b; see also 6.2). Furthermore, it has been reported that growth in high concentrations of ND repressed the uptake of ND in Streptococcus faecalis (Neujahr and Varga, 1966) and that growth in high NA concentrations repressed a binding protein for, and active transport of, NA in L. casei (Henderson and Zevely, 1976; see also Section II).

SECTION II : TRANSPORT OF VITAMINS INTO BACTERIA

1. Introduction

The transport of solutes into bacteria necessitates the solutions to two main problems: i) translocation of hydrophilic solutes across the cytoplasmic membrane, a predominantly hydrophobic barrier, and ii) the nature of the coupling between metabolism and transport. Gram-negative bacteria have the additional problem of having a second permeability barrier, the outer membrane, through which solutes must first pass. Furthermore, transport systems are relatively specific, suggesting the involvement of recognition sites for translocation. Cohen and Monod (1957) gave the first clear demonstration that the selective permeability of solutes into bacteria was mediated by stereospecific, cell membrane proteins. The study of bacterial transport has generated an enormous literature which would be neither feasible

TABLE 6. Effect of growth in high or low concentrations of NA or ND on PA and HSF activities and the 28k/30k cell envelope proteins(1)

CULTURE(2) MEDIUM	PA	HSF	28k/30k
NA1	+	+	+
ND1	+	+	+
NA500	-	-	-
ND500	+	+	+

(1) Adapted from Wardlaw et al (1976)

(2) NA1, NA500, ND1 and ND500 are the concentrations ($\mu g\ ml^{-1}$) of NA or ND in the medium.

nor relevant to review here. Consequently, this section will be limited to a brief and selective review of the various modes of transport followed by a review of the transport of vitamins into bacteria.

2. Modes of Transport

Solutes are transported into bacteria by four distinct processes: i) passive diffusion, ii) facilitated diffusion, iii) active transport, and iv) group translocation. These processes are further discussed below.

2.1. Passive Diffusion

In this mode of transport, a solute passes unaided through the cell membrane as a result of random molecular movement and diffusion occurs down an electrochemical gradient. There is no requirement for energy-coupling and the flux of the solute is bidirectional. At equilibrium, there is no net flux of solute and the solute is equally distributed on either side of the membrane. Passive diffusion is not stereospecific and there is no competition between solute and structural analogues (Finean *et al*, 1974; Tristram, 1978). This mode of transport is thought to be the means of translocation of solutes through the non-specific, aqueous pores of the outer membrane (see 3, below).

2.2. Facilitated Diffusion

Virtually all bacterial transport systems operate via interactions with protein components in the cell membranes. The simplest of these is facilitated diffusion and shares certain features with passive diffusion: bidirectional flux of solute, diffusion down an

equilibrium gradient, no energy requirement and no accumulation at equilibrium. However, the rate of translocation is more rapid than in passive diffusion and displays saturation kinetics. Furthermore, the process is highly stereospecific and structural analogues of the solute compete for translocation. These features are consistent with the involvement of protein membrane carriers in the translocation process (Silhavy et al, 1978; Tristram, 1978). This mode of transport is thought to be the means of uptake of glycerol (Silhavy et al, 1978) and NDMN in Sal. typhimurium (Kinney et al, 1979; see also 4.8 below).

2.3. Active Transport

This mode of transport exhibits all the characteristics of facilitated diffusion but with two important exceptions: i) there is an absolute requirement for energy-coupling, and ii) solute is accumulated intracellularly against an electrochemical gradient. Inhibition of the energy-coupling to translocation prevents accumulation of solute, but the system may still function for facilitated diffusion. In each of the three processes so far described, the solute remains unaltered chemically during translocation (Rosen, 1978; Tristram, 1978).

Active transport systems are extremely common in bacteria and are involved in the transport of most types of compounds, e.g. carbohydrates, amino acids, ions, vitamins, etc (Rosen, 1978). The transport system is located in the cytoplasmic membrane and the carrier protein and the proteins involved in energy-production and coupling are all integral proteins of the membrane.

Active transport proceeds by essentially three steps: i) solute recognition and binding to the carrier protein, ii) translocation across the membrane, and iii) coupling to metabolic energy. The exact

mechanisms involved in these steps remain unknown. Most of the studies on uptake are done on E. coli or other gram-negative bacteria. These bacteria have the additional outer membrane, separated from the cytoplasmic membrane by the periplasmic space which contains a number of proteins released by osmotic shock of the cells and which specifically bind certain sugars, amino acids, ions or vitamins. These binding proteins have a single solute binding site and have molecular weights in the range 25,000 to 42,000. There is now much evidence for the involvement of these binding proteins in the transport process, although not all compounds require them for transport (Rosen, 1978; Tristram, 1978).

Translocation across the membrane is facilitated by the carrier protein spanning the membrane and enclosing a water-filled channel within an aggregate of a small number of subunits. Binding of the solute causes a conformational change such as to allow the passage of the solute (Jardetzky, 1966; Singer, 1977). Singer (1977) postulated that binding proteins, where involved, may possess an additional binding site specific for the carrier protein, rather than the solute interacting with the carrier directly. Such interaction between a carrier protein and a binding protein has been found in the His system of Sal. typhimurium (Singer, 1977; Ames and Nikaido, 1978). Singer's model has the important consequence that it predicts that several independent binding proteins could use the same carrier protein.

Energy-coupling to transport is known to be of two distinct types. This was first shown in E. coli by Berger and Heppel (1974) who separated the types thus:

i) Primary active transport where the energy is derived directly from the hydrolysis of high-energy phosphate compounds such as ATP. In

gram-negative bacteria, such coupling is usually found in systems involving binding proteins, e.g. Gln transport in E. coli (Berger, 1973).

ii) Secondary Active Transport, in which the energy is derived from the electrochemical gradient formed according to Mitchell's (1970) Chemiosmotic Hypothesis. This coupling is generally associated with systems not involving binding proteins, e.g. Pro transport in E. coli (Berger, 1973).

2.4. Group Translocation

Group translocation processes are quite distinct from those described above. They consist of one or more enzymes which interact to permit the vectorial metabolism of a solute into the cell. Thus a solute is translocated and metabolised simultaneously. There is a requirement for energy, but accumulation of the compound will only occur if it cannot diffuse out of the cell and is not itself further metabolised.

There are various types of group translocation, but by far the best known and best studied is the phosphoenolpyruvate-phosphotransferase system (PEP:PTS) involved in the uptake of some sugars in many bacteria. The system has been well studied in E. coli and Staphylococcus aureus and is found only in facultative anaerobes. The PEP:PTS has been reviewed recently by Saier (1977) and Hays (1978) and involves four proteins which act sequentially to transfer the high-energy phosphate group of PEP to the sugar as it is translocated, entering the cell as the sugar-phosphate derivative (Figure 3). Enzyme I and HPr are cytoplasmic proteins and are involved in the transport of all PTS sugars. The sugar-specific proteins of the system, the components of the enzyme II complexes, are the enzymes II and III. The enzymes II are always found as integral membrane proteins associated with phospholipid and cations, which are essential for their activity. However, enzymes III

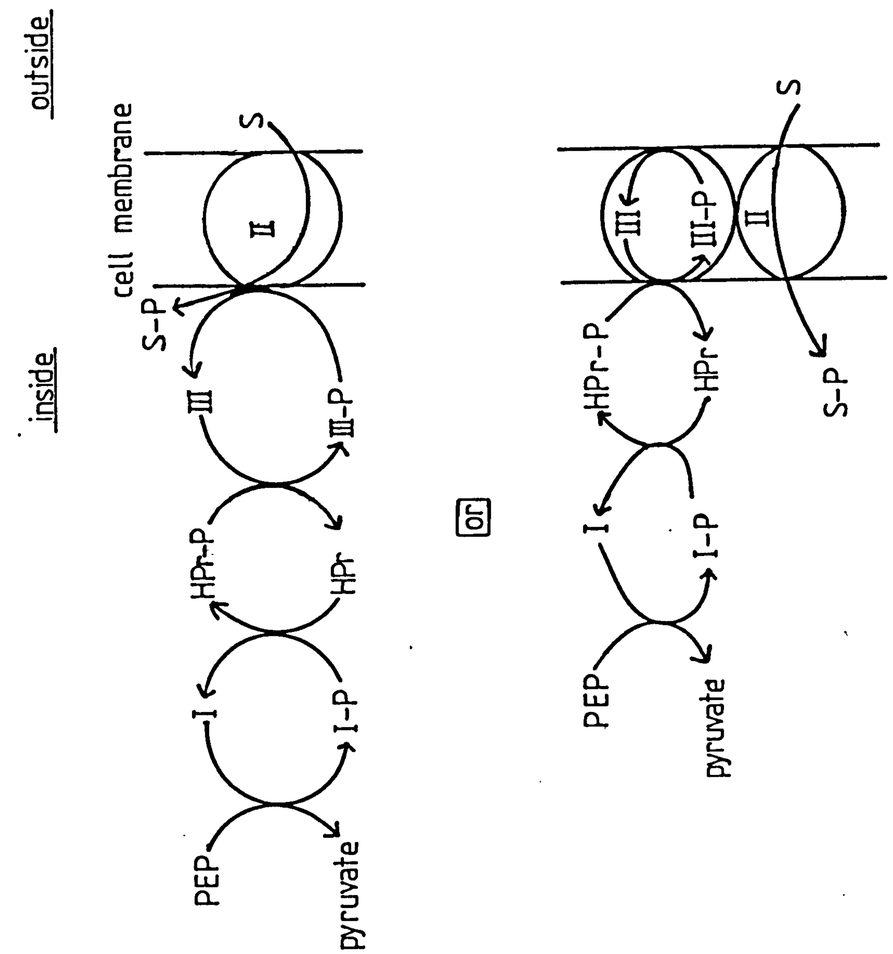


Figure 3. Diagrammatic representation of the PEP:PTS for the uptake of certain sugars.

I, II and III represent enzymes I, II and III respectively.

Abbreviations - P, phosphate; S, sugar; HPr, heat-stable protein.

can be either membrane bound as components of the enzyme II complexes or are cytoplasmic, although loosely associated with the inner face of the membrane. Each sugar is translocated via its own specific pair of enzymes II and III.

Other examples of group translocation processes have been described. Thus, in E. coli the capacity to transport long chain fatty acids is induced concomitantly with the ability to degrade these compounds. Mutants lacking the membrane-bound acyl-CoA synthetase were unable to transport these fatty acids, suggesting that transport may be a group translocation process involving vectorial acylation as the fatty acid crosses the membrane (Hays, 1978).

There is some evidence to suggest that in E. coli and Sal. typhimurium purines, pyrimidines and nucleosides are translocated by group translocation, although there is also some conflicting evidence (Hays, 1978; Saier, 1979). Thus, it has been suggested that extracellular adenosine is first catabolised vectorially on the outer surface of the membrane such that adenine remains extracytoplasmic and the ribose is taken up by the cell. The adenine is then translocated via a second enzyme which simultaneously metabolises it to AMP.

Foster et al (1979a) have recently proposed a similar mechanism for the utilisation of exogenous NAD by Sal. typhimurium. They suggest that the NAD is first metabolised such that NDMN remains extracytoplasmic and AMP is released intracellularly. The NDMN is then translocated by vectorial metabolism by the membrane-bound NDMN glycohydrolase which converts the NDMN to ND and ribose-5-phosphate, both of which are released intracellularly.

3. Role of the Outer Membrane

The outer membrane (OM), like the cytoplasmic membrane, is composed of proteins, LPS and phospholipids, but the components are distributed asymmetrically in the membrane. The outer leaflet of the OM contains all the LPS whilst the inner leaflet contains almost all the phospholipid, with the protein distributed between the two (Smit et al, 1975). Although the protein content of the OM is quite high, SDS-PAGE analysis has revealed only about 25-30 protein bands, of which 3-4 are the major proteins and have molecular weights in the region 32,000-36,000 (Kadner and Bassford, 1978; Nikaido and Nakae, 1979).

The fact that gram-negative bacteria were more sensitive to certain dyes and antibiotics than gram-positive bacteria suggested that the OM may function as a permeability barrier. Nakae and Nikaido (1975) and Decad and Nikaido (1976) showed that the OM had a molecular seiving action in that it was freely permeable to polysaccharides up to a molecular weight of 600, but not to larger ones. The permeability of the OM was shown to be due to the OM proteins by Nakae (1976a). He found that vesicles prepared from LPS and phospholipids extracted from the OM of Sal. typhimurium were only permeable, and to the same limits as intact cells, when a complex of isolated OM proteins were also incorporated. Nakae and Ishii (1978) subsequently showed that individual, major OM proteins were sufficient to make the vesicles permeable. Nakae (1976b) obtained similar results with E. coli and proposed that certain major OM proteins formed aqueous, non-specific transmembrane channels or pores in the OM for the free passage of low molecular weight hydrophilic molecules.

Subsequent studies have shown there are two classes of pores in the OM:

i) Non-specific pores

Lutkenhaus (1977) and Van Alphen et al (1978) have shown that two of the major OM proteins of E. coli form separate pores allowing free diffusion of a wide variety of compounds including glucose, fructose, Cu^{2+}, phosphate, sulphate, Arg, Leu, Ile, Met, His, Thr and adenosine-, guanosine- and cytidine-5'-monophosphates. The rate of permeation of solutes through these pores is affected by the size of the solute, its degree of hydrophobicity and its electrical charge (Nikaido and Nakae, 1979).

ii) Specific pores

Passive diffusion through the non-specific pores is rate-limiting if the solute is above a certain size or if the external concentration is very low (Nikaido and Nakae, 1979). In these cases, specific systems for translocation of the OM would be required and four specific systems have been described: uptake of vitamin B_{12}, iron-siderochrome complexes, maltose and nucleosides. The first three of these have been reviewed recently by Kadner and Bassford (1978). The specific pores are also known to function as receptors for certain bacteriophage and colicins (Braun and Hantke, 1977) which has been of help in the study of the systems.

The maltose specific pore is induced by and required for the uptake of maltose and maltatriose from low external concentrations. High concentrations do not require the specific pore and can enter via other pores. The specific pore for maltose is also the OM receptor for bacteriophage lambda, but has no binding activity for maltose (reviewed by Kadner and Bassford, 1978). Nakae (1979) showed that when the purified maltose pore was used in reconstitution experiments, the pore lost its specificity and was permeable to several compounds.

Heuzenroeder and Reeves (1980) demonstrated, in E. coli, that the specificity of the maltose pore was given by its interaction with the periplasmic maltose binding protein. Mutants lacking the binding protein and general pores, but containing the maltose pore, showed a much reduced specificity of uptake.

4. Transport of Vitamins into Bacteria

Vitamins can be defined as a diverse collection of organic compounds which cannot be synthesised by the organism but which are required in small amounts, usually to form parts of prosthetic groups of enzymes. The study of vitamin uptake in bacteria was largely ignored for many years, mainly due to problems such as low levels of utilisation, low rates of uptake and rapid metabolism. They have recently become more popular either because of the role of OM receptors (vitamin B_{12}) or because of their potential for the isolation of carrier proteins (folate and thiamine). The group of vitamins considered here are the low molecular weight, water soluble vitamins known collectively as the Vitamin B complex.

4.1. Vitamin B_{12} (Cyanocobalamin)

The uptake of B_{12} in E. coli was first described by Oginsky (1952), but was not further studied for some time. DiGirolamo and Bradbeer (1971) showed that the uptake of B_{12} by E. coli was by a bi-phasic process: i) an initial phase that was very rapid, energy-independent and represented binding of the vitamin to an OM receptor protein, and ii) a slower, second phase of energy-dependent intracellular accumulation of B_{12}. Whether or not B_{12} uptake is an active transport

process with accumulation of unaltered vitamin is unclear (Kadner, 1978). Osmotic shock reduces B_{12} uptake by about 85-95% in E. coli (DiGirolamo and Bradbeer, 1971), although only very low amounts of a periplasmic binding protein (less than 10 molecules per cell) could be detected (White et al, 1973). Furthermore, the energy-coupling for B_{12} uptake was found to be via the proton motive force (Bradbeer and Woodrow, 1976) which is usually associated with binding protein - independent systems (see 2.3).

The OM receptor for B_{12} is thought to be present in about 200-300 copies per cell in E. coli (White et al, 1973) and is also the receptor for Colicins E1 and E3 (DiMasi et al, 1973) and for bacteriophage BF23 (Bradbeer et al, 1976). Another OM protein, the tonB gene product, is also required for the second, energy-dependent phase of B_{12} transport, although the nature of this protein is unknown (Bassford et al, 1976).

4.2. Thiamine (Vitamin B_1)

Thiamine transport was first studied in L. fermenti by Neujahr (1963), and in a subsequent series of reports (Neujahr, 1966a,b,c) she showed the system involved active transport and was repressed by growth in high concentrations of thiamine. Although there was rapid intracellular phosphorylation of the thiamine, Neujahr (1966b) showed that the transport and phosphorylation steps were separate.

Thiamine uptake in L. casei is also by active transport (Henderson and Zevely, 1978). Thiamine has been found to bind strongly to a cytoplasmic membrane binding protein which has been solubilised, purified and found to have a molecular weight of 29,000 and a single thiamine binding site (Henderson and Zevely, 1976; Henderson et al, 1977b).

Both the binding and transport activities are repressed by growth in high concentrations of thiamine, but it remains to be clarified whether the binding protein is also the carrier protein (Henderson and Zevely, 1978). In some elegant experiments, Henderson et al (1979a) have provided evidence that three vitamins share a common membrane component for transport in L. casei. Thus thiamine, folate and biotin have separate membrane binding proteins which interact with a common, constitutive membrane component and inhibit the transport of each other. The degree of inhibition is proportional to the amount of binding protein, not to the amount of vitamin. This finding agrees well with the prediction made by Singer (1977) in his hypothesis of translocation (see 2.3).

Thiamine transport has also been studied in E. coli where it appears there may be separate transport systems for the uptake and exit of the vitamin. Uptake of thiamine is by a repressible active transport process, and is followed by phosphorylation in two steps to thiamine pyrophosphate (Kawasaki et al, 1969). A repressible, periplasmic binding protein has also been isolated by Iwashima et al (1971). Thiamine may also be excreted by a separate, active transport process which may serve to regulate intracellular thiamine levels (reviewed by Kadner, 1978; Yamada and Kawasaki, 1980).

4.3. Folates

The term folates encompasses a group of derivatives of the vitamin folate. This group of compounds are involved in pathways of one-carbon transfer. Most of the studies on folate transport have been done on the auxotrophic L. casei. Henderson and Huennekens (1974) showed that the uptake of folates by L. casei was by a repressible

active transport process. They also suggested that several of the folates shared the same carrier protein. Henderson et al (1977a) reported the solubilisation and purification of a cytoplasmic membrane folate-binding protein of molecular weight 25,000. The binding protein had a single folate binding site and was repressible. As discussed in 4.2, this binding protein interacted with a common membrane component for thiamine and biotin transport. Henderson et al (1979b) have shown that folate transport is coupled to phosphate-bond energy either from ATP or some other high energy compound. They speculate that there is a direct interaction of the energy source with some component of the transport system, possibly that shared with thiamine and biotin transport (Henderson et al, 1979a).

4.4. Biotin

Biotin-containing enzymes are involved in carboxylation reactions. The vitamin is not metabolised, it becomes covalently bound to the apoenzyme. Lichstein and Ferguson (1958) were the first to study biotin uptake, in the auxotrophic L. plantarum, and found preliminary evidence for a carrier-mediated process. Waller and Lichstein (1965a,b) showed that uptake in the same bacterium was by a repressible, active transport process, although there was also some uptake of biotin by a facilitated diffusion process.

Henderson and Zevely (1976) have reported that biotin uptake in L. casei is also by a repressible active transport process. As with thiamine (4.2) and folate (4.3) transport in L. casei, biotin transport is via a repressible, cytoplasmic membrane binding protein (Henderson and Zevely, 1976). The levels of this binding protein are much lower than those for thiamine or folate and thus these latter vitamins

strongly inhibit biotin uptake, whilst biotin has little effect on their uptake (Henderson et al, 1979a).

Biotin is accumulated by a repressible, active transport process in E. coli (Prakash and Eisenberg, 1974). Griffith and Leach (1973) reported there was no periplasmic binding protein for biotin in E. coli. Pai (1972, 1973) showed that the biotin transport system and the biotin biosynthetic enzymes in E. coli are under separate regulation, although both are repressible by biotin.

4.5. Pyridoxine (Vitamin B_6)

Vitamin B_6 is a collective term for three closely related factors: pyridoxine, pyridoxal and pyridoxamine. The phosphate derivatives of these are the coenzyme forms which are involved in amino acid metabolism reactions such as transamination, deamination, decarboxylation and racemisation. Mulligan and Snell (1976) reported that Sal. typhimurium transported only pyridoxal or pyridoxine by a facilitated diffusion system, followed by rapid intracellular phosphorylation. They did not find any binding protein. Pyridoxine uptake in E. coli is likewise via facilitated diffusion (Kadner, 1978) and does involve a binding protein (Griffith and Leach, 1973).

Mulligan and Snell (1977) reported facilitated diffusion of pyridoxal or pyridoxamine by Strep. faecalis and pyridoxal by L. casei. The authors also reported that the transport of B_6 derivatives was reflected in the growth requirement. Thus, L. delbrueckii actively transports only the phosphorylated derivatives of B_6, which are also the only forms of the vitamin it uses for growth.

4.6. Pantothenic Acid

The coenzyme form of pantothenate is Coenzyme A which is

important in both fatty acid metabolism and the activation and transfer of acyl groups. Uptake of pantothenate was first described in Pseudomonas fluorescens and E. coli and was an active transport process involving a periplasmic binding protein in both species (reviewed by Kadner, 1978). Both the transport and the binding protein were inducible by pantothenate in P. fluorescens.

Germinario and Waller (1977) showed that pantothenate uptake in L. plantarum was by active transport followed by rapid intracellular phosphorylation.

4.7. Riboflavin (Vitamin B_2)

Riboflavin is metabolised to form the important flavin nucleotide coenzymes which are involved in some dehydrogenations and in the respiratory chain. Riboflavin transport into B. subtilis was by a carrier-mediated process and was dependent on energy (Cecchini et al, 1979). However, it was not possible to determine if the energy was required for the transport or the phosphorylation steps or both. These authors also found that the uptake involved a cytoplasmic membrane binding protein for riboflavin which, along with transport activity, was repressible.

4.8. Nicotinic Acid, Nicotinamide and Nicotinamide Mononucleotide

NA and NM are metabolised to the coenzymes NAD and NADP (see Section I, 6.2) which are of great importance to the cell both in energy-yielding and biosynthetic processes. However, there have been very few studies on the transport of these vitamins.

NA transport into L. casei was reported to be by active transport and to involve a membrane binding protein, both activities

being repressed by NA. The binding protein was said to be more hydrophilic than those for folate, biotin and thiamine in L. casei and did not interact with their uptake (Henderson and Zevely, 1976; Henderson et al, 1979a).

ND uptake was studied in Strep. faecalis by Neujahr and Varga (1966). The whole uptake process (transport plus metabolism) was dependent on energy and was repressible. However, structural analogues were not tested and metabolism of the ND was very rapid.

McLaren et al (1973) reported that E. coli had a much higher affinity for ND than for NA. They measured the simultaneous rates of uptake of the two vitamins. NA uptake was linear whereas ND uptake was much more rapid and was complete by 10 min, their first time sample. They also reported that E. coli did not accumulate ND in mutants lacking ND deamidase. Griffith and Leach (1973) reported that E. coli had a periplasmic binding protein for ND.

In their studies on the utilisation of exogenous NAD by Sal. typhimurium, Foster et al (1979a) suggested that NDMN had two transport systems. The first and main one was a group translocation system whereby the membrane-bound NDMN glycohydrolase translocated the NDMN into the cell and degraded it to ND and ribose-5-phosphate. The second system became effective at external NDMN concentrations of 10^{-4}M and above and is a facilitated diffusion system. Further proof of this second system and its genetic map locus in Sal. typhimurium were detailed by Kinney et al (1979).

OBJECT OF RESEARCH

Two distinct processes are known whereby B. pertussis undergoes a reversible loss of the pathophysiological properties in response to changes in the growth environment:

i) antigenic modulation, described by Lacey (1960) and typically induced by replacing NaCl with an equivalent amount of $MgSO_4$ or by growth at low temperatures, and

ii) NA modulation, described by Pusztai and Joó (1967) and induced by growth in high concentrations of NA.

There are several reports of the effects of antigenic modulation on B. pertussis, but NA modulation has been almost totally neglected. Wardlaw et al (1976) reported that growth in high concentrations of NA but not of ND caused modulation. They also reported the loss of the same two, heat-modifiable, main envelope proteins that are lost in antigenic modulated cells.

Growth in high concentrations of vitamins is known to repress their transport systems in a variety of bacteria (Kadner, 1978). It is possible, therefore, that the envelope proteins lost during modulation of B. pertussis are involved in the uptake of NA, but not ND. Furthermore, certain transport systems and the composition of the cell envelope of gram-negative bacteria may be controlled by cAMP levels (Aono et al, 1978; Saier, 1979). Parton and Durham (1978) have shown that AC levels were markedly reduced in antigenic modulated B. pertussis. There have been no reports on the effect of NA modulation on AC levels in B. pertussis.

The immediate aims of this investigation were, therefore:

i) to determine the characteristics of NA and ND uptake by B. pertussis,

ii) to determine and compare the effects of growth in high concentrations of NA or ND on the subsequent uptake of the vitamins,

the AC level, and certain other activities of B. pertussis, and iii) to suggest possible mechanisms for NA modulation in B. pertussis.

The longer-term aim of this project was to determine if cell envelope proteins of B. pertussis which may function as virulence components in the pathogenic activities of the organism might, from a biochemical standpoint, be definable as transport proteins for NA, but not for ND. This might lead to a better understanding of the pathogenesis of the disease and, in turn, improve the prospects for immunological or chemotherapeutic control.

SECTION I : BACTERIOLOGICAL MATERIALS AND METHODS

1. Species and Strains

Three Phase I strains of B. pertussis were used:

Strain 134, the strain originally used by Pillemer (1950) in the preparation of his stromata-adsorbed protective antigen;

Strain 2992, a vaccine strain from The Wellcome Research Laboratories, Beckenham, Kent;

Strain 18323, the mouse-virulent Kendrick strain used in the mouse-protection test (Kendrick et al, 1947).

One isolate of B. parapertussis was obtained from the culture collection at The Wellcome Research Laboratories, Beckenham.

B. bronchiseptica strains 7632 and 7635 were also obtained from the culture collection at The Wellcome Research Laboratories, Beckenham.

All the above strains were stored either in the freeze-dried state or under liquid nitrogen (Appendix 7).

E. coli B was obtained from the culture collection of the Microbiology Department, University of Glasgow and was maintained on nutrient agar slopes stored at $4^{o}C$.

2. Media

Bordetella organisms were grown on one or more of the following media:

Charcoal agar (CA) (Difco Laboratories, Detroit, Michigan, U.S.A.); Bordet-Gengou agar (Gibco Bio-cult Diagnostics Ltd., Paisley, Scotland) containing 17% (v/v) defibrinated horse blood (BG) (Gibco);

Cohen and Wheeler Blood Agar (CWBA), a solid medium derived from Cohen and Wheeler's liquid medium (Cohen and Wheeler, 1946) and containing 5% (v/v) defibrinated horse blood. The basal solid medium for CWBA was routinely prepared by the Media Production Unit of The Wellcome Research Laboratories, Beckenham and the blood was obtained from the same source. For growth at NA500 or ND500 on these media, NA or ND was added to the medium to a final concentration of 500 $\mu g\ ml^{-1}$.

B. pertussis strains 134 and 2992 were also grown in Stainer and Scholte's defined liquid medium (SS) (Stainer and Scholte, 1971) modified to contain 5 $\mu g\ ml^{-1}$ NA (SS-NA5) for phase I growth. NA-mode growth was obtained by increasing the NA concentration to 500 $\mu g\ ml^{-1}$ (SS-NA500). In some experiments 500 $\mu g\ ml^{-1}$ ND (SS-ND500) was used in place of NA.

E. coli B was grown either on nutrient agar (Oxoid Ltd., Basingstoke, Hants) or in nutrient broth No. 2 (Oxoid).

The composition and preparation of the media are given in Appendix 1.

3. Growth of Cultures

Freeze-dried cultures were reconstituted with 1% (w/v) Casamino acids solution (Appendix 1) and grown on either BG or CWBA for 72 h at 35-37°C. Liquid nitrogen cultures were left to thaw at room temperature and grown as above. Stock cultures on BG, CWBA or CA were stored at 4°C.

i) Batch cultures : solid media

Stock cultures of Bordetella organisms were inoculated onto BG or CWBA and incubated at 35-37°C for 48 h. These were then sub-

cultured onto the various solid media detailed in 2, and incubated either at 35-37°C for 48 h or at 28°C for 66 h. The plates of solid media were usually incubated in a closed plastic box containing a beaker of water to ensure a moist atmosphere.

E. coli B was grown on nutrient agar for 24 h at 35°C.

ii) Batch cultures : liquid media

Seed cultures of *B. pertussis* were obtained by inoculating 200 ml of SS-NA5 in a 500 ml flask with a loopful of culture from a 48 h BG culture. The seed cultures were incubated at 35-37°C for 24 h on an orbital shaker either at 100 rpm (Mk IIIB, L.H. Engineering Co. Ltd., Bells Hill, Stoke Poges, Bucks) or at 150 rpm (Type Rc-4-Sp, Kuehner, Basel, Switzerland). The seed cultures were used to inoculate (5-9% v/v) 200 ml amounts of SS-NA5, SS-NA500 or SS-ND500 in 500 ml flasks. These cultures were incubated for 24 h at 35-37°C on an orbital shaker as above, except for the SS-NA500 cultures used for uptake studies which were grown for 48 h. Further transfers (5-9% v/v inocula) were made from these cultures into flasks of the same medium. At least two sub-cultures in a given medium were made before using the cells for experimental work. The purity of the cultures was determined by Gram staining and by inoculation onto nutrient agar, which should show no growth, and onto BG.

Seed cultures of *E. coli* B were prepared by inoculating 200 ml of nutrient broth No. 2 (Oxoid) in a 500 ml flask with a loopful of culture from a 24 h nutrient agar culture. The seed cultures were incubated at 35°C for 24 h on an orbital shaker as for *B. pertussis*. The seed cultures were used to inoculate (1% v/v) further 200 ml lots of nutrient broth which were incubated for 24 h at 35°C as above. The purity of the cultures was determined by Gram staining.

iii) Semi-continuous culture of *B. pertussis* 2992

B. pertussis 2992 was grown in semi-continuous culture (SCC) essentially as described by Novotny and Cownley (1979). The fermentor vessel, which was of all glass construction, consisted of a Sovirel 1 litre reaction vessel (Cat. No. 4.296-60), a cover with five ports (4.296-03), a stirrer bearing (4.588-02), a stirrer shaft (4.588-33) and a teflon-coated stirrer rotor (4.588-97) (all supplied by V.A. Howe and Co., London). At the bottom of the reaction vessel there was a glass outlet with a screw joint. This was used as a port for harvesting the culture into 1 litre, sterile glass bottles.

The inoculum culture and all additional fresh media were pumped into the reaction vessel (Vario-perspex pump, L.K.B.) through one of the ports in the glass cover. All flexible connections were of silicone rubber tubing (Esco-Rubber Ltd., London) and clamped as required with artery forceps.

The pH of the culture was kept constant at 7.6 by using a combined glass pH-electrode (Ingold, 465-35, Pye Unicam Ltd., Cambridge) which was inserted through one of the cover ports and was connected to a Radiometer TTT2b titrator (V.A. Howe and Co.) that controlled the addition of 1.0N HCl via a Vario-perspex pump (L.K.B.).

The oxygen content of the medium was monitored by a galvanic type (Pb-Ag) pO_2 electrode which was inserted through one of the cover ports and was connected to a pO_2 meter (both the electrode and the meter were supplied by Western Biological Equipment Ltd., Sherbourne, England). The large-diameter impeller with diagonal blades that was used to stir the culture was kept at a constant speed of 400 rpm. This was sufficient to maintain the air saturation above 10%.

The vessel was heated by a 250W Philips infrared lamp controlled by a Thyristat TA5-0 unit (Cressal Manufacturing Co. Ltd., Birmingham) with a contact thermistor ITT-M53W (Standard Telephone and Cables Ltd., Harlow, Essex) affixed to the side of the reaction vessel.

The whole apparatus (shown diagrammatically in Figure 4) was sterilised by autoclaving at 121°C for 60 min and was then housed in a dust-free cabinet.

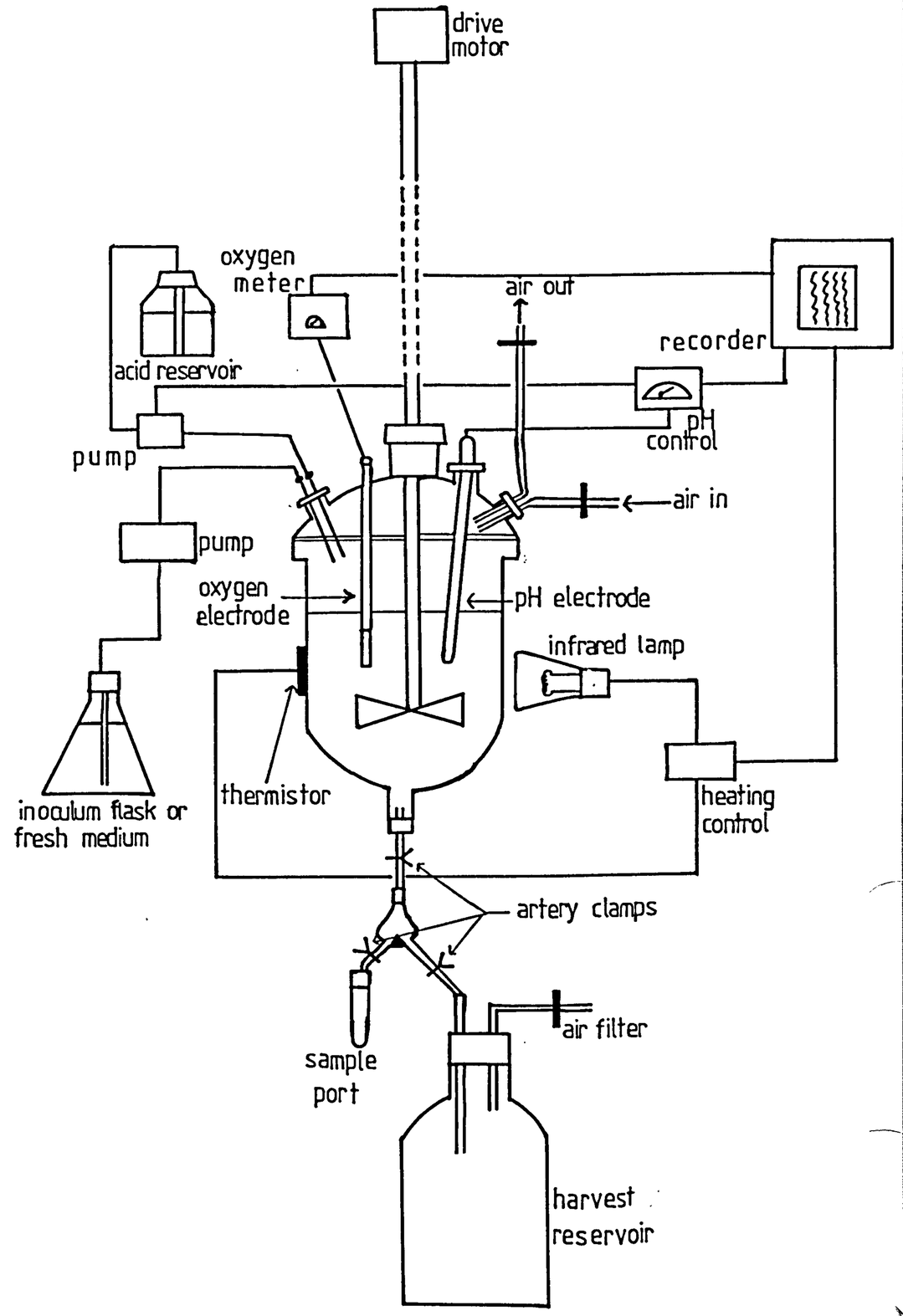
The inoculum for the tank was a 100 ml culture of B. pertussis 2992 grown in SS-NA5 at 35°C for 24 h on an orbital shaker as described in ii) above. Once the inoculum had been pumped into the tank, 600 ml of prewarmed (35°C) SS-NA5 medium was pumped into the tank and the culture incubated at 35°C for 24 h. Approximately 600 ml of the culture was then run off and replaced with 600 ml, prewarmed medium. The culture conditions used in SCC were growth at 35°C or 28°C in SS-NA5 or at 35°C in SS-NA500. At least five subcultures in a particular medium were made before using the cultures for AC assay. The purity of the cultures was checked daily by Gram staining and occasionally by inoculation onto CWBA and nutrient agar.

4. Harvesting the Cultures

The general method used is given below. Variations in the washing and resuspending medium or in the concentration of resuspended cells are detailed in the appropriate section of RESULTS or MATERIALS AND METHODS.

Cultures on solid media were scraped into sterile 0.85% (w/v) NaCl (saline). All cultures, both liquid and solid, were then harvested by centrifugation at 8000 g for 15 min at 4°C either on an MSE High Speed 25 centrifuge or on a Sorvall Superspeed RC2-B centrifuge.

Figure 4. Schematic representation of the apparatus for semi-continuous culture of B. pertussis.



Samples of those culture supernates to be tested for AC activity were decanted into universal bottles and stored on ice. The remainder of the supernate was discarded. The cell pellets were resuspended in phosphate buffered saline, pH 7.3 (PBS) (Appendix 2) and washed once as described above. The cell pellets were resuspended in PBS containing 1% (w/v) monosodium glutamate (PBG) to a concentration of either 50 opacity units (ou) (see 5 below) or 3.0 mg dry weight ml^{-1} (see 6 below).

Cells for experimental use were used immediately. Portions of the batch cultures grown in various liquid media for use in uptake assays were stored, untreated at -20°C. Cell envelopes were prepared from these at a later date (see 9 below) and examined by slab gel electrophoresis (Appendix 6).

5. Standardisation of Bacterial Concentration by Opacity

The concentration of bacterial suspensions was periodically determined by comparison with the Fifth International Opacity Reference Preparation, designated as having 10 international opacity units and supplied by the World Health Organisation International Laboratory for Biological Standards (National Institute for Biological Standards and Controls, Holly Hill, Hampstead, London). The dilution factor necessary to obtain a cell suspension whose density matched that of the reference preparation was calculated and multiplied by 10 to give the concentration in opacity units (ou) of the undiluted cell suspension.

6. Standardisation of Bacterial Concentration by Dry Weight

The concentration of cell suspensions in mg dry weight ml^{-1}

was determined either from a standard curve relating the optical density at 650 nm (OD_{650}) of cell suspensions to their dry weight concentration or from the relationship: OD_{650} 1.4 = 1.0 mg dry weight ml^{-1}. The latter relationship was determined for the Perkin-Elmer Model 6/20 Spectrophotometer (see 7 below) from a standard curve such as that noted above.

Standard curves of OD_{650} versus dry weight were prepared as follows. A 24 h culture of B. pertussis in SS-NA5 was harvested as described in 4 above, washed two or three times with distilled water and finally resuspended in a small volume of distilled water. Weighing boats were prepared by wrapping aluminium foil around the top of a universal bottle and cutting off the excess foil once the cap had been screwed on. These boats were heated at 110°C for at least 24 h before use. Three 1 ml portions of the cell suspension were pipetted into preweighed boats and heated overnight at 110°C before determining the weight of cells.

Dilutions of the cell suspension were made in distilled water and their OD_{650} measured. The dry weight concentration of these cell suspensions was calculated by multiplication of the known dry weight of the original cell suspension by the appropriate dilution factor. A plot of OD_{650} versus dry weight concentration was constructed.

7. Measurement of Optical Density

The optical density of cell suspensions was made at a wavelength of 650 nm either in a SP600 Series 2 Visible Spectrophotometer (Pye Unicam Ltd., York Street, Cambridge) or in a Perkin-Elmer, Model 6/20 Spectrophotometer (Coleman Instruments).

8. Preservation of Cell Suspensions

Portions of cell suspensions to be treated for agglutinogen titres were harvested as in 4 but were washed and resuspended in saline to a concentration of 1.0 mg dry weight ml^{-1}. Formalin (37-39% Formaldehyde solution, AnalaR, British Drug Houses Ltd., Poole, England), was added to a final concentration of 0.25% (v/v) and the suspension left at 37°C for 24 h before being stored at 4°C.

9. Disintegration and Fractionation of Cell Suspensions

9.1. Ultrasonics

Cell suspensions (Section III,3) were disrupted by sonication in an MSE 100 Watt Ultrasonic Disintegrator set at maximum amplitude. *E. coli* B was sonicated for eight 30 sec pulses (giving about 95% lysis as estimated by phase contrast microscopy) and *B. pertussis* 134 was sonicated for fourteen 30 sec pulses (giving about 75% lysis).

Cell lysates were prepared from the disintegrated cells by centrifuging at 20000 g for 30 min at 4°C in an MSE High Speed 25 centrifuge and discarding the pellet. The supernates were assayed immediately for nicotinamide deamidase activity (Section III,3).

9.2. X-Press

B. pertussis 134 was readily disintegrated by passage through an LKB X-Press (Biotech Ltd., Stockholm). The X-Press chamber, piston and valves were pre-cooled in solid carbon dioxide for about 12 min. One valve was inserted into one side of the chamber and about 10 ml of cell suspension pipetted into the other side, followed by the second valve. Three passages in the chamber were made, giving about 95% lysis.

Cell envelopes and lysates were prepared by centrifuging as in 9.1 above. The cell envelope pellet was washed once in saline, resuspended in the same and stored at -20°C. The cell lysates were assayed immediately for nicotinamide deamidase activity (Section III,3).

9.3. Rotary Disintegrator

Suspensions of B. pertussis prepared for assay of AC activity (Section III,2) were disintegrated in a Rotary Disintegrator (Novotny, 1964) and fractionated as described by Novotny and Cownley (1979).

Seven millilitre of a cell suspension in saline was mixed with Ballotini beads until the levels were the same. The thick suspension was disintegrated in the rotary disintegrator for 2 min and then filtered through a sintered glass filter to remove the beads. The beads were washed several times with distilled water to recover quantitatively all cellular material. The final volume was noted.

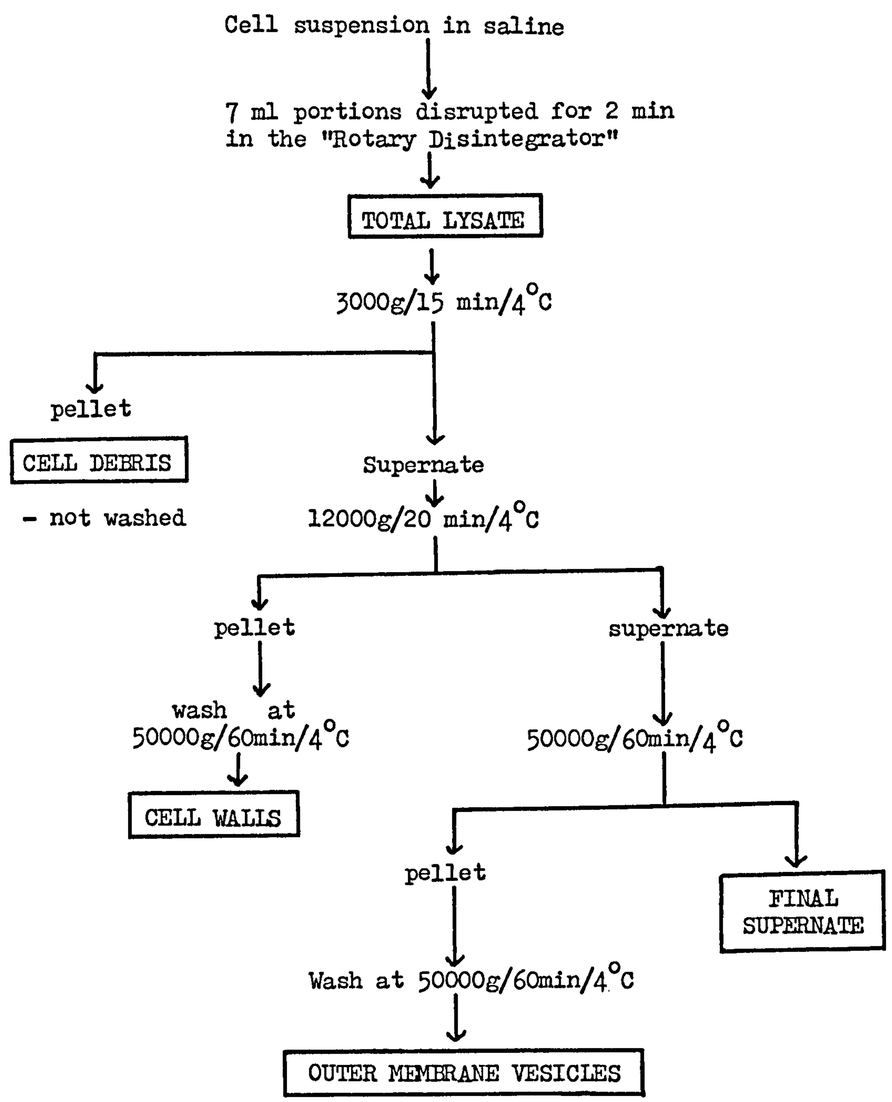
The lysate was then fractionated as shown in Figure 5 by centrifugation in a Sorvall RC2-B Superspeed centrifuge. Fractions were washed and resuspended to a known volume in distilled water and assayed immediately for AC activity and dry weight concentration (by the method detailed in 6 above). The fractions were then stored untreated at -20°C.

SECTION II : BIO-ASSAY MATERIALS AND METHODS

1. Histamine-Sensitising Activity

Seven to nine-week old Ham/ICR mice (Charles River, U.K. Ltd., Manston Road, Margate, Kent) of either sex were injected intraperitoneally, in groups of five, with 0.5 ml of graded doses of heated (56°C for 30 min)

Figure 5. Procedure for fractionation of B. pertussis.



B. pertussis suspensions. Five days later the mice were injected intraperitoneally with 3 mg of histamine dihydrochloride (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) contained in 0.5 ml of sterile saline. Survivors were counted 4 h after challenge.

The results were analysed by the Probit method (Finney, 1964) by means of a computer programme developed in the Department of Epidemiology and Biometrics, School of Hygiene, University of Toronto, Canada. One sample was taken as a standard and the potencies, with the 95% confidence limits, of other samples relative to the standard were calculated. The standard used here was a vaccine of B. pertussis 18334 prepared in this laboratory by Professor A.C. Wardlaw and Dr. R. Parton.

2. Measurement of Agglutinogens

Agglutinogen factors 1, 2 and 3 were assayed in B. pertussis by the method of Novotny and Cownley (1979) using the specific factor sera prepared by them (Novotny and Cownley, 1979).

Serial two-fold dilutions of the sera were made in saline in plastic microtitre haemagglutination trays with U-shaped wells (Linbro) by means of 25 µl Microtitrator diluters. The end well of each row had only saline added and was used as a negative control. Twenty-five microlitre of the formolised cell suspensions (Section I,8) was added to each well. The trays were sealed with self-adhesive membranes, mixed by shaking, incubated at 56°C for 1 h in a waterbath and finally left overnight at room temperature.

Agglutination titres were determined by examining the trays through a Nikon plate microscope and recorded as the reciprocal of the highest dilution of serum giving agglutination.

It should be noted that as it was the antisera that were serially diluted, strictly speaking these assays determined the agglutinin titre of the sera. However, as the titre remained constant and it was the cell agglutinogens that were altered, the results are recorded as agglutinogen titres.

SECTION III : BIOCHEMICAL MATERIALS AND METHODS

1. Uptake of ^{14}C-Nicotinic Acid (NA) and ^{14}C-Nicotinamide (ND)

The general method used is described below, variations are detailed in the appropriate section of RESULTS.

Cell suspensions harvested as described in Section I,4 were prewarmed at 35°C for 10 min in a reciprocal waterbath shaker. Reaction mixtures consisting of 9 ml PBG and either 5.0 μM ^{14}C-NA or 0.5 μM ^{14}C-ND (specific activities 12.5 and 50.0 μCi μmol^{-1} respectively) (Appendix 2) were prepared in universal bottles and prewarmed at 35°C for 10 min, as were the cell suspensions.

Uptake was started by the rapid addition of 1 ml of cell suspension to the reaction mixture by means of a hypodermic syringe and needle. The final cell concentration was either 5 ou or 0.3 mg dry weight ml^{-1}. The reaction mixtures were further incubated at 35°C in the water bath with shaking. At designated times, 1 ml samples were removed and rapidly filtered through cellulose acetate membrane filters of 25 mm diameter and 0.45 μm pore size (Oxoid). The filters had been prewashed and were then post-washed with 5 ml of PBS containing either 100 μM NA or ND (for NA or ND uptake respectively) prewarmed to 35°C.

The filters were then placed in scintillation vials, dried under an infrared lamp and 10 ml toluene scintillant added (Appendix 2).

The samples were counted in a liquid scintillation counter (Automatic Beta Gamma Spectromter NE8312, Nuclear Enterprises Ltd., Edinburgh).

Reaction blanks for uptake assays consisted of reaction mixtures identical to those described above but lacking bacterial cells. Two 1 ml samples from the ^{14}C-NA and ^{14}C-ND blanks were filtered and treated exactly as described above.

The counts per minute (cpm) in the blanks were subtracted from the cpm of the samples and the results for uptake converted to pmol ou^{-1} or pmol mg dry weight^{-1} by means of the formula:

$$\text{cpm} \times {}^{1}/_{\text{C.E.}} \times {}^{1}/_{\text{s.a.}} \times \frac{10^{-6}}{2.22} \times {}^{1}/_{\text{cell concentration}}$$

where: i) C.E. is the counting efficiency of the ^{14}C-NA and ^{14}C-ND solutions (Appendix 2);

ii) s.a. is the specific activity of the ^{14}C-NA and ^{14}C-ND solutions (Appendix 2 and above) in μCi μmol^{-1}; and

iii) cell concentration is that in the reaction mixture expressed either in ou or in mg dry weight ml^{-1}.

Rates of uptake were calculated from unweighted linear regression analysis of plots of uptake versus time.

2. Adenylate Cyclase Assay

Cultures harvested as described in Section I,4 were prepared for AC assay as follows:

i) cultures from solid media were washed once and resuspended in sterile saline to a concentration of 0.5-5.0 mg dry weight ml^{-1};

ii) cultures from SCC were not washed. Cell pellets were resuspended in sterile 0.05M Tris-HCl, pH 7.6 to a concentration of 20-30 mg dry weight ml^{-1} for assay in the absence of an ATP-regenerating

system. The suspensions were stored, untreated at -20°C after assay. When required for assay of AC in the presence of an ATP-regenerating system, the suspensions were first diluted 1:5 in sterile saline;

iii) cultures from liquid batch media were washed once and resuspended in sterile saline to a concentration of 1.0 mg dry weight ml^{-1}.

Intact cells, prepared as above, culture supernates (Section I,4) or cell fractions (Section I,9.3) were assayed for AC activity as described below. The assay was performed in two stages:

i) reaction of substrate (ATP) with the enzyme (AC);

ii) assay for the reaction product, cAMP.

i) Reaction of AC with ATP

The assay used was adapted from that of Hewlett and Wolff (1976). All the reagents detailed below were first prewarmed to 30°C.

a) Forty microlitre of 180 mM N-tris-(hydroxymethyl)-methyl glycine (Tricine) (Sigma), pH 8.0 with or without an added ATP-regenerating system, NA or ND, was pipetted into 12 mm x 100 mm rimless, pyrex test-tubes (Jobling) held in a rack in a waterbath set at 30°C.

The ATP-regenerating system consisted of 20.1 mM creatine phosphate and 1 mg dry weight ml^{-1} creatine phosphokinase (both from Sigma) dissolved in 180 mM Tricine, pH 8.0. NA or ND were added to 180 mM Tricine, pH 8.0 (without an added ATP-regenerating system) to give final concentrations of either 15 μg ml^{-1} or 1.5 mg ml^{-1}.

b) Twenty microlitre of the enzyme solution was pipetted into the test-tubes, vortex mixed and the tubes incubated at 30°C for 5 min. Each sample was tested in duplicate.

c) To start the reaction, 60 μl of a solution of 2 mM ATP (Sigma) and

20 mM $MgCl_2$ was pipetted into the tubes, which were then vortex mixed and incubated for 10 min at 30°C.

d) The final concentrations of reagents in the reaction mixture were: 60 mM Tricine, pH 8.0; 1 mM ATP; 10 mM $MgCl_2$; and where present, 6.7 mM creatine phosphate, 0.3 mg ml^{-1} creatine phosphokinase and 5 or 500 μg ml^{-1} NA or ND. The enzyme was contained in about 0.1 to 5.0 mg dry weight ml^{-1} of cellular material.

e) To stop the reaction, 600 μl of a solution of 50 mM Tris-HCl and 4 mM ethylenediaminetetra-acetic acid (EDTA) (B.D.H.), pH 7.5 was pipetted into each tube which was then vortex mixed and heated at 100°C for 5 min.

f) The tubes were then centrifuged at 1200 g for 20 min at 4°C in an MSE 4L centrifuge fitted with a Multitube Carrier.

g) Six hundred microlitre of each supernate was pipetted into glass, screw-cap liquid nitrogen vials containing 150 μl of a solution of 500 mM Tris-HCl and 40 mM EDTA, pH 7.5, vortex mixed and stored at -20°C.

h) Reaction blanks were prepared exactly as described above, a) - g), but using saline in place of enzyme solution and using double all the volumes mentioned.

ii) Assay for cAMP

The product of the AC reaction, cAMP, was assayed by the cAMP binding protein determination of Gilman (1970) with the cAMP Assay Kit purchased from the Radiochemical Centre, Amersham, England.

The assay was based on the competition between unlabelled cAMP in the sample and a fixed quantity of added ^{3}H-cAMP for binding to a protein which had a high specificity and affinity for cAMP. The amount of ^{3}H-cAMP-protein complex formed was inversely related to the amount of unlabelled cAMP present in the sample. Measurement of the

protein-bound radioactivity enabled the amount of unlabelled cAMP in the sample to be calculated. Unbound cAMP was removed by adsorption onto coated charcoal, followed by centrifugation. A portion of the supernate was removed and counted by liquid scintillation counting and the concentration of unlabelled cAMP in the sample determined from a linear standard curve.

The assay was performed as follows:

a) The contents of the kit were reconstituted as detailed in the instructions accompanying the kit and the reagents stored on ice until required.

b) Test-tubes (12 mm x 100 mm) were numbered and placed in a rack kept at $0^{\circ}C$ in an ice-water bath.

c) Serial two-fold dilutions of the standard cAMP reagent were prepared in 200 µl volumes of the AC reaction blank. Final concentrations of the cAMP in 50 µl were 1, 2, 4, 8 and 16 pmol.

d) One hundred and fifty microlitre of the AC reaction blank was pipetted into test-tubes 1 and 2.

e) Fifty microlitre of the AC reaction blank was pipetted into test-tubes 3 and 4.

f) Starting with the lowest level of standard cAMP, 50 µl of each dilution was pipetted into each successive pair of test-tubes (tubes 5-14).

g) Duplicate 50 µl volumes of each sample to be tested were pipetted into successive pairs of the remaining tubes.

h) Fifty microlitre of the ^{3}H-cAMP reagent was pipetted into all the test-tubes, followed by 100 µl of the binding protein reagent added to all the test-tubes except numbers 1 and 2 (see d) above).

i) All the test-tubes were vortex mixed, covered with a piece of

"cling film" and the ice bath containing the tubes placed in a cold room at $4^{o}C$ for at least 2 h.

j) Charcoal blank: tubes 1 and 2 contained no binding protein and no unlabelled cAMP. The cpm not absorbed by the charcoal were the blank cpm for the assay;

C_o: tubes 3 and 4 contained binding protein but had no unlabelled cAMP. These gave the highest cpm for the cAMP-binding protein complex and were known as the C_o values.

k) At the end of the 2 h incubation, 100 µl of the charcoal reagent was pipetted into the number of tubes (usually about 26) that could be centrifuged in one batch and subsequently processed within 10 min.

l) The tubes were vortex mixed and centrifuged at 1600 g for 5 min at $4^{o}C$ on an MSE 4L centrifuge fitted with a Multitube carrier.

m) Without disturbing the charcoal pellet, 200 µl of each supernate was removed and pipetted into scintillation vials containing 800 µl of distilled water.

n) Ten millilitre of scintillant (Cocktail T, Hopkin and Williams Ltd.) was added to the scintillation vials which were then shaken to mix the contents.

o) The radioactivity in the vials was counted in a Packard liquid scintillation counter.

The cpm in the charcoal blank sample were subtracted from all other samples to give Cx values. For each sample, $^{Co}/_{Cx}$ was calculated (Co was defined in step j) above). A linear standard curve was obtained by plotting $^{Co}/_{Cx}$ versus standard cAMP concentration (pmol per assay tube) and the concentration of unlabelled cAMP (pmol per assay tube) in each sample was calculated from this curve.

The results were converted into AC activities, expressed as

pmol cAMP $min^{-1}ml^{-1}$ (of enzyme solution), by multiplication of the pmol per assay tube results by 90 (see Appendix 3) and by the dilution factor of the enzyme solution, where appropriate. AC activities of intact cells or cell fractions were expressed as pmol cAMP min^{-1} mg dry $weight^{-1}$ by division of the pmol cAMP $min^{-1}ml^{-1}$ results by dry weight concentration (mg ml^{-1}) of the undiluted enzyme solutions.

3. Nicotinamide Deamidase Assay

Cultures of B. pertussis 134, in SS-NA5, and E. coli B, in nutrient broth No. 2, grown for 24 h at 35°C were harvested as in Section I,4, washed once and resuspended in a small volume of 0.05M potassium phosphate buffer, pH 7.0. Cell lysates were prepared as described in Section I,9.1 and 9.2 and the ND deamidase activity in these lysates assayed by measuring the conversion of ND to NA (Pardee et al, 1971). The preparation of the reagents is detailed in Appendix 4.

The assay was performed as follows:

a) The reagents and 12 mm x 100 mm test-tubes were prewarmed to 35°C.
b) Three hundred microlitre of a 1.4 mM ^{14}C-ND (2.0 μCi ml^{-1}) solution was pipetted into the test-tubes, followed by 100 μl of 0.5M potassium phosphate buffer, pH 7.0, and the tubes vortex mixed.
c) To start the reaction, 100 μl of undiluted cell lysate was pipetted into duplicate test-tubes for each sample.
d) The tubes were vortex mixed and incubated at 35°C for 15 min.
e) Reagent blanks were prepared and incubated as above (a) to d)), but using 100 μl of either heated cell lysate (100°C for 6 min) or 0.05M potassium phosphate buffer, pH 7.0 in place of the enzyme.
f) To stop the reaction, the tubes were heated at 100°C for 2 min and then cooled on ice.

g) Two hundred microlitre of a solution containing 0.1M NA and 0.1M ND was added to each tube as markers.

h) The tubes were centrifuged at 1600 g for 20 min at $4^{o}C$ on an MSE 6L centrifuge fitted with a Multitube carrier.

i) From each supernate, a 10 µl volume was removed and spotted onto sheets of Whatmann 3MM chromatography paper (9 inch x 57 cm).

j) The sheets were developed by descending paper chromatography for 12 h in a solvent of n-butanol:distilled water:NH_3 (110:19:1, Pinder et al, 1971).

k) NA and ND spots were located as deep purple spots when the dried sheets were viewed under an ultraviolet lamp (Hanovia Lamps, Slough, England). The spots were marked out in pencil and cut out in strips of 1 inch x 2 cm. Only the NA spot of the reagent blank samples was cut out.

l) The Rf values obtained by this method were 0.22 for NA and 0.69 for ND.

m) The strips cut out in k) were placed in scintillation vials, 10 ml toluene scintillant (Appendix 2) added and the vials counted in a liquid scintillation counter (Nuclear Enterprises).

After the correction of the counts for the blank values, the percentage of counts in the NA spot was calculated. ND deamidase activity was calculated from the percentage conversion of ND to NA and converted to specific activity (nmol NA min^{-1}mg $protein^{-1}$) by measuring the protein concentration of the cell lysate (Section III,4i)) by the method of Lowry et al (1951).

4. Measurement of Protein Concentration

The composition of all the reagents is given in Appendix 5.

i) Cell lysates

The protein concentration of the cell lysates used for ND deamidase assays (Section III,3) was measured by the method of Lowry et al (1951) with bovine serum albumin (BSA) (Sigma) as the standard protein.

a) One millilitre volumes of distilled water were pipetted into duplicate tubes as reagent blanks.

b) Into duplicate tubes were then pipetted two 1 ml volumes of the samples.

c) The BSA standard protein was diluted to give concentrations of 100, 75, 50 and 25 $\mu g\ ml^{-1}$ and 1 ml volumes of each dilution pipetted into duplicate tubes.

d) Five millilitre of Reagent D was added to all tubes (a)-c)) which were then vortex mixed.

e) After 10 min incubation at room temperature, 0.5 ml of Reagent C was added to each tube, with immediate mixing.

f) The tubes were then left at room temperature for 30 min, for full colour development, before measuring the optical density at 750 nm of each of the solutions against the reagent blanks (a)).

g) A standard curve of OD_{750} versus BSA concentrations was plotted and the protein concentrations of the cell lysates determined from that curve.

ii) Cell envelopes

The protein concentration of the cell envelopes prepared as in Section I,9.1 and 9.2 were determined by the method of Lowry et al (1951) as modified by Herbert et al (1971) with BSA as the standard protein.

a) The BSA standard protein was diluted to give concentrations of 400, 300, 200, 100, 50 and 25 $\mu g\ ml^{-1}$.

b) Into duplicate test-tubes were pipetted 0.5 ml distilled water as reagent blank, 0.5 ml of each dilution of the BSA standard and 0.5 ml of each sample.

c) To all test-tubes was then added 0.5 ml of 1.0N NaOH.

d) The tubes were vortex mixed, heated at 100°C for 5 min and then left at room temperature.

e) Once cool, 2.5 ml of Reagent D was pipetted into each test-tube and the tubes left at room temperature for 10 min.

f) Finally, 0.5 ml of Reagent C was added to each tube, with immediate mixing and the tubes left at room temperature for 30 min to allow full colour development.

g) The OD_{750} of each tube was measured against the reagent blank and a standard curve plotted as for Cell Lysates (Section III, 4i)).

5. Slab Gel Electrophoresis

i) Cell envelopes from X-pressed *B. pertussis*

Cell envelopes from 24 h batch cultures of *B. pertussis* 134 grown in SS-NA5, SS-NA500 or SS-ND500 were prepared by X-Pressing cell suspensions as described in Section I,9.2. These were analysed by slab gel electrophoresis by the method of Laemmli (1970) as modified by Ames (1974). Periodically, intact cells of *B. pertussis* 134 were analysed by slab gel electrophoresis in an identical manner to cell envelopes.

Electrophoresis was done in the presence of sodium dodecyl sulphate (SDS) in a discontinuous gel and buffer system. The separating and stacking gels contained 11% (w/v) and 5% (w/v) acrylamide respectively (B.D.H.). Both the gels and buffers contained 0.1% (w/v) SDS.

The dimensions of the slabs were approximately 8.0 x 7.0 x 0.3 cm, the separating gels being 6.0 x 7.0 x 0.3 cm. Details of reagents, preparation of gels, staining and destaining are given in Appendix 6.

To prepare samples for application to the gels, 0.5 ml of the sample, adjusted to approximately 1.0 mg protein ml^{-1}, was added to 0.5 ml of 0.125M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.002% (w/v) bromophenol blue. The mixture was heated at 100°C for 5 min prior to application of 25 µl to the gel.

Electrophoresis was done in a Uniscil slab gel electrophoresis unit (Universal Scientific Ltd., London) at a constant current of 15 mA per gel for approximately 3 h. Staining and destaining was by the method of Weber and Osborn (1969), destaining being accomplished with several changes of the destaining solution.

To provide a series of reference molecular weights for the gel bands, the following mixture of proteins was prepared and run under identical conditions: bovine serum albumin (Molecular weight 67,000, Sigma), ovalbumin (43,000, Sigma), bovine chymotrypsinogen (25,700, Miles Servac, Maidenhead, Berks), trypsin (23,000, Armour Pharmaceutical Co., Eastbourne, Sussex) and horse-heart cytochrome c (11,700, Koch Light, Colnbrook, Bucks).

ii) Cell fractions prepared by rotary disintegration

The cell fractions of *B. pertussis* strains 134 and 2992 prepared by rotary disintegration and differential centrifugation (Section I,9.3) were analysed by slab gel electrophoresis in the Tris-glycine system of Laemmli as described above. These gels were prepared and run by Dr. P. Novotny of The Wellcome Research Laboratories, Beckenham, Kent.

6. Sucrose Density Gradient Centrifugation

These gradients were prepared and run by Dr. P. Novotny (Wellcome) jointly with myself.

Sucrose solutions of 70, 64, 58, 52 and 15% (w/v) in 30 mM Tricine, pH 7.8 were prepared. The gradient was constructed in 35 ml centrifuge tubes as follows: 3 ml cushion of Fluorinert FC-48, 3 ml 70% sucrose, 5 ml 64%, 5 ml 58%, 5 ml 52% and 5 ml 15%. Two millilitre of the OMV sample was mixed with 1 ml of the 15% sucrose solution and the 3 ml layered onto the gradient. Paraffin oil was layered on the top of all tubes until they were all of the same weight.

The gradient was centrifuged in a Sorvall centrifuge fitted with a vertical head rotor (Sorvall), at 21000 rpm for 30 min at 4°C and then at 15000 rpm overnight at 4°C. The gradient was collected from the top of the tube by expression using Fluorinert FC-48. Two millilitre fractions were collected and the OD measured in a Uvicord II (LKB). The specific gravity of each fraction was measured in a densitometer (Bellingham and Stanley Ltd., London).

7. Metabolism of ^{14}C-Nicotinic Acid (NA) and ^{14}C-Nicotinamide (ND)

Cultures of *B. pertussis* 134 grown in SS-NA5 for 24 h were harvested, washed and resuspended as described in Section I,4, except that the cells were resuspended in PBG to a final concentration of 5.0 mg dry weight ml^{-1}. Reaction mixtures were prepared for NA and ND uptake as described in Section III,1 except that double the volumes were used and the specific activity of ^{14}C-NA was 50.0 μCi μmol^{-1}.

Uptake of ^{14}C-NA or ^{14}C-ND was initiated by the rapid addition of 2 ml of cell suspension followed by incubation at 35°C for

3 min. Uptake was then stopped and the cells extracted by pouring the whole reaction mixture into 20 ml of 95% ethanol at 70°C and heating for 20 min at 70°C.

The ethanol extract was centrifuged at 10000 g for 30 min at 4°C on an MSE High Speed 25 centrifuge. The supernate was decanted into a flask and stored on ice. The pellet was re-extracted by re-suspension in 1 ml of distilled water, added to 4 ml of 95% ethanol at 70°C and heated for 30 min at 70°C. This extract was centrifuged at 3000 g for 30 min at room temperature on an MSE Superminor centrifuge and the supernate combined with that of the first extract.

The ethanol extracts were concentrated by evaporation to dryness on a Rotary Evaporator (Büchi Rotavapour-R, Orme Scientific Ltd., Manchester). These were resuspended in 5 ml of distilled water to which 0.5 ml of a marker solution containing 0.1M NA, 0.1M ND and 0.05M NAD was added.

Thirty microlitre amounts of each extract were spotted onto sheets of Whatmann 3MM chromatography paper (9 inch x 57 cm) and 5 µl of 0.075M NAMN (Sigma) and 10 µl of 0.089M NDMN (Sigma) as markers were also applied to each spot. The sheets were developed by descending paper chromatography for 12 h in a solvent of Iso-butyric acid:NH_3: distilled water (66:1:33, pH 4.2; Kinney et al, 1979). Spots were located by viewing the dried sheets under an ultraviolet lamp (Hanovia Lamps), marked out in pencil and cut out in strips of 1 inch x 1 cm. The Rf values obtained by this method were 0.84 (ND), 0.71 (NA), 0.41 (NAD and NDMN) and 0.23 (NAMN).

The strips were placed in scintillation vials, 10 ml of toluene scintillant (Appendix 2) added and the vials counted in a liquid scintillation counter (Nuclear Enterprises).

8. Measurement of Glutamate Oxidation

Batch cultures of B. pertussis 134 grown in SS-NA5 for 24 h at 35°C were harvested as in Section I,4, but were washed once and resuspended in 0.05M Tris-HCl buffer, pH 7.3 to a concentration of 1.0 mg dry weight ml^{-1}. The cell suspensions were left at room temperature for 1-2 h and then stored on ice.

Oxidation of glutamate was measured by following the rate of oxygen uptake by the cells on Clarke Oxygen Electrode System (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, U.S.A.), a Lauda-Thermostat unit (MGW Lauda, West Germany) and a flat bed chart recorder (Kipp and Zonen, Holland).

Three millilitre of cell suspension was pipetted into one of the chambers, maintained at 37°C, and the oxygen probe inserted. The endogenous rate of oxygen uptake was monitored for a few minutes after which 0.3 ml of a 0.2M solution in distilled water of glutamate was injected into the chamber. The increased rate of oxygen uptake was monitored for a few minutes before the addition to the chamber of 0.3 ml of one of a variety of solutions, in distilled water, of NA or ND analogues or energy-inhibitors. The effect of these additions on the rate of oxygen uptake was monitored.

RESULTS

SECTION I : THE UPTAKE OF NICOTINIC ACID AND NICOTINAMIDE BY *B. PERTUSSIS*

1. Growth Curve and Dry Weight Standardisation of *B.pertussis* 134

The growth of *B. pertussis* 134 in SS-NA5 and SS-NA500 liquid media was followed by measuring the OD_{650} of samples of the cultures at various times. The results show (Figure 6) that NA500 causes a reduction in the rate of growth of *B. pertussis* 134. This was in contrast to what was reported by Pusztai and Joó (1967) and was not due to pH changes in the media before or after growth (data not shown). Twenty-four hour cultures of *B. pertussis* 134 grown in SS-NA5 were used for the uptake experiments described below. The results of these experiments were expressed in terms of the dry weight of the cells by means of a standard curve relating OD_{650} of cell suspensions to dry weight (Figure 7). It will be seen that an OD of 1.0 corresponds to a dry weight of 580 $\mu g\ ml^{-1}$.

2. Kinetics of NA and ND Uptake by *B. pertussis* Grown in SS-NA5

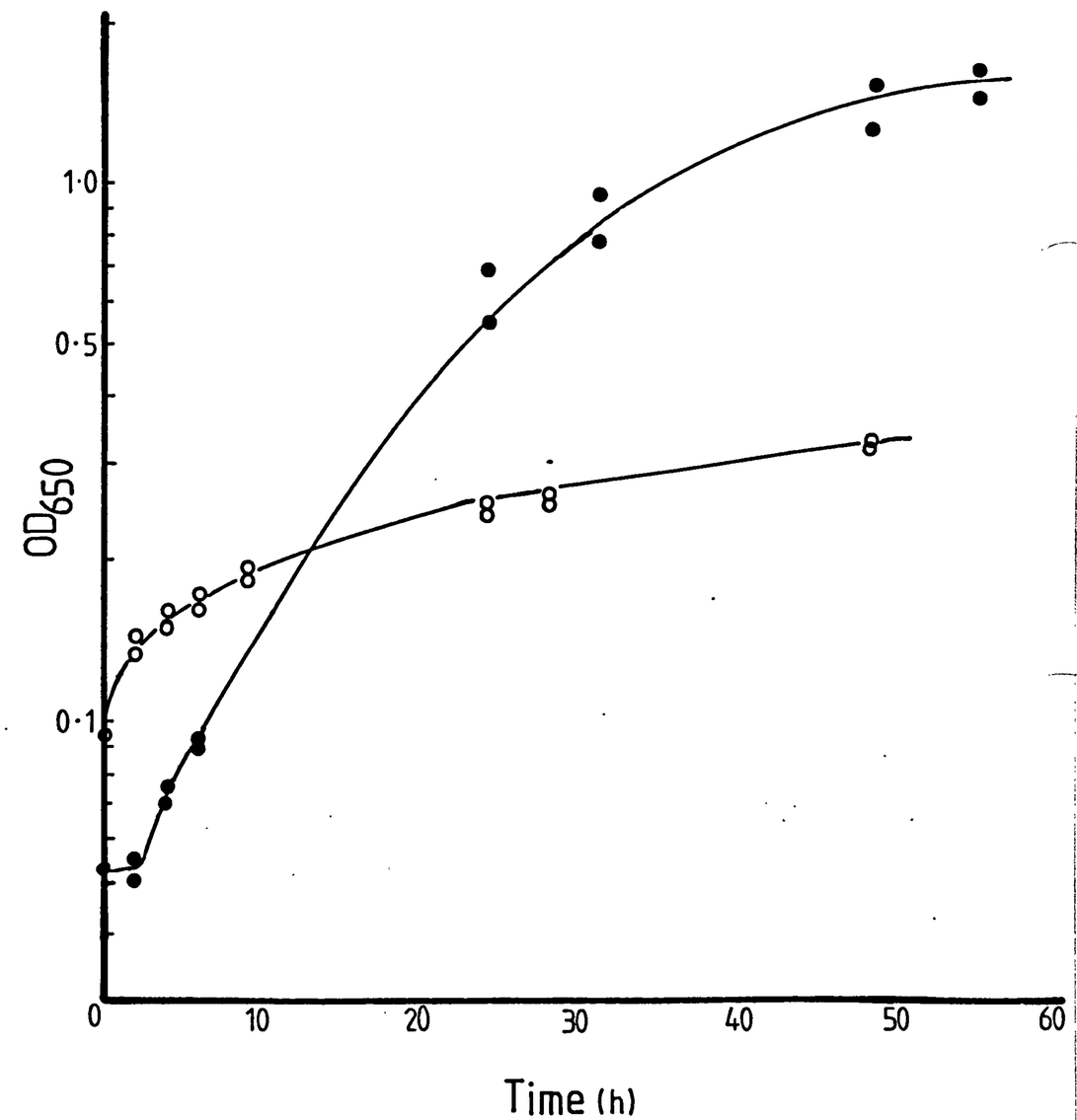
2.1. Definition of Uptake

The word uptake as used here means the total amount of radioactive material accumulated intracellularly at defined times from an external, radioactively-labelled substrate (Heichal *et al*, 1979). It is, therefore, the sum process of transport into the cell plus any subsequent metabolism (Figure 8). Thus, in all of the uptake experiments to be described, there is no distinction made between the transport step and the metabolic step(s). This situation exists

Figure 6. Growth curves of B. pertussis 134 in SS-NA5 and SS-NA500.

SS-NA5 ●——●

SS-NA500 ○——○



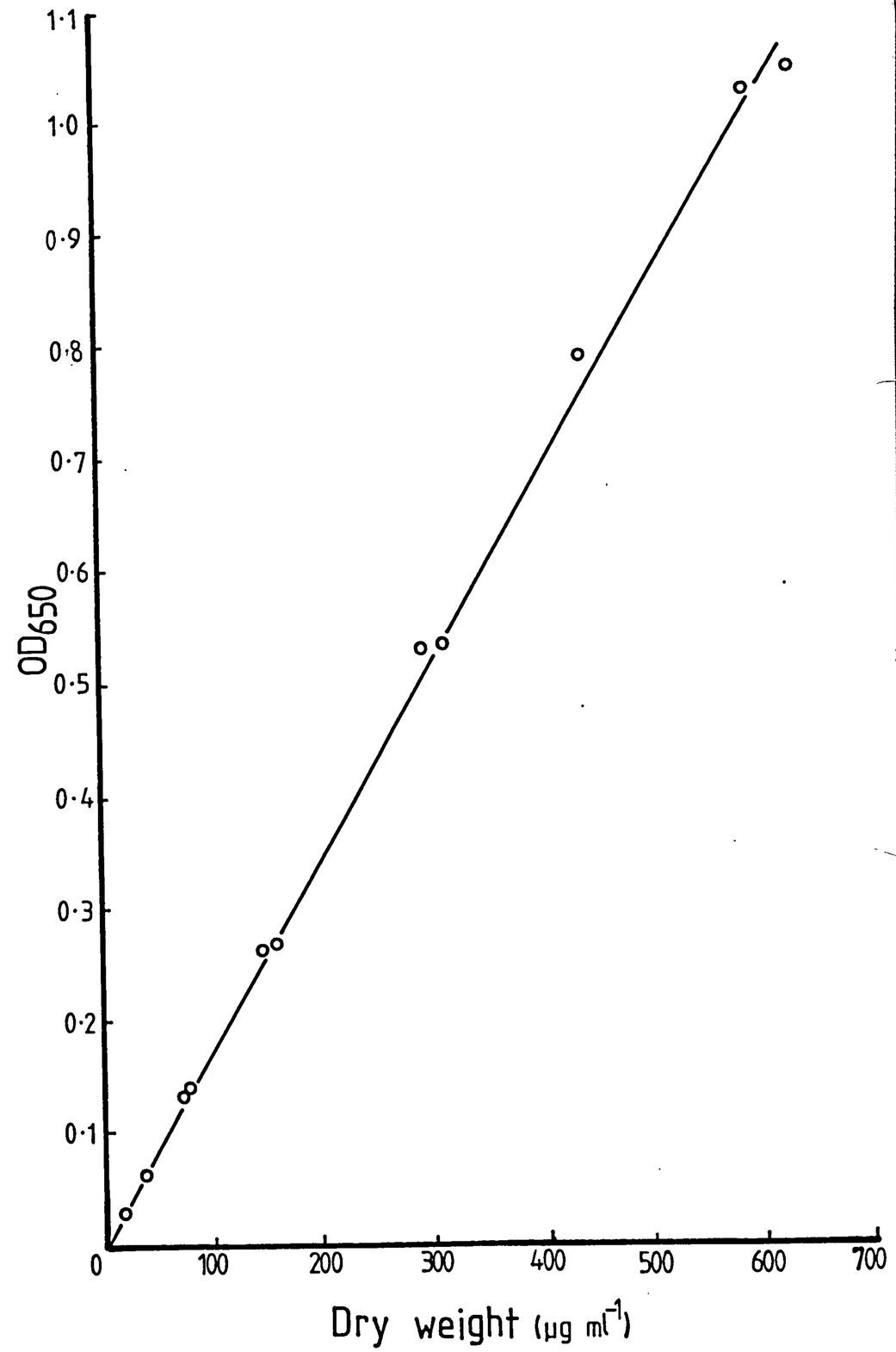


Figure 7. Standard curve relating OD_{650} to dry weight (µg ml^{-1}) of B. pertussis 134.

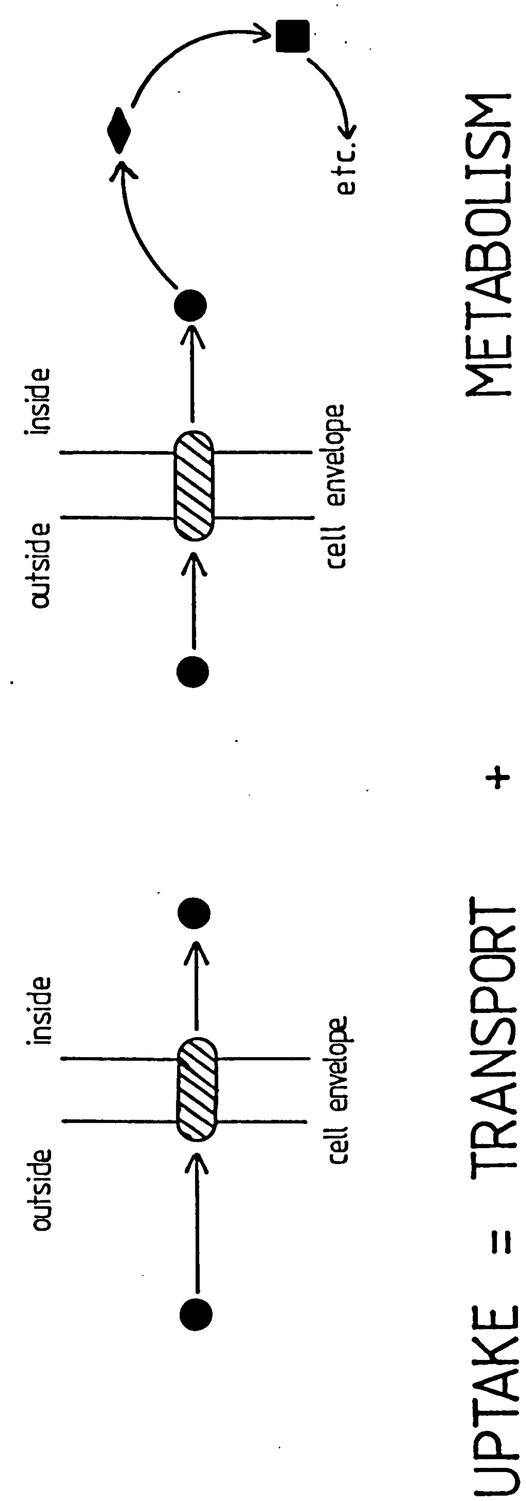


Figure 8. Diagram representing the definition of uptake.

because, unlike that with E. coli, there are no mutant strains of B. pertussis which are incapable of metabolising NA or ND; neither are there analogues of NA or ND which are known to be taken up but not metabolised.

The concentrations of ^{14}C-NA and ^{14}C-ND used in uptake experiments was about 5 to 10x the Km values for NA or ND uptake (see 2.7 and Table 8). At these concentrations, the uptake systems were effectively saturated and the rates of uptake of the vitamins were not dependent on their external concentrations. The 10-fold difference in the concentrations of ^{14}C-NA and ^{14}C-ND used in the uptake experiments is thus explained, as the Km values for NA and ND uptake are very different (Table 8).

2.2. Time Course

The uptake of both NA and ND was linear for at least 30 min (Figure 9). Furthermore, the rates of uptake of both vitamins were almost identical. This extended period of linearity was almost certainly due to the post-transport metabolism of the vitamins thereby trapping them within the cell. In a separate experiment not reported here, it was shown that the rate of uptake of ND over the period 0-1 min was no different from that over the period 1-30 min.

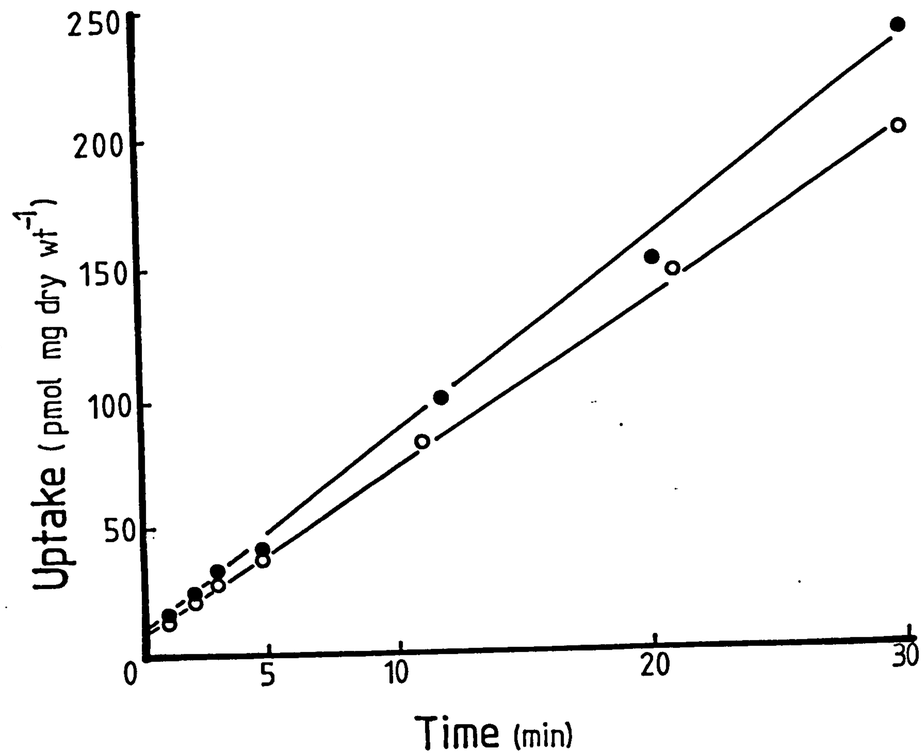
2.3. Effect of Cell Concentration

This experiment was done during preliminary kinetic studies and the conditions of uptake differed slightly from those used in later studies. A 48 h culture of B. pertussis 134 grown in SS-NA5 was used and the initial ^{14}C-NA and ^{14}C-ND concentrations in the uptake medium were both 3.3 μM. A single sample was taken at 60 min, preliminary

Figure 9. Time course of uptake of NA and ND.

●——● ^{14}C-NA, 5.0 μM

○——○ ^{14}C-ND, 0.5 μM.



experiments having shown this was within the range of linearity of uptake. Figure 10 shows that over the range of cell concentrations tested, the uptake of both vitamins was linear and was almost identical. For subsequent experiments, a final cell concentration of 0.3 mg dry weight ml^{-1} was taken.

2.4. Effect of Temperature

The uptake of both NA and ND was dependent on temperature as shown in Figure 11. There was no uptake of either vitamin at $0^{o}C$. Note that the $35^{o}C$ and $0^{o}C$ uptake lines of either vitamin intersect on the y-axis. This is because the blank preparations used for the uptake measurements did not contain organisms. Thus, there was no measure of vitamin bound to the cell envelope, but not transported. The point where the lines meet on the y-axis is an approximate measure of this binding.

2.5. Effect of Glutamate

Both NA and ND were found to require a source of energy for their uptake (Figure 12). *B. pertussis* does not attack carbohydrates and instead derives its energy from the oxidation of amino acids. Glutamate appears to be the preferred amino acid and was found here to support the uptake of both NA and ND. No other amino acids were tested.

2.6. Effect of Potassium Ions

Preliminary studies in which the PBG of uptake assay media was replaced by a 0.05M Tris-HCl-saline buffer, pH 7.3, containing 0.5% (w/v) glutamate (TNG) showed there was no uptake of either NA or ND in the TNG. However, addition of 0.4 mM KCl to the TNG ($TNG.K^{+}$) resulted

Figure 10. Effect of cell concentration on uptake of NA and ND.

●—● ^{14}C-NA, 3.3 μM

○—○ ^{14}C-ND, 3.3 μM.

The 48 h culture in SS-NA5 was harvested, washed and the cell pellet resuspended to a concentration of 8.0 mg dry weight ml^{-1} in PBS containing 0.5% (w/v) glutamate. Appropriate dilutions of this suspension were made in the same medium.

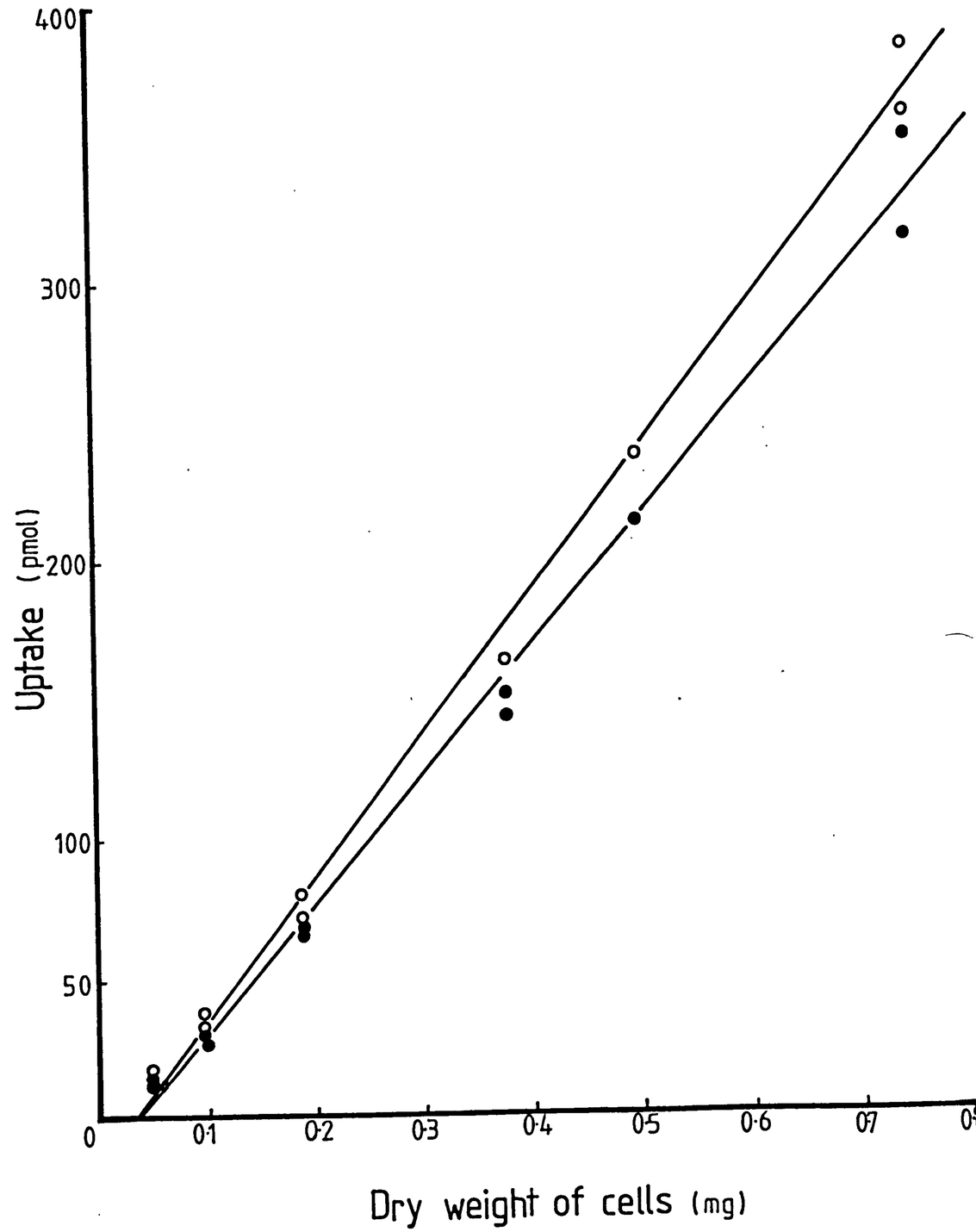


Figure 11. Effect of temperature on NA and ND uptake.

Cells for 0°C uptake were stored at 0°C for 10 min before assay and were washed on the filter with wash solution at 0°C.

●——● ^{14}C-NA, 5.0 μM

○——○ ^{14}C-ND, 0.5 μM.

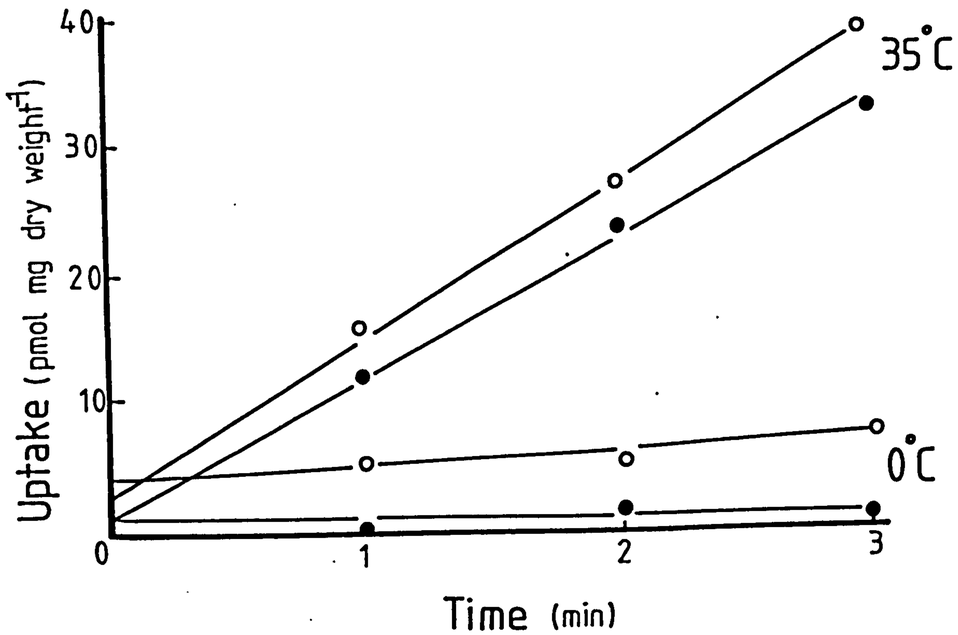
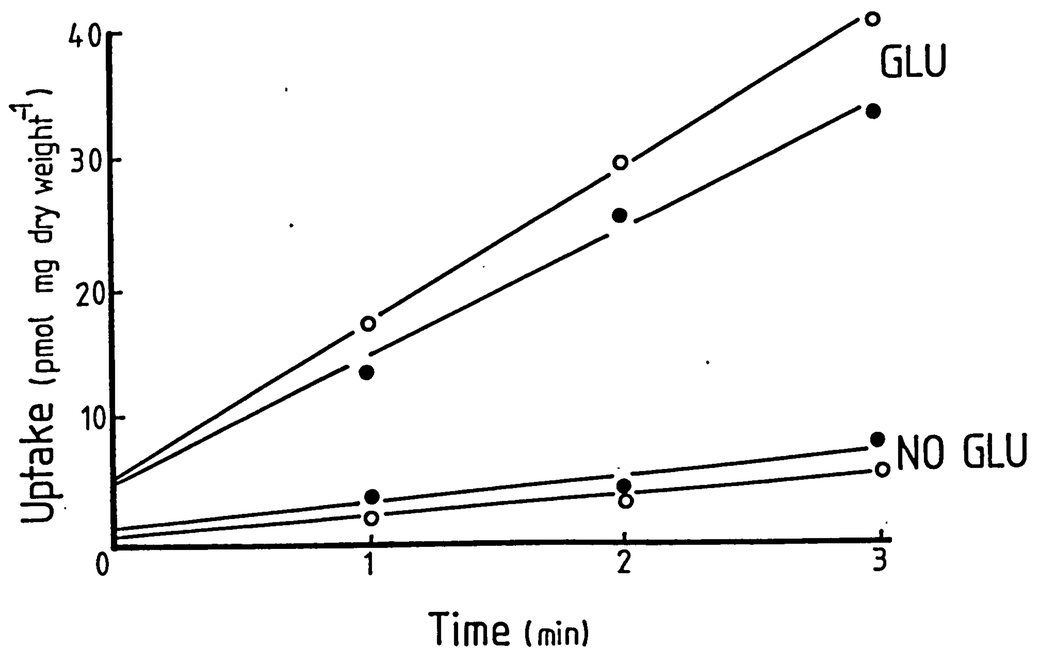


Figure 12. Energy-requirement of NA and ND uptakes.

Cell suspensions to be tested for NA and ND uptake in the absence of glutamate were harvested, washed and re-suspended in PBS to a concentration of 3.0 mg dry weight ml^{-1} and then preincubated for 30 min at 35°C before use in the assay. The reaction mixture for these cells contained 9 ml of PBS in place of PBG.

●—● ^{14}C-NA, 5.0 μM

○—○ ^{14}C-ND, 0.5 μM.



in almost complete recovery of the uptake of both vitamins (Table 7). The concentration of K^+ in PBS is 2.2 mM.

The requirement for K^+ was further established by following the uptake of NA and ND over the period 1-3 min in either a potassium phosphate-saline-glutamate buffer or a sodium phosphate-saline-glutamate buffer. Figure 13 shows that the uptake of both NA and ND was dependent on the presence of K^+.

The requirement, if any, for Na^+, which was present in all uptake assay media, was not determined. The effect of other ions on the uptake of NA and ND was not investigated.

2.7. Effect of External Vitamin Concentration

A range of NA and ND concentrations was prepared from the standard 1 mM ^{14}C-NA and ^{14}C-ND solutions (Appendix 2) by the addition of unlabelled vitamin. Each range of concentration had the same amount of ^{14}C-NA or ^{14}C-ND, but had varying amounts of unlabelled vitamin added.

The rate of NA or ND uptake over 1-3 min was determined for each external vitamin concentration. Plots of these rates against external vitamin concentration show that the uptake of both NA and ND exhibits saturation kinetics (Figure 14 and Figure 15). Double-reciprocal plots of these results (Lineweaver and Burke, 1934) were used to calculate the Km and Vmax values for NA and ND uptake (Table 8). The table shows that NA and ND have the same Vmax value for their uptake, but have very different Km values. The Km value is defined as that concentration of substrate required to give half the maximal rate of uptake. Thus, B. pertussis clearly has a much greater affinity for ND than for NA. The Km and Vmax values reported here fall within the

Table 7. Requirement for K^+ for the uptake of NA and ND

	UPTAKE AT 60 min (pmol mg dry weight^{-1}) OF:(1)	
ASSAY MEDIUM	NA	ND
PBS	17	13
PBG	414	467
TNG	14	15
TNG.K^+	365	298

(1) Cell pellets were washed once in 0.05M Tris-HCl-saline, pH 7.3 and resuspended in TNG. The cell concentration in the assay medium was 0.2 mg dry weight ml^{-1} and the ^{14}C-NA and ^{14}C-ND concentrations were 3.3 µM and 3.8 µM respectively. The concentration of glutamate in either PBG or TNG was 0.5% (w/v). A 1 ml sample was removed from the uptake assay medium at 60 min.

Figure 13. Effect of K^+ on NA and ND uptake

Cell suspensions were washed once either in 0.05M potassium phosphate-saline buffer, pH 7.2, or in 0.05M sodium phosphate-saline buffer, pH 7.2 (see Appendix 2 for their preparation) and resuspended in the same buffer containing 1% (w/v) glutamate to a concentration of 3.0 mg dry weight ml^{-1}.

●——● ^{14}C-NA, 5.0 μM

○——○ ^{14}C-ND, 0.5 μM.

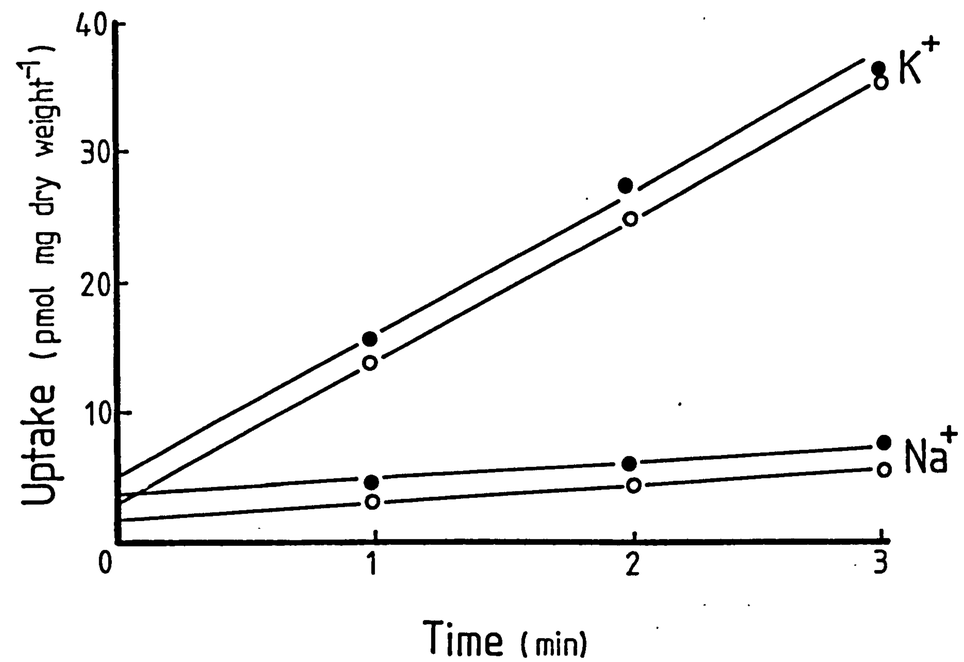


Figure 14. Effect of external NA concentration on NA uptake

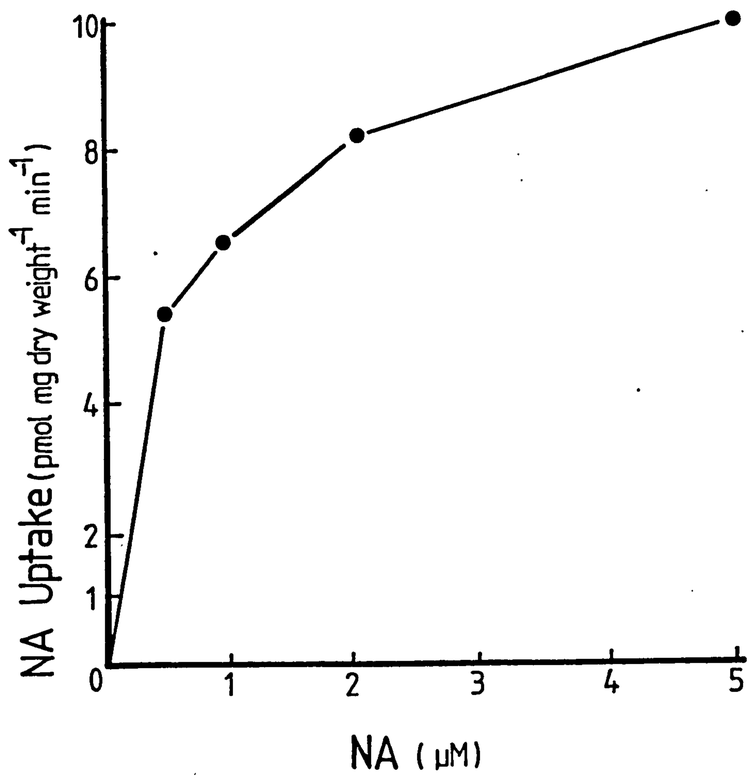


Figure 15. Effect of external ND concentration on ND uptake

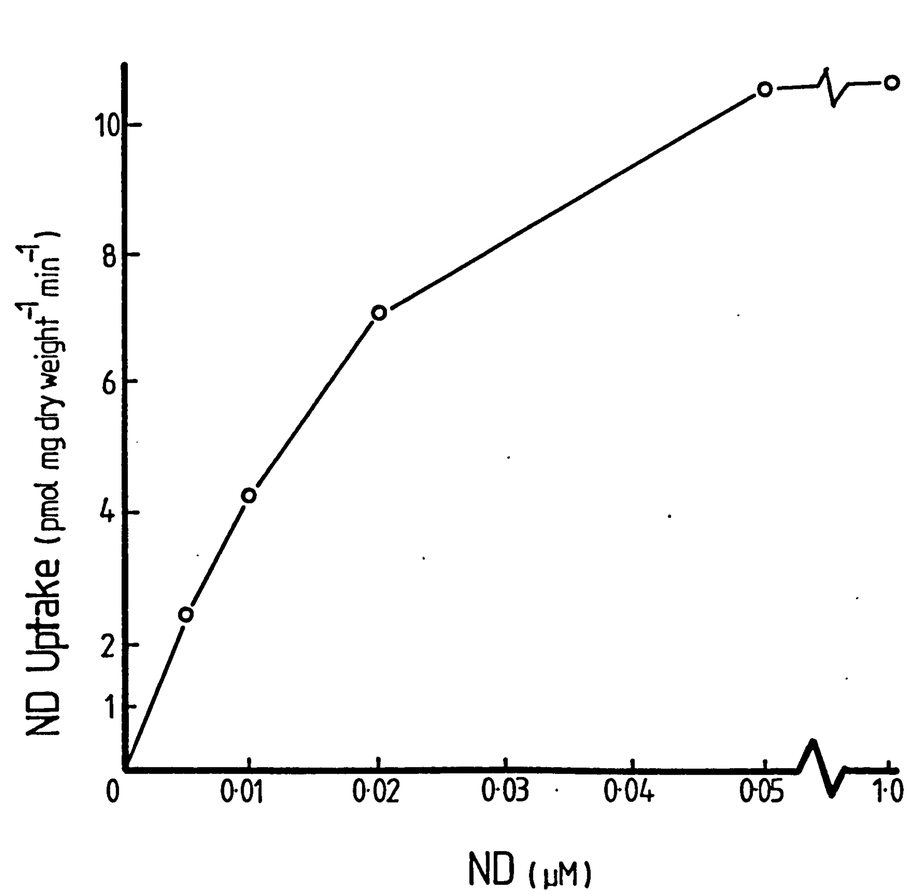


Table 8. Km and Vmax values for the uptake of NA and ND

VITAMIN	Km (μM)	Vmax (pmol mg dry weight^{-1}min^{-1})
NA	1.0 (± 0.2)(1)	13.5 (± 2.4)
ND	0.04 (± 0.009)	18.4 (± 3.8)

The values for NA uptake were the means of 6 determinations and those for ND the means of 4 determinations.

(1) ± Standard Error of Mean.

range of such values reported for other vitamin uptake systems in other bacteria (Kadner, 1978).

3. Metabolism of NA and ND

The metabolism of NA and ND has been examined in some detail in E. coli and Sal. typhimurium. Some of these metabolic enzymes have been observed in many other bacterial species and it appears likely that the metabolism of NA and ND is performed via a pathway common to many bacterial species.

3.1. Metabolism under Uptake-Assay Conditions

The definition of uptake given earlier included both the transport and the metabolism of NA and ND. It would have been desirable, therefore, to have known to what extent these vitamins were metabolised in the conditions employed for uptake assays.

Several attempts were made to determine the extent of that metabolism, but these were unsuccessful. This was principally due to the fact that only very low levels of the vitamin were taken up by the cells and consequently there was only a low amount of radioactive material associated with the cells. It was not possible, therefore, to recover sufficient radioactive material from intact cells for analysis by paper chromatography.

In two experiments, instead of filtering and extracting the cells, the whole reaction mixture was extracted with 95% ethanol at 70°C. This gave sufficient counts for chromatographic analysis, but the vast majority of these counts were due to external vitamin. However, the results obtained were quite informative in a qualitative manner, although it was not possible to calculate the percentage

metabolism of vitamin from the results. Figure 16 shows the chromatogram for NA uptake, and Figure 17 the chromatogram for ND uptake. From these figures it appeared that ND was rapidly converted to NA, but that neither this NA nor the NA from NA uptake was appreciably metabolised. However, this may have been due to a lack of sensitivity of the assay as only low levels of vitamin are taken up. However, in view of the relatively large amount of NA formed from ND, yet little or no appreciable metabolism of that NA, it may be tentatively suggested that the further metabolism of NA is the rate-limiting step in NA and ND metabolism. This would be in agreement with reported results for E. coli and Sal. typhimurium.

3.2. Nicotinamide Deamidase

The observation described above that B. pertussis 134 rapidly converts ND into NA suggested that the organism contained the enzyme nicotinamide deamidase. This enzyme was therefore assayed directly in cell lysates of B. pertussis 134, using E. coli B as a positive control. The results are shown in Table 9. Also given there are the levels of this enzyme in cell lysates of other bacteria. From the table it is clear that not only does B. pertussis contain this enzyme, but it has one of the higher activities so far reported.

4. Effect of Growth Conditions

4.1. Preliminary Studies

B. pertussis 134 was grown for 48 h at 35°C on CA, CA-NA500, BG and BG-NA500 and assayed for the effect of growth on these media on the rates of NA and ND uptake. The cells were used after a single

Figure 16. Chromatogram of extract of whole reaction mixture for NA uptake.

Unlabelled markers

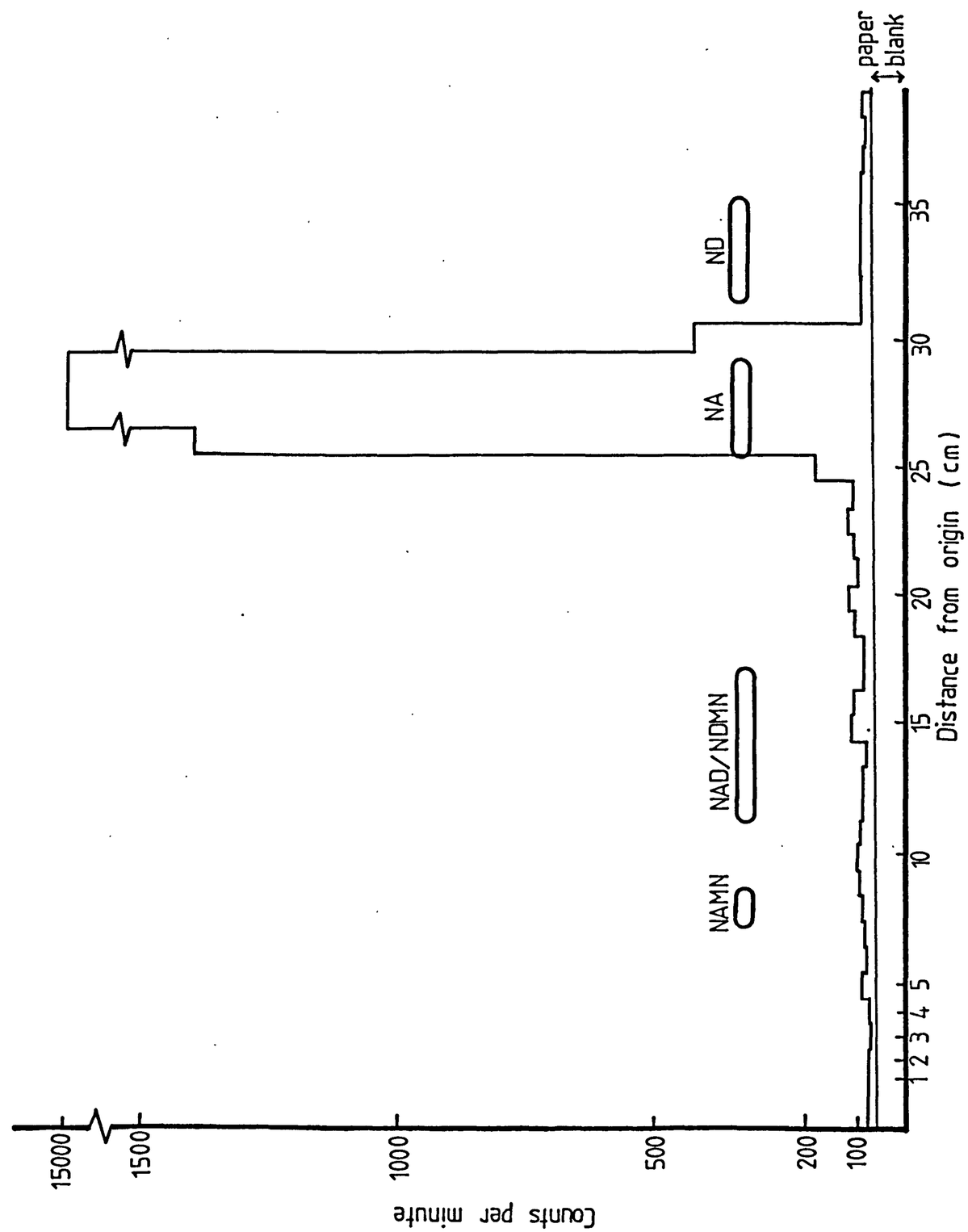
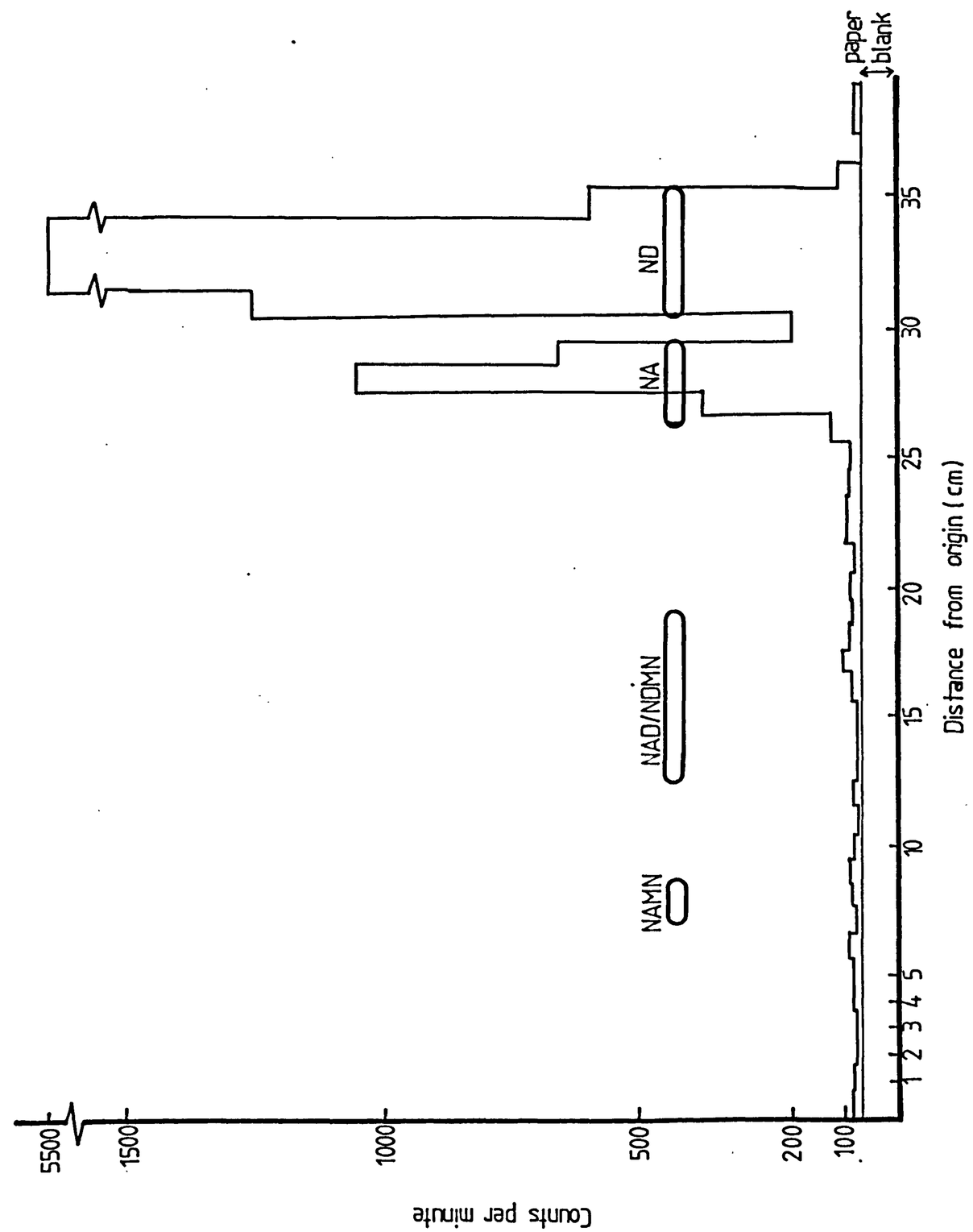


Figure 17. Chromatogram of extract of whole reaction mixture for ND uptake.



Unlabelled markers

Table 9. Nicotinamide deamidase activities in *B. pertussis* and other bacteria.

ORGANISM[1]	NICOTINAMIDE DEAMIDASE[2]
Mycobacterium tuberculosis[3] *var. hominis*	3.2 - 4.3
Escherichia coli B[4]	3.1
Bordetella pertussis 134[4]	0.6 - 0.8
Lactobacillus plantarum[5]	0.2
Salmonella typhimurium LT-2[6]	0.2
Mycobacterium tuberculosis[3] *var. bovis*	0.1 - 0.2
Lactobacillus casei[5]	0.01
Leuconostoc mesenteroides[5]	0.0
Lactobacillus fructosus[5]	0.0

(1) The organisms are listed in descending order of activity.

(2) Specific activity, expressed as nmol NA formed min^{-1} mg $protein^{-1}$.

(3) Kasařov and Moat (1972).

(4) My experimental results.

(5) Ohtsu *et al* (1967).

(6) Foster *et al* (1979b).

subculture on the medium in question.

In these early experiments, the cell concentration was standardised by opacity unitage and uptake was followed by sampling at very long time intervals: 25, 100 and 180 min. Furthermore, the experiments were not repeated.

Figures 18 and 19 show that growth on either CA-NA500 or BG-NA500 had no effect on the rate of uptake of either NA or ND compared with cells grown on CA or BG. This was an unexpected result and so the cultures were analysed for loss of the 28k and 30k SDS-PAGE bands and for loss of HSF activity (Wardlaw et al, 1976) to ensure that these cultures from CA-NA500 and BG-NA500 had modulated.

Figure 20 shows that the 28k and 30k gel bands were lost when the cells were grown on CA-NA500 or BG-NA500. Table 10 shows that the HSF activity of the cells grown on CA-NA500 or on BG-NA500 was also very much reduced. Thus, by these two criteria, the cells grown on CA-NA500 and BG-NA500 had modulated but had retained the capacity for uptake of both NA and ND. Whatever the explanation for this, the results do suggest that the uptake system(s) for the two vitamins cannot be related to either the 28k/30k gel bands or to the components responsible for HSF activity.

4.2. Growth in Stainer and Scholte Liquid Media

Further experiments on the effect of growth conditions on NA and ND uptake were performed with cultures of B. pertussis 134 which had been grown for 24 h in SS-NA5 or SS-ND500 or for 48 h in SS-NA500. Growth in SS-ND500 had no apparent effect on the growth rate of the cultures compared with growth in SS-NA5.

Cell envelopes prepared from such cultures were analysed by

Figure 18. Effect of growth on CA and CA-NA500 on NA and ND uptake.

The cell pellets were prepared for uptake as usual except that the cell concentration in the assay medium was 5 ou, the glutamate concentration was 0.5% (w/v) and the ^{14}C-NA and ^{14}C-ND concentrations were both 1.6 µM.

The uptake was expressed as pmol $(ou.ml)^{-1}$ because ou is an expression of concentration (e.g. equivalent to mg dry weight ml^{-1}) and thus (ou.ml) is an expression of absolute amount of material (e.g. equivalent to mg dry weight) (Wardlaw, 1980).

●——● NA uptake, CA-grown cells

♦——♦ NA uptake, CA-NA500-grown cells

○——○ ND uptake, CA-grown cells

◊——◊ ND uptake, CA-NA500-grown cells

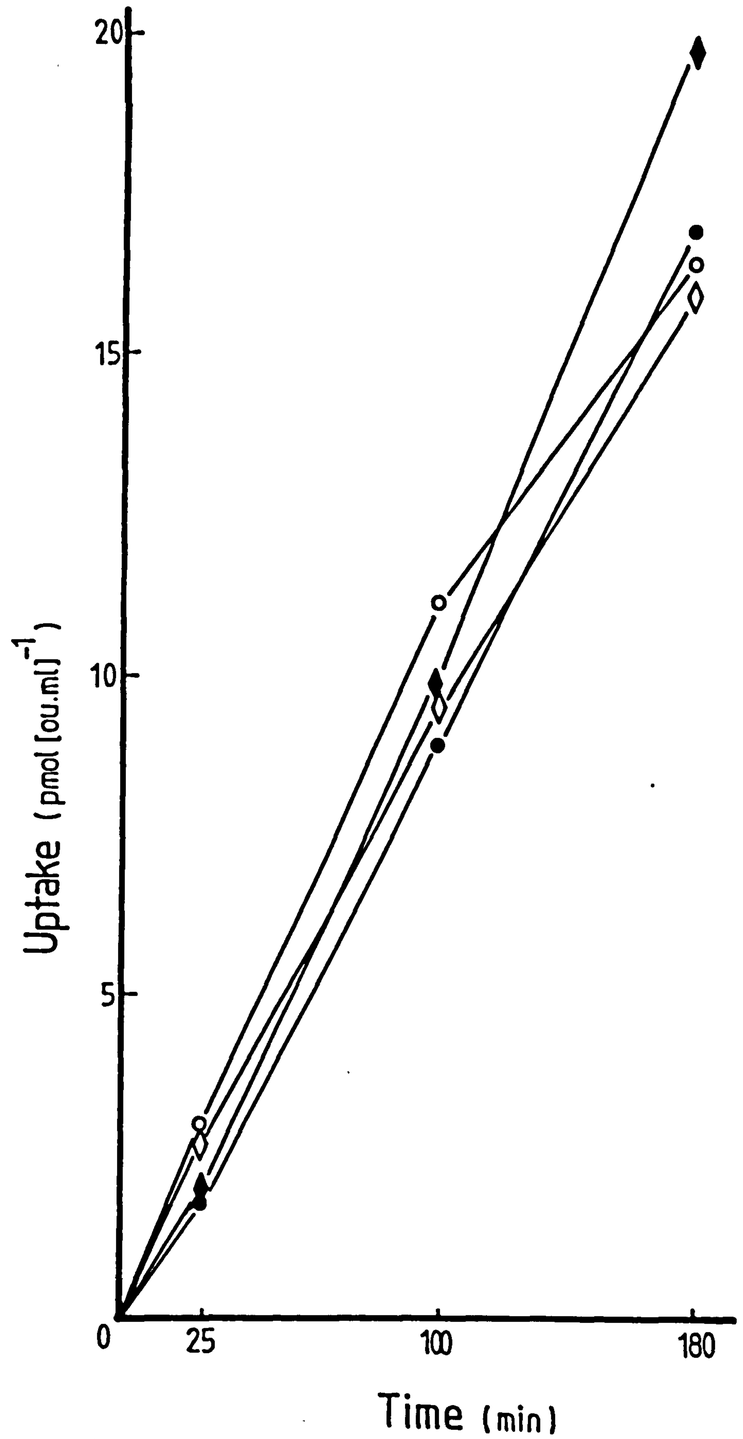


Figure 19. Effect of growth on BG or BG-NA500 on NA and ND uptake.

Assay conditions were as described in Figure 18.

●——● NA uptake, BG-grown cells.

♦——♦ NA uptake, BG-NA500-grown cells.

○——○ ND uptake, BG-grown cells.

◊——◊ ND uptake, BG-NA500-grown cells.

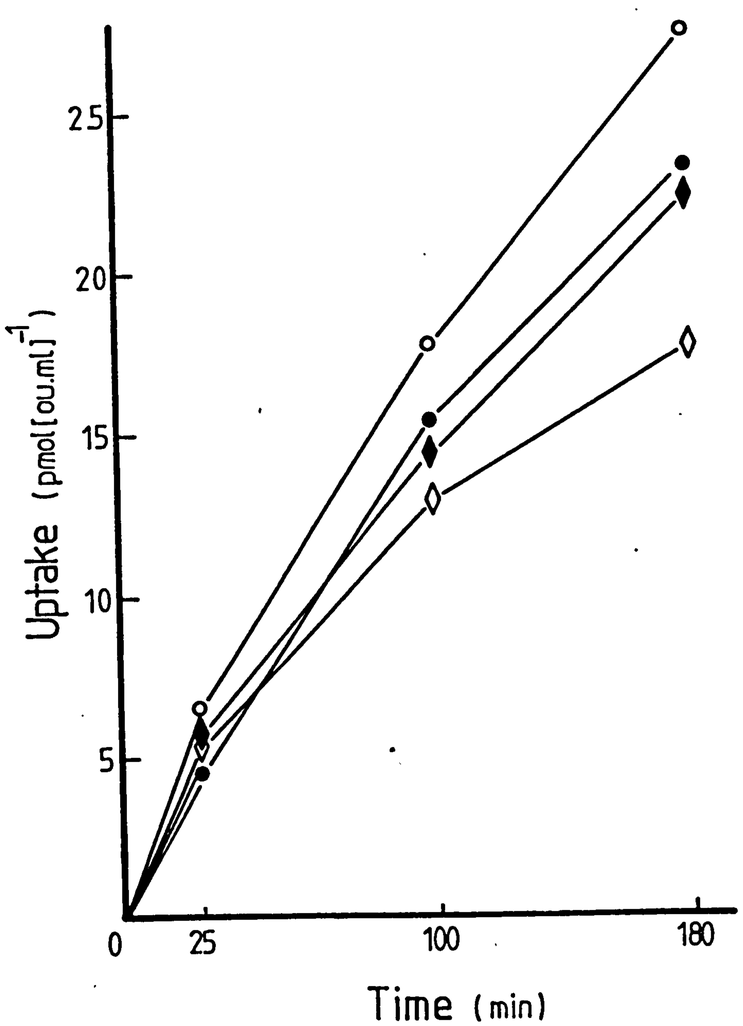


Figure 20. SDS-PAGE profile of intact cells of B. pertussis 134 grown on CA, CA-NA500, BG or BG-NA500.

Track no.	Growth medium
1	CA
2	CA-NA500
3	BG
4	BG-NA500

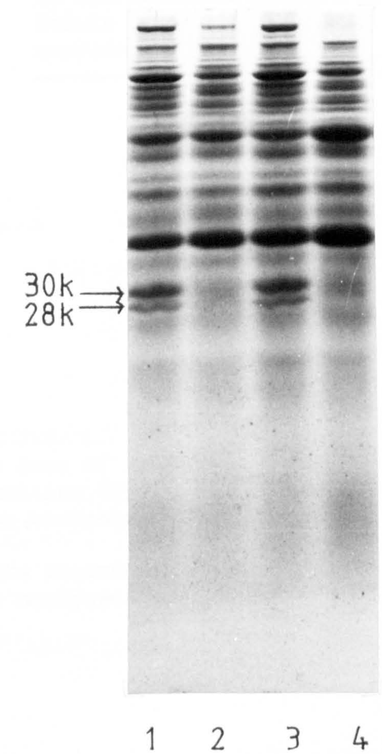


Table 10. Loss of HSF activity from B. pertussis 134 grown on CA-NA500 or BG-NA500.

CULTURE MEDIUM	Potency of vaccine relevant to the standard vaccine of strain 18334 (with 95% confidence limits)	HSD_{50} (1)
CA	0.438 (0.265, 0.718)	0.73
CA-NA500	0.104 (0.062, 0.208)	3.06
BG	0.323 (0.193, 0.527)	0.99
BG-NA500	0.0 (2)	>16.0

(1) HSD_{50}: The dose of sample (ou.ml per mouse) required to sensitise 50% of the test animals to subsequent challenge with histamine.

(2) No deaths were recorded at any of the doses tested, so the results could not be analysed by the Probit method.

SDS-PAGE. Only those cultures grown in SS-NA500 showed a loss of the 28k and 30k gel bands (Figure 21). The gels show, in addition, that there also appear to be changes in other envelope proteins of higher molecular weight in these cultures; whereas cell envelopes from cultures of SS-NA5 or SS-ND500 appeared to be identical (Figure 21). A detailed analysis of changes in envelope proteins was not undertaken.

NA and ND uptakes over the period 1-3 min were assayed with these organisms. Table 11 shows that the uptake of both vitamins by cells grown in SS-NA500 was markedly reduced compared with that by cells grown in SS-NA5. NA uptake by cells grown in SS-ND500 appeared to be no different from that by cells grown in SS-NA5; whereas the uptake of ND by cells grown in SS-ND500 appeared to be slightly reduced, but not as markedly reduced as ND uptake by cells grown in SS-NA500.

These uptake results were analysed statistically by two methods: a) the Paired-sample t-test (Campbell, 1974) (Appendix 8a), and b) the Mann-Whitney U-test (Campbell, 1974) (Appendix 8b). The t-test is based on an assumption of normally distributed data, whereas the U-test is non-parametric and has no such requirement. The t-test, however, is more sensitive to smaller differences between groups than is the U-test.

For validity of the t-test, there is also a requirement for homogeneity of variances of the groups of data to be compared. On this basis, the t-test could not be used to analyse the data for NA or ND uptake by cells grown in SS-NA500 (Appendix 8a). However, the t-test was valid for the analysis of NA or ND uptake by cells grown in SS-ND500. The results (Appendix 8a) showed there was no significant difference ($P > 0.05$) in the rate of NA uptake by cells grown in SS-ND500 as compared with cells grown in SS-NA5, whereas there was a significant

Figure 21. SDS-PAGE profile of cell envelopes of B. pertussis 134 grown in SS-NA5, SS-NA500 or SS-ND500.

Track no.	Growth medium
1	SS-ND500
2	SS-NA5
3	SS-NA500
4	SS-NA5

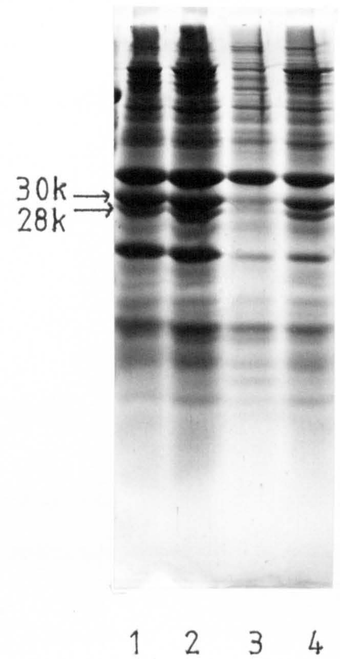


Table 11. The effect of growth in SS-NA5, SS-NA500 or SS-ND500 on NA and ND uptake

GROWTH CONDITIONS	MEAN[1] RATE OF UPTAKE (pmol min^{-1} mg dry $weight^{-1}$) OF:	
	NA[2]	ND[2]
SS-NA5	9.1	8.7
SS-NA500	3.1	2.2
SS-NA5	8.8	8.3
SS-ND500	7.1	4.7

(1) Individual rate values are given in Appendix 8a.

(2) ^{14}C-NA concentration was 5.0 μM, and the ^{14}C-ND concentration was 0.5 μM.

difference ($P < 0.05$) in the rates of ND uptake.

The U-test has no requirement for homogeneity of variances. This test showed a significant difference ($P < 0.05$) in the rate of uptake of either NA or ND by cells grown in SS-NA500 as compared with cells grown in SS-NA5 (Appendix 8b). However, unlike the t-test, the U-test showed no significant difference ($P > 0.05$) in the rate of uptake of either NA or ND by cells grown in SS-ND500 as compared with those grown in SS-NA5 (Appendix 8b).

The conclusions from these statistical analyses were that growth in SS-NA500 causes a significant decrease in the rates of uptake of both NA and ND. This was not found with cultures from CA-NA500 or BG-NA500 media. By contrast, growth in SS-ND500 had no effect on the rate of uptake of NA, whereas there was a marginal effect on the rate of uptake of ND.

5. Effect of Structural Analogues of NA and ND

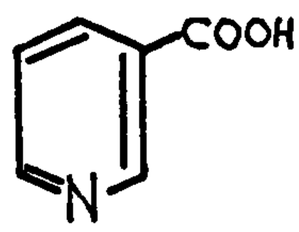
A list of the analogues used and their structures is given in Table 12.

5.1. Effect of Analogues on Uptake

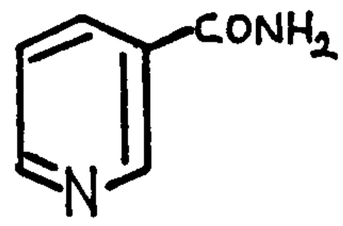
Preliminary experiments were performed with the analogues given in Table 12. These were tested for inhibitory activity at 10 x the molar concentration of ^{14}C-NA or -ND used in the uptake assay. The percentage inhibition was calculated by comparison of that uptake with a negative control containing no added analogue and having only radioactively labelled vitamin present. Positive controls, where inhibition of the vitamin uptake by the vitamin itself is measured (e.g. ^{14}C-ND and ND), were not performed in these preliminary experi-

Table 12. Structural analogues of NA and ND used in uptake-inhibition studies.

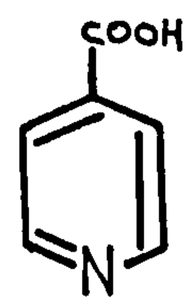
a) Preliminary Experiments:



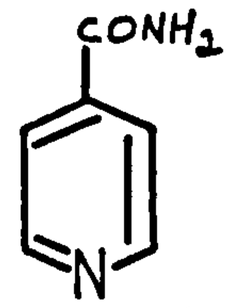
NA



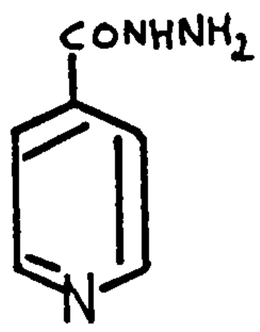
ND



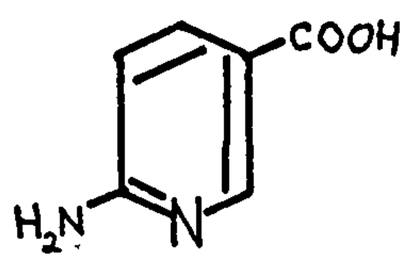
Iso-NA(1)



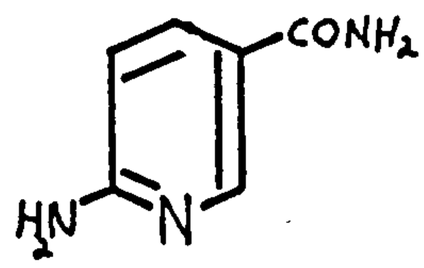
Iso-ND(1)



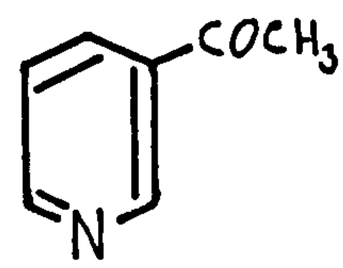
Isoniazid(1)



6-amino-NA(2)

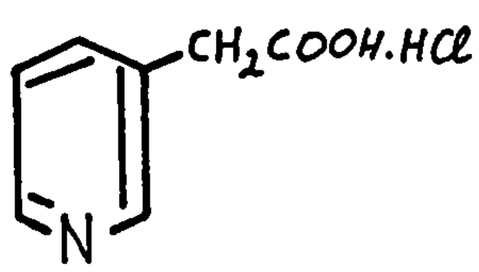


6-amino-ND(2)

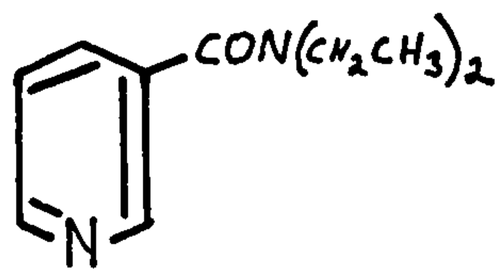


3-acetyl-pyridine(1)

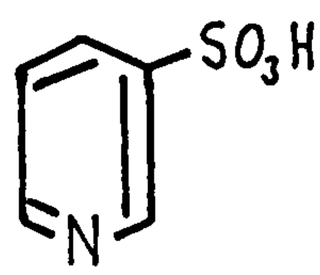
b) Further experiments:



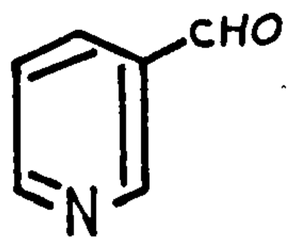
3-Pyridyl-Acetic Acid HCl(3)



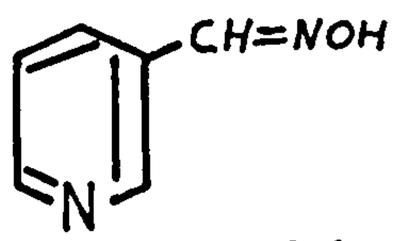
N-N-Diethyl-nicotinamide(2)



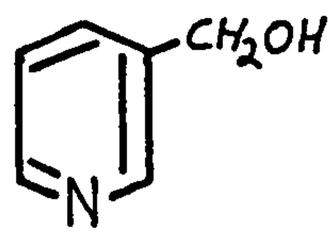
3-Pyridine-Sulfonic Acid(3)



3-Pyridine-Carboxaldehyde (3PC)(3)



3-Pyridine-Aldoxime (3PA)(3)



3-Pyridyl-carbinol(3)

(1) Obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

(2) Obtained from Sigma Chemical Co., St. Louis, U.S.A.

(3) Obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset.

ments, but would have been expected to give 90% inhibition.

Although not complete, therefore, the results of these experiments suggested that the analogues tested may not be fully effective inhibitors of ^{14}C-NA or -ND uptake, although iso-NA gave a slight inhibition of ^{14}C-NA uptake (Table 13). ND was found to inhibit ^{14}C-NA uptake and vice versa.

ND is structurally very similar to NA (Table 12) and yet the degree of inhibition these vitamins had for one another was much less than the expected positive control values. Thus, the low degree of inhibition found with the other analogues may be due to their being less structurally related to NA or ND (Table 12). The uptake system(s) for NA and ND may be sufficiently specific that only certain derivatives of position 3 of the pyridine ring (such as NA and ND themselves) would be effective as inhibitors. Thus, in a second series of experiments a set of such analogues (Table 12) were tested. Positive controls were included in these experiments and the analogues tested at 100 x the molar concentration of the appropriate vitamin.

The results obtained showed that ^{14}C-NA and -ND uptakes had the same pattern of sensitivity to analogue inhibition (Table 14). Both vitamins were completely inhibited by 3-pyridine-carboxaldehyde (3PC) and 3-pyridine-aldoxime (3PA), partially inhibited by 3-pyridyl-carbinol and not inhibited by 3-pyridyl-acetic acid.HCl, N-N-diethylnicotinamide or 3-pyridine-sulfonic acid. The positive controls gave inhibition values very close to the expected value of 99% inhibition for a 100-fold excess of unlabelled vitamin. Thus, none of the analogues tested were found to inhibit ^{14}C-NA or -ND uptake without also inhibiting the other.

As in the preliminary experiments, NA was found to inhibit ^{14}C-ND uptake and vice versa. However, in each case the degree of

Table 13. Preliminary experiments on the inhibition of NA and ND uptake by structural analogues.

ANALOGUE[2]	% INHIBITION OF UPTAKE OF:[1] NA	ND
NA	nt[3] (positive control)	34-39
ND	49-56	nt (positive control)
Iso-NA	15-11	nt
Iso-ND	nt	0
Isoniazid	11-14	0
6-amino-NA	0	nt
6-amino-ND	nt	0
3-acetyl-pyridine	nt	0

^{14}C-NA concentration: 5.0 μM ^{14}C-ND concentration: 0.5 μM

Analogue concentration: 50.0μM Analogue concentration: 5.0μM

(1) The lower and upper figures in the table refer to the percentage inhibition at 2 and 4 min respectively. The analogues were added to the uptake assay medium before addition of the cells to start uptake.

(2) Analogues were prepared in PBG to a concentration of 500 μM and dilutions were made in PBG.

(3) nt: not tested.

Table 14. Effect of analogues on NA and ND uptake

ANALOGUE[2]	% INHIBITION OF UPTAKE OF:[1] NA	ND
NA	98.1 (positive control)	88.7
ND	67.9	97.8 (positive control)
3-Pyridylacetic Acid.HCl	0.0	6.3
N-N-Diethyl-nicotinamide	0.0	3.4
3-Pyridine-sulfonic acid	2.2	2.5
3PC	96.0	100.0
3PA	96.9	100.0
3-Pyridyl-carbinol	50.4	63.2

^{14}C-NA concentration: 5.0 μM ^{14}C-ND concentration: 0.5 μM

Analogue concentration: 500.0μM Analogue concentration:50.0μM

(1) These results are the means of several assays where samples were taken at 2, 3 or 4 min and the percentage inhibition calculated relative to a negative control. The analogues were added to the uptake assay medium before addition of the cells to start uptake.

(2) Analogues were prepared in PBG to a concentration of 500 μM and dilutions were made in PBG.

inhibition was less than the corresponding positive control. This observation was of relevance to the question of whether or not NA and ND share the same transport system.

Thus, when the analogues were tested at concentrations 100 x that of the vitamins, there was no apparent difference in their effects on ^{14}C-NA and -ND uptakes. 3PC, 3PA and the positive controls were then retested at molar ratios of analogue to vitamin of 100, 10 and 1 (Figures 22 and 23). The positive controls gave, as expected, about 99, 90 and 50% inhibitions at these molar ratios. Expression of the results in terms of the molar ratios required to give 50% inhibition (this value is 1 for the positive controls) clearly showed that 3PC and 3PA had in fact quite different effects on ^{14}C-NA and -ND uptake (Table 15). Thus, ^{14}C-ND uptake was more sensitive to inhibition by either 3PC or 3PA than was ^{14}C-NA uptake. Furthermore, 3PC was a stronger inhibitor of the uptake of either vitamin than was either 3PA or the respective positive controls. These results could be explained in terms of different Km values (and therefore in affinity) for the uptake of 3PC, 3PA, ^{14}C-NA and -ND. However, 3PC gave complete inhibition of ^{14}C-ND uptake over the whole range of molar ratios tested (Figure 23). The reason why 3PC was so effective an inhibitor of ^{14}C-ND uptake was unknown, but seemed unlikely to be due to chemical reactivity of the aldehyde group. Benzaldehyde is structurally very similar to 3PC (Table 12) and yet had no inhibitory effect on ^{14}C-ND uptake at any of the molar ratios tested (Figure 23, Table 15).

5.2. Effect of Analogues on Cell Growth

The effect of the above analogues on the growth of *B. pertussis* 134 was examined by addition of the analogues to SS-NA5 liquid

Figure 22. Inhibition of ^{14}C-NA uptake by 3PC, 3PA and NA at various molar ratios of analogue to vitamin.

The ^{14}C-NA concentration was 5.0 μM.

o——o : positive control, i.e. unlabelled NA

×——× : 3PC

●——● : 3PA

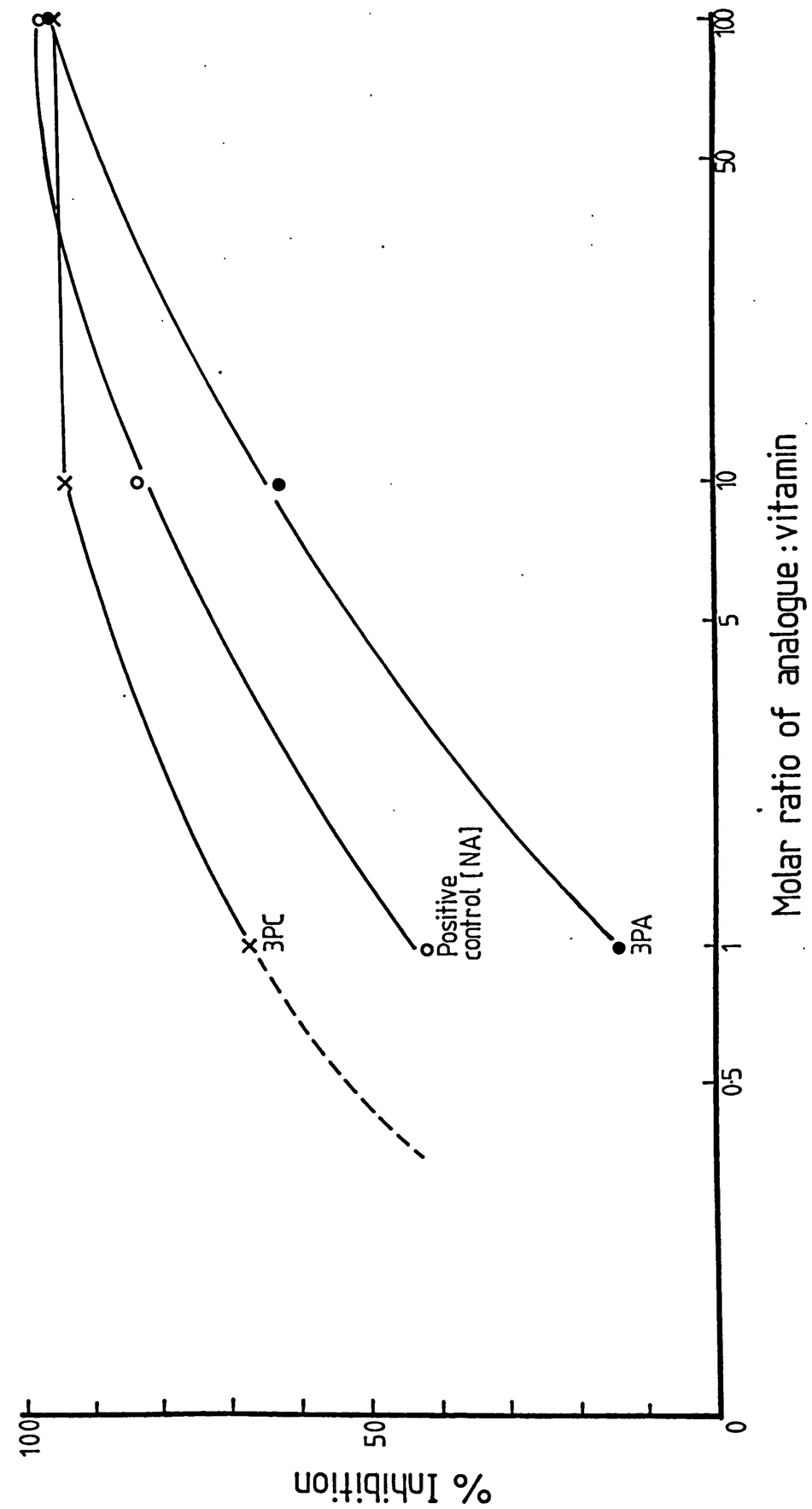


Figure 23. Inhibition of ^{14}C-ND uptake by 3PC, 3PA, benzaldehyde and ND at various molar ratios of analogue to vitamin.

The ^{14}C-ND concentration was 0.5 μM.

o——o : positive control, i.e. unlabelled ND.

×——× : 3PC

●——● : 3PA

□——□ : Benzaldehyde

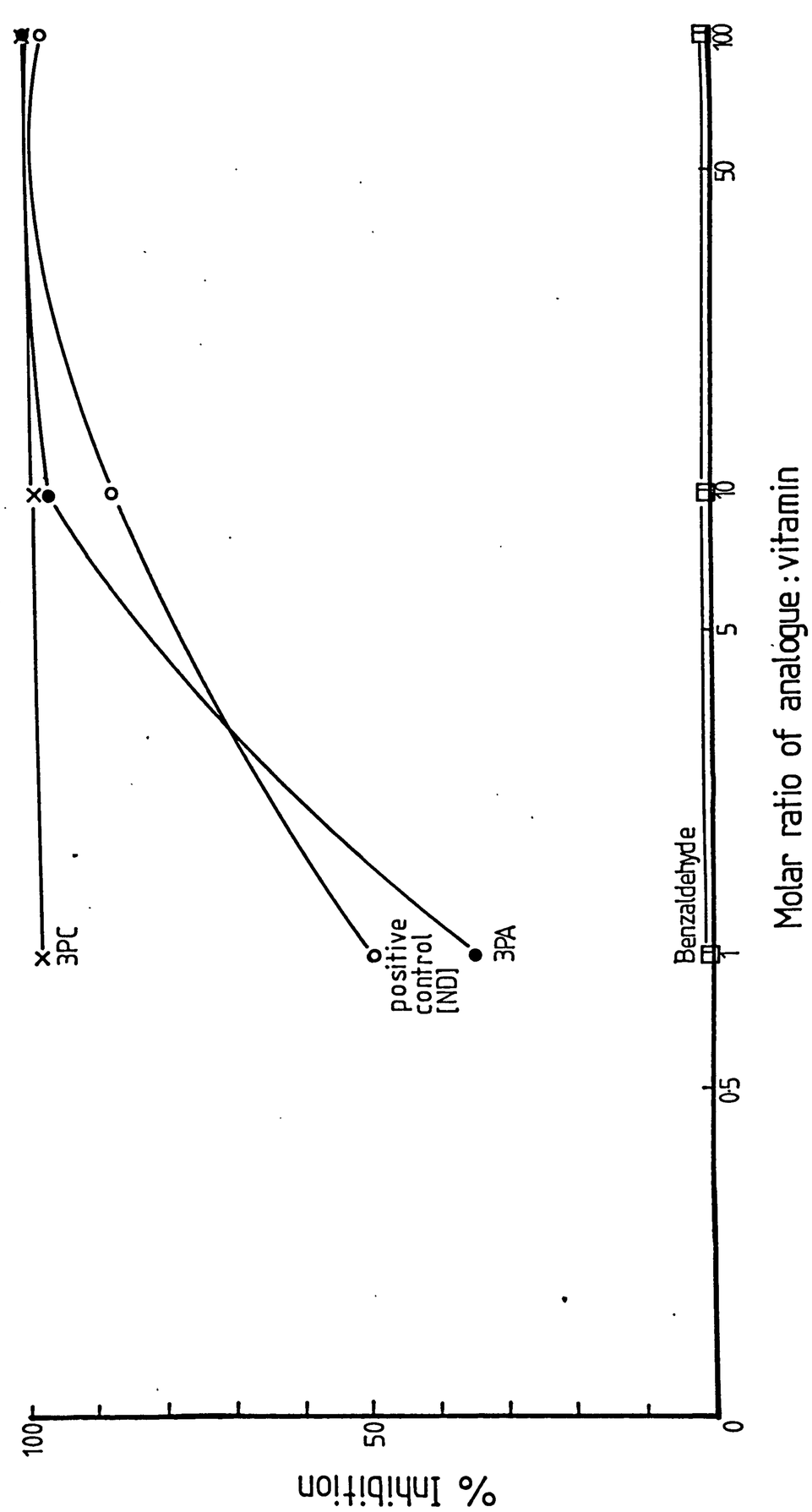


Table 15. Comparison of the inhibitory effects of 3PC, 3PA, benzaldehyde, NA and ND on ^{14}C-NA and ^{14}C-ND uptakes.

	Molar ratio of analogue to vitamin required to give 50% inhibition of the uptake of:	
ANALOGUE	NA	ND
NA	1.4 (positive control)	nt(1)
ND	nt	1.05 (positive control)
3PC	0.56(2)	< 0.1(3)
3PA	5.2	1.5
Benzaldehyde	nt	> 100(4)

(1) nt : not tested.

(2) Obtained by extrapolation of the inhibition curve.

(3) Complete inhibition of ND uptake was found over the range of molar ratios tested.

(4) No inhibition of ND uptake was found over the range of molar ratios tested.

medium at 12 x the concentration of the NA also present. OD_{650} readings were taken at 24 h. Table 16 shows that of the analogues tested, only 3-pyridine-sulfonic acid had an inhibitory effect on growth of B. pertussis 134 under these conditions. However, this analogue was one that had no effect on either NA or ND uptake, even when tested at 100 x the vitamin concentration (Table 14).

5.3. Effect of Analogues and Energy-Inhibitors on Glutamate Oxidation

Glutamate is required for the uptake of both NA and ND. It was possible that the analogues inhibiting NA and ND uptake may have exerted their effect by a general inhibition of glutamate metabolism rather than by a specific inhibition of vitamin uptake. To test this idea certain of the analogues used above were tested for their ability to inhibit the oxidation of glutamate. Table 17 shows that none of them had any inhibitory effect on glutamate oxidation. Thus, those analogues which inhibited NA and ND uptake most probably did so by some specific interaction with the uptake system(s).

It is worthy of note that 3-pyridine-sulfonic acid, which had no effect on the uptake of NA or ND (Table 14) or on the oxidation of glutamate (Table 17), did inhibit the growth of B. pertussis 134 in SS-NA5 medium. This was not a pH effect as 500 μM solutions in PBG of all the analogues tested had pH values of 7.0 to 7.1 (data not shown). The rate of glutamate oxidation declined as the storage time of the cells on ice increased.

Sodium azide and potassium cyanide were found to have no effect, at the concentrations tested, on glutamate oxidation (Table 17). This confirmed the results of Dobrogosz et al (1979) who reported that B. pertussis contained an azide- and cyanide-insensitive cytochrome d_{629} oxidase complex in its respiratory chain.

Table 16. Effect of NA and ND analogues on the growth of *B. pertussis* 134 in SS-NA5.

ADDITION TO SS-NA5[1]	OD_{650} AT 24 h
None	1.404
NA	1.479
ND	1.326
3-Pyridyl-acetic acid.HCl	1.392
N-N-Diethyl-nicotinamide	1.347
3-Pyridyl-sulfonic acid	0.240
3PC	1.332
3PA	1.440
3-Pyridyl-carbinol	1.350

(1) The concentration of NA in SS-NA5 is 40.7 μM.
Analogues were added to give a final concentration of 500 μM i.e. 12.3 x the NA concentration present.

Table 17. Effect of NA and ND analogues and of energy-inhibitors on glutamate oxidation by B. pertussis 134

INITIAL RATE[1] of GLUTAMATE OXIDATION ($\mu l\ O_2$ mg dry weight^{-1}min^{-1})	ADDITION	CONCENTRATION OF ADDITION (μM)	POST-ADDITION RATE OF GLUTAMATE OXIDATION ($\mu l\ O_2$ mg dry weight^{-1}min^{-1})
5.3	3PC	417	5.9
5.3	3PA	417	4.8
3.8	3-Pyridine-sulfonic acid	417	3.5
2.9	3-Pyridyl-carbinol	417	3.3
2.8	Benzaldehyde	417	3.0
1.9	Sodium azide	67,000	2.5
0.9	Potassium cyanide	833	1.2
0.9	Hibitane	Approximately a $^{1}/_{1000}$ dilution	0.0

(1) Initial concentration of glutamate was 18.2 mM.

Inhibition of the oxidation of glutamate was achieved with a dilute solution of hibitane, a cationic detergent which probably acted by disruption of the phospholipid layers of the cell envelope.

SECTION II : THE EFFECT OF NICOTINIC ACID MODULATION ON ADENYLATE CYCLASE AND THE AGGLUTINOGENS

1. Adenylate Cyclase in *Bordetella* Species

Preliminary studies were made in which AC was assayed in intact cells of representative strains of the three species of *Bordetella*: *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. *B. pertussis* was grown for 48 h, and *B. parapertussis* and *B. bronchiseptica* for 24 h, on CWBA.

The results in Table 18 show that all three species contain AC which was measurable in the intact cells. The activity of AC varied from species to species and from strain to strain, and the AC activity of *B. pertussis* 18323 was particularly low. These AC assays were performed without addition of an ATP-regenerating system (see 2 below).

2. Comparison of Adenylate Cyclase Levels in *B. pertussis* Grown on CWBA or in SS-NA5

Hewlett *et al* (1978) reported that the activity of AC in intact cells of *B. pertussis* was much higher in cells grown on solid media containing blood than in liquid media without blood. They showed that this increased activity was due to a protein activator of the AC of *B. pertussis* which was present in the red blood cells of some animals (Hewlett *et al*, 1978, 1979a).

Table 18. AC levels in intact cells of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*

SPECIES	STRAIN	AC ACTIVITY (nmol min^{-1} mg dry $weight^{-1}$)
B. pertussis	134	0.70
	2992	0.15
	18323	0.02
B. parapertussis	Wellcome	0.45
B. bronchiseptica	7632	0.24
	7635	0.25

The results in Table 19 agree with this observation. B. pertussis was grown for 48 h either on CWBA or in SS-NA5 and the levels of intact-cell AC assayed in the absence of an ATP-regenerating system.

Both strains tested had much higher AC activities when grown on CWBA than in SS-NA5. It is noteworthy that the two strains had very similar AC activities when grown in SS-NA5, but had quite different activities when grown on CWBA. Presumably this is a result of different interactions with the protein activator in the blood.

Hewlett et al (1979b) have suggested that this protein activator is present as a contaminant of the ATP-regenerating system. Consequently, the activation of the AC by the ATP-regenerating system may be due mainly to the presence of the contaminating activator. As shown later (3.7), the AC in intact cells from various growth conditions were activated to the same extent in the presence of the ATP-regenerating system. Thus, the presence or absence of this system is not important when comparing the effect of growth conditions on the AC of intact cells. Preliminary experiments were done in the absence, and later experiments in the presence of an ATP-regenerating system.

3. Effect of Growth Conditions on Adenylate Cyclase

3.1. Preliminary Studies

Intact-cell AC levels were assayed, in the absence of an ATP-regenerating system, in B. pertussis 134 and B. pertussis 18323 grown on CWBA under various conditions. All cultures were grown for 48 h except those at 28°C, which were grown for 66 h. Cells were assayed for AC at the first subculture on the medium.

Table 19. Comparison of AC levels in B. pertussis grown on CWBA or in SS-NA5.

STRAIN	MEDIUM	AC ACTIVITY (nmol min^{-1} mg dry $weight^{-1}$)
134	CWBA	0.70
	SS-NA5	0.02
2992	CWBA	0.15
	SS-NA5	0.02

Table 20 shows that the growth conditions had a very marked effect on the intact-cell AC activities. B. pertussis 134 shows a loss of activity when grown on CWBA-NA500 or at 28°C. Growth at this lower temperature was one of the methods used by Lacey (1960) to obtain antigenically modulated cells. Growth on CWBA-ND500 had no effect on AC activity in strain 134.

B. pertussis 18323 also showed a loss of AC activity when grown on CWBA-NA500 (Table 20). The AC activity of strain 18323 grown on CWBA was very low in comparison with other strains (Table 18) and was at the level of AC found in strain 134 grown in SS-NA5. However, when grown on CWBA-ND500, there was a marked increase in the AC activity of strain 18323, raising it to a level comparable with that of other strains grown on CWBA (Tables 18 and 20).

3.2. Batch Cultures

Strains 134 and 2992 were grown in Stainer and Scholte's liquid medium (Stainer and Scholte, 1971), with variations in the NA and ND content, for 24 h in shake flasks. AC activity was assayed, in the presence of an ATP-regenerating system, in intact cells, cell lysates and culture supernates. The cells were used for assay after at least two subcultures in the appropriate medium. The growth rate of B. pertussis 2992 did not appear to be affected by NA500, unlike strain 134 (Section I,1).

The results in Table 21 show that the AC activity of intact cells of both strains was markedly reduced by growth in SS-NA500. In these cultures, the AC activity was also much reduced in cell lysates and culture supernates. Thus, the decreased activity of intact cells cannot be due to excretion of AC or to impermeability of the cell

Table 20. Effect of growth conditions on intact-cell AC activity of *B. pertussis* 134 and *B. pertussis* 18323 grown on CWBA

STRAIN	GROWTH CONDITIONS	AC ACTIVITY (nmol min^{-1} mg dry $weight^{-1}$)
134	CWBA. 37°C	0.70
	CWBA - NA500	0
	CWBA - ND500	0.72
	CWBA. 28°C	0
18323	CWBA. 37°C	0.02
	CWBA - NA500	0
	CWBA - ND500	0.33

Table 21. Effect of growth conditions on AC levels in B. pertussis grown in batch culture.

STRAIN	GROWTH CONDITIONS	AC ACTIVITY		
		INTACT CELLS(1)	TOTAL LYSATE(1)	CULTURE SUPERNATE(2)
134	SS-NA5	13.4	47.4	46.5
	SS-NA500	0.1	0.2	0.05
	SS-ND500	11.8	53.0	17.3
2992	SS-NA5	7.6	62.8	48.8
	SS-NA500	0.02	0.5	0.2
	SS-ND500	17.6	52.7	44.3

(1) nmol min^{-1} mg dry $weight^{-1}$

(2) nmol $min^{-1} ml^{-1}$

envelope to ATP. Growth in SS-ND500 had no effect on the AC activities of either strain.

The intact-cell AC activities in Table 21, and subsequent tables, are about an order of magnitude greater than those presented in Tables 18, 19 and 20. This difference is most probably due to 48 h cultures having been used in these earlier studies (Tables 18-20) whereas all subsequent cultures were harvested at 24 h. Hewlett et al (1979b) have shown that the AC activity starts to decline after about 18 h growth in SS medium.

Lacey (1960) stated that about seven cell divisions were required for antigenic modulation to become established. Pusztai and Joó (1967) did not comment on whether or not cell division was required for NA modulation. The results shown in Table 22 provide some evidence that cell division is required for NA modulation to become established in terms of loss of AC activity. Cultures assayed for AC after a single subculture in SS-NA500 (having been transferred from an exponential SS-NA5 culture) had slightly reduced levels of AC activity compared with those of SS-NA5 cultures. The AC activity was not markedly reduced until the second subculture in SS-NA500, i.e. after approximately 8 cell divisions.

3.3. Semi-Continuous Cultures

B. pertussis 2992 was grown in various conditions in either batch culture or SCC. The main difference between these two cultural conditions was that the pH was kept constant at pH 7.6 in SCC. The cells were subcultured every 24 h and used either for AC assays or treated with formaldehyde and stored for agglutinogen tests.

Table 22. Effect of subculture in SS-NA500 on the AC activity of *B. pertussis* 134 and *B. pertussis* 2992

STRAIN	GROWTH CONDITIONS	SUBCULTURE NUMBER	AC ACTIVITY		
			INTACT CELLS(1)	CELL LYSATE(1)	CULTURE SUPERNATE(2)
134	SS-NA5	1	12.2	nt(3)	87.4
		2	13.4	47.4	46.5
	SS-NA500	1	7.7	nt	7.4
		2	0.1	0.2	0.05
2992	SS-NA5	1	27.3	nt	89.3
		2	7.6	62.8	48.8
	SS-NA500	1	2.9	nt	7.1
		2	0.02	0.5	0.2

(1) nmol min^{-1} mg dry $weight^{-1}$

(2) nmol min^{-1} ml^{-1}

(3) not tested.

The cells were given at least five subcultures in each type of medium before being used for AC assays. This was to allow the cultures to become established in the new growth conditions. Growth in SS-ND500 was not tested in SCC. As with the batch cultures, growth in SS-NA500 had no apparent effect on the growth rate of strain 2992 (Section II,3.2).

AC activity was assayed, in the presence of an ATP-regenerating system, in intact cells and culture supernates. The results show (Table 23) that when grown in SCC, B. pertussis 2992 did not lose AC activity. This was after five subcultures in SS-NA500 in SCC, whereas strain 2992 lost AC activity after two subcultures in SS-NA500 in batch culture (Table 22). However, strain 2992 did lose AC activity when grown at 28°C in SCC. This was not due to excess excretion of AC as levels of the enzyme in culture supernates were also reduced. Nor was it due to impermeability of the cell envelope to ATP as cell lysate AC levels were also reduced.

Thus a subtle difference in the type of cultural condition has resulted in a loss of ability to modulate when grown in SS-NA500, although there was no effect on the ability to modulate when grown at 28°C. SDS-PAGE analysis of these cultures confirmed that the 28k and 30k gel bands were not lost when grown in SS-NA500 in SCC but were lost when grown at 28°C.

3.4. Comparison of Batch and Semi-Continuous Cultures of Strain 2992

Villarejo et al (1978) reported that the cAMP content of E. coli grown in continuous culture was five times greater than that when grown in the same medium in batch culture. On the other hand, Harman and Botsford (1979) found that the rate of cAMP synthesis in

Table 23. Effect of growth conditions in SCC on the AC activity of *B. pertussis* 2992.

GROWTH CONDITIONS	SUBCULTURE NUMBER	AC ACTIVITY		
		INTACT CELLS(1)	CELL LYSATES(1)	CULTURE SUPERNATE(2)
SS-NA5$_1$.37°C	10	4.6	22.8	nt(3)
SS-NA500	5	5.7	12.4	0.05
SS-NA5$_2$.37°C	6	11.5	48.4	0.2
SS-NA5.28°C	6	0.6	0.9	0

(1) nmol min^{-1} mg dry $weight^{-1}$

(2) nmol min^{-1} ml^{-1}

(3) not tested

Sal. typhimurium was greater when grown in batch culture than in continuous culture.

Table 24 shows that when B. pertussis 2992 was grown in SS-NA5 in batch culture, the AC activity was greater than when grown in SCC. Thus, the cAMP content and AC activity of a particular strain grown in a given medium and at the same temperature may vary according to the exact type of cultural conditions, e.g. batch or SCC.

3.5. Inhibition by Nicotinic Acid and Nicotinamide

Kather and Simon (1979) found that the AC of human fat cells was inhibited by NA and postulated that this may be due to an interaction of NA with the insulin effector system rather than an interaction with the AC itself.

In a single preliminary experiment, NA and ND were added to assay mixtures set up to measure the AC activity in cell lysates of batch cultures of strains 134 and 2992 grown in SS-NA5. Assays were done in the absence of an ATP-regenerating system. Table 25 shows that low levels (0.04 mM, 5 $\mu g\ ml^{-1}$) of either NA or ND had no effect on the AC activity. Higher concentrations (4 mM, 500 $\mu g\ ml^{-1}$) of either NA or ND inhibited AC to about 50-60%.

3.6. Cellular Distribution

Hewlett et al (1976) reported that about 90% of the cellular AC of B. pertussis was extracytoplasmic and probably located in the periplasm. This was confirmed by Cowell et al (1979). Novotny and Cownley (1979) have described the preparation of various fractions of B. pertussis, including outer membrane vesicles. It was of interest, therefore, to determine the distribution of AC in these fractions and

Table 24. Comparison of AC activity in batch culture and SCC of *B. pertussis* 2992 grown in SS-NA5

CULTURE CONDITIONS	SUBCULTURE NUMBER	AC ACTIVITY		
		INTACT CELL(1)	CELL LYSATE(1)	CULTURE SUPERNATE(2)
Batch	2	7.6	62.8	48.8
SCC_1(3)	10	4.6	22.8	nt(4)
SCC_2(3)	6	11.5	48.4	0.2

(1) nmol min^{-1} mg dry $weight^{-1}$

(2) nmol min^{-1} ml^{-1}

(3) SCC_1 and SCC_2 refer to the first and second SS-NA5 cultures respectively (see Table 23).

(4) not tested.

Table 25. Inhibition of B. pertussis cell lysate AC by NA and ND

STRAIN(1)	ADDITIONS TO(2) ASSAY MIXTURE	AC ACTIVITY (nmol min^{-1} mg dry $weight^{-1}$)	% (3) INHIBITION
134	NONE	0.19	-
	NA5	0.25	0
	NA500	0.11	42.1
	ND5	0.19	0
	ND500	0.08	57.9
2992	NONE	0.17	-
	NA5	0.13	23.5
	NA500	0.08	52.9
	ND5	0.24	0
	ND500	0.07	58.8

(1) Cells were grown for 24 h in SS-NA5 batch cultures.

(2) Figures give the initial vitamin concentration in $\mu g\ ml^{-1}$.

(3) Calculated as a percentage of the control having no additions to the assay mixture.

the effect of growth conditions on that distribution.

3.6.1. Electron-microscope and SDS-PAGE Analysis of Cellular Fractions.

Batch cultures of B. pertussis strains 134 and 2992 and SCC's of strain 2992 were disintegrated and fractionated to yield: i) cell debris (CD): unbroken cells and fragments of Ballotini beads; ii) cell walls (CW): peptidoglycan layer and cell membrane plus some outer membrane material (Figure 24); iii) outer membrane vesicles (OMV): portions of the outer membrane which tend to form "droplets" (Figure 25); and iv) the 50,000 g supernate (F.SN): cell cytoplasm and periplasm (Novotny and Cownley, 1979). CW and OMV fractions of strains 134 and 2992 from batch culture or SCC and from various growth conditions were virtually the same as those shown in Figure 24 and Figure 25.

SDS-PAGE analysis of the fractions was also done. The gels for the cell lysates, OMV and F.SN fractions from batch cultures of strains 134 and 2992 are shown in Figure 26 and Figure 27. These gels indicate that OMV fractions contain between 3 to 5 main bands and several minor bands. The number of bands is much less than for the cell lysate fractions. The gels also show that growth in SS-NA500 in batch culture caused the loss of the 2 main envelope bands, 28k and 30k, in both strains. Growth in SS-ND500 had no effect on these bands. Growth at 28°C in batch culture also had no effect on the bands or AC, but this was becuase the cultures were used at the first subculture and so had not had enough cell divisions to permit modulation.

3.6.2. Adenylate Cyclase Distribution

The activity of AC in each of the cellular fractions was assayed in the presence of an ATP-regenerating system. Dry weights of all the fractions were measured and used for calculation of the percentage

Figure 24. Electron micrograph of the CW fraction of B. pertussis 2992 grown at 28°C in SCC and negatively stained with phosphotungstate (X 40,000).

CM : Cytoplasmic membrane.

PG : Peptidoglycan layer.

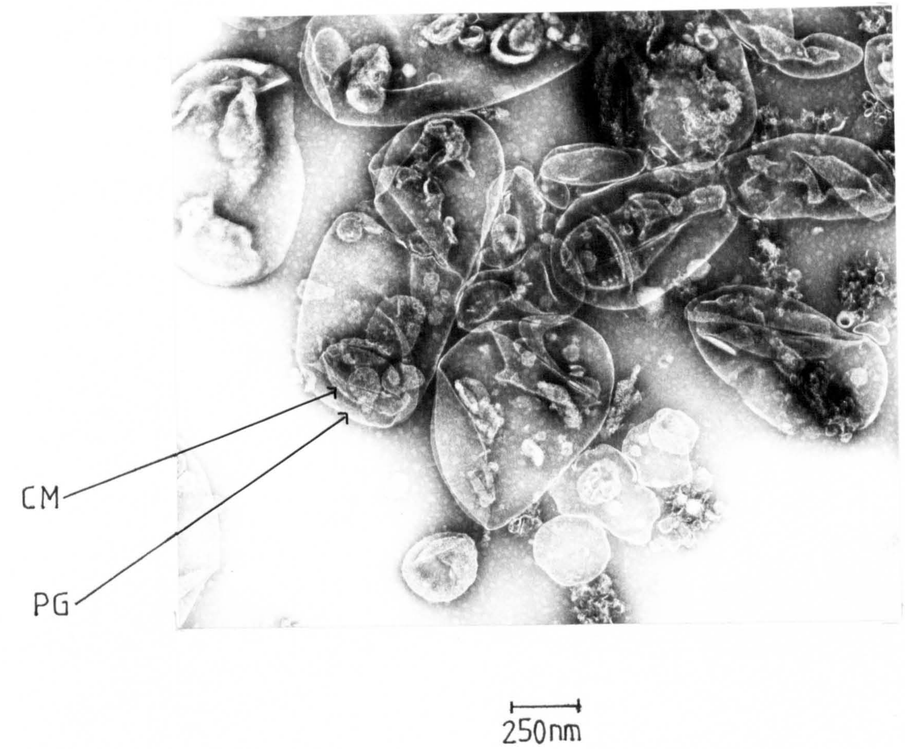
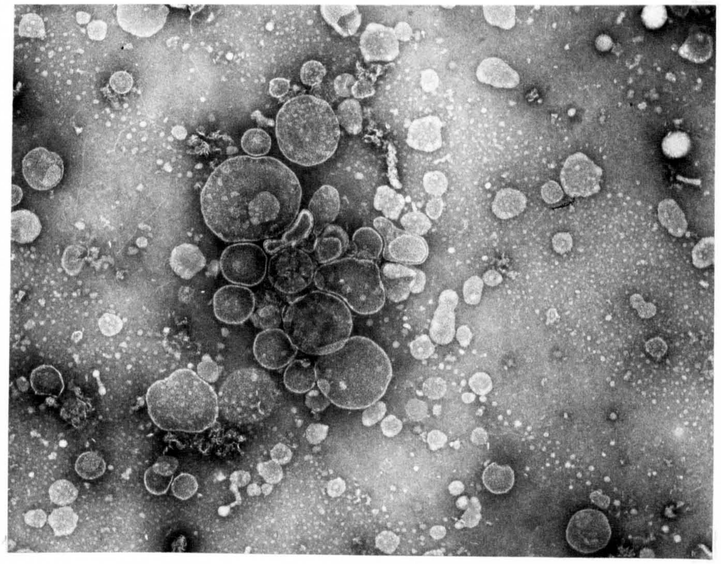


Figure 25. Electron micrograph of the OMV fraction of *B. pertussis* 2992 grown in SS-NA500 in SCC.

Preparation and magnification as for Figure 24.



250nm

Figure 26. SDS-PAGE profile of fractions of B. pertussis 134 grown in various conditions in batch culture.

Track no.	Fraction	Growth Conditions
1	Cell Lysate	SS-NA5
2	" "	SS-NA500
3	" "	SS-28°C
4	" "	SS-ND500
5	OMV	SS-NA5
6	"	SS-NA500
7	"	SS-28°C
8	"	SS-ND500
9	F.SN	SS-NA5
10	"	SS-NA500
11	"	SS-28°C
12	"	SS-ND500

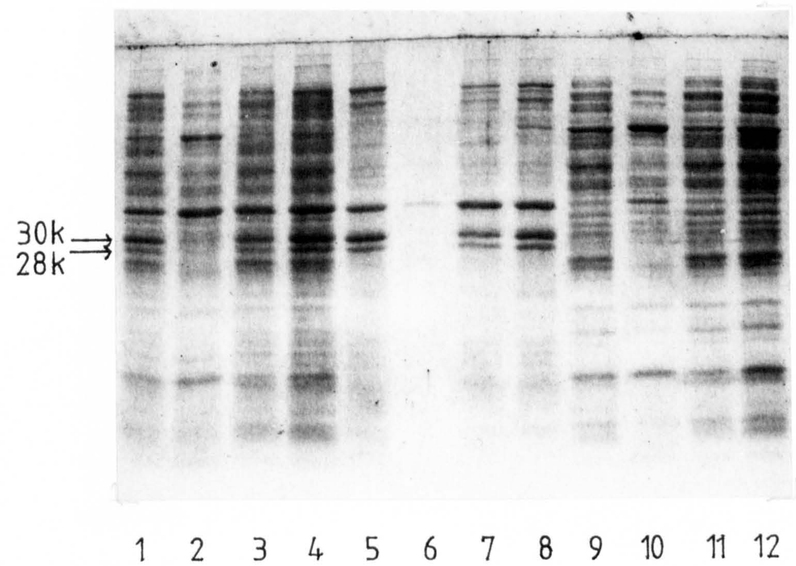
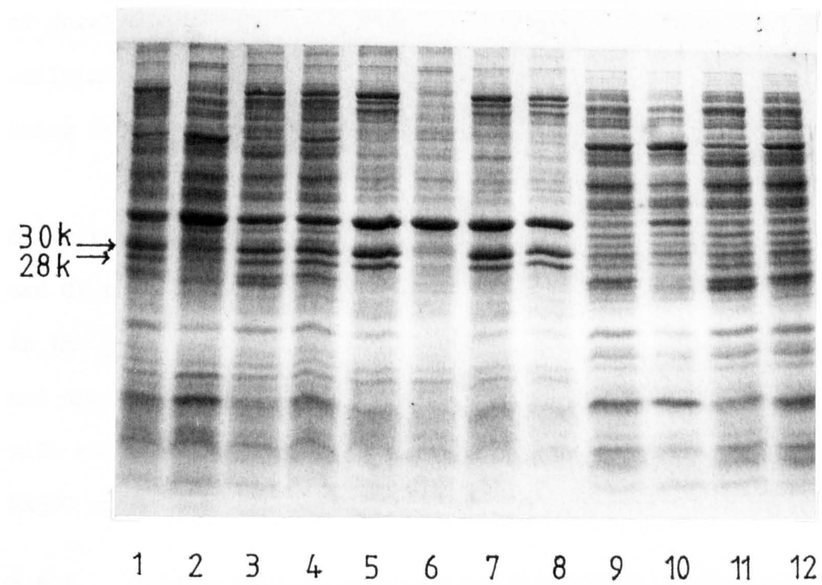


Figure 27. SDS-PAGE profile of fractions of B. pertussis 2992 grown in various conditions in batch culture.

Track no.	Fraction	Growth Conditions
1	Cell Lysate	SS-NA5
2	" "	SS-NA500
3	" "	SS-28°C
4	" "	SS-ND500
5	OMV	SS-NA5
6	"	SS-NA500
7	"	SS-28°C
8	"	SS-ND500
9	F.SN	SS-NA5
10	"	SS-NA500
11	"	SS-28°C
12	"	SS-ND500



distribution of AC in the cell fractions (see also 3.7). The dry weight data (not shown here) also confirmed the report of Novotny and Cownley (1979) that the OMV contained about 10% of the dry weight of the cells.

Table 26 gives the results for batch cultures of strains 134 and 2992, and Table 27 gives the results for SCC's of strain 2992. These results show that most of the AC activity is found in the F.SN fraction (50000 g supernate) and are thus in agreement with the results of Cowell et al (1979). It is noteworthy that between 3 and 13% of the cellular AC activity was in the OMV fraction, the exact percentage being dependent on the strain and growth conditions.

Growth in SS-NA500 in batch culture caused a change in the distribution of the AC in that there was a lower percentage in the OMV and CW fractions of both strains. There was a corresponding increase in the percentage in the F.SN fractions. It is not known whether or not the reduction in the CW fractions represents loss of AC associated with contaminating OM. The distribution of AC in strain 2992 grown in NA500 or at 28°C in SCC was unaltered.

3.6.3. Sucrose Density Gradient Analysis of Outer Membrane Vesicles from Strain 2992

Sucrose density gradient analysis of the OMV fractions was done to confirm that the small percentage of AC activity in these fractions was indeed associated with the OMV and was not a contaminant of the suspending fluid.

Figure 28 shows the results of sucrose density gradient analysis of OMV from B. pertussis 2992 grown in SCC. Each sample gave a clearly visible band and the OMV from the three growth conditions had

Table 26. Effect of growth conditions in batch culture on the cellular distribution of AC in B. pertussis strains 134 and 2992

STRAIN	GROWTH CONDITIONS	% ACTIVITY			
		CD(1)	CW	OMV	F.SN
134	SS-NA5	30.9	9.6	2.8	56.7
	SS-NA500	33.6	6.2	1.8	58.5
	SS-ND500	36.2	13.2	6.2	43.9
2992	SS-NA5	39.0	13.8	5.4	41.8
	SS-NA500	38.0	5.1	0.7	56.2
	SS-ND500	32.0	15.5	6.0	46.4

(1) Cell debris fractions not washed.

Table 27 Effect of growth conditions in SCC on the cellular distribution of AC in B. pertussis 2992

GROWTH CONDITIONS	% AC ACTIVITY			
	CD	CW	OMV	F.SN
SS-NA5	14.4(1)	17.6	12.8	55.3
SS-NA500	39.3(2)	20.8	13.2	26.7
SS-NA5	26.6(2)	23.9	10.1	39.4
SS-28°C	23.1(2)	36.2	9.8	31.0

(1) CD fraction washed

(2) CD fraction not washed.

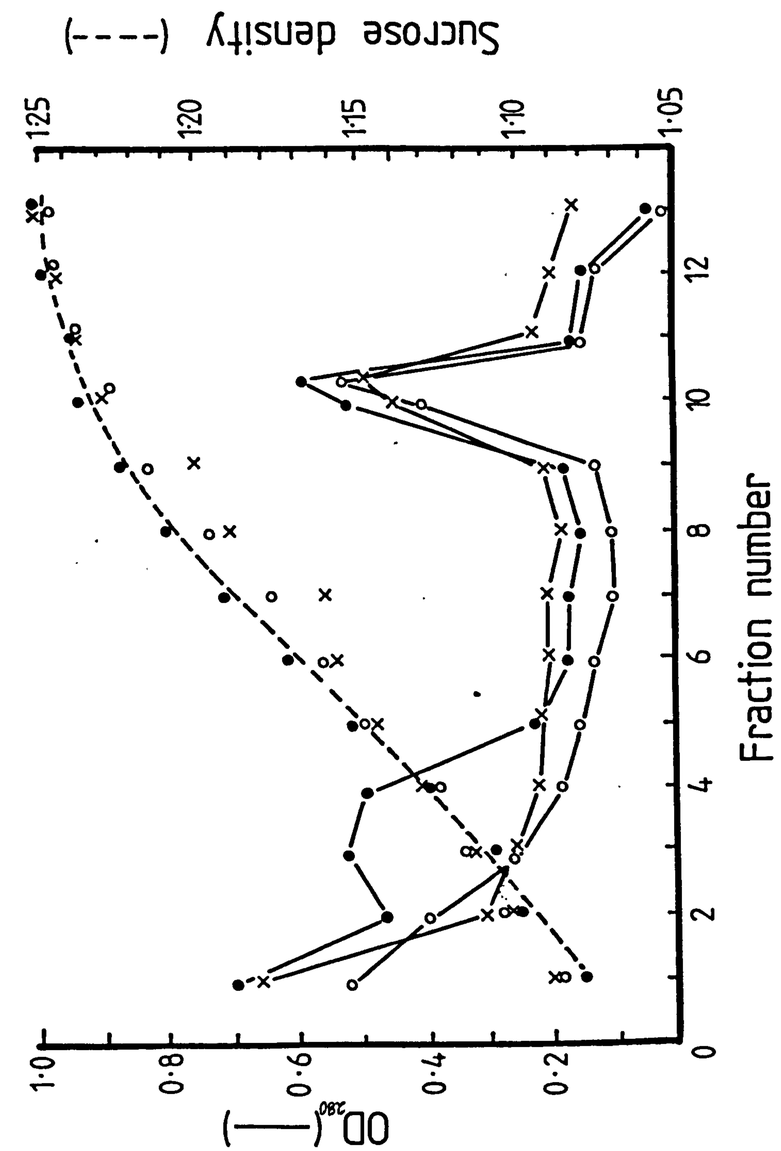


Figure 28. . Sucrose density gradient analysis of OMV fractions of semi-continuous cultures of <u>B. pertussis</u> 2992

×——× SS-NA5$_1$

o——o SS-NA500

●——● SS-NA5$_2$

identical density values of 1.236. This value agreed well with the figure of 1.218-1.224 reported by Novotny and Cownley (1979) obtained on a metrizamide gradient. The lower density peak in OMV from SS-NA500 probably represents contaminating cytoplasmic membrane.

The AC activity in a few of the gradient fractions from each sample was assayed in the absence of an ATP-regenerating system. Table 28 indicates that the AC activity was only associated with the OMV peak. This association is revealed more clearly in Figure 29. The OMV fraction from strain 2992 grown at 28°C in SCC was run in a separate experiment from those above. The OMV had a density of 1.230 which agreed very well with that for the other samples. All of the gradient fractions were assayed for AC activity in the absence of an ATP-regenerating system. Figure 29 shows that the AC activity coincided exactly with the OMV peak.

3.7. Effect of an ATP-Regenerating System on the Cellular Distribution and Activity of Adenylate Cyclase of Strain 2992

Hewlett and Wolff (1976), in their original report on the AC purified from culture supernates of *B. pertussis*, found that the presence or absence of an ATP-regenerating system had no effect on the activity of the enzyme. However, Endoh *et al* (1980), using crude culture supernates as the source of AC, found that an ATP-regenerating system caused a large stimulation of AC activity. They concluded that this difference in activity was due to the presence of ATPase activity which was counteracted by the ATP-regenerating system.

Hewlett *et al* (1978, 1979a) reported the isolation of a protein from rabbit erythrocytes which activated the AC of *B. pertussis*.

Table 28. AC activity of the sucrose density gradients of *B. pertussis* 2992 OMV fractions

GROWTH CONDITIONS	AC ACTIVITY (pmol min^{-1} ml^{-1}) IN FRACTION NUMBER: (1)			
	1	2	10	11
$SS\text{-}NA5_1$	0	0	410	680
SS-NA500	0	0	11	52
$SS\text{-}NA5_2$	0	0	170	140

(1) The fraction numbers referred to are those in the sucrose density gradients given in Figure 28.

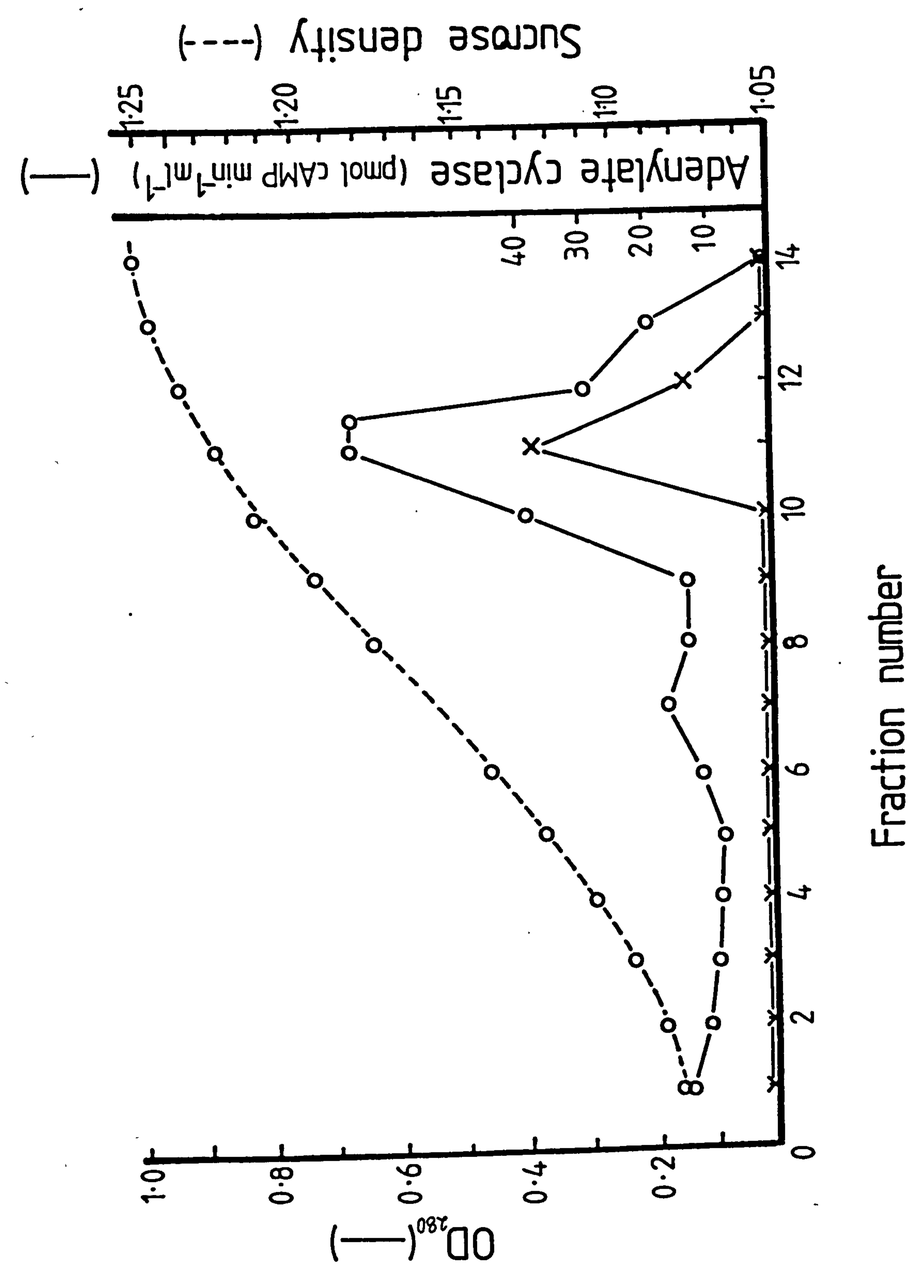


Figure 29. Sucrose density gradient and AC activities of the OMV fraction of B. pertussis 2992 grown at 28°C in SCC

o——o : OMV

×——× : AC activity

The degree of activation was about the same (approximately 200-300-fold) whether intact cells or cell lysates were the source of AC. However, the degree of activation was much lower (approximately 7-fold) if soluble (100,000 g supernate) AC from intact cells was used. Furthermore, if this soluble AC was purified, there was no activation of the AC activity and they concluded that some other, unidentified factor was also involved in the activation process.

The effect of an ATP-regenerating system on AC activities in intact cells and cell fractions of strain 2992 grown in SCC is shown in Table 29. The results indicated that AC activities in intact cells, and the CD, CW and OMV fractions were stimulated to about the same extent, although the limits were quite wide. However, the degree of stimulation of AC from these sources was much greater than that for the AC in the F.SN fraction (50,000 g supernate).

The fact that the various cell fractions were stimulated to quite different extents by the ATP-regenerating system would obviously affect the calculated cellular distribution of AC if measured in its absence. Table 30 shows a comparison of the cellular distribution of AC in strain 2992 grown in SCC obtained by measuring AC in the presence or absence of an ATP-regenerating system and shows that the distributions were indeed different. In the absence of the ATP-regenerating system, there was a very much lower percentage of AC in the CD, CW and OMV fractions and a much increased percentage in the F.SN. The OMV fractions had only 2-3% of the AC activity, when assayed in the absence of the ATP-regenerating system. The reason why SS-NA5_1 OMV had such a high percentage of AC and a corresponding decrease in F.SN percentage is unknown.

Table 29. Effect of an ATP-regenerating system on the AC activities of intact cells and fractions of B. pertussis 2992 grown in SCC.

GROWTH CONDITIONS	ENZYME SOURCE	AC Activity (nmol min^{-1} mg dry $weight^{-1}$) with the ATP-regenerating system: PRESENT	ABSENT	-FOLD INCREASE
$SS-NA5_1$	intact cells	4.6	0.01	460
	CD	20.4	0.08	255
	CW	24.8	0.13	191
	OMV	37.4	1.10	34
	F.SN	21.0	0.48	44
SS-NA500	intact cells	5.7	0.004	1425
	CD	35.3	0.13	272
	CW	16.7	0.10	167
	OMV	20.0	0.06	333
	F.SN	5.3	0.45	12
$SS-NA5_2$	intact cells	11.5	0.02	575
	CD	81.4	0.47	173
	CW	63.8	0.14	456
	OMV	48.0	0.26	185
	F.SN	34.2	1.17	29
SS-28°C	intact cells	0.6	0.005	120
	CD	1.8	0.04	45
	CW	2.1	0.02	105
	OMV	0.7	0.01	70
	F.SN	0.5	0.12	4

Table 30. Comparison of the cellular distribution of AC in *B. pertussis* 2992 assayed in the presence or absence of an ATP-regenerating system.

ATP-REGENERATING SYSTEM	GROWTH CONDITIONS	% AC ACTIVITY			
		CD	CW	OMV	F.SN
PRESENT	$SS-NA5_1$	14.4(1)	17.6	12.8	55.3
	SS-NA500	39.3(2)	20.8	13.2	26.7
	$SS-NA5_2$	26.6(2)	23.9	10.1	39.4
	SS-28°C	23.1(2)	36.2	9.8	31.0
ABSENT	$SS-NA5_1$	3.3(1)	5.2	21.1	70.3
	SS-NA500	5.7(2)	4.7	1.6	88.0
	$SS-NA5_2$	9.5(2)	3.2	3.4	83.9
	SS-28°C	6.0(2)	4.7	1.9	87.4

(1) CD fraction washed.

(2) CD fraction not washed.

4. Effect of Growth Conditions on the Agglutinogens

Pusztai and Joó (1967) demonstrated that agglutination of B. pertussis with whole cell antisera raised against normal cells was lost when cells had been grown in NA500 liquid medium. Lacey (1960) demonstrated a similar loss for his antigenic modulated cells, but these differed from the NA500-modulated cells in that the agglutinating antigen lost from X-mode cells was replaced by a different antigen in C-mode cells. There have been no reports on the effect of cultural conditions on the heat-labile serotype agglutinogens of B. pertussis. Table 31 shows the effect of various growth conditions in batch culture on the agglutinogens of strains 134 and 2992. These were cells which had had at least two subcultures in the medium before being tested and are the same cultures that were used for the AC assays given in Table 21.

Growth in SS-ND500 had no effect on the agglutinogens of either strain, whereas growth in SS-NA500 caused a loss of Factors 2 and 3 and an increase in the titre of Factor 1 in both strains. Factor 1 is the agglutinogen found in all B. pertussis strains and the reason for the increase in its titre in SS-NA500 grown cells is unknown.

The effect of various growth conditions in SCC on the agglutinogens of strain 2992 is shown in Table 32. Under these growth conditions, strain 2992 did not modulate in SS-NA500 and there was no loss of Factors 2 and 3 and no effect on Factor 1 titre. The cells modulated, however, when grown at 28°C in SCC and again there was a loss of Factors 2 and 3 only. This time there was no effect on Factor 1 titre. The data in Table 32 also suggest that Factor 3 was lost earlier than Factor 2. They also provide good evidence for the requirement for cell

Table 31. Effect of growth conditions in batch culture on the agglutinogens of *B. pertussis* strains 134 and 2992

STRAIN	GROWTH CONDITIONS	RECIPROCAL TITRE		
		FACTOR 1	FACTOR 2	FACTOR 3
134	SS-NA5	32	256	32
	SS-NA500	512	0	0
	SS-ND500	32	32	32
2992	SS-NA5	64	128	64
	SS-NA500	1024	0	0
	SS-ND500	16	128	32

Table 32. Effect of growth conditions in SCC on the agglutinogens of B. pertussis 2992

GROWTH CONDITIONS	SUBCULTURE NUMBER	RECIPROCAL TITRE FACTOR 1	FACTOR 2	FACTOR 3
$SS\text{-}NA5_1$	1	64	64	16
	2	64	128	32
	3	128	64	16
	4	128	64	32
	5	128	64	16
	6	128	256	32
	7	64	128	32
	8	128	128	64
	9	16	32	nt
	10	nt (1)	nt	nt
SS-NA500	1	128	64	32
	2	256	256	64
	3	128	64	16
	4	128	128	32
	5	nt	nt	nt
	6	256	128	32
$SS\text{-}NA5_2$	1	128	64	16
	2	128	128	16
	3	128	128	16
	4	512	512	32
	5	512	512	32
	6	nt	nt	nt
SS 28°C	1	64	128	32
	2	256	256	32
	3	128	128	16
	4	512	64	0
	5	128	0	0
	6	nt	nt	nt
	7	512	0	0

(1) not tested.

division for antigenic modulation to occur (i.e. growth at 28°C) as the Factors were only lost at the fourth subculture.

DISCUSSION

SECTION I : THE UPTAKE OF NA AND ND BY B. PERTUSSIS

Previous to this work, the only report on the kinetics of uptake of either NA or ND by any species of bacteria was by Neujahr and Varga (1966) on the uptake of ND by Strep. faecalis. These authors reported that the ND was rapidly metabolised and thus the properties they reported of pH- and energy-dependence, stimulation by K^+ and repression by ND related to the overall process of uptake as defined earlier (Figure 8). McLaren et al (1973) reported a comparison of the simultaneous rates of uptake of NA and ND by E. coli. They found that uptake of ND reached a maximum within 10 min, whereas NA uptake was slower and linear for at least 90 min, and that E. coli had a greater affinity for ND than for NA. As above, though, these authors also measured uptake as opposed to transport. To study the transport of NA and ND as the initial step of their uptake, it would be necessary to have either mutants of B. pertussis which were incapable of metabolising NA or ND, or have analogues of NA or ND which would be transported but not metabolised. None of the above were available and therefore only the overall process of uptake (Figure 8) could be investigated.

Attempts to determine the extent of metabolism of NA and ND within the period of uptake assays were made difficult by the low levels of uptake at these early times. A preliminary experiment, not reported in RESULTS, had suggested that NA was rapidly metabolised to form NAD, ND and NA. This would agree with the findings of Chaffin et al (1979) and Foster and Moat (1980) that almost all the radioactively labelled intracellular material from NA or ND uptake was recovered as NA, ND and NAD. This implies the presence of a fully functional PNC (Figure 2). The metabolism experiments reported here for B. pertussis suggested that ND was rapidly converted to NA (Figure 17), although it was not

possible to detect subsequent metabolism of the NA (Figures 16 and 17). B. pertussis was found to contain quite high levels of ND deamidase (Table 9) which would account for the conversion and also supplied further evidence of a fully functional PNC VI.

Prior to comparing the effects of growth in NA500 and ND500 media on NA and ND uptake, it was necessary to define the general features of uptake of these vitamins in normally grown (i.e. SS-NA5) cells. These features would be applicable to the overall process of uptake.

The uptake of both NA and ND was linear for at least 30 min and the rates of uptake of both vitamins were almost identical. This is in contrast to the results of both McLaren et al (1973) with E. coli, who found different rates of uptake, and with those of Neujahr and Varga (1966) who reported that ND uptake in Strep. faecalis was 85% complete by 20 min and essentially complete by 60 min. McLaren et al (1973) measured uptake at a single, equimolar concentration of ND and ND.

The uptake of both vitamins was dependent on temperature and a source of energy. B. pertussis utilises amino acids rather than carbohydrates and Glu supported the uptake of both vitamins. It was not possible, of course, to state whether the Glu requirement was for the transport step as well as for the metabolic steps, for which ATP is known to be required. The ability of other amino acids to support uptake of NA and ND was not tested.

Both vitamins also demonstrated a marked dependence for K^+ ions for uptake. No other report of a definite dependence on K^+ ions for vitamin uptake could be found, although Neujahr and Varga (1966) suggested that K^+ stimulated ND uptake by Strep. faecalis. Neither have there been any reports of a K^+ requirement for enzymes of the PNC, although many of the assays for these enzymes include K^+ (Imsande, 1961).

However, K^+ ions are important to bacteria in regulating the intracellular osmolarity as well as activating many enzymes.

In the experiments to determine the K^+ requirement, Na^+ was always present, along with Glu, and it may be that one or the other (or both) of the ions was required for the transport or metabolism of Glu. Jebb and Tomlinson (1951) have reported that oxidation of Glu by B. pertussis was much greater in a phosphate buffer than in a borate buffer. Although they did not detail the cationic composition of these buffers, the former usually includes a potassium salt and the latter a sodium salt. Schellenberg and Furlong (1977) reviewed the multiple transport systems in E. coli for Glu and Asp. One of the Glu-specific systems was dependent on Na^+ for transport of Glu. The effect of other ions, such as Mg^{2+}, Ca^{2+}, Mn^{2+} or F^-, on NA and ND uptake by B. pertussis was not tested.

The uptake of both NA and ND exhibited saturation kinetics and the Km and Vmax values for the overall uptake were estimated (Table 8). These values were found to be within the range of those for most other bacterial vitamin transport systems (Kadner, 1978). The Km for ND uptake by Strep. faecalis was 0.17 μM (Neujahr and Varga, 1966), which was greater than the 0.04 μM found here for ND uptake. The Vmax values for NA and ND uptake were very similar, whilst the Km value for NA uptake was 25-fold greater than that for ND uptake. Thus, B. pertussis appears to have a much greater affinity for ND than for NA, as was found in E. coli (McLaren et al, 1973).

However, the most striking feature of NA and ND uptake by B. pertussis was the similarity of their rates of uptake and their identical responses, in the kinetic studies detailed above, to all conditions tested. In view of the rapid metabolism of ND to NA, this suggests that NA and ND uptakes have the same rate-limiting step,

probably one of the enzymes responsible for the further metabolism of NA. Thus the same Vmax values for NA and ND uptake would be related to the balance of transport and metabolism controlled by the rate-limiting step. The different Km values indicate binding of NA and ND to different uptake components prior to this step. These different components could be transport system proteins such as binding or carrier proteins. Alternatively, the Km values could reflect binding of ND to ND deamidase, for which NA has little or no affinity. NAPRTase, which converts NA to NAMN, has been found to be the rate-limiting enzyme in NAD biosynthesis in E. coli (Imsande and Pardee, 1962) and may be the overall rate-limiting factor for NA and ND uptake in B. pertussis.

In view of the differential effect of NA500- and ND500-growth on cell envelope components (Wardlaw et al, 1976), it would be important to know whether or not these vitamins share the same or separate transport systems, followed by common metabolism. The results discussed above provide little evidence one way or the other, with the exception of the different Km values. McLaren et al (1973) suggested that NA and ND have separate uptake processes in E. coli and further suggested that ND was free to diffuse through the cell envelope. Griffith and Leach (1973), however, reported the presence of a ND binding-protein released from E. coli by osmotic shock. Whether or not this is a binding protein involved in transport is unknown, but its presence argues against free diffusion of ND. Neither Henderson and Zevely (1976) nor Neujahr and Varga (1966) compared the uptakes of NA and ND. Chaffin et al (1979) found that Candida albicans could utilise ND but not NA, even although the yeast contains ND deamidase. This was because of a lack of transport of NA and suggests that NA could not use the same transport system as ND.

Henderson et al (1979a) studied the transport of several

vitamins into L. casei and suggested that folate, biotin and thiamine each had their own separate cytoplasmic membrane binding-proteins which interacted with a common, membrane-bound protein for transport. This latter component was constitutive, whilst the binding proteins were repressible. NA transport, which also involved a repressible binding-protein, did not interact with the system for these other vitamins.

Thus, the results from other bacteria suggest that NA and ND would have separate transport systems or perhaps, as above, separate binding proteins and a common carrier protein. As the results for NA and ND uptake by B. pertussis did not provide an answer to this, the effect of various structural analogues of NA and ND on their uptake was assayed. No previous report of the effect of such analogues on NA or ND uptake or metabolism by any bacteria was found.

Preliminary experiments were disappointing in that none of the analogues tested affected uptake, even at 10x the vitamin concentration (Table 13). Although NA gave a slight inhibition of ND uptake and vice versa, no positive controls were done. NA and ND are substituted at position 3 of the pyridine ring. The analogues tested in these early experiments were substituted at other positions of the pyridine ring and the uptake systems may be sufficiently specific for this to prevent the analogues from being effective as inhibitors. All the same, the non-effect of the analogues was unexpected as Iso-NA and Iso-ND are closely related structurally to NA and ND respectively (Table 12). Furthermore, the 6-amino derivatives of NA and ND are known to be transported and metabolised to 6-amino-NAD by E. coli, and to be inhibitory to growth (Cobb et al, 1977).

A further set of analogues which were derivatives of position 3 of the pyridine ring (Table 12) were tested at 100x the vitamin

concentration for inhibition of NA and ND uptake. NA and ND showed the same pattern of inhibition by these analogues in that 3 had no effect on either vitamin, 3-pyridine-carbinol partially inhibited both NA and ND uptake and 3PC and 3PA completely inhibited both NA and ND uptakes, as did the respective positive controls. These results suggest that the uptake system(s) for NA and ND are relatively specific in that only a small number of analogues gave inhibition. The transport systems for most of the other vitamins studied have been shown to be inhibited by analogues. The same pattern of inhibition by the analogues could be explained by NA and ND sharing the same transport system, or the analogues having an inhibitory effect on metabolic enzymes as well as on one or both of their separate transport systems.

That NA and ND have separate transport systems was suggested by two pieces of evidence: the effect of NA on ^{14}C-ND uptake and vice versa, and the degree of inhibition of NA or ND uptake by various molar ratios of analogue to vitamin. When tested at 100x the vitamin concentration, NA inhibited its own uptake to a greater extent that it did ND uptake. Likewise, ND inhibited its own uptake greater than it did NA uptake. Further, NA inhibited ND uptake to a greater extent than the reverse situation, and this in spite of the fact the cells have a greater affinity for ND than for NA.

3PC and 3PA were also tested for inhibition of NA and ND uptake at 10x and 1x the vitamin concentration (Figures 22 and 23). Again, ND uptake was always inhibited to a greater extent than NA uptake at any given molar ratio of analogue to vitamin for either 3PC or 3PA. The inhibition of NA uptake by either analogue decreased as the ratio of analogue to vitamin decreased. The same decrease was found with the positive control, as expected. 3PC was a much stronger

inhibitor of either NA or ND uptakes than was 3PA. Inhibition of ND uptake by 3PA decreased in line with the ratio of 3PA to ND, as with the positive control. However, 3PC completely inhibited ND uptake at any of the ratios tested. Thus, 3PC clearly acts at two sites, one unique to ND uptake and the other involved in NA uptake, either uniquely or in common with ND uptake. The unique site of action on ND uptake could be either ND deamidase or some component of a separate ND transport system. The inhibitory effect of 3PC on ND uptake seemed unlikely to be due to chemical reactivity of the aldehyde group, since benzaldehyde, which resembles 3PC in this respect, had no effect at all on ND uptake. This observation also confirms the high specificity of the ND uptake system.

To explain the effect of these analogues on NA and ND uptake, along with the earlier observations on the general features of their uptake, 3 possible models for their transport system(s) coupled to common metabolism are shown in Figure 30. On the assumption that ND deamidase is a cytoplasmic enzyme, group translocation of NA would be a special form of model 2. Group translocation of NA is a possibility to be considered as some of the NAPRTase is periplasmic in E. coli (Baecker et al, 1978) and presumably the NAMN formed is taken up by a group translocation process similar to that for NDMN in Sal. typhimurium (Foster et al, 1979a). Furthermore, uptake of adenine by E. coli may be by group translocation via an enzyme very similar in action to NAPRTase (reviewed by Kadner, 1978).

If transport was the common, rate-limiting step for both NA and ND uptake, then either models 1 or 3 would be correct. Model 3 will be considered later. With regard to model 1, it is difficult to envisage how NA and ND can both inhibit their own uptake to a greater

Figure 30 : Possible models for the transport and metabolism of NA and ND by B. pertussis.

: non-specific pores

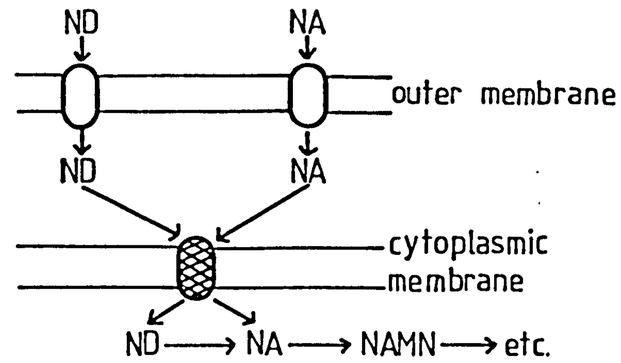
: carrier protein

: ND binding-protein

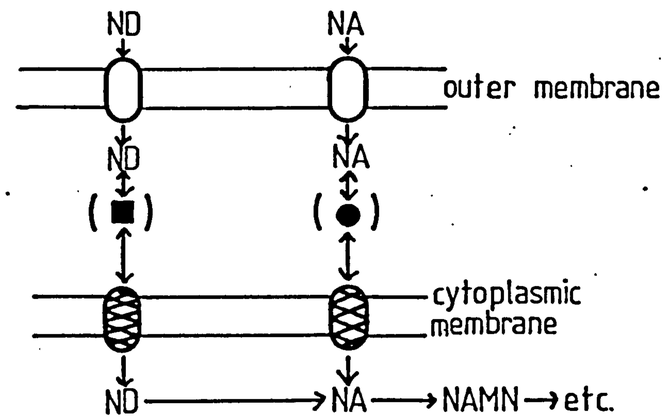
: NA binding-protein

( ) : binding-proteins may or may not be involved in the transport process.

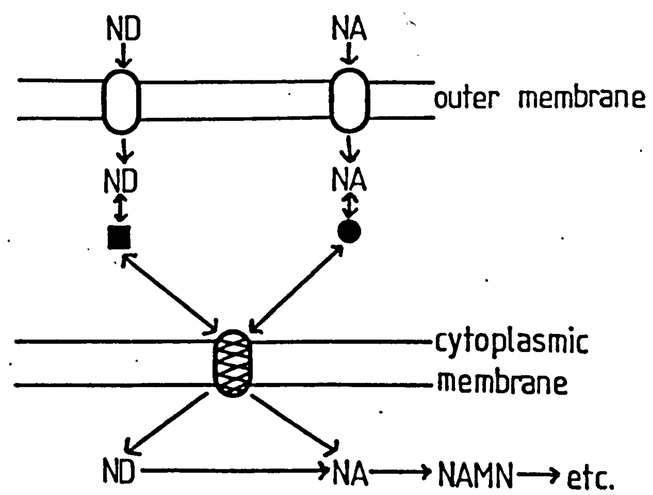
MODEL 1



MODEL 2



MODEL 3



degree than they inhibit each other. Were they to share the same carrier protein, without interaction with binding proteins, then it would be expected that at least one of the vitamins would completely inhibit the uptake of the other, when tested at a molar ratio of 100. However, this was not observed and on these grounds alone, model 1 appears to be incorrect.

If it is one of the metabolic steps that is rate-limiting, then either model 2 or model 3 would be applicable. In model 2, as NA and ND have separate transport systems, and in view of the apparent high degree of specificity of uptake, NA and ND may be expected to have little effect on each others transport system. Therefore, the inhibitory effect of NA on ^{14}C-ND uptake, and vice versa, would be due mainly to intracellular metabolism of the vitamins. As ND is rapidly metabolised to NA, the apparent inhibition would be a function of the intracellular ratio of labelled to unlabelled NA. If the ND deamidase reaction is, as seems likely, very rapid, this ratio will be dependent on the relative rates of transport of NA and ND into the bacterium. It was observed that NA inhibited ND uptake greater than the reverse process. To account for this, the rate of NA transport would have to be greater than that for ND. Although no data for this suggestion were available for B. pertussis, it is the reverse from that found in E. coli where ND uptake was much more rapid than NA uptake (McLaren et al, 1973).

Model 3 is based on the observations of Henderson et al (1979a), described earlier, and the suggestion of Singer (1977) that two or more binding proteins could interact with a common membrane-bound carrier protein. In this model, the carrier protein may be constitutive and the binding proteins repressible. The model depicts periplasmic

binding proteins, as found for vitamin binding-proteins in other bacteria. However, the binding proteins could also be located in the cytoplasmic membrane. Henderson et al (1979a) stated that only vitamin-binding-protein complexes competed for the carrier protein. Free vitamin did not compete with this binding, nor with the interaction of the other vitamins and their specific binding-proteins. The authors also stated that the different vitamin binding-proteins were present in different concentrations and that the degree of inhibition observed was proportional to the concentration of binding protein. Thus, a vitamin having a higher concentration of its specific binding-protein than another inhibits that vitamin to a greater extent than the reverse process. The authors further suggested that the common carrier protein was present in insufficient amounts to support maximum rates of transport for combinations of vitamins. Thus, as each binding protein has access to a finite amount of carrier protein, complete inhibition of the uptake of one vitamin by another is not observed.

The results for 3PC and 3PA inhibitions of NA and ND uptake do not discriminate between models 2 and 3. In view of the apparent specificity of uptake, it may be suggested that 3PC and 3PA interact with the specific component of either NA or ND transport (i.e. the binding proteins) but not with both. For inhibition of both NA and ND uptakes, however, the analogues must also inhibit one or more of the metabolic enzymes. The inhibitory effect of 3PC must be due to interaction of the 3PC with either the ND-specific transport component or ND deamidase, neither of which should interact with NA.

If the assumption is made that the NA binding-protein is present in greater amount than the ND binding-protein, then model 3 fits well to the observed results that NA inhibits ND uptake greater

than the reverse and that neither vitamin completely inhibits the uptake of the other. This model has the added advantage that it requires no assumption regarding the nature of the rate-limiting step for uptake, which could be either translocation via the common carrier or a common metabolic enzyme. It is suggested, therefore, that from the results available, model 3 is the best representation of NA and ND uptake by B. pertussis, although model 2 cannot be discarded. Assays for the presence of binding-proteins for NA and ND in B. pertussis and the effect of 3PC and 3PA on these and on ND deamidase and NAPRTase would be useful in determining which, if any, of the models is correct.

Growth in high vitamin concentrations is known to cause repression of the transport systems for biotin, folate, thiamine and riboflavin (Kadner, 1978; Cecchini et al, 1979). Henderson and Zevely (1976) reported that NA transport into L. casei was repressible, and Neujahr and Varga (1966) reported that ND uptake by Strep. faecalis was also repressible. Thus, growth of B. pertussis in high concentrations of NA or ND may be expected also to cause repression of their uptake.

A few preliminary experiments were performed, with strain 134, in which 500 $\mu g\ ml^{-1}$ NA was added to either BG or CA medium. The cells from these media showed no loss of uptake of either NA or ND (Figures 18 and 19), although they had lost the 28k/30k gel bands (Figure 20) and had reduced levels of HSF (Table 10). By these latter criteria, the cells were considered to have modulated (Wardlaw et al, 1976). This result suggested that the uptake systems for NA and ND were not equivalent to either the 28k/30k gel bands or the cell envelope component responsible for HSF activity.

All subsequent studies were done with cells grown in SS-NA500 and these showed a marked reduction in the uptake of both NA and ND, both reduced by about the same extent. The discrepancy between liquid and solid NA500 cultures was not examined in detail. One possible suggestion is that significant amounts of the excess NA are absorbed by constituents of the solid media and thus become inaccessible to the cells. The lowered NA concentration may then be sufficient to repress other factors, but not NA and ND uptake. It is known that the biosynthetic enzymes and transport system for biotin in E. coli are separately repressed by different concentrations of biotin in the medium (reviewed by Kadner, 1978). Reduction in the uptake of both NA and ND by growth in SS-NA500 could be due to repression of the NA transport system or binding protein as well as repression of the rate-limiting enzyme. NAPRTase is subject to repression in E. coli and Sal. typhimurium (Baecker et al, 1978; Foster et al, 1979b), although it is only a modest degree of repression, mediated via NAD as the corepressor (reviewed by Foster and Moat, 1980). Alternatively, in model 3, the common carrier protein may be repressible by NA.

Growth in SS-ND500 was quite distinct in its effect on NA and ND uptake from growth in SS-NA500. There was no effect at all on NA uptake, whilst there appeared to be a marginal effect on ND uptake. Neither was there any loss of HSF activity nor the 28k/30k gel bands, when grown in ND500. Growth in ND500 would perhaps have been expected to cause repression of the ND transport system. A modest repression of the same may be the cause of the marginal effect on ND uptake.

SECTION II : NA MODULATION AND ITS EFFECT ON AC AND AGGLUTINOGENS

Adenylate cyclase is an important enzyme in the regulation of cellular metabolism, in certain bacteria, through the action of its product, cAMP (Pastan and Adhya, 1976; Peterkofsky, 1976). Hewlett and co-workers have studied the AC of B. pertussis and found that it differs in certain properties from other bacterial AC's, most notably in that about 90% of the enzyme is extracytoplasmic, with a large proportion of this in the periplasm (Hewlett and Wolff, 1976; Hewlett et al, 1976). B. parapertussis and B. bronchiseptica also have an extracellular AC (Julina-Zlateva et al, 1979), but Endoh et al (1980) could find no intact cell-associated enzyme in these two species. All the AC activity was in the culture supernate of the cells grown in SS medium. By contrast, it was found here that B. parapertussis and B. bronchiseptica grown on CWBA had quite high levels of intact cell AC. The reason for the discrepancy may be due to differences in strains or cultural media.

B. pertussis AC can apparently exist in either a high or low activity state dependent on the presence of a protein activator (Hewlett et al, 1978). The activator is found in the erythrocytes of certain animal species and cultures of B. pertussis grown on BG have much greater AC activity than those grown in SS (Hewlett et al, 1978; Table 19). ATP-regenerating systems are often included in AC assays, but their stimulatory effect on AC (Table 29; Endoh et al, 1980) may be due mainly to being contaminated with the activator (Hewlett et al, 1979b). The activation of the AC is also dependent on some unknown regulator in the cell envelope. Solubilisation and purification of the AC leads to a loss of response of the AC to the activator (Hewlett et al, 1979a; Table 29). The AC of B. pertussis thus resembles

hormone systems, and indeed insulin has recently been reported to activate specifically the AC of E. coli (Abou-Sabe' and Reilly, 1978). As activation of the AC of B. pertussis occurs at 4°C and persists after removal of the activator by washing (Hewlett et al, 1979a), it may be that the activator binds strongly to the regulator and by some unknown means activates the AC. In this regard, it may be relevant that Cowell et al (1979) concluded that the periplasmic AC of B. pertussis was loosely associated with membrane surfaces having an extracytoplasmic exposure. Activation of the AC in the OMV and CW fractions (Table 29) may indicate the presence of this regulator in the OM and cytoplasmic membranes of B. pertussis.

The activities of intact-cell AC varied between species and strains of Bordetella grown on CWBA, and thus exposed to the activator. Parton and Durham (1978) had found a similar variation between six Phase I strains of B. pertussis, assayed in the presence of an ATP-regenerating system. However, strains 134 and 2992 grown in SS had very similar AC activities. This may indicate that strains vary in the amount of the AC regulator that they possess, giving rise to variations in activities of AC on exposure to the activator.

Parton and Durham (1978) had reported the reduction of AC during antigenic modulation of B. pertussis and speculated that this may be the means of control of antigenic modulation. In view of this suggestion and the observation that NA, but not ND, causes modulation (Wardlaw et al, 1976) these growth conditions were tested for their effect on the AC of B. pertussis. Batch cultures of strains 134 and 2992 in NA500 media showed a large reduction in intact-cell AC, whereas ND500 growth had no effect on the AC. This reduction was not due to excess excretion of AC or decreased permeability of the cell envelope

as the AC levels in culture supernates and cell lysates was also much reduced.

Nor did inhibition of the AC explain the discriminatory effect of NA500 and ND500 growths, on two counts. First, preliminary experiments suggested that high concentrations of either NA or ND in the AC assay inhibited the AC in cell lysates of NA5-grown cells. As metabolism of both vitamins would result in high concentrations of NAD, this latter may be the inhibitory molecule, as has been found for the AC of Mycobact. smegmatis (Lee, 1978). Second, evidence was found that modulation in NA500 media only occurred after a certain number of cell divisions. This requirement for cell division has also been reported for antigenic modulation (Lacey, 1960) and indicates that both modulations arise from repression of certain cellular components. It should be noted, however, that the recent studies of Idigbe (1979) have suggested that, in antigenic modulation, the affected properties are lost within 10 h of transfer from X- to C-mode media. This was a greater rate of loss than that predicted from repression of the synthesis of the properties and dilution of the remaining activities by cell division. Thus, repression alone may not fully explain antigenic modulation.

As both NA and antigenic modulations occur in SS media in the absence of the protein activator, it seems that the protein activator and membrane regulator are not involved in the repression of AC synthesis, but only in the control of its activity. However, it is possible that the presence or absence of the regulator is closely related to that of AC. Six strains of B. pertussis grown in C-mode media had very little variation in their AC activities, when assayed in the presence of an ATP-regenerating system, compared with the wide variation in their

X-mode AC activities (Parton and Durham, 1978). Furthermore, Hewlett et al (1979b) reported that the AC of Phase IV strains of B. pertussis were not responsive to the activator, as well as having very low AC activities. Phase variation is thought to be due to a series of random mutations.

Thus, it appears that the AC of B. pertussis is subject to three controls: i) inhibition, possibly by NAD or some other factor, ii) activation, mediated by the protein activator and membrane regulator, and iii) repression, as occurs in NA modulation and in antigenic modulation.

Control of the activity and amount of the AC in E. coli and Sal. typhimurium by growth in various sugars is well documented. Control of the activity of AC in E. coli is intimately linked to the transport of various sugars and control is exerted by two means:

i) uptake of sugars by the PEP:PTS leads to phosphorylation of a membrane protein which is not a component of the PEP:PTS (reviewed by Saier, 1979; Feucht and Saier, 1980). This protein mediates regulation of both AC and certain other transport proteins. The phosphorylated protein interacts with and thereby activates the AC. The non-phosphorylated protein interacts with and inactivates the other transport proteins, usually those for inducible, non-PTS sugars. Uptake of sugars such as glucose via the PTS leads to mainly the non-phosphorylated form of the protein. This system was used to explain control of lactose uptake by glucose.

ii) It is also known that transport of some non-PTS sugars in E. coli, e.g. lactose, also inhibits the AC. Peterkofsky and Gazdar (1979) have shown that this control occurs only where the transport of the sugar is driven by the PMF and there is co-transport of protons.

They suggested that the AC is related to some membrane protein responsible for the maintenance of the PMF. Transport of the sugar reduces the proton gradient; this causes a conformational change in the membrane protein, which in turn inhibits the AC.

The amount of AC in E. coli and Sal. typhimurium is subject to control by repression. Rates of cAMP synthesis, measured in the absence of the growth sugar, were found to be dependent on the sugar present during growth (Botsford and Drexler, 1978; Harman and Botsford, 1979). Repression of the AC is mediated by the cAMP-CRP (cAMP receptor protein) complex, which is normally involved in the induction of other genes (Pastan and Adhya, 1976; Botsford and Drexler, 1978).

Two further observations on the effect of growth conditions on the AC of B. pertussis were made that were noteworthy. First, B. pertussis 18323 grown on CWBA had very low levels of intact cell-associated AC. The level was, in fact, as low as the levels of strains 134 and 2992 grown in SS media, and thus non-activated. Growth on CWBA-NA500 caused repression of the AC as with the two other strains. However, when grown on CWBA-ND500, there was a 15-fold increase in the AC activity over that of CWBA-grown cells, whereas the AC of strains 134 and 2992 was unaffected by growth on CWBA-ND500. The reason for this effect of ND500 on strain 18323 is unknown. As the cells grown on CWBA do have AC, albeit low activity, ND500 growth may affect the membrane regulator protein, perhaps by derepression or neutralisation of some medium inhibitor. This strain was also reported not to lose PA or agglutinogens when grown in C-mode media (Adams, 1970) and not to lose agglutinability when grown in NA500 media (Pusztai and Joó, 1967). The effect of growth in NA500 on other pathophysiological properties of this

strain was not reported. Thus, clearly this strain is atypical in its response to certain growth conditions and would be worthy of more detailed study.

Second, growth of strain 2992 in SCC had a different effect on the AC than growth in batch culture. The activities of intact cell and cell lysate AC were slightly reduced and that of culture supernate AC much reduced compared to those from batch cultures. Similar differences in cAMP content or rate of synthesis were found between batch and continuous cultures of E. coli (Villarejo et al, 1978) and Sal. typhimurium (Harman and Botsford, 1979). Thus, the AC activities are dependent on cultural conditions, as well as on growth media. The response of strain 2992 to growth in NA500 was drastically altered by growth in SCC in that there was no loss of AC, agglutinogen factors 2 and 3 (see below) or the 28k/30k gel bands, whereas these components were lost when strain 2992 was grown in NA500 in batch culture. The effect of SCC was apparently specific to NA modulation as the above components were all lost when strain 2992 was grown at 28°C in SCC. Growth at this temperature is one of the means of causing antigenic modulation (Lacey, 1960). This suggests that NA modulation and antigenic modulation are distinct processes. The reason why strain 2992 does not respond to growth in NA500 in SCC, yet does in batch culture, is unknown. However, as growth at 28°C in SCC does cause the loss of AC, perhaps the SCC conditions themselves affect some cellular component other than AC such that its interaction with NA does not cause modulation. This suggests that AC may not be involved in the regulation of NA modulation.

The cellular distribution of AC and the effect of growth conditions on this was also investigated. In view of the differential

stimulation of the AC activities in the various cell fractions when assayed in the presence of an ATP-regenerating system (Table 29), it was not surprising that the absolute figures for percentage distribution of activity varied depending on the assay conditions (Table 30). However, the order of distribution of AC in the fractions was unaltered by the assay conditions. Most of the AC was found in the soluble fraction, with small amounts also found in the CW and OMV fractions. Association of some of the AC with OMV was confirmed by sucrose density gradient analysis of the OMV. AC activity in the gradients correlated exactly with the position of the OMV. How much of the AC activity in the CW and CD fractions was due to the contaminating OM, which may be a large amount, was unknown. The distribution of AC was also found to vary slightly between strains and between batch culture and SCC. Growth in NA500 in batch culture also caused an altered distribution of AC in that there was a drop in the percentage in the CW and OMV fractions and a corresponding rise in the soluble AC fraction. This altered distribution is distinct from the reduction in AC levels caused by growth in NA500. Growth at 28°C in SCC did not appear to cause an altered distribution of the AC, thus indicating another difference between NA and antigenic modulations.

The distribution of AC reported here agrees well with that of other authors. Hewlett _et al_ (1976) found that 90% of _B. pertussis_ AC was soluble and was extracytoplasmic, with a large proportion of that being periplasmic. They based their conclusions on results of assays of intact cells for AC and the treatment of intact cells with trypsin. Intracellular enzymes were not assayable in intact cells and were not affected by the treatment of intact cells with trypsin. The remaining 10% or so of the AC was said to be intracellular. Cowell

et al (1979) examined the distribution of AC in B. pertussis after osmotic shock, sphaeroplast formation and cell lysis and separation into membrane and soluble fractions. No attempt was made, however, to separate the cytoplasmic and outer membranes. Their results confirmed that the majority of the AC was soluble and extracytoplasmic, but that the periplasmic AC was loosely bound to the exposed membrane surfaces. However, their fractionation procedures resulted in a loss of about 40% of the AC activity in cell lysates.

The effect of growth conditions on the agglutinogens of B. pertussis was also examined. These are OM proteins (Novotny and Cownley, 1979) and are known to be lost on antigenic modulation (Adams, 1970). Pusztai and Joó (1967) showed that growth in NA500 media led to the loss of agglutination of B. pertussis by whole cell antisera, but the effect on individual agglutinogens was not tested. Growth of strains 134 and 2992 in NA500 in batch culture led to the loss of factors 2 and 3, but caused an increase in the titre of factor 1, the species-specific agglutinogen. Growth in ND500 had no effect on any of the factors. Factor 1 is clearly under a separate control from that of factors 2 and 3. The reason for the increase in factor 1 titre is unknown. It may be that excess factor 1 is synthesised to replace the lost material, but SDS-PAGE analysis of NA500 OMV's shows a definite loss of protein and gel bands. Alternatively, factor 1 may be rearranged on the cell surface such as to cause an apparent increase in titre. These results further suggest that factor 1 is not related to the PA, HSF, AC, 28k/30k gel bands or the NA or ND uptake systems, all of which are lost or reduced in NA modulated cells. Furthermore, Julina-Zlateva et al (1979) have reported that B. pertussis AC is serologically distinct from agglutinogen factors 2 and 3.

No loss of factors 2 and 3 was found with strain 2992 grown in NA500 in SCC, as expected. However, growth at 28°C in SCC resulted in the loss of factors 2 and 3, but had no effect on the titre of factor 1. Adams (1970), however, found that factor 1 was lost along with factors 2 and 3 in two strains grown on solid media at 25°C or with $MgSO_4$ in place of NaCl (i.e. C-mode conditions). The cause of the difference between our results with factor 1 is unknown, although cultural and strain differences may be involved.

Agglutinogen titres were assayed in each daily harvest of strain 2992 grown at 28°C in SCC. The titres of factors 2 and 3 did not start to reduce until the fourth subculture, thus confirming the need for a certain number of cell divisions for antigenic modulation. It was also found that the loss of factor 3 slightly preceded that of factor 2. A sequential loss of agglutinogens in the order 3-2-1, and their reappearance in the order 1-2-3, had been suggested in B. pertussis (Cameron, 1967; Stanbridge and Preston, 1974), although the reason for this phenomenon is unexplained.

SECTION III : THE POSSIBLE MECHANISM OF NA MODULATION

NA modulation has received very little attention since its description by Pusztai and Joó (1967), although Wardlaw et al (1976) described the differential effect of growth in high concentrations of NA or ND. These studies and those presented here indicate that at least 6 features of the cell are lost or greatly reduced during growth of B. pertussis in NA500, but not in ND500 media:

i) PA

ii) HSF

iii) Agglutinogen factors 2 and 3

iv) 28k/30k gel bands

v) AC

vi) NA and ND uptake.

The first 4 of these activities are located in the cell envelope, and are probably in the outer membrane (Imagawa et al, 1979; Novotny and Cownley, 1979; Wardlaw and Parton, 1979; my results). The AC is mainly extracytoplasmic, being distributed between the periplasm and the culture supernate. However, a small amount of AC is located in the OM, as well as a small amount of intracellular AC. An unknown membrane component, possibly also located in the OM, is required for the activation of the AC. The components of the NA and ND uptake systems that are lost are unknown. They may possibly be the NAPRTase or other NAD biosynthetic enzymes, or transport system components such as a membrane carrier protein or binding proteins.

Thus growth in NA500, but not ND500 medium affects a variety of unrelated properties of B. pertussis, several of which may be located in the OM. An explanation of NA modulation, therefore, requires answers to two main questions:

i) Why does growth in ND500 have such a different effect from growth in NA500 medium, and

ii) Why does growth in NA500 medium repress certain features which appear to be unrelated to the uptake of NA?

The answer to the first question must be that NA and ND interact with separate cell envelope components and that NA also interacts with some regulatory component, also in the cell envelope, with which ND does not interact. ND is rapidly metabolised to NA and thus growth in either NA500 or ND500 medium would yield high levels of NAD.

As NAD is the end-product of NA and ND metabolism, this compound would be expected to be the co-repressor for end-product repression effects of high concentrations of NA or ND. However, such high concentrations clearly have very different effects and thus NAD cannot be the effector. Interactions of NA and ND with separate components of the cell envelope must be the explanation of their differential effects. That NA reacts with a regulatory component distinct from the transport system is suggested from the preliminary experiments on the effects of growth on BG- or CA-NA500, where uptake was unaffected whilst HSF, AC and 28k/30k gel bands were lost.

The answer to the second question is not quite so straightforward. Several other vitamin uptake systems are known to be repressed by growth in high concentrations of the vitamin, including that of NA in L. casei and ND in Strep. faecalis (see DISCUSSION, Section I). It is not unreasonable, therefore, to expect the same repression of NA or ND uptakes in B. pertussis. However, growth in NA500 media has a pleiotropic effect on certain cellular properties. This can only be explained by the regulatory component with which NA interacts (see above) either directly or indirectly regulating the expression of several, apparently unrelated cellular properties. The results presented here do not provide sufficient evidence to determine the nature of the component or the mechanism of regulation. However, from the observations made here and elsewhere on antigenic and NA modulations, it is possible to offer some speculations on the mechanism of NA modulation.

A striking feature of NA and antigenic modulations is that all the components lost, with the exception of NA and ND uptake in NA modulation, are lost conjointly. There was no instance where one

component, e.g. HSF, was retained whilst all the others were affected. However, when grown on solid media containing NA500, NA and ND uptakes were not lost, whilst HSF, 28k/30k gel bands and AC were reduced. The uptakes of the two vitamins were markedly reduced, along with these other properties on growth in NA500 liquid media. It was suggested (DISCUSSION, Section I) that repression of NA and ND uptakes may therefore be controlled by higher concentrations of NA in the growth medium than are required for repression of the other properties.

The composition of the OM of E. coli and Sal. typhimurium is known to be very responsive to the growth environment and is easily modified by changes in the growth medium or temperature (Lugtenberg et al, 1976; Braun, 1978). Furthermore, many of the OM proteins and transport systems in these bacteria are controlled by cAMP levels in the cells (DISCUSSION, Section II). This regulation can be either induction (Aono et al, 1978) or repression (Alderman et al, 1979; Mallick and Herlich, 1979) and mutants lacking AC show a pleiotropic loss of the utilisation of sugars and changes in cell envelope protein composition (Pastan and Adhya, 1976).

The presence of AC in B. pertussis and the pleiotropic effect associated with its loss in NA and antigenic modulations is reminiscent of the situation in E. coli. It has been suggested, therefore, that the AC has a regulatory role in B. pertussis and controls the modulation of the cells (Parton and Durham, 1978; Idigbe, 1979). However, the explanation may not be as simple as this and certain evidence has been found which suggests that AC may not necessarily have a regulatory function in the modulations of B. pertussis.

Doubts regarding a regulatory role for AC in B. pertussis were expressed by Hewlett et al (1979b). They suggested that the

relationship of AC activity to the regulation of phenotypic variations could be explained in one of two ways:

i) the AC may have an important regulatory function and its presence or absence controls the loss of certain other cell activities, and

ii) the AC may not have a regulatory role and that the association with the loss of other activities is merely temporal.

The latter possibility seemed the more likely as transfer of the cells from BG to SS media resulted in a very marked reduction in AC activity, possibly by inhibition as well as a lack of the protein activator, but had no effect on other cell activities. Thus, the activity of the AC could be regulated separately and without effect on other cell activities, whilst the presence or absence of the AC was regulated coordinately with that of these other activities.

Growth on solid media containing NA500 did not result in a loss of NA and ND uptake (DISCUSSION, Section I), but did cause the loss of HSF, 28k/30k gel bands and AC activity. Unfortunately, the loss of AC activity was not determined on the same batch of cells as used for the other assays. However, the results suggest that AC activity does not control the loss of the NA and ND uptake systems.

Despite certain similarities in effect of NA and antigenic modulations on B. pertussis, they appear to be quite distinct processes. For instance, HLT is lost on antigenic modulation (Livey et al, 1978), but is unaffected by NA modulation (Pusztai and Joó, 1967). Also, Lacey (1960) reported that in antigenic modulation, not only do the X-mode cells lose the main X-antigen, but this is replaced by the C-antigen. Antisera raised against C-mode cells will agglutinate those cells, but not X-mode cells. In NA modulation, however, there

is no replacement of the main agglutinating antigen and antisera raised against the modulated cells do not agglutinate those cells (Pusztai and Joó, 1967). Furthermore, strain 2992 modulated when grown at 28°C (C-mode conditions) but not in NA500 in SCC. On the basis of this evidence, the two modulations are suggested to be distinct processes.

The role of AC activity and cAMP levels in antigenic modulation remains to be established. However, as strain 2992 modulated in NA500 media only in batch culture, this suggested that AC activity does not control NA modulation and the NA must therefore interact with some other component which mediates the regulation. Other evidence to support this was that NA modulation occurred equally well in the presence or absence of the protein activator of AC. In E. coli, increases in cAMP levels mediate induction of certain functions or repression of others (Pastan and Adhya, 1976). However, with B. pertussis, increasing AC activity alone had no modulatory effect and was not required for NA modulation. Indeed, growth in NA500 would be expected to inhibit the AC activity, via increased levels of NAD (see earlier), as well as repressing its synthesis. This is the opposite of the situation in E. coli where increased levels of cAMP are required for the repression of AC (Botsford and Drexler, 1978).

There appears, therefore, to be some evidence that AC activity does not regulate either NA or antigenic modulations and that the AC is merely one of the factors lost on modulation as a result of some other regulation system.

To account for the simultaneous loss of certain cell properties during NA modulation, it is tempting to suggest, as has Idigbe (1979) for antigenic modulation, the existence of a "virulence operon" in B. pertussis. On the basis of the above discussion, this

operon would include PA, HSF, agglutinogens 2 and 3 and the 28k/30k gel bands, but not HLT. Furthermore, and contrary to Idigbe's (1979) conclusion, the operon would not be regulated by AC activity or increased cAMP levels. Indeed, the AC may even be part of the operon, although there is no evidence as yet for a role of the AC in the pathogenesis of B. pertussis. The uptake systems for NA and ND are probably not part of this operon, and may not even be under the same control mechanism.

A model of the possible mechanism of NA modulation is given in Figure 31 and is related to model 3 for the uptake of NA and ND (Figure 30). The model suggests that either free NA or the NA-binding-protein complex interact with a separate regulatory component which, by steps unknown, represses the synthesis of the proteins of the virulence operon, including AC.

Suggestions as to the nature of any alternative regulatory system to AC activity in B. pertussis would be purely speculative. Perhaps the next most likely candidate would be glutamine synthetase (GS). In E. coli and Sal. typhimurium, GS is involved in the regulation of the metabolism of nitrogenous compounds, particularly amino acids (Magasanik, 1977; Saier, 1979). The regulation of the synthesis of enzymes and transport proteins involved in this nitrogen metabolism is a complex affair. It appears that the GS complex itself binds to the DNA, although some other unknown factor is also involved, and thereby regulates protein synthesis (Kustu et al, 1979; Saier, 1979). Furthermore, the synthesis of GS itself is strictly regulated (Saier, 1979).

As B. pertussis does not attack carbohydrates but instead uses amino acids as its source of carbon, nitrogen and energy, it may

Figure 31 : A possible model for NA modulation of B. pertussis

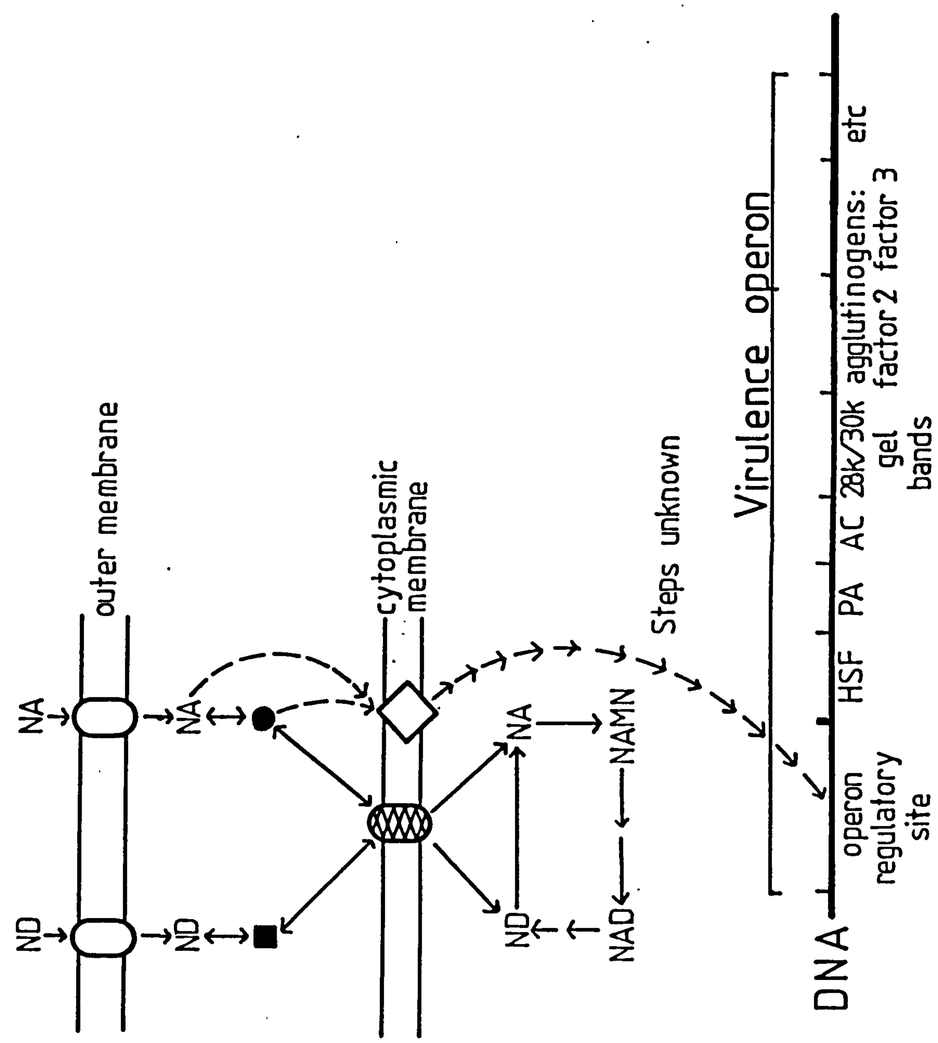
: non-specific pores

: carrier protein

: regulator protein

: ND binding-protein

: NA binding-protein



be that the main regulatory system in B. pertussis is GS rather than AC, although the latter cannot yet be ruled out. Indeed, in E. coli, cAMP is thought to be involved in the regulation of GS and associated enzymes (Pastan and Adhya, 1976). The presence of GS in B. pertussis has recently been reported and the enzyme appears very similar in structure to that found in E. coli (Zakharova et al, 1979).

In conclusion, it is suggested that NA and ND interact with separate cell surface components. The NA system is linked to some regulatory unit such that high external concentrations of NA, but not ND, mediate the repression of a variety of cell activities, some of which may form a "virulence operon" (Figure 31). The nature of the regulatory unit is unknown, but there is some evidence to suggest that it is not AC.

APPENDICES

APPENDIX 1

PREPARATION OF MEDIA

1. 1% Casamino Acids Solution

10.0 g Casamino acids (Difco technical)

0.1 g $MgCl_2.6H_2O$

0.01 g $CaCl_2$

5.0 g NaCl

Dissolve the components in 1 litre of distilled water. Adjust the pH to 7.6 with 1.0N NaOH. Autoclave for 15 min at 121°C.

2. Bordet-Gengou Plates

Dissolve 10.0 g Bordet-Gengou agar base (Gibco Bio-cult Diagnostics Ltd., Paisley, Scotland) in 250 ml 4% (v/v) glycerol. Autoclave at 121°C for 15 min and allow to cool to 50°C. Asceptically add 50 ml defibrinated horse blood (Gibco), mix by gentle rolling and pour approximately 20 ml into 90 mm diameter, triple vent plastic petri dishes. Remove surface bubbles by light flaming. Plates were stored, wrapped in plastic bags, at 4°C and used within one week of preparation.

3. Charcoal Agar Plates

Dissolve 62.5 g charcoal agar medium (Difco) in 1 litre of distilled water. Heat to boiling with frequent stirring and then autoclave at 121°C for 15 min before cooling to 50°C. Pour into plates and store as for BG medium.

4. Stainer and Scholte Medium (Stainer and Scholte, 1971)

1. 10.72 g L-glutamate, monosodium salt (B.D.H.)
2. 0.24 g L-proline (Sigma)

3. 2.5 g NaCl
4. 0.5 g KH_2PO_4
5. 0.2 g KCl
6. 0.1 g $MgCl_2.6H_2O$
7. 0.02 g $CaCl_2$
8. 6.075 Tris base (Sigma)
9. 0.04 g L-cysteine (B.D.H.)
10. 0.01 g $FeSO_4.7H_2O$
11. 0.02 g Ascorbic acid (B.D.H.)
12. 0.005 g Nicotinic acid (B.D.H.)
13. 0.1 g Glutathione, reduced (Sigma)

Dissolve ingredients 1-8 in 800 ml distilled water and adjust the pH to 7.6 with 2.5N HCl. Make the volume up to 990 ml with distilled water and autoclave at 121°C for 15 min.

Dissolve ingredients 9-13 in 10 ml distilled water and sterilise by filtration through a millipore filter of 0.45 μm pore size (Millipore S.A. Molsheim, France). Larger volumes of this vitamin solution can be prepared and stored at 4°C for up to one week. Immediately before inoculation of the medium, 10 ml of the vitamin solution was added to 990 ml of the sterile basal medium.

The above formulation is for SS-NA5. SS-ND500 is prepared exactly as above except that the 0.005 g NA is replaced with 0.5 g ND.

SS-NA500 is prepared as described for SS-NA5, except that the 0.005 g NA is omitted from the formulation. A separate solution of NA is prepared by dissolving 0.5 g NA in 10 ml distilled water and a few drops of 4N NaOH. A further vitamin solution is prepared by dissolving ingredients 9-13, but not NA, in 10 ml of distilled water. Both solutions are filter-sterilised as above. Ten millilitre of each solution is added to 980 ml of sterile basal medium immediately before inoculation.

APPENDIX 2

PREPARATION OF MISCELLANEOUS REAGENTS

1. Phosphate Buffered Saline, pH 7.3

PBS tablets were obtained from Oxoid Ltd., Basingstoke, Hants. One tablet was dissolved in 100 ml distilled water and the PBS autoclaved at 121°C for 15 min.

2. Potassium or Sodium Phosphate-Saline Buffers

a) 0.05M Potassium phosphate-saline buffer, pH 7.2

180 ml 0.2M $K_2HPO_4.3H_2O$

70 ml 0.2M KH_2PO_4

Mix and make the volume up to 1 litre with distilled water. Dissolve 8 g NaCl in the phosphate buffer and autoclave at 121°C for 15 min.

b) 0.05M Sodium phosphate-saline buffer, pH 7.2

180 ml 0.2M $Na_2HPO_4.12H_2O$

70 ml 0.2M $NaH_2PO_4.2H_2O$

Mix and make the volume up to 1 litre with distilled water. Dissolve 8 g NaCl in the phosphate buffer and autoclave at 121°C for 15 min.

3. ^{14}C-NA and ^{14}C-ND Solutions

a) Reconstitution

^{14}C-NA and ^{14}C-ND were obtained in 50 µCi amounts as freeze-dried powders from The Radiochemical Centre, Amersham, Bucks. These were reconstituted in 1 ml of distilled water containing sufficient unlabelled NA or ND to give final concentrations of 1 mM for the

reconstituted vitamins. Thus the reconstituted vitamins had vitamin concentrations of 1 mM, radioactive concentrations of 50 μCi ml^{-1} and specific activities of 50 μCi μmol^{-1}. The solutions were stored at -20°C.

b) Preparation of ^{14}C-NA and ^{14}C-ND for uptake assays

i) NA: 250 μl 1 mM ^{14}C-NA

250 μl 3 mM NA

500 μl distilled water

Vitamin concentration : 1 mM

Radioactive concentration : 12.5 μCi ml^{-1} (2.775×10^7 cpm ml^{-1})

Specific activity : 12.5 μCi μmol^{-1}

50 μl of this solution was added to 9 ml of PBG in uptake assays to give a final concentration of 5.0 μM. 50 μl of the solution was added to 10 ml PBG for the reagent blank solutions for uptake assays.

ii) ND: 200 μl 1 mM ^{14}C-ND

800 μl distilled water

Vitamin concentration : 0.2 mM

Radioactive concentration : 10 μCi ml^{-1} (2.22×10^6 cpm ml^{-1})

Specific activity : 50 μCi ml^{-1}

25 μl of this solution was added to 9 ml of PBG in uptake assays to give a final concentration of 0.5 μM. 25 μl of the solution was added to 10 ml PBG for the reagent blank solution for uptake assays.

c) Determination of counting efficiencies (C.E.)

The C.E. of the solutions prepared in b) above were determined thus:

i) NA: Dilute the solution in b):

50 μl 1 mM ^{14}C-NA, 12.5 μCi ml^{-1}

450 μl distilled water.

Spot 25 µl onto a cellulose acetate membrane filter (25 mm diameter, 0.45 µm pore size, Oxoid), place in scintillation vials, dry under an infrared lamp and add 10 ml toluene scintillant (APPENDIX 2.4). 25 µl of distilled water were similarly treated. Vials were counted in a liquid scintillation counter.

Expected cpm = 0.025 ml x 12.5 µCi ml^{-1}

= 69,375 cpm

C.E. = observed cpm / expected cpm.

ii) ND: Dilute the solution in b):

50 µl 0.2 mM ^{14}C-ND, 10.0 µCi ml^{-1}

950 µl distilled water.

Spot and treat as for NA in i) above.

Expected cpm = 0.025 ml x 10.0 µCi ml^{-1}

= 27,750 cpm

C.E. is calculated as for NA in i) above.

4. Toluene Scintillant

4.0 g PPO (Intertechnique, Uxbridge, Middlesex, England)

0.1 g Dimethyl-POPOP (Intertechnique)

Dissolve the components in 1 litre of toluene (B.D.H.) and transfer to a dark bottle.

APPENDIX 3

CALCULATION OF CONVERSION FACTOR FOR cAMP ASSAYS

a) A 50 µl sample for cAMP assay was removed from the 750 µl volumes of samples stored at the end of AC assays. Therefore, the cAMP content of this 750 µl volume was: (pmol cAMP/assay tube) X $\frac{750}{50}$ pmol.

b) This amount of cAMP was present in the 600 µl removed from the AC assay tubes. The total volume in the AC assay tubes was 720 µl. Thus, the cAMP content of this 720 µl was:

$$\text{(pmol cAMP/assay tube)} \times \frac{750}{50} \times \frac{720}{600} \text{ pmol.}$$

c) This amount of cAMP was formed in the 10 min of the AC assay. Therefore, the rate of cAMP production was:

$$\text{(pmol cAMP/assay tube)} \times \frac{750}{50} \times \frac{720}{600} \times \frac{1}{10} \text{ pmol min}^{-1}.$$

d) The volume of enzyme solution used in the assay was 20 µl. Therefore, the rate of cAMP production per ml of enzyme solution was:

$$\text{(pmol cAMP/assay tube)} \times \frac{750}{50} \times \frac{720}{600} \times \frac{1}{10} \times \frac{1000}{20} \text{ pmol min}^{-1}\text{ml}^{-1}.$$

e) The formula in d) simplifies to:

$$\text{(pmol cAMP/assay tube)} \times 90 \text{ pmol min}^{-1}\text{ml}^{-1}.$$

f) Division of this result by the mg dry weight ml^{-1} of the enzyme solution converts the AC activity into: pmol min^{-1} mg dry $weight^{-1}$.

APPENDIX 4

PREPARATION OF NICOTINAMIDE DEAMIDASE ASSAY SOLUTIONS

1. 0.5M Potassium Phosphate Buffer, pH 7.0

30.5 ml 1.0M $K_2HPO_4.3H_2O$

19.5 ml 1.0M KH_2PO_4

Mix and make the volume up to 100 ml with distilled water.

2. 0.05M Potassium Phosphate Buffer, pH 7.0

152.5 ml 0.2M $K_2HPO_4.3H_2O$

97.5 ml 0.2M KH_2PO_4

Mix and make the volume up to 100 ml with distilled water.

3. 1.4 mM ^{14}C-ND, 2.0 μCi ml^{-1}

100 μl 1 mM ^{14}C-ND, 50 μCi ml^{-1} (APPENDIX 2.3a)

400 μl 1 mM ND

1000 μl 3 mM ND

1000 μl distilled water

Mix and store at 4°C.

APPENDIX 5

PREPARATION OF REAGENTS FOR PROTEIN DETERMINATION

1. Protein Determination of Cell Lysates

Reagent A

2% (w/v) Na_2CO_3 in 0.1N NaOH

Reagent B

1% (w/v) $CuSO_4.5H_2O$ in distilled water

2% (w/v) Sodium potassium tartrate in distilled water.

Mix equal volumes of the two solutions and allow the precipitate to settle before using the supernate.

Reagent C

Folin and Ciocalteau phenol reagent (B.D.H.) diluted 1:1 with distilled water.

Reagent D

To 50 ml of Reagent A add 2 ml of Reagent B. Prepare immediately before use.

Reagent E

Protein standard : 1 mg ml^{-1} Bovine serum albumin (Sigma).

2. Protein Determination of Cell Envelopes

Reagent A

5% (w/v) Na_2CO_3 in distilled water

Reagent B

As in APPENDIX 5.1

Reagent C

As in APPENDIX 5.1

Reagent D

To 50 ml of Reagent A add 2 ml of Reagent B. Prepare immediately before use.

Reagent E

As in APPENDIX 5.1.

APPENDIX 6

SLAB GEL ELECTROPHORESIS

1. Stock Solutions

a) 30% (w/v) Acrylamide solution

30 g acrylamide (B.D.H.)

0.8 g NN'-methylenebisacrylamide (B.D.H.)

Add distilled water to 100 ml. Filter and store in a brown bottle at 4°C.

b) Tris-HCl buffer, pH 8.8

50 ml 1M Tris base (Sigma)

8.1ml 1N HCl

Add distilled water to 100 ml.

c) Tris-HCl buffer, pH 6.8

50 ml 1M Tris base

45 ml 1N HCl

Add distilled water to 100 ml.

d) Tris-glycine buffer, pH 8.3 (10 x concentrated)

144.13 g Glycine

30.28 g Tris base

Add distilled water to 1 litre.

e) Ammonium persulphate

0.8% (w/v) in distilled water.

f) 20% (w/v) Sodium dodecyl sulphate (SDS)

20% (w/v) in distilled water.

g) Bromophenol blue

0.1% (w/v) bromophenol blue (B.D.H.) in distilled water.

h) Solubilising buffer

2.0 ml Tris-HCl, pH 6.8

3.2 ml 20% (w/v) SDS

1.6 ml β-mercaptoethanol

3.2 ml Glycerol

0.32 ml 0.1% (w/v) bromophenol blue

5.68 ml distilled water.

2. Preparation of Gel Plates

Two glass plates (80 mm x 80 mm), separated at two sides with spacers, were sealed on three sides with adhesive tape. The corners were sealed by dipping the prepared plates in molten paraffin wax.

3. Preparation of Separating Gel

14.7 ml 30% (w/v) acrylamide stock solution

15.0 ml Tris-HCl, pH 8.8

0.2 ml 20% (w/v) SDS

10 µl N',N',N',N'-tetramethyl-ethylenediamine (TEMED)

4.0 ml 0.8% (w/v) ammonium persulphate

6.1 ml distilled water.

Mix thoroughly and pipette approximately 14 ml into the gel plates, which should be held vertically in a rack. Overlay with 5% (w/v) ethanol without disturbing the surface of the gel. This removes the meniscus from the gel and leaves a flat surface. Leave the gel to polymerise.

4. Preparation of Stacking Gel

1.7 ml 30% (w/v) acrylamide stock solution

1.25 ml Tris-HCl, pH 6.8

0.05 ml 20% (w/v) SDS

2.5 µl TEMED

1.0 ml 0.8% (w/v) ammonium persulphate

6.0 ml distilled water

Mix thoroughly, but with as little aeration as possible. Pour off the ethanol overlay from the separating gel and stand the plates vertically in a rack. Add stacking gel to within 3-4 mm of the top of the plates. Suspend the sample "combs" in the stacking gel and fill any remaining space with stacking gel. Leave the gels to polymerise and then remove the "combs" with the plates submerged in 'running buffer' (see 5 below).

5. Electrophoresis Conditions

Prepare the running buffer by diluting 1:10 the stock Tris-glycine buffer, pH 8.8 and adding SDS to a final concentration of 0.1% (w/v). Fill the lower electrode tank with approximately 1.5 litre of this solution.

Remove the tape from the base of the gel plates and insert into the upper electrode tank, having first smeared the gel plates and tank gaskets with a liberal amount of vaccuum grease to prevent leakage of buffer. Fill the upper tank with 'running buffer' and then layer 25 µl of samples into the wells in the gels.

Connect the electrode unit to the power supply and run the gels at a constant current of 15 mA per gel for approximately 2.5 h. When the tracking dye reaches the end of the gel, switch off the power. Remove the gels from the plates and immerse in fix-staining solution for 90 min. Destain by soaking the gels in several changes of destaining solution.

6. Fix-Staining Solution

1.25 g Coomassie brilliant blue R250

454 ml 50% (v/v) methanol

46 ml glacial acetic acid.

7. Destaining Solution

50 ml methanol

75 ml glacial acetic acid

875 ml distilled water.

APPENDIX 7

LIQUID NITROGEN STORAGE OF *B. PERTUSSIS* 134

This is the method described by Novotny and Brookes (1975).

a) Dispense 1 ml amounts of glycerol into universal bottles and autoclave at 121°C for 20 min.

b) Pipette 9 ml of a 48 h culture of *B. pertussis* 134 in SS-NA5 into each universal and mix thoroughly. Final glycerol concentration is 10% (v/v).

c) Use a 5 ml syringe and needle to inject approximately 0.5 ml volumes of the culture-glycerol mixture into plastic, liquid nitrogen storage vials with screw caps.

d) Store the vials under liquid nitrogen.

APPENDIX 8

STATISTICAL ANALYSIS

APPENDIX 8a

PAIRED-SAMPLE t-TEST

1. Tabulation of Individual Experiment Results

a) Growth in SS-NA5 and SS-NA500:

NA Uptake (pmol min^{-1} mg dry $weight^{-1}$)		ND Uptake (pmolmin^{-1} mg dry $weight^{-1}$)	
SS-NA5	SS-NA500	SS-NA5	SS-NA500
7.2	2.9	6.2	2.9
11.9	3.2	11.7	1.9
8.2	3.3	8.3	1.8

b) Growth in SS-NA5 and SS-ND500:

NA Uptake (pmol min^{-1} mg dry $weight^{-1}$)		ND Uptake (pmolmin^{-1} mg dry $weight^{-1}$)	
SS-NA5	SS-ND500	SS-NA5	SS-ND500
7.5	6.8	8.0	4.8
5.9	4.6	5.1	2.4
13.1	10.0	11.7	6.8

2. Homogeneity of Variances

a) The variances of each group of data were calculated by the formula:

$$\frac{\Sigma x^2 - (\Sigma x)^2/n}{df}$$

where : n = number values in each group = 3

df = degrees of freedom = 2.

b) Then, $\frac{\text{variance of group 1}}{\text{variance of group 2}}$ yields the "found" F value, for 2,2 df.

c) The "found" F is compared with the tabulated F value, at 2,2 df, at values of P = 0.05 and 0.01. The tabulated F values at these values of P are 19.00 and 99.00 respectively. The analysis is only valid if the variances of groups to be compared have non-significantly different variances, i.e. "found" $F < 19.00$, and therefore $P > 0.05$.

d) <u>NA uptake by SS-NA5- and SS-NA500-grown cells</u>

"found" F = 136.2

Therefore, the test is not valid.

e) <u>ND uptake by SS-NA5- and SS-NA500- grown cells</u>

"found" F = 20.8

Therefore, the test is not valid.

f) <u>NA uptake by SS-NA5- and SS-ND500-grown cells</u>

"found" F = 1.9

Therefore the test is valid.

g) <u>ND uptake by SS-NA5- and SS-ND500-grown cells</u>

"found" F = 2.3

Therefore the test is valid.

3. <u>Paired-Sample t-Test</u>

a) Tabulate the results for the two groups to be compared as follows:

<u>Twin Pair Number</u> :	<u>1</u>	<u>2</u>	<u>3</u>
x (e.g. NA uptake/SS-NA5 cells)	x_1	x_2	x_3
y (e.g. NA uptake/SS-NA500 cells)	y_1	y_2	y_3
z (= x - y)	z_1	z_2	z_3

b) Calculate : mean z, $\bar{z}$

$$\text{variance} = \frac{\sum z^2 - (\sum z)^2/n}{df}, \text{ where } n = 3 \text{ and } df = 2.$$

$$\text{standard deviation} = S_z = \sqrt{\text{variance}}$$

$$\text{"found" } t = \frac{\bar{z}}{S_z / \sqrt{n}} = \frac{\bar{z} \cdot \sqrt{n}}{S_z}, \quad n = 3.$$

c) The "found" t values are compared with the tabulated t values at P = 0.05 (4.303) and at P = 0.01 (9.925) at df = 2. A significant difference between the groups exists if the "found" t value is $>$ 4.303, i.e. $P < 0.05$.

d) <u>NA uptake by cells grown in SS-NA5 and SS-ND500</u>

<u>Twin Pair Number</u> :	<u>1</u>	<u>2</u>	<u>3</u>
NA uptake, SS-NA5 cells :	7.5	5.9	13.1
NA uptake, SS-ND500 cells :	6.8	4.6	10.0
z :	0.7	1.3	3.1

$\bar{z}$ = 1.70 ; variance = 1.56 ; S_z = 1.25 ; "found" t = 2.36.

As the "found" t is less than 4.303 ($P > 0.05$), there is no significant difference in NA uptake by cells grown in SS-NA5 or SS-ND500.

e) <u>ND uptake by cells grown in SS-NA5 and SS-ND500</u>

<u>Twin Pair Number</u> :	<u>1</u>	<u>2</u>	<u>3</u>
ND uptake, SS-NA5 cells :	8.0	5.1	11.7
ND uptake, SS-ND500 cells :	4.8	2.4	6.8
z :	3.2	2.7	4.9

$\bar{z}$ = 3.60 ; variance = 1.33 ; S_z = 1.15 ; "found" t = 5.42.

As the "found" t is greater than 4.303 ($P < 0.05$), the test shows a significant difference in the rate of uptake of ND by cells grown in SS-NA5 or SS-ND500.

APPENDIX 8b

MANN-WHITNEY U-TEST

The results are as tabulated in APPENDIX 8a. The U-test is performed as follows:

a) Arrange the results of each of the pair of groups to be compared in ascending order:

x sample (e.g. NA uptake/SS-NA5 cells) : $x_1, x_2, x_3, x_4, x_5, x_6$

y sample (e.g. NA uptake/SS-NA500 cells) : y_1, y_2, y_3.

In this test, the results tabulated in APPENDIX 8a can be grouped together. Thus there are six values for NA uptake by SS-NA5 cells and six for ND uptake by the cells.

b) Assign to each observation its rank number in the whole array of x and y values. Where two values are identical, they are each assigned the average of the two sequential rank numbers.

c) The sum of rank values for the x sample is R_1, and that for the y sample is R_2.

d) Calculate values of U_1 and U_2 from the formulae:

$$U_1 = n_1 \cdot n_2 + \tfrac{1}{2} \cdot n_2(n_2 + 1) - R_2, \text{ and}$$

$$U_2 = n_1 \cdot n_2 + \tfrac{1}{2} \cdot n_1(n_1 + 1) - R_1, \text{ where}$$

n_1 and n_2 are the number of values in the x and y samples respectively.

e) A significant difference between samples exists if either U_1 or U_2 is less than or equal to the tabulated values of U at $P = 0.05$ for specified values of n_1 and n_2.

For this test as applied to my results, $n_1 = 6$ and $n_2 = 3$. At $P = 0.05$ and $n_1 = 6$, $n_2 = 3$, the tabulated value of U is 1.

Example: NA Uptake by Cells Grown in SS-NA5 and SS-NA500

		Values in ascending order
SS-NA5	:	5.9, 7.2, 7.5, 8.2, 11.9, 13.1
SS-NA500	:	2.9, 3.2, 3.3

		Rank number
SS-NA5	:	4, 5, 6, 7, 8
SS-NA500	:	1, 2, 3

$R_1 = 39, \quad R_2 = 6$

$U_1 = 18, \quad U_2 = 0$

Thus, as U_2 is less than the tabulated U value, there is a significant difference in the rate of NA uptake by cells grown in SS-NA5 or SS-NA500.

Similar calculations were done for the other groups of data. The results were as follows:

a) ND uptake by cells grown in SS-NA5 and SS-NA500

$U_1 = 18 \quad , \quad U_2 = 0$

There is, therefore, a significant difference in the rates of ND uptake by cells grown in SS-NA5 or SS-NA500.

b) NA uptake by cells grown in SS-NA5 and SS-ND500

$U_1 = 13 \quad , \quad U_2 = 5$

There is no significant difference in the rates of NA uptake by cells grown in SS-NA5 or SS-ND500.

c) ND uptake by cells grown in SS-NA5 and SS-ND500

$U_1 = 16 \quad , \quad U_2 = 2$

There is no significant difference in the rates of ND uptake by cells grown in SS-NA5 or SS-ND500.

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