Defining the Metabolic and Growth Responses of Porcine Haemophili to Exogenous Pyridine Nucleotides and Precursors

By T. O'REILLY AND D. F. NIVEN*

Department of Microbiology, Macdonald College of McGill University, 2111 Lakeshore Road,
Ste Anne de Bellevue, Quebec, Canada H9X 1C0

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A variety of biologically important pyridine nucleotides and precursors were examined for their capacities to satisfy the V-factor requirement of 30 strains of porcine haemophili. Of the compounds tested, only NAD, NMN and nicotinamide riboside (NR) supported the growth of all strains; NADP supported the growth of only the type strain of *Haemophilus parasuis*. Further studies with the *H. parasuis* type strain and the neotype strain of *H. pleuropneumoniae* demonstrated that, during growth, these organisms exhibited affinities for NMN that were greater than those for NAD; the affinity of *H. pleuropneumoniae* for NR was similar to that for NMN, whereas *H. parasuis* exhibited relatively low affinity for NR. With either organism, equimolar amounts of NAD and NMN supported the production of approximately equal amounts of biomass whereas growth yields were substantially lower when NR was the pyridine nucleotide source. When either organism was grown in the presence of excess exogenous [*carboxyl*- ^{14}C]NAD, cessation of growth was accompanied by the apparent exhaustion of the NAD supply. Approximately 80% of the radioactivity added as [^{14}C]NAD could be recovered as extracellular [^{14}C]nicotinamide and the majority of the assimilated radioactive material was present intracellularly in the form of a [^{14}C]NAD(P) pool. The results are discussed in terms of the structural features required of a pyridine compound for it to support the growth of porcine haemophili, the capacity of these organisms to compete for pyridine nucleotide sources *in vivo*, and possible mechanisms involved in the assimilation of such compounds.

INTRODUCTION

The porcine haemophili, which include *Haemophilus parasuis* and *H. pleuropneumoniae*, are important respiratory tract pathogens of swine. *H. parasuis* causes Glässer's disease, and *H. pleuropneumoniae* pleuropneumonia. The clinical and pathological findings associated with such infections are well documented (Nicolet & Scholl, 1981; Sebunya & Saunders, 1983). However, little is known about the biochemistry and physiology of the causative organisms.

Haemophili are nutritionally fastidious, and most, including the porcine isolates, require a source of pyridine nucleotides (V-factor) for growth (Kilian, 1976; Kilian *et al.*, 1978). To provide for this requirement, media can be supplemented with NAD. However, a limited number of other pyridine compounds, including NADP, NMN, nicotinamide riboside (NR) and nicotinamide hypoxanthine dinucleotide (NHD), are known to support the growth of at least *H. influenzae* and/or *H. parainfluenzae* whereas nicotinic acid adenine dinucleotide (NAAD), the α -anomer of NAD (α -NAD), 3-acetylpyridine adenine dinucleotide and a mixture of nicotinamide (Nam), D-ribose and AMP are ineffective (Schlenk & Gingrich, 1942; Gingrich & Schlenk, 1944; Bachur & Kaplan, 1955; Lamborg *et al.*, 1958).

Abbreviations: NA, nicotinic acid; Nam, nicotinamide; NAR, nicotinic acid riboside; NR, nicotinamide riboside; NAMN, nicotinic acid mononucleotide; NAAD, nicotinic acid adenine dinucleotide; NGD, nicotinamide guanine dinucleotide; NHD, nicotinamide hypoxanthine dinucleotide; PAD, 3-pyridinealdehyde adenine dinucleotide; QA, quinolinic acid.

Although low concentrations of pyridine nucleotide sources (0.5 to 5 mM) can support detectable growth of *H. influenzae* and *H. parainfluenzae* (Gingrich & Schlenk, 1944), the extent to which haemophili can compete with host tissues for such compounds, and also the *in vivo* source and identity of V-factor, remain unknown. Serum contains NAM (Bernofsky, 1980*a*), but this compound cannot support the growth of haemophili. Although mammalian cells contain significant amounts of NAD(P)(H), the supply of V-factor in extracellular fluids (in these forms) is probably quite low as several types of mammalian cells possess extrinsic NAD(P)⁺ nucleosidases (EC 3.2.2.6) which would catabolize extracellular NAD(P) to yield NAM and (2'-P)ADP-ribose (Artman & Seeley, 1979; Artman & Frankl, 1982; Goodman *et al.*, 1982; Muller *et al.*, 1983; Johnson, 1984). Despite this, haemophili are still very successful at colonizing mucosal membranes, and many can spread through the tissues of the host. This would suggest that haemophili possess extremely efficient mechanisms for the acquisition of suitable pyridine nucleotide sources. The purpose of the present study was to investigate the *in vitro* growth responses of selected porcine haemophili towards such compounds.

METHODS

Organisms and growth conditions. According to the nomenclature of Kilian (1976) and Kilian *et al.* (1978), the organisms used in these studies were the type strain of *H. parasuis* (ATCC 19417) and the neotype strain of *H. pleuropneumoniae* (ATCC 27088). In one experiment, an additional 28 *Haemophilus* strains were also used. These strains included organisms representative of *H. parasuis*, *H. pleuropneumoniae*, *Haemophilus* taxon 'minor group', *Haemophilus* taxon C, and a group of mannitol-positive, urease-negative haemophili (O'Reilly *et al.*, 1984). Cultures were stored and frozen as described previously (O'Reilly *et al.*, 1984) except that organisms to be used as inocula for *K_s* and yield studies were grown in the presence of NADP, NAD, NMN or NR, as appropriate, harvested by centrifugation (40 000 g, 5 min) and resuspended with an equal volume of a sterile solution containing 4 vols growth medium (without added pyridine compound) and 1 vol. 75% (w/v) glycerol, before freezing at -80 °C. Little or no lysis occurred when frozen suspensions were thawed.

The growth medium (TYE) contained 2% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 100 mM-NaCl, 10 mM-KCl, 10 mM-Na₂HPO₄, 10 mM-glucose and KOH to pH 7.4; tryptone and yeast extract from single production lots only were used for these studies. Pyridine nucleotides and precursors were filter-sterilized and added to autoclaved TYE just before inoculation (liquid media) or plate pouring (solid media); media were solidified by incorporation of 1.6% (w/v) agar (Difco). Liquid cultures (10 ml) were grown in 50 ml flasks fitted with foam rubber closures (Canadian Laboratory Supplies).

Frozen inocula were thawed rapidly at 37 °C and either streaked directly on to solid media or diluted tenfold with sterile TYE (without added pyridine compound), samples (0.1 ml) of which were used to inoculate liquid media. Incubation was at 37 °C. Liquid cultures were agitated at 200 r.p.m. Glycerol, used in frozen suspensions, is not used as an energy source by either *H. parasuis* or *H. pleuropneumoniae*.

Preparation of pyridine nucleotides and precursors. NAD and NMN (50 mg lots) were purified by anion-exchange chromatography, as described by Bernofsky (1980*b*), except that a linear gradient (0–2 M, 1 l total volume) of HCOOH was used to elute a 2.5 × 45 cm column. NADP, α-NAD, nicotinamide guanine dinucleotide (NGD), NHD and 3-pyridinealdehyde adenine dinucleotide (PAD) (10–25 mg lots) were purified similarly except that HCOONH₄ was used as eluant.

NR and nicotinic acid riboside (NAR) are not available commercially and were prepared by acid phosphatase (EC 3.1.3.2) treatment of NMN and nicotinic acid mononucleotide (NAMN), respectively. To 50 mg substrate contained in 3 ml 100 mM-CH₃COONH₄/CH₃COOH to pH 5.0, was added 5 mg acid phosphatase. The solution was sterilized by filtration (0.45 µm pore size), transferred aseptically to a sterile bottle, and incubated for 48 h at 37 °C. The reaction was halted by immersing the reaction vessel in boiling water (3 min) and the precipitate removed by centrifugation. Reactants and products were separated and purified by anion-exchange chromatography, as described above (HCOOH gradient), except that NR was eluted with H₂O (300 ml total volume).

Pooled fractions containing any one pyridine nucleotide or nucleoside were lyophilized, and, where necessary, HCOONH₄ was removed with 95% (v/v) ethanol (Bernofsky, 1980*b*). Samples were redissolved in a small volume of HCl (1 mM), and stored at -20 °C. NAAD, NAMN, NAM, nicotinic acid (NA) and quinolinic acid (QA) were used without further purification.

Each pyridine compound was analysed for purity by spectrophotometry (Beckman DU-7 spectrophotometer) and HPLC; the HPLC system consisted of a Waters model 510 pump, model 441 detector (254 nm) and Wisp 710B automatic injector, with the mobile phase (200 mM-NH₄H₂PO₄/NH₄OH to pH 5.1) eluting a 0.4 × 15 cm µBondapak C₁₈ column (Waters) at 2 ml min⁻¹. Chromatograms were recorded on a Hewlett-Packard 3390A

integrator and the calculated peak areas used to estimate compound purity. This procedure, based on that of Kothari & Taylor (1982), gave the following retention times (min): QA, 0.86; NAMN, 1.06; NMN, 1.22; NAR, 1.64; NR, 1.79; NA, 2.13; α -NAD, 6.20; NGD, 6.38; NAm, 7.60; NHD, 8.47; NADP and NAAD, 8.66; PAD, 12.21; NAD, 15.93.

Determination of K_s and yield values. Pyridine compounds were prepared as sterile stock solutions of known concentration (determined spectrophotometrically) and small volumes (0.0–0.1 ml) used to supplement TYE to produce a range of limiting concentrations (Pirt, 1975). After inoculation, samples (0.1 ml) were removed from cultures at known time intervals, and added to 0.1 ml 2% (v/v) formalin in 145 mM-NaCl. The optical densities (660 nm) of such suspensions were determined (Beckman DU-7 spectrophotometer) and corrected for dilution. Initial growth rates (μ) were determined from plots of growth [$\ln(OD_{660} \times 10^3)$] versus incubation time (h). The slope of the line of best fit was calculated by linear regression. Consideration of 4 to 10 data points (depending on μ and/or duration of growth) gave correlation coefficients of 0.97 or greater. Each experiment was monitored in triplicate, and the μ value expressed as the mean $\pm$ SEM. K_s values were determined from secondary plots of reciprocal μ versus the reciprocal of the initial concentration of the pyridine nucleotide source. The data gave straight line plots with probabilities of fit greater than 99%. All experiments were done at least twice.

Yield (Y) values (mg dry weight produced per nmol pyridine nucleotide source supplied) were determined as follows. Using a calibration plot, the optical densities of cultures, after growth had ceased, were used to calculate cell yields (mg dry weight ml⁻¹; mean of three determinations $\pm$ SEM) which were then plotted as a function of the initial concentration (nmol ml⁻¹) of the pyridine compound supplied. For any such plot, the slope of the linear portion, which is equivalent to Y (Pirt, 1975), was calculated using regression techniques. Consideration of four to seven data points gave correlation coefficients greater than 0.98. All experiments were done at least twice.

Analyses. Samples (0.1 ml) from each of three cultures were combined for measurement of pH. A total of 18 cultures was used, with sets of nine being monitored alternately, at each time interval, for either culture pH or growth.

To determine extracellular glucose or NAD concentrations, culture samples (0.1 ml) were centrifuged (2 min, Eppendorf centrifuge) to sediment the organisms and the supernatant fractions retained and stored overnight at -20°C . Six cultures were used, with sets of three being monitored alternately, at each time interval, for either growth or extracellular glucose or NAD. Glucose was determined essentially as described by Niven *et al.* (1977) except that the enzyme mix contained 2500 units glucose oxidase (EC 1.1.3.4) and 1200 units peroxidase (EC 1.11.1.7), and standard glucose solutions contained appropriate dilutions of TYE (lacking glucose). NAD was determined by the cycling assay described by Bernofsky & Swan (1973) and modified by Lilius *et al.* (1979), except that HEPES was the buffer system used, 12.5 units alcohol dehydrogenase (EC 1.1.1.1) were used for each assay, and standard NAD solutions contained appropriate dilutions of TYE. Samples were diluted, as required, with 25 mM-Na₂HPO₄, 100 mM-NaCl, 10 mM-KCl, 5 mM-MgSO₄ and HCl to pH 7.4, such that the amount of NAD to be assayed was consistent with the sensitivity of the method (10–125 pmol).

All experiments were done twice and culture pH and extracellular glucose and NAD concentrations were expressed as means $\pm$ SEM.

Determination of the growth-related metabolic fate of exogenous [¹⁴C]NAD. Bacterial cultures (10 ml) were grown in the presence of [¹⁴C]NAD (16 μM , 2.11 GBq mmol⁻¹). Parallel cultures ([¹²C]NAD) were used to monitor growth.

To monitor cellular assimilation of radiolabel during growth, samples (0.05 or 0.1 ml) were removed from cultures at known time intervals, diluted to 1 ml with sterile TYE (37 $^\circ\text{C}$) and a 0.9 ml portion was filtered and washed (4.5 ml TYE, 37 $^\circ\text{C}$) on prewashed membrane filters (0.45 μm pore size) which were then removed to scintillation vials and air dried.

To determine the fate of extracellular radiolabel, culture samples (0.05 ml) were centrifuged and the supernatant fractions retained and stored overnight at -20°C . Supernatant fractions (5 μl) were spotted (in duplicate) on cellulose TLC plates (160 μm thick; Eastman Chromagram) and overlaid with 5 μl of a solution containing 20 mM-NAm, 15 mM-NR, 15 mM-NMN, 10 mM-NAD and 10 mM-NADP. The nicotinic acid derivatives of these compounds were not included, for neither intact cells nor cell free extracts derived from *H. parasuis* or *H. pleuropneumoniae* are capable of metabolizing such compounds (unpublished data). Plates were developed in the ammonium acetate/ethanol solvent of Pinder *et al.* (1971), under a saturated atmosphere, and the separated pyridine compounds detected by short wavelength UV light. Regions that contained these were removed, by scraping, to scintillation vials, followed by the remainder of the lane material which was also analysed for radioactivity. In addition, for each organism, a sample removed at early stationary phase was treated as above, except that a butanol/NH₄OH/H₂O solvent system (Pinder *et al.*, 1971) was used to develop TLC plates.

Formic acid-soluble and trichloroacetic acid (TCA)-insoluble fractions of stationary phase organisms were analysed to investigate the nature of intracellular radiolabelled compounds and whether or not they were incorporated into cellular materials. For formic acid extraction, 1 ml culture, diluted with TYE (9 ml, 37 $^\circ\text{C}$), was immediately filtered and washed (9 ml TYE, 37 $^\circ\text{C}$) on a prewashed membrane filter which was then removed to a

beaker on ice and agitated with 2.5 ml ice-cold 6 M-HCOOH for 30 min. The extract was removed, the filter and beaker rinsed with cold HCOOH (6 M, 2.5 ml), and the combined formic acid fractions filtered again. The filter was washed with H₂O (10 ml) and the combined filtrates lyophilized (24 h). Dried extracts were dissolved in either 0.125 ml H₂O (*H. parasuis*) or 0.25 ml H₂O (*H. pleuropneumoniae*) and samples (5 µl) removed for determination of radioactivity. Portions (5 µl) of each sample were also subjected to TLC, as described above, and the separated pyridine compounds removed to scintillation vials. For TCA extraction, 1 ml culture was added to 1 ml ice-cold TCA (10%, w/v), the samples were stored on ice for 30 min, and the TCA-insoluble material was collected by filtration and washed twice with H₂O (5 ml). Filters were removed to scintillation vials and air dried.

Aquasol (10 ml; New England Nuclear) was added to samples which were then shaken gently overnight. After equilibration in the dark (> 2 h), the samples were analysed for radioactivity using a Beckman model LS 7500 liquid scintillation counter. Quench was monitored by the H-number (Compton edge) method.

Chemicals. NADP (sodium salt), NAD (grade III), α -NAD (grade II), NGD, NHD (grade I, sodium salt), PAD, NAAD (sodium salt), NMN, NAMN, NAM, NA, QA, AMP (type II, sodium salt), D-ribose, *o*-dianisidine dihydrochloride, thiazolyl blue, phenazine ethosulphate and alcohol dehydrogenase (cat. no. A3263) were obtained from Sigma. [*carboxyl*-¹⁴C]NAD (57 mCi mmol⁻¹; 2.11 GBq mmol⁻¹) was purchased from Amersham and HEPES, acid phosphatase (grade II), glucose oxidase (grade II) and peroxidase (grade II) were from Boehringer-Mannheim. Anion-exchange resin (AG 1-X4, 200–400 mesh, formate form) was supplied by Bio-Rad.

HPLC-grade NH₄H₂PO₄ was used and, whenever possible, all other reagents were of analytical grade; glass-distilled water was used throughout.

RESULTS AND DISCUSSION

Pyridine nucleotide sources supporting the growth of porcine haemophili

When TYE agar plates were supplemented (to 5 µM) with either QA, NA, NAM, NAR, NR, NAMN, NMN, NAAD, NAD, NADP or a mixture of NAM, D-ribose and AMP, only NR, NMN and NAD supplementation allowed luxuriant growth of all 30 *Haemophilus* strains examined. Media supplemented with NADP supported luxuriant growth of only the type strain of *H. parasuis*; the other organisms either failed to grow or produced pinpoint colonies. The NADP used in these studies was purified by anion-exchange chromatography because preliminary studies (not shown) demonstrated that although commercial NADP could support the growth of *H. parasuis* and *H. pleuropneumoniae* in liquid culture, only the former organism could use purified NADP as a pyridine nucleotide source. These results suggest that the appearance of the pinpoint colonies could have been due to growth supported by a NADP degradation product generated during the preparation, storage or incubation of the agar plates. Such studies, and the fact that HPLC analysis of commercial NADP revealed the presence of up to 2.5% NR and 0.5% NMN, would suggest that the capacities of other haemophili to utilize NADP as pyridine nucleotide source should, perhaps, be re-examined using purified compound.

The structural relationships between the pyridine compounds that did, and did not, support the growth of the porcine haemophili indicate that, to promote the growth of these organisms, the pyridine nucleotide source must possess an intact pyridine-ribose bond and the pyridine carbonyl group must be amidated. In support of the latter conclusion, PAD could not support the growth, in liquid culture, of either *H. parasuis* or *H. pleuropneumoniae*, demonstrating that substitution of an aldehyde group, rather than a carboxylic acid (cf. NAAD), for the amide group, also prevents the compound from being utilized. Furthermore, in keeping with the results of Gingrich & Schlenk (1944) and Bachur & Kaplan (1955), we have also demonstrated that NGD and NHD, but not α -NAD, can support the growth of the latter two organisms; these results would suggest that NAD analogues, bearing slight alterations with respect to the adenine moiety, may still function as pyridine nucleotide sources, whereas anomerization about the pyridine-ribose bond renders the molecule ineffective.

In summary, we have identified the structural features required of a pyridine compound for it to support the growth of porcine haemophili, but it remains to be elucidated whether the requirement for such compounds by these organisms reflects their limited capacity for metabolism, membrane transport or both.

K_s and Y values for the pyridine nucleotide sources supporting the growth of porcine haemophili

At a concentration of 5 µM-NAD, growth rate and final cell density for *H. parasuis* and *H. pleuropneumoniae* are unlimited by the NAD supply (this study). Under such conditions, the

Fig. 1. Effect of NAD concentration on the initial growth rate (a) and final cell yield (b) of *H. parasuis*. Growth of the organisms, in TYE medium, was monitored turbidimetrically and the optical density readings were used to calculate initial growth rates and final cell yields. The symbols represent mean values and the SEM is either indicated by vertical bars or encompassed by the size of the symbol.

Fig. 2. Effect of NMN concentration on the initial growth rate (a) and final cell yield (b) of *H. parasuis*. Details are as in the legend to Fig. 1.

specific growth rates (mean $\pm$ SEM) for *H. parasuis* and *H. pleuropneumoniae* were 0.67 ± 0.01 ($n = 16$) and 1.42 ± 0.03 ($n = 14$), respectively. Final cell densities ($\text{mg dry weight ml}^{-1}$; mean $\pm$ SEM) were 1.68 ± 0.04 and 2.41 ± 0.05 , respectively.

Figs 1 and 2 illustrate the effect of decreasing the initial concentrations of NAD and NMN on the initial growth rates (Figs 1a and 2a) and final cell densities (Figs 1b and 2b) of *H. parasuis*. Whereas a reduction in the initial NAD concentration, from approximately 5 μM to approximately 0.5 μM , slightly decreased the growth rate (from 0.67 to 0.57) but had little effect

Fig. 3. Effect of NR concentration on the initial growth rate (a) and final cell yield (b) of *H. parasuis*. Details are as in the legend to Fig. 1.

on the final cell density, a similar decrease in the NMN concentration reduced both growth rate and cell yield. However, when the NAD concentration was decreased further, the final yield of organisms was also reduced. Although growth rates were approximately equal when the NAD and NMN concentrations were approximately 0.5 μM, further decreases required the NMN concentration to be lowered to a much greater extent than was the case for NAD. An initial concentration of approximately 40 μM was required for the growth rate of *H. parasuis* to be unlimited by the supply of NR, and decreasing the NR concentration to below 10 μM decreased the growth rate considerably (Fig. 3a). At all NR concentrations tested, the final yield of organisms appeared to be regulated by its provision although supplying the organisms with 39 μM-NR resulted in a final cell density approaching that obtained in the presence of excess NAD (cf. Figs 1b and 3b). With NADP as pyridine nucleotide source, plots of growth rate versus initial NADP concentration for *H. parasuis* were qualitatively similar to those presented in Figs 1–3, although at 52 μM the growth rate was still limited by the NADP supply (not shown); however, as the NADP concentration approached 52 μM, the presence of other pyridine nucleotide sources, as contaminants of the NADP preparation, became increasingly important, and precluded the continuation of such studies. Furthermore, when initial NADP concentrations were less than 10 μM, growth rates were very low, and, consequently, long incubation times were required for the organisms to enter stationary phase; owing to uncertainty with respect to the stability of NADP during extended incubation at 37 °C, the effect of NADP concentration on final cell yield was not investigated.

Results qualitatively similar to those presented in Figs 1 and 2 were obtained when *H. pleuropneumoniae* was grown in the presence of various initial concentrations of NAD and NMN. However, with NMN, a reduction in growth rate required the NMN concentration to be decreased to very low levels (< 0.05 μM), and, consequently, rapid depletion of NMN prevented accurate estimation of growth rates when the organisms were supplied with such low NMN concentrations. The latter situation was also the case when NR was the pyridine nucleotide source for the growth of *H. pleuropneumoniae*; however, to decrease the final yield of organisms, decreasing the NR concentration to approximately 4 μM was sufficient, whereas the NMN concentration had to be decreased below 1 μM (not shown).

K_s values and, where appropriate, Y values for the pyridine nucleotide sources supporting the growth of the two organisms under study are presented in Table 1, but note that, because of the

Table 1. K_s and Y values for the pyridine nucleotide sources supporting the growth of porcine haemophili

K_s (μM) and Y [mg dry wt (nmol pyridine nucleotide source) $^{-1}$] values are the means from at least two separate experiments; ND, not determined.

Organism	NAD		NMN		NR		NADP	
	K_s	Y	K_s	Y	K_s	Y	K_s	Y
<i>H. parasuis</i>	0.21	4.00	0.03	4.23	0.74	0.46	7.40	ND
<i>H. pleuropneumoniae</i>	0.24	3.63	<0.03	3.90	<0.03	1.06	No growth	

difficulties encountered in the determination of the initial growth rates of *H. pleuropneumoniae*, at low initial NMN and NR concentrations, only upper estimates of the corresponding K_s values are listed.

H. parasuis and *H. pleuropneumoniae* had similar affinities for NAD and for NMN with both organisms exhibiting greater affinity for NMN (Table 1) whilst the Y values for both substrates, and both organisms, were approximately equal. These data would suggest that *H. parasuis* and *H. pleuropneumoniae* possess similar mechanisms for the assimilation of NAD and of NMN, but whether the cellular affinities for these compounds reflect the affinities of transport or metabolic processes is not known. It is interesting to note that the K_s values for NAD and NMN, and also NR, are very similar to the K_m values reported for the transport of NAm by *Escherichia coli* (0.2 μM ; Andersen & von Meyenburg, 1977) and NAm (0.04 μM) and NA (1 μM) by *Bordetella pertussis* (McPheat & Wardlaw, 1980).

H. parasuis and *H. pleuropneumoniae* differed dramatically with respect to their affinities for NR as a pyridine nucleotide source (Table 1). However, it remains unknown whether the affinities of the transport or metabolic processes of these organisms for NR are responsible for the different K_s values. With both organisms, the Y values for NR were substantially less than those for NAD and NMN. This latter finding would suggest that during growth in the presence of NAD or NMN, their assimilation by porcine haemophili does not involve their prior catabolism to NR by means of extracytoplasmic enzymes.

The affinity of *H. parasuis* for NADP was very poor with respect to the affinities for the other pyridine nucleotides supporting growth (Table 1). However, the singularity of this strain with respect to its capacity to utilize NADP would suggest that this organism possesses either a specific process(es) for the assimilation of NADP, or, perhaps more likely, an enzyme (e.g. alkaline phosphatase, EC 3.1.3.1; NAD⁺ pyrophosphatase, EC 3.6.1.22) that can accommodate the 2'-phosphate of NADP, albeit at low affinity.

In summary, the high affinities exhibited by the porcine haemophili for NAD and other pyridine nucleotide sources could explain the capacities of these organisms to compete effectively for such compounds *in vivo*. It is notable that the extrinsic NAD nucleosidases of erythrocytes, from various mammalian species, have K_m values for NAD of greater than 19 μM (Goodman *et al.*, 1982). However, the activities and concentrations of such enzymes could still influence the availability of pyridine nucleotide sources supporting the growth of porcine haemophili, and the growth of these organisms *in vivo* may still have to occur under conditions of pyridine nucleotide source limitation.

Consumption of glucose and NAD during the growth of porcine haemophili in the presence of excess NAD

In Fig. 4, bacterial growth, culture pH and extracellular glucose and NAD concentrations are plotted against time for *H. parasuis*. Qualitatively similar results (not shown) were obtained with *H. pleuropneumoniae*.

Growth of either organism was accompanied by decreases in extracellular glucose concentration and culture pH and, as cultures of either organism reached stationary phase, the glucose supply was essentially exhausted and culture pH attained a constant value (e.g. see Fig. 4). Increasing the initial glucose concentration increased the extent of growth and decreased the

Fig. 4. Consumption of glucose and NAD during the growth of *H. parasuis* in the presence of excess NAD. Growth of the organisms (●) was monitored turbidimetrically and cell-free culture supernatant fractions were assayed for glucose (▲) and NAD (■) by enzymic methods; the open circles (growth) and open squares (NAD concentration) show the results obtained from a parallel series of experiments in which cultures were supplemented with NAD (to $5 \mu\text{M}$) at 13 h. Culture pH (△) was monitored potentiometrically. The symbols represent mean values and the SEM is either indicated by vertical bars or encompassed by the size of the symbol.

final pH (not shown), demonstrating that under the growth conditions used (Fig. 4), the production of biomass was limited by the supply of glucose. However, despite this, and the provision of cultures with excess NAD (cf. Fig. 1), determination of extracellular NAD concentrations revealed that, on cessation of growth, the NAD supply was essentially exhausted (i.e. at or near the limit of detection) (e.g. see Fig. 4a). Rapid disappearance of exogenous NAD also occurred when early stationary phase cultures were supplemented with NAD (e.g. see Fig. 4a). These results could be due to the metabolism of NAD by an extracytoplasmic enzyme(s) to yield an alternative, exogenous, pyridine nucleotide source(s) or the NAD, either intact or via some metabolite, could have been assimilated by the bacteria.

Metabolic fate of exogenous [^{14}C]NAD during the growth of porcine haemophil

Bacteria were grown in the presence of excess [^{14}C]NAD. Owing to limitations with respect to the volumes of culture supernatant fractions that could be applied to TLC plates, and the specific activity of the radiolabelled NAD ($2.11 \text{ GBq mmol}^{-1}$), the initial NAD concentrations had to be increased to $16 \mu\text{M}$.

Fig. 5 shows results obtained with *H. parasuis*; similar results (not presented) were obtained with *H. pleuropneumoniae*. With both organisms, growth was accompanied by the assimilation of radiolabel from the growth medium but net uptake stopped before the organisms entered stationary phase despite the fact that only a small percentage ($\sim 20\%$) of the radiolabel had been assimilated. This effect was emphasized in plots of the logarithm of the cell-associated radiolabel versus incubation time (not shown), and these plots also demonstrated that, at least during mid-exponential phase, the uptake of radiolabel from the growth medium paralleled the growth of the organisms.

Fig. 5. Metabolic fate of exogenous [¹⁴C]NAD during the growth of *H. parasuis*. (a) Growth of the organisms (●) was monitored turbidimetrically and membrane filtration was used to collect organisms for the detection of cellular assimilation of radioactive material (■). (b) The appearance and/or disappearance of extracellular ¹⁴C-labelled pyridine nucleotides and precursors was monitored by subjecting cell-free culture supernatant fractions to TLC in the presence of known pyridine compounds, followed by determination of the radioactivity associated with each spot: △, NADP; ○, NAD; ▲, NMN; □, NR; ▽, NAM.

The radioactivity associated with stationary phase cells of *H. parasuis* and *H. pleuropneumoniae* represented the assimilation of 1.84 and 1.26 nmol NAD (mg dry weight)⁻¹, respectively. As 0.25–0.275 nmol NAD can support the production of 1 mg dry bacterial weight (Table 1), our results indicate that the organisms removed substantially more NAD from the growth media than was required to support the observed extents of biomass production. Analysis of the TCA-insoluble material, derived from stationary phase *H. parasuis* and *H. pleuropneumoniae*, for radioactivity, revealed that these extracts contained only 1–3% of the radioactivity associated with the intact organisms, and at least some of this TCA-precipitable radioactive material could, perhaps, have been derived from enzyme-bound pyridine nucleotides (cf. Bernofsky & Pankow, 1973). TLC analysis of the formic acid-soluble material, derived from *H. parasuis* and *H. pleuropneumoniae*, demonstrated that, of the radioactive material recovered from the TLC plates (85–100% recovery), 97% and 93% of the radioactivity, respectively, co-chromatographed with the pyridine compound overlays. The percentage distribution of the radioactivity amongst these compounds (NADP:NAD:NMN:NR:NAM) was 12:82:2:1:3 for the *H. parasuis* extract, and 18:72:5:3:2 for the *H. pleuropneumoniae* extract. The predominance of NAD and NADP in such extracts is in agreement with the results of similar studies with *E. coli* (Lundquist & Olivera, 1971; Andersen & von Meyenburg, 1977; Hillyard *et al.*, 1981) and *Salmonella typhimurium* (Foster & Baskowsky-Foster, 1980); however, to what extent the low levels of NMN, NR and NAM are representative of intracellular metabolites, as against breakdown products of NAD(P)(H), generated during the extraction and analytical procedures is not known.

It would appear, therefore, that although *H. parasuis* and *H. pleuropneumoniae* assimilated only a small proportion of the available NAD, the accumulated material was not only greater than that required to support the observed production of biomass but also present, intracellularly, predominantly in the form of pyridine nucleotide sources that could be used to support further growth. Indeed, we have demonstrated that either organism, grown to late exponential phase (initial NAD concentration 5 μ M), can undergo at least three divisions (determined turbidimetrically) when the washed organisms are transferred to fresh growth medium lacking an added pyridine nucleotide source (not shown). Lundquist & Olivera (1973) have described analogous results with a NA-requiring auxotroph of *E. coli*.

The appearance and/or disappearance of extracellular 14 C-labelled pyridine nucleotides and precursors during the growth of *H. parasuis* and *H. pleuropneumoniae* in the presence of [14 C]NAD, was monitored by TLC analyses of culture supernatant fractions. In support of the results described above (e.g. see Fig. 4*a*), when the growth of either organism ceased, the supply of [14 C]NAD was essentially exhausted (e.g. see Fig. 5). Interestingly, the disappearance of the [14 C]NAD was accompanied by the transient appearance, particularly during the growth of *H. parasuis*, of extracellular [14 C]NMN, and with both organisms, the majority of the [14 C]NAD available to support growth was degraded to yield extracellular [14 C]NA*m* (e.g. see Fig. 5). Although not shown, the identity of the latter radiolabelled compound was confirmed by TLC, using the butanol solvent system.

The extracellular degradation of [14 C]NAD by bacteria has been investigated by several research workers. Murakawa & Takahashi (1978) reported that an *unc* mutant of *E. coli*, and also the parent strain, produced extracellular NA*m* from [14 C]NAD, and, depending on the organism, traces of NA, NMN and possibly NADP. On the other hand, Hillyard *et al.* (1981) reported that extracellular degradation of NAD by a NA-requiring strain of *E. coli* yielded NA, and Foster *et al.* (1979) have demonstrated that mutants of *S. typhimurium* [defective in QA synthetase or NA phosphoribosyltransferase (EC 2.4.2.11)] produce extracellular [14 C]NMN from exogenously-supplied [14 C]NAD. Although these data could indicate the uptake and intracellular metabolism of NAD, followed by leakage of products from the bacteria, they also suggest that NAD can be metabolized by extracytoplasmic enzymes, yielding products which could then be transported into the bacteria (e.g. see Foster & Moat, 1980). In this respect, Falconer *et al.* (1984) have demonstrated that *S. typhimurium* possesses a membrane-associated NAD pyrophosphatase, which, if oriented extrinsically, could metabolize extracellular NAD to yield NMN (and AMP). Furthermore, Liu *et al.* (1982) have demonstrated that NMN is not metabolized extracellularly by *S. typhimurium*, and that growing organisms can transport the molecule intact.

The transient appearance of extracellular NMN (Fig. 5*b*), and also the K_s and Y values for NMN and NAD (Table 1), suggest that *H. parasuis* possesses mechanisms for the acquisition of NMN and NAD that are similar to those discussed for *S. typhimurium*. Although other interpretations are possible, the K_s values for NMN and NAD could reflect, respectively, the affinity of a transport system for NMN, and the somewhat lower affinity of an extrinsic NAD pyrophosphatase; such an enzyme is present in *H. influenzae* (Kahn & Anderson, 1983). Whereas NMN would be transported intact, NAD would be subjected, initially, to pyrophosphatidic cleavage, yielding NMN to be transported and AMP. Greater activity and/or concentration of NAD pyrophosphatase, with respect to NMN translocase, could then explain the transient extracellular accumulation of NMN. Although a similar transient accumulation of NMN was not so obvious in comparable experiments with *H. pleuropneumoniae* (not shown), the K_s and Y values obtained for NAD and NMN (Table 1) would suggest that mechanisms similar to those described for *H. parasuis* are also operative in *H. pleuropneumoniae*.

The appearance of [14 C]NA*m* extracellularly during the growth of *H. parasuis* and *H. pleuropneumoniae* in the presence of [14 C]NAD (e.g. see Fig. 5*b*), would appear to be anomalous, for these organisms cannot use NA*m* as a pyridine nucleotide source (this study). However, in keeping with these findings, Bachur & Kaplan (1955) have reported that intact cells of *H. parainfluenzae* can degrade NADP, NAD, NMN, NR and NHD by nucleosidase-like reactions. Although such results could indicate that the production of NA*m* by *H. parasuis* and *H.*

pleuropneumoniae was due to the activity of extracytoplasmic NAD⁺ or NMN nucleosidases (EC 3.2.2.5 and EC 3.2.2.14), they do not exclude the possibility that the NAM was produced intracellularly, by an incomplete pyridine nucleotide cycle, and then excreted. However, the findings that *H. parasuis* and *H. pleuropneumoniae*, in the stationary phase of their respective growth cycles, contained substantial amounts of NAD(P), but only traces of NAM, and that the extracellular appearance of NAM ceased before growth terminated (Fig. 5), would suggest that the production of [¹⁴C]NAM from [¹⁴C]NAD was a consequence of extracytoplasmic metabolism. As such a process causes the apparent waste of pyridine nucleotide source, further studies are in progress in an attempt to rationalize these findings in the context of overall cellular activity.

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