Utilization and Metabolism of NAD by *Haemophilus parainfluenzae*

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The utilization of exogenous nicotinamide adenine dinucleotide (NAD) by *Haemophilus parainfluenzae* was studied in suspensions of whole cells using radiolabelled NAD, nicotinamide mononucleotide (NMN), and nicotinamide ribonucleoside (NR). The utilization of these compounds by *H. parainfluenzae* has the following characteristics. (1) NAD is not taken up intact, but rather is degraded to NMN or NR prior to internalization. (2) Uptake is carrier-mediated and energy-dependent with saturation kinetics. (3) There is specificity for the β -configuration of the glycopyridine linkage. (4) An intact carboxamide group is required on the pyridine ring. The intracellular metabolism of NAD was studied in crude cell extracts and in whole cells using carbonyl- ^{14}C -labelled NR, NMN, NAD, nicotinamide, and nicotinic acid as substrates in separate experiments. A synthetic pathway from NR through NMN to NAD that requires Mg^{2+} and ATP was demonstrated. Nicotinamide was found as an end-product of NAD degradation. Nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide were not found as intermediates. The NAD synthetic pathway in *H. parainfluenzae* differs from the Preiss–Handler pathway and the pyridine nucleotide cycles described in other bacteria.

INTRODUCTION

NAD is an essential coenzyme in cellular oxidation–reduction reactions. Most prokaryotes and eukaryotes are capable of synthesizing NAD *de novo* utilizing dihydroxyacetone phosphate and aspartate, formate and aspartate or tryptophan (Foster & Moat, 1980). These pathways produce quinolinic acid (QA) as an intermediate and each terminate in the common pathway shown in Fig. 1. The intracellular biosynthetic pathway of NAD has been well studied in *Salmonella typhimurium* and *Escherichia coli* (Foster & Moat, 1980). In these and most other prokaryotic cells dihydroxyacetone phosphate condenses with aspartic acid to form QA (Chandler & Gholson, 1972; Foster & Moat, 1980). The QA is converted to nicotinic acid mononucleotide (NAMN) in the presence of 5'-phosphoribosyl pyrophosphate (PRPP). The NAMN reacts with ATP to form nicotinic acid adenine dinucleotide (NAAD) which forms NAD with the addition of an amino group from glutamine. This pathway does not exist in *Haemophilus* species that require exogenous NAD for growth.

In addition to the *de novo* synthetic pathways, several salvage pathways known as the pyridine nucleotide cycles (PNCs) have been described that allow some cells to utilize pre-formed pyridine bases such as nicotinamide (Nam), nicotinic acid, or nicotinamide mononucleotide (NMN) when they are available (Foster, 1981; Hillyard *et al.*, 1981). These pyridine bases may be obtained from the breakdown of intracellular NAD or from exogenous sources.

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Abbreviations: QA, quinolinic acid; NAMN, nicotinic acid mononucleotide; NAAD, nicotinic acid adenine dinucleotide; PNC, pyridine nucleotide cycle; NMN, nicotinamide mononucleotide; PRPP, 5'-phosphoribosyl pyrophosphate; Nam, nicotinamide; NR, nicotinamide ribonucleoside; TNAD, thionicotinamide adenine dinucleotide; N[U- ^{14}C]AD, nicotinamide[U- ^{14}C]adenine dinucleotide.

Fig. 1. NAD metabolism in aerobic bacteria. The solid arrows indicate the Preiss–Handler pathway (Preiss & Handler, 1958*a, b*), with the common pathway in bold arrows. The dashed arrows represent alternative PNCs described in *S. typhimurium* (Foster & Moat, 1980; Hughes *et al.*, 1983; Liu *et al.*, 1982). NR can be utilized as an exogenous source of pyridine for the synthesis of NAD via one of the two pathways shown.

There has been little attention paid to the utilization of exogenous NAD or to the synthetic and degradative pathway of NAD in *Haemophilus parainfluenzae* or *Haemophilus influenzae*. These organisms are of interest because they are important human pathogens. They are generally considered to require exogenous β -NAD for growth (Killian, 1980). Nicotinamide ribonucleoside (NR) or NMN can also support growth; however, 3-acetylpyridine adenine dinucleotide, 3-acetylpyridine ribonucleoside, nicotinamide glucoside, nicotinamide pyranoside, α -NAD, nicotinic acid, and Nam do not support growth (Bachur & Kaplan, 1955). These growth requirements suggest that NR or possibly NMN are the metabolites that enter the cell and serve as substrates for the intracellular synthesis of NAD.

The purpose of this study was to characterize the utilization of exogenous NAD and to examine the intracellular metabolism of NAD in *H. parainfluenzae*. We showed that NAD is hydrolysed to NMN and subsequently to NR (Cynamon *et al.*, 1985). The latter compound is internalized and converted intracellularly to NAD via NMN (Sorg *et al.*, 1986).

METHODS

Organism and culture media. The *H. parainfluenzae* strain used in our studies was a clinical isolate obtained from the Microbiology Laboratory at the Veterans Administration Medical Center, Syracuse, NY. This organism grew optimally in $1\ \mu\text{M}$ -NAD and $5\ \mu\text{M}$ -NMN or NR. It did not grow in Nam, NAAD, or NAMN, even at concentrations as high as 100 mM. A chemically defined medium (M1c) which supports rapid growth of *H. influenzae* (Herriot *et al.*, 1970) was used with the following modifications: polyvinyl alcohol, nitroethanol and haemin were omitted; oleic acid was added to a final concentration of $20\ \mu\text{g ml}^{-1}$. Bacteria were titrated on chocolate agar plates (BBL).

Chemicals and reagents. β -NAD, β -NMN, β -NAAD, β -NAMN, α -NAD, α -NMN, Nam, nicotinic acid, ATP, AMP and PRPP were obtained from Sigma. Thionicotinamide adenine dinucleotide (TNAD) was obtained from P-L Biochemicals. Nicotinamide[U- ^{14}C]adenine dinucleotide (N[U- ^{14}C]AD) (uniform labelled adenine) [$\text{carbonyl-}^{14}\text{C}$]NAD, [$\text{carbonyl-}^{14}\text{C}$]nicotinamide and [$\text{carbonyl-}^{14}\text{C}$] nicotinic acid were obtained from Amersham. Their specific activities were $269\ \text{mCi mmol}^{-1}$ ($9.95\ \text{GBq mmol}^{-1}$), $53\ \text{mCi mmol}^{-1}$ ($1.96\ \text{GBq mmol}^{-1}$), $56\ \text{mCi mmol}^{-1}$ ($2.07\ \text{GBq mmol}^{-1}$) and $56\ \text{mCi mmol}^{-1}$ ($2.07\ \text{GBq mmol}^{-1}$), respectively. The [$\text{carbonyl-}^{14}\text{C}$]NAD utilized in the uptake studies was at a final concentration of $0.15\ \mu\text{M}$ and a specific activity of $24\ \mu\text{Ci }\mu\text{mol}^{-1}$ ($0.88\ \text{MBq mmol}^{-1}$) except where indicated.

[$\text{carbonyl-}^{14}\text{C}$]NMN was prepared from [$\text{carbonyl-}^{14}\text{C}$]NAD by treatment with snake venom phosphodiesterase (Calbiochem-Behring) or NAD pyrophosphatase (Sigma) in Tris/HCl buffer pH 7.5. [$\text{carbonyl-}^{14}\text{C}$]NR was made by adding alkaline phosphatase (Calbiochem-Behring) to the above reaction mixture. The labelled NMN was purified by reversed-phase HPLC using a C_{18} $\mu\text{Bondapack}$ column and a Waters system equipped with a UV detector, a fraction collector, and a model 660 solvent programmer. A concave gradient (curve no. 7) from 0 to 30%

was developed over 40 min, using 0.02 M-KH₂PO₄ as the weak solvent and 60% methanol in water as the strong solvent, with a flow rate of 1.0 ml min⁻¹ (Miksic & Brown, 1977). The labelled NR was purified by passing the reaction mixture over a column of anion-exchange resin AG 1-X8 (200–400 mesh) in the formate form (Bio-Rad) using water as the eluent (Kasarov & Moat, 1972). The final product still contained 5–10% Nam as determined by thin layer chromatography (TLC).

TLC. Three TLC systems were used to separate the various NAD metabolites. Solvent system A was 0.05 M-KH₂PO₄ (adjusted to pH 3.0 with concentrated ≈ 17 M-H₃PO₄) 100% (absolute) methanol (9:1, v/v). Solvent system B was 1 M-ammonium acetate (adjusted to pH 5.0 with concentrated HCl/95% ethanol (7:3, v/v). Solvent system C was 0.2 M-LiCl₂. The solid phase for the former two systems was cellulose acetate (Kodak) and PEI cellulose (Merck) for the latter. The *R_F* values for system A were: Nam, 0.84; nicotinic acid, 0.93; NR, 0.75; NMN, 0.78; and NAD, 0.78. For systems B and C respectively the *R_F* values were: Nam, 0.85 and 0.70; nicotinic acid, 0.85 and 0.45; NR, 0.70 and 0.85; NMN, 0.45 and 0.71; NAMN, 0.45 and 0.25; NAD, 0.20 and 0.37; and NAAD, 0.35 and 0.20. System A separated Nam from nicotinic acid and was used to assay nicotinamidase activity. System B separated NAD, NMN, NR, and Nam (Kasarov & Moat, 1972), and was used to assay NAD synthesis and degradation. System C separated the deaminated metabolites NAMN and NAAD from the other compounds and was used to detect their presence.

Specimens and standard markers were loaded in separate lanes on commercially prepared, plastic-backed TLC plates containing fluorescent indicators. The plates were developed in the appropriate solvents, dried, and the standards were visualized under UV light. The lane for each specimen was cut at intervals corresponding to the standards and each section was placed in scintillation counting vials with 10 ml Betafluor (National Diagnostics) for measurement of the radioactivity.

Uptake studies. *H. parainfluenzae* was grown overnight in M1c medium supplemented with 6 μ M-NAD. The cells were washed twice with 50 mM-potassium phosphate buffer, pH 7.0, followed by centrifugation, and then resuspended at a final concentration of approximately 10⁸ c.f.u. ml⁻¹ in the same buffer supplemented with 5 mM-glucose and 10 mM-MgSO₄. The suspension was incubated for 5 min at 37 °C on a rotary shaker. Radiolabelled NAD was then added and triplicate 0.5 ml samples were removed immediately and after 10 min, except where indicated. The samples were filtered through GA6 0.45 μ m membrane filters (Gelman Sciences), which had been soaked in 50 mM-potassium phosphate buffer (pH 7.0) with 1.5 μ M-NAD. The filters were washed twice with 5 ml of the NAD-containing phosphate buffer then placed in scintillation vials containing 10 ml of Filtron-X (National Diagnostics) and radioactivity counted by liquid scintillation. Samples of the cell suspensions were titrated in duplicate on chocolate agar to determine the initial cell count. Results are expressed as ¹⁴C-labelled metabolite uptake as a percentage of the control or as pmol min⁻¹ (10⁸ c.f.u.)⁻¹. The kinetics of uptake were analysed by the method of Lineweaver and Burk (Lineweaver & Burk, 1934; Michal, 1978). Uptake experiments were also done in the presence of various unlabelled NAD metabolites and the rates of uptake for radiolabelled Nam, NR, NMN, and NAD were compared.

NAD breakdown by whole cells of *H. parainfluenzae*. Cells were prepared in the same manner as for the uptake studies described above. The cells were suspended to a final concentration of 8 $\times 10^8$ c.f.u. ml⁻¹ and uptake studies done under three conditions: (1) 10 mM-Mg²⁺ and 5 mM-glucose in 50 mM-phosphate buffer, pH 7.0, at 37 °C; (2) 10 mM-Mg²⁺ and 0.2 mM-2,4-dinitrophenol in 50 mM-phosphate buffer, pH 7.0, at 37 °C, and (3) 50 mM-phosphate buffer, pH 7.0, at 4 °C. The cells were incubated at these temperatures for 5 min and [carbonyl-¹⁴C]NAD with a specific activity of 4.1 μ Ci μ mol⁻¹ (0.15 MBq μ mol⁻¹) was then added to a final concentration of 0.25 μ Ci ml⁻¹ to initiate the reaction. Single 0.5 ml samples were removed at 0, 10, 30, 120, and 300 min and vacuum filtered as in the uptake studies except that the filtrate from each sample was collected before the filters were washed. The ¹⁴C-labelled compounds in the filtrates were analysed by TLC with solvent system B. The filters were washed, placed in Filtron-X, and the radioactivity in the cells was measured.

Crude bacterial extracts. The cell-free extracts used in the metabolism experiments were prepared by growing the organism overnight in Todd-Hewitt broth (BBL) supplemented with 5 μ g ml⁻¹ NAD. The cells were pelleted, washed twice with 50 mM-phosphate buffer pH 7.0 and resuspended in the same buffer. The cells were broken in a Cell Homogenizer MSK (Braun) using 0.1 mm glass beads. The extracts were centrifuged at 6000 *g* for 10 min, then the supernatant was centrifuged at 30000 *g* for 10 min and filtered through a GA6 0.22 μ m membrane filter (Gelman). The typical protein concentration was about 12 mg ml⁻¹ as determined by the Bio-Rad assay (Bio-Rad). The crude extracts were stored at -70 °C.

Enzymic reactions with crude extracts. The standard reaction mixture for the assay of NAD synthesis contained 1–2 mg crude extract protein ml⁻¹ in 50 mM-Tris/HCl buffer, pH 7.0 at 37 °C. MgCl₂ and ATP were added at final concentrations of 2 mM and 4 mM respectively. The appropriate carbonyl-¹⁴C-labelled substrate (NR, NMN, Nam or nicotinic acid) was added to a final concentration of approximately 0.5 μ Ci ml⁻¹ to start the reaction. The specific activities of the substrates were between 44 and 56 μ Ci μ mol⁻¹. When Nam or nicotinic acid were used as substrates, PRPP (12 mM) was added as a source of ribosyl phosphate, and glutamine (2 mM) was added as an amine donor when nicotinic acid was the substrate. A 50 μ l sample was removed from the mixture at 0, 5, 10, and 20 min and heated at 100 °C for 2–3 min to stop the reaction. Each sample was analysed by TLC.

NAD degradation and NMN deamidase activity were assayed using similar concentrations of cell extract, Tris/HCl buffer, and substrate (NAD or NMN) in the absence of Mg^{2+} and ATP. Boiled cell extracts were used as controls.

Extraction of radiolabelled intracellular components from whole cells. *H. parainfluenzae* was grown overnight in M1c broth. The cells were pelleted, washed, and resuspended to a concentration of approximately 5×10^8 c.f.u. ml^{-1} in 50 mM-phosphate buffer pH 7.0, 10 mM- $MgSO_4$, and 5 mM-glucose. The final volume of the suspension was 10 ml. [carbonyl- ^{14}C]NR was added to a final concentration of $0.13 \mu Ci ml^{-1}$ and the cells were incubated at $37^\circ C$. Samples (2 ml) were removed at various times, and the cells were collected on $0.45 \mu m$ membrane filters and washed with phosphate buffer. The filters were placed in a vial with 5 ml cold toluene/water (5:95, v/v) and shaken vigorously to extract the intracellular components (Kay, 1971). The extracts were lyophilized, and the residue was dissolved in 0.2 ml water and analysed by TLC.

RESULTS

Uptake of ^{14}C -labelled metabolite from [carbonyl- ^{14}C]NAD

The uptake of ^{14}C -labelled metabolite from [carbonyl- ^{14}C]NAD by *H. parainfluenzae* at $37^\circ C$ required glucose and was further enhanced by Mg^{2+} (Table 1). There was no measurable uptake of label when the cells were incubated at $4^\circ C$ in the buffer with glucose or with glucose and Mg^{2+} . The uptake of label was linear during the initial 16 min (Fig. 2*a*). By 16 min 25% of the added label had been sequestered by the cells. The initial rate of uptake of ^{14}C -labelled metabolite from [carbonyl- ^{14}C]NAD was measured at various NAD concentrations (Fig. 3*a*). A maximal rate of uptake was approached at concentrations of NAD above $4 \mu M$. A double reciprocal plot of the uptake of ^{14}C -labelled metabolite from labelled NAD as a function of NAD concentration is shown in the inset of Fig. 3*a*). This analysis gives a K_m of $0.55 \mu M$ and a V_{max} of $2.4 pmol min^{-1}$ (10^8 c.f.u.) $^{-1}$.

Effect of metabolic inhibitors on ^{14}C -labelled metabolite uptake from [carbonyl- ^{14}C]NAD

The effect of KCN, 2-deoxyglucose, sodium azide and 2,4-dinitrophenol (DNP) on the uptake of ^{14}C -metabolite from [carbonyl- ^{14}C]NAD is illustrated in Table 1. DNP and 2-deoxyglucose inhibited uptake to a much greater extent than did KCN or sodium azide. Since the ^{14}C -labelled metabolite uptake required glucose and was inhibited by both DNP and 2-deoxyglucose, it appears that uptake is energy dependent.

Table 1. *Effect of glucose and Mg^{2+} or metabolic inhibitors on uptake of ^{14}C -metabolite from [carbonyl- ^{14}C]NAD*

Uptake was measured in the presence of $0.15 \mu M$ -NAD with a specific activity of 48 000 c.p.m. $nmol^{-1}$. The uptake of ^{14}C -labelled metabolite in the presence of glucose and Mg^{2+} was $0.59 pmol min^{-1}$ (10^8 c.f.u.) $^{-1}$. The data are the mean of triplicate samples; SD values were less than 5% of the mean.

Composition of medium	Uptake of ^{14}C -labelled metabolite (% of control)
50 mM-Phosphate buffer, pH 7.0	<2
50 mM-Phosphate buffer, pH 7.0 + 5 mM-glucose	29
50 mM-Phosphate buffer, pH 7.0 + 10 mM- Mg^{2+}	<2
50 mM-Phosphate buffer, pH 7.0 + 5 mM-glucose + 10 mM- Mg^{2+}	100
Metabolic inhibitor*	
20 mM-2-Deoxyglucose	14
1 mM-KCN	89
10 mM-Sodium azide	59
0.2 mM-DNP†	4

* Cells were suspended in 50 mM-potassium phosphate buffer, pH 7.0, 5 mM-glucose and 10 mM- $MgSO_4$. The metabolic inhibitor was incubated for 5 min with the cell suspension prior to addition of labelled NAD (final concentration $0.15 \mu M$ with a specific activity of 48 000 c.p.m. $nmol^{-1}$). The uptake of ^{14}C -labelled metabolite under control conditions was $0.59 pmol min^{-1}$ (10^8 c.f.u.) $^{-1}$.

† This concentration of DNP was not lethal to *H. parainfluenzae* after a 15 min incubation period.

Fig. 2. Uptake of ^{14}C -labelled metabolite from $[\text{carbonyl-}^{14}\text{C}]\text{NAD}$ (a) and $^{14}\text{C}[\text{NR}]$ (b) by *H. parainfluenzae*. (a) The specific activity of $[\text{carbonyl-}^{14}\text{C}]\text{NAD}$ was $48000 \text{ c.p.m. nmol}^{-1}$ and the concentration was $0.15 \mu\text{M}$. Sampling was done immediately after the addition of label and at the times indicated on the abscissa. Each point represents the mean of triplicate samples, the SD of which was less than 5%. (b) The specific activity of $^{14}\text{C}[\text{NR}]$ was $112000 \text{ c.p.m. nmol}^{-1}$ and the concentration was $0.15 \mu\text{M}$. Sampling was done immediately after the addition of label and at the times indicated on the abscissa. Each point represents the mean of duplicate samples.

Table 2. Uptake of ^{14}C -labelled metabolite from $[\text{carbonyl-}^{14}\text{C}]\text{NAD}$ in the presence of pyridine nucleotides

Pyridine nucleotide	Molar ratio: unlabelled compound/labelled NAD*	Uptake of ^{14}C -labelled metabolite†
Control		100
β -NAD	10	29
α -NAD	10	78
	50	33
TNAD	2	73
	4	81
	8	42
	16	10
β -NMN	5	15
	10	8
	50	2
NMN	10	78
	50	85
	100	82
β -NAMN	2	105
	4	117
	8	98
	16	91
Nam	16	89

* The concentration of $[\text{carbonyl-}^{14}\text{C}]\text{NAD}$ was $0.15 \mu\text{M}$ with a specific activity of $48000 \text{ c.p.m. nmol}^{-1}$.

† Expressed as percentage of the control value, which was $0.59 \text{ pmol min}^{-1} (10^8 \text{ c.f.u.})^{-1}$. Each value is the mean of triplicate samples; SD values were less than 5% of the mean.

Effect of pyridine nucleotides on uptake of label from $[\text{carbonyl-}^{14}\text{C}]\text{NAD}$

The ability of pyridine nucleotides to block the uptake of ^{14}C -labelled metabolite from $[\text{carbonyl-}^{14}\text{C}]\text{NAD}$ was studied using varying molar ratios of these compounds (Table 2). The NAD analogues could interfere with the conversion of NAD to NMN/NR and/or with the

Fig. 3. Influence of substrate concentration on uptake of ^{14}C -labelled metabolite from [carbonyl- ^{14}C]NAD and [^{14}C]NR by *H. parainfluenzae*. (a) The concentration of the [carbonyl- ^{14}C]NAD was $0.15 \mu\text{M}$ at a specific activity of $48000 \text{ c.p.m. nmol}^{-1}$. As the concentration of NAD was increased with the addition of unlabelled NAD the specific activity correspondingly decreased. The inset is a Lineweaver-Burk reciprocal plot ($y = 0.43 + 0.23x$) fit by least-squares linear regression analysis. The values of $1/v$ are expressed as $\text{pmol min}^{-1} (10^8 \text{ c.f.u.})^{-1}$ and those of $1/S$ are expressed as $(\text{nmol ml}^{-1})^{-1}$. ^{14}C -labelled metabolite is used to designate that part of NAD which is taken into the cell since it is clear from experiments shown below that NAD is not taken up intact. (b) The specific activity of [^{14}C]NR was $112000 \text{ c.p.m. nmol}^{-1}$, which remained constant over the concentration range shown. Sampling was done 2 min after the addition of label. The inset is a Lineweaver-Burk reciprocal plot ($y = 0.12 + 0.016x$) fit by least-squares linear regression analysis. The values of $1/v$ are expressed as $\text{pmol min}^{-1} (10^8 \text{ c.f.u.})^{-1}$ and those of $1/S$ are expressed as $(\text{nmol ml}^{-1})^{-1}$.

uptake of ^{14}C -labelled metabolite. The nucleotides could interfere with the uptake of labelled compound but not with the conversion of NAD to NMN/NR (Kahn & Anderson, 1986). TNAD decreased ^{14}C -labelled metabolite uptake to a greater extent than α -NAD, probably because the former is a better substrate for nucleotide pyrophosphatase. β -NMN blocked ^{14}C -labelled metabolite uptake far better than α -NMN. β -NAMN and Nam reduced ^{14}C -labelled metabolite uptake by approximately 10% at molar ratios of 16. The results suggest that the transport system has specificity for the β -configuration of the glycosidic linkage of Nam nucleoside and an intact carboxamide group.

Comparison of uptake of label from [carbonyl- ^{14}C]NAD and N[U- ^{14}C]AD

The uptake of ^{14}C -metabolite from [carbonyl- ^{14}C]NAD and from N[U- ^{14}C]AD in the presence of unlabelled NAD, NMN, and AMP is shown in Table 3. When N[U- ^{14}C]AD was used, the uptake of label was not decreased in the presence of NMN, but was decreased with unlabelled NAD, and markedly decreased in the presence of 5- and 10-fold molar concentrations of AMP. When [carbonyl- ^{14}C]NAD was used, the uptake of label was markedly diminished in the presence of NMN but decreased only slightly in the presence of AMP. These data are consistent with the hypothesis that NAD is initially cleaved to NMN and AMP, prior to transport, and that NR or NMN is the actual metabolite that enters the cell.

Table 3. Uptake of ^{14}C -labelled metabolite from $\text{N}[U\text{-}^{14}\text{C}]\text{AD}$ or $[\text{carbonyl-}^{14}\text{C}]\text{NAD}$ in the presence of NAD, NMN, or AMP

Compound*	Addition	Molar ratio	Uptake of ^{14}C -labelled metabolite†
$\text{N}[U\text{-}^{14}\text{C}]\text{AD}$		Control	100
	NAD	10	27
	NMN	10	119
	AMP	5	14
	AMP	10	14
$[\text{carbonyl-}^{14}\text{C}]\text{NAD}$		Control	100
	NMN	10	7
	AMP	10	81

* The concentration of both labelled compounds was $0.15\ \mu\text{M}$ with a specific activity of $48\,000\ \text{c.p.m. nmol}^{-1}$.

† Expressed as percentage of the control value, which was $0.62\ \text{pmol min}^{-1}\ (10^8\ \text{c.f.u.})^{-1}$ for $\text{N}[U\text{-}^{14}\text{C}]\text{AD}$ and $0.47\ \text{pmol min}^{-1}\ (10^8\ \text{c.f.u.})^{-1}$ for $[\text{carbonyl-}^{14}\text{C}]\text{NAD}$. Each value is the mean of triplicate samples; SD values were less than 5% of the mean.

Uptake of $[\text{carbonyl-}^{14}\text{C}]\text{NR}$, and $[\text{carbonyl-}^{14}\text{C}]\text{Nam}$

The uptake of ^{14}C -labelled metabolite from $[\text{carbonyl-}^{14}\text{C}]\text{NR}$ over time is shown in Fig. 2(b). Compared to the uptake from $[\text{carbonyl-}^{14}\text{C}]\text{NAD}$ shown in Fig. 2(a) the uptake of label from NR was faster at the same concentration. The effect of NR concentration on the initial rate of uptake is illustrated in Fig. 3(b). A maximal rate of ^{14}C -metabolite uptake was approached at NR concentrations above $0.4\ \mu\text{M}$, compared with $4\ \mu\text{M}$ for NAD. The K_m for transport of label from NR as derived from a double reciprocal plot (Fig. 3b, inset) was $0.14\ \mu\text{M}$ and the V_{max} was $8.5\ \text{pmol min}^{-1}\ (10^8\ \text{c.f.u.})^{-1}$. Although the K_m is similar to that for NAD, NR is taken up at about a fourfold greater rate than NAD under saturation conditions. $[\text{carbonyl-}^{14}\text{C}]\text{Nam}$ was not taken up by *H. parainfluenzae* (data not shown).

*NAD breakdown by whole cells of *H. parainfluenzae**

When whole cells of *H. parainfluenzae* were incubated with an excess of labelled NAD in the presence of $0.2\ \text{mM}$ -DNP, increasing concentrations of NR were found in the extracellular filtrates (Fig. 4a). The concentration of NR increased in a linear fashion over 5 h. The cells converted approximately one third of the NAD to NR, and about one third to Nam. The rest of the NAD was unchanged. NMN was not detected. When uptake was allowed to proceed (Fig. 4b), no NR was found in the extracellular filtrates. About half of the original radioactivity was converted to Nam over 5 h and the remainder was NAD. At $4\ ^\circ\text{C}$, the NAD was unaltered by incubation with *H. parainfluenzae*. Cell membrane preparations of *H. parainfluenzae* break down NAD to NR and adenosine (data not shown). These results indicate that NAD is hydrolysed to NR extracellularly prior to uptake (Kahn & Anderson, 1986). The amount of Nam found in the extracellular fluid was decreased when uptake of the NAD metabolite (i.e. NR or NMN) was inhibited. The extracellular Nam probably resulted from intracellular metabolism of the small amount of NR that is taken up in the presence of DNP (see Table 1).

Synthesis of NAD

The results of NAD synthesis experiments using cell extracts are shown in Fig. 5. NAD was rapidly produced when NR was used as a substrate, with NMN appearing as a transient intermediate. The reaction required ATP and Mg^{2+} . The reaction did not proceed at $4\ ^\circ\text{C}$ or when boiled cell extracts were used. NAD was produced from NMN (not shown) and both ATP and Mg^{2+} were required for the reaction. A large amount of Nam was also present in the reaction mixtures. About half of the labelled Nam found at the final time point was present in the preparation of the labelled NR used as the substrate. The additional Nam was probably produced by the action of nucleoside phosphorylase, an enzyme previously described in *H. parainfluenzae* (Bachur & Kaplan, 1955).

Fig. 4

Fig. 4. TLC of extracellular filtrate from *H. parainfluenzae* incubated with [carbonyl- ^{14}C]NAD. Whole cells of *H. parainfluenzae* (8×10^8 c.f.u. ml^{-1}) were incubated with $60 \mu\text{M}$ -[carbonyl- ^{14}C]NAD (specific activity 8300 c.p.m. nmol^{-1}) and 10 mM - Mg^{2+} at 37°C . Samples ($500 \mu\text{l}$) were filtered at various times. Samples ($20 \mu\text{l}$) of filtrate were analysed by TLC. The amount of radioactive label taken up by the cells (4×10^8 c.f.u.) at each time is indicated in parentheses. (a) Conditions which inhibit uptake. DNP (0.2 mM) was present in the reaction mixture. (b) Conditions which promote uptake. Glucose (5 mM) was present in the reaction mixture. □, NAD; ■, NR; ○, Nam; ●, NMN.

Fig. 5

Fig. 5. *In vitro* synthesis of NAD from NR by *H. parainfluenzae* extract with 4 mM -ATP and 2 mM - Mg^{2+} in 50 mM -Tris/HCl buffer, pH 7.0 and [carbonyl- ^{14}C]NR (approx. $0.5 \mu\text{Ci ml}^{-1}$). The products were analysed by TLC system B. The amount of radioactivity at each time point is about 7000 c.p.m. per $10 \mu\text{l}$ sample. By the time the t_0 sample can be sampled and boiled, over half of the NR has been converted to NMN or Nam. The reaction continues until all the remaining NR is converted to NAD. □, NAD; ○, Nam; ●, NMN; ■, NR.

When TLC system C was used to analyse the reaction products, neither NAMN or NAAD was found as an intermediate. Similarly, when NMN was incubated with the cell extracts without the ATP and Mg^{2+} , Nam was produced, but no NAMN was detected. NAD was not produced from Nam or nicotinic acid, even with the addition of PRPP and glutamine. Both Nam and nicotinic acid were unaltered in this system. *Staphylococcus aureus* cell extracts synthesized NAD from either nicotinamide or nicotinic acid under the same conditions (data not shown).

NAD was also synthesized in whole cells of *H. parainfluenzae* incubated with NR, as evidenced by TLC analysis of the toluene extracts. NAD and Nam were the only intracellular metabolites detected.

Degradation of NAD

Nam was the end-product of NAD metabolism both in toluene extracts of whole cells and in cell extracts incubated with NAD. Nicotinamidase activity was not detected in either system. A small amount of NR (about 5% of the radioactivity) appeared transiently as an NAD breakdown product in the cell extract system, but it was converted within 5 min to Nam,

probably by nucleoside phosphorylase. The NR found in the cell extract experiments may have resulted from the nucleotide pyrophosphatase that breaks down NAD prior to uptake of NR. There was no spontaneous breakdown of NAD in the boiled extract controls.

Nam was also the only product found in the extracellular media after incubating the cells with labelled NAD (Fig. 4*b*). When NAD uptake was inhibited by deleting the glucose and adding DNP, NR was found in the extracellular media, and less Nam was produced (Fig. 4*a*). These results indicate that NAD glycohydrolase activity is intracellular, while hydrolysis of NAD to NR occurs extracellularly.

DISCUSSION

Since exogenous NAD, NMN, and NR support growth of *H. parainfluenzae* it was hypothesized that NAD is not taken up intact. The results of the uptake studies with [*carbonyl*-¹⁴C]NAD and N[U-¹⁴C]AD indicate that NAD is enzymically degraded to NMN or NR prior to being internalized. Further support for this hypothesis is provided by the greater reduction in uptake of ¹⁴C-labelled metabolite by unlabelled β -NMN than by unlabelled β -NAD at comparable molar ratios, and by the higher rate of uptake for labelled NR. Also the differential effects of AMP and NMN on the uptake of ¹⁴C-labelled metabolite from labelled NAD argue for cleavage of NAD prior to uptake. The affinities of the transport system for NR and NAD are similar as indicated by the K_m values of these compounds, but the V_{max} for NR uptake is several fold higher than for NAD uptake. Hydrolysis of NAD appears to be the rate limiting step for utilization of exogenous NAD. These conclusions are supported by the characterization of a nucleotide pyrophosphatase from *H. influenzae* (Kahn & Anderson, 1986) that catalyses the hydrolysis of NAD to NMN and AMP. This enzyme was able to hydrolyse structural analogues of NAD where the pyridine ring was modified; however, compounds with modifications of the adenine ring were very poor substrates (Kahn & Anderson, 1986). The enzyme required a diesterified pyrophosphate group. Both ADP and AMP inhibited enzyme activity and the former compound inhibited growth of *H. influenzae* in the presence of NAD.

The breakdown of NAD to NR occurs extracellularly, by the action of nucleotide pyrophosphatase at the cell surface (Kahn & Anderson, 1986). The products of this enzyme are NMN and AMP. Probably NMN is rapidly hydrolysed to NR, perhaps by the action of alkaline phosphatase at the cell surface. The Nam nucleotide/nucleoside uptake system appears to have the following characteristics: (1) it is energy-dependent and carrier-mediated with saturation kinetics, (2) it is selective for the β -configuration of the glycosidic bond, and (3) it requires a carboxamide group on the pyridine ring. These characteristics may be useful in designing specific antimetabolites that can block uptake of NR and/or inhibit subsequent intracellular NAD synthesis and therefore growth of *Haemophilus* species that require exogenous NAD.

The intercellular NAD synthetic pathway in *H. parainfluenzae* has not previously been reported but it has been described for *H. influenzae* (Kahn & Anderson, 1986), *H. pleuropneumoniae* and *H. parasuis* (O'Reilly & Niven, 1986*b*). Cell fractions derived from *H. parasuis* and *H. pleuropneumonia* can synthesize NAD from NMN or NR in the presence of ATP (O'Reilly & Niven, 1986*b*). The catabolism of NAD, NMN or NR yielded Nam (O'Reilly & Niven, 1986*a, b*). These organisms have adapted to an environment in which NAD, NMN or NR is supplied by the host or by other micro-organisms. The observation that NR is taken up rather than NAD means that NAD must be resynthesized inside the cell. The results of these experiments suggest a synthetic pathway for NAD in *H. parainfluenzae* (Fig. 6), which is similar to that of other *Haemophilus* species that require exogenous NAD but otherwise unique among prokaryotes.

Salmonella typhimurium has also been shown to utilize NR for growth and NAD synthesis (Liu *et al.*, 1982). However, *S. typhimurium* first phosphorylates NR to NMN, which is then converted to NAMN by direct deamination (Cruz *et al.*, 1974; Hughes *et al.*, 1983), or by sequential conversion of NR to Nam, nicotinic acid and NAMN (Hughes *et al.*, 1983; Liu *et al.*, 1982), as shown in Fig. 1. The NAMN is then converted to NAD through the common pathway. In our experiments there was no evidence of NAMN or NAAD formation in *H. parainfluenzae*. Nam and nicotinic acid remained unaltered in the *in vitro* system.

Fig. 6. Proposed pathway for NAD metabolism in *H. parainfluenzae*. Enzyme activities identified: 1, nicotinamide ribonucleoside kinase; 2, NMN adenylyltransferase; 3, nucleoside phosphorylase; 4, NAD glycohydrolase. RP, ribose 5'-phosphate.

The proposed NAD pathway is consistent with the *in vitro* growth requirements of *Haemophilus* species requiring exogenous NAD. They will not grow on QA, which is an intermediate in all *de novo* NAD synthetic pathways that have been described. *H. parainfluenzae* also cannot utilize Nam, nicotinic acid or NAMN for growth, which is evidence against a pyridine nucleotide cycle in this organism.

The presence of a unique pathway among *Haemophilus* species that require exogenous NAD and the absence of a PNC suggest the possibility that NR analogues might be able to inhibit the growth of these organisms by blocking NR entry into the cell, by inhibiting intracellular NAD synthesis, and/or by serving as a substrate for the synthesis of an NAD analogue that has defective coenzyme activity.

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