Inhibition of Nicotinic Acid and Nicotinamide Uptake into *Bordetella pertussis* by Structural Analogues

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3-Pyridine-carboxaldehyde and 3-pyridine-aldoxime were effective and specific inhibitors of the uptake of both nicotinic acid (NA) and nicotinamide (ND) by *Bordetella pertussis*, although neither compound inhibited the growth of the bacteria in liquid medium or the oxidation of glutamate by washed suspensions. In contrast, the following pyridine derivatives did not inhibit uptake of NA or ND: iso-NA, iso-ND, isoniazid, 6-amino-NA and 6-amino-ND, 3-acetyl-pyridine, 3-pyridyl-acetic acid, *N,N*-diethyl-ND and 3-pyridine-sulphonic acid. 3-Pyridyl-carbinol was inhibitory, but less so than the first listed compounds.

INTRODUCTION

Bordetella pertussis, the causative organism of whooping cough, has a single vitamin requirement which can be satisfied by either nicotinic acid (NA) or nicotinamide (ND). In a previous paper (McPheat & Wardlaw, 1980), we reported that [^{14}C]NA and [^{14}C]ND were taken up by washed suspensions of *B. pertussis* at almost identical rates and that the uptake of both was dependent on temperature, an energy source and potassium ions. The vitamins differed only in their K_m values for the overall uptake process of transport plus subsequent metabolism, the bacteria having a much greater affinity for ND than for NA.

The uptake of several other vitamins by bacteria has been found to be inhibited by appropriate structural analogues (riboflavin: Cecchini *et al.*, 1979; pantothenate: Germinario & Waller, 1977; folates: Henderson & Huennekens, 1974; thiamin: Kawasaki *et al.*, 1969, Yamada & Kawasaki, 1980; biotin: Prakash & Eisenberg, 1974). There appears to be no such information for NA or ND in *B. pertussis* or any other bacterial species.

In this paper, we report that two structural analogues of NA and ND, 3-pyridine-carboxaldehyde (3PC) and 3-pyridine-aldoxime (3PA), were efficient inhibitors of vitamin uptake whereas a number of related compounds were not.

METHODS

Chemicals. Radiolabelled nicotinic acid and nicotinamide, [^{14}C]NA and [^{14}C]ND, were obtained from Amersham. Unlabelled NA and ND were from BDH. Iso-NA, iso-ND, isoniazid and 3-acetyl-pyridine were from Koch-Light. 6-Amino-NA, 6-amino-ND and *N,N*-diethyl-nicotinamide were from Sigma and 3-pyridyl acetic acid HCl, 3-pyridine sulphonic acid, 3PC, 3PA and 3-pyridyl carbinol were from Aldrich. Where available, chemicals were of analytical grade.

Organism and growth conditions. *Bordetella pertussis* 134 was grown in Stainer & Scholte's (1971) liquid medium modified to contain $5\text{ }\mu\text{g NA ml}^{-1}$ ($40\text{ }\mu\text{M}$) and harvested as previously described (McPheat & Wardlaw, 1980).

Inhibition of uptake by analogues. Uptake of NA and ND was measured by filtration at timed intervals of portions of an assay mixture containing *B. pertussis*, phosphate buffer, glutamate and either [^{14}C]NA or [^{14}C]ND (McPheat & Wardlaw, 1980). Analogues were added to the assay mixture, usually at concentrations of 1-, 10- or

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Abbreviations: NA, nicotinic acid; ND, nicotinamide; 3PA, 3-pyridine-aldoxime; 3PC, 3-pyridine-carboxaldehyde.

100-times the molar concentration of the radiolabelled vitamin, and prior to the initiation of uptake by the addition of *B. pertussis* 134. Percentage inhibition of uptake was calculated by comparison of the amount of uptake in the presence and absence of the analogue.

Glutamate oxidation assay. *Bordetella pertussis* 134 was grown and harvested as before but was resuspended in 50 mM-Tris/HCl, pH 7.3 to a concentration of 1.0 mg dry wt ml⁻¹. The cells were stored at room temperature for 1 h to allow removal of endogenous energy stores and were then kept on ice until required for the assay. Cell suspension (3 ml) was pipetted into the chamber of a Clarke oxygen electrode and equilibrated to 37 °C. The endogenous rate of oxygen uptake was measured prior to the measurement of the rate of glutamate oxidation by the addition of 0.3 ml 0.2 M-monosodium glutamate. A further addition (0.3 ml) of the test solution was then made and the effect on oxygen uptake was monitored. The chamber was then emptied and washed with buffer. The above sequence of additions was then repeated for each test solution, as the rate of glutamate oxidation slowly declines during storage of the cell suspension on ice.

RESULTS

Inhibition of NA and ND uptake into B. pertussis by analogues

In preliminary experiments, a set of analogues of NA and ND was tested at 10-times the molar concentration of the vitamin present. The vitamin concentrations themselves were chosen such that they were between 5- to 10-times their respective K_m values for uptake (McPheat & Wardlaw, 1980). This was done to ensure that the rate of vitamin uptake was maximal and not affected by changes in vitamin concentration. Under these conditions, analogues having a similar K_m value to the test vitamin would be expected to show about 90% inhibition of uptake of the vitamin when present in a 10-fold excess. The results (not tabulated) were as follows: [¹⁴C]NA uptake was inhibited by a 10-fold excess of unlabelled NA (84%), ND (53%), iso-NA (13%) and isoniazid (13%); 6-amino-NA was not inhibitory. However, [¹⁴C]ND uptake was inhibited only by a 10-fold excess of unlabelled ND (92%) and NA (36%); iso-ND, isoniazid, 6-amino-ND and 3-acetyl-pyridine were not inhibitory.

A second set of analogues, which resembled NA and ND more closely in being 3-substituted pyridines, was tested for inhibition of [¹⁴C]NA and [¹⁴C]ND uptake. Furthermore, in view of the low degree of inhibition observed in the preliminary experiment, the analogue-to-vitamin molar ratio was increased to 100-fold. Under such conditions, analogues having a similar K_m to the vitamin should give inhibition values of about 99%.

The uptake of both [¹⁴C]NA and [¹⁴C]ND was strongly inhibited by 3-pyridine-carboxaldehyde (3PC) and 3-pyridine-aldoxime (3PA), while 3-pyridyl-carbinol was much less effective (Table 1). Also, as expected NA inhibited ND uptake and vice versa. None of the other compounds inhibited the uptake of either NA or ND.

3PC and 3PA were further tested for inhibitory action on NA and ND uptake at analogue-to-vitamin ratios of 1, 10 and 100 (Table 2). The positive controls (i.e. [¹⁴C]NA/NA and [¹⁴C]ND/ND) gave values close to the expected inhibitions of 99, 90 and 50%. When compared in such a manner, it was found that whereas 3PA and ND were less effective inhibitors of NA

Table 1. *Effect of 3-substituted pyridines on uptake of [¹⁴C]NA and [¹⁴C]ND*

[¹⁴C]NA and [¹⁴C]ND concentrations were 5.0 and 0.5 µM, respectively. Analogues were tested at 500 µM (for NA uptake) and at 50 µM (for ND uptake).

Pyridine derivative	Substituent at position 3	Inhibition of uptake (%)	
		NA	ND
NA	-COOH	98	89
ND	-CONH ₂	68	98
3-Pyridyl-acetic acid HCl	-CH ₂ COOH.HCl	0	6
<i>N,N</i> -Diethyl-nicotinamide	-CON(CH ₂ CH ₃) ₂	0	3
3-Pyridine-sulphonic acid	-SO ₃ H	2	3
3PC	-CHO	96	100
3PA	-CH=NOH	97	100
3-Pyridyl-carbinol	-CH ₂ OH	50	63

Table 2. Inhibition of [^{14}C]NA and [^{14}C]ND uptake by 3PA, 3PC and benzaldehyde at various analogue-to-vitamin molar ratios

Vitamin*	Analogue-to-vitamin molar ratio	Inhibition of uptake (%)				
		NA	ND	3PA	3PC	Benzaldehyde
NA	100	98	68	97	96	NT
	10	84	53	63	94	NT
	1	42	NT	14	68	NT
ND	100	89	98	100	100	0
	10	36	92	97	99	0
	1	NT	50	35	98	0

NT, Not tested.

* [^{14}C]NA and [^{14}C]ND concentrations were 5.0 and 0.5 μM , respectively.Table 3. Effect of NA and ND analogues and of energy inhibitors on glutamate oxidation by *B. pertussis*

The glutamate concentration used was 18 mM.

Addition	Concentration (μM)	Rate of glutamate oxidation [$\mu\text{l O}_2 \text{ min}^{-1} (\text{mg dry wt})^{-1}$]	
		Initial	Post-addition
3PC	420	5.3	5.9
3PA	420	5.3	4.8
3-Pyridine-sulphonic acid	420	3.8	3.5
3-Pyridyl-carbinol	420	2.9	3.3
Benzaldehyde	420	2.8	3.0
Sodium azide	67000	1.9	2.5
Potassium cyanide	830	0.9	1.2
Hibitane	1/1000 dilution	0.9	0.0

uptake than was NA itself, 3PC was more effective. Likewise for ND uptake, 3PA and NA were less effective inhibitors than ND itself and 3PC was more effective. Indeed, 3PC inhibited ND uptake almost completely at all molar ratios tested. Thus, 3PC was the strongest inhibitor found of both NA and ND uptakes. Furthermore, the results presented in Table 2 suggest that ND uptake was more sensitive to inhibition, by either 3PA or 3PC, than was NA uptake.

The inhibition of ND uptake by 3PC was apparently not due to a chemical reactivity of the amino group on ND with the aldehyde group on 3PC since benzaldehyde, which is structurally very similar to 3PC, had no inhibitory effect on ND uptake (Table 2).

Inhibition of growth of B. pertussis by NA and ND analogues

The analogues shown in Table 1 were added to a final concentration of 500 μM to the *B. pertussis* growth medium containing 40 μM (5 $\mu\text{g ml}^{-1}$) NA. Measurements of the A_{650} at 24 h showed that only 3-pyridine-sulphonic acid inhibited growth (results not given).

Inhibition of glutamate oxidation by B. pertussis by NA and ND analogues

Uptake of both NA and ND by *B. pertussis* requires a source of energy which can be fulfilled by glutamate, the main carbon and energy source for *B. pertussis* (McPheat & Wardlaw, 1980). It was therefore desirable to examine the effect of the NA and ND structural analogues on glutamate oxidation to ensure that their inhibitory effect on vitamin uptake was not secondary to an effect on the energy source.

None of the NA and ND analogues, at the highest concentrations used previously, inhibited glutamate oxidation by washed suspensions of *B. pertussis* (Table 3). Sodium azide and

potassium cyanide were also non-inhibitory, as has been previously reported (Ezzell *et al.*, 1979). Only hibitane, a cationic detergent, inhibited glutamate oxidation.

The rate of glutamate oxidation by *B. pertussis* declined during the storage of the washed cell suspension on ice while the experiment was being done. This is shown by the steadily decreasing 'initial' rates of oxidation (Table 3). Consequently, the 'initial' rate of glutamate oxidation was measured prior to each analogue addition (see Methods). Hibitane was also found to completely inhibit oxidation by fresh cell suspensions having a high rate of glutamate oxidation (data not presented).

DISCUSSION

Of a total of 12 structural analogues of NA and ND which were examined, only 3PA and 3PC were effective inhibitors of [^{14}C]NA or [^{14}C]ND uptake. The previously reported similarities between NA and ND uptake (McPheat & Wardlaw, 1980) were further extended in the present work by the finding that there was no analogue which specifically inhibited either NA or ND uptake. Furthermore, NA inhibited ND uptake and vice versa.

ND, once taken up, is rapidly metabolized to NA by *B. pertussis* (unpublished) and consequently the two vitamins show a common metabolism, possibly by the same enzymes as found in many other bacteria (Foster & Moat, 1980). As the process of uptake is taken to comprise transport into the bacteria followed by metabolism of the substrate, it is possible that a common metabolism of NA, ND, 3PA and 3PC could explain the similarities in the uptake of NA and ND by *B. pertussis* and their inhibition by these analogues.

Cobb *et al.* (1977) reported that the 6-amino derivatives of NA and ND were metabolized to 6-amino-NAD, which inhibited growth as it was not an electron acceptor. We report here that 6-amino-NA and 6-amino-ND were not inhibitory to the uptake of NA and ND, respectively. It would appear, therefore, that the transport components of the uptake systems of NA and ND of *B. pertussis* are sufficiently specific to exclude these 6-substituted pyridines.

The question of whether NA and ND share the same transport system into *B. pertussis* remains unsettled. However, the results reported here show that ND uptake was more sensitive to analogue inhibition than was NA uptake (Table 2), and that it was particularly sensitive to inhibition by 3PC. As only one metabolic step separates ND and NA (Foster & Moat, 1980), such differences are more likely to result at the level of transport into the cell.

The exact nature of the transport system(s) for NA and ND into *B. pertussis* merits further study in view of the fact that high concentrations of NA (but not of ND) in the growth medium yield cells that lack protective antigen and histamine-sensitizing factor (Pusztai & Joó, 1967; Wardlaw *et al.*, 1976). Of particular interest would be an investigation for specific outer membrane pores, such as that reported for vitamin B₁₂ in *Escherichia coli* (for review, see Sennet & Rosenberg, 1981), or for periplasmic binding-proteins, such as that reported for ND in *E. coli* (Griffith & Leach, 1973). Such structures, which may be vital to the growth of the bacteria, may also be important in pathogenicity because of their location in the outer layers of the cell.

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