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Pyridine Nucleotide Cycle of *Salmonella typhimurium*: Isolation and Characterization of *pncA*, *pncB*, and *pncC* Mutants and Utilization of Exogenous Nicotinamide Adenine Dinucleotide

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Mutants of *Salmonella typhimurium* LT-2 deficient in nicotinamidase activity (*pncA*) or nicotinic acid phosphoribosyltransferase activity (*pncB*) were isolated as resistant to analogs of nicotinic acid and nicotinamide. Information obtained from interrupted mating experiments placed the *pncA* gene at 27 units and the *pncB* gene at 25 units on the *S. typhimurium* LT-2 linkage map. A major difference in the location of the *pncA* gene was found between the *S. typhimurium* and *Escherichia coli* linkage maps. The *pncA* gene is located in a region in which there is a major inversion of the gene order in *S. typhimurium* as compared to that in *E. coli*. Growth experiments using double mutants blocked in the de novo pathway to nicotinamide adenine dinucleotide (NAD) (*nad*) and in the pyridine nucleotide cycle (*pnc*) at either the *pncA* or *pncB* locus, or both, have provided evidence for the existence of an alternate recycling pathway in this organism. Mutants lacking this alternate cycle, *pncC*, have been isolated and mapped via cotransduction at 0 units. Utilization of exogenous NAD was examined through the use of [^{14}C]carbonyl-labeled NAD and [^{14}C]adenine-labeled NAD. The results of these experiments suggest that NAD is degraded to nicotinamide mononucleotide at the cell surface. A portion of this extracellular nicotinamide mononucleotide is then transported across the cell membrane by nicotinamide mononucleotide glycohydrolase and degraded to nicotinamide in the process. The remaining nicotinamide mononucleotide accumulates extracellularly and will support the growth of *nadA pncB* mutants which cannot utilize the nicotinamide resulting from the major pathway of NAD degradation. A model is presented for the utilization of exogenous NAD by *S. typhimurium* LT-2.

Genetic and biochemical investigations using *Escherichia coli* (5, 6, 22, 23) and *Salmonella typhimurium* (10) have shown that the de novo pathway for the biosynthesis of NAD is similar, if not identical, in both of these organisms (see Fig. 1). *E. coli* degrades intracellular NAD and recycles it via a pathway referred to as the pyridine nucleotide cycle, shown in Fig. 1 (1). Mutants of *E. coli* blocked at certain steps in the pyridine nucleotide cycle (*pnc*) have been isolated and identified. The *pncA* locus, responsible for the synthesis of nicotinamide deamidase (nicotinamidase), was mapped at 39 min on the recalibrated *E. coli* linkage map (39). The *pncB* locus, responsible for the synthesis of nicotinic acid phosphoribosyltransferase (NAPRTase), has not been accurately mapped in either *E. coli* or *S. typhimurium*.

Since biochemical and genetic similarities exist between *E. coli* and *S. typhimurium*, it might be presumed that the pyridine nucleotide cycle in *S. typhimurium* is functionally similar to that in *E. coli*. However, *S. typhimurium* has not been studied with regard to the genetics of its pyridine nucleotide cycle.

In *E. coli*, the pyridine nucleotide cycle has been investigated in some detail. However, several points remain unclear with regard to how this cycle actually functions. Gholson et al. (11) reported that exogenous NAD did not support the growth of a *nadB pncA* double mutant of *E. coli*, whereas a *nadB* mutant grew well on this cofactor. These investigators proposed that ex-

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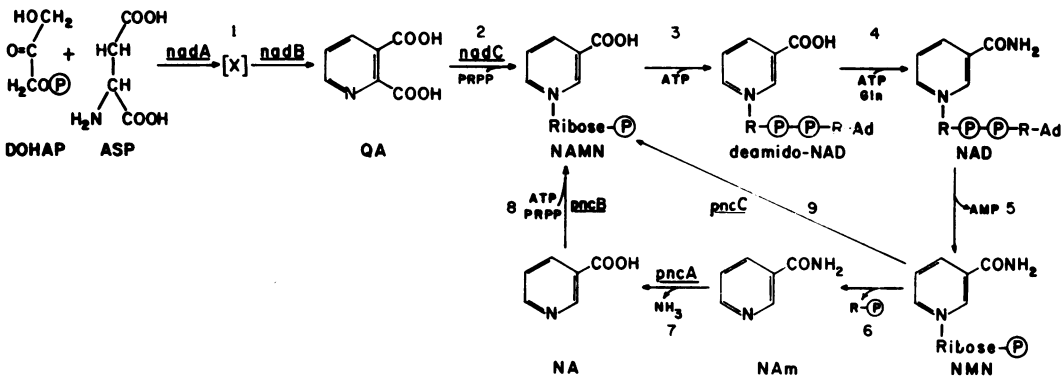

FIG. 1. *De novo pathway of NAD biosynthesis and the pyridine nucleotide cycle. The relationship of known genetic markers is shown. The "X" in the de novo pathway indicates one or more intermediates that have not, as yet, been fully characterized. DOHAP, Dihydroxyacetone phosphate; ASP, aspartate.*

ogenous NAD is not utilized directly by *E. coli*, but is converted to nicotinamide (NAm) during uptake. NAm could then be converted to nicotinic acid (NA) and then to NAD. Degradation and recycling of exogenous NAD would explain the ability of NAD to support the growth of mutants blocked in the de novo pathway (*nadA*, *nadB*, or *nadC*) and its inability to support the growth of a double mutant simultaneously blocked in the de novo pathway and the pyridine nucleotide cycle (*nadB pncA*). However, despite careful research, the enzyme NAD glycohydrolase, which cleaves NAD to NAm and ADP-ribose, has never been found in *E. coli* (2). Intracellularly, *E. coli* can potentially degrade NAD to nicotinamide mononucleotide (NMN) via a pyrophosphorylase (8) or DNA ligase (18) NMN can then be converted to NAm via an NMN glycohydrolase (2). Manlapaz-Fernandez and Olivera (19) suggest the presence of an NMN deamidase which would convert NMN to NA mononucleotide (NAMN), an intermediate common to the de novo pathway and the pyridine nucleotide cycle. The presence of NMN deamidase should permit a bypass of the *pncA* mutant, allowing NAD to support the growth of a *nadB pncA* mutant. Investigations by Gholson et al. (11) indicate that NAD does not support the growth of the *nadB pncA* mutant, presumably because exogenous NAD is not converted to NMN. Therefore, if exogenous NAD is transported into the cell, neither NAD pyrophosphorylase nor DNA ligase could be involved in the process.

Using various techniques, mutants of *S. typhimurium* blocked at various sites in the de novo pathway and in the pyridine nucleotide cycle were obtained. The *pncA* and *pncB* loci have been mapped using interrupted mating procedures and have been analyzed with regard to their enzymatic deficiencies. The *pncC* locus has been mapped by conjugation and cotransduction. The utilization of exogenous NAD by *S. typhimurium* has been investigated through the use of growth experiments and labeling studies using both [^{14}C]carbonyl-NAD and [*U*-^{14}C]adenine-NAD.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used in this investigation (Table 1) were derivatives of *S. typhimurium* LT-2.

Chemicals and reagents. All reagents were of analytical quality. The NA analog, 6-aminonicotinic acid (6-ANA), was the gift of R. Lemmon and G. J. Tritz, Kirksville College of Osteopathic Medicine, Kirksville, Mo. The nicotinamide analog, 6-aminonicotinamide (6-ANAm), was purchased from Sigma Chemical Co., St. Louis, Mo. Both analogs were checked for purity by using chromatographic techniques described previously (16, 17). [^{14}C]carboxyl-labeled NA, [^{14}C]carbonyl-labeled NAD, and [*U*-^{14}C]adenine-labeled NAD were purchased from Amersham Searle Corp., Arlington Heights, Ill. NA, NAm, quinolinic acid (QA), NAMN, NMN, NA adenine dinucleotide (deamido-NAD), and NAD were all purchased from Sigma Chemical Co. and were checked for purity by using the paper chromatography methods described previously (16, 17). Ethyl methane sulfonate (EMS) was purchased from Eastman Organic Chemical Co., Rochester, N.Y.

Culture medium. The minimal E medium of Vogel and Bonner (23), supplemented with 0.4% glucose, was used. Minimal lactose base medium was used to test for carbohydrate utilization. This medium contains 0.02% $MgSO_4 \cdot 7H_2O$, 0.4% KH_2PO_4, 0.5% K_2HPO_4, and 0.35% $NaH(NH_4)PO_4 \cdot 7H_2O$. Amino acids were supplemented at a concentration of 40 μg/ml. All Nad$^-$ mutants were grown in minimal medium supplemented with NA or NAm at a final concentration of 1.67 μg/ml. Other intermediates in the pyridine nucleotide cycle were added as indicated in the Results section. All vitamins and intermediates were sterilized

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TABLE 1. *Bacterial strains used*

Strain	Genotype	Source
PS147	*pro met purE*	J. S. Gots[a]
SA797	*purC proA46 ilv-405 rha-461 str*^{r} M-10 *fla-56 fim*	K. E. Sanderson[b]
HU180	*metP760 gal*	J. S. Gots
trpA49	*trpA49*	K. E. Sanderson
Hfr		
SA534 137 cw[c]	*serA13 rfa-3058*	K. E. Sanderson
SA464 117 cw	*serA13 rfa-3058*	K. E. Sanderson

[a] Department of Microbiology, University of Pennsylvania, Philadelphia.

[b] Salmonella Genetic Stock Centre, University of Calgary, Calgary, Alberta, Canada.

[c] Designated transfer origin in minutes and direction of transfer: cw, clockwise; ccw, counterclockwise.

by filtration using membrane filters with a pore size of 0.45 μm.

Isolation of analog-resistant mutants. Analog-resistant mutants were obtained by plating 10^8 cells on minimal medium containing 50 μg of either 6-ANA or 6-ANAm per ml.

Enzyme assays. Nicotinamidase was measured as described by Pardee et al. (20). NAPRTase was measured using the procedure described by Imsande (15). Crude extracts were prepared by suspending 4 g (wet weight) of cells in 5 volumes of 0.1 M phosphate buffer (pH 7.5) and sonically disrupting for 5 min using 30-s bursts with a Branson Sonifier. The cell suspension was then centrifuged for 15 min at 10,000 × *g*, and the supernatant extract was used immediately.

Transduction and conjugation. The method used for generalized transduction was that of Hartman et al. (13). The generalized transducing phage P22 was obtained from J. S. Gots, Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia. Conjugation was performed as described by Sanderson (21). Counterselection was accomplished using 500 μg of streptomycin per ml.

Mutagenesis and mutant enrichment. Mutagenesis with EMS and mutant enrichment were conducted as previously described (10) with the exception that penicillin G was used instead of ampicillin.

[^{14}C]NAD labeling studies. An overnight culture of the strain to be tested was diluted 1:20 in fresh minimal medium supplemented with either NA or QA and allowed to grow to a cell density of 2 × 10^8 cells per ml. This suspension was centrifuged and washed twice with minimal E medium lacking carbohydrate. A final suspension of 2 × 10^8 cells per ml was made using vitamin-free minimal medium. [^{14}C]carbonyl-labeled NAD with a specific activity of 53 μCi/μmol was added to a final concentration of 0.4 μCi/ml. [*U*-^{14}C]NAD with a specific activity of 50 μCi/μmol was added to a final concentration of 0.2 μCi/ml. All cultures were incubated in a rotary shaking water bath at 37°C. Samples of 2.0 ml were assayed at specified time intervals by passage through a membrane filter syringe apparatus with a 0.45-μm filter (Millipore Corp., Bedford, Mass.). The filter was washed three times with cold E buffer and placed in a centrifuge tube, and 1.0 ml of cold 40% acetic acid was added. The tube's contents were then blended in a Vortex mixer for 30 s and centrifuged. A 0.5-ml sample of the supernatant solution was counted in a Beckman LS133 liquid scintillation system. A 20-μl volume of the cell-free incubation medium was analyzed by chromatography on 1-inch Whatman no. 1 filter paper using either propanol–water (4:1) ascending or 95% ethanol–1 M ammonium acetate (7:3; pH 5.0) descending. Unlabeled pyridine nucleotides were analyzed simultaneously. The radioactive strips were cut into 10 segments corresponding to the R_f positions and placed in 10 ml of complete scintillation medium containing 5.0 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-2-(5-phenyloxazolyl)benzene per liter of toluene (Yorktown Research, South Hackensack, N.J.) plus 106 ml of Beckman Solubilizer Formula BBS-3 (Beckman Instruments, Fullerton, Calif.).

RESULTS

Isolation of 6-ANAm-resistant mutants. Mutants of *E. coli*, initially isolated as 6-ANAm resistant, were found to lack nicotinamidase activity and were designated *pncA* mutants (G. J. Tritz, personal communication). 6-ANAm-resistant mutants of *S. typhimurium* were isolated as described in Materials and Methods to obtain *pncA* mutants similar to those in *E. coli*. Mutants of strain PS147, resistant to 6-ANAm, were isolated and found to be resistant to concentrations of the analog as high as 500 μg/ml. Sensitive strains of PS147 were inhibited by 6-ANAm, but the addition of NA readily reversed the inhibitory effect (Table 2), indicating that the inhibitory effect of 6-ANAm on *S. typhimurium* is a result of its metabolism via the pyridine nucleotide cycle as shown in *E. coli* (1). However, as shown below, most of the 6-ANAm-resistant mutants of *S. typhimurium* did not prove to be deficient in nicotinamidase activity.

Cross-feeding of Nad$^-$ mutants by analog-resistant mutants. Analog-resistant mutants lacking an enzyme in the pyridine nucleotide cycle should accumulate the intermediate preceding the missing step. The intermediate produced by these analog-resistant mutants should support the growth of mutants blocked in the de novo pathway of NAD biosynthesis.

TABLE 2. *Growth response to 6-ANAm*

Growth condition		Growth of strain:	
6-ANAm (μg/ml)	NA (μg/ml)	PS147	JF74[a]
0	0	+	+
50	0	−	+
500	0	−	+
500	1.23	+	+

[a] JF74 is an analog-resistant mutant of PS147.

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To test this hypothesis, Nad⁻ mutants of *S. typhimurium* were streaked on minimal medium perpendicular to a single streak of an analog-resistant mutant. Both 6-ANAm- and 6-ANA-resistant mutants produced soluble products which supported the growth of all three classes of Nad⁻ mutants (Table 3).

Determination of enzyme deficiency in analog-resistant mutants. Virtually all of the 6-ANAm-resistant mutants of *E. coli* proved to be nicotinamidase deficient (Tritz, personal communication). It was assumed that mutants of *S. typhimurium* resistant to the same analog would also be deficient in nicotinamidase activity. The results obtained revealed that this was not the case. Several 6-ANAm-resistant isolates of *S. typhimurium* had normal levels of nicotinamidase activity but possessed lower than normal NAPRTase activity (JF38, JF52, and JF43) (Table 4). These results indicated that the genetic lesion was in the *pncB* locus rather than in the *pncA* locus even though these mutants were isolated on the basis of their resistance to 6-ANAm.

TABLE 3. *Cross-feeding of Nad⁻ mutants by analog-resistant mutants*

Strain	Pertinent genotype	Cross-feeding		
		nadA39	*nadB16*	*nadC19*
HU180	*pnc*⁺	–	–	–
JF36	*pncB1*	+	+	+
JF37	*pncB2*	+	+	+
JF38	*pncB3*	+	+	+
JF39	*pncB4*	+	+	+
JF40	*pncB5*	+	+	+
JF41	*pncB6*	+	+	+
JF50	*pncA16*	+	+	+
JF57	*pncA19*	+	+	+
JF58	*pncA20*	+	+	+

TABLE 4. *Enzyme activities of various pyridine nucleotide cycle mutants of S. typhimurium*

Strain	Pertinent genotype	Resistance[a] to:		Sp act[b]	
		6-ANA	6-ANAm	NAPRTase	Nicotinamidase
HU180	*pncA*⁺ *pncB*⁺	–	–	6.12	11.20
JF38	*pncB3*	+	+	1.02	11.61
JF43	*pncB9*	+	+	3.04	10.3
JF46	*pncB12*	+	+	0.90	10.79
JF52	*pncB18*	+	+	1.53	11.15
JF44	*pncA10*	–	+	5.50	2.63
JF49	*pncA15*	–	+	6.32	0.59
JF57	*pncA19*	–	+	5.51	3.03
JF72	*pncA19* *pncB21*	+	+	1.39	2.55

[a] Resistance to the analogs is denoted as follows: +, resistant; –, sensitive.

[b] Expressed as nanomoles of product formed per hour per milligram of protein.

Isolation of *pncA* mutants. Despite the results reported above, it was still considered that some 6-ANAm-resistant isolates should be *pncA* mutants. Therefore, additional mutants resistant to 6-ANAm were isolated. These mutants were scored on minimal medium containing either 6-ANAm or 6-ANA. Less than 10% of the mutants isolated were resistant to 6-ANAm while remaining sensitive to 6-ANA. Such results would be expected in mutants lacking nicotinamidase (*pncA*) but still possessing NAPRTase activity (*pncB*⁺). All 6-ANA-sensitive, 6-ANAm-resistant mutant strains tested possessed reduced nicotinamidase levels (Table 4).

Construction of double mutants blocked at *nadA* and *pncB*. Strain HU180 is a *gal* strain of *S. typhimurium*. The *nadA* locus can be cotransduced with *gal* at a frequency of approximately 12% (10). A 6-ANAm-resistant strain of HU180 was transduced with phage grown on strain JF28 (*nadA39*). Selection for *gal*⁺ transductants was performed on minimal lactose base plates containing 0.2% galactose and 10^{-2} M QA. The *gal*⁺ transductants obtained were screened for the presence of both the *nadA* and *pncB* lesions by plating them on MLB galactose supplemented with either QA or NA. The double mutant, *nadA39 pncB3* (JF53), grew on the QA-supplemented medium but not on the NA-supplemented medium. Subsequently, several double mutants were constructed using mutants isolated as resistant to 6-ANAm or 6-ANA. For mapping purposes, additional *nadB pncB* mutants were obtained by treating a *pncB* mutant with EMS and selecting for the *nadB* lesion by using the penicillin enrichment procedure described in Materials and Methods. The characteristics of these mutants are shown in Table 5.

Construction of double mutants blocked at *nadA* and *pncA*. To further prove the nature of the *pncA* mutants, the *nadA* lesion was introduced through cotransduction, and subsequently it was demonstrated that the growth of the resultant double mutant was supported by NA but not by NAm. The same procedure was used to generate these *nadA pncA* mutants as was used to construct the *nadA pncB* double mutants, with the exception that the *gal*⁺ transductants were scored on MLB galactose medium supplemented with either NA or NAm. From 10 to 20% of the *gal*⁺ transductants proved to be *nadA pncA* double mutants. The characteristics of these double mutants are shown in Table 6.

Mapping of the *pncB* locus. Mapping *pncB*

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TABLE 5. *Construction of nad pncB mutants*

Analog-resistant strains			*nad pncB* strains constructed from the analog-resistant strain		
Parent strain no.	Analog used	Analog-resistant strain no.	Strain no.	Pertinent genotype	Mode of construction
HU180	6-ANAm	JF38	JF53	*nadA39 pncB3*	Cotransduction
HU180	6-ANA	JF47	JF54	*nadA39 pncB13*	Cotransduction
HU180	6-ANAm	JF52	JF55	*nadA39 pncB18*	Cotransduction
HU180	6-ANAm	JF51	JF56	*nadA39 pncB17*	Cotransduction
trpA49	6-ANAm	JF43	JF69	*nadB61 pncB9*	EMS mutagenesis
trpA49	6-ANAm	JF43	JF70	*nadB63 pncB9*	EMS mutagenesis
trpA49	6-ANAm	JF43	JF71	*nadA66 pncB9*	EMS mutagenesis

TABLE 6. *Construction of nad pncA mutants*

Parent strain no.	6-ANAm-resistant strain no.	*nad pncA* strains constructed from the 6-ANAm-resistant strain		
		Strain no.	Pertinent genotype	Mode of construction
HU180	JF57	JF59	*nadA39 pncA19*	Cotransduction
HU180	JF58	JF60	*nadA39 pncA20*	Cotransduction
trpA49	JF49	JF63	*nadB51 pncA15*	EMS mutagenesis
trpA49	JF49	JF64	*nadA52 pncA15*	EMS mutagenesis
trpA49	JF49	JF65	*nadC54 pncA15*	EMS mutagenesis
trpA49	JF49	JF66	*nadA56 pncA15*	EMS mutagenesis

mutants of *E. coli* on the basis of 6-ANA resistance poses difficulties because of the high frequency of spontaneous mutants that arise which are resistant to this analog (R. Lemmon, personal communication). Introduction of the *nadB* locus into a *pncB* mutant of *S. typhimurium* enabled us to accurately map the *pncB* locus. Transfer of the *pncB*$^+$ locus into a *nadB pncB* F$^-$ cell through conjugation allowed the F$^-$ recombinant to grow on NA. Strains SA464 and SA534 were used to map the *pncB* locus of JF69. Results were obtained which consistently placed the *pncB* gene at 38 min on the *S. typhimurium* linkage map (Fig. 2 and 3). Accuracy of the mapping procedure was determined by simultaneously transferring the *trpA*$^+$ gene and comparing the observed map time with the published map time of 52 min (21). The *trpA* locus consistently mapped at 52 ± 1 min.

Mapping of the *pncA* locus. The *pncA* locus in *E. coli* was mapped at 39 min by Dickinson and Sundaram (3, 9). The placement of this gene was found to be between the *trp* and *his* operons. Interrupted mating experiments were performed between JF63, a *nadB pncA trpA* mutant of *S. typhimurium*, and the Hfr strains SA464 and SA534. Our results indicate that the *pncA* locus in this organism maps at 42 min and is transferred after the *gal* operon and before the *trp* operon (Fig. 4 and 5). This appears to be a significant difference from the map location reported for *pncA* in *E. coli*.

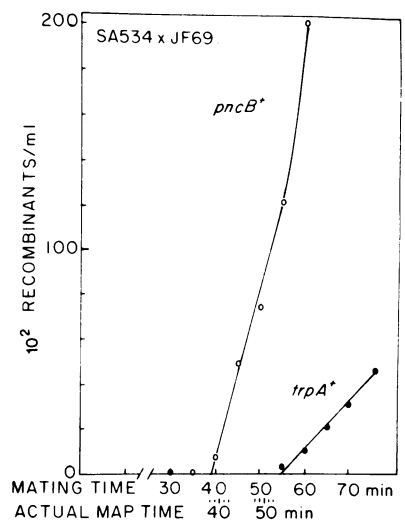

FIG. 2. *Interrupted mating between SA534 and JF69. pncB*$^+$ *recombinants* (○), *trpA*$^+$ *recombinants* (●).

Growth of JF53 on pyridine nucleotide cycle intermediates. Gholson et al. (11) reported that a *pncA nadB* double mutant of *E. coli* did not grow on NAD and concluded that NAD had to be degraded to NAm via the pyridine nucleotide cycle before it could be used to support growth. *E. coli* may possibly contain an NMN deamidase that converts NMN to NAMN (19). Assuming that the NMN deamidase functions in vivo, the *nadB pncA* mutant should

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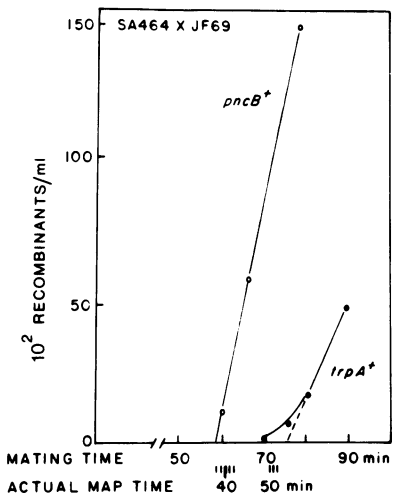

FIG. 3. *Interrupted mating between SA464 and JF69. pncB⁺ recombinants* (○), *trpA⁺ recombinants* (●).

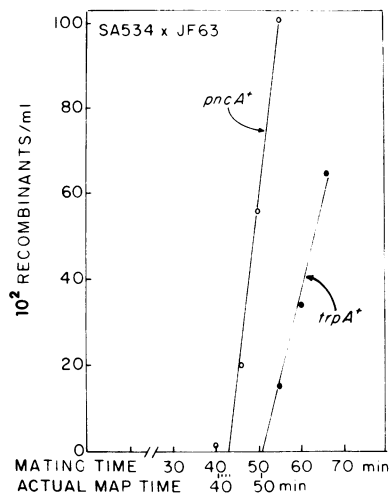

FIG. 4. *Interrupted mating between SA534 and JF63. pncA⁺ recombinants* (○), *trpA⁺ recombinants* (●).

grow on NAD or NMN since the NMN deamidase would bypass the *pncA* block in the pyridine nucleotide cycle. Growing these *nadB pncA* mutants on pyridine nucleotide intermediates presents difficulties in that higher concentrations of these intermediates are usually required to obtain maximum growth. Increasing the concentrations of these compounds provides a greater chance that growth-supporting amounts of NAm or NA may be present. Using a *nadA pncB* mutant for growth on the pyridine nucleotides should eliminate this contamination problem, since neither NA nor NAm can support the

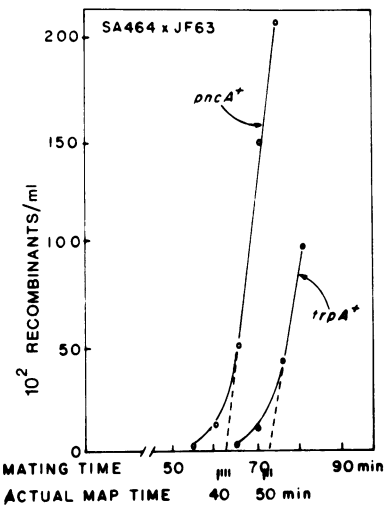

FIG. 5. *Interrupted mating between SA464 and JF 63. pncA⁺ recombinants* (○), *trpA⁺ recombinants* (●).

growth of this double mutant. Loop inoculation of growth media was performed using strains JF28 (*nadA*), JF53 (*nadA pncB*), JF59 (*nadA pncA*), and JF73 (*nadA pncA pncB*). QA, NAMN, and NMN supported maximum growth of strains JF53, JF59, and JF73 (Table 7). By comparison, NAD barely supported growth of these mutants even at high concentrations, probably due to the leaky nature of the enzymatic block. No growth was observed with NA or NAm. To be certain that growth was not due to revertants, cultures exhibiting growth were streaked on media containing NAm or NA, depending upon the genotype involved. No revertants were observed.

At 10^{-4} M, NMN supported good growth of the *nadA pncB* mutant, suggesting that some alternate route of NMN utilization must be present. The presence of NMN deamidase in *S. typhimurium* would explain how NMN was able to bypass the block at *pncB*. If the pyridine nucleotide cycle functions as depicted in Fig. 1, then NAD should also support growth of the *nadA pncB* double mutant by degradation to NMN and subsequent conversion to NAMN via the proposed NMN deamidase. The data presented in Table 7 are not consistent with this line of reasoning. Even at 10^{-3} M, NAD barely supported the growth of *nadA pncB* or *nadA pncA* double mutants, whereas growth of the *nadA* single mutant was maximal at 10^{-5} M NAD. Exogenous NAD is either being transported very slowly by the double mutant or it is being degraded to metabolites that *pnc* mutants cannot utilize for NAD synthesis within the cell.

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TABLE 7. *Effect of various precursors and derivatives of NAD on the growth of mutant strains of S. typhimurium LT-2*[a]

Growth factor	Concn (M)	Growth of: JF28 (*nadA*)[b]	JF53 (*nadA pncB*)	JF55 (*nadA pncB*)	JF59 (*nadA pncA*)	JF73 (*nadA39 pncA19 pncB21*)
QA	10^{-3}	0.9	1.0	1.0	1.0	1.0
NA	10^{-3}	1.0	0.1	0	1.0	0
	10^{-4}	1.1	0	0	1.0	0
	10^{-5}	1.0	0	0	1.0	0
NAm	10^{-3}	1.0	0.2	0.2	0.3	0
	10^{-4}	0.9	0	0	0	0
	10^{-5}	1.1	0	0	0	0
NAMN	10^{-4}	1.0	1.0	0.9	1.0	0.9
NAD	10^{-3}	1.1	0–0.1	0–0.1	0–0.1	0–0.1
	10^{-4}	1.1	0	0	0	0
	10^{-5}	1.0	0	0	0	0.1
NMN	10^{-4}	1.0	1.0	0.9	0.9	1.0
	10^{-5}	1.0	0.5	0.4	0.5	0.5

[a] Growth is expressed as a ratio of optical density at 540 nm for pyridine nucleotide tested/optical density at 540 nm for 10^{-5} M NA. All measurements were taken after 24 h of incubation at 37°C.

[b] Strain number and pertinent genotype tested.

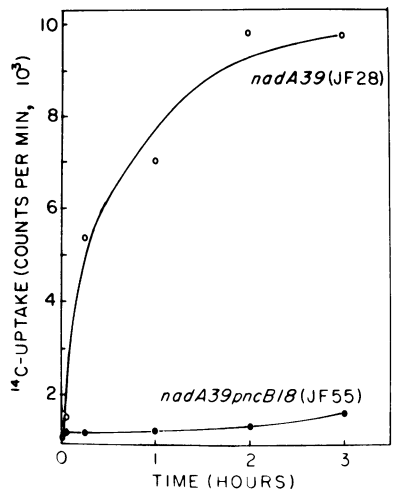

FIG. 6. *^{14}C uptake studies using [^{14}C]carbonyl-labeled NAD. Suspensions of JF28 and JF55 were made in vitamin-free minimal medium at a concentration of 2 × 10^8 cells per ml. [^{14}C]carbonyl-labeled NAD with a specific activity of 53 μCi/μmol was added to a final concentration of 0.4 μCi/ml. Samples of 2.0 ml were collected at the specific time intervals by syringe filtration through a 0.45-μm filter followed by washing with three 5-ml volumes of E buffer. Intracellular pool constituents were released with 1.0 ml of cold 40% acetic acid. After centrifugation, 0.5 ml of the supernatant was counted as described in the text.*

[^{14}C]NAD uptake studies. Studies using both [^{14}C]carbonyl-labeled NAD and [*U*-^{14}C]-adenine-labeled NAD were performed to investigate the nature of NAD uptake. Figure 6 illustrates that when [^{14}C]carbonyl-NAD was used, the *nadA* single mutant rapidly accumulated a significant amount of intracellular label, whereas the *nadA pncB* double mutant took up a small amount of label after the 3 h of incubation. These results show that the NAm portion of NAD is transported into cells possessing an intact pyridine nucleotide cycle. The question that remains is whether or not the adenine moiety of NAD is transported with the NAm as intact NAD. Analysis of experiments using [*U*-^{14}C]adenine-labeled NAD revealed no uptake by either the *nadA* single (JF28) or the *nadA pncB* double mutants. The results of these experiments indicate that exogenous NAD is degraded during transport, resulting in the uptake of the pyridine moiety, while the adenine portion remains exterior to the cell. Chromatography of cell extracts revealed that all detectable intracellular label was in the form of [^{14}C]NAD. No label was found in any other pyridine nucleotides. Chromatographic examination of the cell-free culture medium obtained during the [^{14}C]carbonyl-labeled NAD uptake experiments revealed that both the single (JF28) and the double (JF55) mutants accumulated [^{14}C]NMN extracellularly during the 3-h incubation period. Figure 7 illustrates that as [^{14}C]NMN accumulated in the culture medium there was a simultaneous decrease in extracellular [^{14}C]NAD. Spontaneous conversion of [^{14}C]NAD to [^{14}C]NMN was not observed in control experiments using uninoculated culture medium containing a concentration of [^{14}C]NAD equivalent to that used with JF28 and JF55 cultures. The results obtained in these experiments provide evidence that NAD is degraded to NMN and AMP at the cell surface. The majority of NMN remains in the culture medium, but a small amount of NMN must be transported inside the cell to support growth of the single mutant. However, as shown in the growth experiment presented in Table 7, this NMN must not enter the cell in its intact form, since exogenously supplied NMN can support the growth of the *nadA pncB* mutant whereas exogenously supplied NAD cannot. NMN glycohydrolase has been reported to be an integral component of the cell membrane of *E. coli* (2). Assuming that NMN glycohydrolase is a mem-

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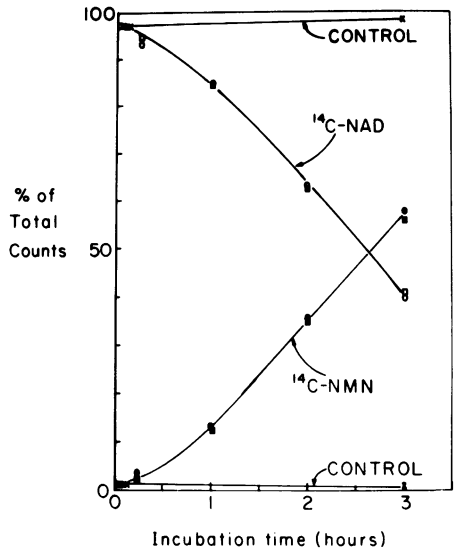

FIG. 7. *Conversion of [^{14}C]NAD to [^{14}C]NMN by mutant strains of S. typhimurium. Amounts observed in cell-free incubation medium after paper chromatographic separation using either propanol-water (4:1) or 95% ethanol-1 M ammonium acetate (7:3) adjusted to pH 5.0. JF28 (nadA39), ○, ●; JF55 (nadA39 pncB18), □, ■.*

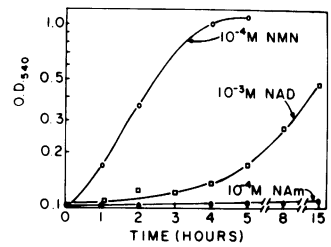

FIG. 8. *Growth of nadA pncB strain (JF55) in minimal medium supplemented with NAm (●), NMN (○), and NAD (□) using an initial inoculum of approximately 10^8 cells per ml. Before inoculation, the cells were starved of their intracellular NAD to prevent growth due to carryover.*

brane-based enzyme in *S. typhimurium*, it may be proposed that some of the NMN resulting from the degradation of extracellular NAD is transported by NMN glycohydrolase and, in the process, is degraded to NAm. This model would explain the results obtained in both the growth and labeling experiments presented thus far.

Growth of JF55 (*nadA pncB*) on NAD. The previous labeling experiments showed that approximately 50% of the NAD added was degraded to NMN by the *nadA pncB* mutant (JF55) after 3 h of incubation. A major difference between this study and the previous growth experiments was the size of the inoculum. Loop inocula were used for the growth experiments. An initial cell density of 10^8 cells per ml was used for the labeling experiments. If the model proposed above is correct, inoculation of NAD growth medium using a high cell density of the *nadA pncB* mutant should result in slow growth due to the accumulation of extracellular NMN, which could then enter the cell by a means other than the activity of NMN glycohydrolase. The results shown in Fig. 8 support this hypothesis. An initial cell density of 10^8 cells per ml was made in each of the growth media, using a culture of JF55 starved of QA for 2.5 h to deplete the intracellular NAD pool. Growth of the double mutant in 10^{-4} M NMN occurred immediately, whereas growth in the presence of 10^{-3} M NAD did not occur until 4 h after inoculation, reflecting the gradual conversion of NAD to a concentration of NMN high enough to enter the cell through an alternate uptake system. This does not infer the induction of an alternate route, merely the utilization of one already present. This NMN must then be converted to NAMN by NMN deamidase to support growth of the double mutant.

Isolation of *pncC* mutants. The previous data have shown that some alternate recycling pathway must exist and function in *S. typhimurium*. This alternate pathway most probably involves NMN deamidase as suggested for *E. coli*. However, attempts to assay for NMN deamidase activity have proven unsuccessful thus far. Nevertheless, it was believed that a mutant could be isolated from a *nadA pncA* strain of *S. typhimurium* which would exhibit growth on NA-supplemented minimal medium but not on NMN-supplemented minimal medium. This mutant should lack the alternate pathway via NMN deamidase and the lesion designated *pncC*. Strain JF66 (*nadA56 pncA15*) was treated with EMS as described in Materials and Methods. Previous to penicillin treatment, starvation of the *nadA pncA pncC* mutants was accomplished in NMN-supplemented medium. Following penicillin treatment, the survivors were incubated for 24 h in NA-supplemented medium prior to plating on a similar medium. The resultant colonies were replica plated onto media supplemented with either NA or NMN. Out of approximately 1,200 colonies tested, 12 proved unable to grow on NMN. Two of these, *pncC7* (JF75) and *pncC11* (JF76), were picked for further characterization.

Mapping of the *pncC* mutation. The results of numerous interrupted mating experiments revealed that the *pncC* gene was located close to the transfer origin of SA534 at 0 min. Subse-

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quently, cotransduction experiments were performed between JF75 and DK1, a *thr* mutant of JF66. The results presented in Table 8 show that the *thr* marker and *pncC* are closely linked, confirming the map position of *pncC* at 0 min.

DISCUSSION

The *pncA* and *pncB* loci of *S. typhimurium* LT-2 represent the structural genes for nicotinamidase and NAPRTase, respectively. Mutants lacking these enzymatic activities were isolated as mutants resistant to either 6-ANAm or 6-ANA. All 6-ANA-resistant strains tested proved to be *pncB* mutants. Interestingly, only 5 to 10% of the 6-ANAm-resistant clones isolated were *pncA* mutants; the other 90% were *pncB*. We also tested the analogs nicotinamide hypoxanthine dinucleotide, isonicotinamide, and *N*-methyl nicotinamide for their ability to inhibit the growth of wild-type *Salmonella*. None of these analogs exerted any effect on the growth of the wild type.

Interrupted mating data placed *pncA* at 42 min and *pncB* at 38 min on the *S. typhimurium* linkage map. Sanderson and Hartman (22) have recently published a revised linkage map of *S. typhimurium* in which 100 map units are used to designate map positions instead of the 138 minutes used previously (21). The new map positions for *pncA* and *pncB* were calculated as suggested by Sanderson (personal communication). The difference in map time between the *pnc* marker and the known marker (*trp*) was multiplied by the ratio 100/138. The results place *pncA* at approximately 27 map units and *pncB* at approximately 25 map units. Figure 9 reflects the newly reported map positions of these genes as well as the recalibrated positions of the *nad* genes previously reported (10) in *S. typhimurium*. Both the *pncA* and *pncB* loci are located between the *gal* and *trp* operons in this organism. Information obtained with *E. coli*, however, places *pncA* between the *trp* and *his* operons (16; Fig. 9). Further comparison of the two linkage maps reveals that the *pncA* gene in both organisms appears to lie near an analogous

TABLE 8. *Cotransduction of pncC with thr*

Donor strain[a]	Recipient strain	Selected marker	Unselected marker	No. scored	Cotransduction (%)
DK1	JF76	*pncC*$^+$	*thr*	110	28
JF76	DK1	*thr*$^+$	*pncC*	150	19

[a] DK1 (*nadA56 pncA15 thr-1*); JF76 (*nadA56 pncA15 pncC11*).

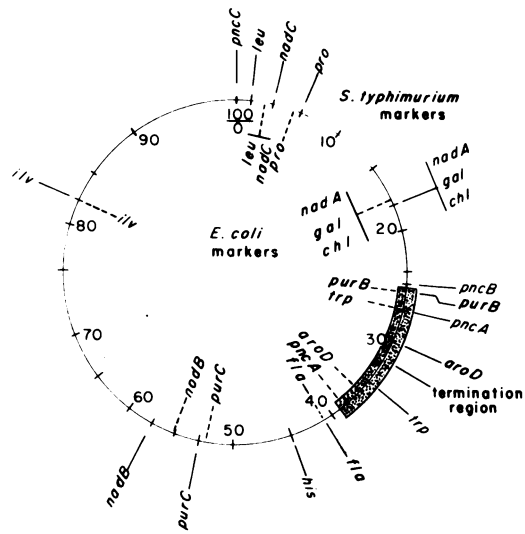

FIG. 9. *Recalibrated linkage maps of S. typhimurium (outer markers) and E. coli (inner markers) showing the relative map positions of nad and pnc loci. Data indicating the location of aroD in S. typhimurium are somewhat conflicting, as explained by Sanderson and Hartman (22). The darkened area represents the inverted region.*

aro locus. The gene order in *S. typhimurium* is *gal-trp-aroD-pncA-his*. The above comparison of the arrangement of these loci provides further evidence that there is a major inversion of this region of the *S. typhimurium* genome as compared to *E. coli* (22). Sanderson and Hartman (22) have estimated the inversion to extend from 25.5 to 35.5 map units. Our data indicate that the inversion must extend further, to 39 units, the map position of *pncA* in *E. coli* (Fig. 9). This is approximately 3 units longer than previously estimated (22). Interestingly, the termination region for replication lies at the approximate center of this inverted region. Additional studies examining the order and location of various genes in this region may reveal the genetic processes by which these two organisms evolved.

Andreoli et al. (2) reported that NMN glycohydrolase, a component of the pyridine nucleotide cycle, is bound to the *E. coli* cell membrane. Recently, Baecker et al. (4) have provided evidence that another enzyme of the pyridine nucleotide cycle, NAPRTase, is located in the periplasmic space of *E. coli*. We have proposed a model for NAD utilization based upon the results obtained using *S. typhimurium* (Fig. 10). This model requires either the membrane location of another pyridine nucleotide cycle enzyme, NAD pyrophosphorylase, or an exoenzyme with similar properties. Our results indi-

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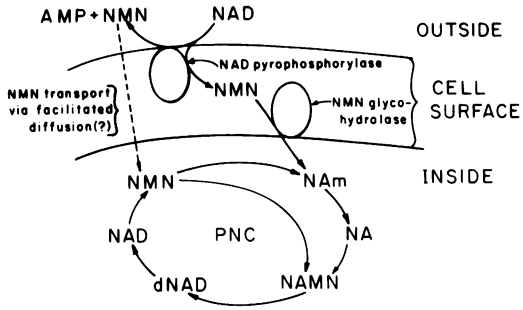

FIG. 10. *Model for the utilization of NAD by S. typhimurium. See text for explanation. For abbreviations, see Fig. 1.*

cate that NAD does not support the growth of *nadA pncA* or *nadA pncB* mutants when small inocula are used. Growth of these double mutants is achieved, however, when they are depleted of their intracellular NAD pool and used at high cell densities for inoculation. NAD is degraded efficiently by large numbers of cells, and the resultant NMN is of sufficient concentration to enter the double mutants by means other than NMN glycohydrolase transport. This is not to suggest induction of an NAD pyrophosphorylase-like enzyme but rather the cumulative effect of a large number of cells degrading NAD to NMN. This NMN can support growth of the double mutants presumably through conversion to NAMN by an NMN deamidase. Small numbers of cells cannot degrade exogenously supplied NAD to the extent necessary, and, therefore, growth of the double mutants is not observed. The small amount of NMN resulting from NAD degradation under the condition of small inoculum size is too dilute to be transported by any means other than NMN glycohydrolase. The NAm resulting from NMN glycohydrolase transport cannot support the growth of either *nadA pncA* or *nadA pncB* mutants due to the nature of the *pnc* blocks. Higher concentrations of extracellular NMN probably allow facilitated diffusion of NMN across the membrane. The possibility of an inducible transport system cannot be overlooked, however.

The in vivo evidence presented indicates that exogenous NAD is degraded to NMN at the cell surface. NMN, then, either is degraded to NAm during transport by NMN glycohydrolase or, if the concentration of NMN is high enough, diffuses across the membrane. Once across the membrane, NMN supports the growth of double mutants presumably via deamidation to NAMN by an NMN deamidase. The isolation of *nadA pncA pncC* mutants which do not utilize NMN provides further evidence for the existence of NMN deamidase in *S. typhimurium*. The *pncC* gene was mapped at 0 units on the genetic map of S. *typhimurium* (Fig. 9). This mutant should provide a means of isolation and characterization of NMN deamidase in *Salmonella*.

Acknowledgement

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