BIOSYNTHESIS OF NAD AND NICOTINIC ACID BY CLOSTRIDIUM BUTYLICUM [1,2]

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The classical tryptophan-niacin pathway present in molds (Bonner and Yanofsky, 1951), mammalian tissues (Nishizuka and Hayaishi, 1963), and the bacterium Xanthomonas pruni (Wilson and Henderson, 1963) does not appear to be operative in most bacteria. Nutritional and radioactive labeling studies with both growing and resting cells of lactic acid bacteria (Volcani and Snell, 1948), Bacillus subtilis (Yanofsky, 1954), Escherichia coli (Ortega and Brown, 1960), Mycobacterium tuberculosis (Mothes, et al., 1961; del Rio Estrada and Patino, 1962; Gross, et al., 1963; Albertson and Moat, 1965) and Clostridium butylicum (Isquith and Moat, 1965) indicated that both aspartate and glycerol could serve as precursors of nicotinic acid (NA) in these organisms. Carbons 2 and 3 of aspartate are incorporated into the ring structure while carbon 4 is incorporated into the carboxyl group as shown by its release as CO_2 upon chemical decarboxylation of NA. All three carbons of glycerol are incorporated into the ring of NA.

Similar results have been obtained in several plants (Leete, 1965). Carbon by carbon degradation proved that the label from aspartate was incorporated into positions 2, 3 and 7 and that from glycerol into positions 4, 5 and 6 of NA. Yeast appear to be unique in that they are capable of utilizing tryptophan under aerobic conditions whereas under anaerobic conditions, aspartate and glycerol serve as precursors of NA (Ahmad and Moat, 1965).

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[2] A preliminary report of this work has been presented at the 1966 meeting of the American Society of Biological Chemists.

We wish to report here investigations on the biosynthesis of NA in a cell-free system obtained from Clostridium butylicum. Crude extracts of C. butylicum were found to incorporate ^{14}C from uniformly labeled aspartate and glycerol in the same manner as did growing cultures. Other metabolites closely related to glycerol were also able to serve as precursors of NA in the crude extract. However, upon fractionation of the extract, only aspartate, acetyl CoA and formate gave rise to an increase in NA production. Radioactivity studies showed that ^{14}C is incorporated into NA from labeled aspartate, acetate and formate. Although the mechanism of condensation of these precursors into the ring structure of NA has not been elucidated, it has been shown that quinolinic acid (QA) is an intermediate in the pathway. The partially purified preparation will convert QA, nicotinic acid mononucleotide (deamido-NMN) and deamido-NAD to NAD and NA. The absence of an enzyme capable of converting free NA to the nucleotide and the presence of enzymes capable of degrading NAD to NA derivatives explains the accumulation of NA or its derivatives in growing cultures of C. butylicum.

Materials and Methods

C. butylicum (ATCC# 42510) was grown in the medium of Lampen, et al. (1943) under anaerobic conditions. All compounds used were of analytical grade. Log phase cells were harvested and washed in cold distilled water at 10,000 x g for 15 min. at 4 C in a Servall RC-2 refrigerated centrifuge. For preparation of cell-free extracts, the cell pellet was resuspended in 0.05 M phosphate buffer, pH 7.4 containing 10^{-4} M EDTA and 10^{-4} M glutathione, to a final concentration of 10% wet weight cells and disrupted in a 10 KC Branson sonifier. Cellular debris was removed by centrifugation at 10,000 x g for 30 min. at 4 C followed by dialysis of the supernatant for 12 hrs. in the cold against 0.05 M phosphate buffer, pH 7.4. Partial purification of the cell-free sonicate was achieved by addition of 0.1% $MnCl_2$ (v/v) 0.25 M at 4 C and removal

of the nucleic acid precipitate by centrifugation. The resultant supernatant was then fractionated with enzyme grade $(NH_4)_2SO_4$. The precipitate obtained at 60% saturation was resuspended in 0.05 M phosphate buffer, pH 7.4, and dialyzed in the cold against the same buffer until free of $SO_4^=$.

The isolation and preparation of NA for counting of radioactivity in a Baird Atomic Proportional Low Level Counting System was accomplished by the method of Ahmad and Moat (1966). NA was determined by microbiological assay using Lactobacillus arabinosis 17-5 as the test organism (Snell and Wright, 1941) as well as by the chemical method of Konno, et al. (1957). NAD was assayed by the enzymatic method of Racker (1950) and by the CN-addition method of Colowick, et al. (1951). Protein was determined by the method of Lowry, et al. (1951). The methods of Preiss and Handler (1958) were employed for the chromatographic identification of products and intermediates.

Results and Discussion

The results in Table I indicate the effect of aspartate, glycerol and closely related metabolites on the synthesis of NAD and NA by cell-free extracts of C. butylicum. QA, deamido-NMN and deamido-NAD, intermediates common to the tryptophan pathway, were also stimulatory. The effect of QA was PP-ribose-P dependent. Tryptophan and other intermediates of the tryptophan path way, prior to QA, were unable to increase the formation of NAD and NA in this system. The failure of this system to form NAD from free NA or nicotinamide indicates the lack of a pyrophosphorylase capable of recycling these products and provides an explanation for the accumulation of these two compounds in the supernatant of growing cultures of C. butylicum. This finding also suggests that neither of these compounds is an intermediate in the formation of NAD but are, rather, degradation products of NAD. Using the method of Brown and Stadtman (1960), the enzymatic degradation of NAD was shown to be due to the

TABLE 1

Effect of Various Substrates on NAD and NA
Synthesis by a Crude Cell-free Extract of C. butylicum

Additions(s)	μmoles NAD	μmoles NA
Control	0.02	0.06
Quinolinate (QA)	0.80	2.11
QA less PP-ribose-P	0.02	0.06
Deamido-NMN	0.57	1.46
Deamido-NAD	0.51	1.34
Acetate and CoA	1.27	3.36
Succinate and glycerol	0.69	1.83
Aspartate and acetate	1.27	3.36
Aspartate and β-alanine	1.18	3.00
Aspartate and glycerol	0.69	1.81
Aspartate and glyceraldehyde-3-P	0.96	2.50
Aspartate and pyruvate	1.07	2.73
Aspartate	0.04	0.12
Pyruvate	0.10	0.27

The incubation mixture contained (in μmoles): substrates 8, PP-ribose-P 2, glutamine 20, $MgCl_2$ 20, ATP 10, potassium phosphate buffer 200 (pH 7.4), sodium pyruvate 4, crude cell-free extract 1.0 ml (23 mg protein N) in a total volume of 3.0 ml. Incubation was at 37 C for 6 hrs. under N_2 in a shaking water bath. Control value represents incubation mixture minus substrate(s). Reaction was stopped by boiling for 1 min. Samples were centrifuged at 10,000 x g for 30 min. and the supernatant assayed for NAD and NA.

presence of active pyro- and monophosphatases. An active glycohydrolase could not be demonstrated.

Upon partial purification of the extract (ca. 40-fold) glycerol no longer served as a precursor to NAD and NA. The data in Table 2 indicate that only a combination of aspartate, acetyl CoA and formate gave rise to an increase in NA using the purified fraction. Thiamine pyrophosphate, pyridoxal phosphate, pyruvate and trace amounts of NAD were required for maximal activity. However, further study is necessary to elucidate the role of these factors in the system.

After incubation of the reaction mixture with UL-^{14}-C-aspartate and separation by two-dimensional chromatography, radioactivity was detected in the intermediates and products shown in Table 3.

TABLE 2

Synthesis of NA by Crude and Partially Purified Extracts

System	Addition(s)	μmoles NA/mg protein N
Crude sonicate	Control	0.04
	Quinolinate and PP-ribose-P	0.59
	Aspartate and acetate	0.52
60% $(NH_4)_2SO_4$ fraction	Control	0.00
	Aspartate, acetate, CoA, HCOOH, HCO_3^- (complete system)[1]	18.86
	" " less HCO_3^-	17.15
	" " less HCO_3^- and HCOOH	7.70
	" " less CoA	3.43
	" " less acetate	0.00
	" " less aspartate	0.00
	" " less acetate, HCOOH and HCO_3^-	0.00
	" " less acetate, HCOOH, HCO_3^- and CoA	0.00

The complete system contained (μmoles): aspartate 8, ammonium acetate 4, CoA 2, sodium formate 4, sodium bicarbonate 4, sodium pyruvate 4, PP-ribose-P 4, glutamine 20, $MgCl_2$ 20, ATP 30, potassium phosphate buffer 200 (pH 7.4), thiamine pyrophosphate 1, pyridoxal phosphate 1, NAD 0.025, partially purified extract 1.0 ml (47.4 mg protein N) in a total volume of 3.7 ml. Control values represent incubation mixture minus substrates. Incubation and collection of samples as described in Table 1.

TABLE 3

Chromatographic Identification of Products and Intermediates formed during Synthesis from Aspartate, Acetyl CoA, and Formate[1]

Reference cmpd.	Rf of Ref. cmpd.[2]	Rf of Detected cmpd.[2]	cpm/ml eluted
QA	0.41	0.43	1648
NAD	0.13	0.14	228
deamido-NAD	0.11	0.11	812
NA	0.75	0.76	344
Nicotinamide	0.80	0.82	72
deamido-NMN	0.23	0.24	104
NMN	0.27	0.26	32
Aspartate	0.19	0.19	5856
Unknown (ninhydrin-positive)	-	0.30	788

[1] Reaction mixture: Partially purified extract, UL-^{14}C-aspartate, acetyl CoA, HCOOH, and cofactors listed in Table 2.

[2] Solvent systems: pyridine: water (2:1) in the first dimension. Ethanol: ammonium acetate (7:3), pH 4.85 in the second dimension. Rf values are those for the ethanol: ammonium acetate system.

TABLE 4

Radioactive Labeling of NA from Various Substrates

System[1]	Compound added Specific activity (mc/mM)	NA picrate	Pyridine picrate	CO_2
		cpm/μmole	cpm/μmole	cpm/μmole
Growing cells	UL-^{14}C-Aspartate (100)	289.9	203.1	76.1
	UL-^{14}C-Glycerol (12)	330.0	318.8	0.0
Crude extract	UL-^{14}C-Aspartate (100)	344.0	262.0	78.4
	UL-^{14}C-Glycerol (12)	97.5	98.3	0.0
60% Ammonium sulfate fraction	UL-^{14}C-Aspartate (50)	820.2	536.0	260.6
	UL-^{14}C-Acetate (12)	242.8	250.1	0.0
	^{14}C-Formate (4)	88.9	76.8	0.0
	^{14}C-Carbonate (5)	0.0	0.0	0.0
	Ul-^{14}C-Pyruvate (2.72)	0.0	0.0	0.0
	UL-^{14}C-Glycerol (12)	0.0	0.0	0.0

[1]In both growing cell and crude cell extract experiments the labeled substrate was used in conjunction with the other as unlabeled substrate, i.e. UL-^{14}C-aspartate and unlabeled glycerol. The same method applies to the use of 60% ammonium sulfate fraction, i.e. UL-^{14}C-acetate plus unlabeled aspartate, formate, and carbonate. Cofactors added are the same as listed in Table 2.

The incorporation of radioactivity into NA from UL-^{14}C-aspartate and UL-^{14}C-glycerol by growing cells and crude extracts, and from labeled aspartate, acetate, and formate by the partially purified extract is presented in Table 4. Although pyruvate stimulated NA formation, UL-^{14}C-pyruvate was not incorporated into NA. Its effect may be due to its capacity to act as a hydrogen acceptor. From the evidence presented, it is concluded that C. butylicum forms NAD via the condensation of formate, acetate and aspartate. The first detectable intermediate is QA, suggesting that this compound is common to this pathway and the classic tryptophan-niacin pathway. Since several prior steps in the tryptophan pathway require molecular oxygen, an anaerobic organism such as C. butylicum obviously must utilize the alternative pathway shown in Figure 1.

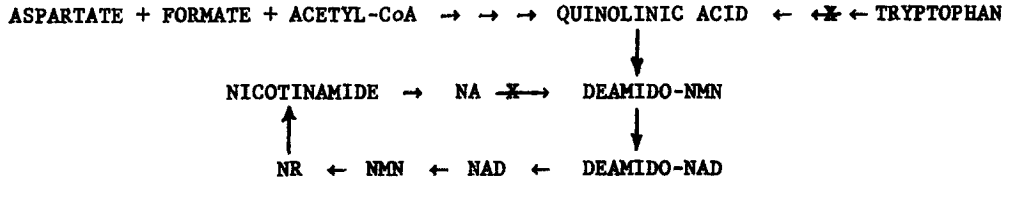

Figure 1. Pathway to NAD and its derivatives in Clostridium butylicum.

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