Studies on Pyridine Nucleotide Biosynthesis

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Until recently it had been generally accepted that the formation of diphosphopyridine nucleotide in biological systems starts from nicotinamide, with nicotinamide riboside and nicotinamide mononucleotide as intermediates (Singer & Kearney, 1954). Much of the earlier work on whether nicotinic acid or nicotinamide is the more efficient precursor of diphosphopyridine nucleotide has been controversial, but Preiss & Handler (1958*a, b*) unequivocally demonstrated that biosynthesis of diphosphopyridine nucleotide could also proceed by a mechanism alternative to the nicotinamide pathway referred to above, as follows:

It is known that micro-organisms (Hughes & Williamson, 1953; Oka, 1954; Halpern & Grossowicz, 1957; Sundaram, Rajagopalan & Sarma, 1958), the insect *Corcyra cephalonica* St. (Sundaram, Rajagopalan & Sarma, 1959; Sundaram, Radhakrishnamurthy & Sarma, 1954) and the pigeon and the chick (Rajagopalan, Sundaram & Sarma, 1958) possess the enzyme nicotinamide deamidase. Consequently, nicotinic acid is an important end product of metabolism of nicotinamide in these organisms. With the demonstration of the nicotinic acid pathway for biosynthesis of diphosphopyridine nucleotide, it appeared likely that nicotinamide deamidase might function not only by converting nicotinamide into nicotinic acid as an end product but also as a means whereby the free nicotinic acid needed for biosynthesis of diphosphopyridine nucleotide might be formed from nicotinamide. The

work reported in this paper relates to the biosynthesis of diphosphopyridine nucleotide in *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides* 9135.

EXPERIMENTAL

Materials

The strain of *Saccharomyces cerevisiae* used was maintained by fortnightly subculture on a malt-agar medium. For growth experiments cells from a 24 hr. culture were used for seeding. *Leuconostoc mesenteroides* 9135, kindly supplied by Dr B. C. Johnson, University of Illinois, Urbana, was maintained by fortnightly subculture on a

medium containing, in 100 ml.: glucose, 1 g.; sodium acetate, 2 g.; Difco yeast extract, 0.5 g.; liver extract, 0.6 g.; peptone, 0.5 g.; K_2HPO_4 , 50 mg.; KH_2PO_4 , 50 mg.; $MgSO_4 \cdot 7H_2O$, 20 mg.; NaCl, 1.0 mg.; $MnSO_4 \cdot 4H_2O$, 1.0 mg.; $FeSO_4 \cdot 7H_2O$, 1.0 mg.; agar, 2 g.; the pH was adjusted to 6.8–7.0.

N-Methylnicotinamide was a gift from Dr H. P. Sarett. [*carboxy*- ^{14}C]Nicotinic acid and [*carboxy*- ^{14}C]nicotinamide were obtained through the courtesy of Dr B. C. Johnson.

Methods

Metabolic products of nicotinic acid and nicotinamide in growing cultures of Saccharomyces cerevisiae. *S. cerevisiae* was grown for 18 hr. on the medium of Atkin, Williams, Schultz & Frey (1950) supplemented with nicotinic acid or nicotinamide at 10 $\mu g./ml.$ The culture medium, after concentration, was analysed for metabolites by paper chromatography as described by Sundaram *et al.* (1958).

Deamidation of nicotinamide by Saccharomyces cerevisiae and Leuconostoc mesenteroides. Cells of *S. cerevisiae*, grown on the above described medium, and of *L. mesenteroides* grown on the medium of Krehl, De La Hueraga, Elvehjem & Hart (1946) supplemented with nicotinic acid (0.25 $\mu g./ml.$), were harvested, washed with aqueous 0.9% NaCl by centrifuging and dispersed in this medium to give a suspension containing 200 mg. of cells/ml. The suspensions were assayed for nicotinamide deamidase by the microbiological method described by Sundaram *et al.* (1958). Cell-free extracts of *S. cerevisiae* and *L. mesenteroides*

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were obtained by grinding the frozen cells with sand in a chilled mortar, extracting the ground mass with ice-cold water and centrifuging the slurry to sediment sand and cell debris.

Biosynthesis of diphosphopyridine nucleotide by cultures of *Saccharomyces cerevisiae*. *S. cerevisiae* was grown for 18 hr. on the basal medium supplemented, where necessary, with nicotinic acid or nicotinamide. The cells were harvested and washed with 0.9% NaCl by centrifuging at 3000 g for 15 min. and assayed for pyridine nucleotides by the following method. The cells were treated with 5% (w/v) trichloroacetic acid and homogenized for 2 min. in a Potter-Elvehjem homogenizer with a Teflon pestle (A. H. Thomas Co., Philadelphia, Pa., U.S.A.). The homogenate was centrifuged at 8000 g for 10 min. and the supernatant, after suitable dilution with 5% trichloroacetic acid, was assayed by the fluorimetric method of Carpenter & Kodicek (1950) with butan-2-one; *N'*-methylnicotinamide was the internal standard. Under these conditions, *S. cerevisiae* does not accumulate *N'*-methylnicotinamide as a metabolite of either nicotinic acid or nicotinamide. Hence the value obtained in the fluorimetric method was taken to represent diphosphopyridine nucleotide (DPN) (see below).

The culture medium of *S. cerevisiae*, after harvesting of cells, was steamed for 15 min. and assayed microbiologically for nicotinic acid plus nicotinamide and for nicotinic acid alone with *Lactobacillus arabinosus* 17-5 and *Leuconostoc mesenteroides* 9135 respectively (Snell & György, 1950).

Biosynthesis of diphosphopyridine nucleotide by suspensions of *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides*. Cells of *S. cerevisiae* grown on the unsupplemented basal medium were harvested by centrifuging at 3000 g for 15 min., washed three times with sterile 0.9% NaCl by centrifuging and suspended in sterile water. *L. mesenteroides* was grown on the medium of Krehl *et al.* (1946) supplemented with suboptimum amounts of nicotinic acid (10 µg./ml.), harvested, washed with sterile 0.9% NaCl and suspended in sterile water as for *S. cerevisiae*. The incubation system contained: 31 mM-glucose; 5.3 mM-glutamine; 12.5 mM-KH₂PO₄ (K₂HPO₄ with *L. mesenteroides*); 2 µmoles of nicotinic acid or nicotinamide were added where necessary and 1 g. fresh wt. of the organism in a final volume of 5 ml.; KCl was added to make the medium equivalent to a 0.152 M solution of di-ionic electrolyte. All the reagents were sterilized at 10 lb./in.² for 10 min. before use. Incubation was in 50 ml. conical flasks plugged with sterilized cotton wool in a mechanical shaker at 30° for 18–20 hr. The incubation mixture was then treated with 5% (w/v) trichloroacetic acid and homogenized. The homogenate was centrifuged at 8000 g for 10 min. and the supernatant, after suitable dilution with 5% trichloroacetic acid, was assayed for pyridine nucleotides. The experiments on pyridine nucleotide synthesis were repeated seven or eight times. The same pattern of results was observed throughout, though with minor quantitative differences. The deamidation of nicotinamide was also followed in these experiments with *S. cerevisiae*. Samples of the mixtures which contained nicotinamide were withdrawn at intervals of 1, 2 and 20 hr., heated in a boiling-water bath for 10 min. and assayed for nicotinic acid with *L. mesenteroides*.

Experiments with [¹⁴C]nicotinic acid and [¹⁴C]nicotinamide. The experimental conditions were similar to those in the previous experiment, except that the incubation mixture, where necessary, contained 2 µmoles of ¹⁴C-labelled nicotinic

acid or nicotinamide (1 µC). After incubation, trichloroacetic acid was added to a final concentration of 5% and the mixture was homogenized. A portion of the supernatant obtained by centrifuging at 8000 g was taken for estimation of pyridine nucleotides. Another portion was extracted with peroxide-free ether to remove trichloroacetic acid, evaporated to dryness in a vacuum desiccator and taken up in a small volume of water. After centrifuging, the supernatant was chromatographed with 95% ethanol-m-ammonium acetate (7:3, v/v), the solution having been adjusted to pH 5.0 with HCl, for 20 hr. on Whatman no. 1 paper alongside reference standards of nicotinic acid, nicotinamide and DPN. The chromatogram was air-dried and cut into 1 cm. segments along the direction of flow of the solvent, and radioactivity in the segments was measured in stainless-steel planchets by using a Geiger counter tube with mica window (thickness 2.1 mg./cm.²) connected to a Panax scaler (Panax Equipment Co., Mitcham, Surrey). A second chromatogram was radioautographed on Ilford Ilfex X-ray film, the period of exposure being 4 weeks.

RESULTS

Metabolic products of nicotinic acid and nicotinamide formed by *Saccharomyces cerevisiae*. Analyses of the culture medium of the organism by paper chromatography revealed that nicotinic acid accumulated as the chief metabolic end product. *N'*-Methylnicotinamide and nicotinuric acid were absent and where nicotinamide had been the supplement it was almost completely deamidated.

Deamidation of nicotinamide by *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides*. The data in Table 1 show the extent of deamidation of nicotinamide by cell extracts and resting cells of *S. cerevisiae* and *L. mesenteroides*; suspensions of *S. cerevisiae* as well as cell-free extract, prepared by grinding the cells with sand, had deamidase activity. On the other hand, *L. mesenteroides* 9135, which can grow only in the presence of nicotinic acid (Johnson, 1945) was devoid of this enzyme.

Table 1. Deamidation of nicotinamide by resting cells and cell extracts of *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides* 9135

Washed fresh cells (100 mg.) were incubated with 6 µmoles of nicotinamide in 2 ml. of 0.1 M-phosphate buffer, pH 7.5, for 1 hr. With cell extract, 0.5 ml. of 10% (w/v) extract of fresh cells of *S. cerevisiae* was similarly incubated, in a final volume of 2 ml. The free nicotinic acid was estimated microbiologically with *L. mesenteroides* 9135.

System	Nicotinic acid released (µm-moles/hr.)
<i>S. cerevisiae</i> , resting cells	1120
<i>L. mesenteroides</i> , resting cells	1.4
<i>S. cerevisiae</i> , cell extract	430*
<i>L. mesenteroides</i> , cell extract	Nil

* Amount/mg. of extract nitrogen.

Biosynthesis of diphosphopyridine nucleotide by cultures of Saccharomyces cerevisiae. *S. cerevisiae* synthesizes DPN equally well from nicotinic acid and nicotinamide (Table 2). The analysis of the culture medium shows that the organism rapidly metabolizes nicotinamide to nicotinic acid, which is the only form of the vitamin finally present in the medium. Little nicotinamide would therefore be available for conversion into DPN by the nicotinamide pathway; since the synthesis of DPN is about the same whether nicotinic acid or nicotinamide has been the original supplement it is reasonable to infer that the nicotinic acid pathway is normally used for the coenzyme synthesis in

S. cerevisiae. Paper chromatography and paper electrophoresis of the pyridine nucleotide-containing extracts revealed the presence of only one quaternary nicotinamide derivative corresponding to DPN. No spot corresponding to nicotinamide mononucleotide was detected. Thus what is being measured by the fluorimetric method is DPN.

Biosynthesis of diphosphopyridine nucleotide by resting cells of Saccharomyces cerevisiae and Leuconostoc mesenteroides 9135. Suspensions of both *S. cerevisiae* and *L. mesenteroides* synthesize DPN when the vitamin is supplied in the acid form (Table 3). Nicotinamide is about equally efficient as a precursor of DPN in *S. cerevisiae*, whereas in

Table 2. *Biosynthesis of diphosphopyridine nucleotide and deamidation of nicotinamide by cultures of Saccharomyces cerevisiae*

S. cerevisiae was grown for 20 hr. on medium (100 ml.) supplemented where necessary with 1 mg. of nicotinic acid or nicotinamide. The cells were harvested and assayed for diphosphopyridine nucleotide and the culture medium was analysed microbiologically for nicotinic acid with *L. mesenteroides* and for nicotinic acid + nicotinamide (if any) with *Lactobacillus arabinosus*.

Supplement to growth medium	Fresh wt. of cells (g.)	DPN content (μg.)	DPN synthesized (μg.)	Nicotinic acid in culture medium (μg.)	
				<i>L. arabinosus</i>	<i>L. mesenteroides</i>
Nil	1.7	306	—	—	—
Nicotinic acid	1.7	759	453	737	750
Nicotinamide	1.6	759	453	827	824

Table 3. *Biosynthesis of diphosphopyridine nucleotide by suspensions of Saccharomyces cerevisiae and Leuconostoc mesenteroides*

Suspensions of *S. cerevisiae* and *L. mesenteroides* were incubated as described in the Experimental section, with supplement, where necessary, of nicotinic acid or nicotinamide (2 μmoles in 5 ml.). The incubation mixture was treated with trichloroacetic acid and assayed for diphosphopyridine nucleotide.

Organism	Supplement to incubation mixture	DPN content (μmoles/g. fresh wt. of cells)	DPN synthesis (μmoles/g. fresh wt. of cells)
<i>S. cerevisiae</i>	Nil	0.37	—
	Nicotinic acid	0.89	0.52
	Nicotinamide	0.83	0.46
<i>L. mesenteroides</i>	Nil	0.22	—
	Nicotinic acid	0.47	0.25
	Nicotinamide	0.23	0.01

Table 4. *Deamidation of nicotinamide by suspensions of Saccharomyces cerevisiae under conditions of diphosphopyridine nucleotide biosynthesis*

Suspensions of *S. cerevisiae* were incubated as described in Table 3 with supplement where necessary of nicotinamide (250 μg./5 ml.). At intervals of 1, 2 and 20 hr. portions of mixture were withdrawn, heated in a boiling-water bath and assayed for nicotinic acid.

Supplement	Time (hr.)	Nicotinic acid in 5 ml. of incubation mixture (μg.)	Nicotinic acid formed by deamidation of nicotinamide* (μg., by difference)
None	1	0	214
Nicotinamide	1	214	
None	2	8	205
Nicotinamide	2	213	
None	20	35	255
Nicotinamide	20	290	

* Vitamin utilized for diphosphopyridine nucleotide synthesis is not included in these figures.

L. mesenteroides there is practically no synthesis from the amide. It is reasonable to presume that *L. mesenteroides* adopts exclusively the nicotinic acid pathway for the synthesis of DPN because there is no nicotinamide deamidase in this organism. This deficiency is also reflected in the inability of nicotinamide to support the growth of *L. mesenteroides*. *S. cerevisiae* utilizes nicotinamide as well as nicotinic acid for the biosynthesis of DPN. The deamidation of nicotinamide is rapid under the conditions used, most of the amide being converted into the acid within 1 hr. (Table 4). This supports the conclusion that *S. cerevisiae* utilizes nicotinic acid normally for biosynthesis and that when nicotinamide is the form of the vitamin provided, the organism rapidly deamidates it and metabolizes it to DPN by the nicotinic acid pathway.

Experiments with [14 C]nicotinic acid and [14 C]nicotinamide. Figs. 1 (a) and (b) show that the radioactivity on the chromatograms is mainly associated with nicotinic acid, confirming a rapid deamidation of nicotinamide by *S. cerevisiae*. Moreover, the radioactivity associated with the

DPN spot is of the same order of magnitude in both, suggesting that nicotinamide is used as efficiently as nicotinic acid for biosynthesis of DPN. The identity of the material in spot B could not be determined. If this spot represents one of the intermediates in DPN synthesis, such as a riboside or a ribotide, the fact that the spot did not fluoresce on exposure to ammonia-butan-2-one would indicate that it is a derivative of nicotinic acid and not of the amide (Preiss & Handler, 1958a).

DISCUSSION

Preiss & Handler (1958b) established that yeast, like human erythrocytes, possesses enzymes for synthesizing pyridine nucleotides from nicotinic acid via the nicotinic acid pathway and that yeast autolysates formed the pyridine nucleotide co-enzymes from nicotinic acid but not from nicotinamide. The present investigation has shown that yeast can synthesize DPN from nicotinamide as readily as from nicotinic acid. This is in accord with the high nicotinamide-deamidating activity of *S. cerevisiae*. Nicotinamide deamidase is also present in beer yeast (Oka, 1954). It is reasonable to conclude therefore that the nicotinamide-deamidating enzyme had been destroyed in the yeast autolysates of the American workers.

Although the enzymes for the synthesis of DPN by the nicotinamide pathway are present in yeast (Hundley, 1954), the results of Preiss & Handler (1958b) and of the present study show that it is the nicotinic acid pathway that is normally responsible for the synthesis of DPN in yeast. Similarly, in human erythrocytes, even though the full complement of enzymes of the nicotinamide pathway is available, the nicotinic acid pathway is probably the predominant route for synthesis of DPN (Preiss & Handler, 1958a).

In *L. mesenteroides*, an organism devoid of nicotinamide deamidase and utilizing only nicotinic acid for growth, the nicotinic acid pathway accounts for the formation of DPN. This mechanism of the synthesis of DPN seems quite widespread; in organisms in which nicotinamide deamidase is present, it may be the normal route of the formation of DPN.

SUMMARY

1. *Saccharomyces cerevisiae* when grown in the presence of either nicotinic acid or nicotinamide accumulates free nicotinic acid as the chief end product. *N'*-Methylnicotinamide and nicotinuric acid are not formed.

2. Resting cells of *S. cerevisiae* as well as a cell-free extract have nicotinamide-deamidase activity. Cells of *Leuconostoc mesenteroides* 9135 are devoid of the deamidase.

Fig. 1. Formation of pyridine nucleotides from [14 C]nicotinic acid (a) and [14 C]nicotinamide (b) by suspensions of *S. cerevisiae*. Radioautographs of incubation system supplemented with [14 C]nicotinic acid and [14 C]nicotinamide. A, DPN; B, unknown; C, nicotinic acid. Solvent system: 95% ethanol-m-ammonium acetate, pH 5.0 (7:3).

3. *S. cerevisiae* can synthesize diphosphopyridine nucleotide from nicotinamide as well as from nicotinic acid and can rapidly deamidate nicotinamide under both growing and resting conditions. Cells of *L. mesenteroides* form diphosphopyridine nucleotide from nicotinic acid but not from nicotinamide.

4. Experiments with [^{14}C]nicotinic acid and [^{14}C]nicotinamide confirm that the biosynthesis of diphosphopyridine nucleotide is effected by suspensions of *S. cerevisiae* almost as efficiently from nicotinamide as from nicotinic acid and that there is a rapid deamidation of nicotinamide.

5. It is concluded that the nicotinic acid pathway of biosynthesis of diphosphopyridine nucleotide is the predominant mechanism under normal conditions in *L. mesenteroides* and *S. cerevisiae*.

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The Amino Acid Sequence around the Reactive Serine Residue of some Proteolytic Enzymes

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The three endopeptidases of the pancreas, chymotrypsin, trypsin and elastase, are stoichiometrically inhibited by diisopropyl phosphorofluoridate, which combines with an unusually reactive serine residue in the enzymes. In chymotrypsin and trypsin this residue has been shown to be present in the sequence Gly.Asp.Ser.Gly (Turba & Gundlach, 1955; Schaffer, Simet, Harshman, Engle & Drisko, 1957; Oosterbaan, Kunst, Van Rotterdam & Cohen, 1958; Dixon, Kauffman & Neurath, 1958). [For definitions of

the abbreviations used for amino acids in this paper see *Biochem. J.* (1957), **66**, 6.] That elastase contains the same sequence was concluded from experiments in which a partial acid hydrolysate of the diisopropoxy[^{32}P]phosphinyl derivative of elastase was subjected to paper ionophoresis and shown to contain the same radioactive peptides as did similar hydrolysates of the diisopropoxy[^{32}P]phosphinyl derivatives of chymotrypsin and trypsin (Hartley, Naughton & Sanger, 1959). The present paper describes some further experiments with two-dimensional ionophoresis which confirm the above-mentioned result.

Considerable difficulties were encountered when we attempted to identify the various radioactive peptide spots present in these hydrolysates. The

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