SYNTHESIS OF PYRIDINE NUCLEOTIDES IN YEAST

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The pyridine nucleotides in crude extracts of yeast undergo a complex series of reactions which include degradation, synthesis, oxidation-reduction, and interconversion. Usually, the most prominent reaction seen is the destruction of DPN. This presumably is caused by the action of DPN nucleosidase, as the disappearance of DPN is not accounted for by comparable increases in the other pyridine nucleotides. The extent of this destruction is variable and depends upon the length of time the cells have been refrigerated before an extract is prepared. Increasing storage time results in decreasing DPN nucleosidase activity in the extract. Storage also lowers the endogenous levels of DPN and DPNH, although it does not appear to affect the levels of TPN and TPNH. The addition of an ATP-generating system to the crude extract markedly stimulates the synthesis of pyridine nucleotides, and the data presented here show that the rate of DPN synthesis from ATP and endogenous precursors can greatly exceed that of DPN degradation. The use of aged cells, in which destruction is minimized, simplifies the study of pyridine nucleotide synthesis in extracts.

A strain of *Saccharomyces cerevisiae,* isolated from commercial yeast (Red Star Yeast & Products Co., Milwaukee, Wis.) was cultured aerobically for 12 hr according to the procedure outlined by Duell and co-workers [1]. After the cells were harvested by centrifugation, they were resuspended (15 g, wet weight/l) in fresh medium supplemented with 100 g glucose/l and stirred slowly at 30°C for 3 hr and 45 min, during which time the culture doubled in mass. The cells were then centrifuged, suspended in distilled water, filtered on 0.45 μ Type MF filters (Millipore Corp., Bedford, Mass.), and washed again with distilled water. The cells were stored as a filtered cake at 4°C before they were used. To extract the cells (12 g wet weight), they were suspended in 30 ml of pH 7.5 buffer (0.1 M KH_2PO_4-0.1 M tris-(hydroxymethyl)aminomethane), transferred to a 75 ml glass homogenization flask containing 0.3 ml of Antifoam B (Sigma Chem. Co., St. Louis) and 20 cc of 0.5 mm diameter glass beads, and shaken at 4000 rpm for 60 sec in a Braun MSK cell homogenizer. All operations were conducted in the cold.

In the control experiment (no additions to homogenate), the above buffer was diluted 1:1 with water and 10 ml was added to the homogenized extract. The mixture was decanted into a vacuum flask, which was evacuated for 1 min; the extract was then transferred to a water-jacketed beaker kept at 0°C, and stirred with a magnetic stirring bar. In the experiment with the ATP-generating system, the 10 ml of diluted buffer also contained 0.15 mmole of ATP (pH 7.5), 0.5 mmole of sodium phosphoenolpyruvate, 0.5 mmole of $MgCl_2$, 0.5 mmole of KCl, and 1.6 mg of rabbit muscle pyruvate kinase (equivalent to 1000 units as measured by the manufacturer, Sigma Chemical Company, at 37°C). The homogenates contained 24 mg of protein per ml as determined by the method of Lowry and associates [2], and microscopic examination revealed a small proportion of unbroken cells. The extracts were not centrifuged so that samples could be taken as early as possible. Zero time is taken as the moment that homogenization began.

At various time intervals, duplicate 0.7 ml samples were mixed with cold acid (0.5 M $HClO_4$, final concentration) or cold alkali (0.1 M NaOH, final concentration) and immediately frozen in a mixture of methyl Cellosolve and Dry Ice. These were later

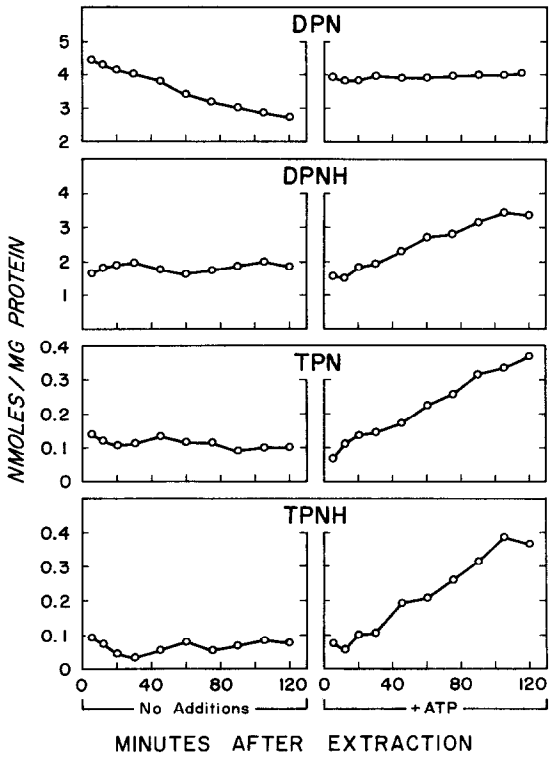

Fig. 1. Pyridine nucleotides in yeast extracts kept at 0°C. Extraction was begun at zero time by shaking with glass beads for 60 sec. Both extracts contained (final quantities/ml): 24 mg of protein and 70 μmoles tris-(hydroxymethyl)aminomethane-70 μmoles KH_2PO_4 buffer, pH 7.5. "No Additions" = unsupplemented extract. "+ATP" = extract supplemented at 2 min with (final quantities/ml): 3 μmoles of ATP (pH 7.5), 10 μmoles of sodium phosphoenolpyruvate, 10 μmoles of $MgCl_2$, 10 μmoles of KCl, and 32 μg of pyruvate kinase.

thawed and treated as described elsewhere [3], and the pyridine nucleotides were assayed fluorometrically using specific enzymatic procedures [3]. The extracts were also analyzed by a cycling procedure essentially as outlined by Slater and co-workers [4], and the same results were obtained. The data shown represent the fluorometric determinations and are not corrected for recoveries (about 70% for TPNH and DPNH, and 90% for TPN and DPN, respectively [3]).

Fig. 1 shows the results obtained with cells stored for 5 weeks at 4°C. At this age the TPN and TPNH content of these cells is the same as when they were first harvested (data not shown); however, the DPN and DPNH content is about one half. In addition, the DPN in the freshly harvested cells underwent a rapid destruction in the extract, whereas after ageing the destruction is much less pronounced (fig. 1, "No Additions"). Another difference is that DPNH destruction is not found with the aged cells, and its level appears to oscillate about a constant value. TPN and TPNH may also be oscillating, but this is less certain.

The addition of ATP and an ATP-generating system to the crude extract markedly increases the yield of all the pyridine nucleotides (fig. 1, "+ATP"). At 0°C, this biosynthesis is approximately linear for 100 min, and the average rates are (pmoles/min/mg protein): TPN+TPNH, 5.8; DPN+DPNH, 21.3. During this period, the increases in DPNH, TPN, and TPNH do not occur at the expense of DPN, indicating that DPN is being replaced at a rate comparable to the rates at which the other pyridine nucleotides are formed. Moreover, if the rate of DPN destruction in the presence of ATP is the same as in its absence, then the total rate of DPN synthesis is even greater by that amount.

The rapid replenishment of DPN in an amount equivalent to that used suggests the presence of DPN precursors as well as the existence of a mechanism which controls the level of DPN. Dietrich and Muniz [5] have reported that in rat liver the pathway of biosynthesis of DPN from nicotinamide is subject to feedback regulation through the inhibition of nicotinamide ribonucleoside pyrophosphorylase by DPN in what appears to be an allosteric mechanism [6]. It is of interest that ATP, which does not participate in the reaction catalyzed by this enzyme, is required for activity. Such a mechanism might at least partially account for the maintenance of the DPN level in the present experiment, and studies of the regulation of pyridine nucleotide biosynthesis in yeast are currently being undertaken. From the results so far obtained, it is apparent that analysis of DPN alone is an insufficient measure of pyridine nucleotide biosynthesis, as prominent effects can be found in the pyridine nucleotides derived from DPN.

Concerning the synthesis of TPN and TPNH in the ATP-supplemented extract, the question arises as to whether TPN is first synthesized from DPN and then converted to TPNH, or whether TPNH is synthesized directly from DPNH. The sequence of phosphorylation followed by reduction has been generally accepted since

Kornberg [7] first described the isolation of DPN kinase from autolysates of yeast. Although this kinase could phosphorylate DPNH, the reaction did not proceed as readily as with DPN. Moreover, the DPN kinases prepared from pigeon liver [8], rat liver [9], and, to some extent, *Azotobacter* [10] are inhibited by DPNH. Nevertheless, it was recently shown [11] that yeast mitochondria contain a DPN kinase which readily phosphorylates DPNH and which is active in intact mitochondria as well as in the damaged mitochondria produced by the homogenization procedure used in the present study. This mitochondrial DPN kinase is specific for DPNH [11] and appears to be distinct [12] from the cytoplasmic DPN kinase described by Kornberg [7]. Because the extracts used in the present study were not centrifuged, the mitochondrial DPN kinase could have been at least partly responsible for the results obtained.

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References

[1] E.A.Duell, S.Inoue and M.F.Utter, J. Bacteriol. 88 (1964) 1762.
[2] O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, J. Biol. Chem. 193 (1951) 265.
[3] C.Bernofsky, submitted for publication.
[4] T.F.Slater, B.Sawyer and U.Sträuli, Arch. Intern. Physiol. Biochim. 72 (1964) 427.
[5] L.S.Dietrich and O.Muniz, (Abstr.) Federation Proc. 25 (1966) 747.
[6] L.S.Dietrich, L.Fuller, I.L.Yero and L.Martinez, J. Biol. Chem. 241 (1966) 188.
[7] A.Kornberg, J. Biol. Chem. 182 (1950) 805.
[8] T.P.Wang and N.O.Kaplan, J. Biol. Chem. 206 (1954) 311.
[9] H.Oka and J.B.Field, J. Biol. Chem. 243 (1968) 815.
[10] A.E.Chung, J. Biol. Chem. 242 (1967) 1182.
[11] C.Bernofsky and M.F.Utter, Science 159 (1968) 1362.
[12] A.M.M.Griffiths and C.Bernofsky, unpublished data.