NADPH Generation in *Aspergillus nidulans*: Is the Mannitol Cycle Involved?

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A cyclic pathway of NADPH generation involving interconversion of mannitol and fructose has been proposed to occur in fungi. In *Aspergillus nidulans* three enzymes of this proposed mannitol cycle (hexokinase, NADP-mannitol dehydrogenase and mannitol-1-phosphate phosphatase) were shown to be localized exclusively in the cytosol. Two isoenzymes of the fourth enzyme (mannitol-1-phosphate dehydrogenase) were detected and shown to be localized respectively in the mitochondrion and the cytosol. The mitochondrial isoenzyme appeared to be present on the outer face of the inner mitochondrial membrane. No evidence was found for a coordinated change in the maximal activities of the enzymes of the proposed mannitol cycle in extracts prepared from mycelia grown on six different carbon, and three different nitrogen sources nor for any increase in these activities induced by growth on NO₃⁻. Studies of this type in which other NADP-linked dehydrogenases were measured showed that for most carbon sources tested growth on NO₃⁻ increased the maximal activity of NADP-isocitrate dehydrogenase as well as that of glucose-6-phosphate and 6-phosphogluconate dehydrogenases but had little effect on the maximal activity of NADP-malate dehydrogenase (decarboxylating). Our studies provide no support for the operation of the mannitol cycle, or for the proposed role of this cycle in NADPH generation in *A. nidulans*.

INTRODUCTION

Provision of NADPH is a crucial requirement for the continued operation of anabolic pathways such as fatty acid synthesis (Walker & Woodbine, 1976), sterol synthesis (McCorkindale, 1976) and purine synthesis, which utilize this coenzyme as a source of reducing equivalents. NADPH is also used in fungi and other micro-organisms to make oxidized nitrogen sources, e.g. NO₃⁻, available for amino acid, purine and pyrimidine synthesis (Lehninger, 1975). In non-photosynthetic micro-organisms it is usually considered that NADPH is generated primarily by the oxidation of glucose 6-phosphate to ribulose 5-phosphate using glucose-6-phosphate and 6-phosphogluconate dehydrogenases. However, oxidation of isocitrate by NADP-isocitrate dehydrogenase and of malate by NADP-malate dehydrogenase can also contribute to NADPH generation. More recently, Hult & Gatenbeck (1978) proposed a cyclic pathway (Fig. 1) in which interconversion of fructose and mannitol is linked to transfer of reducing equivalents from NADH to NADPH. The presence of the enzymes of the mannitol cycle has been shown in a number of Deuteromycetes (Hult et al., 1980) and evidence suggesting its operation as a mechanism for NADPH generation has been obtained in studies using *Alternaria alternata* (Hult & Gatenbeck, 1978). However, doubts have been expressed about the operation of this cyclic pathway (McCullough et al., 1986) based on the very unfavourable *Kₘ* of mannitol dehydrogenase for mannitol (Niehaus & Dilts, 1982) and on the lack of a coordinated

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change in the maximal activities of two enzymes of the proposed cycle in response to a change in carbon growth-substrate (Strandberg, 1969).

We have attempted to obtain further insight into the status of the mannitol cycle and into the proposed role of this and other mechanisms for NADPH generation. Our studies have involved definition of the subcellular localization of the enzymes of the mannitol cycle in *Aspergillus nidulans* and determination of the effect of growth of *A. nidulans* using a range of carbon and nitrogen sources on the maximal specific activities of the enzymes of the mannitol cycle and of some other NADP-linked dehydrogenases.

**METHODS**

*Organism and growth.* *A. nidulans* (strain R21: pabaA1 yA2) was grown in a defined medium containing salts and minerals, p-aminobenzoate and Tween 80 as described previously (Osmani et al., 1981) except that the phosphate concentration was increased to 60 mM in order to minimize changes in pH. The nitrogen source (NO_3\(^-\), NH_4\(^+\) or urea) was added at a final concentration of 70 mM and the carbon source, as specified, at a final concentration of 20 mM. When carbon sources other than glucose were used the medium also contained 0.5 mM glucose to accelerate conidial germination. Control studies demonstrated that addition of 0.5 mM glucose to the medium in the absence of another carbon source permitted only minimal growth. Furthermore, this addition caused no significant changes in the measured enzyme levels during growth on the various carbon substrates tested. Growth was initiated by addition of 0.5 ml of a conidial suspension (10^6 conidia ml^-1) and the inoculated medium (600 ml) was then shaken in 2 l baffled flasks at 150 r.p.m. and 37 °C for 18-24 h in a Gallenkamp Orbital shaker. Growth yields were in the range 2-9 g mycelium per 600 ml medium depending on the carbon source. The growth yield showed little dependence on the nitrogen source.

*Preparation of cell-free extracts.* Mycelium was harvested by filtration through muslin and then washed thoroughly with distilled water. Cell-free extracts were prepared by suspending the mycelium in 10 vols 10 mM-Tris/HCl, pH 7.3, containing 2 mM-MgCl_2, 250 mM-sucrose, 2 mM-dithiothreitol, 1 mM-EDTA and 0.01 mM-phenylmethylsulphonyl fluoride (Tris/Mg\(^{2+}\)/sucrose buffer) by gentle manual homogenization. The mycelial suspension was then subjected to N_2 cavitation at 4 °C in a Parr cell disruption bomb using an equilibration time of 30 min and a pressure of about 14 MPa (2000 p.s.i.). The resulting preparation was centrifuged at 21 000 g and 4 °C for 30 min to remove cell debris and the supernatant fraction was used for assay of total enzyme activities. N_2 cavitation under these conditions disrupts intracellular organelles as well as cells and therefore allows measurement of total enzyme activity even if the enzyme is normally localized within an organelle.

For preparation of cell-free extracts containing intact organelles mycelium grown on a glycerol/NH_4\(^+\) medium was suspended in 10 vols 200 mM PIPES/KOH, pH 6.4, containing 250 mM-sucrose (PIPES/sucrose buffer). Novozym 234 [40 mg (g mycelium)^{-1}] (Novo Industries) was added and the suspension was incubated at 20-25 °C for approximately 30 min with gentle shaking. The progress of cell wall digestion was monitored by diluting a small sample of the suspension into distilled water and examining for release of intracellular contents under the light microscope. When lysis could be observed by this method mycelium was recovered by filtration through muslin and thoroughly washed first with PIPES/sucrose buffer and then with Tris/Mg\(^{2+}\)/sucrose buffer. The washed mycelium was suspended in 10 vols of Tris/Mg\(^{2+}\)/sucrose buffer also containing 0.2% (w/v) bovine serum albumin and was subjected to N_2 cavitation as described above but using a pressure of about 3.5 MPa. The resulting cell-free extract was filtered through four layers of muslin to remove cell debris and intracellular organelles were isolated by differential centrifugation. In most of the studies the extract was subjected to
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centrifugation at 12000 g for 20 min to obtain a heavy particulate fraction (F2) and then at 100 000 g for 60 min to obtain a light particulate fraction (F3) and a 100 000 g supernatant fraction (F4). In some studies the second centrifugation was omitted and the cell-free extract was fractionated only into the heavy particulate and 120 000 g supernatant fractions. The particulate fractions were resuspended in 1 vol Tris/Mg²⁺/sucrose buffer.

Mitoplasts were prepared from the heavy particulate fraction (F2) by resuspension in the Tris/Mg²⁺/sucrose buffer, or in 100 mM-potassium phosphate, pH 7.4, followed by a gentle homogenization using a hand-driven Potter–Elvehjem homogenizer essentially as described by Comte & Gautheron (1979). The mitoplasts were collected by centrifugation at 12000 g for 20 min and resuspended in Tris/Mg²⁺/sucrose buffer.

Enzyme assays. Glucose-6-phosphate (EC 1.1.1.49) and 6-phosphogluconate dehydrogenases (EC 1.1.1.44) were assayed as described by Horecker & Smyrniotis (1955); NADP-isocitrate dehydrogenase (EC 1.1.1.42) as described by Kelly & Hynes (1982); NAD-isocitrate dehydrogenase (EC 1.1.1.41) as described by Osmani & Scroton (1983); NADP-malate dehydrogenase (decarboxylating) (EC 1.1.1.40) as described by McCullough & Roberts (1974); NADP-mannitol dehydrogenase (EC 1.1.1.67) as described by Niehaus & Dils (1982) but using 200 mM-mannitol; hexokinase (EC 2.7.1.1) as described by Joshi & Jagannathan (1966); mannitol-1-phosphate dehydrogenase (EC 1.1.1.17) as described by Hankinson & Cove (1975); citrate synthetase (EC 4.1.3.7) as described by Srere (1969); and mannitol-1-phosphate phosphatase (EC 3.1.3.22) as described by Hult & Gatenbeck (1979) but using 2 mM-mannitol-1-phosphate. The specificity of this latter enzyme assay was established by measurement of the rate of phosphate production from fructose 6-phosphate and sorbitol 6-phosphate under identical conditions. The rate of dephosphorylation of these analogues was found to be less than 5% and 8% respectively of the rate of dephosphorylation of mannitol 1-phosphate.

The sodium salts of mannitol 1-phosphate and sorbitol 6-phosphate were prepared from the commercially available barium salts by addition of an equimolar amount of Na₂SO₄. The BaSO₄ precipitate was removed by centrifugation for 10 mins at 4000 g.

Electrophoresis. Cellulose acetate strip electrophoresis was done as described by Osmani & Scroton (1983). Activity was released from the mitochondrial fraction by exposure to N₂ cavitation at about 14 MPa and the fractions were then concentrated 25-fold (cell-free extract and cytosol) or 200-fold (mitochondria) using a Millipore CX-30 immersible ultrafiltration unit. Samples (1 µl) were applied to the cellulose acetate strip. Electrophoresis was done in 19 mM-potassium phosphate buffer, pH 6-5, for 1 h using a current of 4 mA per strip. Mannitol-1-phosphate dehydrogenase activity was visualized using the modified agar overlay method described by Osmani & Scroton (1983). The activity stain contained 100 mM-HEPES/NaOH, pH 8.1, 5 mM-mannitol, 0.25 mM-NAD, 0.1 mg phenazine methosulphate ml⁻¹ and 0.1 mg 3-(4,5-dimethylthiazolyl-2,5-diphenyltetrazolium bromide) ml⁻¹. Activity bands were developed by incubation for 20 min at 20 °C in the dark.

Statistical significance of observed differences. This was assessed using Student's t-test.

Chemicals. All biochemicals were obtained from Sigma unless otherwise indicated. Chemicals were AnalaR grade.

RESULTS

Subcellular distribution of the enzymes of the proposed mannitol cycle

The subcellular distribution of the four enzymes of the proposed mannitol cycle together with the distribution of two marker enzymes in A. nidulans mycelium grown on a glycerol/NH₄⁺ medium are shown in Fig. 2. In order to permit valid comparisons, the data are expressed as the specific activity of the fraction relative to that of the cell-free extract (= 1) as a function of the percentage of total cell protein present in that fraction (De Duve et al., 1955). The percentage recoveries of activity in the separated fractions when compared with those present in the initial cell-free extract (F1) are 73% (hexokinase), 87% (mannitol-1-phosphate dehydrogenase), 86% (mannitol-1-phosphate phosphatase), 90% (NADP-mannitol dehydrogenase), 81% (citrate synthetase) and 65% (adenylate kinase) respectively. For all the enzymes except adenylate kinase the extent of recovery in the separated fractions was in an acceptable range. For hexokinase, mannitol dehydrogenase and mannitol-1-phosphate phosphatase the data indicate a totally cytosolic localization with no activity being present in the heavy particulate (12000 g precipitate) (F2) or light particulate (100000 g precipitate) (F3) fractions (Fig. 2). Such a result was expected for hexokinase which is a marker for the 100 000 g (F4) fraction. Mannitol-1-phosphate dehydrogenase showed a more complex distribution with significant activity being present in both the F2 and F4 fractions. The activity of this enzyme which appeared in the F3 fraction may result from incomplete separation of the mitochondria and microsomes. A similar distribution of citrate synthetase, a mitochondrial marker enzyme, was observed between the F2
and F3 fractions but markedly less of this latter enzyme was present in F4 (Fig. 2). Further evidence supporting the presence of unique mannitol-1-phosphate dehydrogenases in the F2 and F4 fractions was provided by electrophoretic analysis of the distribution of the activity of this enzyme in a cell-free extract containing disrupted organelles, and in the isolated fractions. The result of such an analysis (Fig. 3) clearly indicated the presence of two activity bands in the cell-free extract (track C of Fig. 3). The faster-moving band arose from the mannitol-1-phosphate dehydrogenase isoenzyme in the cytosolic (F4) fraction since its mobility corresponded to the only activity band observed when a similar analysis was done on this isolated fraction (track A of Fig. 3). Similarly, the slower moving band in the cell-free extract (track C) was identified as the mitochondrial isoenzyme since its mobility corresponded to that of the only band observed when the analysis was done on an isolated F2 fraction (track B of Fig. 3). All bands resulted from mannitol-1-phosphate dehydrogenase activity since they were not observed if mannitol 1-phosphate was omitted from the incubation medium (data not shown).

The localization of mannitol-1-phosphate dehydrogenase within the mitochondrion was less clear. Fig. 4 indicates that the activity of this enzyme in F2 was fully expressed in the absence of a detergent such as Triton X-100 with a decrease in activity being observed as the Triton X-100 concentration was increased. Similar results were obtained when Nonidet P-40 was used in place of Triton X-100 (data not shown). In contrast, in accord with previous observations (Osmani & Scrutton, 1983), the activity of the matrix enzyme citrate synthetase was fully expressed only in the presence of detergent. This is demonstrated in Fig. 4 which also shows that different ranges of Triton X-100 concentration increased citrate synthetase, and decreased mannitol-1-phosphate dehydrogenase activities. The decrease in mannitol-1-phosphate dehydrogenase activity seen for the F1 and F2 fractions was not due to a general sensitivity of this enzyme to detergents since no such decrease was observed for the F4 fraction (Fig. 4).

We examined whether mannitol-1-phosphate dehydrogenase was localized in the mitochondrial inter-membrane space or on the outer mitochondrial membrane by determining the effect

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Fig. 2. Subcellular localization of the mannitol cycle enzymes and of adenylate kinase in *A. nidulans* grown on a glycerol/NH₄⁺ medium. ■, Mitochondrial (F2) fraction; □, microsomal (F3) fraction; □, cytosolic (F4) fraction. The data are means of three experiments with bars indicating SEM.
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Fig. 3. Analysis by cellulose acetate strip electrophoresis of the isoenzymes of mannitol-1-phosphate dehydrogenase in the cell-free extract (track C) and in the separated mitochondrial (track B) and cytosolic (track A) fractions. Arrows indicate bands of mannitol-1-phosphate dehydrogenase activity. The pattern shown is representative of that obtained in two other similar experiments.

Fig. 4. Effect of Triton X-100 on the activity of mannitol-1-phosphate dehydrogenase in the cell-free extract (●) and the mitochondrial (○) and cytosolic (▲) fractions, and of citrate synthetase in the mitochondrial fraction (■). The data shown are for a single experiment with determinations done in duplicate but are typical of those obtained in two further similar experiments.

of mitoplast preparation on retention of this enzyme in the particulate fraction. In these studies, citrate synthetase was used as a marker enzyme for the mitochondrial matrix and adenylate kinase as a marker for the mitochondrial inter-membrane space. The data shown in Fig. 2 indicate that a substantial portion of adenylate kinase activity in *A. nidulans* is localized in the mitochondrion but do not exclude the presence of a cytosolic isoenzyme.
Table 1. Effect of homogenization and sonication on citrate synthetase, adenylate kinase and mannitol-1-phosphate dehydrogenase activity in the heavy particulate (F2) fraction

The F2 fraction was prepared from 7.2 g mycelium. The resuspended mitoplasts held at 2 °C were subjected to four 30 s periods of sonication separated by 60 s cooling periods and particulate matter was then separated by centrifugation at 36000 g for 20 min. Citrate synthetase, adenylate kinase and mannitol-1-phosphate dehydrogenase were assayed in the absence and presence of 0.1 % (v/v) Triton X-100. The data shown in parentheses indicate the activities observed when the assays were done in the presence of Triton X-100. The data are the means of duplicate determinations which did not differ by more than 5% from a single experiment. They are representative of the results obtained in two other similar experiments. No consistent change in the pattern of release shown here was observed if homogenization was increased to eight strokes or if the mitoplasts were sonicated for six periods of 30 s.

<table>
<thead>
<tr>
<th>Total activity (μmol h⁻¹)</th>
<th>Citrate synthetase</th>
<th>Adenylate kinase</th>
<th>Mannitol-1-phosphate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial fraction (F2)</td>
<td>21.8 (75.4)</td>
<td>69.5 (34.7)</td>
<td>43.4 (17.4)</td>
</tr>
<tr>
<td>After suspension in buffer and homogenization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36000 g pellet (mitoplasts)</td>
<td>16.5 (79.4)</td>
<td>38.3 (23.2)</td>
<td>39.1 (8.7)</td>
</tr>
<tr>
<td>36000 g supernatant fraction</td>
<td>6.1 (3.7)</td>
<td>21.4 (16.1)</td>
<td>7.1 (5.4)</td>
</tr>
<tr>
<td>36000 g supernatant fraction obtained after sonication of 36000 g pellet</td>
<td>31.1 (34.1)</td>
<td>7.0 (4.3)</td>
<td>4.8 (3.5)</td>
</tr>
</tbody>
</table>

Suspension of the F2 fraction in either phosphate or Tris buffer followed by homogenization as described in Methods caused minimal release of citrate synthetase to the supernatant fraction but substantial release of adenylate kinase as would be expected for the proposed localizations of these two enzymes in the mitochondrion. In addition, expression of the maximal activity of citrate synthetase in the 12000 g pellet obtained after homogenization was dependent on addition of Triton X-100 but that of adenylate kinase was not. Instead, addition of Triton X-100 decreased the activity of adenylate kinase both in the F2 fraction and in the 12000 g pellet obtained after homogenization although the extent of this decrease was less marked than that observed for mannitol-1-phosphate dehydrogenase (Table 1). For mannitol-1-phosphate dehydrogenase suspension in phosphate or Tris buffer followed by homogenization caused a release of activity which was less than that observed for adenylate kinase but still greater than the minimal extent of release observed for citrate synthetase (Table 1).

We also examined the effect of sonication on the mitoplasts obtained by homogenization of the F2 fraction. This procedure, which would be expected to cause disruption of these particles, gives, as predicted, substantial release of the matrix marker citrate synthetase, but only minimal release of either adenylate kinase or mannitol-1-phosphate dehydrogenase (Table 1).

Total activities of the enzymes of the proposed mannitol cycle in A. nidulans grown on various carbon and nitrogen sources

We evaluated the operation of the mannitol cycle as a transhydrogenation mechanism for NADPH generation by comparing the maximal activities of the constituent enzymes of the proposed cycle expressed in mycelia grown on different carbon and nitrogen sources. Studies of this type done using six carbon and three nitrogen growth substrates are summarized in Table 2 with the carbon growth-substrates being classified as catabolite repressing or derepressing according to Arst & Bailey (1977). In these studies change of pH during growth was minimized by using increased buffering capacity and the final pH of the medium was in all cases in the range 7.0-7.8. The assay conditions used expressed the maximal activities of hexokinase and mannitol-1-phosphate dehydrogenase but underestimated this parameter for mannitol-1-phosphate phosphatase and mannitol dehydrogenase. For the phosphatase the $K_m$ for mannitol-1-phosphate was determined as 1.0 ± 0.1 mM (data not shown). Hence the assay concentration (2 mM) routinely used in these studies underestimated the maximal activity of the phosphatase by approximately 30%. For mannitol dehydrogenase a complex initial rate/substrate
Table 2. Activities of enzymes of the mannitol cycle in A. nidulans grown on various carbon and nitrogen sources

Mycelia were grown, cell-free extracts prepared and enzyme activities measured as described in Methods. The values shown are means ± SEM of at least four determinations.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Nitrogen source</th>
<th>Hexokinase</th>
<th>Mannitol-1-phosphate dehydrogenase</th>
<th>Mannitol-1-phosphate phosphatase</th>
<th>Mannitol dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Carbon catabolite repressing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>NO₃</td>
<td>4.6 ± 0.9</td>
<td>1.6 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>NH₄</td>
<td>7.3 ± 0.9*</td>
<td>3.3 ± 0.1*</td>
<td>3.9 ± 0.3*</td>
<td>0.4 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>7.8 ± 0.2*</td>
<td>7.8 ± 0.9*</td>
<td>9.7 ± 0.7*</td>
<td>0.9 ± 0.02*</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>NO₃</td>
<td>5.5 ± 0.4</td>
<td>3.7 ± 0.6</td>
<td>6.3 ± 0.6</td>
<td>1.6 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>NH₄</td>
<td>3.0 ± 0.1*</td>
<td>6.4 ± 0.1*</td>
<td>13.9 ± 1.5*</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>14.4 ± 1.9*</td>
<td>5.8 ± 0.3*</td>
<td>13.6 ± 0.7*</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Acetate</td>
<td>NO₃</td>
<td>4.3 ± 1.6</td>
<td>1.8 ± 0.4</td>
<td>2.9 ± 0.3</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>NH₄</td>
<td>1.9 ± 0.2*</td>
<td>0.9 ± 0.2*</td>
<td>2.0 ± 0.4*</td>
<td>0.2 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>3.3 ± 0.4</td>
<td>2.7 ± 0.5*</td>
<td>5.2 ± 0.2*</td>
<td>0.3 ± 0.1</td>
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<tr>
<td>(b) Carbon catabolite derepressing</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Glycerol</td>
<td>NO₃</td>
<td>6.1 ± 0.7</td>
<td>3.5 ± 0.8</td>
<td>4.9 ± 1.3</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>NH₄</td>
<td>9.0 ± 0.1*</td>
<td>6.5 ± 0.7*</td>
<td>3.9 ± 0.9</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>4.8 ± 0.6</td>
<td>3.1 ± 0.7</td>
<td>5.2 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>NO₃</td>
<td>7.6 ± 2.9</td>
<td>2.5 ± 0.2</td>
<td>5.3 ± 0.3</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>NH₄</td>
<td>7.1 ± 0.03</td>
<td>9.4 ± 1.8*</td>
<td>7.0 ± 0.2*</td>
<td>2.7 ± 0.1*</td>
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<td></td>
<td>Urea</td>
<td>20.2 ± 4.1*</td>
<td>3.8 ± 1.4</td>
<td>6.4 ± 0.1*</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Ethanol</td>
<td>NO₃</td>
<td>5.5 ± 0.9</td>
<td>2.2 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>NH₄</td>
<td>5.2 ± 0.5</td>
<td>4.9 ± 0.3*</td>
<td>5.7 ± 0.4*</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>5.3 ± 1.0</td>
<td>2.4 ± 0.3</td>
<td>3.4 ± 0.3</td>
<td>0.2 ± 0.03</td>
</tr>
</tbody>
</table>

* P < 0.05 or better with respect to specific activity observed in extracts of NO₃-grown mycelium.

concentration relationship was observed with apparent substrate activation occurring at high mannitol concentration (data not shown). The mannitol concentration (200 mM) used in these studies caused suboptimal substrate activation but it is not possible to quantificate satisfactorily the extent to which maximal activity was underestimated.

The data in Table 2 can be analysed in several ways. First we can compare the effect of different nitrogen growth substrates on the maximal specific activities of enzymes expressed during growth on a given carbon source. The statistical analysis given both in Table 2 and in Table 3, is based on this comparison. Where statistically significant differences were observed (Table 2) the maximal specific activities in extracts of mycelia grown on NH₄ or urea as nitrogen source were typically increased as compared with those found for extracts prepared from mycelia grown using NO₃ as nitrogen source. This relationship has previously been reported for mannitol-1-phosphate dehydrogenase by Hankinson & Cove (1975). The only consistent exception to this generalization was observed for growth on acetate where the maximal specific activities of all four enzymes were significantly lower in extracts prepared from mycelia grown on NH₄ as compared with those grown on NO₃ as nitrogen source. However, a similar relationship was not observed between these activities in extracts of mycelia grown on acetate/NO₃ and acetate/urea media.

Second, for extracts of mycelia grown on a given nitrogen source there was no consistent pattern in the maximal specific activities of the enzymes of the proposed mannitol cycle which could be related to the metabolic relationship of the carbon source for growth to the oxidative pentose pathway. For example, growth on ethanol or acetate as carbon source did not consistently increase the maximal specific activities of the enzymes when compared to the values found in glucose-grown mycelia regardless of the source of nitrogen used (Table 2).
Table 3. Activities of NADP-linked dehydrogenases in A. nidulans grown on various carbon and nitrogen sources

Mycelia were grown, cell-free extracts prepared and enzyme activities measured as described in Methods. The values shown are means ± SEM of at least four determinations.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Nitrogen source</th>
<th>Glucose-6-phosphate dehydrogenase</th>
<th>6-Phosphogluconate dehydrogenase</th>
<th>NADP-isocitrate dehydrogenase</th>
<th>NADP-malate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Carbon catabolite repressing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>NO₃⁻</td>
<td>6.4 ± 1.2*</td>
<td>3.0 ± 0.1*</td>
<td>0.5 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>1.2 ± 0.2*</td>
<td>0.3 ± 0.04*</td>
<td>0.02 ± 0.01*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>1.5 ± 0.1*</td>
<td>0.4 ± 0.05*</td>
<td>0.5 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>NO₃⁻</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>0.05 ± 0.01*</td>
<td>0.1 ± 0.01*</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>1.3 ± 0.1*</td>
<td>0.9 ± 0.1</td>
<td>0.02 ± 0.05*</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Acetate</td>
<td>NO₃⁻</td>
<td>2.9 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>0.8 ± 0.1*</td>
<td>0.3 ± 0.04*</td>
<td>0.7 ± 0.1*</td>
<td>0.02 ± 0.01*</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>1.9 ± 0.1*</td>
<td>0.3 ± 0.05*</td>
<td>1.4 ± 0.1*</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>(b) Carbon catabolite derepressing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>NO₃⁻</td>
<td>2.3 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>0.6 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>1.7 ± 0.1*</td>
<td>1.4 ± 0.1*</td>
<td>0.02 ± 0.01*</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>1.3 ± 0.2*</td>
<td>1.0 ± 0.1*</td>
<td>0.02 ± 0.04*</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>NO₃⁻</td>
<td>1.0 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>0.2 ± 0.02*</td>
<td>1.0 ± 0.01*</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>3.1 ± 0.3*</td>
<td>1.7 ± 0.1</td>
<td>0.37 ± 0.08*</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Ethanol</td>
<td>NO₃⁻</td>
<td>1.3 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.01</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>0.8 ± 0.02*</td>
<td>0.8 ± 0.03*</td>
<td>0.2 ± 0.02*</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>1.0 ± 0.05</td>
<td>0.6 ± 0.04*</td>
<td>0.1 ± 0.01*</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>(c) Uncertain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>NO₃⁻</td>
<td>0.8 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>0.7 ± 0.05</td>
<td>0.6 ± 0.1</td>
<td>0.4 ± 0.05</td>
<td>0.05 ± 0.01</td>
</tr>
</tbody>
</table>

ND, Not detectable.
* P < 0.05 or better with respect to specific activity observed in extracts of NO₃⁻-grown mycelium.

Finally, the levels of the enzymes of the mannitol cycle were not subject to significant carbon catabolite repression. While the maximal activities of mannitol-1-phosphate phosphatase and mannitol dehydrogenase were in many cases higher in extracts obtained from mycelia grown on the derepressing substrate, these increases were small, rarely exceeding 2- to 3-fold in magnitude. Furthermore, they were not observed when urea was used as the nitrogen source (Table 2).

We also studied the effect of growth on these carbon and nitrogen sources on the maximal specific activity of NADP-linked dehydrogenases. Data for four such enzymes are summarized in Table 3. The expected elevation in maximal activity caused by growth on NO₃⁻, as compared with NH₄⁺, as nitrogen source was seen for both glucose-6-phosphate and 6-phosphogluconate dehydrogenases in extracts from mycelia grown on all the carbon sources used except succinate. For mycelia grown on glucose, glycerol, ethanol or acetate as carbon source a similar effect on the maximal activities of both these dehydrogenases was observed when the comparison was made for extracts prepared from mycelia grown on NO₃⁻ and urea as nitrogen sources. However, when the pentose sugars xylose or arabinose were used as carbon source the maximal activities of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in extracts of mycelia grown on urea as nitrogen source were comparable to, or greater than, those found for the mycelia grown on NO₃⁻ (Table 3).

Growth on NO₃⁻ also increased the maximal specific activity of NADP-isocitrate...
NADPH generation in Aspergillus nidulans

Table 4. Effect of carbon and nitrogen source on the subcellular distribution of NADP-isocitrate dehydrogenase and NADP-malate dehydrogenase

Fractions were prepared and enzymic activities measured as described in Methods. Values in parentheses are the percentage of the total activity present in each fraction. The total activity values given represent the mean values obtained from duplicate determinations in three similar preparations. The variation between the means was less than 10%. The integrity of the mitochondria in these preparations was indicated by the observation that 92-99% of total NAD-isocitrate dehydrogenase activity was recovered in the 15000 g precipitate.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Nitrogen source</th>
<th>Total NADP-isocitrate dehydrogenase activity</th>
<th>Total NADP-malate dehydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15000 g precipitate</td>
<td>15000 g supernatant</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>NO₃⁻</td>
<td>1.5 (5)</td>
<td>27.7 (95)</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>0.5 (65)</td>
<td>0.3 (35)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>NO₃⁻</td>
<td>7.3 (21)</td>
<td>27.9 (79)</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻</td>
<td>0.4 (44)</td>
<td>0.5 (56)</td>
</tr>
<tr>
<td>Acetate</td>
<td>NO₃⁻</td>
<td>2.8 (2)</td>
<td>147.6 (98)</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>1.7 (6)</td>
<td>26.3 (94)</td>
</tr>
<tr>
<td>Succinate</td>
<td>NO₃⁻</td>
<td>2.8 (10)</td>
<td>24.6 (90)</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>0.9 (6)</td>
<td>13.9 (94)</td>
</tr>
</tbody>
</table>

dehydrogenase in extracts prepared from mycelia grown on some of the carbon sources tested when compared with the levels obtained in extracts from mycelia grown on urea or NH₄⁺ as nitrogen source. This effect was seen most clearly for mycelia grown on glycerol, acetate and ethanol but was also apparent for glucose as carbon source when NH₄⁺ was used as nitrogen source. No such effect of NO₃⁻ was found in extracts obtained from mycelia grown on xylose or arabinose as carbon sources, while use of urea as the nitrogen source for growth caused a more marked increase in the maximal specific activity of NADP-isocitrate dehydrogenase than was observed for glucose-6-phosphate or 6-phosphogluconate dehydrogenases.

No such effects of NO₃⁻ or urea were observed on the maximal specific activity of NADP-malate dehydrogenase in the mycelial extracts regardless of the carbon source used. With the exception of mycelia grown on ethanol as carbon source the maximal specific activity of NADP-malate dehydrogenase was 10-fold lower than that observed for the other NADP-linked dehydrogenases, including mannitol dehydrogenase, tested in this study.

Glucose-6-phosphate and 6-phosphogluconate dehydrogenases are exclusively localized in the cytosol in A. nidulans as in other eukaryotic cells. Hence measurement of maximal specific activity in the cell-free extract is a valid indicator. However, the situation is less simple for NADP-isocitrate dehydrogenase for which previous studies (Osmani & Scrutton, 1983) have shown the presence of distinct mitochondrial and cytosolic isoenzymes. We therefore measured the maximal activity of this enzyme in mitochondrial (15000 g precipitate) and cytosolic (15000 g supernatant) fractions prepared by a simple differential centrifugation procedure. The data obtained are expressed in Table 4 as total activities [units (g mycelium)⁻¹] rather than specific activities since the effect of a change in nitrogen source is seen more clearly. However, qualitatively similar results are obtained if the results are expressed as specific activities. In fractions prepared from mycelia grown on glucose, glycerol and acetate, the major part of the increase in NADP-isocitrate dehydrogenase activity caused by growth on NO₃⁻ resulted from induction of the cytosolic isoenzyme. Some increase in the total activity of the mitochondrial isoenzyme was observed, especially in mycelia grown on glycerol, but its contribution was less important. Hence for all three carbon growth-substrates growth on NO₃⁻ caused an increase in the cytosolic : mitochondrial activity ratio with the effect being most marked in mycelia grown on glucose as carbon source (Table 4). The smaller increase in this ratio when acetate was used as carbon source resulted, as shown previously (Osmani & Scrutton, 1983), from induction of the
cytosolic isoenzyme of NADP-isocitrate dehydrogenase. When succinate was the carbon source, NO$_3^-$ as nitrogen source caused only a small further enhancement in the activity of the dominant cytosolic isoenzyme. Hence although the extent of increase in the activity of the mitochondrial isoenzyme caused by growth on NO$_3^-$ was comparable to that obtained for the other carbon growth-substrates, this increase made a relatively minor contribution to the total NADP-isocitrate dehydrogenase activity. Hence the data shown in Table 4 are compatible with the comparable total maximal specific activities found for this enzyme in mycelia grown on NO$_3^-$ and NH$_4^+$ when succinate was used as carbon growth-substrate (Table 3).

NADP-malate dehydrogenase (decarboxylating) is also present in the particulate and supernatant fractions (J. Campa, S. A. Osmani & M. C. Scrutton, unpublished observations). The failure to observe an increase in the maximal specific activity of this enzyme in the cell-free extract of mycelia grown on NO$_3^-$ as nitrogen source could be due to compensating changes in the particulate and supernatant fraction activities, but the data in Table 4 demonstrate that such compensation does not occur. No significant difference was observed in the particulate activity for mycelia grown on NO$_3^-$ and NH$_4^+$ regardless of the carbon growth-substrate used. The effects on the cytosolic activity were also small except when acetate was used as carbon growth substrate. In this case induction by NO$_3^-$ was comparable to that found for NADP-isocitrate dehydrogenase (Table 4) and accounted for the statistically significant increase in the maximal specific activity of this enzyme found in the cell-free extract of acetate-grown mycelia (Table 3).

DISCUSSION

The data presented in this report suggest that the increase in the maximal specific activities of NADP-linked dehydrogenases caused by growth of *A. nidulans* on an oxidized nitrogen source (NO$_3^-$) may be a more widespread phenomenon than has been suggested by previous studies (Hankinson & Cove, 1974; Kelly & Hynes, 1982). Our data showed a NO$_3^-$-induced increase in the maximal specific activity of glucose-6-phosphate and 6-phosphogluconate dehydrogenases when glucose was used as carbon source which is comparable in magnitude to that reported by Hankinson & Cove (1974). Moreover, this was observed for all other carbon growth-substrates tested with the exception of succinate provided that the comparison was made with mycelia grown using NH$_4^+$ as the reduced nitrogen source (Table 3). In contrast to the report of Kelly & Hynes (1982) we observed an increase in the maximal specific activity of NADP-isocitrate dehydrogenase induced by growth on NO$_3^-$ provided that glucose, glycerol, acetate or ethanol were used as carbon source (Table 3). This effect was primarily due to an increase in the activity of the cytosolic isoenzyme (Table 4). No comparable increase in the maximal specific activities of NADP-malate dehydrogenase (decarboxylating) or mannitol dehydrogenase were observed under these conditions and there was no evidence for a coordinated increase in the maximal specific activities of the enzymes of the proposed mannitol cycle caused by growth on NO$_3^-$ as nitrogen source. The effect of an increase in demand for NADPH imposed by growth on NO$_3^-$ appears therefore to be confined to three of the NADP-linked dehydrogenases studied here suggesting that these enzymes are responsible for meeting this demand. Our data provide no support for the postulates that either the mannitol cycle (Hult & Gatenbeck, 1978) or a pyruvate–malate cycle (McCullough et al., 1986) might provide a mechanism for generation of NADPH by indirect transfer of reducing equivalents from NADH.

However, the situation is less clear-cut than the above analysis would suggest since the results obtained using a second reduced nitrogen source (urea) resemble only in part those obtained for mycelia grown on NH$_4^+$. The results differed most notably in that mycelia grown on the pentose sugars L-arabinose and D-xylose showed significantly higher maximal specific activities of glucose-6-phosphate, 6-phosphogluconate and NADP-isocitrate dehydrogenases when urea was used as nitrogen source than those found in mycelia grown using NO$_3^-$ (Table 3). The origin of this latter effect is unclear but is unlikely to be related to an alteration in the pH of the growth medium since this parameter did not change significantly for growth on any of the carbon sources used when urea or NO$_3^-$ was used as nitrogen source. Similarly, under the conditions used
here a minimal decrease in pH (less than 0.5 units) occurred during growth in media containing NH$_4^+$ (as ammonium sulphate) as the nitrogen source.

Our data offer little support for the postulate (Kelly & Hynes, 1982) that during growth on acetate NADPH for reductive biosynthesis is provided primarily by NADP-isocitrate dehydrogenase and NADP-malate dehydrogenase. The extents of induction of NADP-isocitrate dehydrogenase and NADP-malate dehydrogenase observed here (Table 3) as a consequence of growth on acetate are comparable to those reported by Kelly & Hynes (1981, 1982). However, the observed increases in the maximal specific activities of these two enzymes together with the twofold decrease detected in the maximal specific activity of glucose-6-phosphate and 6-phosphogluconate dehydrogenases gave rise to a situation in which mycelia grown on acetate or ethanol as carbon source with NO$_3^-$ as nitrogen source contained comparable maximal activities of glucose-6-phosphate, 6-phosphogluconate and NADP-isocitrate dehydrogenase with NADP-malate dehydrogenase (decarboxylating) being present at 10–25% of that activity (Table 3). The pattern observed in mycelia grown on these carbon sources with NH$_4^+$ or urea as nitrogen source was not as clear-cut but there was no evidence in any of these mycelia that NADP-isocitrate and/or NADP-malate dehydrogenases represent the dominant NADP-linked dehydrogenase. The continued utility of the enzymes of the oxidative pentose phosphate pathway in mycelia grown on acetate or ethanol was predictable since synthesis of ribose 5-phosphate, as well as of NADPH, from these carbon sources is a requirement for growth and cell division. Where the demand for NADPH exceeds that for ribose 5-phosphate the pentose phosphate pathway may still be a viable option since cyclic operation of this pathway causes only a minimal loss of carbon from the system.

In mycelia grown on glycerol and NH$_4^+$ all the necessary enzymes of the proposed mannitol cycle were present in the cytosol and except for mannitol-1-phosphate dehydrogenase were exclusively localized in this compartment. In addition an exclusively cytosolic localization has also been shown for NADP-mannitol dehydrogenase in mycelia grown on glucose, acetate or succinate with NO$_3^-$ or NH$_4^+$ as the nitrogen source and on alanine as carbon and nitrogen source (N. S. Scrutton & M. C. Scrutton, unpublished observations). The maximal activities of these enzymes did not, however, increase in a coordinated manner under conditions where NADPH demand was increased or show any coordinated response in mycelia grown on a variety of carbon substrates (Table 2). While our data do not exclude the operation of a mannitol cycle in A. alternata as proposed by Hult & Gatenbeck (1978), they do not support the concept that such a cycle is a feature of fungal metabolism in any other species which contains all the relevant enzymes.

The subcellular fractionation data (Fig. 2) and the isoenzyme analysis (Fig. 4) are however consistent with the postulate that mannitol-1-phosphate dehydrogenase might play a role in the transfer of reducing equivalents between the cytosol and the mitochondria (Boonsaeng et al., 1977). This enzyme was localized in both the mitochondria and the cytosol and unique isoenzymes were present in these two sites (Figs 2 and 4). The mitochondrial isoenzyme was not localized in the matrix of this organelle since its activity was fully expressed in the absence of detergent (Fig. 3), or in the intermembrane space since it was not released in significant amounts by mitoplast preparation (Table 1). Localization on the outer mitochondrial membrane seems unlikely since the fragments of this membrane would probably not be precipitated by centrifugation under the conditions used (Comte & Gautheron, 1979). Hence the enzyme is probably bound to the outer face of the inner mitochondrial membrane in a manner comparable to that proposed for the NADH oxidase of fungal mitochondria (Watson & Smith, 1967).

While these findings may suggest a role for one of the enzymes involved in mannitol synthesis our studies have failed to evaluate the significance of mannitol in the physiology of A. nidulans. None of our data exclude the proposed involvement of this compound in osmoregulation (Jennings & Austin, 1973) but other observations, notably the failure of osmotic stress to cause any marked change in mannitol concentration (Beever & Laracy, 1986), appear incompatible with this postulate. Significant further progress may therefore depend on isolation of mutants defective in enzymes of mannitol metabolism, or development of a selective inhibitor for one of these enzymes.
REFERENCES


