Assay of steady-state level of glucose-6-phosphate

S. Brody

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Abstract
Assay of glucose-6-phosphate
Table 1. Distribution of total protein in subcellular fractions of Neurospora mycelia.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Centrifuge force (x 1,000 g)</th>
<th>Time (minutes)</th>
<th>Protein (mg) recovered in fraction per 100 mg total Protein *</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>2</td>
<td>10</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>M2</td>
<td>1 b</td>
<td>20</td>
<td>28 ± 12</td>
</tr>
<tr>
<td>P</td>
<td>120</td>
<td>120</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>S</td>
<td>su</td>
<td></td>
<td>49 ± 13</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td>100**</td>
</tr>
</tbody>
</table>

* Average and standard deviation of four experiments with wild-type (74A). Protein was measured by a biuret method after samples were dissolved in 8 M urea containing 0.1 N NaOH.
** Average recovery in the sum of the fractions was 92% of the protein in the filtered homogenate (H1). About 3 gm protein is obtained in H1 Per 200 gm fresh mycelium.

The fine structure of mitochondria prepared by this method is indistinguishable from mitochondria in hyphoe in electron micrographs of the sections stained with osmium tetroxide and uranyl acetate. Nuclear preparations, although apparently free of mitochondria, are variable in size and shape, and contain some ruptured nuclei.

Systematic and empirical variation of the conditions for cell disruption and fractionation may be necessary in order to study enzyme localization. Malate dehydrogenase is found exclusively associated with mitochondria in situ by histochemical methods and electron microscopy. On the other hand, the recovery of malate dehydrogenase in the mitochondrial fraction is a direct and linear function of the sucrose concentration of the isolation medium. = = = Department of Biological Sciences, Stanford University, Stanford, California. 94305.

Brody, S. Assay of steady-state level of glucose-b-phosphate.

A. General scheme: Extraction by hot ethanol, chromatography of extract to isolate glucose-6-P, elution, and enzymatic assay of glucose-b-P using glucose-6-P dehydrogenase and TPN. The amount of TPNH formed (measured at 340 μM) is equivalent to the input amount of glucose-b-P. Sensitivity: can detect the steady state glucose-b-P level in 50–100 mg of wild-type lyophilized material.

B. Procedure: Only freshly harvested cultures, which were capable of at least a doubling in dry weight, were used. These cultures were washed well and placed in a 125-ml Erlenmeyer flask. 60 ml of 80% ethanol, was added and the mixture gently heated until boiling. Boiling was continued for 5-8 minutes, the fluid then immediately filtered by use of a filter flask, and the extract was concentrated in a vacuum rotary evaporator to approximately 1-2 ml. This opaque and somewhat syrupy solution was chilled and centrifuged at 8,000 x g for 10 minutes, achieving partial clarification. The volume of the extract was then measured exactly, and the extract was stored at -15°C if it was not to be used immediately. The extracted mycelium was dried overnight at approximately 100°C and the residual dry weight measured.

(> 4 mg glucose-6-P in the extract can be measured at this point; however, accuracy and sensitivity are reduced due to the high level of particulate matter which contributes a large blank at 340 μM. Secondly, the purest possible glucose-b-P dehydrogenase must be used, otherwise other substances in the crude extract will serve as substrates for impurities in the enzyme preparation. Thirdly, any inhibitors present in the crude extract will affect the values obtained.)

To overcome these problems, a portion of the extract (0.4-0.5 ml) was applied as a streak to a large sheet of Whatman No. 1 paper and then subjected to ascending chromatography in an n-butanol:Acetic acid:Water (2:1:1) system for approximately 2 days. A marker strip of one edge, as well as a sample of Na2glucose-6-P, were located by AgNO3 staining and the area corresponding to the glucose-b-P eluted in 2-3 ml water. Other areas of the chromatogram were eluted also.

The assay procedure: Different aliquots of the eluate were separately made up to 2.5 ml with 0.1 M Tris ± 0.01 M MgCl2 buffer pH 7.5, 0.60 PM TPN were made to 2.5 ml with water, and the reaction was started by the addition of 0.2 international units of glucose-b-P dehydrogenase (Boehringer-Manheim was a good source). After 20 minutes or more, the change in OD340 was determined for each sample, as well as for the samples incubated without enzyme (i.e., appropriate blanks). A standard curve of known amounts of Na2glucose-6-P . 2H2O was run with every series of determinations, and the values obtained were used for subsequent calculations.

This assay procedure can also be used for the detection of any fructose-b-P or glucose-l-P in the eluate by adding the appropriate isomerase (Boehringer, also) to the reaction mixture (after the glucose-b-P has been completely converted to 6-phosphogluconic acid) and determining the subsequent OD340 changes.

Extraction and elution of added glucose-b-P indicated 85-90% recovery for the entire procedure. Determinations of the wild-type glucose-6-P level were usually done on approximately 1 gram lyophilized powder (under the conditions stated above), whereas more material and more concentrated extracts were needed for determinations of fructose-b-P.
C. Notes: 1. Lyophilized material can be used only if the culture has been lyophilized immediately after harvesting. Frozen and thawed cultures which were extracted, or lyophilized and then extracted, gave variable results, possibly due to gly-
cogen breakdown. Also aerobic cultures grown to the point of carbon-source exhaustion had lower levels of glucose-6-P.
Therefore, only actively growing cultures can be used for the assay.

2. Extraction by cold 1 M HClO₄ (and subsequent neutralization with cold KOH) gave comparable results to the ethanol
extractions, as did extraction with cold 10% TCA. In both cases the glucose-6-P could be assayed properly only after inhibitors
were removed by chromatography.

3. Extracts are not concentrated to dryness since phosphorylated compounds occasionally adhere to glass surfaces.

4. Almost complete clarification can be obtained by 100,000 x g for 90 minutes.

5. Glucose-6-P in extracts tends to precipitate somewhat, particularly on paper which has not been treated with EDTA.

b. Core must be taken to avoid small bits of paper in the eluate as they subsequently interfere with the assay.

7. Other sources occasionally contained significant amounts of 6-phosphogluconic acid dehydrogenase (thereby doubling all values obtained), as well or traces of isomerasers.

8. It is not known in what form the glucose-6-P is isolated, so a molecular weight of 340 (i.e., Na₂glucose-6-P.2H₂O) was assumed.

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Brody, at temperature-sensitive mutant strains for isolation of additional mutants at a given site.

For purposes of correlation with the results of studies on N. crassa, strain col-2 (Y5331), it was desirable to isolate additional strains altered at col-2 locus and to examine the properties of their glucose-6-P dehydrogenases. However, the isolation of a particular
colonial strain would involve numerous mutant hunts and extensive
mapping and/or biochemical screening since the colonial phenotype can be due to mutation at many different genes (40 at least). The selection and isolation of temperature-sensitive revertants circumvented these problems. The rationale for this approach is as follows: revertants from a presumed point mutation in a structural gene may be due to mutations at the original site, elsewhere in the same gene, or at another locus. Quite often compensatory mutations at another site in the gene, the so-called second-rite revertants, lead to the production of temperature-sensitive proteins. Therefore, some of the temperature-sensitive revertants may be second-site revertants. Approximately 40 wild-type revertants were isolated at 25°C and 4 of these were found to be colonial at 35°C. One of these temperature-sensitive strains was the strain desired; i.e., it had a
temperature-sensitive glucose-6-P dehydrogenase.

Another temperature-sensitive revertant, which proved to be a temperature-sensitive suppressor strain, was helpful in that it was used as a source of conidia for the inositol-less mutant selection technique. Selection of mutants in most colonial
strains is difficult due to the lack of conidiation. However, this difficulty is bypassed by harvesting conidia from this suppressor
strain grown at 25°C and then performing the mutant selection at 35°C. Selection of certain mutants in colonial strains may be advantageous, since the altered metabolism of the colonial strains might not allow the growth of certain "leaky" mutants during inositol deprivation. Rockefeller University, New York, New York. 10021.

Pall, M. L. The use of cycloheximide as an inhibitor of protein synthesis in Neurospora.

The antibiotic cycloheximide (Actidione) has been reported to be an inhibitor of protein synthesis in some fungi, higher animals, and higher plants. It is shown here to be an effective inhibitor of protein synthesis in Neurospora crassa.

Wild-type strain 69-1113a was grown for 2 days at 25°C in 20 ml of Vogel's Medium N + 2% sucrose. Cycloheximide was added and the flasks were shaken on a reciprocal shaker for one hour. They were then given a 12-minute pulse of 0.5 µC 14 C L-lysine. The mycelial pads were fractionated according to the procedure of Roberts, et al. (1955 Carnegie Inst. Washington Publ. 607: 207).

<table>
<thead>
<tr>
<th>Concentration of cycloheximide (µg/ml Vogel's medium)</th>
<th>Percent of counts token up incorporated into protein</th>
<th>Percent inhibition of protein synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>63.3 %</td>
<td>93.0 %</td>
</tr>
<tr>
<td>1</td>
<td>4.44 %</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.11 %</td>
<td>98.2 %</td>
</tr>
</tbody>
</table>

Cycloheximide has also been used to study the inducible enzyme tyrosinase. When ethionine is added during the log period, no activity develops. When it is added during the synthetic period, the activity remains at the level reached at the time of addition of the cycloheximide. These results are consistent with the idea that the tyrosinase is de novo protein synthesis and the cycloheximide inhibits any further synthesis of the enzyme. Division of Biology, California Institute of Technology, Pasadena, California, 91109.