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

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

B. Procedure: Only freshly harvested cultures[1], 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[2] was oddsd ond the mixture gently heated until boiling. Boiling was continued for 5-B minutes, the fluid was then immediately filtered by use of a filter flask, and the extract was concentrated in a vacuum rotary evaporator to opproximotely I-2 ml.3 This opaque and somewhat syrupy solution was chilled and centrifuged at 8,000 x g for 10 minutes, achieving partial clarification.[4] 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 opproximotely 100°C and the residual dry weight measured.

(The level of glucose-6-P in the extract con be measured at this point; however, accuracy and sensitivity ore reduced due to the high level of particulate matter which contributes a large blank at 340 mμ. 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 pre-p-ovation. 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 ond then subjected to ascending chromatography in a n-Butanol: Acetic acid: Water (2:1:1) system for approximately 2 doys. A marker strip of one edge,5 as well as a sample of No2 glucose-6-P, were located by AgN03 staining and the area corresponding to the glucose-b-p eluted in 2-3 ml water.[6] Other areas of the chromatogram were eluted also.

The assay-oceedure: Different aliquots of the eluate were separately mode up to 2.5 ml with 0. 1 M Tris - 0.01 M $MgCl_2$ buffer pH 7.5 , *0.60* PM TPN were added to each tube, and the reaction was started by the addition of 0.2 international units of glucose-b-p dehydrogenose (Boehringer-Manheim was a good source[7]). After 20 minutes or more, the change in $OD_{340}$ 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 $Na_2$-glucose-6-P · $2H_2O$ was run with every series of determinations, and the values obtained were used for subsequent calculations.[8]

This assay procedure can also be used for the detection of any fructose-b-P or glucose-I-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 $OD_{340}$ change.

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 lyophilired 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 con 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 glycogen breakdown. Also aerobic cultures grown to the point of carbon-source exhaustion had lower levels of glucose-6-P. Therefore, only actively growing cultures con be used for the assay.

2. Extraction by cold 1 M $HClO_4$ (and subsequent neutralization with cold KOH) g ave 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 ore not concentrated to dryness since phosphorylated compounds occasionally adhere to glass surfaces.

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

5. Glucose-6-P in extracts tends to trail 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 obtain), as well or traces of isomerases.

8. It is not known in what form the glucose-6-P is isolated, so a molecular weigh+ of 340 (i.e., $Na_2$.glucose-6-P.$2H_2O$) was assumed.

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