Determination of protein in conidia

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Abstract
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Hedman, S. C. Determination of total phosphorus in Neurospora extracts.

Often one desires to ascertain the total phosphorus content of various Neurospora extracts. The following method has been found applicable for a wide variety of such extracts. This method incorporates various features of previously published procedures as well as some new modifications. There are two parts to the procedure: acid hydrolysis and phosphate determination.

Acid hydrolysis: 0.2 ml of extract (containing 1-10 μg of phosphorus) is placed in an acid-cleaned 15 x 150 mm Kimax test tube. 0.3 ml of 5 N Na<sub>2</sub>SO<sub>4</sub> and 0.9 ml of H<sub>2</sub>O are added. The contents are heated over a small burner until dense white fumes of SO<sub>2</sub> are given off. At this point, the contents of the test tube may be dark-brown to black in color. After cooling the mixture, 0.1 ml of 2 N HNO<sub>3</sub> is added and heat is applied until SO<sub>2</sub> is again given off. This HNO<sub>3</sub> treatment is repeated until the contents of the test tube are colorless. The volume is then brought to 1.5 ml by the addition of H<sub>2</sub>O and the tube is heated in a 100°C water bath for 5 minutes to hydrolyze pyrophosphates. An acid-cleaned glass marble is placed over the top of the test tube to prevent excessive evaporation.

Phosphate determination: To 1.5 ml of hydrolyzed extract are added 1.2 ml of phosphate reagent. The phosphate reagent is made as follows: (a) Stock solution: 50 g of ammonium molybdate.4H<sub>2</sub>O are dissolved in 400 ml of 10 N H<sub>2</sub>SO<sub>4</sub> with constant stirring. After all is in solution, the volume is brought to 500 ml with additional 10 N H<sub>2</sub>SO<sub>4</sub>. This stock solution can be stored for several months at room temperature. (b) Preparation of reagent: The phosphate reagent must be made up fresh for each series of assays. To make 20 ml of such reagent, 2.0 ml of stock solution are added to 14 ml of H<sub>2</sub>O containing 1.0 mg of FeSO<sub>4</sub>.7H<sub>2</sub>O. After the ferrous sulfate is in solution, the volume is brought to 20 ml with distilled water.

After five to ten minutes, the absorbance of each tube is read at 710 μm in 1 cm cuvettes of 1.0 cm path length. A reagent blank is used as a reference. Under these conditions, linearity is observed between absorbance and phosphorus content over the range of 1-10 μg. 10 μg of phosphorus routinely gives an optical density of 0.464 ± 0.010.

The phosphate determination by itself can also be utilized to determine the total orthophosphate content as, for example, when assaying for phosphatase activity. The following compounds do not appear to interfere with this method: tris buffer (0.2 M), trichloracetic acid (20% w/v), bovine serum albumin (400 μg/1.5 ml), CHCl<sub>3</sub>, C<sub>2</sub>H<sub>5</sub>OH, Cleland's reagent (10<sup>-4</sup> M), or sucrose (0.3 M).


During experiments in which the rate of uptake of different sugars by conidia of a number of different strains of Neurospora crassa was to be estimated and referred to their protein content, a quick and reliable method for measuring the protein content of ungerminated conidia was needed. For technical reasons, we first tried to measure their protein content directly by applying a modification of the Biuret method as described by Beisenherz et al. (1953 Z. Naturforschung 8b: 555) to whole conidia.

Individual steps of this modification are as follows: (1) Prepare conidial suspensions of 5 x 10<sup>7</sup> conidia/ml. (2) Precipitate the protein in 1 ml of the conidial suspension with 0.2 ml 50% TCA, shake well and spin down. (3) Wash twice with a mixture of 3 parts ethyl alcohol and one part ethyl ether and a third time with ethyl ether to remove carotenoid color and other lipids. (4) Let the ether evaporate from the pellet and resuspend in 1 ml Biuret reagent. (5) Incubate for 30 min. at room temperature on a shaker. Centrifuge and transfer the supernatant into a 1 cm cuvette. (6) Read the absorption at 546 μm. Add a small amount of KCN powder to the cuvette, stir and wait 1-2 min. until the remaining absorption is constant, and read again. The difference in absorption is due to the protein in the solution (A<sub>p</sub>). (7) Measure the absorption of the Biuret reagent alone before and after addition of KCN. The difference in the blank (Ab) to a calibration curve, obtained for bovine serum albumin, is to obtain mg protein/ml.

Applying this procedure and increasing the incubation time at room temperature from 30 min. to 120 min., a marked increase of the resulting absorption was observed (Fig. 1, curve a). This finding was thought to indicate that not all of the protein in a whole conidium was available freely to the Biuret-reagent. This was confirmed by increasing the temperature of incubation up to 50°C, where a further increase in absorption was observed (Fig. 1, curve b). Neither elongation of incubation-time up to 120 min. nor increase of incubation-temperature up to 50°C had a significant effect on absorption of calibration samples containing bovine serum albumin.

In additional experiments, conidia were therefore disrupted either by treating them 1 to 3 times with the X-press (AB Biox, Box 235, Nacka 2, Sweden) with a 25 ml volume operated at -25 to -35°C at ca. 2000 kg/sq cm, or by smashing them with glass beads (d = 0.45-0.50 mm) for 5 to 10 minutes in a homogenizer.

![Fig. 1](image-url) Values for whole or disrupted conidia (5 x 10<sup>7</sup>/ml) after incubation with the reagent for different periods of time.
Buhl, T. E. Buhl, Tübingen, Germany. The results obtained by these disintegration methods document a further increase in protein content of the supernatant (Fig. 1), curve c for 3 times X-pressing, curve d for glass-bead disintegration). They demonstrate that in the broken cells either protein is available faster to the reagent or additional protein is set free to react with the reagent. Visual observation of the state of the cells by means of phase contrast microscopy showed less than 5% intact conidia after glass-bead disintegration, but co. 50-25% after 1-3 X-pressings, respectively. A finding which may explain the fact that the latter procedure gives a lower protein value than the former. To obtain reliable data on the protein content of Neurospora conidia, the complete disruption of the cells is therefore an essential prerequisite.

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In studies of enzymes induced by lactose and by galactose in Neurospora, it has become apparent that growth conditions must be carefully controlled, and that such cultures containing a single carbon source can provide reproducible conditions well-suited for these studies. The ideal carbon source should cause no repressive interference with the induction process. Glycerol is suitable for such studies, but wild type strains are quite variable in their ability to grow on glycerol under the required conditions. The isolate 105-L5-A (formerly designated L5D, Bates and Woodward 1967 Neurospora News 12: 11) shows greatly improved growth on glycerol when compared with STA4. Crosses of this isolate to wild type 74-OR8-1a (Bates 1967 Genetics 56: 543) yielded a variety of isolates with improved glycerol growth characteristics, although initial selection was for lactose growth. Two of there were crossed (211-L5-a x 341-8A) and an isolate designated 41-L5-A was obtained. This isolate has been used for all subsequent glycerol growth studies.

Growth conditions are: rotary shaking, 3/4 inch radius, 150 cycles per minute, 30 ± 0.5°C, 0.18 M glycerol, Vogel's medium, 200 ml in 500 ml Erlemeyer flasks, mounted at a 30 degree angle. The Vogel's medium is autoclaved at 2 x concentration, and the carbon source is autoclaved separately in 100 ml water. The inoculum is 106 conidio per ml medium. Under these conditions growth is linear for 90 hours (yielding ca. 1.5 g dry weight), and comparisons are made by harvesting at 48 hours (yielding ca. 0.7 g dry weight). The rate of growth with glucose under these conditions is ca. 2 times the rate obtained with glycerol.

In comparison with 411-L5-A, taken as 100%, growth of some wild type strains on glycerol can be grouped in the following way: STA4 and RL-A, 33-36%; ST73 a, RL-a and Em-A (FGSC#691), 58-68%; Em-o (FGSC#692), 103% (all based on total mycelial dry weight at 48 hrs). RL-A and RL-a are Rockefeller-Lindgren isolates obtained from J. F. Wilson. It is apparent that a mating type shows better glycerol growth than doer A for all three strains, except for 411-L5-A. When grown on sucrose under otherwise identical conditions, little difference (±10%) in total growth is observed among these different strains. Another distinguishing characteristic is the orange pigmentation which occurs under these glycerol growth conditions in an inverse relationship to ability to grow on glycerol. The Em-o and 411-L5-A cultures show no evidence of this pigmentation.

Among the isolates obtained along with 411-L5-A, there was a marked correlation between ability to grow on glycerol and reduced production of conidia. For example, 411-L5-A producer only 30-50% of the conidia produced by STA4 when grown and harvested under the same conditions. That this characteristic is not necessarily associated with glycerol growth is shown by Em-o, which conidiate more abundantly than STA4, but which grows well on glycerol. Crosses designed to combine the glycerol growth characteristics with amino acid and inositol requirements are now in progress. The 411-L5-A isolate producer abundant protoperithecia on Westergaard's synthetic cross medium, and up to 90% spore viability, but the mature perithece apparently have low internal pressure, and discharge ascospores weakly.

It appears that the 411-L5-A amino acid auxotrophs have very similar glycerol growth characteristics, but these strains have not been completed. Such characteristics would allow very precisely controlled study of incorporation of labeled amino acids during induction studies. If there isolates appear to be potentially useful to other workers, the set of cultures will be deposited in the Fungal Genetics Stock Center. (Supported by NSF Grant GB 5189). I - I Department of Biology, The Univ. of North Carolina at Greensboro, Greensboro, North Carolina 27412.

Sargent, M. L. and D. Braymer.

Selection of intramural-enzyme mutants.

Selection for mutants with altered enzymes in Neurospora is a formidable task if the enzyme is normally located outside the cell membrane, whether within the wall (intranuclear) or outside of it (extracellular). This difficulty is due to cross-fertilization of hydrolytic products from wild-type conidio to mutant conidio. Our efforts to isolate mutants damaged in the carbohydrates, invertase and trehalase, have yielded a combination of techniques that offers some promise for the selection of altered, intramural enzymes.

One assumption is basic to our strategy: "the products of hydrolysis are not completely equilibrated with the external milieu, but may be preferentially taken up by the cell that performs the hydrolysis." (Marzluf and Metzenberg 1967 Arch. Biochem. Biophys. 120:487), i.e., transfer release is cytotoxic. Marzluf and Metzenberg were unable to detect cytotoxic release of glucose from invertase-hydrolyzed sucrase. However, our reconstitution experiments using the one available invertase mutant (spontaneous) demonstrated that wild-type conidio suffered a 50% inositol-less death 10-100 times faster than the mutant conidio in mixed populations. This result coupled with our ability to select invertase and trehalase mutants suggests that the assumption is valid in certain situations.