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## The separation of malic dehydrogenase isoenzymes of *Neurospora crassa* by polyacrylamide gel electrophoresis

### Abstract

The separation of malic dehydrogenase isoenzymes of *Neurospora crassa* by polyacrylamide gel electrophoresis

Laycock, M. V., H. G. Kølmark and D. Boulter. The separation of malic dehydrogenase isoenzymes of Neurospora crassa by polyacrylamide gel electrophoresis.

for enzyme studies with dehydrogenases are a) electrophoresis time is 40 min. as opposed to 8-20 hr. using starch, b) polyacrylamide gels are smaller and require only a small volume of incubation medium which in this work may be expensive due to the requirement for nicotinamide adenine dinucleotide, c) shorter incubation time resulting in sharp, clearly defined bands, and d) ease of incubation anaerobically since several gels will readily fit into a Thunberg tube. Polyacrylamide gel electrophoresis was carried out according to the method of Ornstein and Davis (Ornstein and Davis, Disc Electrophoresis, Preprinted by Distillation Products Industries, Eastman Kodak Co., 1962) with the modification introduced by Fox, Thurman and Boulter (Fox, Thurman and Boulter, Biochem. J., 87, 29, 1963).

Using starch gel electrophoresis Tsao (Tsao, Science 136, 42, 1962) demonstrated that homogenates of Neurospora crassa contain at least four isoenzymes. This communication reports similar results using polyacrylamide gel electrophoresis. The advantages of the latter technique over starch gel electrophoresis

*Neurospora crassa* (74A) was grown in aerated liquid medium (Fries, 2% w/v sucrose) for 90 hr. at 25°. About 1 g. fresh weight of mycelium was washed with deionized water and the surplus water was squeezed out through several layers of cotton gauze. The mycelium was homogenized with, 1 g./ml., large pore acrylamide solution in a mortar and pestle, the slurry was centrifuged at 2,000 x g. for 10 min. and the supernatant fluid was used as the sample. 0.1 ml. of the sample was layered on top of each gel column and electrophoresis carried out at 2° at 4 mA/gel for about 40 min. so that the bromophenol blue tracking dye moved about 3 cm. The gels were removed from the apparatus and some placed in an incubation medium in Thunberg tubes which were evacuated and then placed in an oven at 34° for 60 min. The remaining gels were fixed and stained for protein by 0.7% (w/v) amido black 10 B. in 7% (v/v) acetic acid.

The incubation medium consisted of tris, 600 mg.; malic acid, 50 mg.; nicotinamide adenine dinucleotide, 10 mg.; methylene blue, 10 mg.; neotetrazolium chloride, 10 mg.; made up to 100 ml. of solution and the pH adjusted to 8.4 with concentrated HCl.

Three strong and one weak formazan bands were separated. A thick band which stained with methylene blue but contained no formazan and was not stained with amido black was also present at the front. No bands were formed in the absence of any one of the components or with the complete incubation medium above 50°, below pH 7.0 or in the presence of oxaloacetic acid.

Gels stained with amido black showed about twenty protein bands; the formazan bands on incubated gels matched with four of the protein bands on amido black stained gels, but further work is necessary to substantiate the conclusion that the matching bands are pure malic dehydrogenase isoenzymes.--The Hartley Botanical Laboratories, The University, Liverpool.

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Stanford *Neurospora* Methods.

The methods listed below are extracted from a working notebook in the laboratory of D. Perkins, Department of Biological Sciences, Stanford University. This laboratory has been shared at various times by E. Barry, B. Maling, P. St. Lawrence, N. Murray, W. N. Strickland, D. Newmeyer, B. N. Bole-Gowda, J. Mayo, J. R. Singleton, K. S. Hsu, H. R. Cameron, and C. Ishitani, all of whom have contributed. Methods have been collected from various published and unpublished sources, many of them in other laboratories, and in most cases it would be difficult to trace and assign credit properly for the original source or for modifications. Inasmuch as we ourselves refer constantly to the notebook, we feel that these extracts from it may be sufficiently useful to others, especially in laboratories not already geared to using *Neurospora*, to warrant making them available, even though undocumented or incompletely documented. This note has not been checked with most of the people involved; it was prepared by N. Murray and D. Perkins. If the consensus is that this sort of summary is worthwhile, additional methods may be described in future issues.

1) Standard strains: Two Oak Ridge wild types from F. J. de Serres and H. E. Brockman are used as standards for inbreeding and reference. These are 74-OR23-1A (FGSC No. 986) and 74-OR8-1a (FGSC No. 532). They were derived from St. Lawrence 74A (see NN 2:25), are homozygous for heterokaryon compatibility factors, and are cytologically favorable.

Two fluffy strains (linkage group II) that originated spontaneously in St. Lawrence background are used for sex-tests and for scoring aberrations according to defective spores projected. These strains are highly fertile as protoperithecial parents and develop perithecia rapidly and simultaneously. Their main convenience is that no conidia are produced, so that contamination risks are minimized. The strains are: flA (fl<sup>P</sup>: FGSC No. 295), and fla (P605: FGSC No. 297).

2) Crosses: The synthetic cross medium (SC) is basically that of Westergaard and Mitchell (1947). Biotin concentration has been increased 100-fold (it probably makes no difference (and phosphate is supplied as K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> rather than as the monobasic salt alone. pH is 6.5 without adjustment.

For 1 liter of SC:

KNO <sub>3</sub>	1.0 g
K <sub>2</sub> HPO <sub>4</sub>	0.7 g
KH <sub>2</sub> PO <sub>4</sub>	0.5 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	1.0 g
NaCl	0.1 g
CaCl <sub>2</sub>	0.1 g
biotin	0.5 mg
trace element solution	(see below)

Trace element solution for SC: We have been using metals in the amounts Bo 0.01 mg, Cu 0.1 mg, Fe 0.2 mg, Mo 0.02 mg, and Zn 2.0 mg per liter final medium (see Beadle and Tatum 1945, *Am. J. Botany* 32:678-686). In the future, we propose to use for SC the trace element solution given by Vogel for Medium N, in the amount 0.1 ml TE solution per liter SC. This will provide the metals in approximately the same concentration, but it differs in containing citric acid.

SC is conveniently prepared as a concentrated stock solution (2x). The unautoclaved stock is stored at 5°C, with 2 ml/l chloroform as preservative. Sucrose (2%) and agar (2%) are added before autoclaving. There is normally some cloudiness from precipitation after autoclaving.

All crosses are routinely carried out at 25°C, in 15 cm tubes, or in Petri dishes. Random ascospores are usually not isolated until at least 18 days after fertilization because germination or allele ratios are not good before this time. Ascospores are heatshocked (30 min. 60°C water bath) without further aging after isolation; no hypochlorite is therefore necessary to kill conidia or hyphae. The ripening of ascospores may be speeded by moving crosses from 25° to 30° for a few days, beginning after ascospores have started to be shot from the perithecia.

Cornmeal agar (Difco) may be used for crosses under special circumstances (e.g., when crosses are unsuccessful on SC). It should be noted that Difco lists two cornmeal agars, with glucose (B114), and without (B286). We have used B114.

3) Minimal medium: Medium N is made up as a 50x stock solution as described by H. Vogel [(1956, *Microb. Genet. Bull.* 13:42-43) see section 12 below], with the following added precautions: In preparing the 50x stock: Do not heat. Add components sequentially. Make certain that everything is dissolved before adding next component (conveniently accomplished in a large flask and a shaker or with a mechanical stirrer).

1% sucrose is used as standard carbon source.

Difco now lists three varieties of *Neurospora* minimal (0324-15, 0460-15, 0817-01), as well as a *Neurospora* complete (0321-15; see N. N. 1:13). We have not used these ourselves, but have suggested them for convenience to school teachers or students who inquire about doing *Neurospora* projects.

4) Color coding of media: Media containing various supplements may be readily coded prior to plating or tubing by the use of Schilling food color (McCormick and Co., Inc., Baltimore and San Francisco). This is added to the medium before autoclaving at approximately 0.05 ml dye per 100 ml medium. The dyes are available from grocery stores in red, orange, yellow, green and blue, and come as 4% solutions in distilled water plus propylene glycol. In our experience these are biologically inert, stable and nontoxic. (Their use was introduced here by P. St. L.).

5) Agar substrate for manipulation and isolation: For the benefit of workers in other countries, it should be mentioned that 4% agar is routinely used here for ascospore manipulation and isolation because 4% is near the limit of solubility for relatively pure agars such as Difco. Our small experience with crude agars suggests that much higher concentrations of them are required to produce an equally stiff gel. This may explain the difficulties mentioned by Prakash (NN 3:11).

6) Stock solutions of supplements:

<u>Supplement</u>	<u>Amount per ml stock solution</u>	<u>Stock solution used per 100 ml medium</u>
p-aminobenzoic acid	0.4 mg.	0.5 ml.
choline chloride	2.0 "	1.5 "
inositol	5.0 "	1.0 "
nicotinamide	5.0 "	0.2 "
Ca-pantothenate	1.0 "	1.0 "
pyridoxine HCl	1.0 "	1.0 "
thiamine	1.0 "	1.0 "
L-arginine	40.0 "	1.25 "
L-histidine HCl	25.0 "	2.0 "
DL-homoserine	10.0 "	2.0 "
indole	1.0 "	2.0 "
L-leucine	5.0 "	4.0 "
L-lysine	20.0 "	2.5 "
L-methionine	10.0 "	5.0 "
L-phenylalanine	10.0 "	2.0 "
L-proline	10.0 "	5.0 "
sulfanilamide	3.4 "	1.0 "
L-threonine	5.0 "	2.0 "
L-arginine	20.0 "	2.5 "
+ L-lysine	40.0 "	
L-isoleucine	3.0 "	3.0 "
+ L-valine	7.0 "	

Individual supplements are dissolved in water and stored at 5°C over chloroform. Adenosine, adenine sulphate, adenylic acid, uracil, and tyrosine are not sufficiently soluble to allow the use of concentrated stock solutions. Adenosine is used at 0.5 mg/ml, uracil at 1.0 mg/ml, and tyrosine at 0.4 mg/ml.

As a rough general rule, we use per ml final medium about 0.2-0.5 mg amino acids, 10 micrograms vitamins, and 0.2-0.5 mg purines and pyrimidines. The concentrations provided are in many cases greater than indicated in the literature as necessary to provide maximum growth in flask assays. The higher concentrations are in a number of cases necessary in crossing and germination media in order to obtain optimal ripening and germination of auxotrophic segregants, e.g., in the case of *nic* mutants, good allele ratios are attained only when supplement in crossing medium approaches 40 µg/ml nicotinamide--several times the amount necessary for optimal growth (P. St. L.). Even when wild type is used as protoperithecial parent, and *nic*<sup>-</sup> to fertilize, recovery of *nic*<sup>-</sup> segregants is better when SC is supplemented.

7) Mating-type tests: Pairs of standard Petri plates, each containing about 15 ml SC agar, are inoculated in the center with the fluffy tester strains. Plates are wrapped in paper towels and incubated 7 days at 25° before tests are made.

Plates are marked on the bottom into suitable sectors, and a small inoculum of each culture to be tested is spotted onto a marked sector. Tests are made on both mating types. The plates are re-incubated at 25° and may be scored for the presence of perithecia after 48-72 hours. If spore patterns are to be checked, the lids may be secured with plastic tape, and plates inverted. (See B.R. Smith, NN 1:14, for a similar method using the mutant spray for mating type tests.)

8) Auxanography: Conidial suspensions (e.g., 0.5 - 1.0 ml in a 75 or 100 mm tube) are made in sterile water. (The conidia should be well distributed by shaking the tube or by pipetting.) A visibly turbid conidial suspension is plated in molten, 1.5% agar minimal medium, and thoroughly mixed by swirling the plate. When the medium has solidified, test substances (usually solids) are spotted at marked points (which are indicated on the base of the plate with wax pencil). A flamed microspatula is used. Tests can be read after 12-24 hours incubation at 34°. With some strains, preincubation for a few hours before spotting may give better results.

9) Preservation of stocks by silica gel: The procedure is described in detail by Perkins, 1962, *Canad. J. Microb.* 8:591 (see Ogata, NN 1:13, for variations). 100 mm tubes are convenient; stock numbers are etched on tubes with a carborundum grinding tool ("Handitool", Chicago Tool and Mfg. Co.). Tubes are sealed with parafilm over a cotton plug, and stored at 5° in closed containers.

10) Cleaning of glassware: Alconox and Event are effective, nontoxic detergents. (Some domestic detergents leave a residue which is very toxic to *Neurospora*--at least they did so ten years ago.) For acid cleaning an undiluted mixture is used consisting of 2 liters concentrated sulfuric acid (tech) plus 100 ml concentrated nitric acid.

11) Control of mites: Mite infestations are readily controlled (and prevented) by using a concentrated solution of Gammexane (Lindane; 1,2,3,4,5,6 Hexachlorocyclohexane) in 95% alcohol; this solution is painted on incubators, shelves, racks, containers, desk-tops, etc. where cultures are to be stored (or containers may be dipped in the solution).

12) With the permission of H. J. Vogel, we include here his directions for preparing Medium N, as given in the *Microbial Genetics Bulletin* 13:42-43, 1956.

In 750 ml. distilled water, dissolve successively with stirring at room temperature (Mallinckrodt "analytical reagents" are satisfactory):

Na <sub>3</sub> citrate, 5 1/2 H <sub>2</sub> O	150 grams	
KH <sub>2</sub> PO <sub>4</sub> , anhydrous	250 grams	
NH <sub>4</sub> NO <sub>3</sub> , anhydrous	100 grams	
MgSO <sub>4</sub> , 7 H <sub>2</sub> O	10 grams	
CaCl <sub>2</sub> , 2 H <sub>2</sub> O	5 grams	Add with stirring
Trace Element Solution (see below)	5 ml.	
Biotin Solution (see below)	2.5 ml.	

The resulting total volume is about 1.00 liter. Chloroform (2 ml.) is added as a preservative, and the 50 times strength medium obtained is stored at room temperature. For use, this medium is diluted 50-fold with distilled water. The resulting single-strength medium is designated N; it has a pH of about 5.8. Medium N is supplemented with a suitable carbon source such as sucrose (20 grams per liter), and the thus supplemented medium is sterilized by autoclaving.

The trace element solution (containing citric acid as a solubilizing agent) is made up as follows: In 95 ml. distilled water, dissolve successively with stirring at room temperature:

Citric acid, 1 H <sub>2</sub> O	5.00 grams
ZnSO <sub>4</sub> , 7 H <sub>2</sub> O	5.00 grams
Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> , 6 H <sub>2</sub> O	1.00 gram
CuSO <sub>4</sub> , 5 H <sub>2</sub> O	0.25 gram
MnSO <sub>4</sub> , 1 H <sub>2</sub> O	0.05 gram
H <sub>3</sub> BO <sub>3</sub> , anhydrous	0.05 gram
Na <sub>2</sub> MoO <sub>4</sub> , 2 H <sub>2</sub> O	0.05 gram

The resulting total volume is about 100 ml. Chloroform (1 ml.) is added as a preservative, and the trace element solution is stored at room temperature.

The biotin solution is prepared by dissolving 5.0 mg. biotin (Merck) in 50 ml. distilled water. The solution obtained is dispensed in test tubes and stored in the frozen state.

To prepare "complete medium," supplement Medium N with a carbon source, 0.5% yeast extract, and 0.5% N-Z-Case (Sheffield). To prepare "minimal" and "complete" slants, use 1% sucrose plus 1% glycerol as carbon source, and solidify with 1.5% agar.

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