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Abstract

Neurospora teaching experiments.

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The following are time-tested Neurospora teaching experiments. All of the strains mentioned are, or soon will be, deposited with the Fungal Genetics Stock Center and should not be requested from the authors (NN editor).

Experiment 1. Growth tests on Neurospora mutants of the methionine-threonine pathway.

Problem: to locate the point of blocking in a series of mutants.

Solutions needed:

1. Liquid minimal medium containing sucrose (2%) and biotin (5ug per liter).

2. Same, containing 50 mg L-methionine per liter.

3. Same, containing 150 mg DL-homocysteine thiolactone per liter.

4. Same, containing 200 mg DL- + Allo-cystathionine per liter.

*5.*Same, containing 50 mg L-cysteine-HCl per liter.

6. Same, containing 40 mg Na thiosulfate +.5H20 per liter.

7. Same, containing 50 mg L-threonine per liter.

8. Same, containing 50 mg DL-homoserine per liter.

9. Same, containing 50 mg L-methionine + 50 mg L-threonine per liter.

Strains needed:

Each student pair will be given a set of 8 cultures labelled with isolation numbers. The cultures are listed below.

Mutant #	Locus	FGSC #	Block
80702	cys-2	125	SO_3^{2-}-----/--->$S_2O_3^{2-}$
39816	cys-10	427	$S_2O_3^{2-}$----/-->Cysteine
26104	me-3	112	Cysteine + Homoserine -----/---> Cystathionine
H98	me-2	283	Cystathionine-----/--->

			Serine + Homocysteine
38706	me-1	560	Homocysteine
			-----/---> Methionine
51504	hs	471	Aspartic acid
			-----/---> Homoserine
35423	thr-2	2	O-Phosphohomoserine
			-----/---> Threonine
25a	wild type	353	(any wild type can be used)

Procedure:

Each strain is to be inoculated into each of the media listed and observed for growth (+or-) after 48 and 72 hours of incubation at 25C. Use 20 ml of medium in the culture flasks provided, plug with cotton, and autoclave at 15 lbs for 10 minutes. At the same time, autoclave 8 tubes containing 2 ml of distilled water to be used for making conidial suspensions for inoculation. After the tubes have cooled, make a conidial suspension of each mutant (visibly turbid) and inoculate the flask with a drop of the suspension.

References:

Horowitz 1950 Adv. Genet. 3:33; Adelberg 1955 p.419 In McElroy and Glass (ed.), A symposium on amino acid metabolism. Johns Hopkins Press, Baltimore. The role of thiosulfate in the pathway of cysteine synthesis is somewhat mysterious. See Leinweber and Monty 1965 J. Biol. Chem. 240:782; and Murray 1965 Genetics 52: 801.

Experiment 2. Demonstration of an enzyme deficiency in a Neurospora mutant.

This experiment consists of two parts: Part A is a growth test in which the phenotypic effect of a mutation that abolishes the synthesis of D-amino acid oxidase is demonstrated. Part B is a test for the enzyme in the mutant and in wild type.

Part A. 18 flasks of supplemented minimal medium will be needed. Nine of the flasks are to be inoculated with mutant #38706, blocked between homocysteine and methionine. These flasks are supplemented as indicated below.

Flask	L-methionine	Flask	D-methionine
1	0 ug/ml	6	4 ug/ml
2	4 ug/ml	7	8 ug/ml
3	8 ug/ml	8	16 ug/ml
4	16 ug/ml	9	32 ug/ml
5	32 ug/ml		

The second set of nine flasks is identical with that listed, except that all flasks contain, in addition, 10 ug of inositol per ml. These flasks are to be inoculated with the triple mutant 38706, 89601, oxD(8). This mutant carries, besides the methionine gene, a block in the synthesis of inositol, and a block in the synthesis of the D-amino acid oxidase. (The inositol requirement is irrelevant to this experiment; it is present because the oxD mutation was isolated by the inositolless-death method).

After the flasks have been autoclaved and cooled, inoculate them and incubate at 25C for 72 to 96 hours. Fish out the mycelial mats, press out the excess medium on paper towels, and determine the wet weight of each.

Part B. This experiment will use a wild type strain and mutant oxD (8) which has been freed of the methionine and inositol requirements by crossing. Grow three cultures of each in 20 ml of minimal medium contained in 125 ml Erlenmeyer flasks. Grow the cultures for 5 days at 25C. Decant the medium and wash each mycelial mat individually by adding 20 to 25 ml of water to the flask, swirling, and decanting. Repeat, using phosphate buffer, 0.1 M, pH 7.2. The cultures may be stored in the deep freeze at this point if so desired. Only four of the cultures will be needed for the assay; keep the extra pair as spares.

To one wild type culture and to one mutant culture, add 5 ml of phosphate buffer containing D-methionine at a concentration of 2 mg/ml. To the other two, add buffer without substrate. Incubate the flasks at 37 for one hour with shaking. Filter the solutions through paper and determine the keto-acids by the dinitrophenylhydrazone procedure which follows:

1. Add 1 ml dinitrophenylhydrazine (0.1% in 2N HCl) to 1 ml filtrate and let stand 5 minutes.

2. Add 2 ml absolute ethanol .

3. Add 5 ml 2.5 N NaOH and shake vigorously to mix. Let stand 5 minutes.

4. Read color in the Klett, using the green filter.

Standard curve: In place of filtrate use 0, 10, 20, 30, 40, and 50 ug of sodium pyruvate.

Calculate the amount of a-keto acid produced from D-methionine by the wild type and mutant strains, assuming that pyruvate is twice as chromogenic, gram for gram, as a-ketomethiobutyrate. What reaction is catalyzed by the D-amino acid oxidase? How can you explain the results obtained in Part A?

Reference: Ohnishi, Macleod and Horowitz 1962 J. Biol. Chem. 237: 138.

Experiment 3. Accumulation of imidozole compounds by a histidine-requiring mutant. Feedback control of biosynthesis.

Into a series of 12 numbered 125 ml Erlenmeyer flasks place 20 ml of minimal medium. To each set of 6 flasks add 0, 0.1, 0.3, 0.5, 1.0 and 2.0 mg, respectively, of L-histidine. Plug the flasks with cotton and sterilize for 10 minutes in the autoclave. Inoculate one set with wild type and the other with histidineless C-84 conidia. Incubate at 25 for 4 days.

Place ½ ml of medium from each flask in which growth has occurred into a series of numbered test tubes. At the some time, set up a series of standards consisting of ½ ml minimal medium plus 0, 10, 25, 50 and 75 ug, respectively, of histidine. Determine imidazole derivatives in the solution by means of the Pauly reaction, as modified by Jorpes (1932). The procedure is explained below. Remove the mycelial mat from the flasks analyzed, press out the moisture on paper towels, and determine the wet weight of each. Record the calculated imidozole concentration for each flask and the amount of imidozole accumulated per gram of mycelium.

Determination of histidine (Jorpes, loc. cit.)

Solutions needed:

1. Sample to be tested.

2. A diazonium solution prepared as follows: to 1.5 ml of a solution containing 0.9 g sulfanilic acid and 9 ml conc. HCl in 100 ml are added 1.5 ml of a 5% sodium nitrite solution. Cool on ice for 5 minutes. Then add 6 ml of the nitrite solution with shaking, cool for 6 minutes, and add water to 50 ml. The diazonium solution should be kept cold. It keeps for 24 hours. Best results are obtained with freshly prepared sodium nitrite solution.

3. 1.1% sodium carbonate solution.

Procedure: To ½ ml of solution 1, add 2 ml of solution 2. After 1-3 hours, add 3 ml of solution 3. Read in a Klett-Summerson colorimeter with a blue filter (400-465mu) 4-8 minutes after addition of solution 3.

References:Haas et al. 1952 Genetics 37: 217; Jorpes 1932 Biochem. J. 26:1507; Ames 1955 p. 357 In McElroy and Glass (ed.), Symposium on amino acid metabolism. Johns Hopkins Press, Baltimore.

Discussion: C-84 (his-1) is blocked between imidazole glycerol phosphate (IGP) and imidozole acetol phosphate (IAP) in the pathway of histidine biosynthesis. As the mutant grows, IGP accumulates in the mycelium and soon its dephosphorylated derivative leaks out and is found in the medium. As a control, wild type (25a) is tested and no imidazole compound is found in the medium. Both mutant and wild type take up the histidine originally added.

The phenomenon of feedback control of biosynthesis is demonstrated when it is noted that the amount of imidozole accumulated per gram of mycelium decreases as the amount of added histidine increases. The end product of a pathway has inhibited an early step in its own synthesis.

Experiment 4. Tyrosinase.

The following experiments demonstrate the thermostability and electrophoretic differences between two allelic tyrosinases of Neurospora. The alleles T^{L} and T^{PR} were chosen for this experiment because they are easily distinguished by both tests. If it is desired to omit the electrophoresis experiment and perform only the thermostability test, then the alleles T^{S} and $T^{L}$are recommended.

Induction: Place 20 ml of ½ strength Vogel's medium containing ½% sucrose into each of ten 125 ml Erlenmeyer flasks. Plug with cotton and sterilize for 15 minutes. Inoculate 5 of the flasks with the strain 4-137 T^{L} and 5 with 65-1434 T^{PR}. Incubate the flasks at 25C for 3 days. Induce 3 flasks of each strain with 2 mg sterile D-phenylalanine (2 mg/ml) per flask using aseptic technique. Leave 2 flasks of each uninduced. Incubate the flasks for an additional 24 hours at 25C on the shaker. Tyrosinase is formed during this time in those flasks containing inducer.

Remove the excess medium by pressing the mycelia between paper towels. Weigh the pooled induced T^{PR} mycelia, and extract the tyrosinase by grinding with sand in a cold mortar, using 4 ml of 0.1 M sodium phosphate buffer, pH 6, per gram of moist mycelium. Do the same with the T^{L} mycelium, and with the uninduced controls, yielding a total of four extracts. Keep the extracts in an ice bath during waiting periods. Spin the extracts at 10,000 x g for 10 minutes or at the highest speed of the clinical centrifuge for 10 minutes, and decant the supernatant, containing the enzyme.

Assay: Add 0.02-0.1 ml crude extract to sufficient sodium phosphate buffer (0.1M, pH 6) to give a final volume of 4 ml, and equilibrate the solution in a water bath at 30C. To start the reaction, rapidly add 1 ml DL-dopa (4 mg/ml in buffer). Shake the tube and read it in the Klett, using the blue filter. Shake the tube again and replace it in the water bath. Five minutes after the first reading was taken, make a second reading on the tube. The difference between the two readings is proportional to the tyrosinase concentration. Demonstrate this proportionality by setting up a concentration series, using either one of the two preparations you have just made. Have at least 5 points, including a zero control, in the series. The readings should be linear with concentration up to about 150 Klett units/5 minutes. Above 150-200 units, the curve departs from linearity owing to the fact that oxygen becomes limiting for the reaction.

Thermostability test: Into 4 Klett tubes place the calculated volume of T^{PR} enzyme which would give a reading of 100-150 Klett units in 5 minutes; make up the volume to 0.5 ml with buffer. Do the same with the T^{L} preparation. Cover the tubes with foil. Keep one tube of each set in an ice bath as a zero control. Set the other six in a water bath at 59C. Remove one tube of each extract after 10 minutes and chill immediately. Repeat after 20 and 30 minutes. To the chilled samples, add 3.5 ml buffer to each tube and proceed with the assay as above. Estimate the half lives of the two enzymes. (More points may be taken on the curves if desired.)

Electrophoresis: Make up 1 liter of 0.05 M sodium phosphate buffer, pH 6. Dissolve 1 gm of bovine serum albumin in 100 ml of the buffer, and pour the solution into an enamel tray. Saturate 8 numbered paper strips in the solution, allow them to drain for a few seconds, and place on the holder of the Spinco Paper Electrophoresis Cell. Mix the BSA solution with the remainder of the buffer and pour into the reservoirs of the cell . Set the

strip holder in position, close the cell, and level the buffer in the reservoirs. The enzyme solutions should contain between 1500 and 3000 Klett units per ml for best results in the electrophoresis. Apply 0.01 ml of each to a paper strip with the wire applicator. (If enough cells are available, use a mixture of the two on a third strip.) Replace the tape on the cell cover, and plug in the cell. Turn on the power supply, and set it for constant current at the rate of 1.25 ma per paper strip. Allow to run for from 16 to 24 hours. Turn off the power supply, unplug the cell, remove the cover and the strip holder. Lay the holder, containing the strips, on a flat surface. Spray the strips with a solution containing either 4 mg/ml of DL-dopa or 0.8 mg/ml of epinephrin. Dopachrome (or adrenochrome) will be formed at the position of the enzyme. Use an atomizer that gives a fine spray; do not flood the strips. Measure the distance migrated.

Reference: Horowitz, et al. 1961 Cold Spring Harbor Symp. Quant. Biol. 26:233. Biology Division, California Institute of Technology, Pasadena, California 91109.