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Abstract
Growth, extraction and assay methods for the enzymes ornithine transcarbamylase, aspartate transcarbamylase and carbamyl phosphokinase from Neurospora
Anthrionic acid in pH 8.1 Tris buffer. The assay was conducted in a quartz cuvette containing 1 ml of the standard assay mixture, 0.05 ml of 0.15 M sodium chorismate solution and water and sample to a total volume of 1.5 ml.

A crude extract of tryp-4 A35, grown four days at 25°C in Vogel’s medium supplemented with 20mg/l indole and 20g/l sucrose, was found to produce 0.15 μM anthranilic acid/hr/mg of protein.

Spectrophotometric assay: The change in optical density at 322.2 kcm due to the utilization of chorismic acid and the production of anthranilic acid was followed in a recording spectrophotometer. The assay was conducted in a 1 cm path length quartz cuvette containing 2 ml of the standard assay mixture, 0.05 ml of 10 μM sodium chorismate solution and water and sample to a total volume of 3 ml. The production of 0.1 μM anthranilic acid produces an optical density change of 0.3. The spectrophotometric assay is unsatisfactory for quantitative determinations of activity prior to ammonium sulfate fractionation. Determinations of anthranilic synthetase may be conducted directly on w-dissolved ammonium sulfate precipitates. Ammonium sulfate is non-inhibitory and at this stage of purification the presence of a small amount is desirable to prevent protein precipitation at 34°C and a consequent spurious optical density rise.

The most highly purified samples of anthranilic synthetase produced in this laboratory contain 45 and 105 components. The behavior of anthranilic synthetase on molecular sieves indicates that anthranilic synthetase is the 105 component.

Anthranilic synthetase seems, therefore, to be a single protein composed of tryp-1 and tryp-2 specified subunits. As it becomes available, pure anthranilic synthetase will be tested for indole-3-glycerol phosphate synthetase activity to determine whether or not this activity, also specified by tryp-1, is located on the same active unit. Department of Microbiology, University of Birmingham, Birmingham 15, England.

Davis, R. H. Growth, extraction and assay methods for the enzymes ornithine transcarbamylase, aspartate transcarbamylase and carbamyl phosphokinase from Neurospora, loci for all of them have been identified: erg-12 (OTC), arg-3 (CPK) and pyr-3 (ATC). A number of genetic and metabolic complications are associated with them; the reader is referred to the pertinent literature. The three enzymes behave as though their synthesis is controlled by the end product of the pathway through a negative feedback mechanism. Assays for the enzymes are reported here for the benefit of investigators who may want to monitor activities in specific experiments. An advantage of these enzymes is that acetone-dried pads, a convenient made of dry-weight determination for a well-dispersed culture, may also be used as a source of enzyme. The methods of growth and extraction reported may be applicable to a number of other enzymes in Neurospora. The speed of some procedures may make trials worthwhile in reference to other enzymes.

Growth, harvest and extraction of mycelia.

Method 1: Stationary cultures, infrequently used by the author, or aliquots of well-dispersed shaken cultures (Davis and Harold 1962 N 172: 18) may be harvested with a suction flask and Buchner funnel with Whatman #1 filter paper. The pods are washed with distilled water, the excess removed by filtration, and the moist pad, still in the funnel, may be dried by pouring reagent grade acetone over it so that all water is removed. Acetone drying should be rapid, and acetone should be added before the filter paper lifts from the funnel surface through drying. The pad is then ground in cold (-10°C) acetone in an Omn-Mixer (Serval); this blender is valuable for its spark-free operation and its choice of cup size) and the powder is collected by suction on a dry filter circle seated firmly in a dry Buchner funnel. The powder is spread out to dry briefly in air, collected in a dry tube, and stored in a freezer (it should be tightly stoppered). At room temperature, however, OTC, ATC and CPK are stable for long periods in the acetone powder form.

Extraction of acetone powders in 0.02 M phosphate (K +) pH 7.0 brings all three enzymes into the soluble phase, and OTC and ATC are quite stable at this pH. 4.5 ml buffer are added to 100 mg of acetone powder in a centrifuge tube and the mixture is stirred occasionally over 15 minutes. More extract is accomplished with a glass homogenizer, but well-dispersed powders make this unnecessary as a rule. The supernatant, after centrifugation at 1,000-20,000 x g, generally contains 4-g6 mg protein per ml. This is a convenient concentration for the assays to be described unless the enzymes are at abnormally high or low levels. Only one extraction need be done for consistent specific activity measurements. For large-scale work, a higher powder-to-buffer ratio is used, initially, but the residue after one centrifugation is re-extracted once or twice. According to the specific needs, the extract may or may not be to be dialyzed. If it must be (for CPK it is essential), dialysis overnight in narrow dialysis tubing against 0.02 M K+ phosphate buffer (pH 7.0) is convenient. Extracts to be assayed for CPK should not be frozen prior to assay without being sure it has no effect.

Method 2 (Used by this investigator only for OTC): This method has been used successfully for detailed kinetic analysis of enzyme activity, size of the arginine pool, and isotope incorporation into protein, the latter being the parameters for which the method was developed. Logarithmically-growing cultures of wild types and arginine mutants have been used. While many investigators have used logarithmic cultures, this one is presented as convenient for small-scale work of certain types.

200 ml of medium with 1.5% sucrose, contained in a round flat-bottomed flask (Pyrex #4060), are inoculated to a standard density, e.g., 40 Klett-Summerson colorimeter units (#42 filter), with fresh, filtered, washed conidia. The culture is aerated vigorously with a hydrated air line through a bent glass tube held in a cotton plug and maintained at 25°C in a water bath. It is in many cases unnecessary to autoclave the medium. After 5-8 hours, and until the material becomes clumped, reliable harvests may be made with an ordinary 10 ml pipette with a reasonably large bore. The culture is most conveniently studied during growth from 0.2 to 2.5 mg dry weight per ml, a range covered between 5 and 14 hours after inoculation. A manifold made with 8-10 small, 3-way needle valves in series allows simultaneous growth of as many cultures. Differences in the rate of aeration, above a rather low value, have no effect on growth rate. It has been noted that reliable harvests cannot be made by pipette for the whole growth cycle; that is, until the sucrose is exhausted. This is mainly because clumping of the mycelium makes
aliquots variable in density. While large conidial inocula are used to give well-dispersed cultures early in growth, larger samples may have to be taken in a graduated cylinder on to minimize sample variability. With the wild type stock (74A) we have used, the tendency of hyphae to clump is greater in media containing ammonium nitrate (e.g., Vogel's) than in those containing nitrate as the sole N source (e.g., Horowitz's). However, the doubling time is longer in nitrate minimal (4.2 hr. at 25°C) than in ammonium nitrate minimal (2.8 hr. at 25°C). The choice of medium will in many cases be dependent upon specific experimental needs. If not, however, the facts given above will be helpful in making a choice.

Harvests are made with a Gelman #123 polypropylene 1-inch filter funnel (Gelman instruments, P. O. Box 1448, Ann Arbor, Michigan), which has a capacity of 25 ml. For both dry weight and enzyme, only one harvest need be made. Whatman #54 filter paper is cut to fit the Gelman filter holder; this fast-filtering, hard paper is used because acetone-dried mycelium can be quantitatively and easily removed from it in one piece with a narrow-ended thin spatula. A portion of the culture containing 7-15 mg dry weight is drawn to a moist pad. The funnel is removed from the filter base, and the disc of material on the filter, still on the filter base, is washed with reagent grade acetone from a wash bottle until all water is removed. Aspiration continues throughout this procedure. The dry filter paper with its pad of mycelium is removed with forceps, and the remaining acetone vapors are allowed to come off in air for 3-5 mins. The disc of mycelium is tied loose at the edges and weighed. Highly reproducible dry weights are obtainable in this way, and monitoring of the density of the culture can be accomplished conveniently.

Logarithmic growth, however, makes the density predictable once the doubling time has been established on semilog paper.

Moist pollen of mycelium may be collected on and scraped quantitatively from Gelman membrane filters (GM-6) with a spatula and placed in perchloric acid for extraction of small-molecular weight materials. The precipitate may be analyzed for protein.

For enzyme extraction, the material used for dry-weight determination (no more than 15 mg) is placed in a small (5 ml) gloss homogenizer with 2 ml 0.02 M K+ phosphate buffer (pH 7.0). Brief but thorough grinding brings OTC into solution; the extract is transferred by pipette to a centrifuge tube and centrifuged at low speed; the supernatant is assayed without dialysis for protein (by small-volume biuret test) and OTC. The extracts may be frozen prior to assay. Reasonably consistent specific activities of OTC are observed. The greater part of the variability can be ascribed to incomplete extraction in the homogenizer. This procedure might be adaptable to many enzymes resistant to acetone drying and which are easily soluble.

Assay of enzymes. The product to be measured in all enzyme incubation mixtures is carbamyl compound (citrulline or ureidosuccinate), and a single colorimetric test (Koritz and Cohen 1954 J. Biol. Chem. 209: 145), described later, is appropriate to all of them.

A. OTC (modified from Davis 1962 Arch. Biochem. Biophys. 97: 185):

\[
\text{L-ornithine} + \text{carbamyl phosphate} \xrightarrow{\text{pH 9.1}} \text{L-citrulline} + \text{inorganic phosphate.}
\]

Using a centrifuge tube or a 13 x 100 mm culture tube, combine, in a final volume of 1.65 ml, 10 µmoles L-ornithine-HCl, 5 µmoles dithioll carbomyl phosphate, 250 µmoles tris(hydroxymethyl)aminomethane-HCl buffer, pH 9.1, and extract (0.3-0.5 mg protein for most wild types). It is convenient to add the buffer and ornithine in one volume (1.0 ml), followed by the addition of extract. Reactions are started by the addition of freshly-dissolved carbamyl phosphate and immediate mixing with stirring rod or shaking. (Carbamyl phosphate is unstable in aqueous solution, and dry powder should be stored in a desiccator over P₂O₅.) Substrates and enzyme should be equilibrated to 25°C before use.

After 5 minutes at 25°C, or longer if circumstances demand it and linearity with time can be assured, 0.25 ml 2M HClO₄ is added with mixing and the incubation mixtures centrifuged after a time to sediment protein. 0.1-0.2 ml of the supernatant (for wild type) is then tested for citrulline in the color test below. The appropriate blank is time-zero, obtained by added HClO₄ prior to carbamyl phosphate. This control will take account of colorogenic materials that may be present in aged preparations of carbamyl phosphate (urea) and in the extract. The non-enzymatic reaction rate is negligible. If large amounts of citrulline are formed in experimental tubes, it is important to know whether or not carbamyl phosphate is limiting.


\[
\text{L-aspartate} + \text{carbamyl phosphate} \xrightarrow{\text{pH 9.1}} \text{L-ureidosuccinate} + \text{inorganic phosphate.}
\]

Using a centrifuge tube, combine, in a final volume of 3.25 ml, 40 µmoles L-aspartate (brought to pH 8.8-9.2 with KOH), 10-20 µmoles dithioll carbamyl phosphate, 500 µmoles glycine-NaOH buffer, pH 9.1, and extract (0.5 to 1.0 mg protein for wild type). It is convenient to add aspartate and buffer in a single volume, followed by the extract. The reaction is started, as in the case of OTC, with freshly-dissolved CAP in a volume sufficient to make the reaction mixture up to the prescribed 3.25 ml, followed by immediate mixing. The reaction is allowed to proceed for 15 min. at 25°C. At the end of the reaction time, 0.5 ml 2M HClO₄ is added with mixing. A time zero control should be used. After centrifugation, the supernatant is tested for US by the Koritz-Cohen colorimetric test. If the extract is dialyzed and the carbamyl phosphate reasonably free of urea, 1 ml of the supernatant (in the case of normal enzyme activity) may be used directly for US determination. If this is not the case, interfering colorogenic materials must be removed from the supernatant. This is done by passing 3 ml of the supernatant through a column of Dowex-50W (H⁺ form, 200-400 mesh, 1 x 1.5 cm), followed by 0.2 ml, then 0.1 ml volume of water, 2 ml of eluate (6 ml total), after stirring, may then be tested for US. This volume is equivalent to 1 ml of the original HClO₄-treated mixture. The Dowex treatment removes cationic materials such as urea and citrulline without binding US.

The colorimetric test for carbomyl compounds of Gerhart and Pardee (1962 J. Biol. Chem. 237: 891) is more sensitive, but more laborious method for US determination. If this is used, however, reaction mixtures may be scaled down to the volume used for OTC assays. The method is very useful for more detailed studies of ATC kinetics, particularly when partially purified extracts are used.)

\[
\text{Mg}^{++} + \text{HCO}_3^- + \text{NH}_4^+ + \text{ATP} \xrightarrow{\text{pH 8.2}} \text{CAP} + \text{ADP}.
\]

In this assay, CAP is measured by including OTC and ornithine in excess in reaction mixtures and measuring the citrulline formed at the end of the reaction time. For crude extracts of most strains, it may be unnecessary to add OTC, since it is present in sufficient activity for measurement of CPK. If it must be added, it may be purified from arg-3 strains by the method of Davis (1965 Biochim. Biophys. Acta 107: 44). Alternatively, crude extracts of arg-3 may be used if the OTC has derepressed sufficiently. The arg-3 strain is used because it has no CPK itself.

Using a 13 x 100 mm tube, combine, in a final volume of 0.5 ml, 50 pmole Tris-HCl (pH 8.4), 8 pmole MgCl$_2$, 20 pmole NH$_4$Cl, 10 pmole KHC03, 3 pmole ATP, Na$_2$, 2.5 pmole L-ornithine HCl, excess OTC (IO-20 units) and rate-limiting amount of diazoylated extract (0.5 mg protein for most wild types). As a rule, a substrate mixture is prepared by adding buffer to the NH$_4$Cl, KHC03, MgCl$_2$, and ornithine. (The KHC03 should not be exposed to acid pH.) The ATP is then added and the pH of the mixture adjusted to pH 8.2 (the 18 is usually unnecessary if pH 8.4 Tris buffer is used). This mixture is kept in the cold; just before use, OTC is added. The concentration of ingredients should be such as to contain the requirements of a single reaction mixture in 0.2 ml. The mixture is then distributed to tubes containing experimental extracts (0.3 ml, with water) with a ZOO-lambda micropipette, the tubes shaken, and immediately put in a $37^\circ$C water bath. Incubation continues for 30 minutes, after which the reactions are stopped in the order in which they were started by adding 0.1 ml 2M HClO$_4$ with a 100-lambda micropipette. The tubes are allowed to stand at room temperature or on ice for 5-10 minutes. They are then centrifuged at low speed to remove protein, and 0.4 ml of the supernatant (2/3 of the acidified reaction mixture) is tested for citrulline in the Koritz-Cohen test, scaled down by one-half. Once again, the appropriate control is a time zero blank.


Reagents: 50% (v/v) H$_2$SO$_4$; 3% diacetylmonoxime (2,3-butanedione monoxime, Eastman Organic Chem., Cat. No. 86. (Store in darkness); 0.4% p-diphenylamine sulfonic acid sodium salt (Eastman Organic Chem. Cat. No. 5897. (Store in darkness); 1 N HCl; 1% K$_2$SO$_4$ (Store in cold).

Method: Standard, matched Klett-Summerson colorimeter tubes are used, as the final color determination may be made directly in them. Samples from control and experimental reaction mixtures (ATC and OTC) are made up to 2.0 ml if necessary. A colorimetric blank (water) and a standard (0.1 pmole citrulline or 1.0 pmole US) were used as controls. All further steps should be carried out in the dark, where indicated, and in any case out of direct, bright light.

To each 2 ml sample, 4.5 ml of a mixture made just prior to use (without exposure to bright light) in the proportions of 4.0 ml H$_2$SO$_4$, 0.2 ml diacetylmonoxime, 0.2 ml p-diphenylamine sulfonate, and 0.1 ml HCl is added and stirred thoroughly. This may be accomplished by snapping the tubes, by the use of a Vortex mixer, or by bubbling air through them with a glass tube connected to an air line. (In the latter case, the aeration tube should be rinsed between uses). The tubes are capped with capillary stoppers, marbles, or aluminum caps, such that vapors may escape, but water cannot enter the tube.

The rack of tubes is placed in boiling water in the dark (a roasting pan with a cover is convenient) for 10 minutes. After this time, they are removed and cooled in a pan of tap water for 5 minutes or so, again in the dark.

To each tube, 0.2 ml K$_2$SO$_4$ is added quickly with a 0.2 ml serological pipette aimed at the surface of the fluid in the experimental tube. The tubes are mixed thoroughly and quickly, and the tubes are replaced in boiling water in the dark for exactly one minutes. They are then cooled for 5-10 minutes in the dark, and each tube is read against the reagent blank in a Klett-Summerson colorimeter with a #54 filter (maximum transmission, 540 mp). Tubes should be kept in the dark until they are read, and they should be read in a short period of time. The readings are converted to pmole (per tube) by comparison with the standard. The pmoles of product per reaction mixture may then be calculated (after subtraction of the time zero control values) with the knowledge of the volume used in the color test as a proportion of the volume of the HClO$_4$-treated reaction mixture.

Specific activities are generally expressed as pmoles product per mg protein per hour.

In the case of CPK assays, all ingredients of the color test are reduced by one half, and the readings (and color test, if possible) are done in flat-bottomed Klett tubes having minimum volumes of 2.5-3.0 ml.

Wild type (74A) contains the enzymes in the following range of specific activities if the mycelium is grown according to the shake flask method: OTC 10-20 pmole citrulline/mg protein/hour; ATC 2-4 pmole US/mg protein/hour; CPK 0.1-0.2 pmole citrulline/mg protein/hour. - - - Department of Botany, University of Michigan, Ann Arbor, Michigan.


A. Preparation of extract: The mycelium is pressed dry on filter paper and then ground with a little sand in a cold mortar. Sodium phosphate buffer (0.1 M; pH 6.0; IO parts v/w) is added and the extract centrifuged at 10,000 X g for 10 minutes. The supernatant is decanted and used for the enzyme assays.

B. Incubation: The incubation mixture contains 0.9 ml phosphate buffer (pH 6.0. 0.1M) containing 400 µg casein plus 0.1 ml extract. The casein in buffer is incubated at 35°C for 5 minutes and the extract is added. After further 15 minutes at 35°C, 1 ml of 1M perchloric acid is added to stop the reaction. After 10 minutes at room temperature, the tubes are centrifuged and the supernatant is pipetted off. A blank, which the perchloric acid is added before the extract is run for each extract used.