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Abstract
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The experiment below has been used in an advanced biochemistry course for the past four years with considerable success. Part A normally takes three full laboratory periods, starting on a Friday to allow for growth of cultures over the weekend. Part B was adapted from a similar experiment with E. coli described in Cowgill and Pardee (1957 Experiments in biochemical research technique. Wiley, New York)

Objective:

The experiment provides experience in radioactive tracer methodology, in the use of mutant organisms, and in the isolation and characterization of compounds of biological significance.

Equipment and supplies:

Cultures: N. crassa wild type strain 1A and mutant strain pyr-4 (36601).

Medium: Fries basal medium.

Isotope: 6-^{14}C-orotic acid (New England Nuclear Corporation).

Equipment: Flash evaporator

UV spectrophotometer

Clinical centrifuge

Hood

Autoclave

Incubator-shaker

Planchet-type radiation counter

Fraction collector (optional)

Mineralite UV lamp

Chromatography tank

Procedure:

Part A. Incroporation of 6-^{14}C-orotic acid into ribonucleic acids in Neurospora.

Growth procedure: The mold is grown in 125 ml Erlenmeyer flasks. 1 ml of a stock solution of orotic acid-6-^{14}C containing 2.5 x 10^6 cpm/ml is added directly to 125 ml of Fries medium by means of a pipettor. Unlabelled orotic acid (40 mg) is added separately to the medium, and 12.5 ml volumes are pipetted (plugged pipettes) into each of ten flasks. The flasks are then stoppered with cotton plugs and autoclaved for 20 minutes. After cooling, the flasks are inoculated with 0.2 ml of a conidial suspension of the mold, made by dispersing two loopfuls of the conidia in 10 ml of sterile distilled water. Incubation is at 25C for 3 days, by which time conidiation is just commencing. The contents of the flasks are then filtered on a fritted glass funnel with suction and the mycelial residue is washed with 5 ml of water and then soaked in 100 ml of acetone for 15 minutes. The acetone is removed and the mycelium washed with dry ether and allowed to dry. A brittle disc is thus obtained which is suitable for grinding.

Isolation of ribonucleotides: The mycelium obtained from 10 flasks of the wild strain of the mold grown for 3 days on orotic acid-6-^{14}C is ground in a mortar with 120 mesh carborundum powder for 15 minutes. The resulting powder is extracted 3 times with 10 ml of cold, 10% (w/v) trichloroacetic acid, transferring to a 12-ml Pyrex centrifuge tube in the process. The solid residue is washed once with 4:1 (v/v) ethanol-water and then extracted 3 times with boiling 3:1 (v/v) ethanol-ether. The extractions are carried out by suspending the centrifuge tube in a boiling water bath and stirring the contents occasionally. The process is carried out for a half-hour the first time and for 6 minutes the remaining times. The lipid-extracted residue is then washed twice with ether and air dried. Five ml of 1N KOH is then added, and the mixture allowed to stand at room temperature for 24 hours. After centrifugation, the supernatant is transferred to a second 12-ml centrifuge tube, cooled on ice, and acidified to a pH of 3 with concentrated perchloric acid (pH test paper). The resulting precipitate of potassium perchlorate and protein is allowed to coagulate for 10 minutes and then removed by centrifugation. The resulting supernatant of ribonucleotides and suspended protein is filtered through a layer of Celite on a fritted glass filter and brought to a pH of 11 with 1N KOH. The solution of ribonucleotides is thus obtained and is allowed to filter into a 1 x 27 cm column containing Dowex 1 anion-exchange resin (Cl- form) of 200-400 mesh size and 10% cross-linking. The column is developed first with 200 ml of water and then with 200 ml of 2N HCl. The optical density of the latter eluate is determined at 260 mu, and an approximate molar extinction coefficient of 10,000, together with an average molecular weight of 350, is used to estimate the concentration of mixed nucleotides (ca. 5 mg). The solution is then evaporated in vacuo to dryness several times to remove hydrochloric acid, yielding a greyish-white residue.

Hydrolysis of ribonucleotides: The mixed ribonucleotides are taken up in several ml of 0.1N HCl and transferred to a glass-stoppered, 10-ml volumetric flask. The mixture is then blown to dryness by means of a stream of charcoal-filtered air. After careful addition of 0.5 ml of concentrated perchloric acid, the flask is heated on the steam bath behind an explosion shield for 40 minutes. The contents of the flask are then transferred to a 12-ml centrifuge tube with the aid of several small portions of water, and, after centrifugation and transfer of the supernatant to a second graduated centrifuge tube, the precipitate is washed and the washings added to the tube. The solution is then diluted to 5 ml, to provide a perchloric acid concentration of about 1N, preparatory to separation of the mixed bases.

Isolation of the purine and pyrimidine bases: The solution of bases in 1N perchloric acid is allowed to filter into the resin bed of a 1 x 27 cm Dowex 50 column (hydrogen form) 200-400 mesh. Elution with 100 ml of water, collecting 10-mi fractions, serves to remove uracil mixed with perchloric acid. Cytosine usually comes off in an 80-ml volume after ca. 160 ml of 2N HCl has been passed through the column. Guanine is next eluted by 3N HCl in an approximately 120-ml volume after about 60 ml of the eluant has passed through the column, and adenine is then removed by ca. 140 ml of 4N HCl after a forerun with about 60 ml of the acid. Elution should be followed by means of the Beckman spectrophotometer and the OD at 260 mu and at max plotted against the volume collected.

The uracil-perchloric acid solution is carefully taken to 3 ml volume and adjusted to a pH of 11 with 5N KOH. After removal of the potassium perchlorate precipitate, the solution is placed on a 1 x 10 cm Dowex 1 column in chloride form. After the solution has filtered into the resin bed, 100 ml of 0.015N ammonium formate buffer pH 9/1 is passed through, and uracil is then removed in a 75-ml volume after 75 ml of 0.015N ammonium formate buffer of pH 8.0 has filtered through the column. Half-milliliter samples of the separated bases are plated on stainless steel planchets, evaporated dryness and counted. Determine the specific activity of each purine and pyrimidine base. Editor's note: Some of the above procedures involving the use of perchloric acid are dangerous and should be performed by students only under the supervision of an experienced chemist and with the proper safety equipment.

Part B. Isolation of excreted orotic acid from N. crassa strain pyr-4 (36601).

Into a 2-liter Fernbach flask place 700 ml of a medium consisting of Fries basal medium + 0.5 mg/ml yeast extract + 0.1 mg/ml asparagine + 0.05 mg/ml cytidine hemisulfate. Plug the flask and autoclave and, after cooling, inoculate with a conidial suspension of strain 36601. After 3 days' growth, harvest the mycelium, collecting the residual nutrient medium. Store the mycelium at OC. Dilute 0.5 ml of the medium with 2.5 ml of water and read the optical density at 290 mu: the reading should be about 0.2. Assuming that 50% of this absorption is due to orotic acid ($e290 = 6.2 \times l0^3$; mol. wt. of orotic acid monohydrate = 174), how much orotic acid is in the solution? Concentrate the medium to about 200 ml by flash evaporation. Make the solution 0.1N in KOH. After the solution is chilled overnight, potassium orotate should crystallize out. Separate the precipitate by centrifugation, dissolve most of it in as small a volume of boiling water as possible (less than 10 ml), add a little charcoal if much color is still present, and filter while hot. Bring the hot filtrate to 0.1N with HCl and allow orotic acid to crystallize by cooling the solution to 0C overnight. Filter, wash the crystals with cold water and dry them in vacuo.

Identify the crystals as orotic acid monohydrate by some of the following criteria: a) melting point 323C (decomposes); b) spectra at several pH (5); c) equivalent weight and pK_a, by titration; d) paper chromatography. For paper chromatography place 5 ul of 0.1% orotic acid neutralized with NaOH on Whatman No. 1 paper, develop the descending chromatogram with a solution containing 50 ml of n-butanol, 15.8 ml of 95% ethanol, 11.4 ml of formic acid and 22.8 ml of water. Orotic acid gives a spot with an R_f of 0.43 that can be seen under ultraviolet light. Run a sample of authentic orotic acid for comparison. Samples obtained in Part A may also be run against known compounds in this solvent system.

On the basis of the results of Parts A and B, what can you conclude about the role of orotic acid in pyrimidine biosynthesis ?

References:

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