STUDIES ON COCCIDIOIDES IMMITIS

II. PHYSIOLOGICAL STUDIES ON IN VITRO SPHERULATION

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ABSTRACT

Brooks, Lula D. (Arizona State University, Tempe) and William T. Northey. Studies on Coccidoides immitis. II. Physiological studies on in vitro spherulation. J. Bacteriol. 86:12-15. 1963.—Studies on the amino acids assayed in Edamine revealed that the amino acids phenylalanine, tyrosine, and tryptophan, in addition to the amino acid derivatives dihydroxyphenylalanine (DOPA), epinephrine, tyramine monohydrochloride (tyramine HCl), and pyrocatechol play an important role in the Coccidioides immitis conversion process. In the presence of phenylalanine, tyrosine, tryptophan, DOPA, pyrocatechol, dextrose, and biotin, conversion as well as maturation was demonstrated. However, in epinephrine and tyramine HCl combinations, conversion occurred without maturation of the spherules. The availability of increased amounts of melanin precursors is suggested as a possible explanation for the increased susceptibility of the dark-skinned races to disseminated coccidioidomycosis. Conversion and maturation were also noted when lactic and fumaric acids served as carbon sources. The conversion of a limited number of arthropores to spherules and their subsequent maturation in the presence of only phenol and biotin suggest the possibility that the key to the C. immitis conversion process lies in the availability of certain ring structures.

Previous studies in our laboratory (Northey and Brooks, 1962) indicated that a rapid and complete in vitro conversion of the dimorphic fungus Coccidioides immitis could be accomplished by using a simple and readily prepared medium, the principal component of which is Edamine, an enzymatic digest of lactalbumin (Sheffield Chemical, Norwich, N.Y.). The ease with which the conversion process was facilitated through the use of Edamine prompted a series of investigations designed to elucidate the physiological requirements for the in vitro conversion of the fungus based upon the constituents of Edamine as assayed and reported by Sheffield Chemical. With this information as a basis, further studies were conducted with supplements not found in Edamine. These studies were designed not only to ascertain the requirements for in vitro conversions but were intended, as well, to provide an insight into the mechanisms of the in vivo arthropore-spherule conversion of this organism.

MATERIALS AND METHODS

Arthropores and mycelial fragments. Four strains of C. immitis (Rixford and Gilchrist, 1896) were used in this investigation. These strains included the Silvera strain, obtained through the courtesy of H. B. Levine of the Naval Biological Center, Berkeley, Calif., and three human strains obtained through the courtesy of Gilbert Creceius of the Arizona State Public Health Laboratories. Cultures were maintained on plates of Sabouraud Dextrose Agar (Difco) at room temperature for a period of 4 to 6 weeks. The arthropores were then harvested by washing with phosphate buffer and utilized in a concentration of 5,000 to 10,000 spores per 10 ml of culture medium. Arthropores were inoculated into 10-ml samples contained in 30-ml screw-cap tubes. The cultures were then incubated at 35 C on a reciprocating shaker at a rate of approximately 96 excursions per min.

Chemical supplements. Suplex no. 5 (Vitamin B-complex; Rocky Mountain Pharmacal Co., Phoenix, Ariz.), and biotin and vitamin B12 (Nutritional Biochemical Corp., Cleveland, Ohio) were used.

Base salt solution. The base salt medium used during this investigation contained KH2PO4, 0.15 g; K2HPO4, 0.30 g; and MgSO4, 0.010 g; per 100 ml of deionized water. The salt solution was sterilized by autoclaving at 15 psi.
for 15 min. Carbon and nitrogen sources and other test reagents were prepared and added separately.

Carbohydrate source. Glucose, lactose, and galactose were utilized as carbohydrate sources throughout this investigation; 10% stock solutions of each carbohydrate were prepared and sterilized by filtration. Dilutions of the 10% stock glucose, lactose, and galactose solutions were added aseptically to tubes of the basal medium in a final concentration of 1%.

Amino acids and amino acid derivatives. The amino acids, L-glutamic, DL-aspartic, L-arginine monohydrochloride, DL-threonine, DL-isoleucine, DL-phenylalanine, DL-tryptophan, DL-leucine, DL-methionine, L-tyrosine, and amino acid derivatives pyrocatechol, dihydroxyphenylalanine (DOPA), and tyramine monohydrochloride (tyramine HCl), and the hormone epinephrine, were obtained from the Nutritional Biochemical Corp., Cleveland, Ohio.

A quantity of 40 mg of each of the amino acids was autoclaved separately and added to 100 ml of sterile base salt solution. The amino acid derivatives were weighed aseptically in 40-mg amounts and added to 100 ml of sterile base salt solution.

Organic acids. Stock solutions of citric, lactic, malic, succinic, and fumaric acids, in 10% concentrations, were prepared and sterilized by autoclaving at 15 psi for 15 min. A final concentration of 0.1% of each was utilized throughout this study.

Phenol. A stock solution containing 0.5% phenol (chemically pure) in base salt solution was prepared. A working solution containing 0.05% phenol was used in this study.

Results

Effect of total assayed amino acids in Edamine. To determine whether the collective use of listed amino acids (glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine, arginine, aspartic acid, glutamic acid, proline, histidine, tryptophan, phenylalanine, tyrosine, cystine, and methionine), in a base salt solution and a carbohydrate source, would initiate the in vitro spherulation of C. immitis, 5 mg of each amino acid were added to 100 ml of base salt solution containing 1% glucose; 10-ml amounts of this medium were added to screw-capped tubes containing 5 to 10 thousand arthropores, placed on a reciprocating shaker, incubated at 37 C, and read every 2 hr.

The results of this study were disappointing in that, within 24 hr, the arthropores began to convert to spherules with some sprouting of mycelium, and, by the end of 48 hr, the culture was composed principally of mycelial growth.

Effect of individual assayed amino acids. The effect of the use of the individual assayed amino acids was investigated by adding 40-mg amounts of each amino acid to 100-ml quantities of glucose-base salt medium. The cultures were placed on a reciprocating shaker at 37 C and observed at the end of 24-hr intervals. Within 24 hr, the arthropores contained in the glycine, alanine, valine, leucine, isoleucine, arginine, aspartic acid, glutamic acid, proline, histidine, and methionine media showed no conversion, and, by the end of a 48-hr period, a mycelial mat had formed. However, the arthropores in the phenylalanine, tyrosine, and tryptophan media began to convert to spherules at 48 hr. In these amino acid solutions, the spherules did not mature and a mycelial mat formed within 72 hr.

Effect of B-complex vitamins. With the information obtained from studies on the amino acid requirements for in vitro conversions, the amino acids phenylalanine, tyrosine, and tryptophan (which were shown to be effective in initiating the conversion process, together with certain of their derivatives) were tested with the B-complex vitamins in an attempt to elucidate the mechanisms of the conversion process.

The effect of the addition of B-complex vitamins upon the in vitro spherulation of C. immitis in the presence of phenylalanine, tyrosine, tryptophan, tyramine HCl, epinephrine, pyrocatechol, and DOPA dextrose base salt media, was investigated by using a basal working medium containing as a nitrogen source 40 mg per 100 ml of the above amino acids and amino acid derivatives, 0.1% dextrose, 0.5% Suplex no. 5, 0.1 mg per 100 ml biotin, and vitamin B1₂. Portions (10 ml) containing 5 to 10 thousand arthropores were cultured at 37 C on a reciprocating shaker.

As may be seen in Table 1, only biotin, together with phenylalanine, tyrosine, tryptophan, DOPA, and pyrocatechol dextrose base salt solutions, resulted in maturation of spherules within 72 hr. When epinephrine and tyramine
### TABLE 1. Effect of supplemental vitamins on arthrospore-spherule conversion in various dextrose base salt media

<table>
<thead>
<tr>
<th>Dextrose base salt medium* containing</th>
<th>Vitamin supplement</th>
<th>Immature spherules</th>
<th>Maturing spherules</th>
<th>Mature spherules</th>
<th>Mycelium formation</th>
<th>Mycelium increasing</th>
<th>Mycelial mat formed</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Su-plex #5</td>
<td>Biotin</td>
<td>B1</td>
<td>B2</td>
<td>24 48 72</td>
<td>24 48 72</td>
<td>24 48 72</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>Tyrosine</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Tryptophan</td>
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<td>x</td>
<td>x</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DOPA</td>
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<td>Pyrocatechol</td>
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<td>+</td>
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<td>Tyramine HCl</td>
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<td>+</td>
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<tr>
<td>Epinephrine</td>
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<td>x</td>
<td>x</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

* In all of the media used, arthrospore-spherule conversion was initiated by the end of 24 hr.

### TABLE 2. Effect of organic acids on the spherulation of Coccidioides immitis

<table>
<thead>
<tr>
<th>Organic acid*</th>
<th>Immature spherules</th>
<th>Mycelium formed</th>
<th>Mature spherules</th>
</tr>
</thead>
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<tr>
<td></td>
<td>48 72</td>
<td>48 72</td>
<td>48 72</td>
</tr>
<tr>
<td>Lactic</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fumaric</td>
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</tr>
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</tr>
<tr>
<td>Succinic</td>
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<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* All of the organic acids gave similar results when used with phenylalanine, tryptophan, and tyrosine base salt solutions.

† The arthrospore-spherule conversion was initiated within the first 24-hr period in all of the media.

**FIG. 1. Points in the metabolic pathways of phenylalanine and tyrosine capable of stimulating spherulation.**
HCl dextrose-base salt solutions were supplemented with biotin, immature spherules were formed which did not subsequently mature. In all other B-complex- and vitamin B12-supplemented dextrose-base salt solutions, the cultures formed mycelial mats within 72 hr.

Effect of organic acids as carbon sources. To determine the effect of the use of organic acids as carbon sources upon the in vitro spherulation of C. immitis, sterile 10% stock solutions of lactic, malic, fumaric, succinic, and citric acids were prepared. A working medium composed of 40 mg per 100 ml amino acid and 0.1% organic acid in a base salt solution was used throughout this part of the study; 5 to 10 thousand arthrospores were added to screw-capped tubes containing 10 ml of each medium. The cultures were placed on a reciprocating shaker and incubated at 37 C. Only lactic and fumaric acids resulted in conversion and subsequent maturation (Table 2).

Effect of phenol upon the in vitro spherulation of C. immitis. To determine whether the arthrospores of C. immitis would convert to spherules in the presence of phenol, 10 ml of a 0.05% working solution in base salt medium were added to screw-capped tubes containing biotin and a suspension of 5 to 10 thousand arthrospores. The tubes were placed on a reciprocating shaker at 37 C and read at 12-hr intervals.

While the basal medium containing only phenol, biotin, and base salt solution did not result in complete conversion of the arthrospore suspension, nonetheless this simple medium did result in the conversion of a very small percentage of the culture to mature double-walled spherules containing endospores.

Discussion
Studies on the physiological requirements necessary for the in vitro conversion of C. immitis led to some interesting observations. While conversion and subsequent maturation of the spherule could not be accomplished by using only the simple amino acids assayed in Edamine, use of those amino acids containing a ring structure (phenylalanine, tyrosine, and tryptophan, and certain of their derivatives together with biotin) resulted in conversion and maturation within 72 hr.

It is interesting to note that in vitro conversion and subsequent maturation of the spherule stage was accomplished with a number of melanin precursors and with a number of pyrocatecholamines (Fig. 1).

It has been estimated that coccidioidomycosis is more than ten times as likely to become disseminated in the Negro than in the Caucasian (Fiese, 1958). The availability of increased amounts of the pyrocatecholamines or melanin precursors, or both, could serve to explain the increased susceptibility of the dark-skinned races to disseminated coccidioidomycosis. In this respect, the synthesis of melanin has been reported in Blastocladiella emersonii (Cantino and Horenstein, 1955), an aquatic phycomycete. The failure of homogenetic acid to stimulate conversion in these studies cannot be satisfactorily explained at this time.

The ability of the phenol-base salt solution to result in a minimal amount of conversion indicates that the availability of simple ring structures or phenols, or both, may serve to stimulate the fungus-yeast conversion.

Acknowledgment
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Literature Cited
