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A Possible Role of Hydroxypyruvic Acid Reductase In Growth of *A. niger*. (32398)

FRANCIS J. BEHAL
Department of Microbiology, Medical College of Georgia, Augusta

In a study of growth and differentiation in *Aspergillus niger* it has been previously shown that while the operation of the citric acid cycle is essential to the development of spores, growth of the hyphae is dependent on a metabolic pathway that seems to diverge from glycolysis (Embden-Meyerhof), and not upon the pyruvic dehydrogenation system and the reactions of the tricarboxylic acid cycle(1).

With the culture method described below, this mold completes its growth cycle in 80 hours. The lag phase continues to the 20th hour; logarithmic phase begins then and continues until the 58-60th hour. At this point spore formation begins and continues rapidly. Mold pad weight begins to decline at the 80th hour. In a previous paper it was shown that the 40-52 hour interval was critical for spore formation and that the purine analog, 6-ethylthiopurine, if present in the growth medium during this interval would selectively inhibit subsequent spore formation (at 60th hour) without any effect on growth of hyphae or conidiophores. In this paper attention will be focused on the 20-40th hour interval, that is the period when mycelial growth is dominant, prior to conidiophore (stalk) formation. This latter process predominates during the subsequent 40-60 hour period and is specifically inhibited by 6-hydroxy-2-mercaptopurine(2).

In earlier papers the production of hydroxypyruvic acid by this mold was reported and it was postulated that this acid served as a precursor for a series of reactions necessary for hyphal growth(3,4). The data presented below concern further observations on the relationship between hyphal growth and the metabolism of this acid.

Materials and methods. The medium routinely used had the following composition: glucose, 45.0 g; $NaNO_3$, 4.5 g; KH_2PO_4, 3.0 g; KCl, 0.75 g; $MgSO_4 \cdot 7H_2O$, 0.75 g; $FeSO_4$, 0.02 g; water (distilled) to make 1.0 l.; and H_3PO_4, to adjust the pH to 4.0. The inoculum was prepared by mixing 2 mg of spores in 100 ml of a 2% agar solution at 55°C which was then poured into a 20 × 25 cm baking dish and allowed to solidify. Discs were cut from the agar to cover the entire surface of 1.0 ml medium of the depressions (diameter, 21 mm; volume, 1.2 ml) of porcelain spot plates (12 depressions per plate). In contrast with the classical small inoculum

these inoculating disks provided a relatively heavy and uniform distribution of spores over the surface of the medium, which resulted in a pad of uniform age and, therefore, a uniform state of development over its entire surface. Inoculated spot plate cultures were then placed in an incubator providing rapid circulation of air saturated with moisture at 29°C. Because of the acidity of the medium and the rapid rate of growth, bacterial contamination did not observably interfere. Rate and extent of growth were expressed in terms of dried mold pad weight. *A. niger* cell free extracts were prepared by high speed grinding with powdered glass. Hydroxypyruvic acid (HPA) was synthesized as previously described (4) and was assayed colorimetrically (5) and enzymatically(6). HPA reductase was determined by measuring the rate of reduced nicotinamide adenine dinucleotide (NADH) disappearance in a 1.0 cm silica cuvette containing 0.6 μmoles of NADH and 2.2 μmoles of HPA in 3.0 ml of 0.03 M phosphate buffer at pH 6.6. The unit of HPA reductase activity was defined as that amount of enzyme that causes the disappearance of 1.0 μmole of NADH per minute under these conditions. HPA-alanine transaminase was determined according to the method of Willis and Sallach(7). Carbon-14 was counted with a Tracerlab windowless flow counter.

Results and discussion. It was reported earlier that 3-phosphoglyceric acid (PGA) is converted to HPA by this mold(3), and that HPA, serine, and certain related metabolites would reverse the inhibitory action of iodoacetic acid (IAA) on hyphal growth even in the presence of potent tricarboxylic acid cycle inhibitors. If such a sequence of reactions is critical during the 20-40 hour period, data on the production of HPA reductase during this period would be desirable. Mold pads were analyzed for HPA reductase content at various times beginning at the 20th hour after inoculation. The level of HPA reductase activity (units/mg of tissue) reaches a sharp maximum at the 33rd hour, then declines quickly. Triose phosphate dehydrogenase levels (units/mg of tissue) remained constant. The alanine-HPA-aminotransferase level rose only slightly during this period.

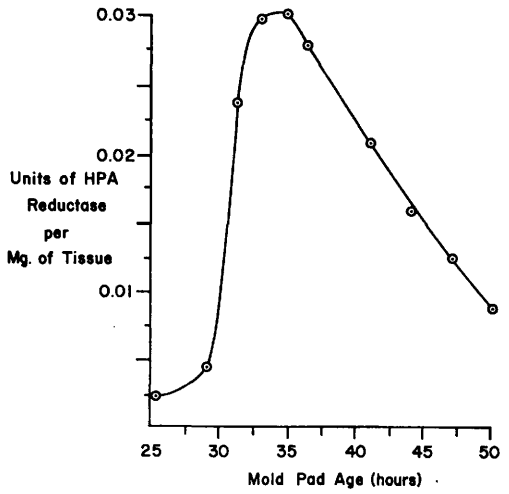

FIG. 1. Hydroxypyruvic acid reductase levels in *A. niger* pads at various time intervals.

In metabolic processes in which HPA is a major intermediate, a number of possibilities exist for conversion of different glucose carbon atoms to CO_2. Since a series of reactions divergent from glycolysis leading to the sequential formation of HPA, serine, formate and glycine, and possibly CO_2 (from formate) was postulated, some preliminary studies were carried out on the metabolic fate of the 1 and 6 carbon atoms of glucose. The above series of reactions would produce CO_2 from the 1 and 6 carbon atoms of glucose. The first experiment involved utilization of glucose 1-C-14 and glucose-6-C-14 as the sole carbon source for growing mold pads through the 80th hour. The culture fluid was chromatographed (paper) at various times and it was found (by autoradiography) that no compounds with C-14 labelling could be demonstrated in the culture medium. This suggested that the bulk of the C-1 and C-6 atoms of glucose either were lost as CO_2 or appeared almost exclusively as cellular material and not as non-volatile fermentation products. Since the latter did not seem probable, a second experiment was done in which the CO_2 evolved from growing mold pads (again with glucose C-1-14 and C-6-14) was trapped in KOH and analyzed for C-14 content.

Each 1.0 ml of medium contained 250

TABLE I. Carbon Dioxide Production by *A. niger* Pads.

	Initial glucose content (per ml)		Glucose metabolized at 40th hr,	Carbon dioxide produced at 40th hr		% Conversion of labelled atom in metabolized glucose to CO_2
	μmoles	c.p.m.	μmoles	μmoles	c.p.m.	
	250		82	155		
1-C-14		4×10^4			9.3×10^3	73
6-C-14		1.2×10^4			2.2×10^3	57

μmoles of glucose. At the 40th hour the average pad (groups of 12) had utilized 82 μmoles of glucose. The CO_2 production at this time reached 155 μmoles per pad or very nearly 2 moles of CO_2 evolved per mole of glucose metabolized. The use of the labelled glucose permitted calculation of the contribution of the glucose C-1 and C-6 atoms to this CO_2 A high percentage of the CO_2 was derived from these atoms, and the conversion (57%) of the 6-C of glucose to CO_2 is consistent with the postulate above that HPA reductase functions in a metabolic branch from glycolysis.

The nature of the proposed metabolic sequence was investigated further by use of additional inhibitor-counteractant studies. The inhibitor was iodoacetic acid (IAA) as in earlier investigations(1). When present in the medium at a concentration of 10 μg/ml growth was completely arrested. Sodium flouride does inhibit sporulation but not growth, even at high concentrations (800 μg/ml); the same action was observed with sodium arsenite(1).

Glyceric acid (GA) was found to be as effective as PGA or HPA in reversing the inhibition of growth by IAA. Thus growth appeared to be dependent on reactions branching from glycolysis at the point of PGA formation. If this branch does indeed involve GA and HPA formation as precursors to other critical compounds required for biosynthesis and growth, it would be expected that some other compounds structurally and metabolically related to GA and HPA might also counteract the inhibition described above. Serine and formate were effective counteractants while glycine, glyoxylate, glycolate, oxalate, gluconate, and 6-phosphogluconate were not. Glycerate, HPA, serine, or formate, at concentrations as high as 10 mg/ml, were not inhibitory in control cultures, and maximum counteraction (of IAA) was observed at concentrations of 4-5 mg/ml.

It would be expected in catabolic processes that the carboxyl carbon of HPA would be converted to CO_2(8). However, the counteraction of IAA inhibition by serine and formate strongly suggests that HPA proceeds through the serine pathway rather than by decarboxylation to yield glycolaldehyde or rearrangement to yield tartronic acid semialdehyde (TAS). There are other mechanisms giving rise to TAS and HPA, such as the glucarate pathway(9). This pathway would explain the conversion of the 1 and 6 carbon atoms of glucose CO_2; however, it does not involve the conversion of HPA or TAS to serine. The sharp peak in production of HPA reductase by this mold and the conversion of PGA to HPA by this mold indicates that HPA is formed as a part of a process directed toward the formation of serine and possibly "one carbon" units, rather than arising from the cleavage of 2-keto-gluconic, glucuronic, or glucaric acids. If the latter were true HPA or TAS would be precursors for pyruvate, glycolaldehyde, and related 2-carbon fragments, none of which counteract IAA. The direct biosynthesis of serine from HPA has been reported(7,10,11).

Thus, it is postulated that certain anabolic reactions utilizing HPA as a precursor play a critical role in hyphal growth of *A. niger*. This involvement of HPA is in contrast to the more common situation where it or TAS is involved in a catabolic reaction sequence.

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Effects of Dietary Ethanol upon Experimental Trypanosomal (*T. cruzi*) Myocarditis.* (32399)

HUGH MILLER† AND WALTER H. ABELMANN (Introduced by M. Finland)
Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital, and Department of Medicine, Harvard Medical School, Boston, Mass.

Ever since Bollinger in 1884(1) attributed chronic cardiac hypertrophy to the excessive intake of beer, the association of primary myocardial disease with chronic alcoholism has led to periodic postulation of an etiologic role of alcohol in the pathogenesis of primary myocardial disease(2-6).

Although acute administration of alcohol had been found to have cardiac effects, both in experimental animals(7,8) and in man(9), we could find no reports of definite heart disease produced experimentally by chronic ingestion of ethanol under controlled conditions when the present studies were being carried out.

Previous attempts in this laboratory to assure chronic intake of ethanol in mice by adding ethanol to drinking water failed because of the animals' failure to drink any but the most dilute solution of ethanol. The success reported by Lieber and associates in feeding rats a liquid diet containing ethanol (10) for other purposes prompted the present investigation, which was designed to study possible effects of chronic ingestion of ethanol on the heart of the healthy mouse, as well as upon acute experimental trypanosomal myocarditis, a model studied previously in this laboratory(11,12).

Material and methods. Animals. Forty-eight weanling, male C3H mice‡ were housed 6 per cage and kept at a temperature of approximately 22°C. They were fed Purina Rat Chow until 21 weeks old and weighing 22 to 28 g. At this time, they were divided into 4 groups (Table II), equal in number and in mean weight (25.1 g), transferred to individual cages and given liquid diets in graduated drinking tubes as the only source of food and water. The mice were inspected daily and weighed weekly.

TABLE I. Composition of Control Diet.

	g/l	% of total cal.
Amino acids	47.35	19
Fats	43.00	42
Sucrose	95.00	38
Salts	10.00	
Choline	.00025	
Methionine	.00150	
Vitamin	.05	
Sod. carrageenate	4.00	

Diets. The diets were adapted from those of Lieber and associates(10). The composition of the control diet, used in Groups 1 and 2, is given in Table I. The ingredients were blended with distilled water to give a final concentration of approximately 1 calorie per ml of diet. The ethanol diet, used in Groups 3 and 4, was identical, except that it contained only

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‡ Jackson Laboratory, Bar Harbor, Maine.