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Mechanism of Fumaric Acid Accumulation in *Rhizopus nigricans*

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ABSTRACT

It is doubtful that the glyoxylate bypass plays a significant role in the accumulation of fumaric acid by fungi, as has been postulated. In high glucose media, which favor fumarate production, isocitrate lyase (threo-D_s isocitrate glyoxylate lyase), which is the key enzyme of the glyoxylate bypass, is strongly repressed. The specific activity of this enzyme remains low as long as glucose is present in the medium, even though fumarate formation proceeds at a high level. In addition, the activity of isocitrate lyase is inhibited by phosphoenolpyruvate, which would be formed from glucose. Alternatively, evidence is presented that bulk accumulation of fumaric acid under aerobic conditions in high glucose media takes place through a C_3 plus C_1 carbon dioxide fixation. CO_2 fixation was measured by the direct incorporation of $NaHC^{14}O_3$ into fumaric acid, and by demonstrating that the specific radioactivity of fumaric acid formed from uniformly labeled C^{14}-glucose was decreased in the presence of nonradioactive carbonate. The extent of decrease in specific radioactivity is in accord with a C_3 plus C_1 CO_2 fixation mechanism.

The accumulation of fumaric acid during the aerobic growth of certain strains of *Rhizopus* species in media of high glucose concentration has been known for a long time. The factors determining fumarate accumulation were clearly established by Foster and Waksman (8, 9) in 1939, and their work has formed the basis of all subsequent studies in this field. These workers pointed out the importance of a high carbon-to-nitrogen ratio and the need for high aeration and a neutralizing agent. They emphasized that glucose was converted to fumarate in quantity only after the available nitrogen supply was exhausted and the carbon needs for cell synthesis were met. A striking effect of zinc ion was noted. Addition of this metal resulted in increased cell synthesis, a more efficient utilization of glucose, and a corresponding decrease in fumarate yield.

However, in spite of this rather clear understanding of environmental factors influencing fumarate accumulation, a completely satisfactory explanation of the metabolic pathway involved has never been advanced. On the basis of isotopic labeling patterns obtained when labeled ethyl alcohol was converted to fumarate by preformed mycelium, Foster et al. (5) postulated that fumarate arose via succinate formed by a Thunberg-Wieland 2-C_2 condensation. The enzymatic basis of such a reaction has never been established, however. With the subsequent discovery of the glyoxylate bypass, Kornberg (10) pointed out that the labeling pattern obtained by Foster et al. was entirely consistent with the operation of this cycle, and postulated that fumarate formation took place via the glyoxylate bypass. These implications were accepted by Foster (4).

This hypothesis has been put to experimental test in this laboratory, and the factors controlling the formation of isocitrate lyase (threo-D_s isocitrate glyoxylate lyase) in fumarate-producing strains of *Rhizopus nigricans* were studied by Wegener and Romano (16). It was found that this enzyme, controlling entrance to the glyoxylate bypass, was strongly repressed by glucose. Yet, fumarate accumulation takes place only in media of high glucose concentration. The present study was undertaken to clarify this discrepancy.

MATERIALS AND METHODS

Organism. R. nigricans 45, a fumaric acid-producing strain described by Foster and Waksman (8), was used.

Growth conditions. The medium consisted of: glucose, 50 g; $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.01 g; $Fe_2(SO_4)_3$, 0.01 g;

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and deionized distilled water to make 1,000 ml. Additions were made to this medium as indicated; zinc, when used, was added as $ZnSO_4 \cdot 7H_2O$ at a final concentration of 5 ppm (1.7×10^{-5} M). The medium was dispensed in 50-ml amounts to 250-ml Erlenmeyer flasks. To promote optimal aeration, flasks were covered with cotton filter discs (Rapid-Flo, Johnson and Johnson Filter Products Div., Chicago, Ill.) instead of the standard cotton plug. After being autoclaved at 10 psi for 30 min, the flasks were inoculated with 1.0 ml of a washed-spore suspension standardized to an optical density of 0.3 at 540 mμ. Incubation was carried out in a New Brunswick Environmental Incubator Shaker at 32 C and 250 rev/min. After 4 hr of incubation to allow spore germination to take place, 1.5 g of sterile $CaCO_3$ was added to all culture flasks.

Chemical determinations. Residual glucose was determined in culture filtrates by the method of Folin and Malmros (3). Fumaric acid was determined gravimetrically as the mercurous salt according to the method of Ölander (14).

Enzyme preparation and assay. Cells were harvested by filtration, washed with distilled water, and suspended in 0.05 M phosphate buffer (*p*H 7.0) to make a thick slurry. This slurry was agitated for 2 min with glass beads (0.2 mm in diameter) in an ice-jacketed Waring Blendor according to the method of Lamanna and Mallette (11). The ruptured-cell suspension was then centrifuged at 14,500 × *g* for 30 min at 4 C; the supernatant fluid was used as the enzyme preparation. Isocitrate lyase assays were carried out at 28 C in a Gilford Recording Spectrophotometer equipped with thermospacers according to the method of Olson (15), whereby the formation of glyoxylate from isocitrate was followed by measuring the rate o formation of glyoxylic semicarbazone at 252 mμ. Protein content of the enzyme preparation was determined by the method of Lowry et al. (12), and specific activity is expressed as micromoles of glyoxylate formed per hour per milligram of protein.

Isotope experiments. Uniformly labeled C^{14}-glucose and $NaHC^{14}O_3$ used in the carbon dioxide fixation experiments were obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Isotopic fumaric acid formed was isolated as follows: 50 ml of culture filtrate was acidified with 2 ml of 12 N HCl and extracted with 50 ml of ether; the ether extract was evaporated to dryness at room temperature; the residue was dissolved in a minimal amount of boiling water, and the fumaric acid was allowed to crystallize on cooling. The fumaric acid crystals were separated by filtration and dried at 65 C. Radioactivity measurements were made with a Nuclear-Chicago gas-flow planchet counter with a window of density not exceeding 150 μg/cm², or with a Packard Liquid Scintillation Spectrometer, as indicated.

RESULTS

Relationship between isocitrate lyase activity and fumarate formation. If the glyoxylate bypass played a significant role in the formation of fumarate, it would be expected that isocitrate lyase, the key enzyme in this pathway, would be present at a high level during active fumarate formation, and that synthesis of the enzyme would precede fumarate accumulation. Therefore, the temporal relationship between glucose utilization, isocitrate lyase formation, and fumarate production was followed carefully, both in the presence and absence of added zinc.

The results obtained in cultures without added zinc are shown in Fig. 1. Fumarate accumulation began after 30 hr and continued to rise through the course of the experiment, eventually reaching a concentration near 20 mg/ml. Glucose was not completely used up during the incubation period, and, as a result, isocitrate lyase specific activity remained at a low repressed level. This activity is much too low to account for the bulk formation of fumarate. Thus, a good yield of fumarate was obtained, even though isocitrate lyase remained at a low level.

The results obtained in cultures with added zinc are shown in Fig. 2. Glucose utilization was much more rapid, and it was exhausted at 44 hr. Fumarate appeared at 24 hr, and then was utilized after the glucose was exhausted. Isocitrate lyase began to be synthesized only when glucose was depleted and after the peak of fumarate formation had been reached, and rose to a high level only when both glucose and fumarate had disappeared. Thus, again, fumarate formation took place before isocitrate lyase synthesis.

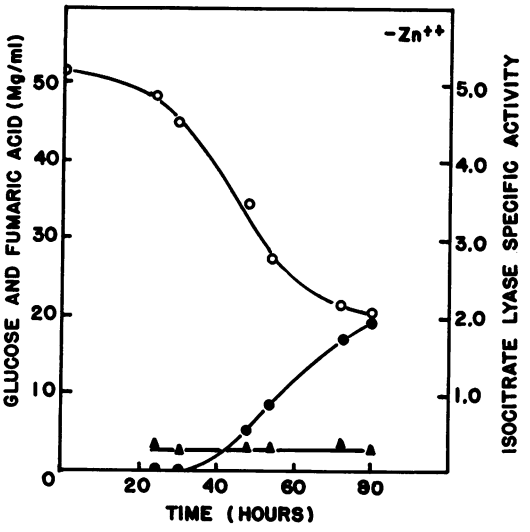

FIG. 1. *Temporal relationship between glucose utilization, fumaric acid formation, and isocitrate lyase synthesis in medium without added zinc. Symbols:* ○, *residual glucose;* ●, *fumaric acid;* ▲, *isocitrate lyase specific activity.*

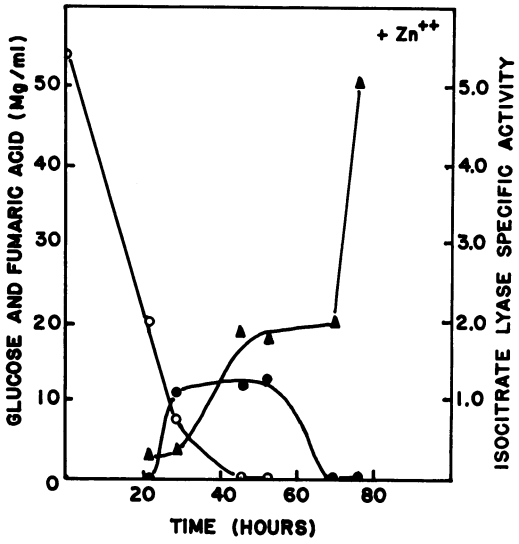

FIG. 2. *Temporal relationship between glucose utilization, fumaric acid formation, and isocitrate lyase synthesis in medium with added zinc. Symbols:* ○, *residual glucose;* ●, *fumaric acid;* ▲, *isocitrate lyase specific activity.*

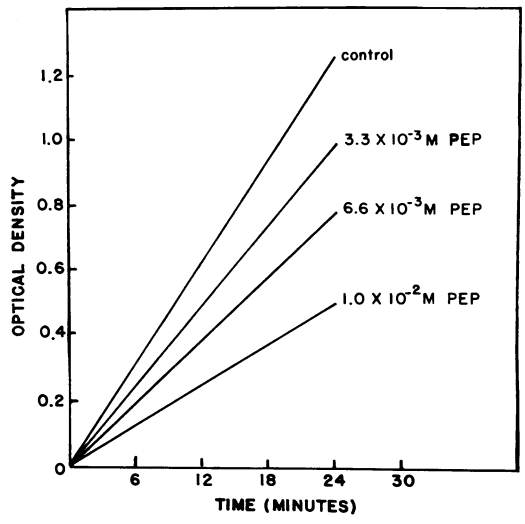

FIG. 3. *Effect of phosphoenolpyruvate (PEP) on activity of isocitrate lyase. Rate of formation of glyoxylic semicarbazone from isocitrate by 0.1 ml of extract of cells grown for 72 hr on medium with added zinc; assay by method of Olson (15).*

Phosphoenolpyruvate inhibition of isocitrate lyase. Ashworth and Kornberg (1) reported that isocitrate lyase of *Escherichia coli* is inhibited noncompetitively by phosphoenolpyruvate, and postulated that phosphoenolpyruvate exerts a fine control of the activity of this enzyme. Figure 3 confirms that phosphoenolpyruvate inhibition also occurs in *R. nigricans*; a tracing of an isocitrate lyase assay in the presence of various concentrations of phosphoenolpyruvate is shown. Thus, not only is the formation of isocitrate lyase repressed by glucose, but the activity of the enzyme is inhibited by phosphoenolpyruvate that would be formed from glucose.

Carbon dioxide fixation. Margulies and Vishniac (13), working with the MX strain of *Rhizopus*, which is principally a lactic acid producer, but which produces some fumarate under aerobic conditions, suggested that fumarate formation takes place via a C_3 plus C_1 carbon dioxide fixation. This possibility has been investigated with two types of experiment: direct incorporation of $NaHC^{14}O_3$ into fumaric acid, and by dilution of C^{14}-glucose carbon by nonradioactive carbonate.

The results of a direct incorporation experiment (Table 1) showed that a significant amount of radioactive bicarbonate was incorporated into the fumaric acid that was isolated, although the tracer had to compete with metabolic CO_2 and excess nonradioactive carbonate that was present as a neutralizing agent. In addition, a significant amount of tracer was lost as CO_2 as a result of acid production. These complicating factors made a quantitative estimate of the amount of carbon in fumaric acid derived from carbonate difficult by the direct incorporation procedure. For these reasons, the isotope dilution procedure was more definitive.

In this latter procedure, 2.5 μc of uniformly labeled C^{14}-glucose was added to each culture before inoculation to adjust the specific activity of glucose to 1 mμc/mg of glucose. Excess nonradioactive calcium carbonate was present during the incubation. If fumarate was formed exclusively by the glyoxylate bypass, the specific activity of the product should be the same as that of the glucose supplied. If, however, CO_2 was fixed, the specific activity of the fumarate formed should be less than that of glucose supplied because of the incorporation of nonradioactive carbon. Moreover, if one of the four carbons of fumarate arose from CO_2, the specific activity in terms of counts per minute per milligram of carbon should be reduced by 25%, and the ratio of specific activity of fumarate to specific activity of glucose should be 0.75. The results of such experiments are shown in Table 2. It is clear that there was a reduction in specific activity, and the degree of reduction is close to the theoretical value. A CO_2 fixation mechanism is thus clearly indicated.

TABLE 1. *Fixation of $NaHC^{14}O_3$ into fumaric acid*[a]

Determination	Result
$NaHC^{14}O_3$ added	10 μc; 4.4×10^6 counts/min
Fumaric acid produced[b]	758 mg
Specific activity of fumaric acid	510 counts per min per mg
Total radioactivity incorporated	3.9×10^5 counts/min
Percentage of total radioactivity incorporated	8.8%

[a] Counted with gas-flow detector with 20% counting efficiency.
[b] Per 50-ml culture.

TABLE 2. *Carbon dioxide fixation measured by decrease in specific radioactivity of fumaric acid formed from uniformly labeled C^{14}-glucose*

Expt	Determination	Specific activity		Decrease in specific activity	Ratio, fumaric acid specific activity/glucose specific activity
		Counts per min per mg	Counts per min per mg of C		
I[a]	Uniformly labeled glucose-C^{14} added	511	1,275	—	—
	Fumaric acid formed				
	48 hr	424	1,034	18.9	.810
	72 hr	423	1,031	19.0	.810
II[b]	Uniformly labeled glucose-C^{14} added	931	2,328	—	—
	Fumaric acid formed				
	48 hr	733	1,788	23.1	.767
	72 hr	737	1,797	22.8	.771

[a] Counted with gas-flow detector.
[b] Counted by liquid scintillation.

DISCUSSION

The data presented here would appear to rule out the glyoxylate bypass as a significant pathway in the bulk accumulation of fumaric acid in media of high sugar concentration. The repressive effect of glucose and the inhibitory effect of phosphoenolpyruvate would preclude extensive operation of the glyoxylate cycle under these conditions.

Alternatively, a C_3 plus C_1 carbon dioxide fixation mechanism is clearly indicated. The importance of CO_2 fixation in molds has long been recognized (6), and Foster and Davis showed that CO_2 was fixed into fumaric acid (7). However, this pathway was considered to be of importance only under anaerobic conditions. The view was favored that, under aerobic conditions, glucose was first metabolized to C_2 fragments, and then two C_2 fragments condensed to form a C_4 dicarboxylic acid which was converted to fumarate (5). It should be pointed out that the experiments leading to this conclusion were carried out with preformed mycelium to which was added ethyl alcohol in the absence of glucose. Under these conditions, the glyoxylate bypass could be operative, since glucose repression would not be manifest, and it has been shown that high levels of isocitrate lyase are induced in *R. nigricans* by ethyl alcohol in replacement cultures (W. S. Wegener and A. H. Romano, Bacteriol. Proc., p. 182, 1961). The significance of the glyoxylate bypass under conditions where a C_2 compound is the sole carbon source is well established. The situation is quite different, however, in media of high glucose concentration.

During active growth in glucose media under aerobic conditions, CO_2 fixation probably plays an important role as a replenishment mechanism by regenerating C_4 tricarboxylic acid cycle intermediates that are drawn off for biosynthesis. When the C to N ratio is high and growth has proceeded to the point that nitrogen becomes limiting, metabolism of glucose and CO_2 fixation would continue, but removal of tricarboxylic acid cycle intermediates for biosynthesis would be curtailed, thus leading to accumulation of C_4 acids. This represents our current hypothesis on the mechanism of fumarate accumulation.

A large number of CO_2 fixation reactions have

now been described in various organisms (17); details of the CO_2 fixation reactions in *R. nigricans* are not yet known. Of the enzyme systems that have been described, phosphoenolpyruvate carboxylase (2) appears to be most logical, because of the irreversibility of the fixation reaction. An investigation of the CO_2 fixation mechanism(s) is now underway.

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