

Evidence for Multiple Forms of Isocitrate Lyase in *Neurospora crassa*

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The effect of carbon source on isocitrate lyase formation was studied in a wild-type strain of *Neurospora crassa* and in a uridine-deficient mutant. A constitutive level of the enzyme was produced in a casein hydrolysate medium. The enzyme was repressed by glucose, although the two strains varied with respect to the degree of glucose repression. Acetate strongly stimulated isocitrate lyase formation. The enzyme formed in the presence of acetate differed in several respects from that formed in glucose-grown cells. Differences were found in *pH*-activity curves, K_m values, and in sensitivity to phosphoenolpyruvate inhibition. Diethylaminoethyl cellulose chromatography allowed separation of two enzymatically active components which showed different rates of heat inactivation. These data indicate the presence of multiple forms of isocitrate lyase in *Neurospora*.

Isocitrate lyase is an enzyme of the glyoxylate bypass, a pathway that has been shown to occur in numerous bacteria, fungi, and higher plants. This pathway is of particular importance in the metabolism of 2-carbon compounds, since it allows a bypass of the decarboxylation steps of the tricarboxylic acid cycle, and allows a mechanism for replenishing C_4 intermediates of the cycle that are drawn off for biosynthesis. Studies on the metabolic significance and mechanism of regulation of the enzymes of this pathway have been reviewed by Kornberg (7) and by Kornberg and Elsdon (8). It can be stated in general that isocitrate lyase is formed under conditions that require the net synthesis of C_4 compounds from acetate, and that acetate has been implicated as an inducer.

In view of the importance of C_2 compounds in the physiology of fungi, the occurrence and control of glyoxylate bypass enzymes in fungi has now been studied in a number of organisms. Thus, investigations on *Aspergillus niger* (2), *Neurospora tetrasperma* and *N. sitophila* (17), and *Rhizopus nigricans* (19) have confirmed that low levels of isocitrate lyase are formed in glucose or sucrose media, and that high levels are induced by acetate. However, in the course of studies on two strains of *Neurospora*, we have noted that isocitrate lyase induced in this organism by acetate has certain properties that differ from the enzyme formed in its absence. These differences form the basis of this report.

MATERIALS AND METHODS

Organisms. *N. crassa* 74A, a wild type described by Webber and Case (18), and *N. crassa* ATCC 11993, a uridine-deficient mutant, were used. These were maintained on Czapek Solution Agar (Difco).

Growth conditions. The basal medium contained: NH_4NO_3 , 1 g; KH_2PO_4 , 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; biotin, 1 μ g; distilled water, 1,000 ml. For growing strain 11993, the uridine-deficient mutant, 0.2 g of uridine per liter was added. When glucose or sodium acetate was used as carbon source, each was added to the basal medium at the concentrations indicated in the experimental section. In the casein hydrolysate medium, NH_4NO_3 was omitted from the basal medium and vitamin-free, salt-free casein hydrolysate was added at a concentration of 10 g per 1,000 ml. Cultures were grown in shake flasks or in a 7-liter New Brunswick Microferm Benchtop Fermentor (New Brunswick Scientific Co., New Brunswick, N.J.), as indicated. For flask cultivation, 50 ml of medium in a 250-ml flask was inoculated with 1 ml of a conidial suspension standardized to an optical density of 0.3 at 550 m μ . Incubation was at 32 C and 250 rev/min on a New Brunswick Environmental Incubator Shaker. For tank cultivation in the fermentor, 100 ml of conidial suspension was used to inoculate 3 liters of medium in a 7-liter tank. Temperature was maintained at 27 C. Aeration was begun at a rate of 3 liters per min for 18 hr, and was then raised to 5 liters per min. Agitation was begun at 250 rev/min for 18 hr, and was then raised to 400 rev/min to minimize clumping of mycelium.

Growth measurement. Cell weight was determined by collecting the mycelium on dried and weighed

Whatman no. 1 filter paper, washing with distilled water, and drying to constant weight.

Chemical and physical determinations. Glucose utilization was measured by determination of residual glucose in culture filtrates according to the method of Folin and Malmros (5). Acetate uptake was determined by measuring the incorporation of ^{14}C -labeled sodium acetate into the mycelium. Counts were made by use of a Nuclear-Chicago gas-flow detector with a window of density not exceeding $150 \mu\text{g}/\text{cm}^2$. Protein determinations of enzyme preparations were made by use of the method of Lowry et al. (11).

Enzyme preparation and assay. Cells were harvested by filtration, washed with distilled water, and suspended in chilled 0.05 M phosphate buffer (pH 7.0) to make a thick slurry. This slurry was agitated for 3 min with glass beads (diameter, 0.2 mm) in an ice-jacketed Waring Blendor according to the method of Lamanna and Mallette (9). The homogenate was centrifuged at $12,300 \times g$ for 15 min at 2 to 5 C. The supernatant fluid represented the crude enzyme preparation.

Isocitrate lyase assays were carried out according to the method of Olson (14), whereby the formation of glyoxylate from isocitrate was followed by measuring the rate of appearance of glyoxylic semicarbazone at $252 \text{ m}\mu$. The reaction mixture contained: 0.03 M cysteine hydrochloride, 0.2 ml; 0.12 M semicarbazide hydrochloride, 0.5 ml; 0.05 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 ml; 0.2 M phosphate buffer (pH varied as indicated), 1.0 ml; 0.074 M NaOH , 0.93 ml; 0.2 M DL-isocitric acid (trisodium salt, Sigma Chemical Co., St. Louis, Mo.), 0.1 ml; enzyme preparation, 0.1 to 0.4 ml; water to make 3.0 ml. In determining Michaelis-Menten constants, concentration of isocitric acid was varied as indicated. Assays were carried out at 28 C with a Gilford recording spectrophotometer equipped with thermospacers. Specific activity of isocitrate lyase is reported as micromoles of glyoxylate formed per hour per milligram of protein.

Chromatography. Partial purification and separation of enzyme activities was carried out by gradient elution diethylaminoethyl (DEAE) cellulose chromatography according to a modification of the procedures of McFadden and Howes (13) and Shiio, Shiio, and McFadden (15). The adsorbent (100 g, Whatman DE-50) was washed successively with 1 liter of 1 N NaOH , 1 liter of 1 N HCl , and five times with distilled water. Fine particles were decanted at each washing step. The adsorbent was then resuspended in 1 liter of 1 N NaOH , filtered on a coarse, sintered-glass funnel, washed with distilled water until free of the base, dried at room temperature, and stored until used for chromatography. It was then suspended in 0.015 M tris(hydroxymethyl)aminomethane (Tris) chloride buffer (pH 6.8) and placed in a column 1 cm in diameter; after settling, the adsorbent formed a column $1 \times 26 \text{ cm}$.

Enzyme samples for chromatography were prepared by homogenizing cell suspensions in 10 ml of 0.015 M Tris chloride buffer (pH 6.8) as described above and centrifuging at $12,300 \times g$ for 15 min at 2 to 5 C. The supernatant fluid was placed on the column, and

elution was carried out at 5 C. Linear gradient elution was accomplished by use of 150 ml of 0.015 M Tris chloride buffer (pH 6.8) in the mixing chamber and 150 ml of 0.33 M Tris chloride buffer (pH 6.8) in the reservoir. An automatic fraction collector (Research Specialties Co., Richmond, Calif.) was used to collect 5-ml fractions.

RESULTS

Effect of carbon source on isocitrate lyase formation. In *N. crassa* 74A, the wild type, there was a pronounced glucose repression of isocitrate lyase formation. This is shown in Fig. 1, where the rates of growth, glucose utilization, and isocitrate lyase formation are plotted. The activity was diluted to a low level during spore germination and initiation of growth; the repression was relieved during the period of rapid growth and glucose utilization. The level ultimately reached is close to that of the spores used as inoculum.

In the presence of acetate as a sole carbon source, isocitrate lyase was induced very rapidly to a high level (Fig. 2). The peak activity was reached after 4 hr, before there was any significant increase in cell weight. This is consistent with the view that, when acetate is present as a sole source of carbon, growth is dependent upon the prior synthesis of glyoxylate bypass enzymes.

There was a difference between wild-type strain 74A and the uridine-deficient mutant 11993 with respect to the degree to which they were repressed by glucose. This is shown in Table 1, where these two strains are compared with respect to enzyme formation on different carbon sources. On casein hydrolysate, the specific activity did not vary over a great range in either organism. This can be considered a constitutive level, since accumulation of neither repressors nor inducers would be expected in this medium. The two strains also responded similarly to acetate, and

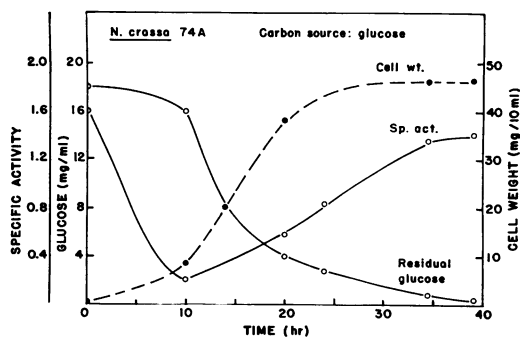


FIG. 1. Relationship among growth, glucose utilization, and isocitrate lyase formation in glucose medium. Tank cultivation; basal medium containing 1.8% glucose.

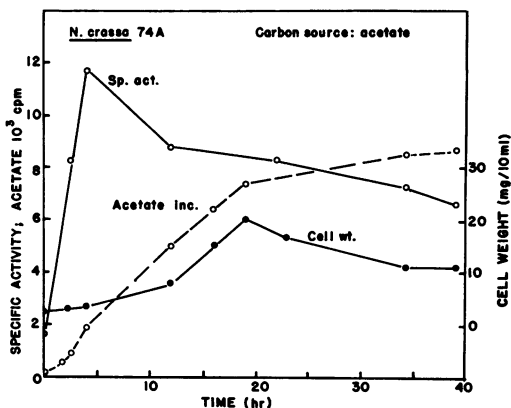


FIG. 2. Relationship among growth, acetate incorporation, and isocitrate lyase formation in acetate medium. Tank cultivation; basal medium containing 0.7% sodium acetate plus 16.6 μC of ^{14}C -labeled sodium acetate per ml. Acetate incorporation was determined on the basis of counts per minute in dried mycelium from a 10-ml sample of culture.

TABLE 1. Effect of carbon source on isocitrate lyase formation

Carbon source	Specific activity ^a				
	0 hr	4 hr	8 hr	24 hr	48 hr
<i>Neurospora crassa</i> 74A					
Casein hydrolysate, 3%	1.6	2.4	2.3	1.7	2.0
Glucose, 1%	1.6	0.9	0.2	0.8	1.8
Acetate, 0.7%	1.6	11.7	10.3	8.1	5.3
<i>N. crassa</i> 11993					
Casein hydrolysate, 3%	1.7	2.6	2.4	1.7	2.6
Glucose, 0.3%	1.7	1.3	1.7	1.7	1.4
Acetate, 0.7%	1.9	14.0	19.2	7.9	9.5

^a Expressed as micromoles of glyoxylate formed per hour per milligram of protein.

rapid induction took place. They responded differently to glucose, however, in that strain 11993 was not as strongly repressed as the wild type. This mutant showed enzyme levels similar to those shown in Table 1 at glucose concentrations up to 2.7%. Thus, it appears that *N. crassa* produces a constitutive level of isocitrate lyase which is inducible by acetate and repressible by glucose to varying degrees, depending upon the strain used.

Influence of pH on activity of enzyme preparations from acetate versus glucose-grown cells. Enzyme preparations from strain 11993 grown on glucose showed a pH-activity curve different from those obtained with acetate-grown cells

(Fig. 3). The acetate-induced enzyme showed a sharp pH optimum at pH 6.8, whereas the enzyme from glucose-grown cells had a broader optimum above pH 7. This was the first indication that the enzymes may be different. Therefore, other parameters of enzyme activity were studied.

Michaelis-Menten constants. K_m values obtained for preparations from glucose-versus acetate-grown cells by the double reciprocal plot method of Lineweaver and Burk (10) are shown in Fig. 4. The values obtained for isocitrate

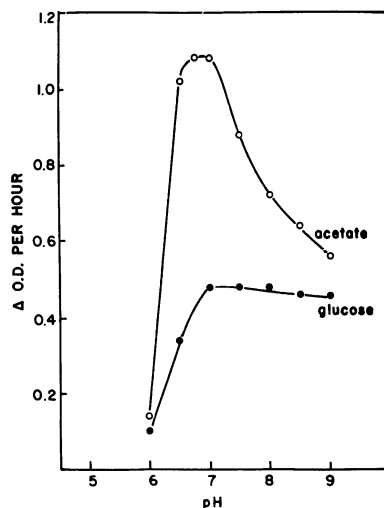


FIG. 3. Effect of pH on isocitrate lyase activity. Strain 11993; ○, grown on basal medium containing 0.7% sodium acetate; ●, grown on basal medium containing 0.3% glucose.

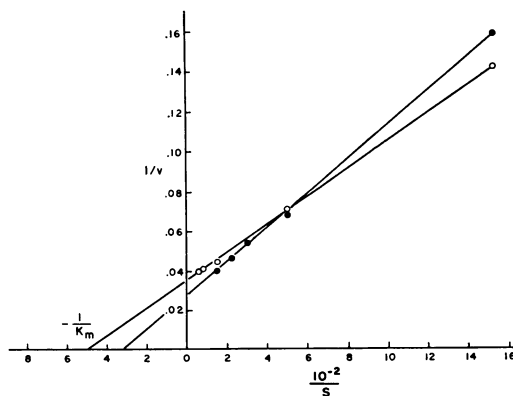


FIG. 4. Lineweaver-Burk plots of effect of substrate concentration on isocitrate lyase activity. Strain 11993; ○, grown on basal medium containing 0.7% sodium acetate; ●, grown on basal medium containing 0.3% glucose.

lyase from glucose- and acetate-grown cells were 3.3 and 1.85×10^{-3} M isocitrate, respectively. Each of these values was reproducible within 5% by use of five different enzyme preparations of each. In addition, partially purified preparations obtained by ammonium sulfate precipitation (fraction between 30 and 45% of saturation) gave values which deviated from the above by 3%. Thus, although the difference between K_m values of the enzymes formed in the presence of acetate and glucose is not great, it is reproducible and significant.

Phosphoenolpyruvate inhibition. Ashworth and Kornberg (1) have shown that isocitrate lyase obtained from *Escherichia coli* is noncompetitively inhibited by phosphoenol pyruvate, and they suggested that this inhibition exerts a fine control in the operation of the glyoxylate cycle. Therefore, the effect of phosphoenolpyruvate on isocitrate lyase of *N. crassa* was investigated. The results obtained with the enzyme from glucose-grown *N. crassa* 11993 are shown in Fig. 5, revealing a pattern of noncompetitive inhibition. The enzyme from acetate-grown cells showed a similar pattern of inhibition (Fig. 6). However, the enzyme from glucose-grown cells was more strongly inhibited. Inhibition data comparing the two enzyme preparations are summarized in Table 2.

DEAE cellulose chromatography. Enzyme preparations from *N. crassa* 11993 grown on casein hydrolysate, glucose synthetic medium, and acetate synthetic medium were subjected to DEAE cellulose chromatography (Fig. 7). All preparations showed two distinct peaks, one at tube 16, and one at tube 24. Significantly, only the peak at tube 16 was greatly enhanced by the presence of acetate in the growth medium. There-

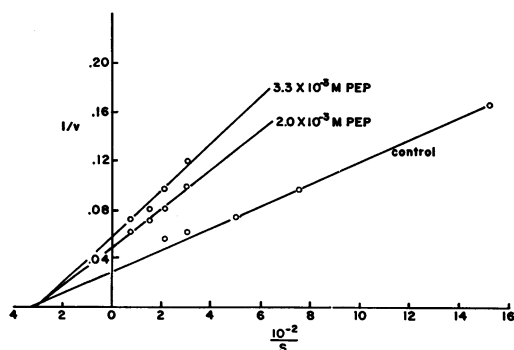


FIG. 5. Lineweaver-Burk plot of effect of substrate concentration on isocitrate lyase activity in presence of phosphoenolpyruvate. Strain 11993 grown on basal medium containing 0.3% glucose.

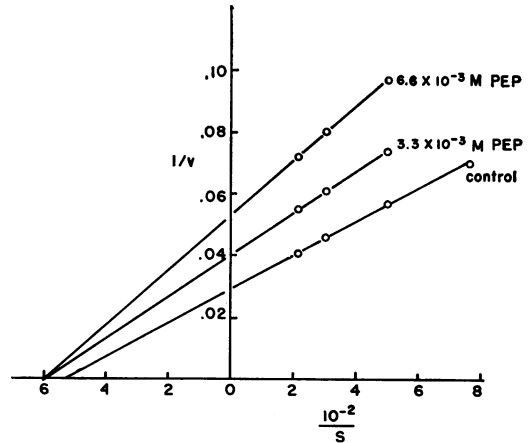


FIG. 6. Lineweaver-Burk plot of effect of substrate concentration on isocitrate lyase activity in presence of phosphoenolpyruvate. Strain 11993 grown on basal medium containing 0.7% sodium acetate.

TABLE 2. Phosphoenolpyruvate (PEP) inhibition of isocitrate lyase of *Neurospora crassa* 11993

Determination	Glucose-grown cells	Acetate-grown cells
K_i at		
2.0×10^{-3} M PEP	3.2×10^{-3} M	—
3.3×10^{-3} M PEP	3.4×10^{-3} M	8.6×10^{-3} M
6.6×10^{-3} M PEP	—	8.2×10^{-3} M
Per cent decrease in activity at 3.3×10^{-3} M PEP.....	49.2	25.7

fore, this peak represents the acetate-induced enzyme.

Thermal inactivation kinetics. Further evidence for the distinct nature of the enzymes separated by chromatography was provided by determination of the rates of heat inactivation. Samples from tubes 15 and 16 and tubes 23 and 24 were pooled and subjected to heat inactivation at 53 C (Fig. 8). It is evident that fractions 15 and 16, whether obtained from acetate- or casein hydrolysate-grown cells, were more sensitive than were fractions 23 and 24. Fractions 15 and 16 showed a half-life of 0.6 min at 53 C, whereas fractions 23 and 24 showed a half-life of 4.1 min.

DISCUSSION

Differences in isocitrate lyase formed in glucose- and acetate-grown cells have been found with respect to pH optima, Michaelis-Menten constants, and degree of phosphoenolpyruvate inhibition. In addition, separation of two activities was achieved by DEAE cellulose chroma-

tography, only one of which was significantly increased by growth on acetate. The enzyme components separated by chromatography showed different rates of heat inactivation. These

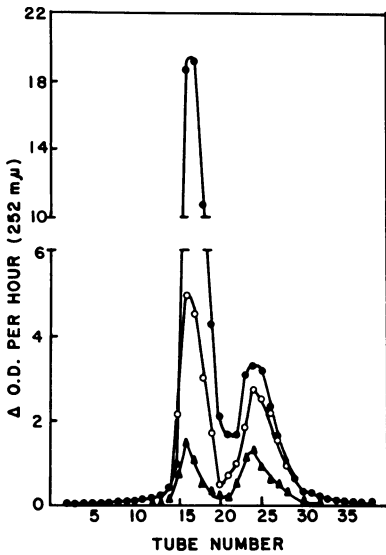


FIG. 7. DEAE cellulose chromatography of enzyme preparations; strain 11993. Symbols: ○, cells grown on casein hydrolysate; ▲, cells grown on glucose medium; ●, cells grown on acetate medium. Enzyme activity was based on change in optical density per hour; a 0.4-ml sample of fraction was used.

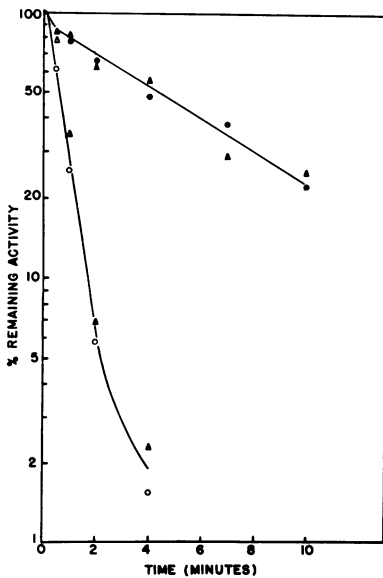


FIG. 8 Thermal inactivation at 53°C. Symbols: ○, fractions 15 and 16 from acetate-grown cells; ▲, fractions 15 and 16 from casein hydrolysate-grown cells; ●, fractions 23 and 24 from acetate-grown cells; ▲, fractions 23 and 24 from casein hydrolysate-grown cells.

data suggest the presence of isocitrate lyase isozymes.

Flavell (4) isolated a number of different *N. crassa* glyoxylate cycle mutants which fell into six complementation groups. It is significant that one of these groups, which could not grow on acetate or be induced by acetate, still produced a low level of isocitrate lyase characteristic of that obtained when the organism was grown on sucrose. The possibility of an impairment in acetate uptake was indicated. However, an alternative explanation might be that the enzymes are distinct and controlled by different gene loci.

It is also interesting to note that the companion enzyme of the glyoxylate cycle, malate synthase, has been shown to be isozymic in *E. coli*. Falmagne, Vanderwinkel, and Wiame (3) have shown that malate synthase induced by glycolate is distinct and separable from the enzyme induced by acetate. Presumably, malate synthase induced by acetate is active in the glyoxylate cycle, acting in concert with isocitrate lyase, whereas the malate synthase induced by glycolate performs a respiratory function by taking part in a dicarboxylic acid cycle that allows the operation of the glycerate pathway (6). Thus, the two enzymes participate in different pathways.

The question thus arises regarding the physiological significance of isocitrate lyase formed in a glucose medium, in which operation of the glyoxylate cycle is not essential. No completely satisfactory explanation can be made at present, although Turian (16) found an active alanine-glyoxylate transaminase in *Neurospora* and *Allomyces* and has suggested that isocitrate lyase may thus play a role in the biosynthesis of glycine. In addition, McCurdy and Cantino (12) have shown that isocitrate lyase and a glycine-alanine transaminase are formed concomitantly in synchronously developing resistant sporangial plants of *Blastocladiella emersonii*, and have ascribed a morphogenetic role to isocitrate lyase. The question of physiological significance in *Neurospora* is being studied further.

ACKNOWLEDGMENT

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