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CONTROL OF ISOCITRATASE FORMATION IN *RHIZOPUS NIGRICANS*

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ABSTRACT

WEGENER, WARNER S. (University of Cincinnati, Cincinnati, Ohio), AND ANTONIO H. ROMANO. Control of isocitratase formation in *Rhizopus nigricans*. J. Bacteriol. 87:156-161. 1964.—A fumaric acid-producing strain of *Rhizopus nigricans* was found to produce a fair level of isocitratase in a casein hydrolysate medium. Glucose repressed enzyme formation. When glucose was utilized during growth, there was a relief of repression, and enzyme synthesis was resumed at a rate equivalent to that found in nonrepressed cells. Zinc stimulated isocitratase formation in glucose-repressed cultures by stimulating growth and glucose utilization, thereby decreasing accumulation of repressor metabolites derived from glucose. The effectiveness of acetate as an inducer was greater on glucose-repressed cells than on nonrepressed cells; cells grown in the presence of glucose formed higher levels of isocitratase when subsequently replaced with an acetate-containing inductive medium than did cells grown without glucose. Moreover, addition of 2 ppm of Zn^{++} during the inductive replacement phase resulted in a twofold increase in isocitratase formation. The hypothesis is submitted that Zn^{++} exerts its action by stimulating ribonucleic acid (RNA) synthesis, thereby facilitating the formation of a specific RNA during induction. Preliminary evidence implicating Zn^{++} in the stimulation of RNA synthesis in this organism is presented.

Isocitratase (D_s-isocitrate glyoxylate lyase) is an enzyme of the glyoxylate bypass, a pathway that has been shown to be of particular significance in the growth of bacteria on two-carbon compounds (Kornberg and Krebs, 1957; Reeves and Ajl, 1961). Studies on the regulation of isocitratase formation and activity in bacteria were reviewed by Kornberg and Elsden (1961); it can be stated in general that isocitratase is formed chiefly under conditions requiring the net synthesis of C_4 compounds from acetate. Kornberg, Gotto, and Lund (1958) found that extremely low levels of the enzyme were formed by *Pseudomonas ovalis* when grown on glucose or succinate; when this organism was transferred to an acetate medium, rapid synthesis of isocitratase preceded growth. In *Micrococcus denitrificans*, succinate has been implicated both as a repressor of enzyme formation and as an inhibitor of enzyme activity (Kornberg, Collins, and Bigley, 1960). Howes and McFadden (1962) found that isocitratase formation was suppressed by glucose in *Pseudomonas indigofera*; these latter workers suggested that an in vivo regulatory role of succinate was unlikely in this organism, since the intracellular concentration of succinate did not vary significantly, even when succinate was added to an ethanol medium.

Kornberg (1959) postulated that the glyoxylate bypass may play an important role in the formation of C_4 acids by fungi. He pointed out that the labeling pattern obtained during formation of fumaric acid from ethanol by *Rhizopus nigricans* (Foster et al., 1949), previously interpreted in terms of a Thunberg-Wieland condensation, was entirely consistent with the operation of the glyoxylate cycle. Foster (1958) accepted these implications and emphasized the probable importance of this pathway in the physiology of fungi. Olson (1954) reported the presence of isocitratase in *Aspergillus niger* and *R. nigricans*; Collins and Kornberg (1960) showed that the enzyme was formed in *A. niger* when grown on acetate, and that the required synthesis of tricarboxylic acid cycle intermediates took place via the glyoxylate cycle. In view of these considerations, and of the relative paucity of information on the regulation of enzyme formation in filamentous fungi, a study of the control of glyoxylate bypass enzymes in fungi was undertaken.

MATERIALS AND METHODS

Organism. R. nigricans 45, a fumaric acid-producing strain described by Foster and Waksman (1939*a*) was used.

Growth conditions. The basal medium consisted of: vitamin-free, salt-free casein hydrolysate (acid-hydrolyzed; Nutritional Biochemicals Corp., Cleveland, Ohio), 5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $Fe_2(SO_4)_3$, 0.01 g; and deionized distilled water to make 1,000 ml. Additions were made to this medium as indicated; zinc, when used, was added as the sulfate, and acetate was added as the sodium salt. 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. Incubation was carried out on a New Brunswick rotary shaker at 250 rev/min in a chamber maintained at 32 C. The media were inoculated with 2.5 ml of a washed-spore suspension standardized to an optical density of 0.3 at 540 mμ.

In replacement experiments, cells were washed with sterile, deionized, distilled water and replaced with 50 ml of the basal medium containing appropriate additions.

Growth measurement. Cell weight was determined by filtering the mycelial mass on dried and weighed Whatman no. 1 filter paper and drying to constant weight at 95 C.

Chemical determinations. Residual glucose was determined by the method of Folin and Malmros (1929), nucleic acids by the method of Ogur and Rosen (1950), and protein by the procedure of Lowry et al. (1951).

Enzyme preparation and assay. Cells were harvested by filtration, washed with distilled water, and suspended in 0.05 M phosphate buffer (pH 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 (1954). The ruptured-cell suspension was then centrifuged at 14,500 × *g* for 30 min at 2 to 5 C; the supernatant fluid was used as the enzyme preparation. Isocitratase assays were carried out with a Beckman DU spectrophotometer equipped with thermospacers at 28 C according to the method of Olson (1959), whereby the formation of glyoxylate from isocitrate was followed by measuring the rate of formation of glyoxylic semicarbazone at 252 mμ. Protein content of the enzyme preparations was determined, and specific activity is expressed as micromoles of glyoxylate formed per hour per milligram of protein.

RESULTS

Formation of isocitratase in casein hydrolysate medium. It was reported previously (Wegener and Romano, 1961) that significant levels of isocitratase were formed by *R. nigricans* in glucose—$(NH_4)_2SO_4$—mineral salts media that favored fumaric acid formation. It was of interest to determine whether the enzyme formed under these conditions represented a constitutive level, or whether it resulted from induction by acetate that may have been formed from the metabolism of glucose. To make a choice between these possibilities, the organism was grown in a casein hydrolysate medium where no accumulation of acetate would be expected. From the results shown in Fig. 1, where the rate of isocitratase formation is compared with growth, it is clear that the enzyme was formed without addition of exogenous inducer and under conditions that would not be expected to produce a significant accumulation of acetate. The rate of formation under these conditions did not become maximal until active growth had begun, and the highest level of enzyme was not attained until the culture approached the stationary phase. This indicates that isocitratase does not play an essential role in the formation of cellular components during growth in casein hydrolysate.

Effect of glucose. Addition of glucose to the casein hydrolysate medium caused a repression

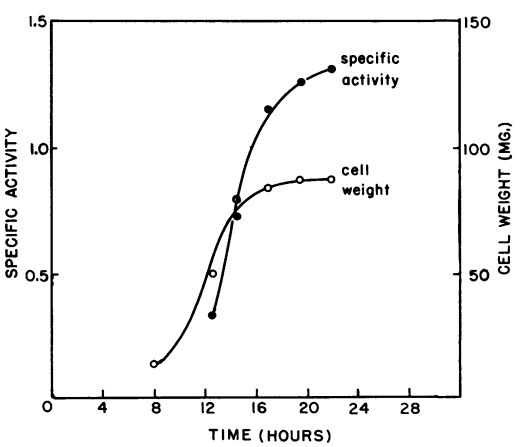

FIG. 1. *Relationship between growth and isocitratase formation. Basal medium containing 1% casein hydrolysate.*

TABLE 1. *Effect of glucose on isocitratase formation*

Glucose in growth medium*	Isocitratase specific activity
M	
0	1.40
0.05	0.76
0.07	0.20
0.10	0.14

* Incubation time: 26 hr.

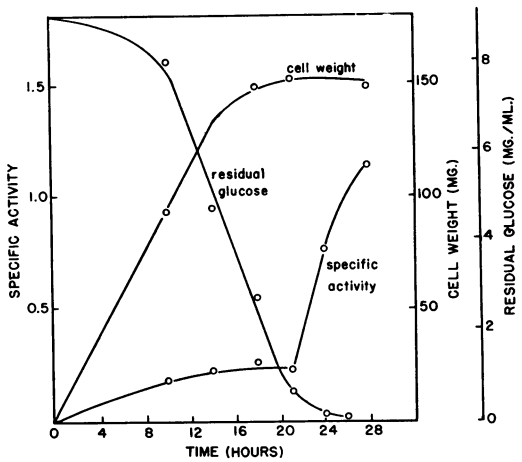

FIG. 2. *Relationship between growth, glucose utilization, and isocitratase formation. Medium: basal plus 0.05 M glucose.*

TABLE 2. *Relief of glucose repression by zinc*

Additions to basal medium*	Isocitratase specific activity	Cell weight	Glucose utilized
		mg	%
None	1.38	94	—
Zn^{++} (2 ppm)	1.44	96	—
Glucose (0.075 M)	0.18	235	66
Glucose (0.075 M) plus Zn^{++} (2 ppm)	1.28	319	98

* Incubation time: 27 hr.

of isocitratase formation in proportion to the concentration of glucose added (Table 1). If the glucose concentration was low enough so that it could be completely utilized during growth, disappearance of glucose resulted in a relief from repression; this is shown in Fig. 2, where rates of growth, glucose utilization, and isocitratase formation are compared in a medium originally containing 0.05 M glucose. It is clear that isocitratase formation was delayed until the glucose concentration fell to a low level. After glucose depletion, isocitratase synthesis proceeded at a rate comparable with that observed in a nonrepressed system (Fig. 1). The maximal enzyme level attained after relief from glucose repression was also comparable with that attained in control cultures grown on casein hydrolysate without glucose. Therefore, the phenomenon of derepression, where there is a greatly accelerated rate of enzyme synthesis after utilization of repressor, was not observed. In many such experiments, the rate of enzyme formation during the period of most active synthesis was fairly constant (Δ specific activity per hr = 0.18 to 0.20); the maximal specific activity after 24 hr of incubation was consistently 1.3 to 1.4. This level of enzyme, obtained without addition of inducer, could be considered a constitutive level.

To rule out the possibility that the observed lower specific activities measured in glucose-repressed cells were caused by accumulation of inhibitors of enzyme activity during growth, heated extracts of glucose-grown cells were added to enzyme preparations from nonrepressed cells in the assay system. There was no diminution in activity under these conditions. Moreover, the admixture of fresh preparations from repressed and nonrepressed cells resulted in an activity equal to the sum of the two individual activities. This would confirm the hypothesis that glucose or a glucose catabolite, in fact, brought about repression of enzyme formation.

Effect of zinc ion. The striking effect of Zn^{++} on the growth and physiological behavior of fungi has been recognized for a long time. Foster and Waksman (1939*b*) showed that the addition of the metal to glucose-containing media provokes an increase in cell synthesis, a more efficient utilization of glucose, and a corresponding decrease in fumaric acid yield in the strain of *R. nigricans* studied here. Therefore, the effect of Zn^{++} on isocitratase formation was investigated. Addition of 2 ppm of Zn^{++} to the casein hydrolysate medium had no significant effect on enzyme formation (Table 2). However, addition of Zn^{++} to a culture containing 0.075 M glucose brought about a relief of the repression exerted by glucose. Moreover, the metal provoked a large increase in cell material synthesized and glucose utilized. Thus, it would appear that Zn^{++} relieves glucose repression by effecting the removal of catabolite repressor(s) derived from glucose through the stimulated incorporation of

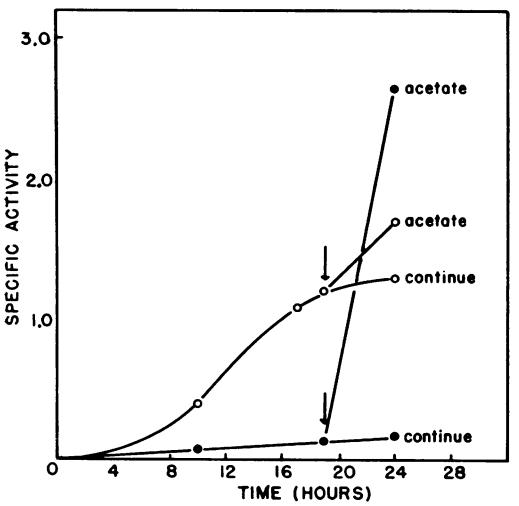

FIG. 3. *Induction of isocitratase by acetate. Symbols:* ○ = *cells grown on basal medium;* ● = *cells grown on basal medium plus 0.1* M *glucose; both replaced with basal medium plus 0.1* M *Na acetate at time indicated by arrow.*

intermediary metabolites into cell material. This effect was found to be specific for Zn^{++}; it could not be duplicated by the addition of Ca^{++}, Mn^{++}, Co^{++}, Cd^{++}, or Cu^{++}.

Induction by acetate. The effect of added acetate was studied as follows. Cells were allowed to grow for 19 hr in casein hydrolysate with or without added glucose; the cells were then washed, replaced with fresh medium containing 0.1 M Na acetate, and incubated for 5 hr more. In this way, the effect of inducer on both nonrepressed and repressed cells could be determined. The results were striking, in that addition of acetate to repressed cells resulted in a much greater degree of induction than was effected by addition of acetate to nonrepressed cells (Fig. 3). The rate of isocitratase formation upon addition of acetate to cells grown in the presence of 0.1 M glucose was five times the rate exhibited by nonrepressed cells. Hence, the degree of induction of isocitratase by acetate appears to be correlated with the degree of repression existing in the system. Greater degrees of repression achieved by increasing concentrations of glucose responded to acetate by greater magnitudes of induction (Table 3).

Effect of Zn^{++} on induction. Induction of isocitratase by acetate was found to be enhanced considerably by addition of 2 ppm of Zn^{++} (Fig. 4). Repressed cells grown in the presence of 0.1 M glucose were washed and replaced with casein hydrolysate containing various additions. While fresh casein hydrolysate alone caused some relief of repression, the presence of Zn^{++} increased enzyme formation twofold; this effect is probably related to the accelerated removal of repressor by Zn^{++}. Acetate induced enzyme formation, increasing the specific activity fourfold over that produced by casein hydrolysate. When, however, the cells were replaced with acetate and Zn^{++}, there was an eightfold stimulation over that

TABLE 3. *Acetate induction of glucose-repressed cells*

Glucose in growth medium	Isocitratase specific activity	
	First growth phase (19 hr)	Replacement inductive phase* (5 hr)
M		
0	1.10	1.70
0.015	0.72	2.00
0.030	0.40	2.30
0.060	0.23	2.50
0.100	0.13	2.65

* Na acetate (0.1 M) added to basal medium.

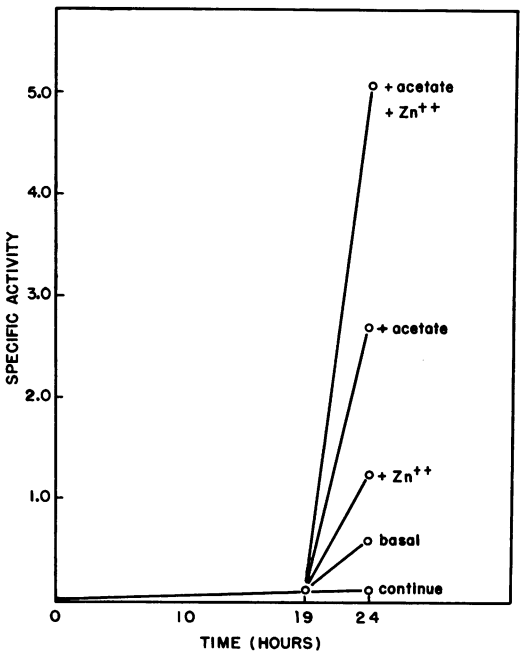

FIG. 4. *Effect of zinc on acetate induction of isocitratase. Cells grown on basal medium plus 0.1* M *glucose; replaced with basal medium containing 0.1* M *Na acetate or 2 ppm of Zn^{++} as indicated.*

TABLE 4. *Effect of acetate and zinc on isocitratase formation*

Additions to basal medium*	Isocitratase specific activity
None	1.48
Zn^{++} (2 ppm)	1.61
Na acetate (0.1 M)	3.76
Na acetate (0.1 M) plus Zn^{++} (2 ppm)	6.51

* Incubation time: 26 hr.

TABLE 5. *Effect of zinc on nucleic acid content*

Additions to basal medium	RNA	DNA
	%	%
Primary growth phase (19 hr)		
Glucose (0.1 M)	6.0	0.51
Replacement phase (5 hr)		
None	7.5	0.54
Na acetate (0.1 M)	7.2	0.56
Zn^{++} (2 ppm)	11.1	0.55
Na acetate (0.1 M) plus Zn^{++} (2 ppm)	10.9	0.56

brought about by casein hydrolysate alone. The inducing effect of acetate was doubled by the presence of Zn^{++}.

This observation may be explainable in terms of a combined effect of Zn^{++} and acetate, whereby Zn^{++} stimulates repressor removal and acetate acts as an inducer. However, Zn^{++} also stimulated the inducing effect of acetate in a nonrepressed medium (Table 4). This would indicate that Zn^{++} plays another role. There is preliminary evidence that Zn^{++} exerts a primary effect by stimulating ribonucleic acid (RNA) synthesis. Results of nucleic acid analysis of cells grown in a casein hydrolysate-glucose medium and replaced with casein hydrolysate with various additions are shown in Table 5. There was an increased RNA content in cultures that were replaced with Zn^{++}, while deoxyribonucleic acid (DNA) content remained fairly constant. These findings are consistent with the concept that induction involves the synthesis of a specific RNA (Pardee and Prestidge, 1961); an increased overall rate of RNA synthesis occasioned by Zn^{++}, then, might facilitate the production of specific RNA during induction. This aspect is being investigated further.

DISCUSSION

R. nigricans forms a fair level of isocitratase when growing in casein hydrolysate; this level may be looked upon as a constitutive level, which may be repressed or induced under appropriate conditions. The phenomenon of catabolite repression (Magasanik, 1961) is manifest in the presence of glucose, and is presumed to result from a repressor pool derived from the metabolism of glucose. When, through the exhaustion of glucose, repressor concentration is lowered beyond a critical level, enzyme formation proceeds at a rate comparable to the maximal rate exhibited in a nonrepressed system. The maximal levels attained without repression and after relief of glucose repression are also equivalent.

Relief of glucose repression by Zn^{++} probably comes about by the stimulation of conversion of the intermediary pool of metabolites into cell material, since increased growth and glucose utilization were measured in the presence of Zn^{++}. The possibility that this metal specifically effects isocitratase synthesis is unlikely since it did not stimulate enzyme formation appreciably in the absence of glucose or an inducer.

It is difficult to advance an adequate explanation for the observation that acetate induced much higher levels of isocitratase in glucose-repressed cells than in nonrepressed cells. The situation may be somewhat analogous to the control of β-glucosidase synthesis in a hybrid yeast studied by MacQuillan and Halvorson (1962); they found that the rate of enzyme synthesis during the inductive phase was much greater after glucose repression than that found for direct induction. These workers postulated that two sites of control of β-glucosidase are present in yeast.

The stimulated induction of isocitratase in the presence of acetate and Zn^{++} may be viewed as an accelerated removal of repressor, both by the metal and by inducer. However, the fact that Zn^{++} is able to stimulate induction in a nonrepressed system as well as a repressed system makes it necessary to assign to this metal a function apart from removal of repressor. Any proposal must account for the fact that in the nonrepressed system, in the absence of inducer, Zn^{++} stimulated enzyme formation only to a minimal degree, while, in the presence of acetate, the inductive process is stimulated twofold. These aspects may be explained by considering Zn^{++} as a stimulator of RNA synthesis. There is evidence that induced enzyme synthesis requires the formation of a specific RNA (Pardee and Prestidge, 1961); therefore, any process that

will stimulate RNA synthesis during the period of induction would be expected to facilitate the induction process. In the absence of inducer, Zn^{++} may also stimulate RNA synthesis, but such a stimulation would be nonspecific in terms of the enzyme studied. Preliminary evidence that Zn^{++} does stimulate RNA synthesis in *R. nigricans* is presented here. These findings are supported by reports of the involvement of Zn^{++} in RNA synthesis in *Nocardia opaca* (Webley, Duff, and Anderson, 1962), and in *Euglena gracilis* (Wacker, 1962). Studies on the effect of Zn^{++} on RNA synthesis are being continued; fungi present a favorable system for studying this phenomenon, in view of the ease with which physiological effects of this metal are accentuated.

ACKNOWLEDGMENT

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