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GLYOXYLATE METABOLISM IN GROWTH AND SPORULATION OF *BACILLUS CEREUS*[1]

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ABSTRACT

MEGRAW, ROBERT E. (Iowa State University, Ames), AND RUSSELL J. BEERS. Glyoxylate metabolism in growth and sporulation of *Bacillus cereus*. J. Bacteriol. **87**:1087-1093. 1964.—Isocitrate lyase and malate synthetase were found in cell-free extracts of *Bacillus cereus* T. The patterns of synthesis of enzymes of the glyoxylic acid cycle were dependent upon the medium in which the organism was grown. Cells grown in acetate or in an acetate precursor, such as glucose, produced enzymes of the glyoxylic acid cycle in greatly diminished quantities, as compared with cells grown in media containing glutamate or yeast extract as principal carbon sources. Glutamate-grown cells had high isocitrate lyase activity but very low malate synthetase activity. Glyoxylate produced in this situation is metabolized by alternate pathways: conversion to tartronic semialdehyde and the latter to glyceric acid, thus providing evidence for a glycerate pathway; and reduction to glycolate (the reverse of this reaction was present at a low rate). Enzymatic activity of the glyoxylic acid cycle declines at the point where sporogenesis begins, indicating a metabolic shift for the synthesis of spore material.

Nakata and Halvorson (1960) demonstrated that, prior to sporulation of *Bacillus cereus* T in a glucose medium, terminal respiration is inhibited, leading to the accumulation of acetate in relatively large amounts. Subsequently, upon the onset of sporulation, the acetate is utilized. This phenomenon suggested the induction of a glyoxylate cycle (Halvorson, 1961) to dissimilate the acetate, but attempts to demonstrate unequivocally the presence of such a cycle were unsuccessful (Gollakota and Halvorson, 1963).

This report will show that the glyoxylate cycle is demonstrable under the conditions employed. The patterns of synthesis of isocitrate lyase and malate synthetase in cells of various presporulation ages and grown in various media are described. Evidence is presented here of alternate pathways for the metabolism of glyoxylate in vegetative cells, via either the glyoxylate cycle (Kornberg and Madsen, 1957) or the glycerate cycle (Kornberg, 1961), depending upon the nature of the growth substrate.

MATERIALS AND METHODS

Organism and culture medium. B. cereus T was grown in a medium containing either monosodium glutamate, potassium acetate, or glucose (filter-sterilized), 0.1%; yeast extract (Difco), 0.2%; ammonium sulfate, 0.2% (0.1% in glutamate medium); and a mineral salts solution prepared according to Nakata and Halvorson (1960).

Inocula were 10^5 viable spores per ml diluted in sterile deionized water and heat-shocked for 30 min at 80 C. The stock spore suspension was grown in a 0.5% glutamate medium as described above, giving essentially complete sporulation. Spores (20 liters) were harvested in a Sharples centrifuge, washed three times in deionized water, and resuspended in 0.05 M potassium phosphate buffer (pH 7.2). The stock spore suspension contained 3×10^8 heat-viable spores per ml. The spore suspension was stored at 0 C.

Growth curves were determined with a Bausch and Lomb Spectronic-20 colorimeter against a culture medium blank at 650 mμ.

Cell extracts. Cell extracts were prepared in tris(hydroxymethyl)aminomethane (tris) buffer (0.05 M, pH 7.0) or potassium phosphate buffer (0.05 M, pH 6.0), both containing 2 μmoles of reduced glutathione per ml, by sonic disruption for 25 min in a Raytheon 10-kw sonic oscillator,

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with a current output of 1.35 amp and tap water as coolant (12 to 15 C). The cellular debris was removed by centrifugation in an SS-1 Servall centrifuge at 35,000 × *g* for 1 hr at 0 C.

Chemicals. DL-Isocitric acid, trisodium salt, thiamine pyrophosphate, glyoxylic acid monohydrate, sodium salt, acetyl-coenzyme A (acetyl-CoA), and coenzyme A (CoA) were obtained from the Sigma Chemical Co., St. Louis, Mo. Acetyl-CoA was also prepared in this laboratory according to the method described by Ochoa (1957), and was lyophilized. Nicotinamide adenine dinucleotide (NAD) and its reduced form (NADH) were products of C. F. Boehringer and Soehne, Mannheim, Germany. Glycolic acid was purchased from Calbiochem. L-Cysteine (free base), L-glycine, L-valine, L-alanine, reduced glutathione, and monosodium glutamate were purchased from Mann Research Laboratory, New York, N.Y. Phenylhydrazine hydrochloride was a product of Matheson Coleman & Bell Co., Inc., East Rutherford, N.J. All inorganic chemicals were of chemically pure or better quality.

Enzyme assays. All colorimetric enzyme assays, with the exception of transaminase determinations, were performed with a Beckman DB recording spectrophotometer.

Isocitrate lyase and malate synthetase assays were modified from the procedure described by Dixon and Kornberg (1959). The isocitrate lyase assay contained (in 3 ml): tris buffer (pH 7.2, 0.2 M), 0.5 ml; $MgCl_2 \cdot 6H_2O$ (2.860%), 0.5 ml; phenylhydrazine·HCl (0.216%), 0.5 ml; cysteine (0.1454%), 0.5 ml; cell-free extract, 0.2 ml; and water. The reaction was started by adding 5 μmoles of sodium isocitrate. Specific activity was defined as the number of micromoles of glyoxylate formed per 10 min per milligram of protein. The malate synthetase assay contained (in 3 ml): tris buffer (0.2 M, pH 7.9), 0.5 ml; $MgCl_2 \cdot 6H_2O$ (2.860%), 0.07 ml; acetyl-CoA, 0.25 μmoles in 0.02 ml; cell-free extract, 0.2 ml; and water. The reaction was started by adding 2 μmoles of sodium glyoxylate. Specific activity was defined as the number of micromoles of acetyl-CoA disappearing per min per milligram of protein.

All enzymatic determinations concerning glyoxylate metabolism in glutamate-grown cells were performed on cells or cell-free extracts from 8-hr cultures.

The manometric assay for glyoxylic acid carboligase and the spectrophotometric procedure for tartronic semialdehyde reductase were performed according to the methods of Krakow, Barkulis, and Hayashi (1961).

Glyoxylic acid reductase and its reverse reaction were assayed according to Zelitch (1955).

Transaminase assays were prepared according to the following protocol: potassium phosphate buffer (1.0 M, pH 8.3), 0.2 ml; cell-free extract, 0.4 ml; pyridoxal phosphate (100 μg/ml), 1.0 ml; L-amino acid (glutamic acid, alanine, and valine; 0.1 M), 0.2 ml; and glyoxylate (0.2 M), 0.4 ml. Duplicates of each were prepared, and one was placed immediately after mixing into a 100-C water bath for 5 min. This tube served as a blank for that particular assay. The other tube was incubated in a 37-C water bath for 60 min, after which it was placed in a 100-C water bath for 5 min. The tubes were cooled and centrifuged to remove any precipitate. The transaminase tubes were then assayed for glycine by a modification of the method of Patton (1935). With 0.1 ml of 95% ethanol was mixed 0.5 ml of the final chloroform extract; 0.1 ml was read at 650 mμ against its blank in the microcuvette of a Beckman model 151 spectrophotometer. The results were read off a standard curve ranging from 0.025 to 0.5 mg of glycine.

Other assays. Formic acid was tested for by the method described by Feigl (1960). Formic acid is reduced to formaldehyde by powdered magnesium in acid, resulting in a purple color when treated with chromotropic acid.

Protein determinations were performed by the method of Lowry et al. (1951).

RESULTS

Isocitrate lyase and malate synthetase were both found to be present in cell extracts of *B. cereus* T when grown in any of several media. Figure 1 shows the patterns of isocitrate lyase synthesis of the organism grown in the presence of different carbon sources. Contrary to that found in many other organisms, isocitrate lyase synthesis is inhibited in *B. cereus* T grown in acetate or in an acetate precursor such as glucose. When grown in glutamate or in yeast extract as sole carbon source, isocitrate lyase activity is highest at the point where microscopic observation shows the organism to be in the granular stage, which is indicative of the onset of sporulation.

It was found that phosphate buffer was inhibitory to isocitrate lyase activity. Figure 2 shows the isocitrate lyase activity of the same

cell-free extract in tris and phosphate buffers. The activity in tris was about 3.5 times that in phosphate buffer.

Table 1 illustrates the amounts of malate synthetase present in cell extracts from cells harvested at 8 hr of growth. The absence of malate synthetase activity in glutamate-grown cells led to studies to elucidate the metabolism of the glyoxylate formed as a result of the high isocitrate lyase activity at this point.

Other pathways for glyoxylate metabolism in microorganisms are the formation of glycine (Campbell, 1956), formate and carbon dioxide (Campbell, 1955), glycolate (Zelitch, 1955), and tartronic semialdehyde and carbon dioxide (Krakow and Barkulis, 1956). Each of these possible pathways was investigated.

In the event that transamination reactions were significant in the dissimilation of glyoxylate, assays were performed at different time intervals to detect any metabolic shifts which may be associated with sporulation. After growth for 5 hr, the cells showed no signs of the onset of sporulation. At 8 hr, the cells showed morphological evidence of beginning sporulation; at 14 hr, the cells were well on their way toward the formation of mature spores.

Alanine and glutamate were included in these assays, owing to their frequency of involvement in transamination reactions. Valine was included because it is present in yeast extract in significant quantities of 3.4% (Difco Laboratories, *personal communication*). Table 2 shows the results of glycine determinations after transaminase assays. The extremely small amounts of glycine formed rule out the possibility that transamination could be significant in glyoxylate metabolism.

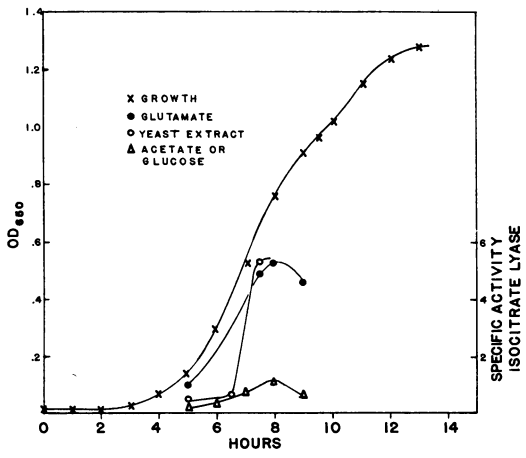


FIG. 1. *Specific activity of isocitrate lyase in Bacillus cereus T as a function of growth. Organisms were grown in media containing glutamate, yeast extract, acetate, or glucose as principal carbon compounds. Carbon compounds were present in 0.1% concentrations, supplemented with 0.2% yeast extract and mineral salts. Yeast extract cultures were supplemented with mineral salts only.*

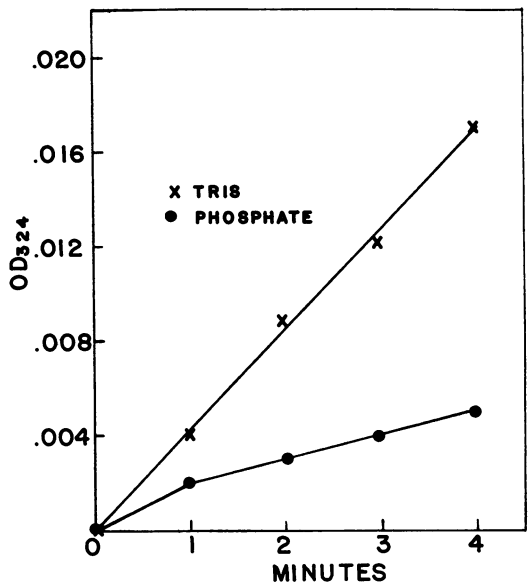


FIG. 2. *Comparison of isocitrate lyase assays of a Bacillus cereus T extract in tris and phosphate buffers. The increase in optical density is directly proportional to the activity of the enzyme, and is a result of the formation of glyoxylic acid phenylhydrozone. See text for details of the assay procedure.*

TABLE 1. *Activity of glyoxylate-cycle enzymes of Bacillus cereus T grown in various carbon compounds**

Compound†	Isocitrate lyase	Malate synthetase
Glucose	1.1	0.18
Acetate	1.5	0.20
Glutamate	5.2	<0.01
Yeast extract	5.3	0.11

* All extracts are from 8-hr cells showing morphological signs of sporulation.

† Concentrations of 0.1%, supplemented with 0.2% yeast extract and mineral salts. The yeast extract culture was supplemented with mineral salts only.

Manometric assays with glyoxylate as substrate were designed to determine whether glyoxylate was being metabolized via reaction 1 or 2, or possibly both.

$$2 \text{ glyoxylate} \rightarrow \text{tartronic semialdehyde} + CO_2 \quad (1)$$

$$\text{glyoxylate} \rightarrow \text{formate} + CO_2 \quad (2)$$

At no time did the stoichiometry achieved in assays suggest that reaction 2 was taking place. Furthermore, the formation of formic acid was ruled out by the qualitative test for formaldehyde and formic acid described by Feigl (1960). The stoichiometry achieved more closely resembled reaction 1, where the disappearance of 1 mole of glyoxylate results in the evolution of 0.5 mole of carbon dioxide. In these studies, the balance was quite variable, according to the manner in which the enzyme was supplied to the system. It was consistently noted that cell-free extracts gave a much lower carbon dioxide yield than that theoretically possible for this reaction. On the other hand, resting-cell suspensions usually produced yields of carbon dioxide closer to the theoretical stoichiometry. The treatment of the cells also had an effect on the reaction. Freshly washed cell suspensions usually produced lower yields than did suspensions prepared from cells which had been frozen (Table 3).

TABLE 2. *Glycine formed through transamination of glyoxylate with L-amino acids by cell-free extracts of Bacillus cereus T*

Hr	Glycine formed (μmoles)		
	Alanine	Glutamate	Valine
5	0	0	0
8	0	0.068	0
14	0.287	0.101	0

TABLE 3. *Stoichiometry of glyoxylic acid carboligase activity in different enzyme preparations**

Enzyme source	CO_2 evolved	Per cent of theoretical yield†
	μmoles	
Cell-free extracts	0.90	30.0
Cell suspensions (fresh)	1.78	58.3
Cell suspensions (stored at −20 C)	2.52	84.0

* Each Warburg flask contained (in 3 ml): 0.2 ml of a 30% suspension of organisms in 0.05 M potassium phosphate buffer (pH 6.0), or 0.2 ml of cell-free extract; 300 μg of thiamine pyrophosphate; 4×10^{-4} M $MgCl_2$; potassium phosphate buffer (0.1 M, pH 6.0) in the main compartment; and 0.2 ml of 20% KOH in the center well. Similar flasks were set up without KOH. The reaction was started after 10-min equilibration with shaking by tipping in 6 μmoles of glyoxylate. Temperature, 31.0 C; gas phase, N_2 . Results represent averages or two or three experiments.

† A theoretical yield is 3 μmoles.

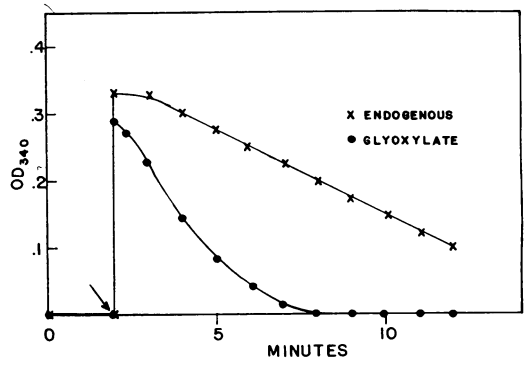


FIG. 3. *NADH oxidation in the presence of glyoxylate and cell extract. The assay method is that referred to in the text, with the exception that 5 μmoles glyoxylate were used. The arrow indicates the point of addition of NADH.*

It was found that *B. cereus* T possesses a glyoxylic acid reductase. Figure 3 shows the rapid oxidation of NADH in the presence of cell extract and glyoxylate. In the carboligase assays, this resulted in the enzymatic reduction of glyoxylate to glycolate in the presence of endogenous NADH, thus removing a portion of substrate from the enzyme system and accounting for the inability to recover theoretical yields of carbon dioxide. When the washed cells were frozen prior to preparing the 30% dilution, there was a marked decrease in reductase activity.

The reverse glyoxylic acid reductase reaction was present (Fig. 4), but at a very low rate.

For evidence of a glycerate cycle which could return the C_3 compound formed to the glycolytic pathway for recycling, it was necessary to demonstrate the enzymatic reduction of tartronic semialdehyde to glyceric acid. The glyoxylic acid reaction product from the glyoxylic acid carboligase assay was treated in the manner described by Krakow et al. (1961). The reaction product was incubated with cell extract at room temperature for 3 hr at pH 7.5. The NADH was

added, and the reaction followed. This incubation period assured the removal of any residual glyoxylate and, at an alkaline pH, the reconversion of any hydroxypyruvate formed to its tautomer, tartronic semialdehyde. The results of this assay are shown in Fig. 5. The oxidation of NADH in the presence of this substrate and cell extract provided evidence for a tartronic semialdehyde reductase, hence, a functional glycerate cycle.

DISCUSSION

The use of phosphate buffer in isocitrate lyase assays makes activity very difficult to detect, especially in extracts from organisms grown in acetate or glucose, where lower activities are observed.

The specific activities seen in acetate- and glucose-grown cells are low compared with those of cells grown in glutamate and yeast extract media, but are comparable to reports of glyoxylate-cycle activity in other organisms (Reeves and Ajl, 1962*a*, *b*; Harrop and Kornberg, 1963).

It is apparent that isocitrate lyase synthesis, regardless of the carbon source in which the organism was grown, is more or less a function of growth, and in every instance has a peak specific activity at the point where the cells present morphological evidence that sporogenesis has begun. At this point, enzyme activity begins to decline. We think that a metabolic shift at this point probably accounts for the cessation of synthesis of glyoxylate-cycle enzymes. The existing enzyme proteins are then probably broken down by proteolytic enzymes (Hardwick and Foster, 1953), and the products are made available for synthesis of spore materials. It was noted through periodic studied of slides stained with Sudan black B that poly-β-hydroxybutyrate (PHB) synthesis begins at a fast rate around 8 hr of growth. This is, in fact, the cause of the granular or mottled effect associated with the onset of sporulation, as evidenced from the work of Lundgren and Bott (1963), Stevenson et al. (1962) and Kominek, Srinivasan, and Halvorson (1963).

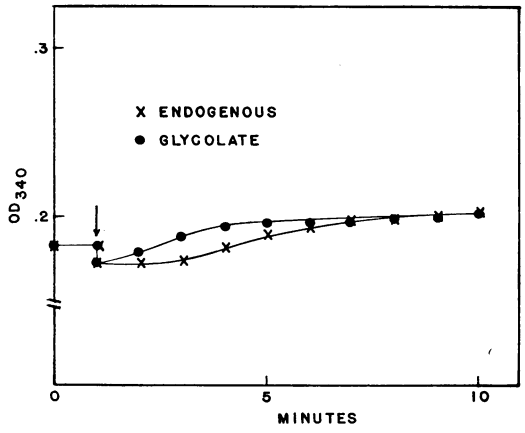


FIG. 4. *NAD reduction in the presence of glycolate and cell extract. The assay method is that referred to in the text, with the exception that tris buffer (0.2 M, pH 8.4) was substituted for sodium pyrophosphate buffer. The arrow indicates the point of addition of NAD.*

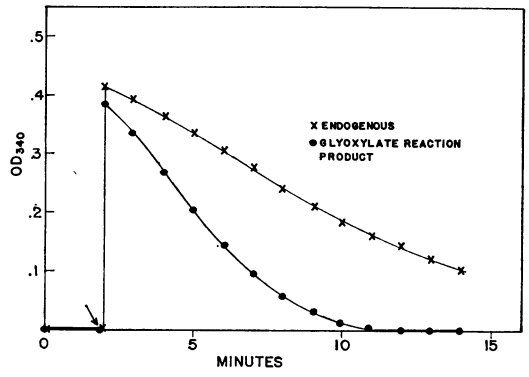


FIG. 5. *NADH oxidation in the presence of glyoxylate reaction product and cell extract. The sample cuvette contained 0.2 ml of cell extract, 0.25 M potassium phosphate buffer (pH 7.5), and 1.3 ml of reaction product containing 0.69 μmole of tartronic semialdehyde (calculated from the CO_2 produced) in 2.5 ml. The reaction was started by adding 0.4 mg of NADH contained in 0.5 ml of water. The arrow indicates point where NADH was added.*

It should be pointed out that the synthesis of isocitrate lyase in yeast extract-salts medium is not exactly applicable to the growth curve in Fig. 1, because total growth in this medium is 55 to 60% that in other media. In this event, the highest specific activity of the enzyme is shown in cells which have been in the stationary phase for 1 hr.

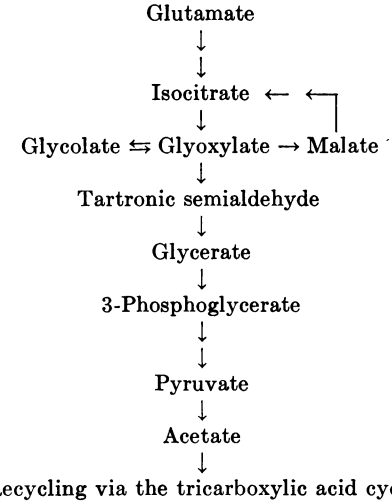
It is exceptional that isocitrate lyase synthesis should be relatively inhibited in media containing acetate or acetate precursors, because this is contrary to observations of other microorganisms (Kornberg, Gotto, and Lund, 1958; Kornberg, 1960; Vanderwinkel et al., 1963). It is probable that the acetate included in the medium

or that which is formed goes into the synthesis of PHB (Sierra and Gibbons, 1962) to serve as an endogenous carbon substrate for endotropic sporulation (Kominek et al., 1963). The high activity in glutamate-grown cells indicates that this compound readily supplies tricarboxylic cycle intermediates, especially isocitrate precursors. Yeast-extract medium had a similar effect, which could be expected in view of the fact that yeast-extract analyses by the manufacturer show glutamic acid to be the most abundant carbon compound present, with a concentration of about 6.5%.

The freezing of washed cells prior to the preparation of cell suspensions apparently has no ill effect on glyoxylic acid carboligase, whereas it was deleterious to glyoxylic acid reductase. Cells which have been frozen over periods ranging from overnight to 3 weeks show about the same effectiveness.

The high rate of endogenous NADH oxidation evident in Fig. 3 and 5 is due to a NADH oxidase indigenous to *B. cereus* T (Hanson, Srinivasan, and Halvorson, 1963*b*). Efforts to remove it by protamine sulfate precipitation and dialysis are only partially successful.

From the data presented in this investigation, it is possible to propose a scheme for the dissimilation of glyoxylate during the growth of *B. cereus* T in various growth substrates.



This organism displays the apparent ability to metabolize glyoxylate via a glycerate cycle or a glyoxylate cycle, depending upon the conditions of growth and the organism's presporulation age. After morphological evidence of sporulation is apparent, glyoxylate production ceases, indicating a shift to another pathway. Evidence has been presented by Hanson et al. (1963*a*) that this pathway is the tricarboxylic acid cycle.

ACKNOWLEDGMENTS

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