ENZYMES OF GLUCOSE AND PYRUVATE CATABOLISM IN CELLS, SPORES, AND GERMINATED SPORES OF *CLOSTRIDIUM BOTULINUM*[1]

RICHARD J. SIMMONS[2] AND RALPH N. COSTILOW

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan

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ABSTRACT

SIMMONS, R. J. (Michigan State University, East Lansing), AND R. N. COSTILOW. Enzymes of glucose and pyruvate catabolism in cells, spores, and germinated spores of *Clostridium botulinum*. J. Bacteriol. **84**:1274-1281. 1962.—An investigation was made of the enzymes of vegetative cells, spores, and germinated spores of *Clostridium botulinum* 62-A to elucidate a pathway of glucose metabolism. Manometric studies were conducted with intact cells, and various enzymes and enzyme systems were assayed in cell-free and spore-free extracts by use of spectrophotometric and colorimetric procedures. Glucose fermentation was found to be inducible; glucokinase was the controlling enzyme. All other enzymes of the Embden-Meyerhof-Parnas (EMP) pathway were found in both induced and noninduced cells, but they were in relatively low concentrations in the latter. This, plus the fact that no glucose-6-phosphate dehydrogenase was detected, led to the conclusion that glucose is catabolized primarily by the EMP system. A number of glycolytic enzymes were also found in extracts of spores and germinated spores of this organism, but the activities were extremely low as compared with activities in cell extracts. A phosphoroclastic-type reaction was readily demonstrated in both glucose-adapted and nonadapted cells, but not in spores and germinated spores. However, both acetokinase and phosphotransacetylase, as well as coenzyme A transphorase, were detected in spores and germinated-spore extracts, although at very low activity levels as compared with cell extracts. The specific activity of diaphorase in spore extracts was about one-half that of corresponding cell extracts, and the activity of reduced diphosphopyridine nucleotide (DPNH) oxidase was actually higher in the spore extracts. In addition, the DPNH oxidase in spore extracts was considerably more heat-stable than that in extracts of cells or germinated spores.

Clifton (1940) reported that *Clostridium botulinum* type A fermented glucose readily, with the production of primarily CO_2 and ethanol and smaller amounts of acetic and lactic acids and hydrogen. However, about 25% of the glucose fermented by resting-cell suspensions was not accounted for, and no attempt was made to elucidate the metabolic pathway. A number of investigations with other clostridia indicate that glucose is catabolized via the Embden-Meyerhof-Parnas (EMP) pathway. Bard and Gunsalus (1950) reported an iron-requiring aldolase of *C. perfringens*, and Shankar and Bard (1955) demonstrated the reduction of diphosphopyridine nucleotide (DPN) by this species with either glucose, glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), or fructose-1,6-diphosphate (F-1,6-P) as substrate. Isotope studies (Paege, Gibbs, and Bard, 1956; Cynkin and Gibbs, 1958) also indicated the EMP pathway to be operative in *C. perfringens*. Martinez and Rittenberg (1959) found that certain strains of *C. tetani* could utilize glucose, and demonstrated the presence of most of the glycolytic enzymes in cell-free extracts. No previous studies of this type have been attempted with *C. botulinum*, to the authors' knowledge.

Other studies in this laboratory demonstrated that germinating spores of *C. botulinum* 62-A fermented glucose to a limited extent, and that a basic level of fermentation apparently did not depend on protein synthesis (Costilow, 1962). Thus, it appeared that the endospores of this organism may contain basic levels of all the en-

[1] Journal article no. 2989, Michigan Agricultural Experiment Station.

[2] Present address: Eli Lilly & Co., Indianapolis, Ind.

zymes required for glucose catabolism. This study was undertaken to elucidate enzymes of glucose catabolism in cells, spores, and germinated spores of *C. botulinum*. Some of the enzymes of pyruvate breakdown and of the flavoprotein group were included for comparative purposes.

MATERIALS AND METHODS

C. botulinum 62-A, obtained from the American Type Culture Collection (ATCC 7948), was used in all experiments. The procedures for sporulation of the culture and for harvesting and storing spore suspensions were those described by Costilow (1962), except that spores were stored in 0.05 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.4) instead of phosphate buffer. A 2% Trypticase (a tryptic digest of casein, BBL) plus 1 ppm thiamine medium was used for growth. Carbohydrates to be used in the growth medium were filter-sterilized and added aseptically. All media were at pH 7.0; the incubation temperature was 37 C.

To obtain germinated spores, 10 to 12 g (wet wt) of spores were suspended in a small volume of 4% Trypticase, heated at 80 C for 20 min, and suspended in 3 liters of 4% Trypticase plus 0.1% sodium bicarbonate in 0.067 M phosphate buffer (pH 7.0). The suspension was incubated at 37 C, and stirred constantly by a steady flow of city gas. In some experiments, 10 μg/ml of chloramphenicol were added, which allowed germination but prevented outgrowth of the germinated spores (Costilow, 1960). A period of 2 to 2.5 hr was required to attain better than 90% germination. The germinated spores were harvested by centrifugation, washed three times with cold distilled water, and resuspended in 0.05 M tris buffer (pH 7.4), for disruption.

Growth response to hexoses was determined by optical density (OD) measurements at 650 mμ with a Bausch and Lomb Spectronic 20 colorimeter; sugar determinations were made on samples removed during the course of growth by a modified anthrone method (Scott and Melvin, 1953).

Extracts were prepared by blending a mixture of 10 to 12 g (wet wt) of spores or 15 to 20 g (wet wt) of cells harvested from a 12-hr culture; 45 g of no. 100 Superbrite glass beads (Minnesota Mining and Manufacturing Co., St. Paul); and 50 ml of 0.05 M tris buffer (pH 7.4), in a high-speed Servall omnimixer (Ivan Sorvall Inc., Norwalk, Conn.). The spores were heated at 80 C for 20 min, just prior to disruption, to destroy any enzyme from vegetative cells. The mixture was cooled in an ice bath before blending, and held in the bath during the blending period of 10 to 12 min. The extracts so obtained were clarified by centrifugation at 30,000 $\times$ *g* for 1 hr in the cold, and dialyzed against distilled water at 4 to 5 C for 15 to 18 hr. Extracts used in measuring the phosphoroclastic reaction and in measuring aldolase were not dialyzed, since this resulted in complete irreversible inactivation of the former system and a great loss in aldolase activity. Protein was estimated by the Folin-phenol method of Lowry et al. (1951).

Warburg techniques were used to follow gas evolution from hexoses by resting-cell suspensions.

Glucokinase was determined by the method of Klein (1953), except that 5×10^{-3} M NaF was added to reaction mixtures as suggested by Saltman (1953), and reducing sugar was measured by the anthrone method of Scott and Melvin (1953). Glucokinase activity was also determined by the method of Horecker and Wood (1957).

Aldolase was determined by the method of Sibley and Lehninger (1949), modified by addition of 10^{-6} M Fe^{++} as suggested by the results of Bard and Gunsalus (1950). The same procedure was used to assay the over-all reaction involving phosphohexoseisomerase, phosphofructokinase, and aldolase, except that G-6-P was used as substrate instead of F-1,6-P, and 20 μmoles each of adenosine triphosphate (ATP) and $MgCl_2$ were added to the reaction mixtures. The system involving both aldolase and 3-phosphoglyceraldehyde dehydrogenase activities was demonstrated by measuring the rate of DPN reduction spectrophotometrically in the presence of arsenate with F-1,6-P as substrate. Reaction mixtures contained 0.1 M tris buffer (pH 7.4), 1 ml; F-1,6-P, 5 μmoles; arsenate, 20 μmoles; DPN, 1 μmole; dialyzed extract; and water to 3 ml.

The reaction sequence involving phosphoglyceromutase, enolase, and pyruvate kinase was assayed by measuring the rate of pyruvate formation from 3-phosphoglycerate. Reaction mixtures contained 0.2 M phosphate buffer (pH 7.5), 5 ml; adenosine diphosphate (ADP), 20 μmoles; $MgSO_4$, 10 μmoles; 3-phosphoglycerate, 25 μmoles; dialyzed extract; and water to 3 ml. Reaction mixtures were incubated at 35 C and

reactions stopped after 5, 10, and 15 min of incubation by addition of 1 ml of 10% trichloroacetic acid. The pyruvate formed was determined by the colorimetric procedure of Friedemann and Haugen (1943). The reaction was ADP-dependent and was inhibited by NaF in the presence but not in the absence of phosphate.

Extracts were assayed for lactic dehydrogenase by the procedure of Neilands (1955), and for alcohol dehydrogenase by the method of Racker (1955) by using acetaldehyde as substrate and following the rate of reduced DPN (DPNH) oxidation. Endogenous rates were substracted from rates with substrate.

The phosphoroclastic reaction was measured by a procedure similar to that of Koepsel and Johnson (1942). The CO_2 and H_2 formed were measured manometrically; the acetyl phosphate was measured by the method of Lipmann and Tuttle (1945). The reaction was inhibited by arsenite and was both DPN- and phosphate-dependent. The method of Rose et al. (1954) was used to assay for acetokinase per se; phosphotransacetylase was determined by the procedure of Stadtman (1955). However, the latter assays were run on a microscale, with the instrumentation described by Wood and Gilford (1961).

Coenzyme A (CoA) transphorase was assayed by the procedure of Stadtman (1953), except that the reaction was run on a microscale, with the instrumentation of Wood and Gilford (1961).

Diaphorase activity was measured by following the rate of change in OD at 600 mμ resulting from the reduction of 2,6-dichlorophenolindophenol with DPNH as substrate. DPNH oxidase activity was determined by measuring the rate of DPNH oxidation spectrophotometrically at 340 mμ. The reaction mixtures contained 1 ml of 0.067 M phosphate buffer (pH 7.4); 0.3 μmole of DPNH; dialyzed extract; and water to 3 ml.

A Beckman model DU spectrophotometer was used for all spectrophotometric assays unless otherwise indicated. All cofactors and other chemicals used were of commercial origin.

RESULTS

Activities of intact cells and spores; utilization of hexoses during growth. The addition of 0.2% glucose or fructose to a 2% Trypticase-1 ppm of thiamine medium increased the average number of cells produced in 18 hr by 75 and 40%, respectively. The glucose disappeared completely from the medium within 18 hr, while only 65% of the fructose was utilized in that period of time. Neither mannose nor galactose increased growth or was utilized during growth.

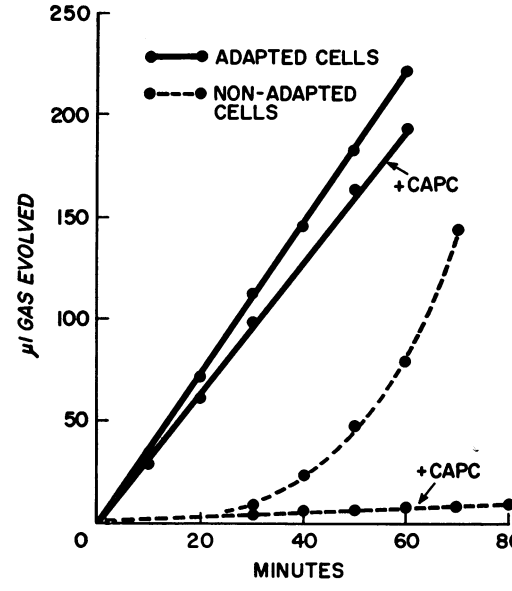

FIG. 1. *Glucose fermentation by cells grown in the presence (adapted) and absence (nonadapted) of glucose. Warburg vessels contained: 1 ml of 0.067 M phosphate buffer (pH 7.0), 0.5 ml of 12% Trypticase, 0.5 ml of 0.06 M glucose, 12 mg of cells, and water to 3 ml. Chloramphenicol* (CAPC; *10 μg/ml) was added where indicated. The temperature was 37 C. The total gas evolved was calculated as CO_2, and the data presented were corrected for the gas evolved in the absence of glucose.*

Glucose fermentation. Cells of *C. botulinum* 62-A, grown in the presence of glucose (glucose-adapted), fermented glucose, whereas those grown in its absence (nonadapted) did not ferment glucose to a significant extent. There was, however, a small volume of gas consistently produced by nonadapted cells in the presence of glucose in excess of endogenous levels. The induced nature of this system is illustrated in Fig. 1. In the presence of a rich amino acid source (Trypticase), the nonadapted cells were induced to ferment glucose. This process was inhibited by chloramphenicol, an inhibitor of protein synthesis. Glucose-adapted cells fermented glucose without an induction lag, and chloramphenicol failed to inhibit to any great extent. Cells grown in the presence of fructose fermented both glu-

TABLE 1. *Comparative activities of enzymes in extracts of vegetative cells, spores, and germinated spores of Clostridium botulinum 62-A*[a]

Enzymes or systems	Relative activities in extracts				
	Vegetative cells		Spores germinated in:		Spores
	Glucose adapted	Nonadapted	Trypticase	Trypticase + CAPC[b]	
Glucokinase[c]	0.25–0.44	0	0	0	0
Phosphohexoseisomerase, phosphofructokinase, and aldolase[d]	0.63	0.19	0.11	0.04	0.04–0.08
Aldolase[d]	1.60–1.85	0.75–0.80	0.50	0.09	0.06–0.11
Aldolase, 3-phosphoglyceraldehyde dehydrogenase[e]	4.2–4.6	1.0–1.4	0.9–1.1	0.6–0.8	0.4–0.5
Phosphoglyceromutase, enolase, and pyruvate kinase[f]	0.30	0.14	0	0	0
Lactic dehydrogenase	0	0	0	0	0
Alcohol dehydrogenase[g]	0.13	0.07	0	0	0
Phosphoroclastic reaction[h]	0.50–0.58	0.50–0.58	0	0	0
Acetokinase[i]	2.0–2.5	1.7–2.0	0.2–0.3	0.2	0.2
Phosphotransacetylase[g]	20	19.3	2.3	1.3	1.1
Coenzyme A transphorase[g]	3.0	2.7	1.4	0.9	0.9
Diaphorase[g]	0.5–0.65	0.54–0.63	0.58–0.60	0.25	0.20–0.40
Reduced diphosphopyridine nucleotide oxidase[g]	0.01–0.02	0.03–0.04	0.025	0.04	0.04–0.06

[a] See Materials and Methods for procedures.
[b] CAPC = chloramphenicol.
[c] Expressed as μmoles of glucose phosphorylated per hr per mg of protein.
[d] Expressed as μmoles of fructose-1,6-diphosphate split per hr per mg of protein.
[e] Diphosphopyridine nucleotide reduction at 340 mμ; change in OD × 10^2 per min per mg of protein.
[f] Expressed as μmoles of pyruvate formed per hr per mg of protein.
[g] Change in OD per min per mg of protein.
[h] Expressed as μmoles of acetyl phosphate formed per hr per mg of protein.
[i] Expressed as μmoles of acetyl phosphate formed per min per mg of protein.

cose and fructose without an inductive lag, but fructose was fermented at a slower rate than glucose. Neither mannose nor galactose was fermented by cells grown in glucose, and these sugars would not induce the glucose-fermenting system.

Enzymes in extracts of cells and spores. Although there has been considerable research on the products of fermentation by *C. botulinum*, no reports were found as to the pathway of glucose fermentation in this species. Initial experiments with extracts of cells grown in the presence of glucose indicated that there was no glucose dehydrogenase or G-6-P dehydrogenase present. Therefore, it was concluded that the EMP pathway was the most likely route of glucose catabolism; the enzymes assayed were those of this system and those believed to be key enzymes in pyruvate metabolism.

Since one of the primary objectives of this work was to compare the relative activities of various enzymes in cells, spores, and germinated spores, the results of the various assays are tabulated in Table 1 so they can be readily compared. Although spores produced in a glucose-free medium were used exclusively in the collection of the data presented here, other experiments demonstrated that the addition of 0.2% glucose to the production medium did not result in an increase in the levels of EMP enzymes in spore extracts. This was expected, since the addition of glucose greatly delayed sporulation, and it had disappeared from the medium at least 48 hr prior to the onset of sporulation. Therefore, the various enzymatic activities observed in the spore extracts may more logically be compared with those observed in nonadapted rather than glucose-adapted cell extracts. The reader is re-

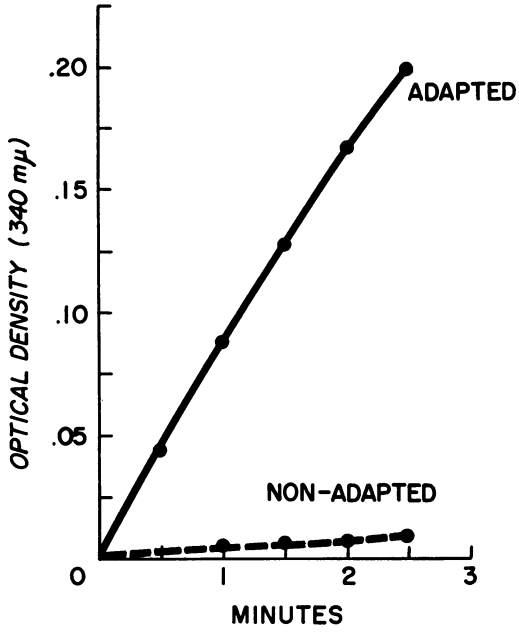

FIG. 2. *Glucokinase activity in extracts of cells grown in the presence (adapted) and absence (non-adapted) of glucose. Reaction mixtures contained: 1 ml of 0.2 M tris buffer (pH 7.4), 5 μmoles of glucose, 10 μmoles of ATP, 10 μmoles of $MgCl_2$, 0.9 μmole of triphosphopyridine nucleotide, 0.01 ml of a 0.01% solution of glucose-6-phosphate (G-6-P) dehydrogenase, 12 mg of extract protein, and water to 3 ml. The G-6-P dehydrogenase was free of hexokinase activity.*

ferred to Table 1 for the data supporting the following statements of results.

Enzymes of the EMP pathway. Glucokinase appears to be the inducible enzyme in glucose fermentation. Extracts of cells grown in the presence of glucose phosphorylated glucose, whereas those grown in the absence of hexose did not (Fig. 2). Extracts of fructose-grown cells also were active on glucose, but had very little capacity to phosphorylate fructose. Neither mannose nor galactose was phosphorylated by the glucokinase.

Glucokinase was not detected in either spore or germinated-spore extracts.

All of the other enzymes of the EMP system were found in extracts of both glucose-adapted and nonadapted cells, but the activities in the former were from two to four times higher than in the latter. This was also true in the case of alcohol dehydrogenase, but no such relationship was observed with any of the other systems measured. Therefore, it appears that the concentrations of glycolytic enzymes in this strain of *C. botulinum* are controlled by a sequential induction process mediated by glucokinase.

The rates of triose production from G-6-P and F-6-P by extracts of cells, spores, and germinated spores were very similar, so only the activities observed with G-6-P involving the enzymes phosphohexoseisomerase, phosphofructokinase, and aldolase, and with F-1,6-P involving only aldolase, are reported in Table 1. The specific activity of aldolase alone was considerably higher than observed with the system, as would be expected. The aldolase had a pH optimum of 7.5 to 8.0, and was greatly stimulated by Fe^{++} and low concentrations of cysteine. The specific rate of triose production from G-6-P by spore extracts was only about one-fourth that of nonadapted cell extracts; the aldolase activity of the spore extracts was about one-tenth that of the corresponding cell extracts. The germinating spore apparently synthesizes these enzymes rapidly, as evidenced by comparing the activities of spores germinated in the presence and absence of chloramphenicol.

The measurements of DPN reduction with F-1,6-P as substrate were complicated by the presence of a DPNH oxidase and, thus, cannot be considered a true reflection of the activities. However, the activities observed with this system (aldolase, 3-phosphoglyceraldehyde dehydrogenase) reflect the high activities in extracts from adapted cells as compared with nonadapted cells, and the relatively lower activities in spores and germinated spores.

The rate of pyruvate production from 3-phosphoglyceric acid involving phosphoglyceromutase, enolase, and pyruvate kinase by extracts of nonadapted cells was only one-half that observed with extracts of glucose-adapted cells. These enzymes were not detected in spore extracts by this technique or by measuring the rate of DPNH oxidation with 3-phosphoglycerate as substrate. The high DPNH oxidase activity interfered greatly with the latter measurement.

Lactic and alcohol dehydrogenases. That pyruvate is reduced to lactate by this strain of *C. botulinum* is unlikely, since assays for lactic dehydrogenase were negative. Also, analyses for lactate in completed fermentation solutions from whole-cell suspensions were negative.

The alcohol dehydrogenase level was very low in nonadapted cell extracts and could not be detected in spore or germinated-spore extracts.

Phosphoroclastic reaction. Cell-free extracts cleaved and oxidized pyruvate, with the production of 1 mole each of CO_2, H_2, and acetyl phosphate per mole of pyruvate. Removal of either phosphate or DPN from the reaction mixture resulted in almost complete inactivity. It was impossible to assess the true function of thiamine pyrophosphate and CoA in the reaction, since attempts to remove these factors by dialysis resulted in almost complete loss of activity, and the addition of these cofactors to the undialyzed extracts showed no effect on the reaction rate. Arsenite (2 μmoles/ml) inhibited the reaction about 50%. The complete phosphoroclastic reaction could not be detected in either spore or germinated-spore extracts.

Acetokinase and phosphotransacetylase. Cell-free extracts contained acetokinase activity in ten times higher levels than found in spore extracts. The enzyme was ATP and Mg^{++} dependent; ADP could not be substituted for ATP. Cell-free extracts also contained an active phosphotransacetylase that was 10 to 20 times higher than that found in spore extracts.

CoA transphorase. Extracts of both cells and spores contained CoA transphorase that catalyzed the transfer of the CoA group between butyryl-CoA and acetate.

Diaphorase. Cell and spore extracts cause a rapid reduction of 2,6-dichlorophenol indophenol with DPNH as substrate, indicating the presence of diaphorase activity. Methylene blue and triphenyl tetrazolium salts would also act as electron acceptors.

DPNH oxidase. Of special interest was the high level of activity (Table 1) and high heat resistance of the spore DPNH oxidase (Table 2). The extracted spore enzyme remained active after heating for 1 min at 80 C, whereas the enzyme extracted from cells did not. However, the heat resistance of oxidase extracted from germinated spores was as low as that of the oxidase extracted from cells. That the decrease in OD at 340 mμ was due to DPNH oxidation and not degradation of the DPNH was demonstrated by testing the DPN formed in the reaction as a substrate for alcohol dehydrogenase. Additions of ethanol and alcohol dehydrogenase caused a rapid and complete restoration in absorption at 340 mμ.

TABLE 2. *Effect of heat on DPNH oxidase*

Heat treatment		Relative activities[a]		
Temp	Time	Cells	Germinated spores[b]	Spores
C	*min*			
—[c]	—	1.8	2.5	5.0
70	1	0.5	0.9	5.5
80	1	0	0	3.5
85	5	0	0	2.8
90	1	0	0	2.0

[a] Activities are in terms of the change in OD × 10^2 at 340 mμ per min per mg of extract protein. Reaction mixtures contained: 1 ml of 0.067 M phosphate buffer (pH 7.4), 0.3 μmole of DPNH, 0.1 ml of extract, and water to 3 ml.

[b] Spores were germinated 2.5 hr at 37 C in 4% Trypticase before extraction.

[c] No heat treatment.

That the DPNH oxidase is a flavoprotein was shown by removal of the flavin component of the enzyme by the acid-ammonium sulfate procedure of Warburg and Christian (1938), then adding back either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). There was no activity without addition of cofactors; FAD (2×10^{-4} M) restored the activity to the original level, and FMN (2×10^{-4} M) partially reactivated the enzyme. Atabrine, a flavin analogue, inhibited the oxidase, whereas KCN (3×10^{-3} M), a cytochrome poison, had no effect on activity. Dipicolinic acid (4×10^{-3} M) did not enhance the activity of spore DPNH oxidase, although it was reported to increase the activity of spore DPNH oxidase of *Bacillus cereus* (Doi and Halvorson, 1961). Other characteristics of the DPNH oxidase noted were that it catalyzed a two-electron reduction of oxygen, did not reduce cytochrome *c*, oxidized reduced triphosphopyridine nucleotide, and was stable on storage at −20 C. It differs from the DPNH oxidase of either *C. perfringens* (Dolin, 1959*a*, *b*) or *C. kluyveri* (Weber and Kaplan, 1954) in one or more of these characteristics.

DISCUSSION

The data presented indicate that glucose fermentation by *C. botulinum* 62-A is via the EMP

pathway and is controlled by a sequential induction process. Thus, glucokinase was found only in cells grown in glucose, and the activities observed with the other glycolytic systems tested were from two to three times higher in extracts of glucose-adapted cells than in extracts of nonadapted cells. A similar relationship was noted with alcohol dehydrogenase, but not with any of the other enzymes tested. It is unlikely that there is any hexose monophosphate dissimilation of sugar by this organism, since no G-6-P dehydrogenase activity was found.

The inducible glucokinase found in these extracts is not unlike that observed in *C. tetani* (Martinez and Rittenberg, 1959), and that found in mutant strains of *Pseudomonas putrefaciens* (Klein, 1953). It is probably not completely absent from cells and spores, since Costilow (1962) observed a basal level of glucose fermentation by heavy suspensions of heat-shocked spores germinating in a buffer in the presence of chloramphenicol; extremely slow glucose fermentation rates were observed in these studies with nonadapted resting cells. This is not unusual. Pollock (1959) pointed out that it is unlikely for an inducible enzyme to be at zero level.

Since glucose fermentation was demonstrated in spores germinated in the presence of chloramphenicol (Costilow, 1962), and data presented here indicate that chloramphenicol does indeed prevent an increase in enzyme level during germination, it is likely that all of the EMP enzymes are present in the spore. Failure to detect glucokinase or enzymes of the lower part of the pathway was probably the result of inactivation or lack of sufficient sensitivity to detect the extremely low levels present, or both. Activities observed in comparable cells (nonadapted) were very low. As mentioned above, the addition of glucose to the sporulation medium failed to increase the activities of the EMP enzymes. The presence of glucose greatly delayed the initiation of sporulation, and the glucose disappeared from the medium long before spores were formed. Thus, an inductive effect could not be observed.

There is increasing evidence that bacterial endospores contain basal levels of a great percentage of the catabolic enzymes found in the vegetative cell. Thus, Goldman and Blumenthal (1961) demonstrated the presence of the enzymes of the EMP pathway in extracts from spores of *B. cereus*, Church and Halvorson (1957) reported a complete glucose-oxidizing system in nongerminated spores of *B. cereus*, Levinson and Hyatt (1960) demonstrated glucose oxidation by germinating spores of *B. megaterium* rendered nonviable by heat or ionizing radiation, and Costilow (1962) demonstrated both glucose and amino acid fermentations by spores of *C. botulinum* 62-A rendered nonviable by ionizing radiation and germinated in the presence of chloramphenicol. When extracted, these catabolic enzymes have no more heat resistance than those of the corresponding vegetative cells. Such information suggests that a percentage of the cell enzymes are "bound" as a part of the heat-resistant protein during sporulation. However, this explanation would not account for those enzymes present in spores in activity levels as high as or higher than found in cells. This group would include the DPNH oxidases found in *C. botulinum* 62-A and in aerobic bacilli (Doi and Halvorson, 1961), alanine racemase (Stewart and Halvorson, 1953), and glucose dehydrogenase (Bach and Sadoff, 1962). Most of these enzymes must be synthesized during sporulation; in fact, the glucose dehydrogenase is not found in cultures of *B. cereus* until sporulation has been initiated. Therefore, the endospore may be more complicated enzymatically than the corresponding vegetative cells.

The high heat resistance of the DPNH oxidase in spore extracts provides a system which might be used for studying heat resistance of spores. After germination, spores as well as the oxidase lose their heat resistance, which indicates that the oxidase of spores, germinated spores, and cells may be the same enzyme with different levels of heat resistance.

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