GLUCOSE METABOLISM OF CELLS, SPORES AND GERMINATED SPORES OF CLOSTRIDIUM BOTULINUM

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ABSTRACT

GLUCOSE METABOLISM OF CELLS,
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OF CLOSTRIDIUM BOTULINUM

By Richard J. Simmons

An investigation was made of some of the enzymes of vegetative cells, spores, and germinated spores of Clostridium botulinum, type A, for purposes of elucidating a pathway for glucose metabolism and of gaining knowledge on the metabolic potential of spores. Manometric studies with whole cells and spectrophotometric and colorimetric assays of enzymes in cell-free extracts showed that glucose or fructose induce the enzyme(s) which is adaptive in the fermentative system, and that glucose is a better inducer and substrate for fermentation than is fructose. Examination of extracts of cells grown in the presence and absence of glucose indicated the presence of all the Embden-Meyerhof-Parnas (EMP) pathway enzymes in both extracts except glucokinase. Glucokinase activity was detected only in extracts of cells grown in the presence of glucose. All EMP enzyme activities were significantly higher in glucose-adapted than in non-adapted cell extracts. It was concluded that in C. botulinum concentrations of glycolytic enzymes may be controlled by a sequential induction process mediated by an inducible glucokinase. The hexose monophosphate pathway may be absent, but this was not conclusively shown. Cell extracts also contained DPN·H oxidase, diaphorase, acetokinase, phosphotransacetylase and coenzyme A transphorase.

EMP enzymes, except glucokinase and enzymes of the lower-part of the pathway, and diaphorase, acetokinase, phosphotransacetylase and coenzyme A transphorase were detected in extracts of spores and germinated spores though in much lower activity levels than found in extracts of cells. However, DPN·H oxidase in spore extracts had a higher activity and a considerably higher heat resistance than a similar enzyme in cell extracts. The results indicate that the spore may contain the whole complement of enzymes found in the corresponding cell, and that a specific enzyme such as the heat resistant DPN·H oxidase may play a significant role in the germination process.

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by

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INTRODUCTION

Spores of Clostridium botulinum are dormant forms of life that have high heat and radiation resistance. Although a wealth of data has been reported on resistance levels, destruction rates, and some of the changes accompanying the transition from spore to vegetative cell, our understanding of the biochemical basis for these changes is limited. Determining the enzyme complement of spores and vegetative cells has been one approach aiding in the resolution of these problems. Studies have been made comparing the respiratory and metabolic enzymes of specific cell stages of the aerobic species, but there is a paucity of data on the anaerobes. Reports have appeared on glucose metabolism of vegetative cells of a few species of clostridia, but relatively little is known regarding the metabolism of C. botulinum, and practically nothing is known of its spore enzyme complement.

This study was undertaken to elucidate some of the enzymes of vegetative cells, spores, and germinated spores of C. botulinum. Although emphasis was placed on enzymes of glucose dissimilation, other enzymes were investigated. These included flavoprotein enzymes concerned in terminal respiration, and enzymes involved in metabolic utilization of acetate and energy production. It was hoped that data derived from these studies would not only elucidate a pathway of glucose dissimilation, but would also contribute knowledge to our understanding of the metabolic potential and biology of spores.

HISTORICAL REVIEW

Glucose Fermentation

Knowledge of glucose metabolism in members of the genus Clostridium is incomplete. Bergey's Manual (Breed, Murray and Smith, 1957) states that a majority of clostridia including C. botulinum ferment glucose. However, since this fact as indicated by Bergey's Manual (Breed, et al., 1957) was based on gas evolution and acid production in a medium containing glucose, this gives no information on the pathway of glucose metabolism.

The early fragmentary evidence on the mechanisms of fermentation suggested a scheme other than the Embden-Meyerhof-Parnas (EMP) pathway. Stone and Werkman (1937), working with Clostridium butylicum, Clostridium sporogenes and Clostridium histolyticum, reported that these organisms did not have an enzyme mechanism capable of producing phosphoglyceric acid in the dissimilation of glucose. Osburn, Brown, and Werkman (1937) showed methylglyoxal formation in the breakdown of glucose by C. butylicum. Methylglyoxal was once regarded as an intermediate in the breakdown of glucose in the so-called Neuberg scheme of alcoholic fermentation.

Clifton (1940) reported that glucose fermentation by washed cell suspensions of C. botulinum yielded primarily ethanol and CO_2, with only traces of H_2, acetate and lactate. Lerner and Pickett (1945) showed that Clostridium tetani ferments glucose with ethanol and CO_2 as main products along with small amounts of H_2 and lactic acid. Iron was essential for the fermentation, with glucose being fermented in direct proportion to the reduced iron present in the culture medium. They suggested that an iron-containing enzyme or coenzyme is essential for glucose fermentation.

Certain clostridia, which normally do not produce lactic acid, are reported to carry out a homolactic fermentation under abnormal conditions. Kempner and Kubowitz (1933) reported that carbon monoxide and cyanide diverted the fermentation by Clostridium butyricum to the homolactic type and that this could be reversed by removal of the inhibitor or by light. Pappenheimer and Shaskan (1944) showed with Clostridium perfrigens that products obtained from glucose dissimilation depended upon iron content of the cells. As iron was decreased, the reaction shifted from an acetic-butyric type with large amounts of CO_2 and H_2 towards a more purely lactic acid fermentation with slight gas formation. Similarly, Hanson and Rodgers (1946) demonstrated that cultures of Clostridium acetobutylicum obtained by serial transfers in low iron medium produced a homolactic fermentation. Such shifts to lactic fermentation would be expected if cytochromes functioned in pyruvate breakdown, but no spectral evidence has been found for these respiratory catalysts in clostridia (Smith, 1954). Lerner and Mueller (1949), working with a mutant strain of C. tetani, showed that cells from an iron-deficient medium and which did not ferment glucose could be activated by glutamine. These data suggest an indirect effect of iron on glucose fermentation.

Recent data, though incomplete, suggest that the EMP pathway is present in members of the clostridia. Bard and Gunsalus (1950) reported an iron-requiring aldolase of C. perfringens, and suggested that since iron is essential for aldolase activity, this affords an explanation of the indispensability of iron for clostridial growth. Furthermore, the occurrence of aldolase as the key enzyme for the conversion of fructose diphosphate to triose phosphates suggested the occurrence of the EMP path.

Shankar and Bard (1955) reported the reduction of diphosphopyridine nucleotide (DPN^+) by glucose, glucose-6-phosphate, fructose-6-phosphate and fructose diphosphate, and this was taken as evidence for the hexose diphosphate pathway.

Further evidence for the EMP pathway was obtained by the use of labelled glucose. Paege, Gibbs and Bard (1956), working with C. perfrigens, showed that the labelling of products was qualitatively that expected of the glycolytic path. Cynkin and Gibbs (1958) while studying pentose metabolism by C. perfringens found ethanol and acetate similarly labelled from glucose 2-C^{14} which is consistent with that expected of the EMP scheme.

Despite reports (Breed et al., 1957) that C. tetani is a non-saccharolytic organism, Martinez and Rittenberg (1959) demonstrated that certain strains utilize glucose in the growth medium, and showed the presence of most of the enzymes of glycolysis in cell-free extracts.

Pyruvate Fermentation

The cleavage and oxidation of pyruvate, termed the phosphoroclastic reaction, results in the formation of acetyl phosphate, CO_2 and H_2. At first it was thought that in this reaction phosphate combined with pyruvate to cause a reversible "phosphoroclastic splitting" leading to acetyl phosphate and formic acid, the acetic acid arising by hydrolysis of acetyl phosphate. Later, Stadtman, Novelli, and Lipmann (1951) showed with extracts of Clostridium kluyveri that coenzyme A, in the presence of suitable enzymes, can accept an acetyl group from pyruvic acid and transfer it to phosphate to give acetyl phosphate. The enzyme which catalyzes

the transfer of the acetyl group or acetate from acetyl phosphate to coenzyme A is called phosphotransacetylase. Acetokinase catalyzes a reversible reaction in which ATP and acetate react to yield ADP and acetyl phosphate. This enzyme was first described by Lipmann (1944) in extracts of Lactobacillus delbrueckii. The metabolic utilization of acetate is brought about through a mechanism involving acetokinase and phosphotransacetylase. The overall reaction is as follows:

$$\text{ATP} + \text{Acetate} \underset{\text{acetokinase}}{\overset{Mg^{++}}{\rightleftharpoons}} \text{ADP} + \text{Acetyl phosphate}$$

$$\text{Acetyl phosphate} + \text{HS-CoA} \overset{\text{transacetylase}}{\rightleftharpoons} \text{Acetyl-SCoA} + \text{iP}$$

$$\text{Net reaction: ATP} + \text{Acetate} + \text{HS-CoA} \rightleftharpoons \text{ADP} + \text{iP} + \text{Acetyl-SCoA}$$

The immediate electron acceptors and the mechanisms of the cleavage of pyruvate are still unknown. The transfer of electrons may result in the evolution of molecular hydrogen either with or without formate as an intermediate, depending on the organism involved. The reaction is superficially similar to the phosphoroclastic system of Escherichia coli which produces acetyl phosphate and formate from pyruvate and phosphate. However, the Clostridium system does not produce H_2 and CO_2 from formate (Koepsell and Johnson, 1942), nor does labelled formate exchange with pyruvate (Wilson, Krampitz, and Werkman, 1948; Hamilton and Wolfe, 1957), although CO_2 readily does (Wolfe and O'Kane, 1955; Shug and Wilson, 1956). Hydrogenase, catalyzing the reaction $H_2 \rightleftharpoons 2H^+ + 2e^-$, is presumed to function in H_2 formation with the clostridia and with E. coli. Wolfe and O'Kane (1953) demonstrated with extracts of C. butyricum that CoA,

cocarboxylase, and Fe^{++} participate in the phosphoroclastic reaction, but that the pyruvate oxidation factor does not. In Clostridium sticklandii folic acid has been shown by Wright and Anderson (1957) to be a specific electron acceptor. The reaction requires substrate levels of CoA and an electron donor such as pyruvate, serine, or methionine, and the dihydrofolic acid formation is associated with increase in acetyl-SCoA and CO_2. No reports are published for the "clastic" reaction by C. botulinum. The scanty data suggests further study of this H_2 producing system.

Pyruvate fermentation varies depending on the species. Rosenfeld and Simon (1950) reported a complete array of products such as acetone, butanol, butyric acid, ethanol and acetic acid by C. acetobutylicum, whereas Kluyver (1931) found the simple conversion of pyruvate to butyrate, acetate, CO_2, and H_2 by C. butyricum. Koepsell, Johnson and Meek (1944) showed that cell-free extracts of C. butylicum formed acetate, CO_2 and H_2 from pyruvate, required phosphate, and produced acetyl phosphate.

Respiratory Flavoproteins

Various soluble systems for the reoxidation of reduced pyridine nucleotides are known. The flavoproteins, DPN·H or TPN·H oxidases, found in yeast and bacteria, catalyze the oxidation of reduced pyridine nucleotides (PN·H) according to equations (1) or (2); oxygen is the electron acceptor.

(1) $PN{\cdot}H + H^+ + O_2 \xrightarrow{\text{flavoprotein}} PN^+ + H_2O_2$

(2) $2\ PN{\cdot}H + 2\ H^+ + O_2 \xrightarrow{\text{flavoprotein}} 2\ PN^+ + 2\ H_2O$

In addition to flavoprotein enzymes that react directly with O_2, bacteria, yeast, and animals contain diaphorases which couple the oxidation of reduced pyridine nucleotide to the reduction of artificial electron acceptors such as methylene blue, 2-6 dichlorophenolindophenol, quinones, and ferricyanide.

Only a few reports have been made on the cell-free DPN·H oxidases and diaphorases in members of the clostridia. Dolin (1959) reported that the DPN·H oxidase of C. perfringens catalyzes the four-electron reduction of O_2 to H_2O, with DPN·H as electron donor, but free peroxide is not an intermediate. Crude extracts have enzymes which catalyze DPN·H dependent reduction of menadione and free flavins, and these reactions are not catalyzed by the four-electron oxidase. Aerobically, the former reactions result in growth inhibitory levels of H_2O_2. Mallin and Seeley (1958) studied the DPN·H oxidase of C. perfringens and suggested that a weak DPN·H peroxidase activity accounts for the low peroxide levels. Dolin (1958) further showed that the oxidase is also a cytochrome c reductase.

The DPN·H oxidase of C. kluyveri catalyzes DPN·H oxidation with O_2 as electron acceptor; with DPN·H as reductant, extracts also catalyze the reduction of cytochrome c (Weber and Kaplan, 1954). When free flavins are removed by dialysis, DPN·H oxidation proceeds according to a two-electron reduction of O_2 (Dolin, 1959).

Spore Enzymes

Enzymic systems and isolated enzymes have been detected in spores of aerobic bacteria, and the following systems have been reported: enzymes of the Embden-Meyerhof pathway (Goldman and Blumenthal, 1961);

glucose dehydrogenase (Bach and Sadoff, 1960); alanine deaminase (O'Connor and Halvorson, 1959); adenosine deaminase and a ribosidase (Powell and Hunter, 1956); catalase (Lawrence and Halvorson, 1954); alanine racemase (Stewart and Halvorson, 1953); and, DPN·H oxidase, diaphorase, cytochrome c reductase, and flavoproteins (Spencer and Powell, 1952; Halvorson, Doi and Church, 1958). There is, however, a lack of data on enzymes of spores of anaerobic bacteria.

MATERIALS AND METHODS

The organism used in all studies was Clostridium botulinum, 62 A, obtained from the American Type Culture Collection. The culture was maintained in the spore state. Spores were produced as described below, washed aseptically, and stored at 5 C in 0.067 M phosphate buffer, pH 7.0.

The sporulating medium was that developed by Day (1960) consisting of 4% Trypticase[1] and 1 ppm thiamine. Vegetative cells were produced in 2% Trypticase containing 1 ppm added thiamine. When carbohydrates were used in the medium they were sterilized separately by passage through a Seitz filter apparatus and then added aseptically to give the concentration desired. These media, without adjustment, were pH 7.0.

For large cultures, a 12 L flask containing 10 L of medium was used; the flask was incubated in a 37 C water bath. Anaerobiosis was attained by passing a slow stream of city gas (methane) first through a sterile water trap and then through the medium. The effluent gases were allowed to escape into a separate water trap flask and then into a ventilating hood. A slight positive pressure was maintained in the system at all times. To establish synchrony of the culture, the following inoculation procedure was followed: one ml of a spore suspension containing approximately 10^8 spores was heat-shocked at 80 C for 15 minutes, inoculated into 40 ml of medium, and incubated for 12 hours; the 12-hour culture was inoculated into 460 ml of medium and incubated for 3 to 4 hours; this 500 ml culture was poured into 1500 ml of medium and again incubated for 3 to 4 hours; finally, the 2 L inoculum was poured into 8 L of medium and incubated for

[1] Baltimore Biological Laboratories, Baltimore, Md.

the desired time period. For vegetative cell production, a 12-hour incubation period was satisfactory as the cells were in the logarithmic growth phase; for spore production the culture was permitted to go 40 to 48 hours when virtually all spores were found. The cells or spores were collected by centrifugation, washed at least 3 times with cold distilled water, and resuspended in 50 ml of .05 M trishydroxymethylaminomethane (tris) buffer, pH 7.4.

Two different germinating solutions were used for spores. One consisted of 4% Trypticase and 0.1% sodium bicarbonate in 0.067 M phosphate buffer, pH 7.0. In some experiments, 10 μg/ml of chloramphenicol were added which allowed germination but prevented outgrowth of the germinated spores. The second germinating solution contained 4% acetate and 0.1% sodium bicarbonate in 0.067 M phosphate buffer, pH 7.0. The same method of attaining anaerobiosis used for sporulation was found suitable for these germinating solutions, and the steady flow of city gas kept the spores in constant agitation. Ten to 12 g wet weight of spores were suspended in a small volume of the germination solution minus the bicarbonate, heated at 80 C for 20 minutes, and suspended in 3 L of the germinating solution. Germination in the Trypticase solution required 2 to 2.5 hours incubation at 37 C, whereas the process was slower in the acetate solution requiring 8 to 10 hours.

To determine utilization of sugars during growth, cultures were grown in flasks containing 0.2% Trypticase, 0.2% sodium thioglycollate, and 0.2% glucose, fructose, galactose or mannose.

At various intervals, samples were removed and turbidities of the cultures were measured at 650 mμ in a colorimeter set to 100% transmission

with an uninoculated medium blank. Sugar determinations were made on samples removed during the course of growth by a modified anthrone method (Scott and Melvin, 1953).

Cell-free extracts were prepared by disruption of the cells with size no. 100 Superbrite glass beads[2] in a high-speed Servall[3] omnimixer. Ten to 12 g wet weight of spores or 15 g wet weight of cells, suspended in 50 ml of 0.05 M tris buffer, were used in the cup along with 45 g of glass beads. The cup was chilled in an ice bath for 10 minutes prior to disruption of the cells, and the ice bath was constantly stirred to allow for good heat transfer during the breaking. The time required for good breakage was 5 to 6 minutes for cells and 10 to 15 minutes for spores. The extracts so obtained were centrifuged at 30,000 rcf for 1 hour to clarify them, and dialyzed against distilled water at 5 C for 15 to 18 hours.

Protein was estimated by the trichloroacetic acid precipitation method of Stadtman et al. (1951), or by the Folin-phenol method of Lowry et al. (1951).

Glucose fermentation by resting cell suspensions was tested by following gas evolution using conventional Warburg techniques.

Glucokinase activity of cell-free extracts was assayed by two methods. One was that of Klein (1953) where glucose is reacted with ATP and Mg^{++}, and then the phosphorylated esters formed in the reaction are precipitated out by addition of $Ba(OH)_2$ and $ZnSO_4$ (Somogyi, 1945). Decrease

[2] Minnesota Mining and Manufacturing Company, St. Paul, Minn.

[3] Ivan Sorvall, Inc., Norwalk, Conn.

in free reducing sugar over that of the control without ATP constitutes net activity (Saltman, 1953). Reducing sugars were determined by the anthrone method of Scott and Melvin (1953). In the second assay, glucose-6-phosphate was identified as the end product of the reaction by coupling glucokinase to the glucose-6-phosphate dehydrogenase system (Horecker and Wood, 1957).

Aldolase was measured by several methods, the one chosen depending upon the particular problem. Mostly, the colorimetric assay of Sibley and Lehninger (1949) was used in which the trioses formed from fructose diphosphate are trapped with hydrazine. The hydrazones are treated with alkali and the color produced after addition of 2-4 dinitrophenylhydrazine and aqueous NaOH is measured at 540 mμ. The optical densities are related to the amount of alkali labile phosphorus formed in the reaction. Aldolase was also measured spectrophotometrically by coupling to diphosphopyridine nucleotide (DPN^+) reduction in the presence of arsenate (Warburg and Christian, 1939), or by coupling to the reduced diphosphopyridine nucleotide (DPN·H) oxidation in the presence of α-glycerophosphate dehydrogenase (Racker, 1947). Phosphohexoseisomerase and phosphofructokinase were assayed by employing any one of the three methods described for aldolase.

A modification of the colorimetric aldolase procedure of Sibley and Lehninger (1949) was used to show triose phosphate isomerase. The method makes use of the fact that about 87% of the color is due to dihydroxyacetone phosphate (DHAP) when the two trioses are present in a 1:1 ratio. Hydrazine prevents the isomerase from operating by fixing both trioses in a 1:1 ratio. The equilibrium after isomerase reaction is overwhelmingly

in favor of DHAP. When hydrazine is omitted, the isomerase converts glyceraldehyde-3-phosphate to DHAP and, hence, more chromogen is formed in the absence of hydrazine than in its presence.

The presence of phosphoglyceromutase, enolase, and pyruvate kinase were determined by following the formation of pyruvate from 3-phosphoglycerate. Pyruvate was estimated by the colorimetric procedure of Friedemann and Haugen (1943). Sodium fluoride, a known inhibitor of enolase, was used to show inhibition of the reaction as well as inhibition of DPN.H oxidation in the presence but not in the absence of phosphate (Warburg and Christian, 1942) using 3-phosphoglycerate as substrate.

Alcohol dehydrogenase was assayed spectrophotometrically by following DPN^+ reduction at 340 mμ with alcohol as the substrate, and by following the oxidation of DPN·H with acetaldehyde as substrate (Racker, 1957). The reduction of pyruvate was tested by following DPN·H oxidation at 340 mμ. Lactic dehydrogenase was assayed using the procedure of Neilands (1955).

The phosphoroclastic reaction of cell-free extracts was tested manometrically by observing the formation of CO_2 and H_2 from pyruvate (Koepsell and Johnson, 1942) under a helium atmosphere, and acetyl phosphate formed in the reaction was measured colorimetrically by the hydroxamic acid method of Lipmann and Tuttle (1945).

DPN·H oxidase was assayed by following the oxidation of DPN·H at 340 mμ, and by oxygen uptake studies using conventional Warburg techniques. Flavoprotein catalysis was shown by inactivating the enzyme by removal of the flavin component with the acid-ammonium sulfate procedure

of Warburg and Christian (1938), and then activating the enzyme by addition of either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). The molar extinction coefficient for DPN·H was taken as 6.22×10^6 cm^2 per mole (Horecker and Kornberg, 1948).

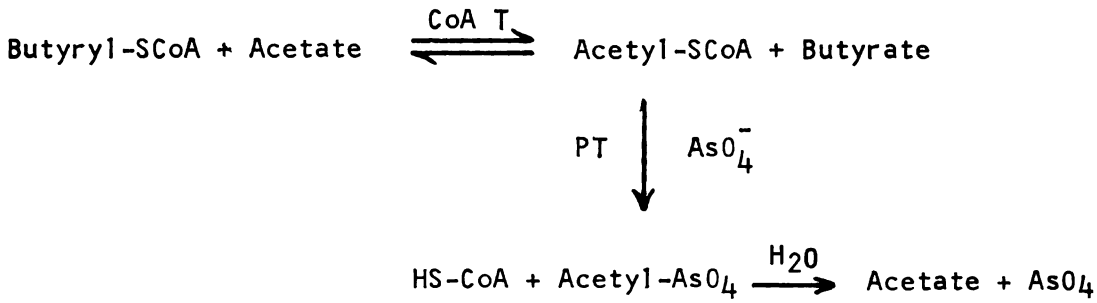
Diaphorase was determined by coupling to various redox dyes. The procedure in these studies was to follow colorimetrically the reduction of 2-6 dichlorophenolindophenol at 600 mμ with DPN·H as substrate. Other assays included observation of methylene blue reduction in an evacuated Thunberg tube using DPN·H as substrate, or following the reduction of triphenyl tetrazolium salts (TTZ). TTZ is colorless and soluble in the oxidized form, and red and insoluble when reduced (Quastel, 1957).

Acetokinase activity was assayed by the colorimetric method of Rose et al. (1954). The procedure is based on the ability of acyl phosphates to form hydroxamic acids rapidly at neutral pH which, in turn, react with trivalent iron to form a bright purplish complex in acid solution (Lipmann and Tuttle, 1945). The absorbancy of the color complex was related to a standard curve using succinic anhydride standards (Lipmann and Tuttle, 1945).

Phosphotransacetylase was measured by a modification (Baldwin, 1961) of a method by Stadtman (1955). The increase in optical density at 232 mμ due to formation of the thiol ester bond is followed; the reaction is:

$$\text{Acetyl phosphate} + \text{HS-CoA} \rightleftharpoons \text{Acetyl-SCoA} + \text{iP}$$

Coenzyme A transphorase (CoA T) was assayed by a method based on the decrease in optical density at 232 mμ when the reaction shown below is coupled with the arsenolysis of Acetyl-SCoA under the influence of phosphotransacetylase (PT) (Barker, Stadtman, and Kornberg, 1955).



The net reaction is an apparent hydrolysis of butyryl-SCoA.

RESULTS

Activities of Intact Vegetative Cells

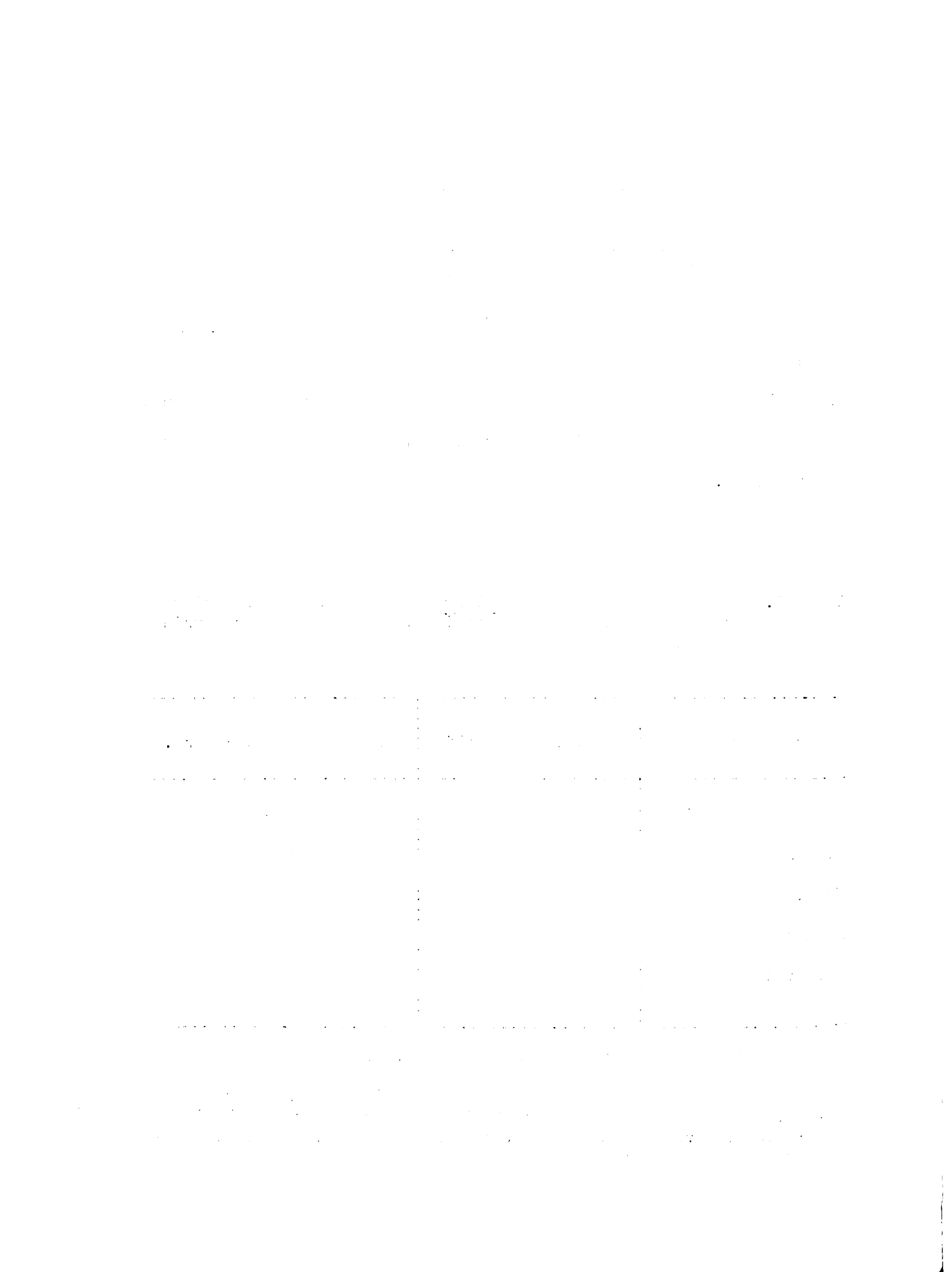
Utilization of sugars during growth. The utilization of glucose, fructose, mannose and galactose was tested by measuring growth response and the disappearance of these sugars during growth of C. botulinum (Table 1). Both glucose and fructose increased the amount of growth, and both sugars were consumed during the test. The presence of mannose and galactose appeared to inhibit growth slightly, and neither sugar was utilized.

TABLE 1. Growth response of C. botulinum to the presence of various sugars, and the per cent utilization of these sugars during growth.

Sugar added*	% Relative growth**	% Sugar utilized, 18 hrs.
None, basal medium	100	- -
Glucose	175	100
Fructose	140	65
Mannose	82	0
Galactose	91	0

*Initial sugar concentration was 0.2%.

**Relative growth was determined by dividing the increase in optical density (O.D.) due to growth in the test culture by the increase in O.D. of the control culture, then multiplying by 100. Values presented are averages of duplicate tubes.



Glucose fermentation by resting cell suspensions. Tests with cell suspensions were employed to obtain information on hexose fermentation. During these studies an extreme sensitivity of cells to oxygen was noted, so precautions were taken to protect cells from oxygen during handling procedures. For convenience, the cells packed during centrifugation of the growth medium were suspended in freshly boiled and cooled 0.2 M phosphate buffer, pH 7.0, containing 0.5% sodium thioglycollate, transferred immediately to Warburg reaction flasks, and gassed with helium for 10 minutes. Endogenous activity was insignificant if care was taken to quickly remove the spent medium by rinsing the pellet of cells with phosphate buffer before resuspending.

Table 2 shows that cells grown in the presence of glucose fermented glucose, whereas cells grown without added glucose did not ferment this sugar to a significant extent. These data suggested that an induced

TABLE 2. Glucose fermentation by cell suspensions of C. botulinum.

Activity	Glucose adapted cells		Non-adapted cells	
	Test	Endogenous	Test	Endogenous
µl Gas produced in 60 min at 37 C	245	20	30	10
µl Gas from glucose	225	- -	20	- -

Each test flask contained: 1 ml of 0.067 M phosphate buffer, pH 7.0; glucose, 10 µmoles; 32 mg dry wt of cells; and water to 3.0 ml. The endogenous control contained the same except for glucose. All flasks were flushed with helium gas for 10 min. Total gas produced was corrected by subtracting the gas released from comparable reaction flasks after tipping in 0.2 ml of 2 M H_2SO_4 initially and at the end of the test.

enzyme(s) must be produced before cells can ferment glucose, and further studies demonstrated that glucose non-adapted cells would adapt to glucose or fructose fermentation in the presence of a rich amino acid source, and that this induction process was inhibited by chloramphenicol, a known inhibitor of protein synthesis (Figure 1). Cells grown in the presence of glucose (Figure 2) or in the presence of fructose (Figure 3) fermented glucose and fructose from zero time without the induction lag, and these activities were not inhibited greatly by chloramphenicol. The data indicated that fructose will act almost as well as glucose as an inducer for the enzyme(s) which is adaptive in this system, but that fructose is a much poorer substrate for fermentation than is glucose.

Activities of Vegetative Cell-Free Extracts

Hexokinase. Extracts prepared from cells grown in a medium containing glucose were tested for their ability to phosphorylate glucose, fructose, mannose and galactose. Figure 4 shows that only glucose was phosphorylated to any extent, while fructose apparently was a poor substrate, and mannose and galactose remained unaltered under the conditions of the test. A different extract of cells grown in the presence of glucose failed to show any activity on fructose. These data suggested the presence of a glucokinase rather than a hexokinase which can ordinarily phosphorylate glucose, fructose and mannose.

Similar experiments were performed using extracts of cells grown in a medium devoid of added sugar, and extracts of cells grown in a medium containing fructose (Table 3). Extracts of cells grown in a

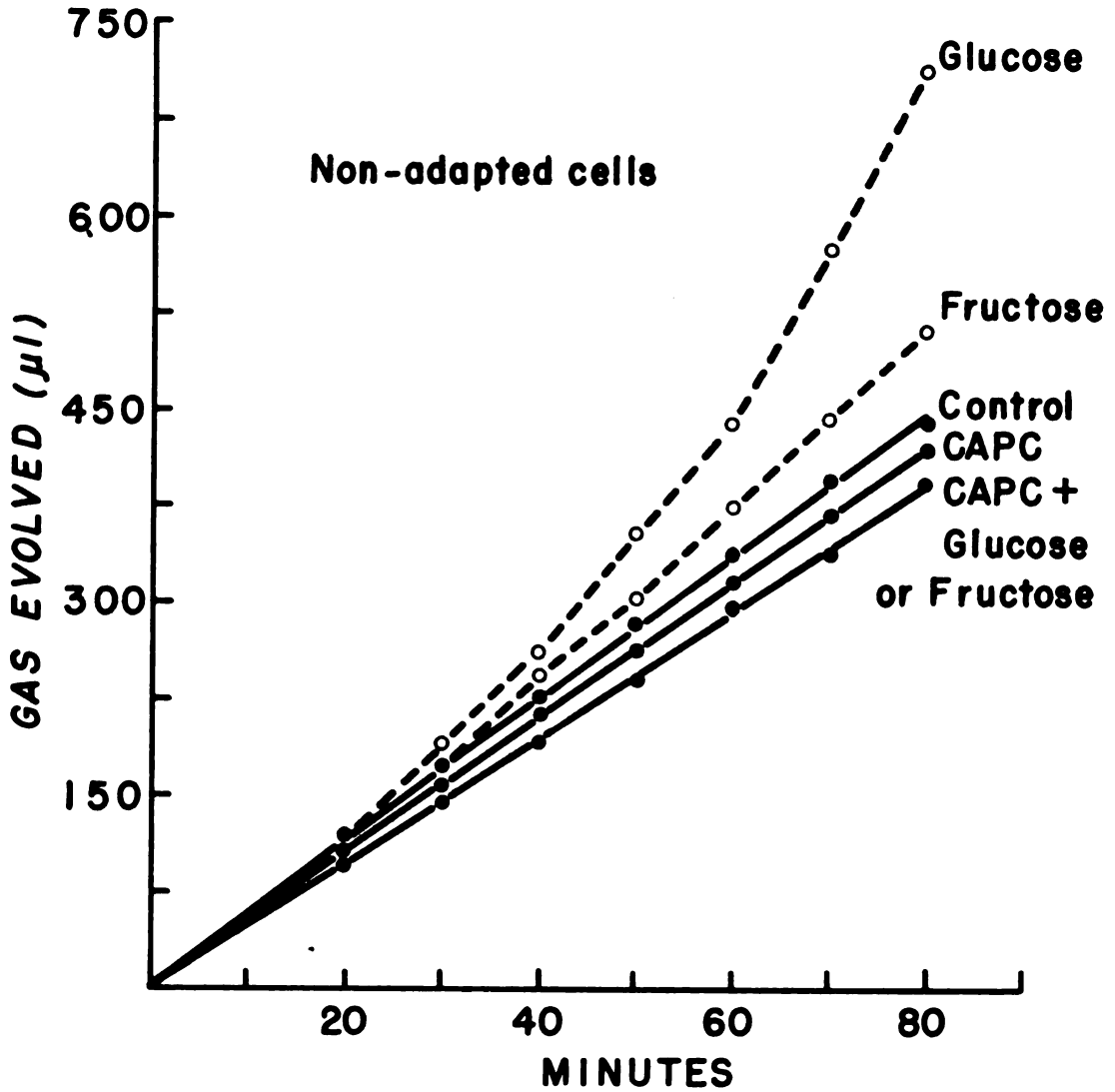


Figure 1. Induction of glucose fermentation and its inhibition by chloramphenicol. Reaction flasks contained: 1.0 ml of 0.067 M phosphate buffer, pH 7.0; 12 mg dry wt of cells; and 2% Trypticase. Where indicated the flasks contained 10 µmoles of either glucose or fructose, or 30 µg chloramphenicol (CAPC). All flasks had water to 3.0 ml.

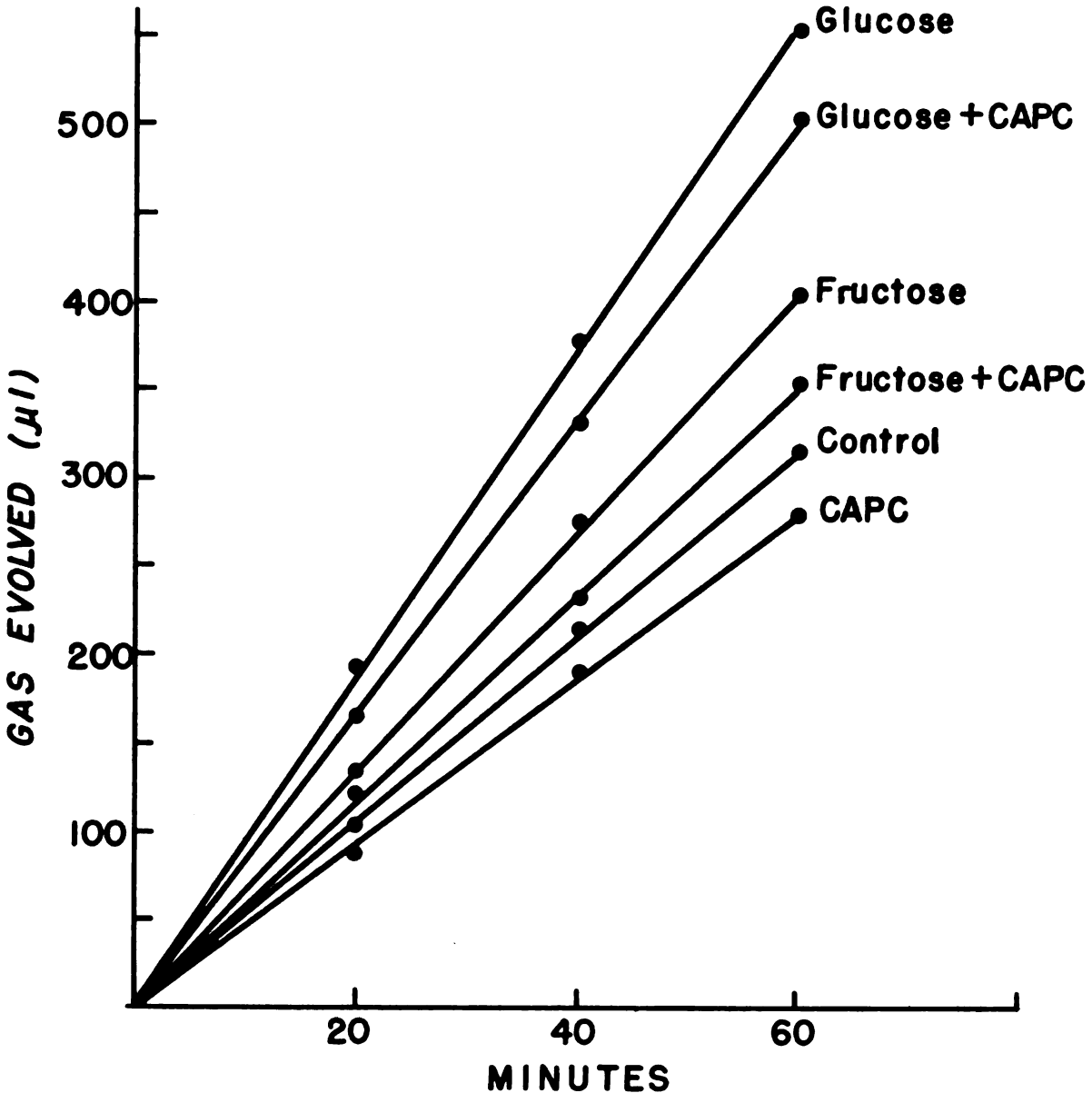


Figure 2. Fermentation of glucose and fructose by cells grown in the presence of glucose. Conditions: see Figure 1.

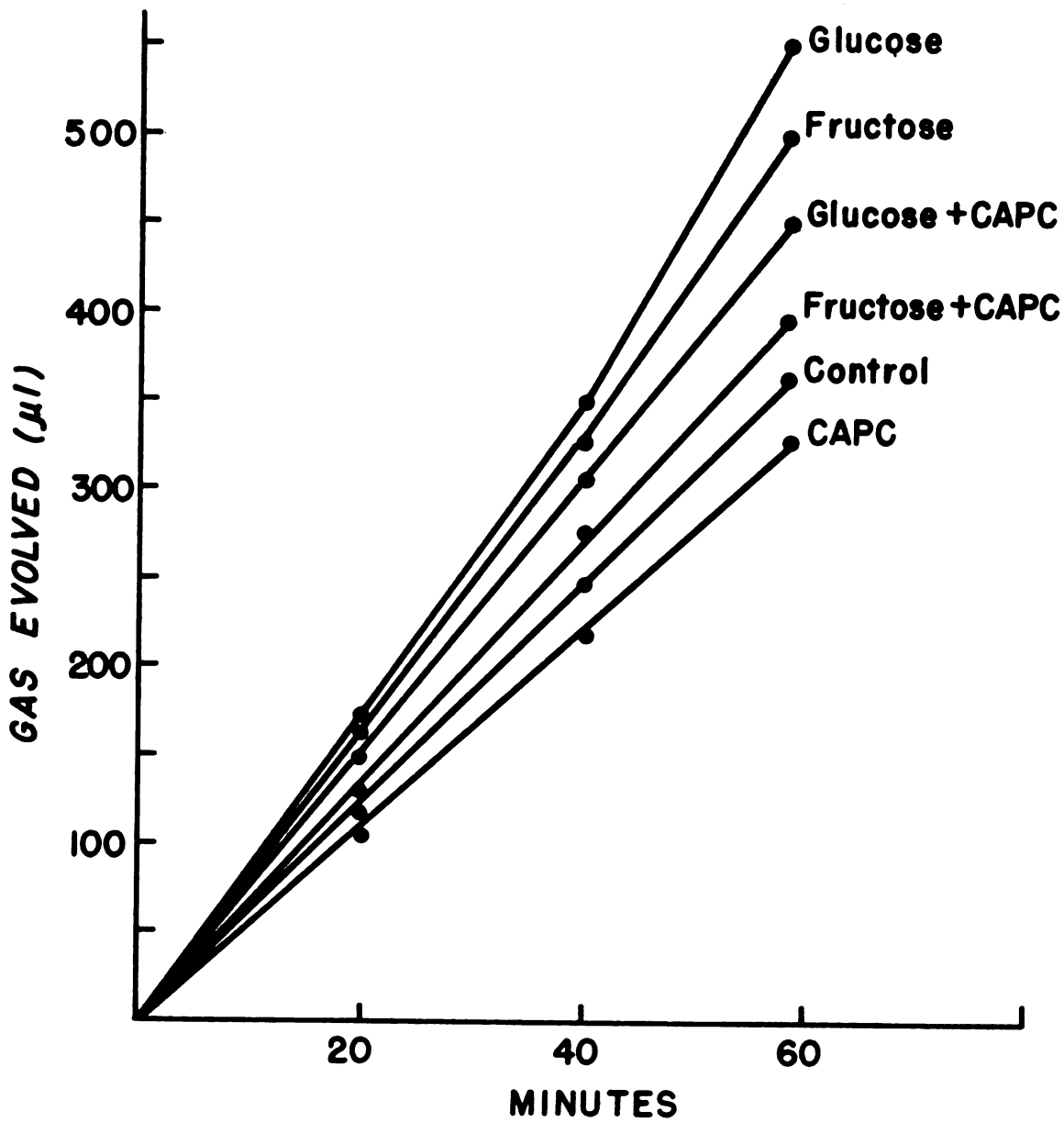


Figure 3. Fermentation of glucose and fructose by cells grown in the presence of fructose. Conditions: see Figure 1.

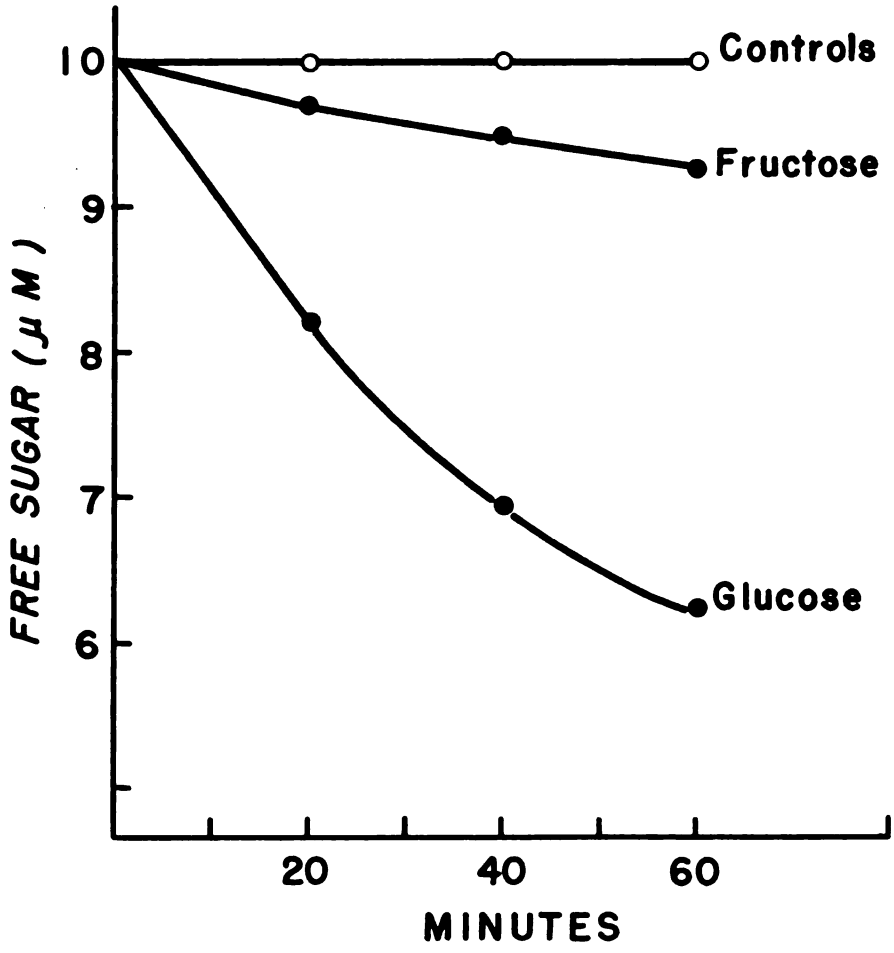


Figure 4. Phosphorylation of hexoses, measured by decrease in free reducing sugar, by a cell-free extract of C. botulinum. Reaction mixture contained: 1.0 ml of 0.067 M phosphate buffer, pH 7.4; sugars, 10 μmoles; Mg^{++}, 10 μmoles; ATP, 20 μmoles; NaF, 15 μmoles; 25 mg of extract protein, and water to 3.0 ml. Control reaction mixtures contained hexose but no ATP. Results with mannose and galactose in complete reaction mixtures were the same as the control.

TABLE 3. Phosphorylation of hexoses by cell-free extracts of C. botulinum.

Extracted cells grown on:	Substrate	Range: Disappearance of substrate (Δ μmoles)*
Basal	Glucose	0
Basal	Fructose	0
Glucose	Glucose	3.5 - 4.0
Glucose	Fructose	0 - 0.9
Glucose	Mannose	0
Glucose	Galactose	0
Fructose	Fructose	0
Fructose	Glucose	2.8
Fructose	Mannose	0
Fructose	Galactose	0

*Net activity as indicated by the total decrease in free reducing sugar over the control without ATP during 60 minutes incubation at 35 C with 25 mg extract protein.

medium devoid of sugar did not possess glucokinase, whereas extracts of cells grown in a medium containing glucose possessed a glucokinase that was active on glucose, had a low activity on fructose, but did not phosphorylate other sugars. Extracts of cells grown in the presence of fructose phosphorylated glucose but not fructose indicating that fructose acted as an inducer for the glucokinase, but was a poor substrate for the enzyme. This type of kinase is similar to the inducible enzyme

observed in C. tetani (Martinez and Rittenberg, 1959), and to that found in mutant strains of Pseudomonas putrefaciens (Klein, 1953).

On the hypothesis that glucokinase, in extracts of cells grown without glucose, may be present in amounts too low for detection by the assay used, a more sensitive test was tried. This second assay measures the glucose-6-phosphate (G-6-P) as the end product of the reaction by coupling to G-6-P dehydrogenase and then following the reduction of TPN^+ at 340 mμ. Extracts of glucose-adapted and non-adapted cells were tested in this way. Figure 5 reveals that the kinase was detected only in extracts of cells grown in a medium containing glucose, which substantiates the earlier evidence that glucokinase is an inducible enzyme in C. botulinum.

Oxidation of hexose phosphates. Extracts of cells grown in the presence of glucose catalyzed a rapid reduction of DPN^+ when fructose diphosphate (F-1-6-P) was used as the substrate (Figure 6). TPN^+ could not be substituted for DPN^+ in the reaction. Similar results were obtained when fructose-6-phosphate (F-6-P) and G-6-P plus ATP and Mg^{++} were substituted in place of F-1-6-P in the reaction mixture, thus indicating the presence of phosphohexoseisomerase, phosphofructokinase, and aldolase in the extract. Iodoacetate, a known inhibitor of glyceraldehyde-3-phosphate dehydrogenase added at zero time resulted in complete inhibition of the reaction. Tests for TPN^+ reduction with G-6-P as substrate were negative indicating the absence of G-6-P dehydrogenase in the extract. Therefore, the reaction sequence involved must be that of the EMP system.

A reaction system resulting in the reduction of DPN^+ with G-6-P, F-6-P, or F-1-6-P as substrate was also demonstrated with extracts of

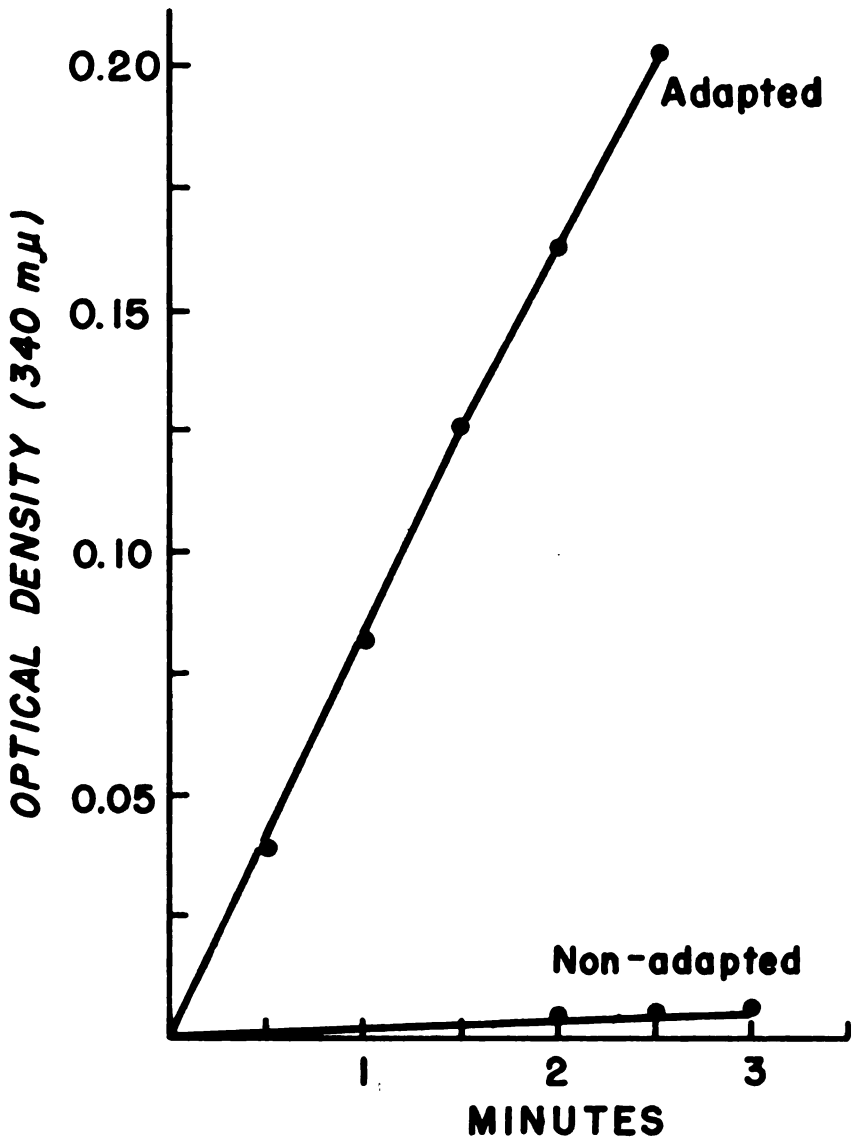


Figure 5. Glucokinase activity in cell-free extracts of glucose-adapted and non-adapted cells. Reaction mixtures contained: 1.0 ml of 0.2 M tris buffer, pH 7.4; glucose, 5 μmoles; ATP, 10 μmoles; Mg^{++}, 10 μmoles; TPN^{+}, 0.9 μmole; glucose-6-phosphate dehydrogenase, 0.01 ml of a 0.01% solution; extract protein, 12 mg; and water to 3.0 ml. The G-6-P dehydrogenase was devoid of hexokinase activity. Reaction temperature was 25 C.

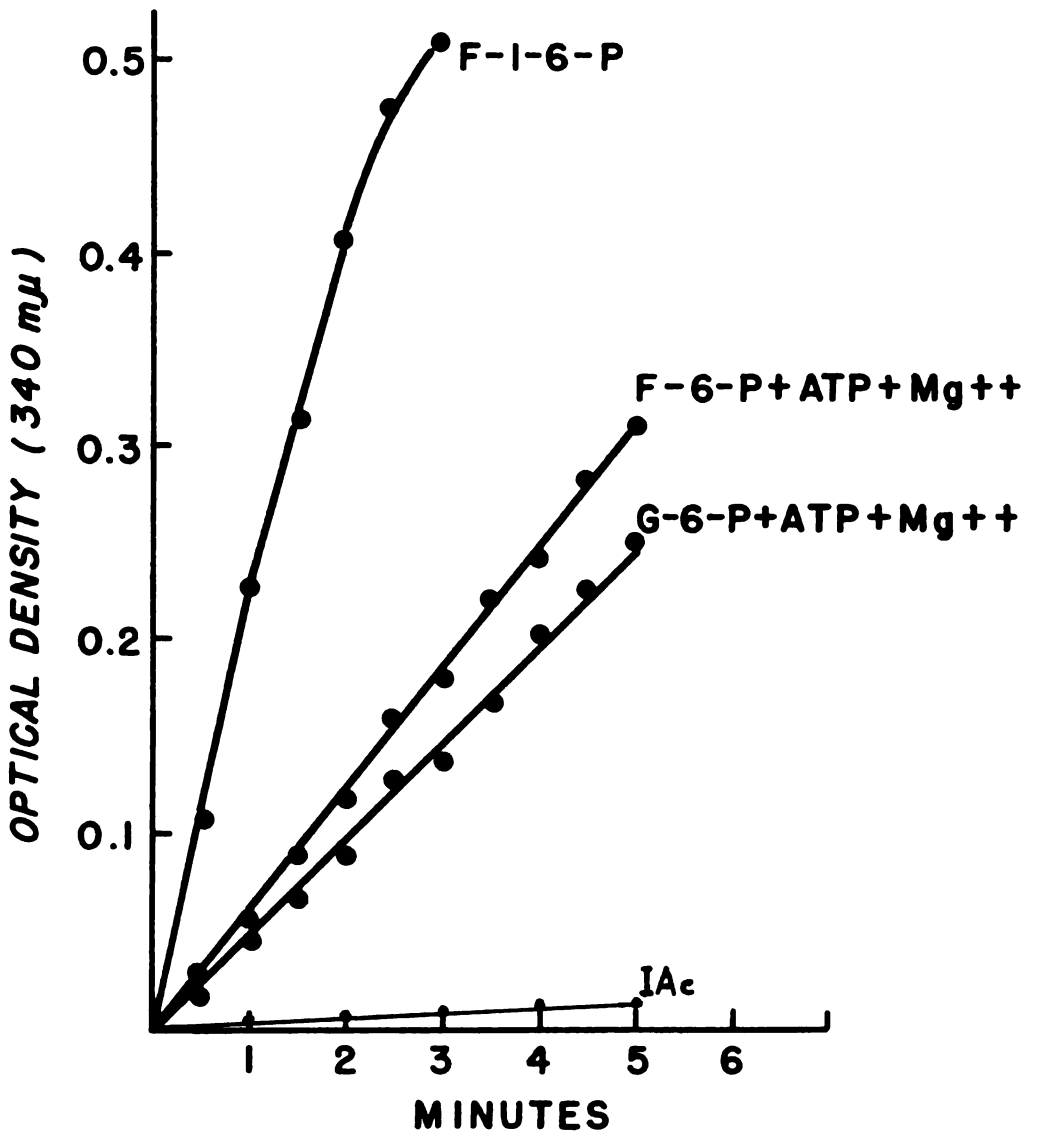


Figure 6. Reduction of DPN^+ by cell-free extracts of C. botulinum with hexose phosphates as substrates. Reaction mixtures contained: 1.0 ml of 0.1 M tris buffer, pH 7.4; hexose phosphates, 5 μmoles; arsenate, 20 μmoles; DPN^+, 1.0 μmole; extract protein, 5.4 mg; and water to 3.0 ml. Where indicated, 20 μmoles each of ATP and Mg^{++}, and 3.0 μmoles of iodoacetate (IAc) were added.

cells grown in the absence of glucose, but the reaction rate was considerably less than that observed with the extracts of glucose-adapted cells.

Phosphohexoseisomerase, phosphofructokinase and aldolase activities were also demonstrated using G-6-P as substrate and measuring DPN·H oxidation in the presence of an excess of α-glycerophosphate dehydrogenase, and by the colorimetric determination of triose phosphates formed.

Aldolase. The clostridial aldolase is inhibited by the metal-binding agent pyrophosphate, and by Zn^{++} and cysteine at high concentrations (Table 4). Zn^{++} at a lower concentration had no effect. A lower concentration of cysteine stimulated aldolase activity which could result from cysteine acting as a reducing agent and thereby protecting the sulfhydryl groups of the aldolase. The inhbition of aldolase at higher cysteine levels was probably due to its ability to act as a metal binder. Ferrous ions caused a three-fold stimulation of activity, and reversed the inhibition caused by cysteine and by pyrophosphate, provided they were added prior to pyrophosphate. Table 5 shows the effect of pH on aldolase activity, the highest activities obtained at pH 7.5 to 8.0. All of these data on aldolase are in complete agreement with those of Bard and Gunsalus (1950) who reported a metallo-aldolase in cell-free extracts of C. perfringens.

Extracts of cells grown in the absence of glucose also had significant levels of aldolase activity.

Triose phosphate isomerase. This enzyme was determined by using a modification of the method of Sibley and Lehninger (1949), as described in the section on methods. Omission of the hydrazine from the assay should produce about a 1.7 fold increase in chromogen over that of an

TABLE 4. Effect of various agents on aldolase of C. botulinum.

Additions	Concn., molar	Specific activity*
None	- -	.83
Fe^{++}	1×10^{-6}	2.40
Fe^{++} + Cysteine	$1 \times 10^{-5} + 1 \times 10^{-4}$	3.12
Fe^{++} + Cysteine	$1 \times 10^{-5} + 4 \times 10^{-3}$	.87
Cysteine	1×10^{-4}	.92
Cysteine	4×10^{-3}	.11
Pyrophosphate	1×10^{-3}	.57
Pyrophosphate	4×10^{-3}	.29
Fe^{++}** + Pyrophosphate	$1 \times 10^{-5} + 1 \times 10^{-3}$	1.10
Zn^{++}	1×10^{-3}	.25
Zn^{++}	1×10^{-6}	.81

*µmoles F-1-6-P split per hr per mg protein.

**Fe^{++} added before pyrophosphate.

Reaction mixtures contained: 1 ml of .1 M tris buffer, pH 7.4; F-1-6-P, 5 µmoles; hydrazine, 56 µmoles; 0.4 mg extract protein using an extract dialyzed against water for 12 hrs; and water to 2.5 ml. After 15 min incubation at 35 C, 1 ml aliquots were added to 2 ml of 10% trichloroacetic acid and analyzed for chromogen formed.

TABLE 5. Effect of pH on aldolase of C. botulinum.

pH of assay	Specific activity*
6.0	.70
6.5	.81
7.0	1.01
7.5	1.19
8.0	1.07
8.5	.95

*Units and conditions same as in Table 4.

assay with hydrazine. Data in Figure 7 show that about a 1.6 fold increase in color was obtained when the activity was tested in the absence of hydrazine.

Phosphoglyceromutase, enolase and pyruvate kinase. In the EMP scheme, 3-phosphoglycerate (3-PG) leads directly to pyruvate, and so it seemed feasible to test for phosphoglyceromutase, enolase, and pyruvate kinase by following the formation of pyruvate from 3-PG. Pyruvate was determined by the double extraction procedure of Friedemann and Haugen (1943), and was tentatively identified as the benzene and carbonate soluble 2-4 dinitrophenylhydrazone. Figure 8 not only shows the conversion of 3-PG to pyruvate, but also its inhibition by sodium fluoride (NaF) which was undoubtedly due to the inhibition of enolase. The extract used was of cells grown in the presence of glucose.

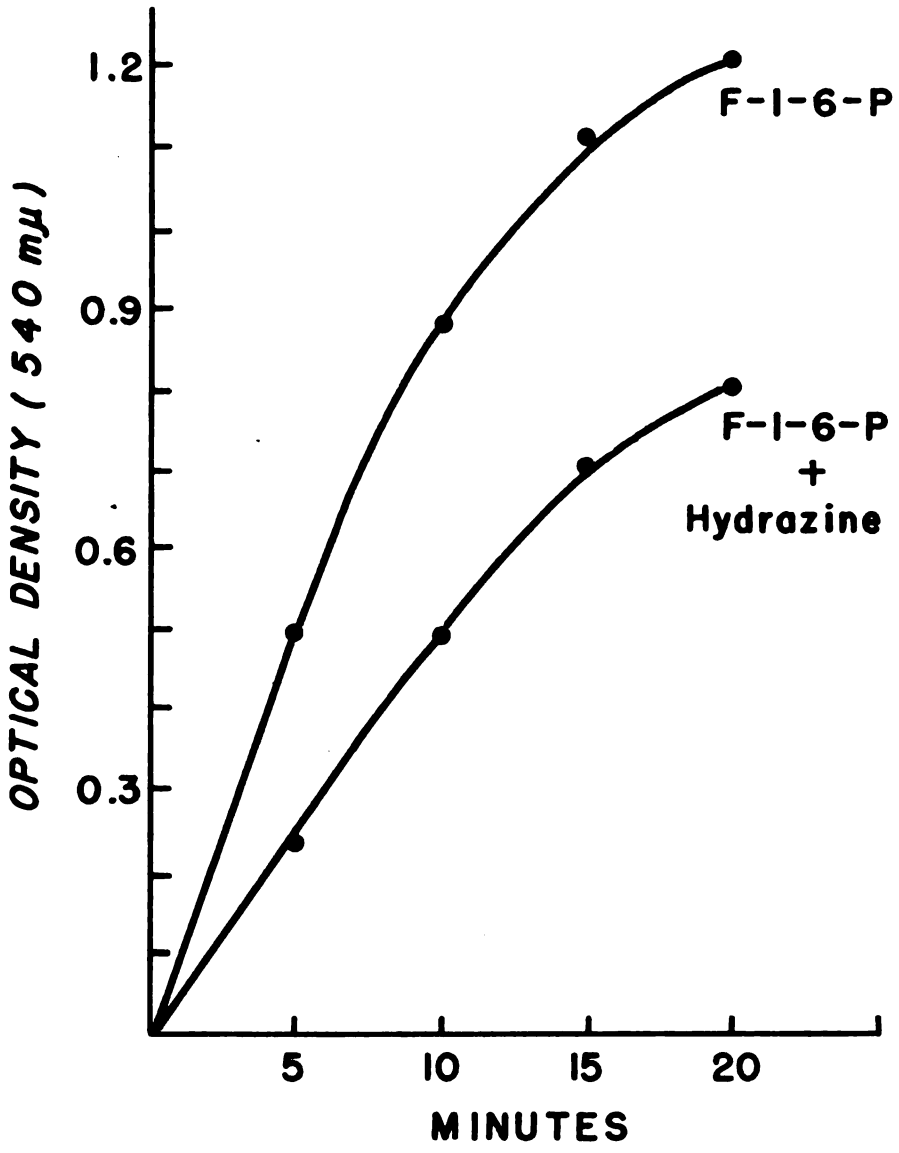


Figure 7. Chromogen formation from fructose diphosphate in the presence and absence of hydrazine. Reaction mixture contained: 3.0 ml of 0.1 tris buffer, pH 7.4; F-1-6-P, 5 μmoles; 0.75 ml of 0.56 M hydrazine; 2.0 mg extract protein using an extract dialyzed against water for 18 hours; and water to 7.5 ml. Incubation temperature was 35 C. One ml aliquots were removed at time intervals indicated, added to 2.0 ml of 10% trichloroacetic acid, and analyzed for chromogen formation.

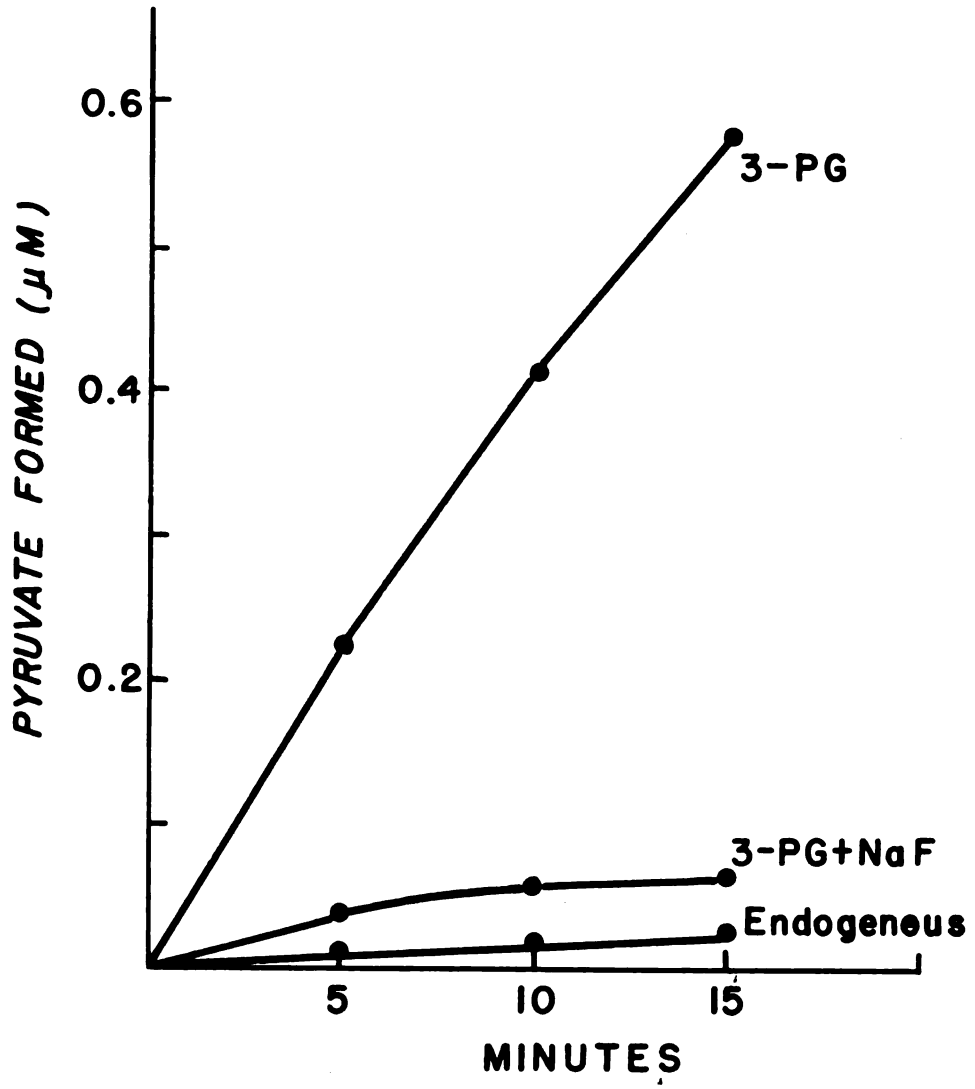


Figure 8. Conversion of 3-phosphoglycerate (3-PG) to pyruvate by cell-free extracts of C. botulinum. Reaction vessels contained: 0.5 ml of 0.2 M phosphate buffer, pH 7.5; ADP, 20 μmoles; Mg^{++}, 10 μmoles; and extract protein, 8 mg. NaF, 5 μmoles, and 3-PG, 25 μmoles were added where indicated. Volumes were adjusted to 2.0 ml. Incubation temperature was 35 C. Reactions were stopped by addition of 1.0 ml of 10% trichloroacetic acid.

Figure 9 presents further evidence for enolase by showing fluoride inhibition of DPN·H oxidation by extracts of glucose-adapted cells with 3-PG as substrate in the presence but not in the absence of phosphate (Warburg and Christian, 1942). When 15 μmoles of NaF were preincubated with the reaction mixture containing inorganic phosphate prior to addition of DPN·H, oxidation of the DPN·H proceeded at the endogenous rate. With the same system, but minus inorganic phosphate, the oxidation rate was equal to that in the absence of fluoride.

Evidence for 3-PG fermentation by extracts of cells grown in the absence of glucose was obtained by measuring the rate of DPN·H oxidation and correcting for endogenous activity. The fermentation rate was much slower than observed with extracts of glucose-adapted cells, but was significant.

Pyruvate fermentation. DPN·H oxidation by extracts of glucose-adapted cells was observed when pyruvate was used as the substrate, the rate being similar to that seen when 3-PG was used as the substrate. These data do not distinguish the probable routes of pyruvate reduction such as direct reduction to lactate, or decarboxylation to acetaldehyde and then reduction to ethanol, so a more comprehensive study of pyruvate breakdown was undertaken. That pyruvate was reduced to lactate seemed unlikely since all attempts to show lactic dehydrogenase in extracts were negative. Also, analyses for lactate in completed fermentation solutions, using whole cell suspensions, were negative. However, a pathway to ethanol became apparent when it was shown that extracts contained alcohol dehydrogenase. This activity could be shown by following DPN^+ reduction at 340 mμ with ethanol as substrate (Figure 10), since the

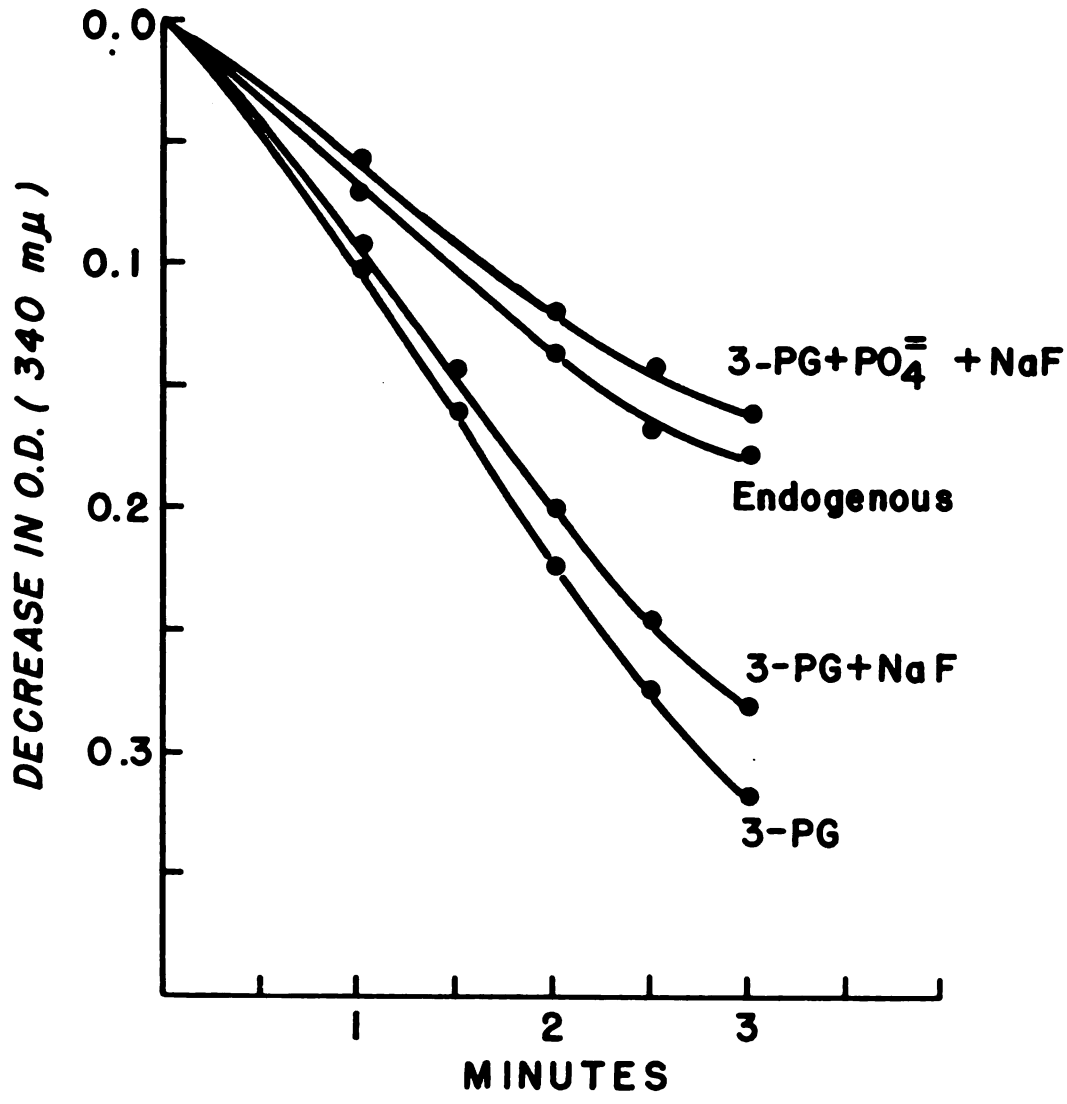


Figure 9. DPN·H oxidation, and its inhibition by fluoride, by a cell-free extract of C. botulinum with 3-phosphoglycerate (3-PG) as substrate. Reaction mixture contained: 1.0 ml of 0.1 M tris buffer, pH 7.4; Mg^{++}, 10 μmoles; cysteine, 20 μmoles; ADP, 10 μmoles; DPN·H, 0.6 μmole; and water to 3.0 ml. Where indicated 4 μmoles of 3-PG, 15 μmoles NaF, and 10 μmoles inorganic phosphate were added.

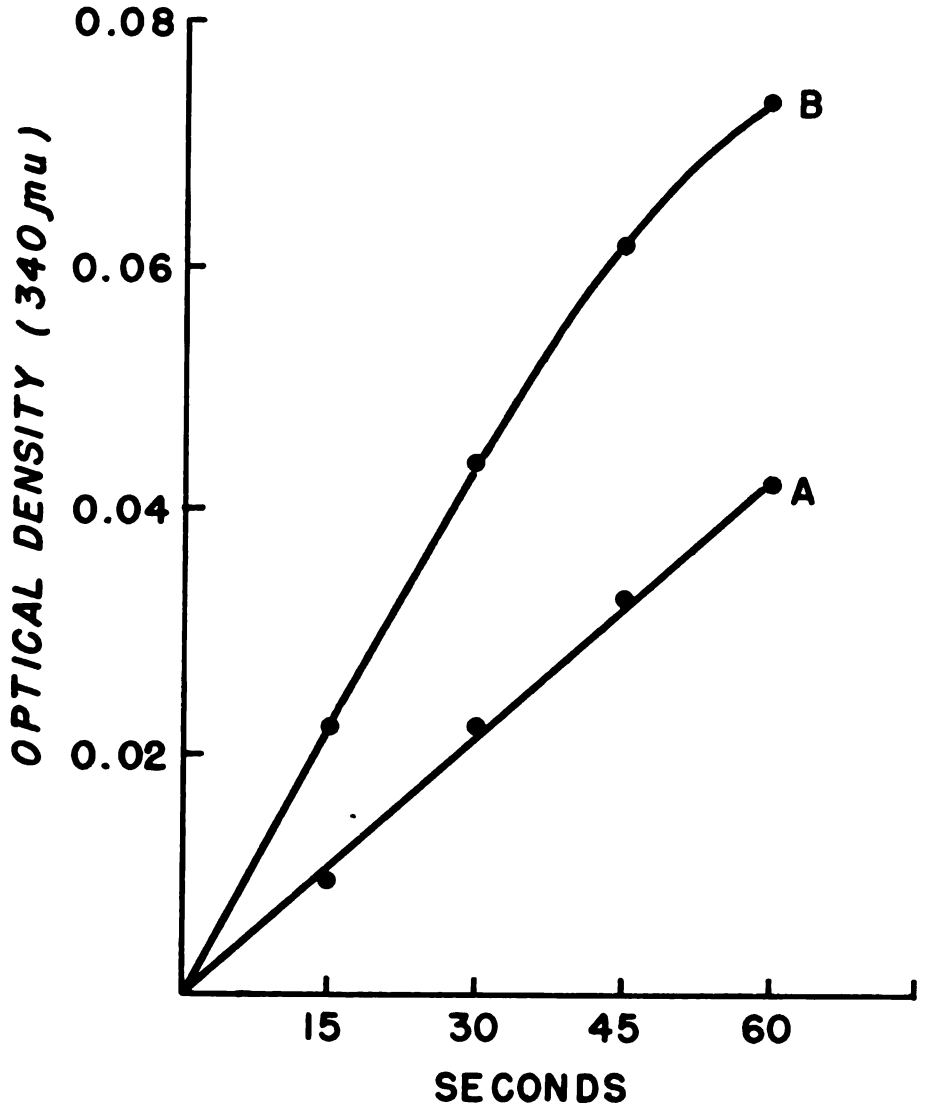


Figure 10. Alcohol dehydrogenase activity in cell-free extracts of C. botulinum. Reaction mixture contained: 1.0 ml of 0.2 M pyrophosphate buffer, pH 8.5; ethanol, 200 µmoles; DPN^+, 2.0 µmoles; and water to 8.0 ml. No activity was observed in the absence of ethanol. A = 2.0 mg extract protein; B = 4.0 mg extract protein.

accumulation of DPN·H without a lag indicated that alcohol dehydrogenase was present in fresh extracts in sufficient amounts to mask the competing endogenous DPN·H oxidation. The activity appeared higher when the reaction was run in the reverse direction, following DPN·H oxidation with acetaldehyde as substrate (Figure 11).

The pathway of pyruvate breakdown was further studied by testing the ability of cell-free extracts to cleave and oxidize pyruvate by the phosphoroclastic reaction. Table 6 shows the products formed in the cleavage of pyruvate, as well as some of the factors affecting the reaction. It appears that one mole each of CO_2, H_2, and acetyl phosphate is formed from one mole of pyruvate. The slightly higher amounts of CO_2 evolved is probably due to side reactions since there was good correlation between the amounts of H_2 and acetyl phosphate produced. The removal of either DPN^+ or phosphate from the reaction mixture resulted in almost complete inactivity, so these factors appeared to be essential. Dialysis of the extract resulted in almost complete loss of activity making it impossible to assess the true function of thiamine pyrophosphate and coenzyme A in the reaction. It may be that the crude extract contained excess CoA and ThPP, and so additions of these cofactors would show no effect on the reaction rate.

Arsenite inhibits the reaction about 50% when phosphate and DPN^+ are present. Exposure of the extract to air resulted in the loss of ability to produce acetyl phosphate and H_2, and only a small amount of CO_2 was evolved. This finding is similar to that of Wolfe and O'Kane (1955) who worked with cell-free extracts of C. butyricum. They reported that the CO_2 exchange with pyruvate survived aging, whereas the acetate exchange is only slightly retained indicating more stability of the CO_2

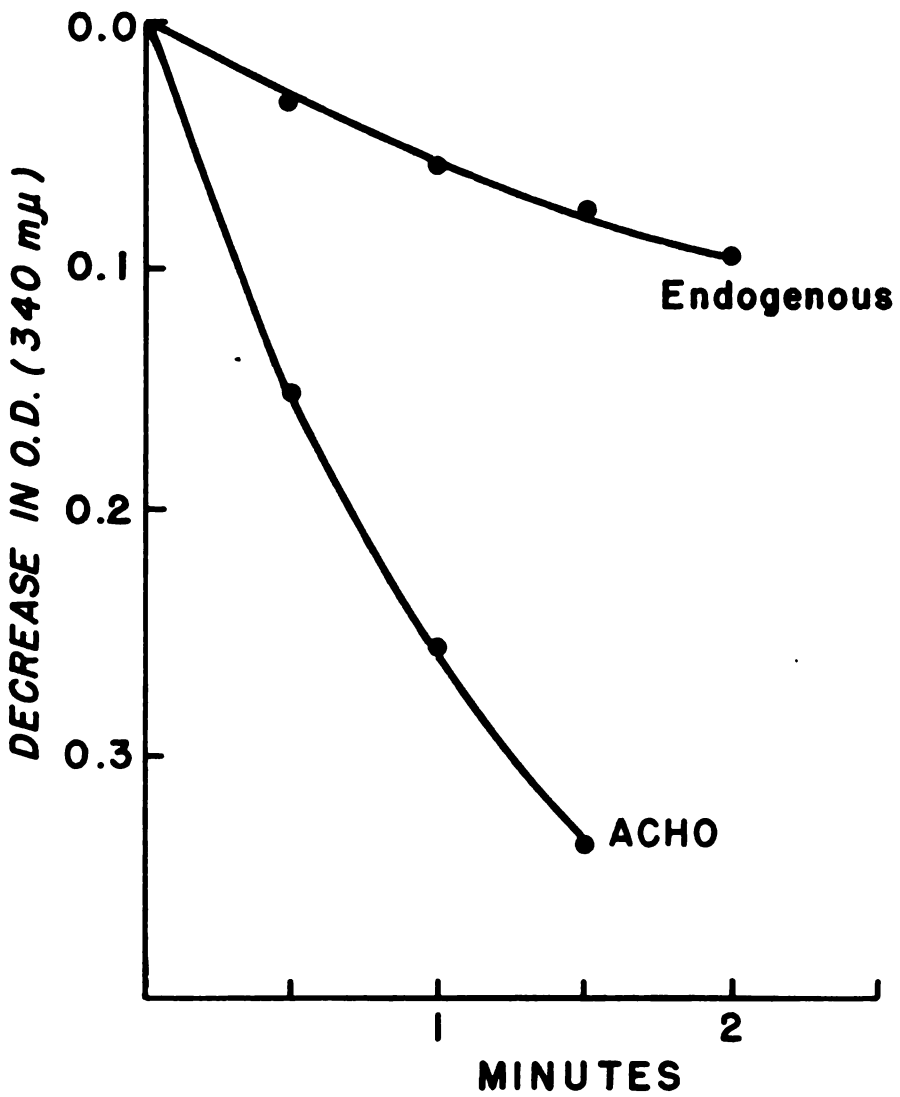


Figure 11. DPN·H oxidation by cell-free extracts of C. botulinum with acetaldehyde (ACHO) as substrate. Reaction mixture contained: 1.0 ml of 0.1 M pyrophosphate buffer, pH 7.0; acetaldehyde, 5 μmoles; DPN·H, 1.0 μmole; extract protein, 2.0 mg; and water to 3.0 ml.

exchange reaction. Ferrous ion was required, but had no affect on the CO_2 exchange with pyruvate. They suggested that in the forward reaction Fe^{++} functions in H_2 production after CO_2 has been liberated.

As pointed out in the historical review section, certain clostridia produce chiefly lactic acid in an iron-deficient medium instead of the

TABLE 6. Products formed by and the effect of some factors on the phosphoroclastic reaction.

Deletions, additions or special treatments	µmoles products formed in 30 min		
	CO_2	H_2	Acetyl phosphate
Complete sytem*	12.0	8.2	7.2
- Enzyme	0.0	0.0	0.0
+ Arsenite, 5 µmoles	5.9	4.3	4.0
- CoA, ThPP	11.1	7.9	7.0
- DPN	0.7	0.5	0.5
- Phosphate buffer**	1.6	1.3	1.1
Dialyzed extract, 8 hrs at 4 C	1.4	0.6	0.5
Extract exposed to air, 1.5 hrs at 35 C	2.2	0.0	0.0

*Complete reaction mixture contained: 1 ml of 0.2 M phosphate buffer, pH 6.9; pyruvate, 100 µmoles; 25 mg extract protein; DPN, 2 µmoles; coenzyme A (CoA), 0.3 µmole; thiamine pyrophosphate (ThPP), 0.5 µmole; and water to 3.0 ml. Cups for measuring H_2 had 0.2 ml of 20% KOH in the center well; incubation temperature was 35 C. Acetyl phosphate was determined by the hydroxamate method of Lipmann and Tuttle (1945).

**Tris buffer used in place of phosphate buffer.

usual mixture of H_2, CO_2, solvents, acetic, lactic, and butyric acids. This could be explained by the fact that in iron deficiency, the pyruvate formed by glycolysis cannot be removed by the phosphoroclastic reaction, a Fe^{++} requiring system, and hence is reduced to lactate.

DPN·H oxidase. A high endogenous DPN·H oxidation was noted during a number of assay procedures using dialyzed extracts. This suggested the presence of a DPN·H oxidase, and inactivation studies (Table 7) indicated that the activity was enzymatic and not due to non-specific action on the DPN·H by the extract. That the decrease in optical density at 340 mμ was due to DPN·H oxidation and not degradation of the DPN·H was shown by testing the DPN^+ formed in the reaction as a substrate for

TABLE 7. Effect of heat on a DPN·H oxidizing system in cell-free extracts of C. botulinum.

Heat treatment	Relative activity*
None	4.80
60 C, 1 min	3.30
65 C, 1 min	1.98
70 C, 30 sec	0.90

*Change in optical density per min x 10^2

Reaction mixtures contained: 1 ml of 0.067 M phosphate buffer, pH 7.4; DPN·H, 0.3 μmole; 0.1 ml extract; and water to 3.0 ml. Change in O.D. at 340 mμ was followed; reaction temperature was 25 C.

alcohol dehydrogenase. Additions of ethanol and alcohol dehydrogenase caused a rapid and complete restoration in absorption at 340 mμ (Figure 12), and this was due to DPN^+ reduction in the alcohol dehydrogenase system. TPN·H could also serve as electron donor for the oxidase; cyanide, a cytochrome poison, did not inhibit the activity, and cytochrome c was not reduced.

Oxygen uptake studies showed that 0.041 μmole O_2 per min per mg protein was used in the reaction, while 0.043 μmole DPN·H per min per mg protein was oxidized during spectrophotometric studies at 340 mμ. The results indicated a two-electron reduction of oxygen by the DPN·H oxidase which should result in formation of hydrogen peroxide.

Proof of flavoprotein catalysis was shown by removal of the flavin component of the oxidase, and then adding back either FAD or FMN. There was no activity without addition of cofactors (Table 8); FAD restored the activity to the original level, and FMN partially reactivated the enzyme. Addition of FAD to dialyzed extracts resulted in a 2 to 3 fold increase in activity, while FMN caused only a slight increase.

Diaphorase. The ability of cell-free extracts to cause a rapid reduction of 2-6 dichlorophenolindophenol with DPN·H as substrate indicated the presence of diaphorase activity (Table 9). Extract alone did not reduce the dye. Addition of FAD or FMN stimulated the activity slightly; heating the extract for one minute at 65 C resulted in loss of one-half the initial activity. Methylene blue and triphenyl tetrazolium salts could also act as electron acceptors.

Enzymes involved in metabolic utilization of acetate. Since acetyl phosphate was formed in the phosphoroclastic cleavage of pyruvate, interest

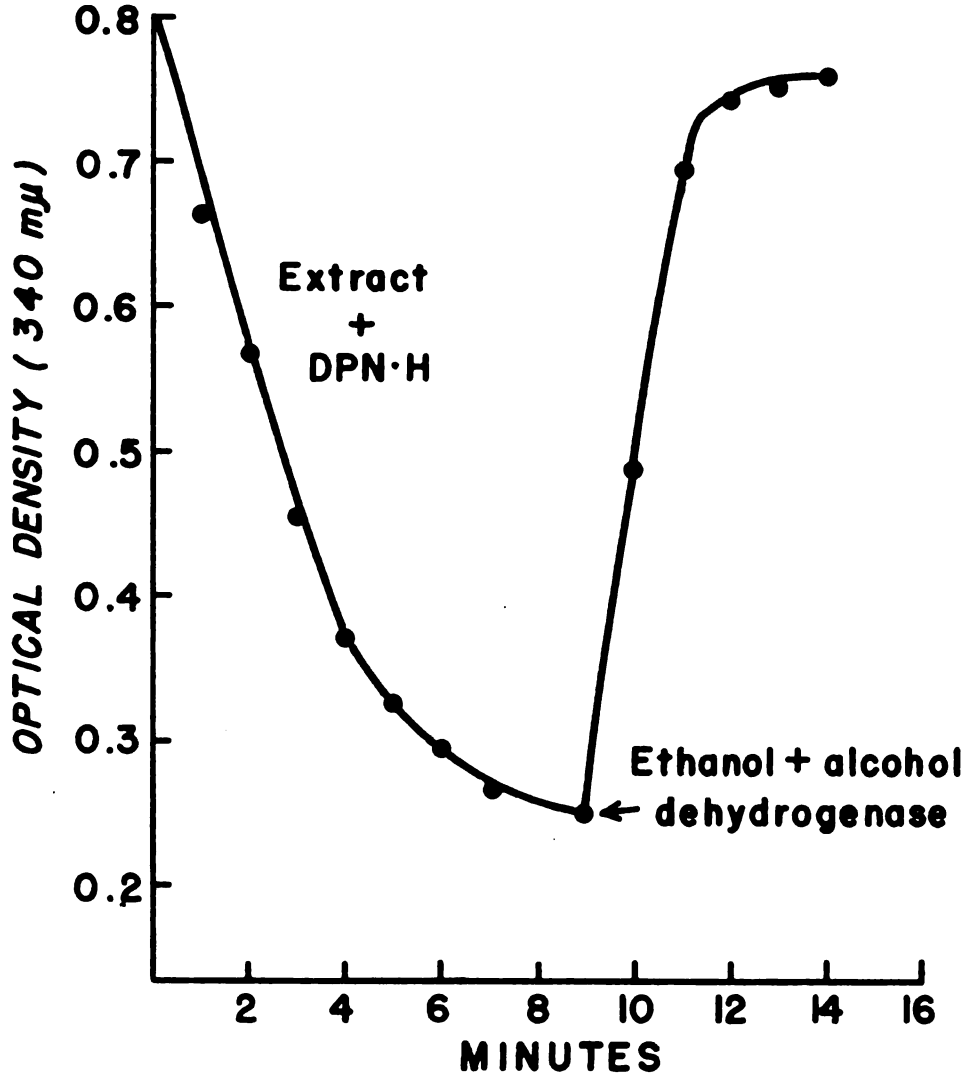


Figure 12. Reduction of DPN^+, formed in the DPN·H oxidase reaction, by alcohol dehydrogenase. Reaction mixture contained: 1.0 ml of a 0.2 M phosphate buffer, pH 8.0; DPN·H, 0.5 μmole; extract protein, 6.0 mg; and water to 3.0 ml. After reaction stopped, the test solution was heated to inactivate the oxidase and then 50 μmoles of ethanol and 0.05 ml of a 0.01% solution of alcohol dehydrogenase were added.

TABLE 8. Cofactor requirement for the DPN.H oxidase in cell-free extracts of C. botulinum.

Cofactor added	Concn., µmole	Specific activity*
None	- -	0
FAD	0.6	.080
FMN	0.6	.044

*µmoles DPN·H oxidized per min per mg protein

Reaction mixture contained: 1 ml of 0.067 M phosphate buffer, pH 7.4; DPN·H, 0.3 µmole; 1.2 mg extract protein, plus cofactors as indicated, and water to 3.0 ml. Change in O.D. at 340 mµ was followed; reaction temperature was 25 C. The flavin component was removed from the oxidase by the acid-ammonium sulfate treatment of Warburg and Christian (1938).

TABLE 9. Diaphorase activity in cell-free extracts of C. botulinum.

Treatment	Relative activity*
None	.44
FAD added	.56
FMN added	.49
Extract heated 1 min at 65 C	.23

*Change in optical density per min.

Reaction mixture contained: 2.0 ml of 0.1 M phosphate buffer, pH 7.4; DPN·H, 0.3 µmole; FAD or FMN where indicated 0.06 µmole; 0.05 ml extract; 2-6 dichlorophenolindophenol and water to 4.0 ml. Change in optical density at 600 mµ was followed; reaction temperature was 25 C. Extract was dialyzed against distilled water at 5 C for 15 hours.

was focused on the enzyme(s) involved in its formation, and the possible role of acetyl phosphate in energy production. Phosphotransacetylase which catalyzes the transfer of acetyl from acetyl phosphate to coenzyme A, and acetokinase which couples acetyl phosphate to ATP formation have been implicated in these mechanisms, so tests were performed to measure these activities.

Data in Table 10 shows that extracts contain high acetokinase activity dependent upon Mg^{++} and ATP. ADP could not be substituted for ATP, and CoA appeared to lower the activity, but not significantly.

TABLE 10. Phosphorylation of acetate by acetokinase in cell-free extracts of C. botulinum.

Factor added	Specific activity*
None	0.0
Mg^{++}	0.0
ATP	0.0
ATP + Mg^{++}	2.0
ADP	0.0
ADP + Mg^{++}	0.0
CoA + ATP + Mg^{++}	1.6

*μmoles acetyl phosphate formed per min per mg protein.

Reaction mixtures contained: 1.0 ml of 0.1 M tris buffer, pH 7.4; potassium acetate, 200 μmoles; hydroxylamine, 600 μmoles; 0.27 mg extract protein; and where indicated, $MgCl_2$, 15 μmoles; ATP, 20 μmoles; ADP, 15 μmoles, coenzyme A, 0.03 μmole, and water to 1.0 ml. Extract was dialyzed against water at 5 C for 15 hours. Incubation temperature was 30 C; reaction was stopped by addition of 1.0 ml of 10% trichloroacetic acid.

Table 11 demonstrates that cell-free extracts contain an active phosphotransacetylase. Apparently C. botulinum forms acetyl-coenzyme A by the coupled action of acetokinase and transacetylase.

TABLE 11. Phosphotransacetylase in cell-free extracts of C. botulinum.

System	Specific activity*
Complete	18.9
with 2x protein	20.0
less CoA	0.0
less acetyl phosphate	0.0

*Change in optical density per min per mg protein.

Reaction mixtures contained: 0.02 ml of 0.1 M tris buffer, pH 8.0; reduced CoA, 0.06 µmole; dilithium acetyl phosphate, 10 µmoles; 0.32 µg extract protein, or as indicated; and water to 0.2 ml. Reaction temperature was 25 C. Change in optical density at 232 mµ was followed due to formation of the thiol ester bond of acetyl coenzyme A.

Role of coenzyme A in fatty acid activation. With the knowledge that cell-free extracts of C. botulinum have enzymes capable of forming acetyl-coenzyme A, it seemed that extracts may also contain enzymes involved in fatty acid synthesis, especially since acetyl-coenzyme A has been implicated in fatty acid activation. C. kluyveri extracts have been shown by Stadtman and Barker (1950) to contain enzymes that transfer

the phosphoryl group from acetyl phosphate to fatty acids containing from 3 to 8 carbon atoms. Furthermore, Stadtman (1953) showed that coenzyme A and phosphotransacetylase are required, along with an enzyme called coenzyme A transphorase that transfers the SCoA group from acetate to several acids such as propionic or butyric, to name a few.

Table 12 shows that extracts of C. botulinum contain coenzyme A transphorase. The enzyme catalyzed the transfer of the SCoA group between butyryl-SCoA and acetate.

TABLE 12. Coenzyme A transphorase in cell-free extracts of C. botulinum.

System	Specific activity*
Complete	2.7
less enzyme	0
less butyryl-SCoA	0
less acetate	0

*Change in optical density per min per mg protein.

Reaction mixtures contained: potassium acetate, pH 8.0, 30 μmoles; potassium arsenate, pH 8.0, 150 μmoles; butyryl-SCoA, 0.01 μmole; phosphotransacetylase, 0.01% of a 300-fold purified enzyme from Peptostreptococcus eldsdenii; 16 μg extract protein; and water to 0.15 ml. Reaction temperature was 25 C. The decrease in optical density at 232 mμ due to the arsenolysis of acetyl-SCoA was followed.

Activities of Spore and Germinated Spore Extracts

One objective of this study was to determine whether spores of C. botulinum contain enzymes, and, if so, how their activities might compare to those found in cell-free extracts. Secondly, it seemed desirable to compare relative enzyme activities of germinated spores to those of the cell and spore, since these data could give information on enzyme synthesis in germinating spores. In general, there is a lack of data on enzymes of spores and germinated spores of anaerobes. Other than the report that a glucose-fermenting system present in spores is activated upon germination (Costilow, 1960), there is no conclusive evidence for a glycolytic pathway in spores of C. botulinum.

In this study, glucokinase was not detected in either spore or germinated spore extracts even when employing the sensitive assay of coupling to the G-6-P dehydrogenase system. It should be pointed out, however, that the spores studied were produced in a medium devoid of glucose or fructose. Attempts to sporulate the culture in the presence of glucose were not very successful.

Attempts to show aldolase activity in spore extracts by coupling to DPN^+ reduction at 340 mμ in the presence of arsenate were not completely satisfactory. Long lags in activity were often observed, and when DPN^+ reduction was noted, the small changes in optical density reflected extremely low activity. It was felt that aldolase activity might be better tested by the more sensitive procedure of coupling to DPN·H oxidation in the α-glycerophosphate dehydrogenase system. Any trioses formed should contain more dihydroxyacetone phosphate (DHAP) than glyceraldehyde-3-phosphate since the equilibrium of isomerase favors DHAP. While trying

this assay, it soon became apparent that spore-extracts contained an active DPN·H oxidase that greatly interferred with the test. The oxidase activity was high enough to mask some of the DPN·H accumulation in the first assay, and appeared as a high endogenous rate in the second assay.

The method which overcame the interferences mentioned was the colorimetric aldolase procedure of Sibley and Lehninger (1949). Reaction times were selected which allowed accumulation of measurable quantities of triose phosphates. Table 13 shows that low, but significant aldolase activity was detected, that Fe^{++} stimulates activity about four-fold, while controls were essentially negative. When F-6-P or G-6-P plus

TABLE 13. Aldolase, phosphohexoseisomerase, and phosphofructokinase in extracts of spores and germinated spores of C. botulinum.

Substrates and cofactors added	Range of specific activities*	
	Spore	Germinated spores**
None	.01	.02
G-6-P, ATP, Mg^{++}, Fe^{++}	.047 - .07	.05 - .10
F-6-P, ATP, Mg^{++}, Fe^{++}	.035 - .08	.06 - .11
F-1-6-P	.060 - .11	.33 - .50
F-1-6-P, Fe^{++}	.250 - .45	.50 - .60

*μmoles F-1-6-P split per hour per mg protein.

**Spores germinated in Trypticase solution for 2.5 hours.

Reaction mixtures contained: 1.0 ml of 0.1 M tris buffer, pH 7.4; substrates, 5 μmoles; hydrazine, pH 7.5, 56 μmoles; 2.0 mg extract protein; and water to 2.5 ml. Cofactors were added as indicated: ATP, 5 μmoles; Mg^{++}, 15 μmoles; and Fe^{++}, 1 μmole. Incubation temperature was 35 C; reactions were stopped by additions of 2.0 ml of 10% trichloroacetic acid, and 1.0 ml aliquots were removed and analyzed for triose phosphate chromogen.

ATP and Mg^{++} were substituted for F-1-6-P, trioses were formed thus indicating the presence of hexoseisomerase and phosphofructokinase in spore extracts. Similarly, these three enzymes were detected in germinated spore extracts.

Enzymes of the final steps in glycolysis were not detected using conventional methods, but detection of hexoseisomerase, phosphofructokinase and aldolase, plus the demonstration of DPN^{+} reduction with F-1-6-P as substrate indicates that at least some of the EMP enzymes are present. More sensitive methods might demonstrate the remainder of the enzymes of the glycolytic system.

The detection of DPN·H oxidase in spore extracts prompted further study of the enzyme since a similar activity was found in cell-free extracts. Of special interest was the high level of activity and high heat resistance of the spore DPN·H oxidase (Table 14). The extracted

TABLE 14. Effect of heat on DPN·H oxidase in extracts of spores and germinated spores of C. botulinum.

Heat treatment	Relative activities*		
	Spore	Germinated spores	
		#1	#2
None	5.0	4.1	2.5
70 C, 1 min	5.5	4.0	0.7
80 C, 1 min	3.5	2.0	0
85 C, 5 min	2.8	0.8	0
90 C, 1 min	2.2	0	0

*Change in optical density per min x 10^2.

#1 = Spores germinated in acetate solution for 8 hours.

#2 = Spores germinated in Trypticase solution for 2.5 hours.

Reaction mixtures contained: 1 ml of 0.067 M phosphate buffer, pH 7.4; DPN·H, 0.3 µmole; 0.1 ml extract; and water to 3.0 ml. Change in O.D. at 340 mµ was followed; reaction temperature was 25 C.

enzyme from the spore remained active after heating for 1 minute at 80 C, whereas the enzyme from the cell did not. Of the spore enzymes demonstrated in this study, DPN·H oxidase was the only one exhibiting a high heat resistance.

Evidence presented in Table 14 suggests that the heat stability of the DPN·H oxidase is lost upon germination of the spores. Germination of spores in an acetate solution was slow and incomplete, and hence the heat resistance of this preparation was probably attributable to contamination with spore DPN·H oxidase from spores which did not germinate. In the Trypticase germinating solution spores germinated very well in 2.0 to 2.5 hours, and the heat resistance of extracted oxidase was low, similar to that of the oxidase extracted from cells, i.e., these oxidases were not active after heating 1 minute at 80 C. Whether the oxidase of spores, germinated spores, and vegetative cells is the same enzyme with different levels of heat resistance was not determined since this difficult problem was beyond the scope of this study.

The DPN·H oxidase of spores and germinated spores appears to be a flavoprotein since quinacrine (atabrine), a flavin analogue (Hellerman et al., 1946) inhibits the enzyme and FAD causes a slight stimulation of activity. The stimulation of spore DPN·H oxidase by FAD is not pronounced as it is with the cell DPN·H oxidase. KCN, a cytochrome poison, had no affect on activity. Dipicolinic acid (DPA) has been reported to stimulate the spore DPN·H oxidase of B. cereus (Doi and Halvorson, 1961), but it did not stimulate the clostridial spore oxidase. A summary of these findings is found in Table 15.

TABLE 15. Effect of various agents on the DPN·H oxidase in extracts of spores and germinated spores of C. botulinum.

Additions	Concentrations, M	Specific activities*		
		Spore	Germinated spores	
			#1	#2
None	- -	5.5	4.1	2.5
FAD	6×10^{-4}	6.0	4.4	3.3
FMN	6×10^{-4}	5.8	4.1	3.0
KCN	3×10^{-3}	5.6	4.1	2.6
DPA	4×10^{-3}	5.5	4.2	2.5
Atabrine	1×10^{-3}	3.3	2.0	1.3
Atabrine	1.5×10^{-3}	1.8	- -	- -
Atabrine	2×10^{-3}	0.8	- -	- -

*Change in optical density per min per mg protein x 10^2.

#1 = spores germinated in acetate solution.

#2 = spores germinated in Trypticase solution.

All reaction mixtures contained: 1.0 ml of 0.067 M phosphate buffer, pH 7.4; DPN·H, 0.3 µmole; extract and water to 3.0 ml. Extracts were dialyzed against distilled water at 5 C for 12 hours. Reaction temperature was 25 C; decrease in optical density at 340 mµ was followed.

Spore and germinated spore extracts possess diaphorase activity (Table 16) similar to that of vegetative cells in that 2-6 dichlorophenolindophenol, methylene blue and triphenyl tetrazolium salts can all act as electron acceptors when DPN·H is the substrate. Another

TABLE 16. Diaphorase in extracts of spores and germinated spores of C. botulinum.

Treatment	Relative activities*		
	Spore	Germinated spore #1	#2
None	.40	.37	.60
FAD added	.45	.40	.64
FMN added	.41	.37	.61
Extract heated 1 min at 65 C	.22	.19	.28

*Change in optical density per min.

#1 = Spores germinated in acetate solution.

#2 = Spores germinated in Trypticase solution.

Reaction mixtures contained: 2.0 ml of 0.1 M phosphate buffer, pH 7.4; DPN·H, 0.3 μmole; FAD or FMN, as indicated, 0.06 μmole; 0.1 ml extract; 2-6 dichlorophenolindophenol and water to 4.0 ml. Change in optical density at 600 mμ was followed; reaction temperature was 25 C. Extracts were dialyzed against distilled water at 5 C for 15 hours.

similarity is the heat resistance of these systems; the half-life for all diaphorase preparations studied was approximately 1 minute at 65 C.

One difference was the apparent lower specific activity of the spore diaphorase as compared with the cell or germinated spore activities which were similar.

Acetokinase could readily be detected in both spore and germinated spore extracts. The enzyme is ATP and Mg^{++} dependent (Table 17), and is significantly lower in activity than that found in vegetative cell extracts.

TABLE 17. Acetokinase activity in extracts of spores and germinated spores of C. botulinum.

Acetate plus factor added	Incubation time, minutes	Specific activity*	
		Spore	Germinated spore**
None	10.0	0	0
Mg^{++}	10.0	0	0
ATP	10.0	0	0
ATP + Mg^{++}	2.5	.26	.35
ATP + Mg^{++}	5.0	.25	.35
ATP + Mg^{++}	10.0	.26	.34
ADP + Mg^{++}	10.0	0	0

*µmoles acetyl phosphate formed per min per mg protein.

**Spores germinated in Trypticase solution for 2.5 hrs.

Reaction mixtures contained: 1.0 ml of 0.1 M tris buffer, pH 7.4; 0.9 mg spore extract protein, or 1.0 mg germinated spore extract protein; potassium acetate, 200 µmoles; hydroxylamine, 600 µmoles; and, where indicated, $MgCl_2$, 15 µmoles; ATP, 20 µmoles; ADP, 15 µmoles; water was added to 1.0 ml. Extracts were dialyzed against distilled water at 5 C for 18 hours. Incubation temperature was 30 C; reactions were stopped by addition of 1 ml of 10% trichloroacetic acid.

Spore and germinated spore extracts contained phosphotransacetylase (Table 18) and coenzyme A transphorase (Table 19) as shown by the very sensitive assays used for their detection.

TABLE 18. Phosphotransacetylase in extracts of spores and germinated spores of C. botulinum.

System	Specific activity*	
	Spore	Germinated spore**
Complete	1.09	2.3
with 2x extract protein	1.00	2.2
less CoA	0.0	0.0
less acetyl phosphate	0.0	0.0

*Change in optical density per min per mg protein.

**Spores germinated in Trypticase solution for 2.5 hrs.

Reaction mixtures contained: 0.02 ml of 0.1 M tris buffer, pH 8.0; reduced CoA, .06 μmole; dilithium acetyl phosphate, 10 μmoles; 2.2 μg extract protein, or as indicated; and water to 0.2 ml. Reaction temperature was 25 C. The change in O.D. at 232 mμ was followed due to formation of the thiol ester bond of acetyl coenzyme A.

TABLE 19. Coenzyme A transphorase in extracts of spores and germinated spores of C. botulinum.

System	Specific activity*	
	Spore	Germinated spore**
Complete	0.9	1.4
less enzyme	0	0
less butyryl-SCoA	0	0
less acetate	0	0

*Change in optical density per min per mg protein.

**Spores germinated in Trypticase solution for 2.5 hours.

Reaction mixtures contained: Potassium acetate, pH 8.0, 30 μmoles; potassium arsenate, pH 8.0, 150 μmoles; butyryl-SCoA, 0.01 μmole; phosphotransacetylase, 0.01% of a 300-fold purified enzyme from Peptostreptococcus eldsdenii; 22 μg extract protein; and water to 0.15 ml. Reaction temperature was 25 C. The decrease in optical density at 232 mμ due to the arsenolysis of acetyl-SCoA was followed.

Table 20 is presented so that the reader can easily compare activities found in the extracts of spores, germinated spores and vegetative cells. Points which should be noted are as follows:

a. Glucokinase appears to be an inducible enzyme since it is readily found in cells grown in the presence of glucose, but is absent or of extremely low activity in cells grown in medium devoid of added sugar. The enzyme was not detected in extracts of spores or germinated spores, but it should be recalled that the spores used in this study were not produced in glucose-containing media.

TABLE 20. Comparative activities of some enzymes in extracts of vegetative cells, spores, and germinated spores of C. botulinum.

Enzymes or Systems	Range of specific or relative activities in extracts of:					
	Vegetative Cells		Spores germinated in:			Spores
	Glucose adapted	Non-adapted	Acetate	Trypticase	Trypticase + CAPC	
Glucokinase[1]	.25-.44	0	0	0	0	0
Phosphohexoseisomerase and phosphofructokinase[2]	.63	.19	.06	.11	.04	.04-.08*
Aldolase[3]	1.6-1.85	.75-.80	.33	.50	.09	.06-.11
Aldolase, triose isomerase and G-3-P dehydrogenase[4]	.23	.05	.025	.04	.03	.02
Phosphoglyceromutase, enolase, and pyruvate kinase[5]	.055	.02	0	0	0	0
Acetokinase[6]	2.0-2.5	1.7-2.0	.2-.3	.21-.33	.20	.20-.25
Diaphorase[7]	.5-.65	.54-.63	.32-.37	.58-.60	.25	.20-.40
DPN·H oxidase[7]	.01-.02	.03-.04	.041	.025	.04	.04-.06
Phosphotransacetylase[7]	20	19.3	not tested	2.3	1.3	1.1
Coenzyme A transphorase[7]	3.0	2.7	not tested	1.4	0.9	0.9

*Assayed with Fe^{++} in reaction mixture.

1 = µMoles glucose phosphorylated per hour per mg protein.
2 = Coupled to aldolase reaction since aldolase was in excess. The units of specific activity are same as for aldolase.
3 = µMoles F-1-6-P split per hr per mg protein.
4 = Coupled to DPN^+ reduction at 340 mµ; change in O.D. per min.
5 = Coupled to DPN·H oxidation at 340 mµ; change in O.D. per min.
6 = µMoles acetyl phosphate formed per min per mg protein.
7 = Change in O.D. per min per mg protein.

b. Activity of enzymes of glycolysis from extracts of non-adapted cells were significantly lower than those from glucose-adapted cells. The rate of DPN^+ reduction with F-1-6-P as substrate was only 0.05 for extracts of non-adapted cells compared with 0.23 for extracts of glucose-adapted cells. The low activity was probably due to a low level of G-3-P dehydrogenase in extracts of non-adapted cells since aldolase (Table 20) was found to be quite active in these extracts.

c. Enzyme activities of spore and germinated spore extracts were much lower than those of cell extracts with the exception of diaphorase and DPN·H oxidase activities. The specific activity of the DPN·H oxidase was 2x to 4x higher in the extracts from spores than in cell extracts.

d. Activities of enzymes from germinated spores were the same or only slightly higher than those from spore extracts depending upon the conditions of germination. Extracts from spores germinated in acetate solution had activities similar to spore extracts, while extracts of spores germinated in the more complete germinating solution (Trypticase) had activities slightly higher than those from spore extracts. Spores germinated in Trypticase plus chloramphenicol yielded extracts with enzyme activities that were as low as those of spore extracts. These data suggested that very little, if any, synthesis of the enzymes studied is necessary for the initial phases of spore germination.

DISCUSSION

Demonstration of the individual reactions and enzymes of the EMP scheme is evidence for the presence of this pathway for the dissimilation of glucose by C. botulinum. The absence of glucose-6-phosphate dehydrogenase would seem to negate the possibility of the shunt pathway, but isotope studies should be run with intact cells before any final conclusion is reached.

Glucose fermentation apparently is mediated by a specific and inducible glucokinase, and the presence of glucose in the growth medium results in higher levels of most of the enzymes of glycolysis. Such control of enzyme synthesis by induction is often encountered in catabolic pathways, and is recognized as an important regulatory mechanism permitting the cell to minimize excessive protein synthesis (Pardee, 1959). As a matter of cell economy, the organism may control the concentrations of glycolytic enzymes by a sequential induction process mediated by the glucokinase. Sequential induction refers to the increase in activity of a whole series of enzymes on the addition of a new compound such as glucose, in this case.

Unpublished work of Costilow (1961) indicated that spores of C. botulinum germinated in an acetate solution fermented glucose slowly, and the rate was not inhibited by chloramphenicol; and data presented in this study indicated that there might be an extremely slow glucose fermentation by intact non-adapted cells. This may be due to the presence of a basal level of a fermentative pathway, probably the EMP pathway, in these spores and in cells. Even assuming that the glucokinase in this pathway is an inducible enzyme, it probably is present at a

basal level. Pollock (1959) points out that it is unlikely for an induced enzyme to be at a zero level, and that frequently low levels of induced enzymes are detected in a non-adapted system under proper assay conditions.

The finding of the above-mentioned fermentative activity on glucose by spores germinated in the presence of chloramphenicol adds more credence to the idea that the EMP pathway, although inactive until germination, is present in spores even though all of the enzymes of this pathway were not detected in this study. Failure to detect enzymes of the lower-half of the EMP pathway in spores may have been due to the lack of adequate sensitivity in the assay procedures used. An active DPN·H oxidase partially masked attempts to assay for enzymes by coupling to DPN·H oxidation, and this was the most sensitive assay used for these enzymes.

Enzymes of glucose metabolism have been demonstrated in spores and germinated spores of the aerobic bacilli. In B. cereus, strain T, a mixture of the hexose monophosphate (HMP) shunt and the Entner-Doudoroff pathways of glucose oxidation have been found in spores (Church and Halvorson, 1957; Halvorson and Church, 1957); while the isotopic studies of Goldman and Blumenthal (1960), with the same strain, and the studies of Amaha and Nakahara (1959) with B. coagulans have shown that most of the glucose is metabolized via the EMP pathway during germination and outgrowth. However, such enzymes are not required by spores for germination and outgrowth. The deamination of alanine to pyruvate and subsequent oxidation of the pyruvate has been shown to be the primary metabolic activity in spores of B. cereus during germination

(Halvorson and Church, 1957). C. botulinum can grow, sporulate and germinate in the absence of glucose, so a glucose fermenting system is not required in spores and cells of this organism.

Such data indicate that spores may contain the whole complement of enzymes found in the corresponding vegetative cells but in much lower activity levels than found in cells. At the same time specific enzymes such as the diaphorase and soluble DPN·H oxidase found in the spores of C. botulinum as well as in the spores of aerobic bacilli (Spencer and Powell, 1952; Doi and Halvorson, 1961) in higher levels than similar enzymes of the corresponding cells, and glucose dehydrogenase (Bach and Sadoff, 1960) found in sporulating B. cereus but not in vegetative cells may play significant roles in germination and outgrowth of spores. The role of DPN·H oxidase in spores undoubtedly is that of recycling DPN·H to DPN^+. The presence of similar systems and enzymes such as diaphorase and DPN·H oxidase in aerobic and anaerobic spores indicates that spores of widely different species may have similar germination processes.

The DPN·H oxidase of C. botulinum appears to be unlike those reported for C. perfringens, C. kluyveri and B. cereus. Dolin (1959) reported that the DPN·H oxidase of C. perfringens catalyzes a four-electron reduction of O_2 to water, and this activity is lost upon storage at -20 C. Loss of oxidase activity led to conversion of the enzyme to a cytochrome c reductase. Dolin (1959) was not able to show reversible removal of the flavin prosthetic group from the oxidase. C. kluyveri catalyzes both DPN·H and TPN·H oxidation with oxygen as electron acceptor; with DPN·H as reductant, the enzyme catalyzes reduction

of cytochrome c. The main pathway of electron transport in spores of B. cereus is through a soluble DPN·H oxidase that is stimulated by dipicolinic acid and requires FMN for full activity after the flavin component is stripped from the enzymes, whereas FAD gives 70% of the rate of FMN. By contrast, the oxidase of C. botulinum catalyzes a two-electron reduction of oxygen, is stable to storage, does not act as a cytochrome c reductase, uses DPN·H or TPN·H as reductant, and is not stimulated by dipicolinate. Also, after removal of the flavin portion from the oxidase, FAD is required for full activity and FMN only partially restores the activity of the enzyme.

The high heat resistance of the DPN·H oxidase in spore extracts provides a system which might be used for studying heat resistance of spores. After germination the 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. If the heat resistance of extracted spore enzymes results from the same physical-chemical phenomena responsible for the much greater heat resistance of spores, then a heat stable spore enzyme and its heat labile form in germinated spores or vegetative cells could be purified and used to obtain basic information on mechanisms of spore resistance. The enzyme has properties of stability upon storage, is in high levels in spores and cells, and is easily assayed. All these are considerations favoring the use of the DPN·H oxidase as a system for studying spore resistance. It would also be of interest to follow the appearance of the heat resistant oxidase during the transition of the cell into a heat resistant spore.

Apparently very little, if any, enzyme snythesis occurs in the first stage(s) of spore germination. The specific activities of enzymes from spores germinated in the presence of chloramphenicol were not accompanied by increased specific activities over that of enzymes from spore extracts. However, increases in specific activities of enzymes from spores during germination in the absence of chloramphenicol were noted, and there must have been considerable enzyme synthesis during the swelling and elongation process. The specific activities of enzymes in extracts of vegetative cells ranged from 10 to 20 times higher than those of extracts of spores.

SUMMARY

A study was made of some of the enzymes of vegetative cells, spores and germinated spores of C. botulinum, type A, emphasizing the elucidation of a pathway for glucose dissimilation and the metabolic potential of spores.

Growth studies showed that both glucose and fructose increased the amount of growth and were utilized during growth, but that galactose and mannose appeared to inhibit growth slightly and were not utilized.

Manometric studies with cell suspensions showed that cells grown in the presence of glucose fermented glucose, whereas cells grown without glucose did not. Glucose-non-adapted cells would adapt to glucose or fructose fermentation in the presence of a rich supply of amino acids, and this induction process was inhibited by chloramphenicol, a known inhibitor of protein synthesis. Cells grown in the presence of glucose or fructose fermented these hexoses without the induction lag and these activities were not inhibited significantly by chloramphenicol. The data indicate that glucose or fructose can induce the enzyme(s) which is adaptive in this system, and that glucose is a better inducer and substrate for fermentation than is fructose.

Results obtained with cell-free extracts demonstrated the presence of an inducible glucokinase. This enzyme was induced by either glucose or fructose, but there was very little activity with fructose as substrate, and no activity with mannose or galactose. Further studies with cell extracts demonstrated the presence of practically all enzymes of the EMP pathway with the exception of glucokinase which was found only in extracts of glucose-adapted cells. The enzyme activities of these enzymes

were much higher in glucose-adapted than in non-adapted cell extracts. The absence of glucose-6-phosphate dehydrogenase seemed to exclude the possibility of the hexose monophosphate shunt being active in C. botulinum, but these data are not sufficient to completely validate this conclusion.

Data were presented for the possible routes of pyruvate fermentation. Acetyl phosphate, CO_2, and H_2 were formed from pyruvate in the phosphoroclastic reaction. Lactic dehydrogenase was not detected, but a pathway to ethanol was apparent as alcohol dehydrogenase was shown.

Cell extracts also contained DPN·H oxidase, diaphorase, acetokinase, phosphotransacetylase and coenzyme A transphorase. DPN·H oxidase and diaphorase are believed to function in terminal respiration; coenzyme A transphorase is implicated in fatty acid activation; and, the presence of phosphotransacetylase and acetokinase indicates that C. botulinum can activate acetate as acetyl-CoA or store energy from the oxidation of pyruvate as ATP by these reactions.

The spores used in this study were produced in a medium devoid of glucose or fructose, therefore it was not surprising to find them devoid of glucokinase. However, extracts of these spores did have activities attributable to phosphohexoseisomerase, phosphofructokinase, aldolase, triose isomerase and glyceraldehyde-3-phosphate dehydrogenase. The EMP enzymes were in very low levels in the spore extracts, and failure to show enzymes of the lower-half of the EMP scheme was probably due to lack of adequate sensitivity in the assay procedures used.

Spore and germinated spore extracts contained DPN·H oxidase, diaphorase, acetokinase, phosphotransacetylase, and coenzyme A transphorase.

The DPN·H oxidase from extracted spores has considerable heat resistance that apparently is lost upon germination.

The specific activities of enzymes in extracts of spores were much lower than those in extracts of cells with the exception of DPN·H oxidase and diaphorase. Activities of enzymes from spores germinated in the presence of chloramphenicol were the same as those from spore extracts. However, increased specific activities of enzymes from spores germinated in the absence of chloramphenicol were found, so considerable enzyme synthesis must occur during the swelling and elongation process.

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