CARBOHYDRATE TRANSPORT AND METABOLISM IN RESTING

SUSPENSIONS OF CLOSTRIDIUM PERFRINGENS: TYPE A

BY

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We accept this thesis as conforming to the
required standard

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ABSTRACT

Suspensions of *C. perfringens*, when grown on a peptone-free, semi-defined medium, have been shown to remain resistant to autolysis for extended periods of time. The stability of these suspensions has been compared with that of cells grown on complex media.

Extracts of cells grown on this semi-defined medium were found to contain all of the enzymes of the Embden-Meyerhof pathway of glycolysis, in addition to lactic acid dehydrogenase and the pyruvate-lysatic system, but no evidence of glucose-6-phosphate dehydrogenase activity could be demonstrated. Evidence has been presented for the implication of the Embden-Meyerhof pathway as the major pathway of glucose degradation by this organism.

Resting suspensions of *C. perfringens* were shown to transport radioactive glucose and mannose, but not other carbohydrates by a common mechanism and accumulate glucose to concentrations several hundred times those found in the external medium. The transport system was found to be an enzymatic, energy-dependent, temperature-sensitive, and highly specific mechanism which was saturated at high substrate
concentrations. The carbohydrate was found to be accumulated as an equilibrium mixture of phosphorylated hexoses. The phosphorylation mechanism involved in accumulation was demonstrated to be other than the soluble hexo-kinase.
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INTRODUCTION

The metabolism of glucose, the major soluble carbohydrate in mammalian systems, by an organism pathogenic to man, is of obvious interest. While the seriousness of the infection by *C. perfringens* has been greatly diminished in recent years by improved clinical technique, cases of "gas-gangrene" have not yet become unknown. This danger, plus the increasing recognition of *C. perfringens* as a causative agent in food poisoning, have motivated numerous attempts to define the physiology and metabolism of this organism. In addition, the characterization of this organism as micro-aerotolerant in a genus that contains only obligate anaerobes warrants intensive investigation of its metabolism.

An integral part of the degradation of a carbohydrate is the initial passage of the carbohydrate through the semi-permeable membrane to enter the cell. However, for meaningful studies of transport, uniform, resting suspensions of the organism are essential. An insight into the preparation of such stable suspensions could also provide information about the widespread survival of the organism in the austere environment of the soil.

A knowledge of the metabolic fate of the transported glucose is of great theoretical interest and possibly of
practical value, due to the pathogenic nature of the organism.

It was the object of this investigation to devise a method of preparing resting cell suspensions of *C. perfringens*, an anaerobic saccharolytic pathogen, to study the metabolism of glucose by this organism, and to elucidate the system of entry of carbohydrates into the cells.
LITERATURE REVIEW

The importance of *Clostridium perfringens* as a pathogen, both in gas gangrene and in food poisoning, and its general importance as a Gram-positive anaerobe, have resulted in fairly extensive studies of the metabolism of the organism.

I. **Metabolism of Carbohydrates and Amino Acids**

1. **Metabolism of carbohydrates**

The degradation of glucose by *C. perfringens* has been studied by many workers. For reasons that will become apparent, much of this work on carbohydrate dissimilation was associated with the metallic-ion requirements for growth and for toxin production.

It has been known since 1932 (Hastings and McCoy) that Fe\(^{++}\) must be added to milk medium in order to insure the gaseous stormy fermentation that is characteristic of the organism. *C. perfringens* has long been recognized as being a saccharolytic, heterofermentative organism producing lactate, acetate, butyrate, ethanol, CO\(_2\) and H\(_2\) from glucose (Friedemann and Knieciak, 1932). Pappenheimer and Shaskan (1944) looked more closely into the fermentation products of glucose, and the effects of the Fe\(^{++}\) concentrations. As the Fe\(^{++}\) ion concentration of the medium approached zero, lactic acid production approached 2 moles of lactic acid per mole of glucose.
fermented. Thus low Fe\(^{++}\) concentration shifted the normal heterofermentation to a homofermentative production of lactic acid.

These workers also demonstrated the coincidence of optimum Fe\(^{++}\) concentration for minimum lactate production, maximum growth, and maximum toxin production. Thus, Fe\(^{++}\) is required for the production of acetate and butyrate from pyruvate, the last precursor common to both the volatile fatty acids and to lactic acid.

Pappenheimer and Shaskan (1944) also demonstrated that while iron-deficient medium gave homofermentative glycolysis, iron-free medium produced by \(\alpha\alpha\)'-dipyridyl treatment, would not support growth. On this basis they assumed that iron played a role in the actual glycolytic pathway to pyruvate as well as in the heterofermentative steps beyond pyruvate.

Bacon (1949) indicated that carbon monoxide would also cause a shift from heterofermentation to homofermentation, and other workers (Lerner and Pickett, 1945; Kubowitz, 1934) showed that high cyanide concentrations caused an inhibition of gas production of other Clostridia. It is known that \(\alpha\alpha\)'-dipyridyl affects inorganic iron, while CO and CN bind heme iron.

While investigating the dual function of iron, Bard and Gunsalus (1950) found that free ionic iron was required for fructose-1,6-di-phosphate aldolase. Removal of Fe\(^{++}\) from cell free extracts completely inhibited aldolase activity and addition of Fe\(^{++}\) or Co\(^{++}\) reactivated the enzyme. They concluded that the presence of aldolase activity indicated that the Embden-Meyerhof pathway of glucose degradation was present. In addition, they suggested that the
enzymes 3-phosphoglyceraldehyde dehydrogenase, triose-phosphate isomerase, and ethanol dehydrogenase were operative in the organism; however, they did not present definitive evidence.

In a series of publications, Shankar and Bard (1952, 1955a, 1955b) studied the effects of \( \text{Mg}^{++} \) and \( \text{Co}^{++} \) on the growth of \( \text{C. perfringens} \). By adding various ions to deionized medium (1952), they demonstrated, as Webb (1948) had done, that \( \text{Mg}^{++} \) deficiency (1955a) produced filamentous cells. They also showed that, with \( \text{Mg}^{++} \)-deficient cells, there was a considerable decrease in gas evolution during growth. In an unsuccessful attempt to demonstrate a deficiency of Embden-Meyerhof pathway enzymes, they qualitatively indicated the existence of hexokinase, phosphohexoseisomerase, phosphofructokinase, aldolase, and 3-phosphoglyceraldehyde dehydrogenase. In addition, they showed a shift to homolactate fermentation from heterofermentation upon addition of excess \( \text{Co}^{++} \), thus indicating that \( \text{Co}^{++} \) interfered with formation of the heterofermentative system (1955b).

Ivanov (1954) examined the hexokinase of \( \text{C. perfringens} \) in cell-free extracts and cell suspensions. In extracts, the pH optimum was shown to range from 7.0 - 8.0, and \( \text{Mg}^{++} \) and \( \text{Co}^{++} \) were found to activate the enzyme.

The ability of \( \text{C. perfringens} \) to grow under higher oxygen tensions than other \( \text{Clostridia} \) is well documented (Fredette, Planté, and Roy, 1967; Prévot, 1966). Hirano et al. (1954) compared the aerobic and anaerobic degradation of glucose by \( \text{C. perfringens} \) and \( \text{Escherichia coli} \), and found that while sodium azide stimulated aerobic degradation of glucose by \( \text{E. coli} \), it prevented the accumulation of pyruvate
from glucose by \textit{C. perfringens}. Bard (1952) also looked at the respiration of glucose and found that whole cells degraded glucose in the presence of oxygen by a mechanism showing a double dependence on iron, similar to that of the fermentation of glucose to acetate.

Attempts to define the major pathways of hexose and pentose metabolism, using radioactive carbohydrates labelled at specific carbon atoms, have been carried out. Paege, Gibbs, and Bard (1956) used \textsuperscript{14}C-glucose preparations labelled at the C-1, C-2, C-6 and C-3 and C-4 positions respectively and degraded the end products of glucose degradation to show the fate of each carbon. They concluded that the major route of glucose dissimilation was indeed via the Embden-Meyerhof pathway. However, the specific activity of the ethanol produced was lower than that of acetate, indicating that \textsuperscript{12}C-ethanol from some other source had diluted the label from the C-1, C-2 and C-6 of glucose. Later, however, Cynkin and Gibbs (1958) used cells grown on pentoses, and were able to show that the ethanol and acetate products had nearly identical specific activities. Either the Embden-Meyerhof pathway is the major pathway, or growth on a pentose "turns off" the additional source of \textsuperscript{12}C-ethanol.

Cynkin and Delwiche (1958) studied the enzymes of ribose dissimilation in cell-free extracts of \textit{C. perfringens}. It had previously been shown (Cynkin and Gibbs, 1957) that the organism could ferment ribose, but not xylose or arabinose, to products similar to those of glucose fermentation. Cynkin and Delwiche were able to demonstrate the existence of ribokinase, phosphopentoisomerase and
the production of hexose-monophosphate from ribose-5-phosphate. The production of hexose-monophosphate from the phosphorylated pentose indicated, they claimed, the existence of trans-aldolase and trans-ketolase activity. No glucose-6-phosphate dehydrogenase activity, either di- or tri-phospho-pyridine nucleotide (NAD or NADP) linked was found in either glucose or pentose grown cells, indicating that the hexose-monophosphate pathway of glucose degradation was not present (Wood 1961).

In a related organism, C. tetani, which is not normally considered to be saccharolytic, an inducible glucokinase activity and the required enzymes to reduce NAD with fructose 1,6-diphosphate as substrate were found and this implicated the presence of the Embden-Meyerhof pathway. Oxidation of reduced NAD (NADH₂) by cell-free extracts with pyruvate as substrate suggested that either a lactic dehydrogenase or a pyruvate decarboxylase was operative also (Martinez and Rittenberg, 1959).

In the saccharolytic, thermophilic, obligate anaerobe, C. thermo-saccharolyticum, all of the enzymes of the Embden-Meyerhof pathway, as well as the pyruvate-elastic system, have been demonstrated (Lee and Ordal, 1967).

The mechanism of the heterofermentative breakdown of pyruvate has been studied extensively and reviewed by several workers (Koepsell and Johnson, 1943; Mortlock, Valentine, and Wolfe, 1959; Valentine, 1964). The non-heme iron containing component of the pyruvate-elastic or phosphoro-elastic system, ferredoxin, has been identified in and purified from several different Clostridia.
and other anaerobes which produce molecular hydrogen. Ferredoxin has also been found in *C. acidi-urici* which does not produce hydrogen during growth (Valentine, 1964). The physical and chemical properties of ferredoxin are well established and have been excellently reviewed (Malkin and Rabinowitz, 1967).

The pyruvate-clastic system is considered to be the general mechanism of pyruvate cleavage in *Clostridia* and produces carbon dioxide, hydrogen and acetyl-CoA, the latter being used subsequently for synthesis of acetate and butyrate (Valentine and Wolfe, 1963). Neither the pyruvate-clastic system, nor the presence of ferredoxin have been demonstrated in *C. perfringens*.

A NADH$_2$ oxidase has been isolated and characterized from *C. perfringens* (Dolin, 1959a) and found to be cyanide insensitive and independent of H$_2$O$_2$ as an intermediate. The enzyme was shown to catalyze a four electron reduction of O$_2$-H$_2$O using NADH$_2$. Although *Clostridia* have not been shown to contain cytochromes, Dolin showed cytochrome c reductase activity (Dolin, 1959b) in the purified NADH$_2$-oxidase preparation.

2. Metabolism of amino acids

Most proteolytic *Clostridia* ferment amino acids in a coupled-oxidation-deamination reaction known as the Stickland reaction (Nisman, 1954). However, most saccharolytic *Clostridia*, and particularly *C. perfringens*, do not use the Stickland mechanism (Nisman, 1954).
Woods and Trim (1942) showed that *C. perfringens* was able to degrade only 5 amino acids. Serine, cystine, cysteine and threonine were metabolized to produce CO$_2$, NH$_3$ and H$_2$, while arginine produced NH$_3$ and H$_2$, but no CO$_2$. In 1952, Tytell demonstrated the degradation of arginine to orthinine, CO$_2$ and NH$_3$ by the organism.

The glutaminase of *C. perfringens* has been purified and characterized (Hughes and Williamson, 1952). Evidence for transaminase activity has been presented by Hicks (1954), who found that aspartate and $\alpha$-keto glutarate produced alanine and CO$_2$, but no $\gamma$-aminobutyrate as would be expected if a simple transamination, followed by decarboxylation had occurred. It was concluded that aspartate and $\alpha$-ketoglutarate formed only catalytic amounts of oxaloacetate and glutamic acid, and that aspartate and pyruvate resulted from decarboxylation of the transaminated oxaloacetate. This provided oxaloacetate, to keep the system in operation, as well as the major product, alanine.

The reduction of nitrate to nitrite by washed cell suspensions, with glucose or ethanol as substrate, has been shown to occur and to be inhibited by KCN, iodoacetamide, and urea (Hicks, 1965).

Fuchs and Bonde (1957) studied the sulphur metabolism of *C. perfringens* and have found that neither sulphate, sulphite or thiosulphate could supply the organism's sulphur requirements. Cysteine, cystine or homocysteine were demonstrated to satisfy these requirements, depending on the strain used, while sulphate, thiosulphate, cysteine, cystine and glutathione were shown to be degraded by the organisms to produce H$_2$S.
11. The Transport of Metabolites

The importance of the process by which microorganisms pass metabolites through their membranes, and the processes by which these membranes can maintain the cell's internal environment, has met with increasing recognition within the last fifteen years.

Systems have been described which are highly specific and which are able to concentrate metabolites to levels several thousand times that of the external environment.

1. Metabolite transport in mammals

The numerous studies of permeation of membranes of various mammalian cells by amino acids, ions, and carbohydrates have been most adequately reviewed (Wilbrandt and Rosenberg, 1961; Quastel, 1965; Albers, 1967).

2. Metabolite transport in bacteria

a. Amino acid transport

The transport of amino acids by microorganisms has been extensively studied and reviewed (Holden, 1962; Kepes and Cohen, 1962; Britten and McClure, 1962).

The workers of the Pasteur Institute (Cohen and Rickenberg, 1956) paralleled their studied on the transport of \( \beta \)-galactosides with investigations into the nature of amino acid transport. They
developed a model that accounted for the concentration of amino acids in a specific, energy-dependent process. Britten and McClure (1962) defined several specific systems for amino acid concentration by *E. coli*. Recently, the transport and accumulation of amino acids by *Pseudomonas aeruginosa* was shown to be mediated by several permeases specific for structural "families" of amino acids (Kay, 1968). As well, the movement of amino acids across the cell membrane was shown to be energy-independent, while the accumulation process on the inside of the membrane is found to be an energy-dependent process.

b. Carbohydrate transport

i. Galactose and galactosides

The galactoside permeation system of *E. coli* is one of the most well defined, both as to specificity, control, and mechanism. The workers of the Pasteur Institute (Rickenberg, Cohen, Buttin and Monod, 1956) showed the existence of an inducible system that selectively transported $^{14}$C-thiomethyl-β-D-galactopyranoside ($^{14}$C-TMG) into the cell, in a process that followed saturation kinetics and was labile to enzyme inhibitors. Because of these enzyme-like properties, they coined the word "permease" to describe the transport function. Kepes (1957) demonstrated the energy-dependence of the process and did kinetic studies (Kepes, 1960) on the exit and entrance processes, using the rapid Millipore filtration technique. In later work by Koch (1964), cells preloaded
with TMG at a low temperature, then warmed to allow exit, were shown to contain an exit process that was facilitated by energy inhibition, and an energy-independent entrance process. Further kinetic studies demonstrated that energy was utilized to alter the rate constant of exit \( (K_e) \) in a manner such that exit was prevented, thus allowing concentration of the substrate within the cells (Winkler and Wilson, 1966).

The specificity of the various systems for galactose and galactosides have been studied fairly extensively. Ganeson and Rotman (1966) reviewed the evidence for two TMG permeases in *E. coli*, as well as a permease for a methyl-\( \beta \)-D-galactose. TMG-permease I, the original permease studied by the Pasteur group (Rickenberg, Cohen, Buttin and Monod, 1956) transported \( \alpha \)- and \( \beta \)-D-galactopyranosides and it was induced by compounds containing an unsubstituted galacto-pyranose ring. The second system was induced by galactinol and transported TMG but not lactose. The third galactoside permease transported methyl-\( \beta \)-D-galactopyranoside but not TMG. A system for the transport of D-galactose by *E. coli* has also received much attention (Horecker, Thomas, and Monod, 1960a; 1960b; Osborn, McLellan and Horecker, 1961) and found to exhibit kinetics of exit and uptake very similar to those of the TMG permease I.

ii. Glucose

In contrast to the galactoside permease system, a permease for glucose in *E. coli* has been described where removal of the energy supply was shown to cause an accumulation of \( \alpha \)-methyl-
glucoside (αMG), a non-metabolizable analogue of glucose (Hoffee, Englesberg and Lamy, 1964). The glucose transport system in E. coli had earlier been shown, by competition and kinetic studies (Cohen and Monod, 1957; Kessler and Rickenberg, 1963), to accumulate αMG. Hagihira, Wilson and Lin (1963) have shown further that mutants defective in glucose uptake cannot concentrate αMG. In competition studies with substituted derivatives of αMG, the same workers showed the specificity of the system depended upon the substituents on C-2, C-3, and C-6 of the αMG. Addition of an exogenous energy source by Hoffee et al. (1964) resulted in a depression of accumulation. These results were presented as evidence for the existence of an energy-requiring exit reaction in glucose transport. The specificity of αMG uptake has also been studied by these workers and it was shown that a ten-fold excess in concentration of βMG or glucose competed very strongly with αMG uptake, while maltose allowed 68% of normal accumulation and mannose, deoxy-glucose, and sucrose each allowed 80% of normal activity. Other carbohydrates had either little effect or stimulated αMG uptake. These results have been confirmed by Halpern and Lupo (1966).

Studies of a galactose-negative strain of E. coli showed that galactose was entering via the glucose permeation system (Rogers and Yu, 1962). Although the strain lacked galactokinase, as much as 50% of the galactose was phosphorylated in the pool, suggesting an accumulation mechanism based on phosphorylation during transport.

III. Carbohydrate accumulation mechanisms and components

The study of carbohydrate transport in Gram-positive
organisms has been limited to the coccus Staphylococcus aureus (Egan and Morse, 1966). Extensive competition experiments defined permease functions, while counterflow experiments and genetic evidence indicated a common carrier protein for a number of carbohydrates. Studies with a mutant of S. aureus indicated that the ability to transport eight carbohydrates could be lost by a single mutation (Egan and Morse, 1965). It was also found that with lactose, maltose, sucrose and αMG, phosphorylation of the carbohydrates was coincident with transport (Hengstenberg, Egan and Morse, 1967; 1968). Wang and Morse (1968) characterized similar pleiotropic carrier mutants for E. coli and Aerobacter aerogenes. These mutants have been demonstrated to lack either of two protein components of the phosphotransferase system (Tanaka and Lin, 1967; Tanaka, Fraenkel and Lin, 1967; Simoni et al., 1967).

Attempts to isolate and characterize components of carbohydrate transport systems have been relatively successful (Fox and Kennedy, 1965; Kolber and Stein, 1966).

3. Carbohydrate transport in yeast and fungi

The mechanism and specificity of the transport of carbohydrates by yeast has recently received a great deal of attention. It has long been recognized that Cobaltous, Nickelous and Uranyl ions could inhibit carbohydrate metabolism by yeast. Hurwitz and Rothstein (1951); Van Steveninck (1966) and Van Steveninck and Dawson (1967) have implicated polyphosphates as the energy source for carbohydrate transport in yeasts, as Nickelous ions interfered
with polyphosphate structure, and inhibited transport. In addition, these workers have claimed the existence of an active, induced, energy-dependent transport system for galactose, using the same carrier mechanism as that used in non-induced, facilitated diffusion.

The specificity of the carbohydrate transport system in yeast has been studied to a considerable extent (Scharff and Kraemer, 1962; Cirillo, 1962; Cirillo, 1968). From competition studies on the uptake of L-sorbose and D-xylose using 25 different carbohydrates, Cirillo (1968) has defined the specific structural requirements of the constitutive yeast monosaccharide transport system. He demonstrated that the conformation and substituents of every carbon except C-2 contribute to the carbohydrates' ability to compete with the transport of the non-metabolized sugars, sorbose and xylose. In addition, Cirillo (1968) demonstrated that the sugar must be in the pyranose form and that while removal of the anomeric 1-OH has little effect on transport, substitution at this position completely destroyed competitive activity. Multiple alterations at carbon atoms other than C-2 had an effect greater than the sum of their individual effects.

A study of the mechanism of transport of carbohydrate by yeast cells under anaerobic conditions, by kinetic analysis, and a tentative model, based on carrier mediated diffusion, for anaerobic carbohydrate transport in general, has been published (Scharff and Kraemer, 1962). From a comparison of rate constants for transport and for purified hexokinase, these workers concluded
that hexokinase did not play an important, direct part in the anaerobic transport of sugars by yeast. They have suggested that metabolism plays essentially no part in anaerobic sugar transport. Further, carrier facilitated diffusion, being the mechanism of transport in erythrocytes and anaerobic yeast, is the method of transport for all cells capable of anaerobic metabolism.
MATERIALS AND METHODS

1. Organisms and Media

*Clostridium perfringens* (BP6K) was used throughout this study.

1. Complex medium

The composition of the complex medium was as follows: yeast extract, 2 gm; proteose peptone, 5 gm; sodium ascorbate, 0.2 gm; and FeSO$_4$.7H$_2$O (0.5% solution), 1.0 ml per liter. These were dissolved in distilled water, the solution was neutralized with 1N NaOH and 160 ml of 0.5 M phosphate buffer (pH 7.2) were added. The volume was adjusted to 1 liter, and the medium was autoclaved. Sterile solutions of glucose and MgSO$_4$.7H$_2$O were added to 0.7% and 0.02% respectively.

2. Semi-defined medium

This medium was a modification of that described by Boyd, Logan and Tytell (1948). Stock solutions were prepared as follows: 2.0 mg of biotin were dissolved in 100 ml of distilled water, 10 mg of riboflavin and 20 mg of Ca-d-pantothenate were dissolved in 100 ml of distilled water, and 50 mg of uracil and 87 mg of
adenine sulfate were dissolved in 50 ml of 0.2N HCl. In addition, 20 mg of pyridoxine monohydrochloride were dissolved in 100 ml of distilled water, and a solution containing 10 gm of MgSO₄·7H₂O and 0.5 gm each of FeSO₄·7H₂O, NaCl and MnSO₄·4H₂O, per 100 ml of distilled water, was prepared. All stock solutions were stored at 4°C.

To replace the individual amino acids of the Boyd, Logan and Tytell (1948) medium, 16.7 gm of acid hydrolyzed casein and 0.83 gm of L-tryptophan were used per liter. These, together with 0.25 gm of sodium ascorbate, were dissolved in distilled water by using low heat and agitation. After cooling, the pH was adjusted to 7.2 with 1N NaOH.

Five ml of the salts solution, 11.25 ml of the uracil and adenine solution, 5.0 ml of the riboflavin and pantothenate solution, 2.5 ml of the pyridoxine solution, and 0.25 ml of the biotin solution were mixed, diluted to 100 ml with distilled water and the pH adjusted to 7.2 with 1N NaOH. This mixture was added to the neutralized amino acid solution, 100 ml of 0.5 M phosphate buffer (pH 7.2) were added, and the total volume was adjusted to 1000 ml with distilled water.

During autoclaving, a heavy precipitate formed and this was allowed to settle out for 12 hrs at 4°C. The supernatant fluid was then decanted into one liter Nalgene bottles, bubbled with N₂ for 15 min, then frozen and stored at -20°C.

Before use, the medium was thawed, filtered through a 0.3 μ Millipore filter and dispensed into sterile, water-jacketed
reaction vessels. Sterile solutions of glucose or other substrates were added aseptically.

3. Stock cultures

Cultures were grown in the complex medium supplemented with 0.5% acid hydrolyzed casein, until an optical density (O.D.) at 660 μμ of 1.2 was reached, then sterile glycerol was added to 15%. The mixture was dispensed aseptically in 2.0 ml amounts into sterile tubes, frozen and held for as long as several months at −20°C (Pivnick et al., 1964).

Cultures were routinely checked for purity by streaking onto blood agar plates which had been previously spread with gas-gangrene antitoxin (Connaught Medical Research Lab., Toronto). Control plates, without antitoxin, were also streaked with the cultures. Plates were incubated for 24 hrs at 37°C, under N₂ and also aerobically. The plates were then examined for colony morphology and partial inhibition of haemolysis in the presence of antitoxin.

4. Growth of inoculum

A stock culture was thawed by slowly warming it and 0.5 ml was added to a tube of Roger's meat medium with the desired carbohydrate substrate, and incubated at 37°C. The culture was used as an inoculum when it was actively gassing (2.5 hrs incubation).
II. Growth Conditions

Cultures were usually grown in 100 or 500 ml water-jacketed reaction vessels heated to 37°C by a temperature controlled circulating waterpump. Sterile nitrogen was passed over the surface of the medium at 50 ml/min to keep the cultures anaerobic. Sterile N₂ was bubbled through the medium for 20 min prior to the addition of a 5% inoculum from an actively gassing meat tube culture. With the semi-defined medium, to ensure a rapidly growing culture with a minimum of carry over from the meat medium, the cultures were grown, with stirring, to an O.D. of 1.0 at 660 μm (approximately 3 hrs). A 5% inoculum from this culture was transferred to a second flask of semi-defined medium prepared as the first, and grown to the desired O.D. at 660 μm.

On occasion, the inoculated meat tube was held in ice in an insulated, water-jacketed reaction vessel until a timer-controlled circulating waterpump warmed the reaction vessel to 37°C and initiated active growth of the culture. After 2.5 hrs growth, a timer-controlled air-pump aseptically forced approximately 2 ml of the supernatant fluid of the actively gassing meat tube into 100 ml of the semi-defined medium in a water-jacketed reaction vessel. The medium was maintained under anaerobic conditions by bubbling sterile N₂ through it. Simultaneous with inoculation, the medium was warmed to 37°C by a timer-
controlled circulating waterpump. When the growing culture reached an O.D. of 1.0 at 660 µm, it was used to inoculate a second reaction vessel of the semi-defined medium. No significant difference was observed between the cells resulting from manual or automatic subculture.

During growth of the second culture in the semi-defined medium or the culture in complex medium, aliquots were removed aseptically and the O.D. at 660 µm was measured. In addition, the pH of the medium was determined and an aliquot was frozen for the quantitative determination of glucose.

III. Stability of Cell Suspensions

Cultures were grown to an O.D. at 660 µm of 1.75 in the complex medium and to 0.75 in the semi-defined medium and harvested by centrifugation at room temperature at 5,000 × g. The cells were washed by resuspending them in 0.85% NaCl (pH 7.2) and centrifuging again at 5,000 × g. This washing procedure was repeated and then the cells were resuspended to the desired O.D. at 660 µm in 0.05 M tris(hydroxymethyl)amino methane-HCl (tris-HCl) buffer (pH 7.2) and held under sterile N₂ in a water-jacketed reaction vessel at 37 C. The surface of the suspension was flushed with sterile N₂ at a rate of 50 ml per min and samples of the cell suspension were removed at various time intervals. Aliquots of the samples were diluted with tris-HCl buffer (pH 7.2) for the determination of O.D. at 660 µm. Other
aliquots were centrifuged at 4°C for 10 min at 10,000 x g and ultra-violet (UV) adsorption spectra from 200 to 300 μm were determined on the resulting supernatant fluid with a Beckmann model DBG recording spectrophotometer.

IV. Enzyme Assays

1. Cell-free extracts

Cultures were grown to an O.D. at 660 μm of 0.6 in the semi-defined medium with 0.7% glucose. The cultures were then transferred to a screw-capped 300 ml plastic centrifuge bottle. The surface of the culture was flushed with N₂ for 5 min and the bottle was sealed and centrifuged at room temperature at 5,000 x g for 7 min. The pellet was resuspended in the following solution, which had previously been bubbled with N₂ to remove oxygen: 0.2 M tris-HCl (pH 7.5), 10⁻³ M MgCl and 6 x 10⁻⁴ M cysteine-HCl. The suspension was transferred, with a 5.0 ml, lightly greased, glass syringe fitted with a 3-inch #21 needle, to a thick-walled, 16 mm centrifuge tube with a rubber stopper. The tube had previously been made anaerobic by flushing it with N₂ using two #20 needles inserted in the rubber stopper (anaerobic centrifuge tube). The suspension was centrifuged at room temperature, at 5,000 x g for 7 min, the supernatant fluid was removed, and the pellet was resuspended in the anaerobic buffer, Mg⁺⁺, cysteine solution. Aliquots were transferred by a
syringe and needle, as described previously, to several anaerobic centrifuge tubes. After centrifugation for 7 min at 5000 x g, the supernatant fluids were removed and the pellets were flushed with N₂ for 5 min. The needles were removed from the stopper and the tubes were stored at -70 C.

Cell-free extracts were prepared from these cells within a maximum time interval of one week. There was no apparent loss of enzyme activity during this storage period at -70 C. As required, the cell pellets were thawed and resuspended to 50 times the growth concentration (approximately 12 mg dry weight/ml) by the addition of the anaerobic buffer-Mg²⁺-cysteine solution. The suspension was subjected to 2.5 min of sonic oscillation at 88 watts (Bromwill BP-11, 1/8 in. dia. probe, Bromwill Industries, Rochester, N.Y.). Sonication was performed in anaerobic centrifuge tubes which were maintained in an ice-water bath. Nitrogen was flushed over the surface of the suspensions at 50 ml per min, and sonication was applied for 1/2 min intervals, with alternate 1/2 min intervals for cooling. The sonicate was centrifuged in the anaerobic centrifuge tube for 7 min at 10,000 x g and the supernatant anaerobic extract was transferred by means of a 5.0 ml lightly greased, glass syringe fitted with a 3 inch #21 needle to a second anaerobic centrifuge tube, where it was kept under N₂ at 0 C. A sample of each cell-free extract was frozen and subsequently assayed for protein content.
2. Enzyme assays

In the following series of assays, with the exception of enolase and the pyruvate-clastic system, the reduction of NADP or NAD, or the oxidation of NADH₂ were followed spectrophotometrically by observing the change in O.D. at 340 m\text{\textmu}m. All assays, except that for the pyruvate-clastic system, were carried out at 37 C.

Anaerobic assays were prepared in 2.5 or 1.5 ml total volumes in 3.0 ml, 1 cm light-path, quartz cuvettes (Beckmann Instruments Inc., Fullerton, California) with circular openings sealed with a rubber serum stopper. To remove oxygen, the reaction mixtures were equilibrated with nitrogen by flushing the surface of the solutions with \text{\textN}_2 at the rate of 20 ml per min, using two #21 hypodermic needles inserted through the rubber serum stoppers. All solutions, which were added anaerobically, were equilibrated under \text{\textN}_2 at 0 C and added through the serum stopper via a lightly greased, 1.0 ml glass syringe fitted with a 3-inch #21 needle.

Aerobic assays were set up in 1.0 or 0.5 ml total volume systems in quartz cuvettes with 1 cm light-path of the appropriate size. Anaerobic cell-free extracts were used for all assays in order to limit enzyme degradation by protein oxidation.
The buffer used in all assays, except the pyruvate-clastic system, was tris-HCl (pH 7.5).

Control reaction mixtures were prepared for each enzyme assay to ensure that the observed reactions were dependent upon the presence of the various substrates employed. Enzyme activities were corrected for the control values and expressed as μmoles of substrate utilized x 10^2/min/mg of protein.

a. Hexokinase was assayed in an aerobic system of 1.0 ml total volume containing: buffer, 80 μmoles; glucose (fructose, mannose or galactose), 5 μmoles; adenosine 5'-triphosphate (ATP), 4 μmoles; NADP, 0.1 μmoles; and commercial glucose-6-phosphate dehydrogenase, 5.35 μg. The reaction was initiated by the addition of the anaerobic extract.

b. Glucose-6-phosphate dehydrogenase was assayed in an anaerobic, 2.5 ml system containing: buffer, 240 μmoles; glucose-6-phosphate, 1.0 μmoles; and MgCl_2, 20 μmoles. After equilibration at 37°C with N_2 for 7 min, anaerobic extract was added and the reaction started by the addition of anaerobic NADP or NAD.

c. Phosphohexose isomerase was assayed in an anaerobic system of 1.0 ml total volume, containing: buffer, 60 μmoles; fructose-6-phosphate, 5 μmoles; glucose-6-phosphate dehydrogenase, 0.3 μg; NADP, 0.125 μmoles; and anaerobic extract.
d. Phospho-fructokinase was measured in a 2.5 ml total volume under anaerobic conditions containing: buffer, 200 µmoles; ATP, 10 µmoles; α-glycerol phosphate dehydrogenase, 20 µg; aldolase, 20 µg; MgCl₂, 30 µmoles; and NADH₂, 0.25 µmoles. After equilibration at room temperature under N₂ for 7 min, the reaction was started by the addition of anaerobic extract.

e. Fructose-1,6-diphosphate aldolase was assayed by a modified procedure of Groves et al. (1966) in an anaerobic system of 2.5 ml total volume containing: buffer, 120 µmoles; potassium acetate, 240 µmoles; cobalt chloride, 1.68 µmoles; cysteine-HCl, 0.24 µmoles; α-glycerophosphate dehydrogenase, 20 µg; and triose-phosphate isomerase, 20 µg. After equilibration for 7 min at 37 C, under N₂ anaerobic extract and 0.25 µmoles of NADH₂ were added and the reaction was started by the addition of 10 µmoles of fructose-1,6-phosphate.

f. Triose-phosphate isomerase was measured in an anaerobic system of 2.5 ml total volume containing: buffer, 240 µmoles; iodoacetamide, 10 µmoles; and 20 µg of α-glycerol phosphate dehydrogenase. The mixture was equilibrated at room temperature under N₂ for 7 min, then anaerobic extract and 0.25 µmoles of NADH₂ were added. The reaction was started by the addition of 7.5 µmoles of anaerobic 3-phospho-glyceraldehyde.
g. Three-phosphoglyceraldehyde dehydrogenase was measured by a modification of Krebs' (1955) procedure in an anaerobic, 2.5 ml system containing: buffer, 240 μmoles; cysteine-HCl, 1.5 μmoles; sodium arsenate, 30 μmoles; sodium fluoride, 30 μmoles; and 3-phosphoglyceraldehyde, 7.5 μmoles. After equilibration at room temperature for 7 min with N₂, anaerobic extract was added and the reaction mixture equilibrated under N₂ for a further 5 min at room temperature and 2 min at 37°C. The reaction was initiated by the addition of 0.25 μmoles of NAD. In a parallel experiment, 10 μmoles of iodoacetamide was added to the reaction mixture prior to the first equilibration with N₂ (Krebs, 1955).

h. Three-phospho-glyceric acid kinase was measured in a 1.5 ml total volume system under anaerobic conditions. The reaction mixture contained: buffer, 60 μmoles; 3-phosphoglyceraldehyde dehydrogenase, 50 μg; ATP, 1.5 μmoles; MgCl₂, 12.5 μmoles; cysteine-HCl, 9 μmoles; and 7.5 μmoles of 3-phosphoglycerate. After equilibration at room temperature under N₂ for 7 min, 0.15 μmoles of anaerobic NADH₂ were added and the assay started by the addition of the anaerobic extract.

i. Mutase. The activity of this enzyme was assayed in an anaerobic system with 1.5 ml total volume containing:
buffer, 60 μmoles; MgCl₂, 15 μmoles; ATP, 0.75 μmoles; 2,3-
diphosphoglyceric acid, 0.625 μmoles; 2-phosphoglyceric acid, 
7.5 μmoles; cysteine-HCl, 9 μmoles; 3-phosphoglyceraldehyde 
dehydrogenase, 20 μg; and 3-phosphoglycerate kinase, 20 μg.
After 7 min under N₂ at room temperature, 0.15 μmoles of anaerobic 
NADH₂ were added, and the reaction initiated by the addition of 
anaerobic extract.

j. Pyruvate kinase was assayed in an anaerobic 2.5 ml 
system by a modification of the procedure of Bücher and 
Pfleiderer (1955). The reaction mixture contained the following: 
buffer, 100 μmoles; KCl, 187 μmoles; MgCl₂, 20 μmoles; lactic 
dehydrogenase, 1 μg; and ADP, 0.125 μmoles. After equilibrating 
with N₂ for 7 min at room temperature, anaerobic extract and 
0.5 μmoles of NADH₂ were added and the reaction started by the 
addition of 2.5 moles of anaerobic phosphoenol pyruvate.

k. Lactic dehydrogenase was assayed in a 1.5 ml 
system under N₂, that contained the following: buffer, 40 μmoles; 
NADH₂, 0.15 μmoles; and sodium pyruvate, 5 μmoles. After 7 min 
at room temperature, the reaction was started by the addition of 
an aerobic extract.

l. NADH₂ oxidase was measured under both aerobic and 
an aerobic conditions.
(i) The aerobic activity of NADH\textsubscript{2} oxidase was measured in a 2.5 ml system containing 30 umoles of buffer and anaerobic extract. The reaction mixture was mixed in air and warmed to 37 C, after which the reaction was started by the addition of 0.45 umoles of aerobic NADH\textsubscript{2}.

(ii) The activity of the oxidase was measured in the absence of molecular oxygen by using a 2.5 ml system containing 250 umoles of buffer equilibrated under N\textsubscript{2} for 7 min at 37 C. Anaerobic extract was added, and the reaction was started by the addition of 0.45 umoles of anaerobic NADH\textsubscript{2}.

m. Enolase was measured by following the change in O.D. at 230 \textmu m, according to the method of Westhead (1966). An aerobic system of 0.5 ml total volume was used and it contained the following: buffer, 20 umoles; MgCl\textsubscript{2}, 1 u mole; and 2-phospho-glycerate, 2.5 u moles. After warming to 37 C, the assay was initiated by the addition of diluted anaerobic extract.

n. Pyruvate-clastic system. This enzyme system was assayed by a modification of the method of Lovenberg et al. (1963), which was based on the assay of Lipmann and Tuttle (1945) for acetyl phosphate. A 10 ml reaction mixture containing the following: phosphate buffer (pH 6.5), 250 u moles; sodium pyruvate, 100 u moles; and coenzyme A, 0.35 mg; was bubbled with N\textsubscript{2} for 5 min at room temperature, and then for 2 min at 30 C. The reaction was
started by the addition of the anaerobic extract and \( N_2 \) was flushed over the surface of the reaction mixture during the experiment. One ml samples were removed at various time intervals and added to a mixture of 100 \( \mu \)moles of acetate buffer (pH 5.4) and 14 mg of neutralized hydroxylamine-HCl in a total volume of 2.0 ml. After mixing, the suspension was allowed to stand at room temperature for 10 min, after which 1.0 ml of 3N HCl, 1.0 ml of 10% trichloroacetic acid (TCA) and 1.0 ml of 5% \( \text{FeCl}_3 \cdot 6\text{H}_2\text{O} \) in 0.1N HCl were added with vigorous mixing. The mixtures were centrifuged for 5 min at 12,000 \( \times g \) and the supernatant fluid was examined for O.D. at 540 \( \mu \text{m} \). A molar extinction coefficient was calculated from a standard curve obtained by measuring the O.D. at 540 \( \mu \) of solutions of the complex of ferric ions and the hydroxamate of succinic anhydride (Lipmann and Tuttle, 1945).

V. Carbohydrate Transport

1. Preparation of anaerobic cell suspensions

Cultures were grown in the semi-defined medium with 0.6% sodium pyruvate and 0.1% glucose unless otherwise indicated. The cells were harvested and washed twice with semi-defined medium plus 0.6% pyruvate under \( N_2 \), as described for the preparation of cell-free extracts. After the final washing, the cells were resuspended to an O.D. at 660 \( \mu \text{m} \) of 0.28 (0.078 mg dry weight/ml) in the semi-defined medium plus 0.6% sodium pyruvate,
held under $N_2$ and at 18 C in a water-jacketed reaction vessel. The O.D. at 660 mu was monitored during the course of the experiment to ensure that the cell density remained constant.

2. Assays for $^{14}C$-carbohydrate incorporation

A small reaction vessel consisting of a shortened 18 mm test-tube was fitted with a rubber stopper, a #18 hypodermic needle for $N_2$ inlet, and an open port for sampling, addition of substrates and cells, and $N_2$ exit. The reaction vessel contained a small magnetic stirring bar driven by an underwater magnetic-stirrer (Bromwill Industries, Rochester, N.Y.), was flushed with $N_2$ at 50 ml per min, and was warmed to 30 C in a water-bath.

$^{14}C$-labelled carbohydrate substrates were prepared in 1.0 ml volumes of the semi-defined medium and placed in a small tube which was fitted with a rubber stopper, with a $N_2$ inlet needle and an open port. The substrate solution was held in this tube on ice, under nitrogen, for 5 min and then warmed to 30 C for 3 min. Four ml of the anaerobic cell suspension was transferred with a 5.0 ml syringe fitted with a 3-inch #21 hypodermic needle, to the anaerobic, 30 C reaction vessel. The cell suspension was warmed to 30 C, stirred for 3 min and the reaction was started by the addition of the 30 C, anaerobic substrate solution, using a 1.0 ml syringe fitted with a 3-inch #21 needle. At time intervals, 1.0 ml aliquots of the reaction mixture were removed with a similar 1.0 ml syringe and needle.
The samples were analyzed for incorporation of $^{14}$C-labelled carbohydrates into the whole cells by the method of Britten and McClure (1962). Cells were filtered onto a Tracerlab E8B precipitation apparatus (Tracerlab, Waltham, Mass.), containing a 0.45 μ Millipore filter, and quickly washed with 2.0 ml of semi-defined medium containing 0.6% sodium pyruvate.

Samples were also added to test-tubes containing equal volumes of ice-cold 5% TCA, held at 0°C for 15 min and filtered on the precipitation apparatus containing a 0.45 μ Millipore as above. The tubes were rinsed twice with ice-cold 5% TCA and the rinse solutions were added to the filter (Britten and McClure, 1962). This procedure was used to determine the incorporation of the $^{14}$C-label into the cold TCA insoluble proteins and nucleic acids (Roberts et al., 1955).

To study the competitive inhibition of carbohydrate transport, a 100-fold excess of the $^{12}$C possible competitor was added to the $^{14}$C-labelled carbohydrate solution and the rate of total incorporation of $^{14}$C into the whole cells was compared to the rate of incorporation of $^{14}$C in the absence of the $^{12}$C-carbohydrate.

In cases where the $^{14}$C-carbohydrate did not accumulate in the cells against a concentration gradient, extreme caution in filtering was necessary in order to prevent the retention of high levels of background radioactivity in the filtered samples, which would obscure the relatively small changes in radioactivity.
being measured. The Millipore filters were pre-washed with 2.0 ml of semi-defined medium containing the $^{12}\text{C}$-substrate at the same concentration as the $^{14}\text{C}$-labelled substrate in the reaction mixture to reduce specific adsorption to the filter. The danger of retaining small volumes of the reaction mixture in the filter was reduced by the use of higher than normal suction rates, and washing the filtered cells 3 times with 1.0 ml volumes of the wash solution, instead of once with 2.0 ml, as in normal incorporation experiments. Care was taken to keep the filters on the precipitation apparatus for equal lengths of time before they were removed for drying and counting. Control values for non-specific retention of label by the filters were determined by performing parallel experiments for total incorporation using 5.0 ml of semi-defined medium containing 0.6% sodium pyruvate, radioactive substrate, and no cell suspension. The filters were carefully washed and dried as above, and the values that were obtained were subtracted from the values for total incorporation into cells.

$^{14}\text{C}$-labelled carbohydrates were added at the various concentrations and specific activities indicated in the Results and Discussion.

3. Determination of the nature of the accumulated $^{14}\text{C}$-carbohydrate

A reaction mixture was prepared as described for the study
of total incorporation of $^{14}$C into cells. The entire reaction mixture was removed with a 5.0 ml syringe fitted with a #21 hypodermic needle at 0.5 min after addition of the $^{14}$C-labelled substrate. The mixture was injected onto a 0.45 $\mu$m Millipore filter, and washed once with 2.0 ml of semi-defined medium containing 0.6% sodium pyruvate. The filtrate from the reaction mixture was recovered and held at 0°C for analysis. The filter was quickly removed and immersed in a beaker containing 3.0 ml of ice-cold 5% TCA. The filter and TCA solution were placed in an 18 mm test-tube, the beaker was rinsed with 2.0 ml of ice-cold 5% TCA, and the rinse solution was added to the test-tube. After vigorous agitation of the filter in the TCA solution, the solution was removed and the filter was further extracted with two washings of 2.0 ml of ice-cold 5% TCA, which were added to the original solution. The combined solutions were centrifuged at 12,000 x g for 15 min at 4°C, to remove the precipitated material and the supernatant fluid was extracted four times with equal volumes of cold ethyl ether to remove the TCA. The remaining aqueous solution, containing the pooled $^{14}$C-carbohydrate, was concentrated to a small volume by warming to 30°C in a flash-evaporator (Laboratory Glass and Instruments Corp., New York, N.Y.) with the condenser cooled in an ice-water bath. The concentrated solution was subjected to analysis by electrophoresis and paper chromatography. An aliquot of the material was fractionated by preparative paper electrophoresis and the separated fractions were eluted with distilled water and
de-phosphorylated with bacterial alkaline phosphatase. The de-phosphorylated preparations were concentrated and subjected to analyses by paper electrophoresis and paper chromatography. The filtrate of the original reaction mixture was assayed for total radioactivity and then concentrated to a small volume and the nature of the radioactive compounds was determined by the same procedures as used for the accumulated radioactive compounds.

The recovery of the radioactivity in all of the samples was monitored during the preparative and analytical procedures, and negligible losses occurred.

VI. Assay of Radioactivity

1. Millipore filters

Dried Millipore filters were placed in the vials containing 5 ml of scintillation fluid (Liquifluor, New England Nuclear Corporation) and the vials were assayed for radioactivity in a Nuclear Chicago liquid scintillation spectrometer model 725.

2. Chromatograms and electrophoretograms

Radioactive chromatograms were scanned by running 1-inch strips through a Nuclear Chicago model C 100 B Actigraph II with a gas flow counter and a model 1620 B Analytical Count ratemeter equipped with a chart recorder.
The radioactivity of compounds from electrophoretograms and chromatograms were quantitatively determined by cutting out the areas where label had been detected, drying under an infrared lamp, and counting in the liquid scintillation counter.

VII. Chromatography and Electrophoresis

Paper chromatography was routinely performed on substrate solutions and extracted pool materials by spotting samples on Whatman No. 4 paper, and running them by the descending technique with the solvent system of Grado and Ballou (1961) as follows: ethyl-acetate/pyridine/saturated aqueous boric acid (60/25/20).

Electrophoresis was carried out with a water-cooled apparatus similar to a Resco model E-800-2B equipped with a Resco model 1911 power supply. The buffer system routinely employed was 0.1 M ammonium carbonate (NH₄HCO₃-NH₂COONH₄-Analar) (pH 8.6) and samples were spotted onto either Whatman No. 4 paper or Whatman No. 3 paper for preparative electrophoresis. The maximum voltage of 750 volts was applied for 1.5 to 2.0 hrs.

After drying chromatograms overnight at 40 C and electrophoretograms for 20 min at 100 C, they were developed in the following manner. Carbohydrates were detected by dipping chromatograms in 0.12% m-periodate in acetone and spraying with 0.18% benzidine in acetone (Cifonelli, 1954). Reducing sugars were detected by dipping chromatograms in 0.5% AgNO₃ in acetone and developing in 0.5N NaOH in 70% Na₂S₂O₃·5H₂O (Smith, 1960).
VIII. **Analytical and Preparative Techniques**

1. **Dry weight**

The dry weight of cell suspensions was determined by centrifuging a known volume of the suspension, resuspending the pellet in distilled water and re-centrifuging. The pellet was resuspended in one-tenth of the original volume in distilled water. A measured aliquot of this concentrated suspension was placed in a pre-dried, pre-weighed aluminum dish. The dish and sample were dried at 95°C for 2.5 hrs, then placed in a vacuum dessicator at room temperature for 24 hrs. The pans were weighed, and again placed under vacuum at room temperature. This final procedure was repeated until the weight of the samples was constant.

2. **Protein**

Protein content of samples was determined according to the method of Lowry *et al.* (1951). The protein standard used was egg albumin five times recrystallized.

3. **Optical density measurements**

Optical densities in the visible range were determined using
a Beckman model B spectrophotometer. Values for optical
densities in the ultra-violet range were taken from spectra
obtained using a Spectronic 505 or Spectronic 600 spectrophotom­
eter (Bausch and Lomb). To record the change in O.D. with time,
for enzyme assays, a Gilford recording spectrophotometer, model
2000, was used.

4. Dephosphorylation of carbohydrates

The removal of phosphate from phosphorylated intermediates
was carried out by incubation with a commercial bacterial
alkaline phosphatase in 0.1 M tris-HCl buffer (pH 8.0) at 37 C.

5. Glucose

Samples were analyzed for glucose content with the enzymatic
Glucostat procedure (Worthington Biochemical Corp., Freehold,
N.J.).

IX. Chemicals, Enzymes and Substrates

All chemicals, enzymes and substrates were purchased from
commercial sources. Where necessary the substrates were neutralized
with NaOH. Barium was removed from substrates by treatment with
Dowex-50 H⁺ and the resulting free acids were neutralized with
NaOH.
$^{14}$C-U-maltose, $^{14}$C-U-D-ribose, $^{14}$C-I-D-galactose, $^{14}$C-I-lactose and $^{14}$C-U-αMG were obtained from the Nuclear Chicago Corp. (Des Plaines, Ill.); Me-$^{14}$C-thio-D-galactoside and $^{14}$C-U-glucose were purchased from Schwarz Bioreserach Corp. (Orangeburg, N.Y.).

All labelled and unlabelled carbohydrates were found to be chromatographically homogeneous by the ethyl acetate/pyridine/saturated aqueous boric acid solvent system.
RESULTS AND DISCUSSION.

I. Growth and Resting Cell Suspensions

The tendency of Gram-positive organisms, both anaerobic and facultatively anaerobic, to undergo autolysis has long been recognized (Jones, Stacey, and Webb, 1949). This rapid autolysis occurs in both stationary phase cultures and resting cell suspensions and has prevented extensive metabolic studies from being carried out with \textit{C. perfringens} and similar microorganisms. Jones, Stacey, and Webb (1949) defined the system of autolytic enzymes in \textit{C. perfringens} and in \textit{Staphylococcus citreus}, as consisting of a ribonuclease and two proteolytic enzymes.

It has been noted that, when in a magnesium-deficient, enriched medium, \textit{C. perfringens} formed filamentous cells which were resistant to autolysis (Webb, 1948). Boyd, Logan, and Tytell (1948) demonstrated that the production of toxins by \textit{C. perfringens} could be repressed, by growing the organism in a defined medium. It was assumed that possibly a correlation existed between the production of exotoxins and autolytic enzymes. A modification of Boyd, Logan, and Tytell's medium was employed and the stability of cells grown in this semi-defined medium was compared with the stability of cells grown in the complex medium. To prevent
loss by precipitation, magnesium was added to the complex medium after autoclaving.

1. Growth curves

Cultures grown in the enriched and in the semi-defined media, with glucose as the energy source, produced somewhat different growth curves. After a considerable lag, rapid growth to a high cell density (O.D. 660, approximately 4.5) was evident in the enriched medium (Fig. 1). The hydrogen ion concentration rose very rapidly during logarithmic growth (Fig. 1) and, as there was excess glucose present during the stationary phase, low pH was most likely the factor which limited growth. Calculations from a logarithmic plot of the cell density (optical density at 660 nm) versus time indicated that the generation time was approximately 48 min (Fig. 2).

Microscopic observations of Gram-stained preparations of the cells from the complex medium demonstrated the typical square-ended, relatively short thick, Gram-positive bacilli, described by other workers (Breed, Murray, and Smith, 1957). During log phase growth, the cultures produced considerable quantities of gas and gave off the rancid odor characteristic of a mixture of acetic and butyric acids.

Neither high cell density, high acid concentration, nor low glucose concentration limited growth in the semi-defined medium
Fig. 1. Growth of *C. perfringens* in complex medium plus 0.7% glucose. O—O, O.D. at 660μ; △—△, pH.
Fig. 2. Growth of *C. perfringens* in complex medium plus 0.7% glucose. Logarithmic plot of O.D. 660 versus time.
Rather, depletion of some required growth factor from the medium was more likely. When compared with the 2-3 hrs lag in growth, routinely observed in the complex medium, the lag in both the primary and secondary culture in the semi-defined medium was negligible. The generation time of the secondary culture in the semi-defined medium was 42 min, which was significantly shorter than the 51 min generation time of the primary culture (Fig. 4). Microscopic observation of logarithmic phase cells from the second culture in semi-defined medium revealed drastically altered cellular morphology, although the Gram-reaction of the cells was still positive. The majority of the cells were filamentous and much narrower than the cells from the complex medium. The lengths of the filaments varied considerably. In addition, the gas produced by cultures grown in the semi-defined medium was greatly reduced, and the rancid odor was not detectable.

2. Stability of cell suspensions

Resting cell suspensions prepared from cells grown in the semi-defined medium, and suspended in 0.05 M tris-HCl (pH 7.4) and $10^{-3}$ M Mg$^{++}$ were stable to autolysis as determined by measuring the O.D. of the suspensions at 660 nm. Cells which had been grown in the enriched medium lysed rapidly and a 60% decrease in O.D. 660 occurred during the course of a 3 hrs experiment (Fig. 5).

In preliminary experiments, harvesting and resuspending of the
Fig. 3. Growth of C. perfringens in secondary culture in semi-defined medium plus 0.7% glucose. ○, O.D. 660; △, pH; □, glucose concentration.
Fig. 4. Growth of *C. perfringens* in semi-defined medium plus 0.7% glucose. Logarithmic plot of O.D. \(660\) versus time.

- o–o, primary culture; △–△, secondary culture.
cells from the complex medium were carried out under various conditions of anaerobiosis, agitation, and temperature. In addition, the cells were resuspended in various concentrations of phosphate and tris-HCl buffers, as well as in \(0.05 \text{ M tris-HCl (pH 7.4)}\) with \(10^{-2} \text{ M Mg}^{++}\), 5\% mannitol, 0.5\% sorbitol, or 3.5\% sucrose. The rate of cell lysis was not affected by any of these attempts to produce stable cell suspensions. Suspensions of these cells in \(0.05 \text{ M tris-HCl (pH 7.4)}\) containing 5\% galactose or 0.05\% glucose remained relatively stable, however the pH of the medium decreased in both cases, demonstrating that galactose and glucose were being metabolized. In the absence of galactose or glucose, cell lysis commenced immediately, and this indicated that the activity of the autolytic enzymes were, essentially, completely repressed by the presence of an adequate energy source.

The concentration of casein hydrolysate in the semi-defined medium was the limiting factor of growth, if the primary culture of the semi-defined medium was allowed to reach the stationary phase. A tendency to form carbonaceous storage products has been observed in other organisms (Dawes and Ribbons, 1962; Doudoroff and Stanier, 1959), when grown under conditions of carbon excess and nitrogen limitation. No direct evidence of such a product has been demonstrated in \textit{C. perfringens}. Utilization of a carbonaceous storage product could have served the same purpose in repressing the activity of autolytic enzymes as the addition of low levels of glucose did in suspensions of cells grown on complex medium.
Fig. 5. Stability of cell suspensions of *C. perfringens* grown with 0.7% glucose in:
- o--o, complex medium; e--e, semi-defined medium.
As the cells in the suspensions lysed and released their intracellular material, the ultraviolet absorption of the suspending fluid increased significantly. The O.D. at 260 \( \mu \text{m} \) in the supernatant fluids, increased by a factor of 4.68, over the zero-hour value with the cells grown in the complex medium (Table I), and by a factor of 4.5 for the cells grown in the semi-defined medium during a 3 hrs period (Table II). However, the zero-time ratios of the concentration of ultraviolet absorbing material (O.D. at 260 \( \mu \text{m} \)) to the cell densities (O.D. at 660 \( \mu \text{m} \)) were found to be 0.0652 for the cells grown on semi-defined medium (Table I), and 0.314 for the suspensions of cells from the complex medium. This high initial concentration of ultraviolet absorbing material in the suspensions of cells from the complex medium demonstrated that a significant amount of lysis had occurred during the washing and resuspension procedures. This tendency to lyse immediately upon resuspension indicated that the cells were induced to synthesize the autolytic enzymes during growth on the complex medium. The fact that the suspension of cells grown in the semi-defined medium did not have this high initial O.D. \( \text{at 260} \) suggested that the synthesis of the autolytic enzymes had been repressed, by some factor, during growth in this medium.

The initial concentration of the cell suspensions could not have conferred stability on the cells grown in the semi-defined medium.
Table I. The autolysis of cell suspensions of *C. perfringens* under starvation conditions.

<table>
<thead>
<tr>
<th>INCUBATION TIME (HR)</th>
<th>OPTICAL DENSITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>660 μm</td>
</tr>
<tr>
<td>cell suspension</td>
<td>supernatant fluid</td>
</tr>
<tr>
<td>0</td>
<td>5.10</td>
</tr>
<tr>
<td>½</td>
<td>5.10</td>
</tr>
<tr>
<td>1</td>
<td>4.85</td>
</tr>
<tr>
<td>2</td>
<td>3.85</td>
</tr>
<tr>
<td>3</td>
<td>2.30</td>
</tr>
<tr>
<td>3 hr/0 hr</td>
<td>0.45</td>
</tr>
</tbody>
</table>
Table II. The stability of cell suspensions of *C. perfringens* under starvation conditions when previously grown in a chemically defined medium.

<table>
<thead>
<tr>
<th>STARVATION MEDIUM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NO ADDITION</th>
<th>$10^{-3}$ M MgCl&lt;sub&gt;2&lt;/sub&gt;</th>
<th>$10^{-2}$ M MgCl&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>INCUBATION TIME (HR)</td>
<td>OPTICAL DENSITY</td>
<td>660 µ&lt;sub&gt;b&lt;/sub&gt;</td>
<td>260 µ&lt;sub&gt;c&lt;/sub&gt;</td>
</tr>
<tr>
<td>0</td>
<td>2.91</td>
<td>0.19</td>
<td>2.70</td>
</tr>
<tr>
<td>1</td>
<td>2.85</td>
<td>0.82</td>
<td>2.79</td>
</tr>
<tr>
<td>2</td>
<td>2.67</td>
<td>1.80</td>
<td>2.87</td>
</tr>
<tr>
<td>3</td>
<td>2.53</td>
<td>2.70</td>
<td>2.88</td>
</tr>
<tr>
<td>6</td>
<td>2.28</td>
<td>4.20</td>
<td>2.96</td>
</tr>
<tr>
<td>18</td>
<td>2.16</td>
<td>9.10</td>
<td>3.34</td>
</tr>
<tr>
<td>3 hr/0 hr</td>
<td>0.87</td>
<td>14.2</td>
<td>1.07</td>
</tr>
<tr>
<td>18 hr/0 hr</td>
<td>0.74</td>
<td>83.5</td>
<td>1.24</td>
</tr>
</tbody>
</table>

<sup>a</sup> - 0.05 in Tris buffer (pH 7.4);

<sup>b</sup> - O.D<sub>660</sub> of cell suspension;

<sup>c</sup> - O.D<sub>260</sub> of supernatant fluid.
as the rate of decrease in O.D. \textsuperscript{660}, with time, for cell suspensions grown on complex medium, increased linearly with increasing initial cell density (O.D. \textsuperscript{660}) (Fig. 6).

One of the major differences between the complex and the semi-defined media was the substitution of acid-casein hydrolysate, for the proteose peptone as the source of amino acids. It has been demonstrated that exotoxins were not produced when the \textit{C. perfringens} was grown on the semi-defined medium (Boyd, Logan, and Tytell, 1948) and the importance of peptides for toxinogenesis by \textit{C. perfringens} type D has been demonstrated (Hauschild, 1965).

If a correlation existed between the production of the exotoxins, extracellular proteolytic enzymes, and autolytic enzymes (Jones, Stacey, and Webb, 1949), all three classes of enzymes may have been coordinately induced by the presence of the peptides in the complex medium. The absence of these substrates of proteolytic enzymes from the semi-defined medium, could have repressed the synthesis of the three classes of enzymes, and thus produced stable cell suspensions.

Attempts were made to improve the stability of suspensions of cells grown in the semi-defined medium by the addition of various concentrations of Mg\textsuperscript{++} to the buffer used to wash and resuspend the cells. Significant changes in the rate of decrease in O.D. \textsuperscript{660} and increase in O.D. \textsuperscript{260} were observed (Table II). Slight autolysis of the suspension in 0.05 M tris-HCl (pH 7.2) was demonstrated with 87\% of the initial O.D. \textsuperscript{660} remaining after 3 hrs. During the same
Fig. 6. The rate of lysis of suspensions of cells grown in complex medium, as a function of the initial cell concentration of the suspensions.
period, the O.D. was increased to 14.2 times the initial value. The addition of $10^{-2}$ M Mg to the resting suspension resulted in 105% of the initial O.D. at the end of 3 hrs, with only a five-fold increase in 260 mp absorbing material. The addition of $10^{-3}$ M Mg gave results nearly identical to those found with the addition of $10^{-2}$ M Mg (Table II).

The addition of the Mg ions may have served to stabilize the cell suspensions in either of two ways, for which no evidence is presented. The existence of low levels of autolytic enzymes in the suspensions of cells grown in the semi-defined medium was evident from the slow, but definite, rate of lysis of the cell suspensions.

Magnesium ions, in the concentrations used, may have directly inhibited the activity of the autolytic enzymes. However, Mg is also known to stabilize the structure of ribosomes in vitro and in vivo (McCarthy, 1962; Tissieres et al., 1959) and therefore the magnesium may have prevented the dissociation of the ribosomes and the release of ribonucleic acid and proteins which would have served as substrates for the ribonuclease and proteolytic enzymes which have been demonstrated to be involved in autolysis (Jones, Stacey, and Webb, 1949).

The ultraviolet absorbing material released by the stable suspensions of C. perfringens (Table II) may have been the degradation products of the endogenous metabolism of the organism,
as has been observed in other microorganisms (Campbell, Gronlund, and Duncan, 1963; Strange et al., 1963). The gradual increase in cell numbers (O.D.\textsubscript{660}) in the stable suspensions (Fig. 2) may have resulted from this utilization of an endogenous storage product.

The lower concentration of Mg$^{++}$, $10^{-3}$ M, conferred adequate stability on the cell suspensions, and was considered to be more suitable for use in routine studies of resting suspensions.

II. Glycolytic Enzyme Assays

The Embden-Meyerhof pathway has often been implicated as being the major pathway of glycolysis in the \textit{Clostridia} (Bard and Gunsalus, 1950; Shankar and Bard, 1955a), however the enzymes of this pathway have been demonstrated definitely in only one \textit{Clostridium}, \textit{C. thermosaccharolyticum} (Lee and Ordal, 1967). One major reason for the lack of a successful demonstration of the complete pathway in \textit{C. perfringens} has undoubtedly been the existence of a strong NADH\textsubscript{2}-oxidase (Dolin, 1959a, 1959b), which interferes with the most efficient procedure for assaying glycolytic enzymes, that of following the oxidation or reduction of pyridine nucleotides spectrophotometrically.

The NADH\textsubscript{2}-oxidase activity in cell-free extracts was considerably greater than the activity of phosphohexose isomerase, phosphofructokinase, and triosephosphate isomerase and more than
ten-fold greater than hexokinase, 3-phosphoglyceraldehyde dehydrogenase, pyruvate kinase, and lactic dehydrogenase. In preliminary experiments, oxidase activity prevented the detection of all enzymes except those which could be linked to NADP reduction via a commercial source of glucose-6-phosphate dehydrogenase.

Attempts were made to decrease the oxidase activity by various procedures. Extended periods of irradiation of cell-free extracts with light at 365 μm (Dolin, 1959b), using the monochrometer of a Bausch and Lomb Spectronic 20 spectrophotometer, failed to reduce the oxidase activity sufficiently. A combination of $10^{-3}$ M atabrine and $10^{-4}$ M $H_2O_2$ reduced the NADH$_2$-oxidase activity to a low level, however, the risk of destroying other enzymes by oxidation with $H_2O_2$ and also the very high absorption at 340 μm of atabrine, combined to severely limit the use of these compounds. Centrifugation at 25,000 x g for 30 min caused practically no reduction in the oxidase activity and confirmed the reported soluble nature of the enzyme (Dolin, 1959a). Because of the enzyme's dependence on molecular oxygen as an electron acceptor for the oxidation of NADH$_2$ (Dolin, 1959a), the possible usefulness of the exclusion of oxygen by preparing cell-free extracts under $N_2$, and of assaying the enzymes anaerobically was examined. This technique reduced the NADH$_2$-oxidase activity of the extracts to less than 1% of the activity.
evident under aerobic conditions (Table III, Fig. 7).

That molecular oxygen was not the physiological electron acceptor for the NADH₂-oxidase has been demonstrated by the lack of involvement of H₂O₂ as an intermediate in the reaction, and by the fact that other enzymes of the organism produced toxic quantities of H₂O₂ when exposed to oxygen (Dolin, 1961).

With the exception of 3-phosphoglyceraldehyde dehydrogenase, each of the enzymes of the Embden-Meyerhof pathway was measured spectrophotometrically by using the product of each reaction, as a substrate for one of the following commercially prepared enzymes: glucose-6-phosphate dehydrogenase, α-glycerophosphate dehydrogenase, or lactic acid dehydrogenase. When required, the product of the enzyme being assayed was converted to the appropriate substrate by the use of a second commercial enzyme preparation. The commercial enzyme preparations were always added in excess, therefore, the enzyme in the cell-free extract was rate-limiting. All of the enzymes of the Embden-Meyerhof pathway were independently shown to be present in the extracts and specific activities, expressed as μmoles x 10² of product formed per min per mg of protein, were calculated (Table III).

Preliminary growth studies demonstrated that the organism would grow readily on mannose, fructose and glucose.

Kinase activity with mannose could not be demonstrated, however, the phosphohexose isomerase was active with mannose-6-phosphate (Table III). The failure to detect the mannose kinase even in the presence of high concentrations of the substrate
Table III. Specific activities of glycolytic enzymes in extracts of *C. perfringens*. The extracts were prepared from cells grown on semi-defined medium plus 0.6% glucose, except where indicated otherwise.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>ENZYME</th>
<th><em>C. perfringens</em></th>
<th><em>C. thermosaccharolyticum</em> (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>specific activity (^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexokinase</td>
<td>glucose</td>
<td>5.87</td>
<td>8.69</td>
</tr>
<tr>
<td></td>
<td>fructose</td>
<td>0.210</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mannose</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Phospho-hexose isomerase</td>
<td>fructose-6-phosphate</td>
<td>21.4</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td>mannose-6-phosphate</td>
<td>13.95</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase in extracts from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>semi-defined medium, log phase</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>semi-defined medium, stat. phase</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>complex medium, log phase</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-fructo kinase</td>
<td>72.5</td>
<td></td>
<td>9.6</td>
</tr>
<tr>
<td>Fructo 1,6-diphosphate</td>
<td>aldolase</td>
<td>3,940</td>
<td>1.84</td>
</tr>
<tr>
<td>Triose-phosphate isomerase</td>
<td>37.7</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td>3-phospho-glyceraldehyde</td>
<td>dehydrogenase</td>
<td>7.9</td>
<td>1.6</td>
</tr>
<tr>
<td>3-phospho-glycerate kinase</td>
<td>726</td>
<td>20.8</td>
<td></td>
</tr>
<tr>
<td>Mutase</td>
<td>210</td>
<td>840</td>
<td></td>
</tr>
<tr>
<td>Enolase</td>
<td>1,416</td>
<td>56,000</td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>5.85</td>
<td></td>
<td>1.28</td>
</tr>
<tr>
<td>Lactic acid dehydrogenase</td>
<td>8.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate-clastic system in extracts from</td>
<td>enriched medium</td>
<td>16.3</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>semi-defined medium</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>semi-defined medium, plus 5 mg Fe(^{++})/ml</td>
<td>6.5</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) - Lee and Ordal (1967); \(^b\) - (10^2) umoles/min/mg protein.
Fig. 7. The activity of NADH-oxidase in aerobic and anaerobic cell-free extracts of C. perfringens.
and extract, indicated that either the assay system was inadequate or a different system for activating this hexose was operative in the organism. A phosphoenol-pyruvate dependent phospho-transferase system has been demonstrated to phosphorylate several carbohydrates in *E. coli* and *Aerobacter aerogenes* (Kundig and Roseman, 1966). The results from mannose transport studies suggest that a phospho-transferase system or, at least a very similar system, is probably the agent for activating mannose for metabolism in *C. perfringens*.

Three-phosphoglyceraldehyde dehydrogenase was measured directly by recording the reduction of NAD in the presence of excess 3-phosphoglyceraldehyde. The enzyme was sensitive to iodoacetamide (Krebs, 1955) and 4 μm/ml of the inhibitor caused 100% inhibition of activity. It was necessary to add 12 μmoles/ml of sodium arsenate and 30 μmoles/ml of sodium fluoride to the assay mixture to obtain measurable activity of the enzyme (Fig. 8). The arsenate ion rendered the reaction irreversible by substituting for phosphate and subsequently forming the unstable product, 1-arseno-3-phosphoglycerate. This product decomposed to 3-phosphoglyceric acid and therefore, prevented the accumulation of the inhibitory end products of the dehydrogenase reaction (Krebs, 1955). Fluoride and arsenate ions inhibited enolase activity by forming a complex with Mg²⁺ (Bücher, 1955), and consequently, this prevented the oxidation of the accumulated
Fig. 8. The activity of 3-phospho-glyceraldehyde dehydrogenase in cell-free extracts of C. perfringens. Control minus 3-phospho-glyceraldehyde, iodoacetamide (4 mM) or the omission of fluoride and arsenate from the reaction mixture inhibits activity completely.
NADH₂ by the subsequent action of lactic acid dehydrogenase. The possibility of interference by 3-phosphoglyceraldehyde dehydrogenase activity in other spectrophotometric assays was negligible when fluoride and arsenate were not present in the reaction mixture (Fig. 8).

A comparison was made of the specific activities of the glycolytic enzymes in \textit{C. perfringens} with those values determined by Lee and Ordal (1967) for glucose-grown cultures of \textit{C. thermosaccharolyticum} (Table III).

In addition to the glycolytic enzymes from glucose to pyruvate, lactic acid dehydrogenase and the pyruvate-clastic system were present in the organism. The method of assaying the pyruvate-clastic system had some undesirable characteristics which should be considered when interpreting the results. The rate of acetyl-phosphate production in the reaction mixture decreased over the duration of the experiment (Fig. 9). This decrease in the rate of product accumulation may have been due to either the conversion of acetyl phosphate to acetic and butyric acids, two of the normal end-products of the metabolism of pyruvate in \textit{C. perfringens} or to the end-product inhibition. Recently, Biggins and Ditworth (1968) demonstrated a control of the pyruvate-clastic system in \textit{C. pasteurianum} extracts by ADP and acetyl phosphate concentrations. Acetyl phosphate was found to be a "product inhibitor" of the clastic reaction. High ADP
Fig. 9. Activity of the pyruvate-clastic system in cell-free extracts of C. perfringens. The role of accumulation of acetyl-phosphate is extrapolated from the initial rate.
concentrations stimulated the activity of the clastic system, probably by lowering the acetyl-phosphate concentration through stimulation of acetate kinase.

The non-linear accumulation of acetyl-phosphate with time precluded exact measurements of the activity of the pyruvate-clastic system, however, linear extrapolations from the initial rates of product accumulation gave comparable estimates of the activity of the system under various conditions (Fig. 9, Table III).

The pyruvate-clastic system was more active in cells grown in the enriched medium than in the semi-defined medium (Table III). Attempts to stimulate the activity of this system by the addition of excess ferrous iron to the semi-defined medium were only partially successful and resulted in an increase in activity of 60%. This increased activity however, was only 40% of the value obtained with cells grown in the enriched medium. This indicated that some factor other than Fe^{++} (required for the synthesis of ferredoxin) was lacking in the semi-defined medium. The presence of both lactic acid dehydrogenase and the pyruvate-clastic system explains the heterofermentative production of lactic, acetic, and butyric acids, as well as the production of carbon dioxide and hydrogen from glucose by cultures of C. perfringens.

The failure to demonstrate the existence of glucose-6-phosphate dehydrogenase activity, either NAD- or NADP-linked, in the extracts, argued against the hexose-monophosphate pathway.
as a major route of glycolysis in this organism. The failure of glucose-grown cells to accumulate acetate under conditions of low Fe$^{++}$ concentration, probably indicated the absence of the phosphoketolase system of glycolysis (De Vries, Gerbrandy, and Stouthamer, 1967). Five-carbon skeletons, for the synthesis of nucleic acids, are probably generated by a reversal of the transketolase-transaldolase system which was demonstrated in C. perfringens by Cynkin and Delwiche (1958).

III. Carbohydrate Transport

An integral step in the utilization of carbohydrates by any cell is the transporting of the carbohydrate across the semi-permeable membrane into the interior of the cell. The ability to transport carbohydrates specifically and rapidly across the membrane and, to a greater extent, the ability to accumulate metabolites within the cell at concentrations many times above that of the external concentration, confer a definite biological advantage upon a microorganism.

The mechanism of transport of $^{14}$C-glucose by C. perfringens was studied with regard to its kinetics, specificity and energy dependence. The ability to accumulate radioactive carbohydrates and the nature of the accumulated product were also investigated. As a result of the variety of techniques used for studying metabolite uptake and for segregating the component functions of "membrane passage and metabolite accumulation", the term "transport" has come to have several meanings (Hengstenberg,
Egan, and Morse, 1968). In these studies, the term transport has been considered to encompass the two functions, although, as will be discussed, attempts were made to separate them in this organism.

1. Utilization of carbohydrates by *C. perfringens*

Growth of *C. perfringens* with various carbohydrates as the source of carbon was studied. Flasks with anaerobic semi-defined medium containing 0.5% of the appropriate carbohydrate were inoculated as for growth studies, and the increase in O.D. at 660 nm was observed over 5.5 hrs, two hours beyond the beginning of stationary phase of glucose-grown cultures (Table IV). Carbohydrates which failed to support growth were added to 3 sequential subcultures of organisms growing on glucose, but no inducible utilization of these carbohydrates was observed.

2. Transport of $^{14}C$-carbohydrates

(i) Transport of $^{14}C$-glucose.

When suspensions of cells grown with 0.5% pyruvate plus 0.1% glucose as the energy sources (pyruvate-grown cells), and resuspended to $7.8 \times 10^{-2}$ mg dry weight/ml (1.4 $\mu$curie/ $\mu$ mole) in the presence of pyruvate and the total $^{14}C$ incorporated into the cells was determined, the $^{14}C$ entered the cells at a linear
Table IV. Growth of *C. perfringens* with various carbohydrates as the source of carbon. Data obtained from two separate trials.

<table>
<thead>
<tr>
<th>CARBOHYDRATE</th>
<th>INITIAL</th>
<th>FINAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.17</td>
<td>0.36</td>
</tr>
<tr>
<td>Sorbose</td>
<td>0.165</td>
<td>0.31</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.2</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.137</td>
</tr>
<tr>
<td>1-Arabinose</td>
<td>0.125</td>
<td>0.16</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.125</td>
<td>0.44</td>
</tr>
<tr>
<td>Ribose</td>
<td>0.04</td>
<td>0.952</td>
</tr>
<tr>
<td>Trehalose</td>
<td>0.125</td>
<td>0.44</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.09</td>
<td>0.45</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.12</td>
<td>0.465</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.05</td>
<td>0.45</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.105</td>
<td>0.435</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.08</td>
<td>0.22</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.12</td>
<td>1.38</td>
</tr>
</tbody>
</table>
rate for 1.5 min, and then the rate decreased during the remainder of the experiment (Fig. 10). As will be demonstrated, the concentration of $^{14\text{C}}$ in the cells at 1.5 min was several hundred times greater than in the external environment.

(ii) Transport of $^{14\text{C}}$-carbohydrates other than glucose.

Of the seven radioactive compounds examined, only glucose and mannose were concentrated in detectable amounts (Table VI). Ribose, maltose, αMG, lactose and galactose were not concentrated in the cells during the intervals studied. Growth of the organism on lactose did not induce an accumulation mechanism for this carbohydrate. Attempts to study the process of equilibration of these sugars across the cell membrane, or to detect concentrations only slightly above the extracellular level, were interfered with by the non-specific retention of radioactivity by the membrane filters even in the absence of cells. The amount of $^{14\text{C}}$ retained was of the same order of magnitude as one would expect to enter the cell if a concentration gradient was not established. The amount of the retained radioactivity was sufficiently erratic to obscure changes in the pool size. This retained radioactivity could not be removed by washing the filters twice with medium, by pre-treating the filter with unlabelled substrate at the same concentration as the $^{14\text{C}}$-substrate, or by varying the
Fig. 10. Early time course of uptake of $8 \times 10^{-6}$ M $^{14}$C-glucose (1.4 μc/μmole) by whole cells of C. perfringens.
temperatures of the filter holder and wash medium. In preliminary experiments, the organism was shown to grow readily with any of these carbohydrates as the carbon source, except αMG, which was not tested. Therefore, it is likely that these compounds entered the cell by facilitated diffusion. However, the limitations discussed above make studies of this type of transport process a technical impossibility at present.

(iii) Temperature dependence of $^{14}$C-glucose transport.

The rate of transport was dependent on the temperature of the reaction mixture as determined by observing the total accumulation of $^{14}$C at 37°C and 30°C. While the maximum size of the pool remained nearly constant, the initial rate of uptake was found to be considerably less at the lower temperature (Fig. 11). Although the cells were grown at 37°C, it was found necessary to routinely observe the uptake of carbohydrates at 30°C to obtain adequate periods of linear uptake. The initial rate of uptake was found to increase by 81% over the seven degree change in temperature (Fig. 11). The $Q_{10}$ of the transport system was estimated from these data to be 2.54. This value compared well with the theoretical value of 2.0 for chemical reactions, and with the value of 2.2 found for components of the glucose transport system in E. coli (Hoffee et al., 1963).
Fig. 11. Total uptake of radioactivity by cells of C. perfringens with 1.25 x 10^{-5} M 14C-U-glucose (1.4 μc/μmole).

○—○, at 37° C; △—△, at 30° C.
(iv) Energy requirement of the transport of glucose.

The processes of accumulation have been shown to be energy dependent for β-galactosides (Kepes, 1957), galactose (Horecker, Thomas, and Monod, 1960a), and amino acids (Kay, 1968). Hoffman, Englesberg and Lamy (1963) demonstrated that a lack of energy caused an increase in accumulation by the glucose transport system in suspensions of E. coli.

Attempts to demonstrate energy dependence of transport in C. perfringens were hampered by the fact that the normal uncouplers of energy for transport systems, such as 2,4-dinitrophenol (Winkler and Wilson, 1966; Horecker, Thomas, and Monod, 1960a), are of little use in limiting the energy yielding reactions of an obligate anaerobe. This could be due to the fact that this organism undoubtedly relies largely on substrate level phosphorylation.

The addition of pyruvate to the cell suspension was demonstrated to be necessary for the achievement of reproducible rates of accumulation (Fig. 12). However, its omission from the resuspension medium did not eliminate accumulation, but did reduce the initial rate of uptake by approximately 50%. After the first 0.5 min of the experiment, the rate of accumulation of 14C was demonstrated to increase rapidly. Addition of 1.5 mM iodoacetamide to the solution without pyruvate during resuspension
Fig. 12. Total incorporation of radioactivity by cell suspension with $8 \times 10^{-6}$ M glucose (1.4 $\mu$C/umole). ○○○, with 0.6% pyruvate; △△△, minus pyruvate, □□□, minus pyruvate, plus $1.5 \times 10^{-3}$ M iodoacetamide.
of the cells did not affect the initial rate of glucose accumulation without pyruvate, but prevented the increase in the rate observed when iodoacetamide was not present (Fig. 12).

The ability of this organism to metabolize five of the amino acids present in the medium (Woods and Trim, 1942), probably accounted for the base level of uptake observed in the absence of added pyruvate. Attempts were made to simplify the resuspension medium in order to define more closely the source of energy for accumulation. Various buffer solutions and combinations of the components of the medium with and without added pyruvate, were used to wash and resuspend the cells but only the complete semi-defined medium with 0.6% pyruvate was found to yield reproducible rates of accumulation.

The increase in the rate of transport with time in the pyruvate-less cells was probably due to the supply of energy from metabolism of the glucose which had entered the cells during the early time course of the experiment. Iodoacetamide has been shown to inhibit the production of energy by glycolysis, through inhibiting 3-phosphoglyceraldehyde dehydrogenase activity (Krebs, 1955).

Active transport in certain systems has been shown to occur by the counter-transport of a similar compound using the same membrane carrier (Wilbrandt, and Rosenberg, 1961). It was difficult to demonstrate that pyruvate or some metabolic product of pyruvate was not involved in counter-transport with glucose, thus providing the energy required for accumulation. However, it is unlikely that a system capable of differentiating between
sugars epimeric in one hydroxyl, as will be shown, would also recognize a low molecular weight acid. Therefore, it would appear that the accumulation of glucose was dependent on energy gained, at least in part, by the metabolism of exogenously added pyruvate.

(v) Kinetics of accumulation of \(^{14}\)C-glucose and \(^{14}\)C-mannose.

Exposure of pyruvate-grown cells to \(^{14}\)C-U-glucose resulted in typical saturation kinetics for total uptake. The rate of uptake of glucose was found to increase linearly with concentration of substrate until 0.01 mM, then the rate of change in velocity with increasing substrate concentration decreased as the velocity approached a maximum (Fig. 13).

When the rates were plotted in the Lineweaver-Burke fashion, a linear relationship between the reciprocal of the velocity of uptake and the reciprocal of the substrate concentration was found (Fig. 14). Three separate trials were performed for glucose (Fig. 15) and the values of \(K_t\) and \(V_{\text{max}}\) for the three trials were estimated and averaged (Table V). A similar response of the rate of uptake of mannose to increasing external concentration of mannose was observed with saturation of the uptake mechanism becoming apparent after 0.016 mM mannose was present (Fig. 16). Values of \(K_t\) and \(V_{\text{max}}\) were estimated from the reciprocal plot of these data (Fig. 17), and compared to those values for glucose uptake (Table V).
Fig. 13. Saturation kinetics of $^{14}$C-glucose (1.4 μc/μmole) incorporation by C. perfringens. Cells at 30 C were exposed for 1 min to increasing concentrations of $^{14}$C-glucose. The rate of total uptake is plotted against concentration of glucose.
Fig. 14. Saturation kinetics of $^{14}$C-glucose (1.4 μc/μmole) incorporation by C. perfringens. Lineweaver-Burke plot of rate of uptake against glucose concentration from trial in Figure 13.
Fig. 15. Saturation kinetics of $^{14}$C-glucose (1.4 μC/μmole) incorporation by C. perfringens. Lineweaver-Burke plot of rate of uptake against glucose concentration for three different trials performed as in Figure 13.
The change from linear response of the rate of uptake to substrate concentration was demonstrated to occur over a very narrow range of substrate concentration, a phenomenon which has been shown to be typical of carbohydrate transport systems (Hoffee, Englesberg, and Lamy, 1964; Egan and Morse, 1966; Horecker, Thomas, and Monod, 1960). This abrupt saturation of the transport system caused technical difficulties in accurately determining the changes in the rate of uptake as the transport system approached saturation. The slight variation in the slope of the three reciprocal plots of velocity versus substrate concentration for glucose and the subsequent variation in the estimates of the $K_t$ and $V_{\text{max}}$ values resulted from this uncertainty.

While the $K_t$ value for mannose transport was over three times as large as the average value for glucose transport, the $V_{\text{max}}$ for mannose was only 33% larger than the average value for glucose (Table V). The $K_t$ values, while not absolute measures of affinity for the substrate, served to indicate that the accumulation mechanism had a much higher affinity for glucose than for mannose. The difference between the $V_{\text{max}}$ values for the transport of the sugars was small enough to indicate that both carbohydrates were accumulated at approximately the same maximum rate. It has been demonstrated that glucose and mannose each inhibited the accumulation of the other, and thus were both accumulated by the same mechanism, probably at the same maximum velocity.

Values of $K_t$ of the order of $10^{-5}$ M have been demonstrated to be typical of microbial carbohydrate transport mechanisms (Egan,
Fig. 16. Saturation kinetics of $^{14}$C-mannose (1.4 µc/µmole) incorporation by C. perfringens. Cells at 30 C were exposed for 1 min to increasing concentrations of $^{14}$C-mannose. The rate of total uptake is plotted against the concentration of mannose.
Fig. 17. Saturation kinetics of $^{14}$C-mannose (1.4 μc/μmole) incorporation by C. perfringens. Lineweaver-Burke plot of the rate of uptake against mannose concentration from Figure 16.
Table V. Saturation kinetics of the glucose-mannose transport system. Estimated values of $K_t$ and $V_{max}$ from trials in Figures 15 and 17.

<table>
<thead>
<tr>
<th>TRIAL</th>
<th>$K_t$ (M)</th>
<th>$V_{max}^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>$2.0 \times 10^{-5}$</td>
<td>38.9</td>
</tr>
<tr>
<td>B</td>
<td>$1.67 \times 10^{-5}$</td>
<td>31.3</td>
</tr>
<tr>
<td>C</td>
<td>$2.38 \times 10^{-5}$</td>
<td>29.5</td>
</tr>
<tr>
<td>Average</td>
<td>$2.02 \times 10^{-5}$</td>
<td>33.2</td>
</tr>
<tr>
<td>Mannose</td>
<td>$7.70 \times 10^{-5}$</td>
<td>44.4</td>
</tr>
</tbody>
</table>

$a$ - μmoles/min/mg dry weight.
and Morse, 1966; Hoffee, Englesberg, and Lamy, 1964; and Horecker, Thomas, and Monod, 1960). It should be noted that this exhibition of saturation kinetics did not differentiate between enzymatic and adsorptive processes of accumulation (Egan, and Morse, 1966).

(vi) Specificity of the transport of glucose and mannose.

As previously mentioned, the demonstration of saturation kinetics for the accumulation of $^{14}\text{C}$-carbohydrates did not differentiate between adsorptive and enzymatic mechanisms. In order to demonstrate that the concentrative mechanism was enzymatic, it was necessary to ascertain that the accumulation of carbohydrates was specific, a property that adsorption would not have exhibited.

When the cells were exposed to $^{14}\text{C}$-U-glucose, or to $^{14}\text{C}$-U-mannose, and the total uptake of $^{14}\text{C}$ observed over 1.5 min, a linear concentration of $^{14}\text{C}$ with time occurred for both sugars (Fig. 18a, 19a). In parallel experiments, unlabelled carbohydrates were added to 100 times the concentration of the $^{14}\text{C}$-substrate, and the rate of total incorporation was compared to the rate observed in the absence of added $^{12}\text{C}$-carbohydrate. The demonstration that for both glucose (Fig. 18) and mannose (Fig. 19) most carbohydrates did not competitively inhibit transport indicated that not all carbohydrates metabolized by the cells were transported
Fig. 18. Competition for glucose uptake in C. perfringens. The rate of incorporation of $8 \times 10^{-6}$ $^{14}$C-glucose (1.4 μCi/μmole) in the presence or absence of $8 \times 10^{-4}$ M $^{12}$C-carbohydrates. A. o—, $^{14}$C-glucose only; □—, $^{12}$C-pentoses added (ribose, xylose, arabinose). B. □—, $^{12}$C-disaccharides except maltose added (lactose, sucrose, trehalose); Δ—, $^{12}$C-maltose.
Fig. 18. Competition for glucose uptake in C. perfringens. The rate of incorporation of $8 \times 10^{-6}$ $^{14}$C-glucose (1.4 μc/μmole) in the presence or absence of $^{12}$C-carbohydrates. C, △, $^{12}$C-fructose; □, $^{12}$C-galactose; □, $^{12}$C-mannose; o, $^{12}$C-glucose control. D, ♣, $^{14}$C-glucose only; ♣, $^{14}$C-maltose; □, $^{14}$C-mannose; o, $^{14}$C-glucose control.
into the cell by a common mechanism, but rather that glucose and mannose were accumulated by mechanisms of a highly specific nature. The mutual inhibition of mannose and glucose concentration by each other (Table VI) demonstrated that they were accumulated or transported by the same mechanism (Wilbrandt, and Rosenberg, 1961).

It was demonstrated that fructose, ribose, and sucrose, all readily metabolized by this organism for growth, did not inhibit uptake of either mannose or glucose, while the disaccharide maltose did reduce the rate of accumulation (Table VI). Thus, the specificity of the accumulation mechanism was not determined solely by the size or number of rings, but was probably determined by the configuration of the substituents on the carbohydrate ring.

At this point it is necessary to consider the distinction between transport and accumulation. These functions have been separated in several metabolite transport systems (Winkler, and Wilson, 1966; Osborn, McLellan, and Horecker, 1961; and Kay, 1968) by observing the specificity of displacement of preloaded pools of one $^{14}$C-carbohydrate when a second, unlabelled carbohydrate was added. Competitive inhibition of initial uptake was considered to act on either the transport mechanism or the accumulation mechanism. However, the displacement of an accumulated pool was only possible if the two compounds shared the same accumulation mechanism.

Stable pools of metabolites were found necessary for this type of study, and techniques for generating these pools are
Fig. 19. Competition for mannose uptake in C. perfringens. The rate of incorporation of $1.5 \times 10^{-5}$ M $^{14}$C-mannose (1.4 μc/μmole) in the presence or absence of $1.6 \times 10^{-3}$ M $^{12}$C-carbohydrates. A. $\bullet$, $^{14}$C-mannose alone; $\triangle$, $^{12}$C-sucrose; trehalose, lactose; $\square$, $^{12}$C-maltose added; $\circ$, $^{12}$C-glucose added; $\circ$, $^{12}$C-mannose control. B. $\square$, $^{12}$C-fructose; $\triangle$, $^{12}$C-αMG added.
Fig. 19. Competition for mannose uptake in C. perfringens. The rate of incorporation of $1.5 \times 10^{-5} \text{ M}^{14}\text{C}-\text{mannose}$ ($1.4 \mu\text{c/umole}$) in the presence or absence of $1.6 \times 10^{-3} \text{ M}^{12}\text{C}-\text{carbohydrates}$. triangle, $^{12}\text{C}-\text{xylose added};$ square, $^{12}\text{C}-\text{arabinose added};$ circle, $^{12}\text{C}-\text{ribose added}.$
Table VI. Accumulation of carbohydrates and competitive inhibition of the rate of uptake of $^{14}$C-glucose ($8 \times 10^{-3}$ mM, 1.4 μc/μmole) and $^{14}$C-mannose ($1.6 \times 10^{-2}$ mM, 1.4 μc/μmole), by a 100-fold excess of these carbohydrates.

<table>
<thead>
<tr>
<th>12C-carbohydrate added in 100-fold excess</th>
<th>Accumulation by C. perfringens</th>
<th>% Inhibition of $^{14}$C-uptake</th>
<th>$^{14}$C-glucose</th>
<th>$^{14}$C-mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maltose</td>
<td>0</td>
<td>33</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Ribose</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Xylose</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>αMG</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Galactose</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fructose</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>79</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
known for other organisms (Kay, 1968; Hoffee, and Englesberg, 1962). The techniques used depended upon the use of non- or slowly metabolizable substrates to form the pools, or the use of mutant strains of the organism that did not immediately degrade the pooled material. Neither non-metabolizable analogues which could be accumulated, nor adequate mutant selection techniques, were available for the study of transport in C. perfringens. Studies of the transport mechanism alone are possible if analogues of carbohydrates that are transported, but not accumulated, are available. The competitive inhibition of the transport of \(^{14}\)C-U-αMG was attempted, but, as mentioned previously, non-specific retention of the radioactivity on the membrane filter obscured the relatively small changes in incorporated \(^{14}\)C levels. The final possible method for segregating transport from accumulation was the use of metabolic poisons to remove the energy supply for those accumulation mechanisms which are energy dependent (Wilbrandt, and Rosenberg, 1961; Winkler, and Wilson, 1966; Hoffee et al., 1963). As will be discussed, it was not possible to completely destroy accumulation through energy limitation. Had it been possible to remove accumulation by this technique, the technical difficulties mentioned previously in observing simple equilibration of substrates across the membrane would have interfered.

Therefore, as it proved impossible to differentiate between the functions of trans-membrane passage and accumulation of carbohydrates in C. perfringens, the terms transport and accumulation will be used interchangeably.
Based on the specificity of inhibition of the uptake of mannose and glucose, the structural requirements of a substrate of the accumulation process were elucidated. The transport mechanism was shown to have the higher affinity for glucose (Table V), with substituents or with any modification in structure reducing its activity. In compounds with any changes other than at the C-2 position, accumulation was completely eliminated.

Epimerization of the -OH at the C-2, to produce mannose, raised the $K_t$ value by a factor of three, but still allowed appreciable accumulation and approximately the same $V_{\text{max}}$ as for glucose accumulation (Table V). As a result of this lowered affinity, the competitive activity of mannose was found to be only 79% that of glucose, for glucose uptake (Table VI). Replacement of the C-1 hydroxyl with any substituents, as in αMG, trehalose, or sucrose, completely destroyed the transport activity. The sugar allose, which is the epimer of glucose at the C-3 hydroxyl, was not available for study of its inhibitory activity.

Epimerization of the C-4 hydroxyl group to form galactose, completely eliminated activity as a competitor for the glucose-mannose accumulation system, and was not itself accumulated by the system. However, substitution of the 4-hydroxyl in the β-configuration, by the formation of maltose, allowed 33% of the inhibitory activity to remain, while accumulation was found to be eliminated. The similar substitution with galactose at the 4 carbon of glucose in the β-configuration to form lactose, completely eliminated both forms of activity in the accumulation system.
Therefore, on the basis of these results, the specificity of the glucose-mannose accumulation system was found to be only partially dependent upon the configuration about C-2, and very sensitive to changes in the configuration about C-4. The system was also found to be very sensitive to substituents at C-1, and substitutions at C-4 resulted in varying degrees of reduction of competitive activity.

Studies by Hagihira, Wilson, and Lin (1963) on the specificity of glucose uptake by E. coli, using derivatives of αMG, demonstrated that substituents on C-2 affected the activity of the carbohydrates. Other workers have demonstrated 30% inhibition of αMG concentration by E. coli by the addition of maltose, while mannose and sucrose each caused a 20% inhibition of activity (Hoffee, Englesberg, and Lamy, 1963). The specificity of the glucose concentration system in E. coli appeared therefore to differ greatly from that demonstrated in suspensions of C. perfringens, in that the configuration about the C-2 in the substrate or competitor of the C. perfringens mechanism was not found to be critical, while changes at this position reduced the rate of accumulation in suspensions of E. coli.

The 'anaerobic transport mechanism' for carbohydrates in yeast, as described by Scharff and Kramer (1962), did not appear in C. perfringens, as fructose was shown to be transported by the yeast system, while it had no activity in the glucose-mannose accumulation system studied. The 'constitutive monosaccharide transport system' of yeast (Cirillo, 1968) is more similar to
the system described for \textit{C. perfringens}. Substitution of the anomeric hydroxyl completely destroyed activity, and, substituents or configurational changes at all other carbons except C-2 decreased the activity of the glucose ring in both systems. However, the carbohydrates were compared as competitive inhibitors of the transport of d-xylose, or of \( \epsilon \)-sorbose, which are not metabolized by yeast. D-xylose was found to be inactive in the system for the accumulation of glucose by \textit{C. perfringens} limiting the value of comparisons between the two systems.

Studies of the specificity of the carbohydrate accumulation system of the Gram-positive \textit{S. aureus}, have indicated that carbohydrate accumulation is very specific, with very little competition for entry between the carbohydrates. Maltose had no detectable effect on the rate of concentration of glucose, and mannose was not tested for activity in the system (Egans, and Morse, 1966), making comparisons with the specificity of \textit{C. perfringens} \textit{uptake} difficult.

3. The pooling of \(^{14}\text{C}\)-glucose

(i) Pool capacity.

In experiments in which both the total incorporation and the incorporation into the cold TCA insoluble material were measured over 40 min, the amount of \(^{14}\text{C}\) in the soluble pool rapidly reached a maximum value (Fig. 20, 21), after which the size of the pool remained relatively constant as the \(^{14}\text{C}\) of both the total
Fig. 20. Formation of a cold TCA soluble, radioactive pool, by cell suspensions with $8 \times 10^{-6}$ M $^{14}$C-U-glucose (1.4 $\mu$C/umole)

- O-O, total incorporation;
- Δ-Δ, soluble radioactive pool;
- □-□, cold TCA precipitate.
Fig. 21. Formation of cold TCA soluble, radioactive pool by cell suspensions in the $1.82 \times 10^{-4}$ M $^{14}$C-U-glucose (1.4 $\mu$C/umole). 0–0, total incorporation; $\Delta$–$\Delta$, cold TCA precipitate; $\square$–$\square$, soluble radioactive pool.
and cold TCA insoluble fractions increased at the same rate. The pool size decreased towards the end of the experiment, presumably due to the metabolism of glucose (Fig. 21).

The decrease in the rate of uptake after the initial 1.5 min, may have been the result of any of several events. The transport system may have become limited by the depletion of exogenous glucose, by the rate of energy production or by the equilibrium constant of the transport process. The $^{14}$C-glucose may have been rapidly metabolized by the cell to intermediates or end-products which were released to the medium, making the system glucose-limiting.

When the pool size was maximal (Fig. 10), the filtrate of the suspension was concentrated and passed through a combined column of Dowex-50-H$^+$ and Dowex-1-formate ion-exchange resins. Essentially all of the radioactivity was eluted frontally with distilled water. When the eluate was chromatographed, it was found to co-chromatograph with the glucose standard. Thus, glucose had not been converted to a degradation product and the system had not become glucose-limiting. When the density of the cell suspension was reduced by one-half in an attempt to prolong the time required to form the maximum pool size, the rate of uptake and the level of the $^{14}$C-pool were also decreased by one-half (Fig. 22). This demonstrated that the rate of accumulation, as well as the total accumulation, was proportional to cell mass and that the transport enzymes were saturated under these experimental conditions.

The decrease in maximum pool size, proportional to the decrease
FIG. 22. Total incorporation of radioactivity by cell suspensions with 2.5 x 10^{-5} M ¹⁴C-U-glucose (1.4 μC/μmole). Δ-Δ, 7.8 x 10^{-2} mg dry weight of cells/ml; O-O, 3.9 x 10^{-2} dry weight of cells/ml.
in cell mass, indicated that some property of the cell limited
the size of the pool of accumulated carbohydrate. Assuming that
the concentration of the carbohydrate was facilitated by an
energy-requiring system, then the size of the pool may have been
determined by the energy supply or by the rate of energy production.
As the cells were resuspended in 0.6% pyruvate at the beginning
of the experiments, and as the rate of accumulation of $^{14}\text{C}$
remained constant over several hours of sequential experiments,
the rate of energy production, rather than the amount of substrate
available, may have been the limiting factor.

An alternative explanation is that the equilibrium constant
of the transport process, a measure of the affinity of the transport
mechanism for the carbohydrate, may have limited the pool size
by defining the maximum ratio of internal to external
concentrations of the carbohydrate. As will be shown later, the
$^{14}\text{C}$ inside the cell was in the form of a charged derivative of
glucose, and therefore was not in a form directly in equilibrium
across the membrane with the glucose. However, the ratio of
the concentration of the derivative to the concentration of free
exogenous glucose determined by the rate constant of the
accumulation mechanism, could have defined the maximum pool
size possible.

(ii) Internal concentration.

During the early time course of transport the $^{14}\text{C}$
entered the cells at a linear rate, and after 1 min, 92% of
the total label was present in the soluble pool (Fig. 20). There was a 15 to 20 sec lag in the incorporation of the \(^{14}\)C into the cold TCA insoluble fraction and this was followed by increasing rate of incorporation for the remainder of the experiment.

The size of the soluble pool of \(^{14}\)C was estimated from the level of soluble \(^{14}\)C at 1.5 min (Fig. 20), after which time the rate of incorporation decreased as the pool capacity was reached (Fig. 10). Based on the assumption that 80% of the cell weight was water (Luria, 1966), and that 10% of this cell was intercellular, the concentration of the \(^{14}\)C in the intracellular water at 1.5 min was calculated to be 396 times the concentration of the extracellular \(^{14}\)C. Losses of \(^{14}\)C as \(^{14}\)O\(_2\) from the system over the course of the experiments were negligible.

A comparison of this concentration ratio with values obtained with other microorganisms was not particularly valuable because of the wild range of published ratios.

Galactose was concentrated by \(10^4\)-fold in *E. coli* (Horecker, Thomas, and Monod, 1960a). Lactose was accumulated 7.2 x \(10^3\) times above the external concentration in *S. aureus* while the same organism was found to concentrate maltose by a factor of 700, sucrose by a factor of 520, and \(\alpha\)MG by a factor of 370 over the extracellular concentration (Egan, and Morse, 1966). These latter values are of a magnitude comparable with the level of accumulation demonstrated in *C. perfringens*. Higher concentration factors have been demonstrated for the accumulation of amino acids by microorganisms, with values running into the tens of thousands (Britten and McClure, 1962).
(iii) Nature of the pooled $^{14}$C.

The accumulation of pools of soluble $^{14}$C which occurred when suspensions of *C. perfringens* were exposed to $^{14}$C-U-glucose has been described as the accumulation of glucose, even though the nature of the pooled material was not known. In *S. aureus*, however, it has been demonstrated that maltose, sucrose, αMG, lactose, and isopropyl-thio galactosides were accumulated as phosphorylated derivatives (Hengstenberg, Egan, and Morse, 1968). In addition, the implication of the phosphoenolpyruvate-dependent phospho-transferase system for the phosphorylation of carbohydrates, in the accumulation of carbohydrates by *E. coli* and *S. typhimurium* has been demonstrated (Tanaka and Lin, 1967; Tanaka, Fraenkel, and Lin, 1967; Simoni et al., 1967). An attempt to elucidate the nature of the $^{14}$C material accumulated within the cell indicated that in *C. perfringens*, the $^{14}$C from glucose and mannose was concentrated as a derivative.

When the $^{14}$C material present in the cold TCA soluble pool at 0.5 min was isolated and concentrated, chromatography of the pool demonstrated no detectable $^{14}$C which chromatographed as free glucose or as free mannose. Rather, in both cases two peaks containing all of the radioactivity were shown to remain near the origin. When aliquots of the pool were separated by electrophoresis, some free carbohydrate was present near the
origin of the electrophoretogram, probably as a result of hydrolysis during preparation or during the actual electrophoresis. The two charged peaks were observed to move the same distance as glucose-6-phosphate and fructose 1,6-diphosphate standards with approximately 65% of the label in the singly-charged fraction. The remainders of the concentrated pools were separated by preparative electrophoresis, and the fractions were recovered and concentrated. Aliquots of these fractions from both $^{14}$C-glucose and $^{14}$C-mannose pools were dephosphorylated with commercial bacterial alkaline phosphatase. The dephosphorylated fractions were concentrated, and then co-chromatographed with fructose, glucose and mannose standards. Upon dephosphorylation, all of the pooled materials chromatographed as uncharged hexoses. The pool of $^{14}$C-glucose was then found to contain monophosphorylated glucose, monophosphorylated fructose and diphosphorylated fructose. The accumulated pool of $^{14}$C from $^{14}$C-mannose contained monophosphorylated derivatives of mannose, glucose and fructose, in addition to a diphosphorylated derivative of fructose. The pooled material isolated after 4 min exposure to $^{14}$C-glucose was found by electrophoresis to be largely in the highly charged fraction, probably as a diphosphorylated derivative of fructose.

It has been demonstrated that cell membranes which are relatively impermeable to charged derivatives of carbohydrates, allow the transport of the free carbohydrates (Hengstenberg, Egan, and Morse, 1968). Thus, an efficient method of concentrating carbohydrates in the cell would be to phosphorylate them on the
inside of the membrane. The uncharged carbohydrate would be transported in by facilitated diffusion, and the charged product of phosphorylation would be unable to leave the cell, thus forming a concentration gradient of carbohydrate. Although an active soluble hexokinase could have phosphorylated the glucose and mannose during accumulation by this organism, evidence has been accumulated for a phospho-transferase or kinase, of the membrane-bound type described for *E. coli* (Kundig, Ghosh, and Roseman, 1964). Comparisons were made of the specificity of the accumulation mechanism with that determined for the hexokinase activity in cell-free extracts. The hexokinase was found to phosphorylate glucose and fructose, with fructose being phosphorylated at a rate 10% that of glucose (Table III). No activity of hexokinase for mannose was observed, even when excess quantities of mannose and extract were added. While the failure to detect this enzyme did not completely disprove its existence, there remained little probability that mannose-kinase was involved in the accumulation of mannose.

Studies of the specificity of $^{14}$C-carbohydrate accumulation mechanism have demonstrated the opposite effect. Fructose if neither accumulated nor has any activity in the inhibition of glucose accumulation, while mannose is accumulated and inhibits the rate of glucose uptake by 70%. It therefore appeared that the phosphorylation of the carbohydrates during accumulation was performed by a mechanism which differed from that of the soluble hexokinase.

The absence of detectable free glucose or mannose in the
pooled material indicated that these carbohydrates were phosphorylated upon transport, implicating a phospho-transferase or kinase associated with the membrane.

This mechanism of accumulation technically cannot be correctly described as a glucose-concentration mechanism, because a derivative of glucose and not free glucose was demonstrated to be accumulated.

4. Mechanism of the accumulation of glucose

The accumulation of glucose and mannose in suspensions of *C. perfringens* has been demonstrated to occur by a system displaying saturation kinetics, temperature sensitivity, and energy dependence. In addition, by its high degree of specificity the reaction appeared to be enzymatic in nature. The mechanism was demonstrated to accumulated glucose to concentrations several hundred times the external concentration, and this pooled material was shown to be in the form of phosphorylated derivatives. The method of phosphorylation was not the soluble hexokinase, but rather was probably due to a membrane-bound phospho-transferase or kinase.

Metabolism of exogenously-added pyruvate or previously accumulated glucose probably supplied the high energy intermediates necessary for phosphorylation. The limited soluble pool size might well have resulted from either the depletion of the reserve of the high energy intermediate, or from the attainment of an equilibrium across the phosphorylating mechanism.
This mechanism as proposed bears more than superficial resemblance to the phosphorylation mechanisms of accumulation by \textit{S. aureus} (Egan, and Morse, 1966) and the phosphoenolpyruvate dependent phospho-transferase system of accumulation of carbohydrates in \textit{E. coli} (Simoni et al., 1967).

The advantages of possession of such an accumulation mechanism to the cell are several. The carbohydrate which served as the most common energy source in the organism's normal environment was efficiently scavenged from the external medium and the energy expended by the accumulation mechanism simultaneously served to activate the carbohydrate for metabolism by glycolysis.
GENERAL DISCUSSION

Necrotic mammalian tissue during pathological conditions, the lower region of the mammalian digestive system under normal conditions, and soil, are known to be the three major ecological systems of \textit{C. perfringens} (Breed, Murray, and Smith, 1957).

There are two major mechanisms by which this organism may survive extended periods of exposure to the deficiency of nutrients in soil. \textit{C. perfringens} is known to form spores, and as certain strains are important causative agents of food poisoning, extensive studies of the formation, survival, and germination of the organism's spores have been published (Collee, Knowlden, and Hobbs, 1961; Weiss, and Strong, 1967). Studies in cultural conditions have indicated that only under alkaline conditions, with a source of carbohydrate present, will spores be formed (Willis, 1964). These conditions occur in the lower region of the mammalian digestive system, so considerable numbers of spores may be excreted in wastes, to ultimately be distributed in the soil.

The maintenance of the integrity, over 18 hrs, of the vegetative cells grown on the peptone-less, semi-defined medium, would seem to provide an alternative explanation for the survival of \textit{C. perfringens} in the austere conditions of
the soil. In the absence of substrates for the proteolytic exo-enzymes, the formation of the autolytic enzyme system would be repressed, and the magnesium present in the soil would repress the small amounts of autolytic activity present. The cells could utilize any carbonaceous or nitrogenous endogenous supply of energy that they may possess, and the viability and integrity of the vegetative cells could be maintained for extended periods of time.

Any glucose or mannose present in the soil, could be immediately accumulated to levels far above the external concentration. Other carbohydrates which would support growth could not be accumulated, but rather could be rapidly transported into the cell by facilitated diffusion.

During growth in necrotic mammalian tissues, the supply of glucose and nutrients would allow rapid growth. Peptides would be present, and the production of the toxins upon which the pathogenicity of *C. perfringens* depends, would be induced. Induction of the autolytic enzymes by the presence of peptides would not destroy the culture, as the presence of an energy source has been shown to repress the activity of the autolytic enzymes.

The accumulated glucose would be metabolized via the Embden-Meyerhof pathway, with the heterofermentative production of lactic, acetic, and butyric acids, in addition to the carbon-dioxide and molecular hydrogen which produce the gaseous disruption of tissue in "gas-gangrene". Five-carbon sugars
for the production of nucleic acids may be produced by the transketolase/transaldolase system, from fructose-6-phosphate and from 3-phospho-glyceraldehyde, in the absence of a hexose-monophosphate pathway or complete pentose cycle.

To provide the energy required for the rapid growth, the maintenance of a low oxidation potential, and the extensive production of exo-toxins, required for the pathogenic state, the organism could concentrate the available glucose from the tissue by the economical means of activating the glucose, through phosphorylation, for glycolysis. Thus, in one step the organism would simultaneously retain the carbohydrate molecule within the cell, and prepare it for further metabolism. This system is twice as efficient as that which has been proposed for the accumulation of β-galactosides in E. coli, by the use of one mole of ATP to concentrate one mole of carbohydrate through altering the affinity of the transport mechanism (Kepes, 1960).

An organism which used this system, would then be required to expend another mole of ATP to phosphorylate the carbohydrate during glycolysis.

The other Gram-positive organism which has been studied for carbohydrate transport, S. aureus, accumulated eight different sugars via permeases highly specific for each carbohydrate, coupled to a general membrane carrier mechanism (Egan, and Morse, 1966). The evolution of C. perfringens into a form which, in nature, grows rapidly only in mammalian
systems, where the major soluble carbohydrate is glucose, may have been related to the loss of the accumulation mechanisms for carbohydrates other than glucose. A study of the specificity of the transport mechanism for non-accumulated carbohydrates in *C. perfringens* would serve to clarify this concept, but has proven to be technically difficult. Perhaps a comparative study of the specificity of transport and accumulation mechanisms of related pathogens and non-pathogens would serve to support evidences of evolutionary trends in microorganisms.
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