THE UNIVERSITY OF MANITOBA

D-GLUCOSE TRANSPORT STUDIES IN A STRAIN OF *CLOSTRIDIUM BOTULINUM*

by

Lucinda E. Gordon

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements for the Degree of

Master of Science

Department of Microbiology

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ABSTRACT

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During growth of vegetative cells of *Clostridium botulinum* MSp^+, derived from successive transfers of log-cells, 50% of the glucose remained in the medium at the end of log growth and only 10% remained when phase-bright endospores were first seen. By contrast, in cultures inoculated with heat-activated spores, as much as 50% glucose was present in the medium when phase-bright endospores appeared.

Kinetic studies of D-glucose uptake and Lineweaver-Burk plot showed that there was a high affinity for the sugar. The transport in the spore-derived suspension was significantly inhibited by sodium fluoride, ATP dependent, and exhibited stereospecificity. With the log-cell derived suspensions, sulfhydryl group reactive agents such as iodoacetate, iodoacetamide and N-ethylmaleimide (NEM) showed various degrees of inhibition, the most potent being NEM, suggesting the involvement of a protein in transport. Various carbon sources were tested for their effects on the uptake of D-glucose which ranged from various degrees to no inhibition, indicating a very specific transport system. Studies showed that the uptake of D-glucose was also pH and temperature dependent.

The phosphorylation of 2-deoxy-D-glucose and fructose was shown to occur by the phosphoenolpyruvate (PEP) phosphotransferase system (PTS), which was protein dependent. It would appear that under certain conditions of growth, *C. botulinum* MSp^+ can transport glucose and fructose by a PEP-dependent system and also by an adenosine triphosphate phosphate (ATP) mediated process.

TO MY WONDERFUL FAMILY

ACKNOWLEDGEMENTS

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INTRODUCTION

INTRODUCTION

The formation of endospores is mainly restricted to genera *Bacillus, Clostridium* and *Sporosarcina*. The bacterial spore shows such marked differences from the mother cell, that it is difficult to imagine that they are interconvertible forms of the same organism (Young 1978).

Endospore formation is considered to be regulated at the level of transcription, which represents a system of sequential induction of genes that are normally repressed during vegetative growth. The transition from a vegetative cell to a spore involves many stages, and during the process the membrane of the mother cell or sporangium grows around and engulfs the prespore, enclosing a portion of the cytoplasm and half of the cellular DNA (Fitz-James and Young 1959). Maturation follows and a dormant body composed of complex internal structures is produced, with the mother cell lysing and releasing the complete spore. In this free state, the spore represents a resting stage and because of its very low rate of metabolism, can survive for decades without an external source of nutrients.

The transition of a spore to a vegetative cell occurs when transferred to suitable nutrients, and involves activation, germination and outgrowth. Activation is described as a 'conditioning' of the spore to germination by means of certain reducing agents, ageing, heat and low pH. Activation apparently involves changes in the configuration of macromolecules but not metabolism (Keynan and Evenchik 1969). Germination is a process of biochemical degradation, triggered by specific inducing agents and is not dependent on RNA or protein synthesis, but

is metabolism initiated. Both activation and germination are considered to be responsible for the termination of the cryptobiotic state of spores. The process of outgrowth is preceded by spore enlargement, elongation and followed by the emergence as a vegetative cell. During outgrowth, new kinds of macromolecules and cell structures are synthesized (Fitz-James and Young 1969).

Although there is remarkable similarity between *Bacillus* and *Clostridium* both from a morphological and physiological aspect, there are fundamental differences between the genera (Day and Costilow 1964). In *Bacillus* species, sporulation is usually not initiated until the end of stationary phase of growth, when there is glucose depletion.

Similarly sporulation in batch cultures of *Clostridum spp.* occurs when glucose is exhausted and growth stops, but Vinter (1969) has reported that sporulation can occur in growing cultures of some clostridial species.

In order to understand committment to sporulation with respect to glucose in a strain of *C. botulinum* MSp^{+}, a comparison of glucose utilization during growth and spore formation was studied, using log cells or clean spores inoculated into a fresh medium. The uptake, and phosphorylation of glucose by PEP and ATP, were also examined.

HISTORICAL

HISTORICAL

Bacterial cells are capable of utilizing biologically important molecules such as glucose from their surroundings, and anaerobic fermentation is the simplest and most primitive type of biological mechanism for obtaining energy from glucose (Lehninger 1970).

Clostridium botulinum Msp^{+} is a non-toxigenic sporogenic mutant of *C. botulinum* type E, obtained by treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). This strain is an obligate anaerobe, saccharolytic with some limited proteolytic activity. Glucose is metabolized by the Embden-Meyerhof Pathway (EMP) yielding acetate which serves as a precursor for the synthesis of poly-β-hydroxy-butyrate (PHB) granules. The PHB granules are degraded to β-hydroxy-butyrate, further dehydrated and reduced, eventually yielding butyric acid and energy for sporulation (Emeruwa 1975).

After growth has stopped completely or almost completely, most cells undergo a series of morphological and structural changes leading to sporulation 8-10 h later. It is generally accepted that sporulation is not usually initiated until the end of the logarithmic phase of growth when there is glucose depletion, nitrogen limitation or amino acid starvation, whereas excess nutrients such as ammonia, glucose and phosphate prevent sporulation. (Freese *et al* 1970; Vinter 1969; and Freese *et al* 1979.)

Reports have established that there are fundamental differences in chemical composition of free spores and of vegetative cells. Sporulation in microorganisms is a *bona fide* example of differentiation in which novel structures are produced *de novo* under genetic

control within a given cell. Studies of this phenomenon can provide well defined model systems for understanding the mechanism whereby irreversible sequential events are ordered and controlled during differentiation (Halvorson 1965).

Glycolysis and oxidative phosphorylation are the major mechanisms of energy generation in cells, but both mechanisms may not be present in a bacterial species. Bacteria such as *C. perfringens*, which metabolize glucose by the EMP, probably do not have a respiratory chain, and cannot carry out oxidative phosphorylation. Not much is known about the mechanism of transport of glucose in anaerobes because only a few *Clostridium spp* have been tested (Futai 1978).

Metabolic Transport

An integral step in the utilization of carbohydrates by these microorganisms is the transport of the molecules across the semi-permeable membrane. The translocation of solutes across membranes is mediated by transport mechanisms. Studies on carbohydrate uptake into bacteria have revealed the presence of several transport systems, which have been defined as facilitated diffusion, active transport and group translocation.

Facilitated diffusion is sometimes referred to as a carrier-mediated transport system in which molecules cross the membranes by carrier proteins specific for the solute. The solute attaches to the membrane-bound carrier that shuttles back and forth between opposite sides of the membrane either by diffusion or by a conformational change (Roseman 1972).

In active transport of carbohydrates by bacteria it is proposed

that membrane permeation by carbohydrates and their accumulation against a concentration gradient are two separate phenomena (Phibbs and Eagon 1970). Postma and Roseman (1976) also suggested that two discrete energy-requiring steps are necessary for sugars that are not substrates of the phosphoenolpyruvate phosphotransferase system (PTS). Solutes that are actively concentrated have the ability to efflux from the cell down the chemical gradient unless metabolic energy is continuously expended to maintain high intracellular levels of such sugars (Postma and Roseman 1976). The net effect of this model, is that carbohydrates are concentrated unaltered within the cell. Permeation is rapidly catalyzed by substrate specific membrane carriers and may be differentiated from concentration by the use of inhibitors that block the supply of metabolic energy (Phibbs and Eagon 1970).

Phosphoenolpyruvate (PEP), long known to be an important intermediate in glycolysis as a major phosphoryl donor in anaerobic ATP generation, has stimulated interest within the last two decades, because of its role in the transport and phosphorylation of sugars in bacterial cells (Anderson and Wood 1969). In group translocation it is proposed that a PEP:PTS functions simultaneously to transport carbohydrates and to trap these substances as phosphate esters (Phibbs and Eagan 1970). This process may or may not require energy. Evidence from many sources suggests that the PTS catalyzes the transport of a "high-energy" phosphoryl group from PEP to a number of sugars. The system, besides its principal role of translocating sugar substrates, also regulates a number of other important

physiological processes such as motility. Group translocation resembles active transport in the sense that the cell appears to accumulate solute but differs because in group translocation the membrane component is acting as an enzyme with vectorial properties. Four proteins are required to catalyze the overall reaction in *Staphylococcus aureus* (Roseman 1972). Although PEP-dependent hexose phosphotransferase systems are found in a wide variety of bacterial genera, a number of investigations have failed to provide firm evidence for such systems in other procaryotic or eucaryotic organisms.

A transport role for PTS in *Escherichia coli* was suggested by Kundig *et al* (1964). Tanaka *et al* (1967) demonstrated genetic evidence of a transport role of the PTS in *Aerobacter aerogenes*. The most convincing evidence for the involvement of the PTS in transport was given by Kaback (1968) who isolated *E. coli* membranes that accumulated phosphorylated derivatives of certain sugars. The most extensive survey for glucose PTS was carried out by Romano and co-workers (1970) on various genera of bacteria. From the results of several surveys, a general pattern has been proposed (Hays 1978).

1. Glucose PTS is not present in strict aerobes.
2. Glucose PTS is found in most facultative anaerobes only if they metabolize hexoses by the EMP yielding two molecules of PEP for each hexose metabolized.
3. PTS is not found in hetero fermentative organisms which use the phosphoketolase pathway or in *Zymomonas mobilis* which uses the anaerobic Entner-Doudoroff pathway, presumably

because these pathways yield only one PEP molecule for each hexose metabolized (McGill and Dawes 1971; Saier 1977).

Anaerobic bacteria probably conserve metabolic energy by utilizing the PTS for sugar uptake. In strictly aerobic organisms however, sugars are generally transported by active transport systems. Even so bacterial cells seem to exhibit so many variations that no generalization with regards to transport can be made at this time. Not all bacteria contain the different types of transport systems. Bacterial species exhibit different modes of transport for various sugars or even the same sugar and any discussion of the process of transport must, therefore, take into account the type of microorganism and the sugar (Roseman 1972).

Glucose

The transport of glucose in virtually all facultative anaerobes is mediated by the PTS (Romano 1970). In the aerobic *Pseudomonas aeruginosa*, the nonmetabolizable analog, 2-deoxy-D-glucose was also transported by facilitated diffusion (Eagon 1971). In *E. coli*, 2-deoxy-D-glucose was accumulated against a gradient as 2 deoxy-D-glucose 6-phosphate (Winkler 1966). A glucose PEP:PTS in *E. coli* was described in Andrews and Lin (1976) who showed that glucose was phosphorylated in transit.

Available information on the transport of carbohydrates by Gram-positive microorganisms is somewhat limited. In 1965 Egan and Morse characterized the system of carbohydrate transport in a pleiotropic transport mutant of *S. aureus*. The transport was accomplished by a system specific for each carbohydrate in

combination with a general protein carrier. The carbohydrates were accumulated as phosphorylated derivatives (Egan and Morse 1966; Saier 1975). In *Bacillus subtilis* glucose was transported by the PEP:PTS inducible by glucose during exponential growth phase, whereas ATP dependent glucokinase remained constant throughout growth (Freese *et al* 1970). Schachtele and Mayo (1973) described a PEP dependent PTS in *Streptococcus mutans*, *S. sanguis* and *S. salivarius* that resulted in the phosphorylation of glucose at carbon-6. Glucose PTS was said to be consitiutive in oral streptococci because decryptified cells of bacteria grown on various sugars readily phosphorylated 2-deoxy-D-glucose. *Streptococcus lactis* is reported to have a constitutive glucose PTS (Thompson 1978). In *C. perfringens* glucose was reported to be transported in resting cell suspensions of the organisms. Competition studies showed that glucose was transported by a highly specific mechanism. The accumulated carbohydrates were present as phosphorylated derivatives and not as free carbohydrates. Data showed that phosphorylation was not mediated by the soluble hexokinase (Groves and Gronlund 1969a and b). Patni and Alexander (1971a) failed to show PEP:glucose PTS activity in *C. thermocellum* and suggested that the initial step in glucose metabolism might be catalyzed by glucokinase.

Fructose

A pathway for D-fructose metabolism in *A. aerogenes* was described by Hanson and Anderson in 1968, which involved a PEP-dependent phosphorylation at C-1, followed by an ATP-dependent phosphorylation of D-fructose 1-P at C-6. In that same year Fraenkel also presented evidence of a similar system in an *E. coli* mutant. He showed that the system is represented in a wide variety of genera of bacteria

including *Pseudomonas alcaligenes*, *Bacillus subtilis* and *Clostridium spp*. The PTS in strictly aerobic bacteria is limited to fructose (Sobel and Krulwich 1973; Sawyer *et al* 1977), whereas the anaerobic clostridia make use of the PTS in conjunction with 1-phospho fructokinase (PFK) to channel fructose into the EMP (Patni and Alexander 1971b).

Galactose

The transport of galactose has been studied in relatively few organisms. In enteric bacteria, galactose is not transported by the PTS, and in *E. coli* K12 it is taken up by at least four distinct active transport systems (Andrews and Lin 1976). Two recent reports suggest that in the absence of an active transport system, some *E. coli* and *S. typhimurium* strains can take up free galactose via a glucose enzyme II, (E_{II}) without a need for enzyme 1 (E_I) or histidine-containing protein (H Pr). This suggests that E_{II} can mediate facilitated diffusion of the galactose but the transport occurred without phosphorylation (Kornberg and Riordan 1972; Postma 1976).

Galactose is a secondary substrate for lactose PTS in *S. aureus* with a K_m 500 times greater than that of lactose (Fox and Wilson 1968). In *C. pasteurianum* galactose uptake is mediated by an electrogenic transport motivated by a membrane potential and/or the transmembrane pH gradient (Booth and Morris 1975).

Mannose

The transport of mannose in membrane preparations obtained from enteric bacteria were found to be phosphorylated by the PTS (Postma and Roseman 1976). Curtis and Epstein (1975) supplied evidence for a

PTS system in *E. coli* which was also active on D-glucose as well as derivatives altered at C-2 positions. Other phosphoryl transferases have been found in *A. aerogenes* such as the acylphosphate:hexose phosphotransferase. This enzyme is constitutive and is able to transfer the phosphoryl group from a wide variety of phosphate derivatives to mannose but PEP is not the phosphoryl donor. (Postma and Roseman 1976).

The mannose transport system in *S. aureus* is via the PTs, is constitutive and not efficient (Friedman and Hays 1977). In *C. perfringens* mannose is actively transported by a highly specific mechanism. Specificity data indicate that phosphorylation occurs at the transport level rather than by soluble hexokinase (Groves and Gronlund 1969a). L-mannose, a sugar not known to occur naturally, is metabolized by *A. aerogenes* through the pathway L-mannose → L-fructose $\xrightarrow{ATP}$ L-fructose 1-P→dihydroxyacetone-P+L-lactaldehyde. This seems to be the only instance in which the first step in the metabolism of a non-derived aldohexose occurs by the isomerization of an aldoketose (Mayo & Anderson 1968).

Lactose

In the transport of lactose in *E. coli* the rate limiting step in its utilization is the translocation across the membrane. The transport system mediates the accumulation of free lactose against a concentration gradient. The sugar is concentrated, without metabolic transformation, by a membrane-bound permease driven by a proton motive force. The lactose is subsequently cleaved by β-galactosidase. From genetic studies of lactose permease, the membrane-bound carrier has been identified as an M protein (Andrews and Lin 1976).

In *S. aureus* lactose is accumulated as lactose phosphate rather than the free disaccharide, and is subsequently hydrolysed by a phospho-β-galactosidase yielding glucose + galactose 6-phosphate (Kennedy and Scarborough 1967; Hengstenberg *et al* 1967, 1970).

Maltose

Maltose enters *E. coli* by a process requiring the participation of a periplasmic binding protein and this transport may depend on chemical energy, such as ATP (Saier 1977; Kellerman and Szmelcman 1974). In *S. typhimurium* maltose is transported by a non-PTS permease but the transport apparently occurs by facilitated diffusion in *S. aureus* and subsequent cleavage by a cellular maltase. The released glucose is mainly metabolized by the PTS (Button *et al* 1973). In *P. fluorescens* maltose transport is mediated by a shock-resistant active transport system (Guffanti and Corpe 1976).

Sucrose

In a strain of *S. aureus* that did not metabolize sucrose, Egan and Morse (1966) found that sucrose was accumulated in a derivatized form. Friedman and Hays (1977) found that the transport system in the strain was constitutive and did not require sugar-specific soluble factors for phosphorylation. In *A. aerogenes*, transport involves uptake and hydrolysis of sucrose, excretion of fructose and re-uptake of fructose via the PTS (Kelker and Anderson 1972).

Transport studies have generally been carried out in whole cells, but metabolic reactions in the cytoplasm have been known to alter the rate of transport and modify the solute transported. To

prevent such effects studies have been carried out on mutants defective in some aspect of transport and also by using non-metabolizable analogs. Rosen and Kashket (1978) stated that many bacterial species have complicated forms of energy metabolism. Some species have sufficient endogenous reserves of metabolizable compounds to energize transport and in some cases endogenous metabolism can continue even in the presence of exogenous substrates (Dawes and Ribbons 1962). In *Euglena gracilis*, it has been reported that endogenous reserve is the main energy source for growth and that exogenous substrates were used only to replenish or increase the supply (Danforth and Wilson 1961). In freshly harvested cells of *E. coli* the high rate of endogenous metabolism tends to mask the effect of an exogenous energy source. Subsequently several procedures have been developed which deplete the energy reserves of *E. coli* (Wilson *et al* 1972; Flagg and Wilson 1976). Species of *Streptococcus* with low endogenous reserves are frequently used for studies on transport (Kanapka and Hamilton 1971; Schachtele and Mayo 1973; Schachtele 1975). These bacteria are unable to take up solute unless an exogenous energy source is provided.

In many bacteria metabolic energy is conserved at the level of the membrane, not in the form of a chemically high energy intermediate, but as an electrochemical gradient of hydrogen (H^+) ions (Rosen and Kashket 1978). A pH gradient has been reported in cells of *C. pasteurianum* which is dependent on an energy source provided by an adenosine triphosphatase (ATP-ase) driven extrusion of protons from the cells (Riebeling and Jungermann 1975; Clark and Morris 1976).

The pH gradient found to be essential for the growing cell, is not found in energy depleted cells and is thought to be required for substrate accumulation and other types of transport processes. The ATP-dependent pH gradient is now postulated to be a general property common to all procaryotes including the strictly anaerobic clostridia which are considered to be among the earliest forms of microorganisms (Riebeling and Jungerman 1976). The multitude of data on transport processes in procaryotes help to explain the diversity between different types of microorganisms. These differences could provide some selective advantages to the bacteria.

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism:

The organism used in this study was a sporogenic non-toxigenic mutant of *Clostridium botulinum* type E.

Media

A Trypticase-glucose-peptone (TPG) medium was used, which contained Trypticase BBL, (5% W/V) proteose peptone Difco (0.5% W/V) D-glucose 22 mM, and sodium thyoglycollate Difco (0.2% W/V). Added to this medium was yeast extract BBL (0.4% W/V) to prepare TPGY. The media were adjusted to pH 7 before autoclaving.

Stock Spore Suspension

The method employed was essentially that of Emeruwa and Hawirko (1972). A spore suspension was inoculated into TPGY broth and incubated for 10 h at 30°C, until most of the spores had germinated. A 10% inoculum of young cells was transferred to TPGY and incubated for 24 to 36 h at 30°C. Cultures showing > 90% spores were centrifuged for 15 min at 4,080 g at 5°C. The pellet was washed three times with deionized water and then treated with a solution of 0.02 M phosphate buffer, pH 7.0, containing 100 µg/ml trypsin (Sigma) and 200 µg/ml lysozyme (Sigma) for 2 to 4 h at 37°C with continuous agitation. The debris was removed by centrifugation at 1000 g for 20 min, 4000 g for 10 min and 10000 g for 5 min. The cleaned spores were suspended in 0.02 M phosphate buffer, pH 7.0, to an absorbance of 0.6 at 600 nm (Coleman Junior 11A, model 6/20A) containing approximately 10^6 spores/ml and stored at 0°C.

Growth Conditions

Test media inoculated with 10% spores obtained from stock spore suspension, in sterile water, activated for 10 mins at 65°C; or a 10% log cell inoculum obtained from 3 successive transfers of 10 h cultures were incubated at 30°C. Growth was monitored by measuring the absorbance at 600 nm and the pH was determined on a radiometer 22. Outgrowth and spore formation was estimated by phase-contrast microscopy. Sporulation was computed as the number of phase-bright endospores (ES) in relation to the total number of cells counted (200 examined).

Carbon Substrate Utilization

Selected carbon sources (22 mM) were used to replace glucose in trypticase peptone broth (TP) prior to inoculation. Log cells were washed twice with the TP broth. Control cultures were grown without added carbohydrate.

Effect of Glucose on Growth and Sporulation

A 10% inoculum (V/V) of log cells or heat-activated spores was added to the test medium in tubes stoppered with Subaseal closures (Griffin & George Ltd., UK) and flushed with CO_2:H_2 (10:1) to maintain anaerobic conditions. Samples were withdrawn at intervals by syringe and analyzed for growth, pH, endospores and residual glucose. Glucose was determined enzymatically by glucostat reagent (Worthington Biochem. Corp, Freehold, N.J., U.S.A.) as outlined in Nowotny (1969).

Assays for ^{14}C-glucose Incorporation during Growth

D-[U-^{14}C] glucose (333 MC_i/mM) Amersham Corp., was added at a concentration of 2 μC_i/ml to the test culture and samples were removed at specified periods. To measure incorporation samples (0.2 ml) were mixed with equal volume of ice cold (10% W/V)

trichloracetic acid (TCA) and held at 0°C for 30 min. The precipitate was collected on 25 mm diameter membrane filters (Gelman, 0.45 uM) and washed 3 times with cold TCA (5% W/V) and twice with cold ethanol. The air-dried filters were placed in 5 ml Scinti-verse scintillation fluid (Fisher Scientific Co.) and counted in a Beckman model SC-230 liquid scintillation spectrometer. For uptake studies the labelled sample (0.2 ml) was collected on membrane filters, washed several times with cold 0.02 M phosphate buffer pH 7.0, air-dried and counted for radioactivity.

Transport Assays

Transport of substrates was carried out according to the method of Groves and Gronlund (1969a). Cells grown to mid-log phase in TPG broth were washed twice with TP broth and then resuspended to an OD of 0.28 at 660 nm. The cell suspension was flushed with N_2 and CO_2 and placed on ice. The OD was measured during the course of the experiment, to ensure that the cell density remained constant. Varying concentrations of ^{14}C-glucose were added to the cell suspension contained in a reaction vessel, prewarmed at 30°C. Samples (0.5 ml) were removed at intervals, filtered, washed with 0.02 M phosphate buffer and placed in Bray's solution (Bray 1960). The test time which was determined by plotting glucose accumulation vs time, with the linear portion of the curve representing the initial reaction rate, was 1 min for transport studies.

To determine the effect of pH, the cells were washed twice and equilibrated in magnesium phosphate (MP) buffer at different pH values before addition to the reaction vessel. Cell suspensions were

preincubated for 5 min before addition of ^{14}C-glucose. Samples were removed and treated as above.

In studying the effect of temperature on the uptake of ^{14}C-D-glucose, the cell suspensions were equilibrated for 5 min at the various temperatures before the addition of ^{14}C-D-glucose. The effects of sulfhydryl group inhibitors and sodium fluoride were determined by their addition to the cell suspensions 2 min before the labelled glucose.

To study the inhibition of D-glucose transport, the unlabelled carbohydrates were added to the ^{14}C labelled D-glucose at 100 times the concentration of the ^{14}C-D-glucose. The rate of total incorporation of ^{14}C into whole cells was compared with the rate of incorporation in the absence of ^{12}C-D-glucose.

Phosphotransferase Assays

For the phosphotransferase assay, cells were harvested after 10-11 h growth in TPG or TPGY by centrifugation at 5000 g for 7 min. PEP:glucose phosphotransferase activity was assayed by a modification of the method of Schachtele and Mayo (1973).

The cells were washed with 0.015 M KCl, suspended in 0.05 M potassium phosphate buffer (pH 6.5) containing 10^{-3} M MP buffer, and stored overnight at -20^{o}C. The cell suspension was then slowly thawed at room temperature. The protein content of 0.1 ml samples was determined with the Folin Ciocalteu reagent by the modified method of Lowry (1951) after extraction with NAOH at 100^{o}C for 5 min (Herbert *et al* 1971).

The reaction mixture contained: 1.0 µmole ^{3}H-2-deoxy-glucose*

(1.0 μC_i/μmole), 1.5 μmole PEP, decryptified cells and MP buffer to a final volume of 1.0 ml. Cells and reagents were pre-warmed to 30°C. The reaction was started by mixing and held at 30°C for 30 min without shaking. After incubation the reaction mixture was rapidly chilled in ice. The cells were removed by centrifugation at 5000 g for 7 min.

The supernatant was analyzed for the amount of ^{3}H-2-deoxy-glucose phosphorylated, by ion exchange chromatography. 0.5 ml of the supernatant was applied to a column (4 x 0.6 cm) of Bio-RAD AG1-X2 anion exchange resin (200-400 mesh) in the chloride form. Free ^{3}H-2-deoxy glucose phosphate was subsequently eluted with 6 ml of 0.1 M NACl + HCl (Gachelin 1970). Samples (0.5 ml), in 10 ml Bray's scintillation fluid were counted for radioactivity in a Beckman LS-230 Scintillation spectrometer.

*18.2 C_i/mM ; Amersham Corp.

RESULTS

RESULTS

PART I

Growth on Various Carbon Sources

The ability of *Clostridium botulinum* Msp^+ to grow on various carbon sources as measured by absorbance (A) 600 nm is shown in Table I. No significant differences were observed except that growth in maltose was increased at 30°C but reduced at 21°C compared to glucose. It was noted that the glucose derivative, α methyl-D-glucoside appeared to be utilised.

Growth and Glucose Utilization

Figs. 1-4 represent the kinetics of growth and glucose utilization using log-cell and spore inocula. In cultures inoculated with successive transfers of log-cells (10 h) into fresh trypticase-peptone-glucose (TPG) broth 15 mM glucose remained in the medium at mid-log phase and 5 mM at the end of exponential growth, when phase-bright endospores appeared. The pH of the medium decreased very rapidly during exponential growth and sporulation (Fig. 1).

However, in cultures inoculated with heat-activated spores 20 mM glucose remained in the medium at mid-log phase and 12 mM at the time phase-bright endopores were seen. The absorbance of the culture continued to rise during the later stage of growth, without evidence of a stationary phase. The pH of the spore-inoculated culture decreased slowly during exponential growth (Fig. 2).

When TPG was supplemented with yeast extract and inoculated with spores 16 mM glucose remained in the medium at mid-log phase and less than 2 mM glucose, when phase-bright endospores appeared. Growth

rate was the same as in the medium without yeast extract, but the utilization of glucose after mid-log phase was much more rapid in the yeast extract supplemented medium. The rate of pH change was the same but occurred earlier than in the medium without yeast (Fig. 3).

When a sub-lethal dose of rifampicin (0.05 µg/ml) was added to the yeast extract supplemented medium 18 mM glucose remained at mid-log phase and 2mM when phase-bright endospores were seen. The rate of growth was reduced, pH change the same, but sporulation was delayed by ~4 h (Fig. 4).

In TPG broth inoculated with log-cells (10%) or spores (10%), and in TPGY media with varying amounts of spore inocula, no significant difference in residual glucose concn occurred at mid-log phase of growth. The pH decreased to 6.5 and phase-bright endospores appeared 8 hours later (Table II).

Cultures inoculated with log-cells or spores in TPG were exposed to ^{14}C-D-glucose and the ~~total~~ incorporation of radioactivity into the cells was determined (Table III). No difference in uptake, or incorporation as determined by TCA precipitation, in log-cells or spore culture was observed up to mid-log phase. Relative incorporation was dependent on the amount of growth as measured by A 600 nm. There was an increase in activity of log cell culture compared to spores at the end of the exponential growth phase however.

Table IV presents the effect of inhibitors of sugar protein carriers, and glycolysis, on growth and spore formation of log cell and spore inoculated cultures. The addition of iodoacetic acid and iodoacetamide to the cells in TPG broth prevented any significant

growth. N-ethyl-maleimide, a known inhibitor of glucose PTS E_{II} in *E. coli*, showed no effect on growth or spore formation.

Figure 1. Growth of and glucose utilization by log-cells in trypticase-peptone-glucose broth.

↓ Time of appearance of phase-bright endospores.

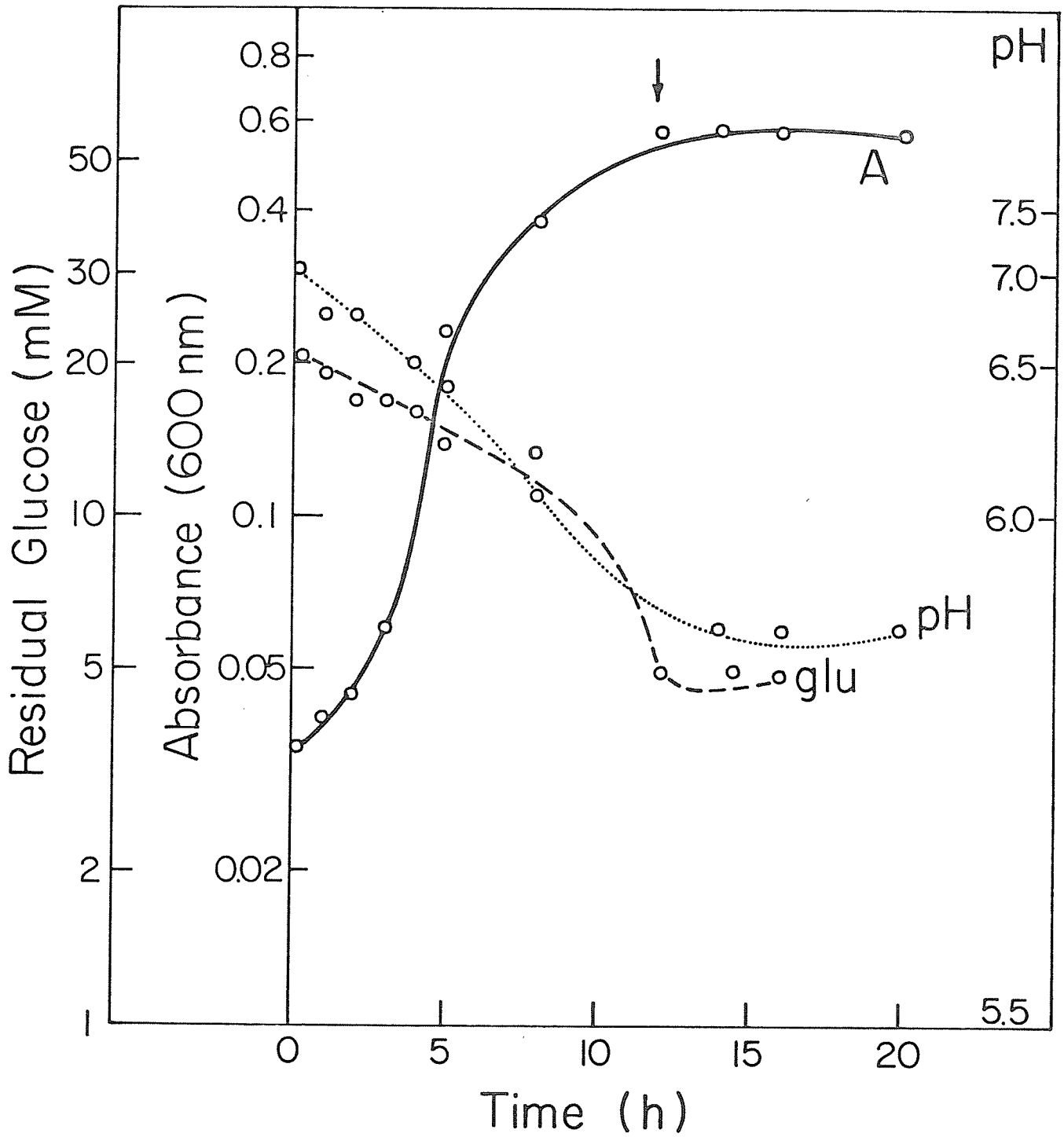
Residual Glucose (mM)
Absorbance (600 nm)
pH
Time (h)
A
pH
glu
50
30
20
10
5
2
1
0.8
0.6
0.4
0.2
0.1
0.05
0.02
7.5
7.0
6.5
6.0
5.5
0
5
10
15
20

Figure 2. Growth of and glucose utilization by spores in trypticase-peptone-glucose broth.

↓ Time of appearance of phase-bright endospores.

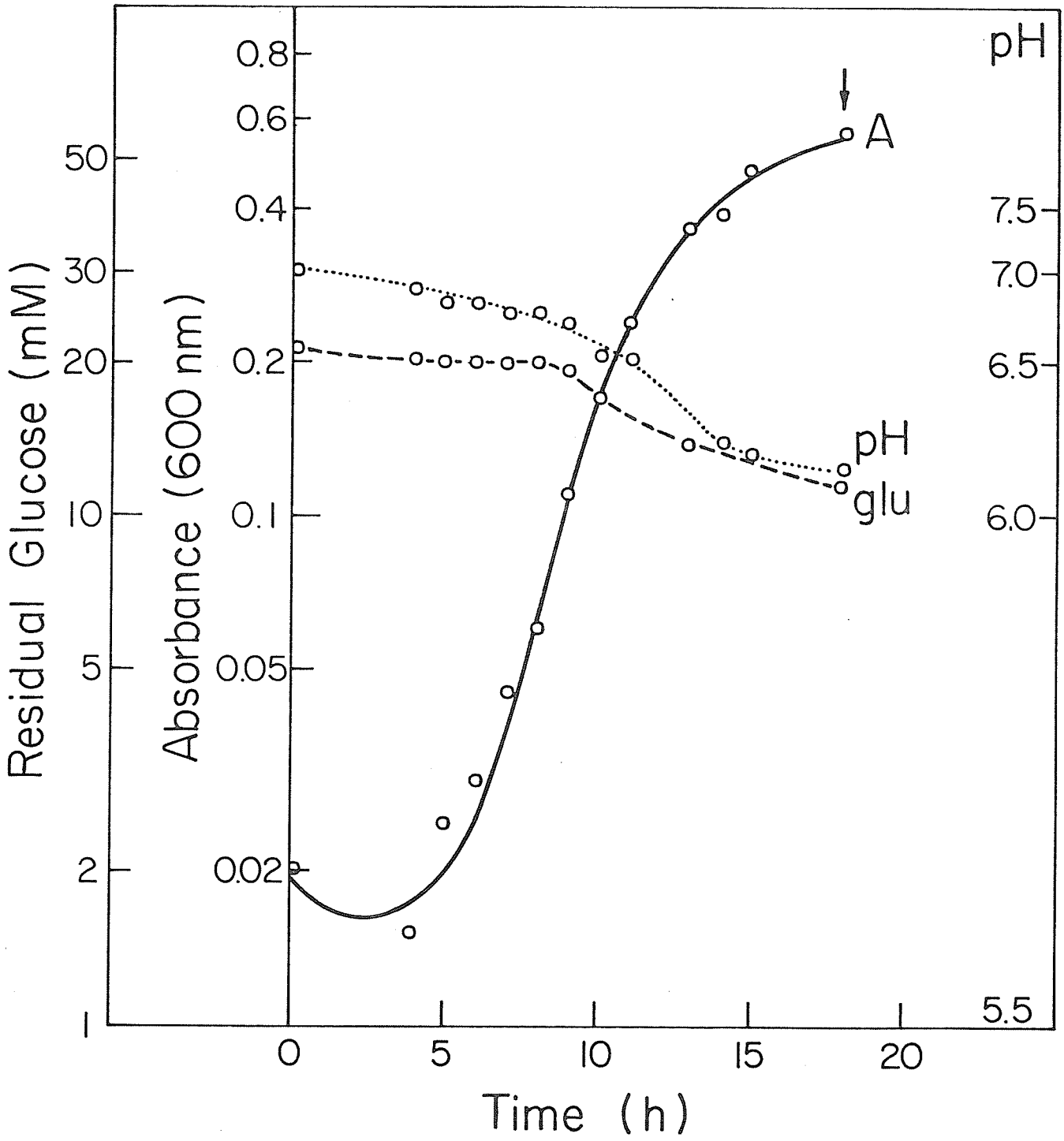
Residual Glucose (mM)
50
30
20
10
5
2
1
Absorbance (600 nm)
0.8
0.6
0.4
0.2
0.1
0.05
0.02
pH
7.5
7.0
6.5
6.0
5.5
A
pH
glu
0
5
10
15
20
Time (h)

Figure 3. Growth of and glucose utilization by spores in trypticase-peptone-glucose-yeast broth.

↓ Time of appearance of phase-bright endospores.

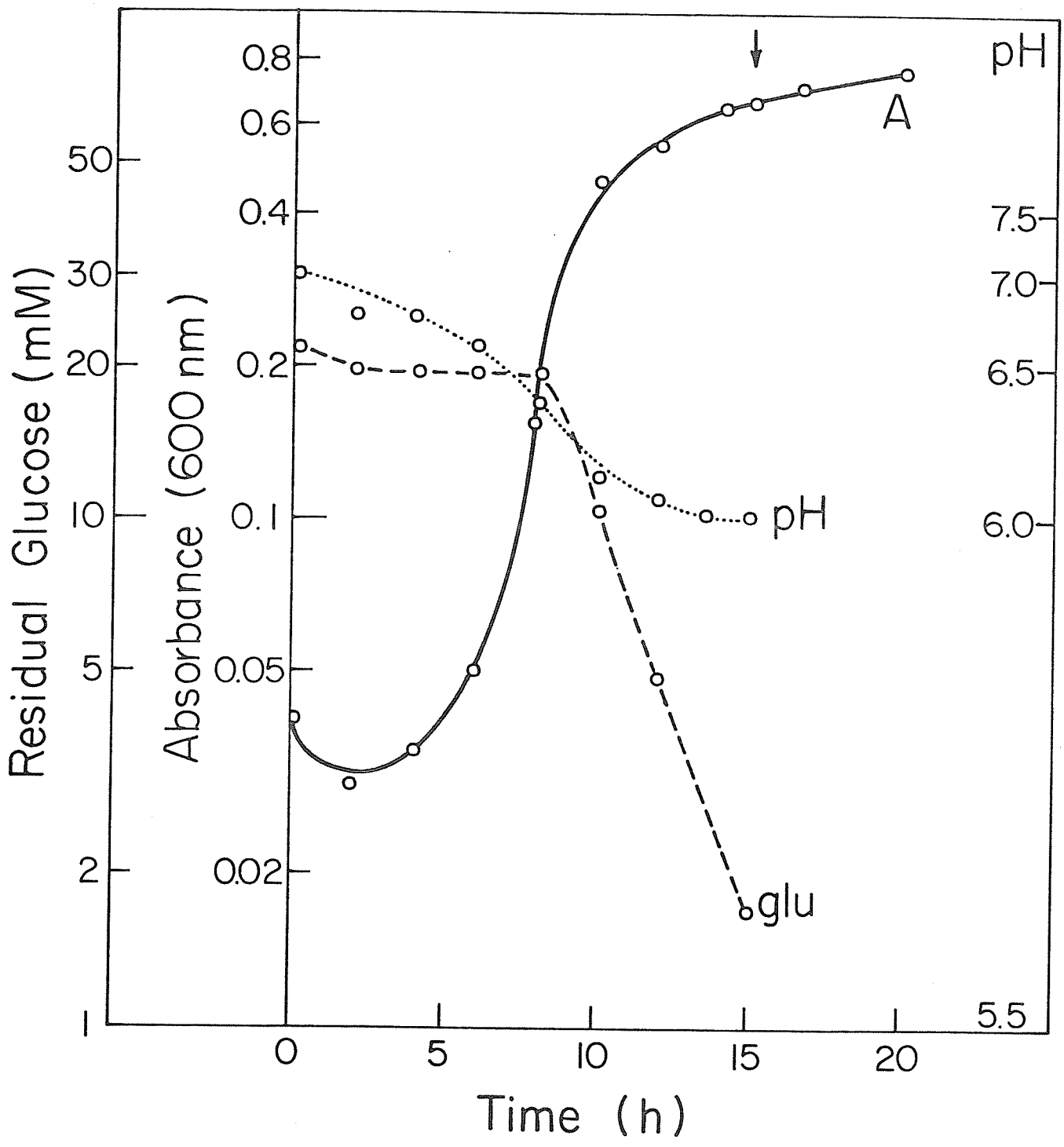
Residual Glucose (mM)
50
30
20
10
5
2
1
Absorbance (600 nm)
0.8
0.6
0.4
0.2
0.1
0.05
0.02
pH
7.5
7.0
6.5
6.0
5.5
A
pH
glu
0
5
10
15
20
Time (h)

Figure 4. Growth of and glucose utilization by spores in trypticase-peptone-glucose-yeast broth with rifampcin.

↓ Time of appearance of phase-bright endospores.

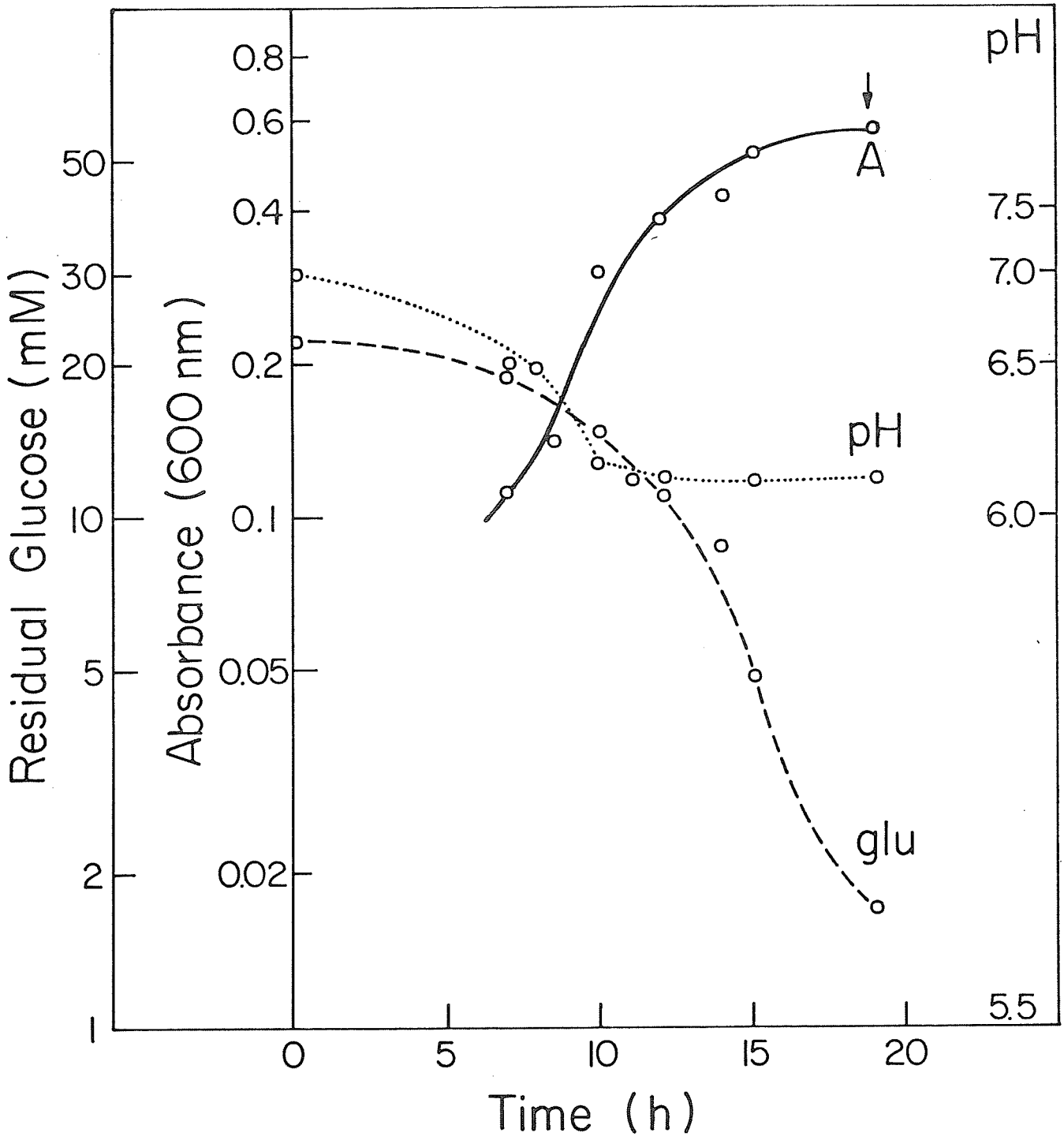
Residual Glucose (mM)
50
30
20
10
5
2
1
Absorbance (600 nm)
0.8
0.6
0.4
0.2
0.1
0.05
0.02
pH
7.5
7.0
6.5
6.0
5.5
A
pH
glu
0
5
10
15
20
Time (h)

TABLE I

Growth[a] on various carbon sources in trypticase peptone broth

Carbon[d] Source (22 mM)	Inocula					
	Log cells[c]				Spores[b]	
			pH			
	30°C	21°C	30°C	21°C	30°C	21°C
Glucose	100	63	6.1	6.2	100	10
Fructose	96	58	6.3	6.4	105	10
Galactose	100	47	6.6	6.7	100	10
Mannose	82	47	6.6	6.6	105	10
α-methyl-glucoside	82	82	6.1	6.4	105	5
Lactose	24	23	6.6	6.7	20	8
Maltose	112	78	5.9	6.2	126	5
Sucrose	110	31	6.8	6.8	110	8
None	24	11	6.9	6.9	20	5

[a] Results given relative to $A_{600\ nm}$ of D-glucose at 30°C.

[b] Reading at 10 h

[c] Reading at 5 h

[d] All monosaccharides were in the D configuration

TABLE II

Effect of inoculum on growth

Inoculum	T (h)	A_{600}	Residual glucose (mM)	pH
		TPG		
Log cells				
(10%)	4†	0.16	16.8	6.5
	12*	0.58	5.4	5.8
Spores				
(10%)	10†	0.05	16.5	6.5
	18*	0.42	11.0	6.1
		TPGY		
Spores				
(10%)	6†	0.11	16.2	6.5
	14*	0.49	0.3	5.8
(5%)	8†	0.24	17.4	6.5
	16*	0.97	0.7	6.1
(1%)	9†	0.10	18.9	6.5
	17*	0.92	1.5	6.1

†Mid-log phase of growth

*Appearance of phase-bright endospores (1%)

TABLE III

Incorporation of ^{14}C-D-glucose during growth

Inoculum	Growth h	Growth A_{600}	Relative Incorporation[a] Whole cell	Relative Incorporation[a] TCA ppt
Log cells				
	0	0.03	1.0	1.0
	1	0.04	10.0	10.0
	2	0.05	27.0	21.0
	3	0.06	56.0	49.0
	4	0.16[b]	98.0	93.0
	8	0.38	343.0	338.0
	16	0.58	558.0	540.0
Spores				
	0	0.02	1.0	1.0
	4	0.01[c]	14.0	13.7
	6	0.03	16.4	16.1
	7	0.05	25.5	24.2
	8	0.06[b]	48.6	45.5
	9	0.11	94.8	89.0
	10	0.17	134.5	120.0
	11	0.37	264.5	262.0

[a] relative cpm

[b] mid-log phase

[c] outgrowth

TABLE IV

Effect of inhibitors on growth[a]

	A_{600}		Sporulation (%)	
Inhibitor (1 mM)	Log cells	Spores	Log cells	Spores
Iodoacetic acid	0.06	0	0	0
Iodoacetamide	0.16	0	0	0
N-ethyl-maleimide[b]	0.86	0.88	80	100
None	0.86	0.98	80	100

[a] 21 h, 30°C

[b] 0.1 mM

PART II

Kinetics of D-glucose Transport

The rate of uptake of D-glucose as a function of concentration is shown in Figs. 5, 7, 8. In cells derived from a log-cell inoculum, the K_m was calculated to be 2×10^{-5} M for D-glucose (Fig. 5) and 5×10^{-6} M for 2-deoxy-D-glucose (Fig. 6). The rate of uptake of D-glucose increased linearly with concentration of substrate until 5×10^{-6} M, then the rate of uptake remained constant as the velocity approached a maximum (Fig. 5).

In cells derived from a spore inoculum, the K_m for D-glucose uptake was 1×10^{-6} M and the linear rate of uptake occurred over a very narrow range at the low glucose concentrations (Fig. 8). It was noted that as the D-glucose concentration approached 10 μM, a substantial increase in uptake occurred with no saturation (Fig. 7).

The result of inhibition studies on the effect of the addition of ^{12}C carbohydrates to the cell suspensions containing ^{14}C-glucose is shown in Table V. Fructose, maltose α-methyl-D-glucoside and 2-deoxy-D-glucose were the effective inhibitors of D-glucose uptake in log-cell derived suspensions and maltose, 2-deoxy-D-glucose, 6-deoxy-D-glucose, α-methyl-D-glucoside and galactose in spore-derived suspensions.

The effect of pH on the uptake of ^{14}C-D-glucose is shown in Table VI. In cells derived from a log-cell inoculum, a maximum increase in transport occurred between pH 6.5-7.0, whereas in the cells derived from spores, no significant change was observed between pH 5.5 and 7.5. The increased effect of temperature on uptake occurred at $20^{\circ}C$ in

both log-cell and spore-derived suspensions (Table VII).

Table VIII presents the effect of various inhibitors on the uptake of D-glucose. The sulphydryl group reactive agents inhibited uptake in cells derived from a log-cell inoculum but had no effect on cells derived from spores. On the contrary, sodium fluoride had no effect on log-cell derived suspensions.

PEP Phosphotransferase Activity

The results of assays carried out with frozen-thawed cells derived from log-cells and spore inocula are shown in Tables IX-XI. In the assays from the log-cell-derived suspensions the phosphorylation of the glucose analog 2-deoxy-D-glucose, increased with the addition of PEP, when the protein concn was 5.0 mg/ml. When ATP was substituted for PEP, the amount of phosphorylation was reduced ~75% in starved cells and ~30% in cells not starved. It was noticed that in the absence of ATP and PEP the activity was reduced ~90% (Table IX).

The fructose PEP phosphotransferase activity of cells (protein 6.0 mg/ml) grown in fructose is shown in Table X. The results indicate that the phosphorylation of ^{14}C-D-fructose increased with the addition of PEP and was reduced ~50% when ATP was substituted for PEP.

In assays with cells derived from a spore inoculum, the PEP: glucose phosphotransferase activity was negligible. In the absence of PEP and ATP the activity was reduced ~20% in both starved and non-starved cells and when ATP was substituted for PEP no significant reduction occurred (Table XI).

Figure 5. Saturation kinetics of ^{14}C-glucose uptake by cells* at mid-log phase in TP broth. Cells were derived from a log-cell inoculum.

Inset: Lineweaver-Burk plot

*340 µg protein/ml

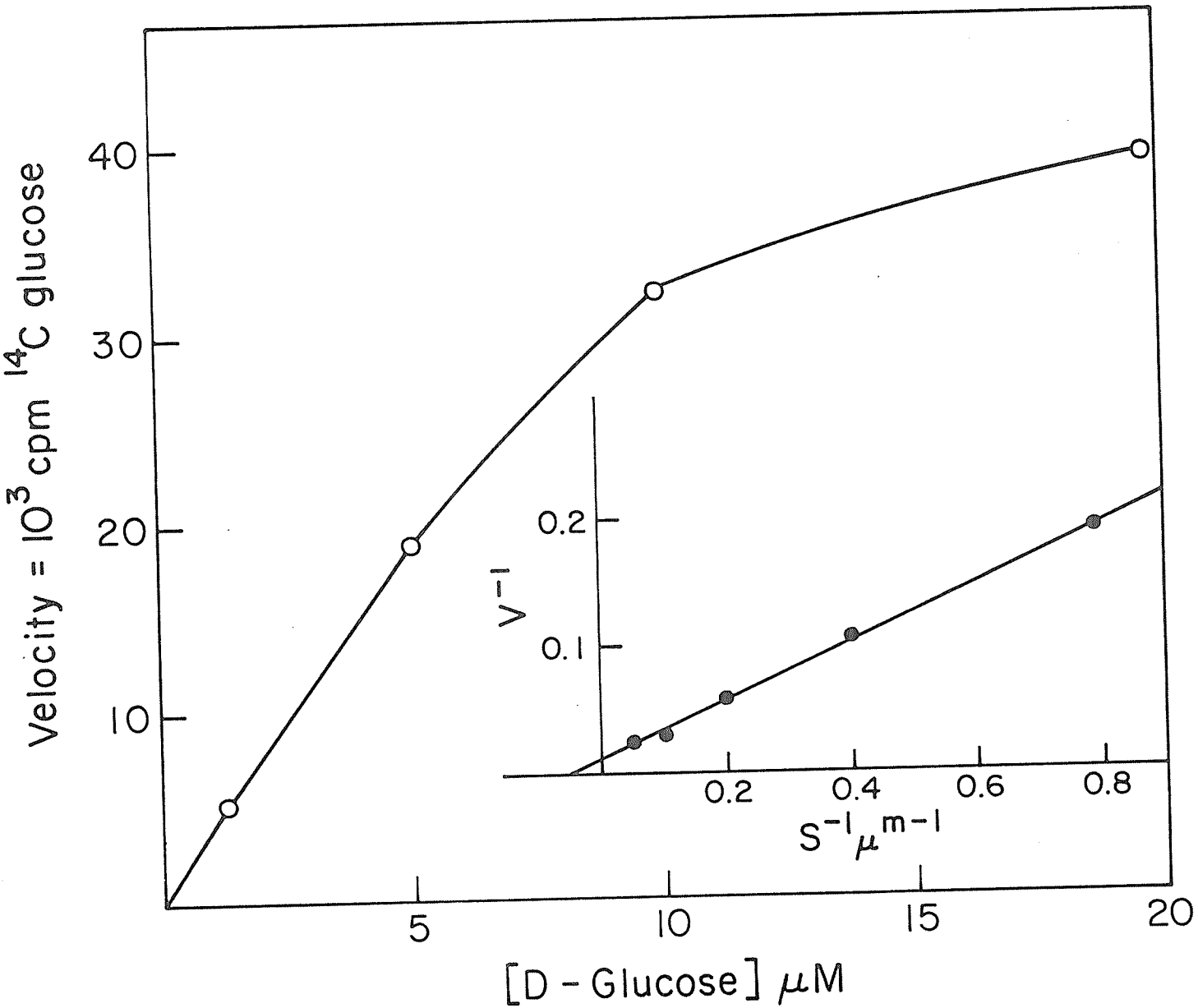
Velocity = 10^3 cpm ^{14}C glucose
40
30
20
10
5
10
15
20
[D - Glucose] μM
V^{-1}
0.2
0.1
0.2
0.4
0.6
0.8
$S^{-1}\mu^{m-1}$

Figure 6. Saturation kinetics of ^{14}C-2-deoxy-D-glucose uptake by cells* at mid-log phase in TP broth. Cells were derived from a log-cell inoculum.

Inset: Lineweaver-Burk plot.

*340 µg protein/ml

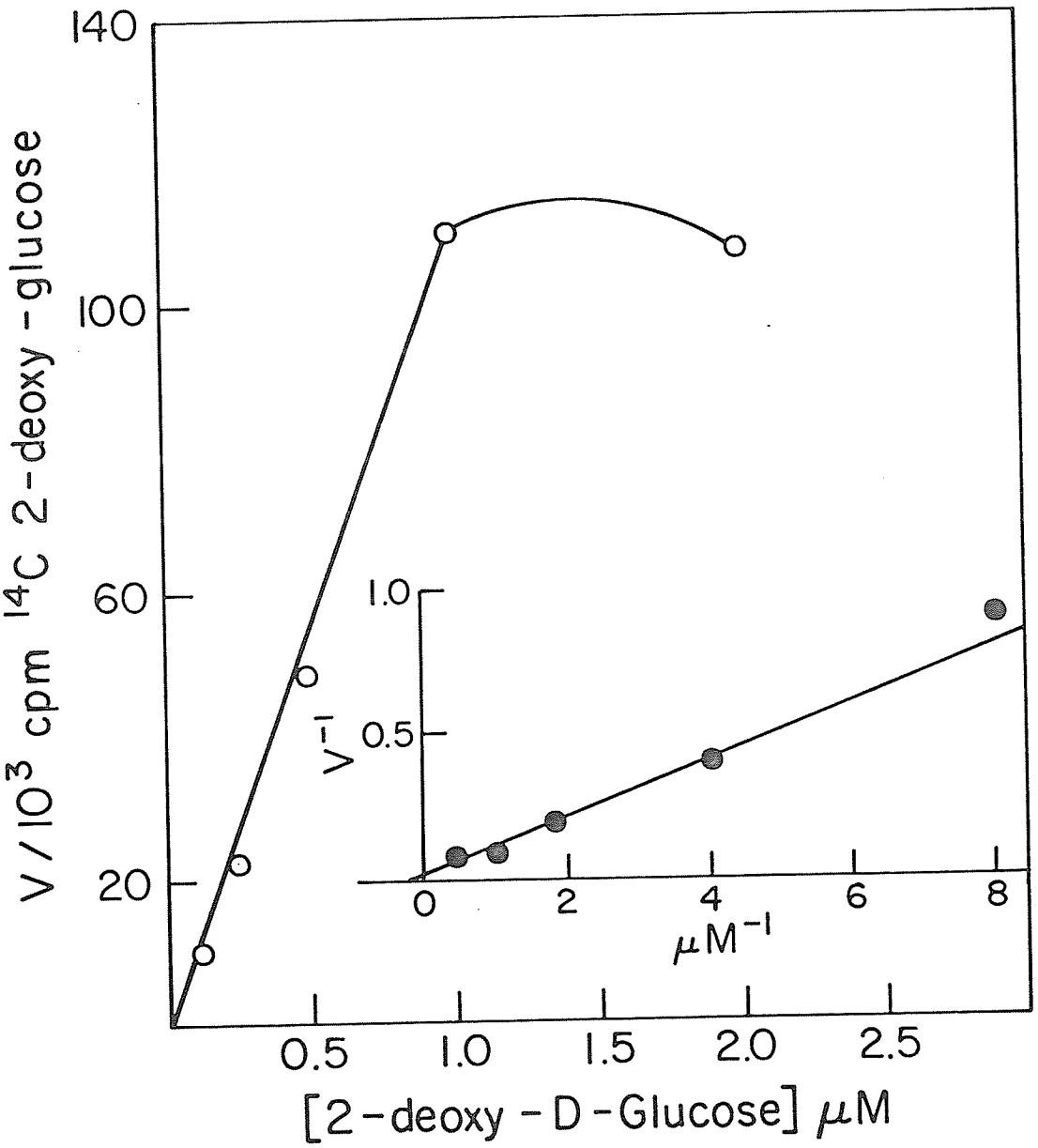
140
100
60
20
V / 10³ cpm ¹⁴C 2-deoxy-glucose
0.5
1.0
1.5
2.0
2.5
[2-deoxy-D-Glucose] μM
1.0
0.5
V⁻¹
0
2
4
6
8
μM⁻¹

Figure 7. Saturation kinetics of ^{14}C-D-glucose uptake by cells* at mid-log phase in TP broth. Cells were derived from a spore inoculum.

Inset: Lineweaver-Burk plot.

*340 µg protein/ml

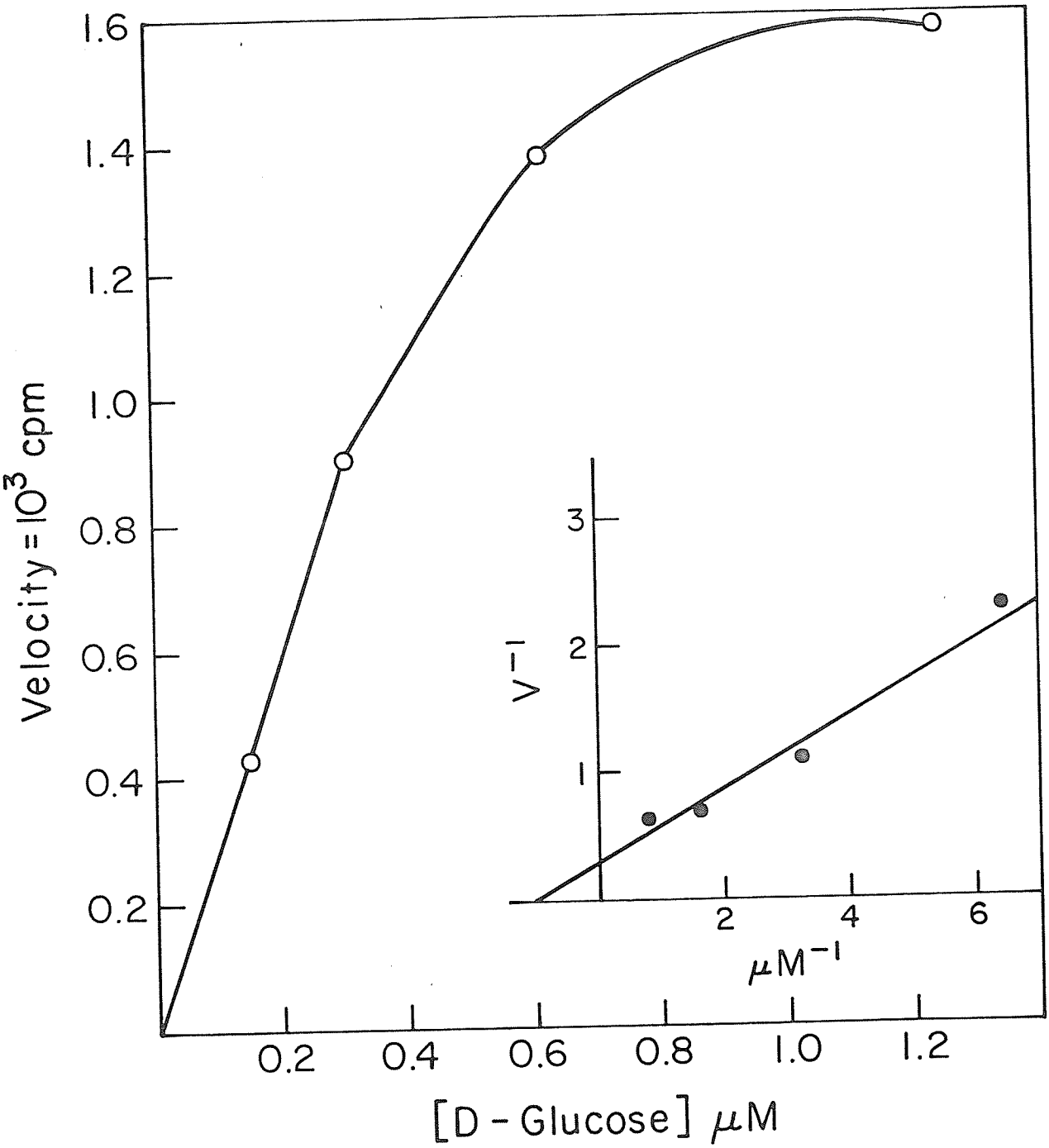
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Velocity = 10^3 cpm
0.2
0.4
0.6
0.8
1.0
1.2
[D - Glucose] μM
3
2
1
V^{-1}
2
4
6
μM^{-1}

Figure 8. Kinetics of ^{14}C-D-glucose uptake by cells* at mid-log phase in TP broth.
Cells were derived from a spore inoculum.

Inset: Lineweaver-Burk plot.

*340 µg protein/ml

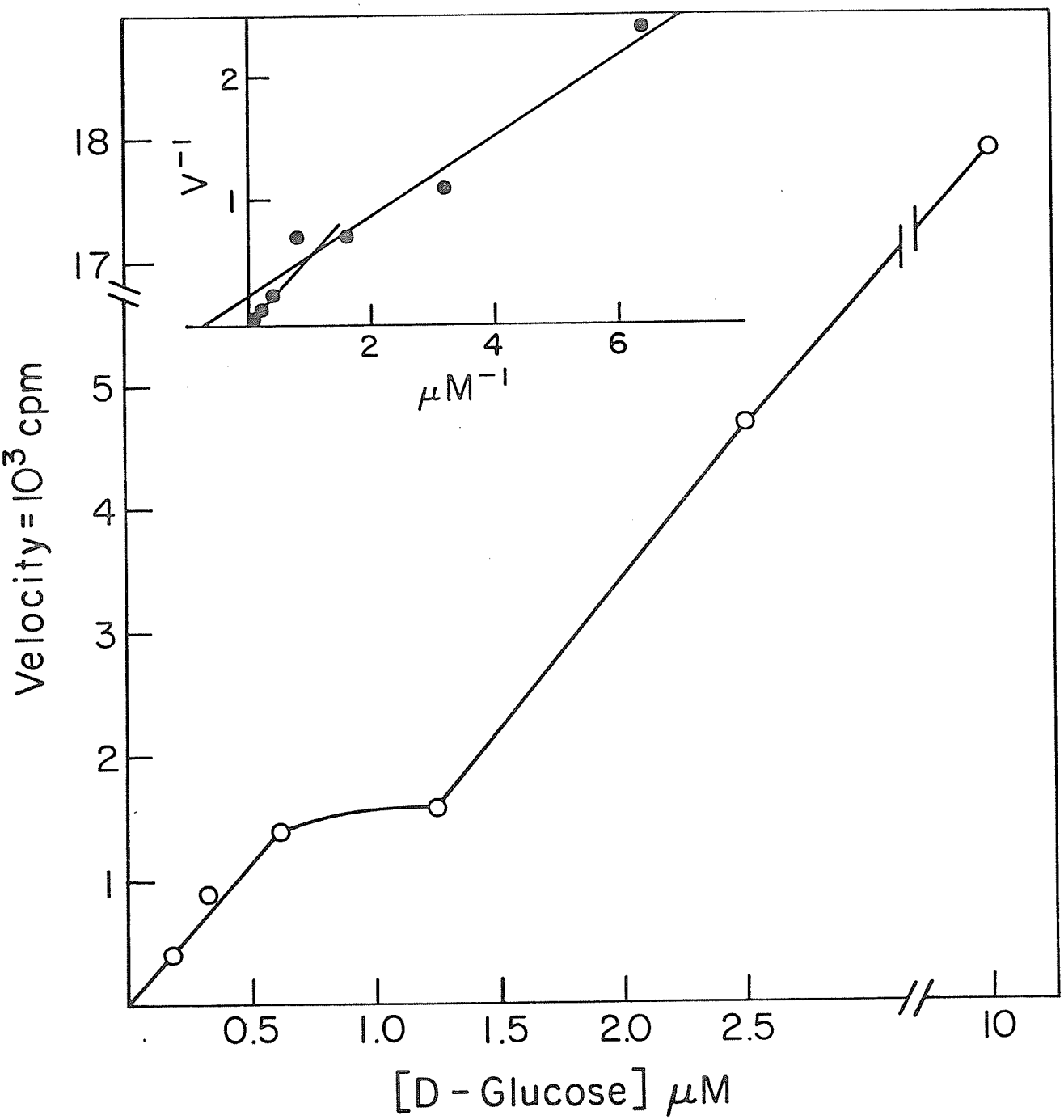
Velocity = 10^3 cpm
18
17
5
4
3
2
1
0.5
1.0
1.5
2.0
2.5
10
[D - Glucose] μM
V^{-1}
2
1
2
4
6
μM^{-1}

TABLE VI

Influence of pH on the transport of D-glucose [a]

pH	(10^3 cpm)[b] Inocula	
	Log cell	Spore
5.5	11.98	4.11
6.0	9.17	4.36
6.5	17.79	3.52
7.0	17.14	2.94
7.5	8.37	4.44

[a] 12.5 μM ^{14}C-glucose

[b] Assay 1 min

TABLE VII

Effect of temperature on the transport of D-glucose[a]

	(10^3 cpm)[b]	
	Inocula	
Temp (^{o}C)	Log Cell	Spore
0	5.75	0.33
20	14.49	4.95
30	10.84	1.38
40	10.55	0.56
50	6.41	1.95

[a] 12.5 μM ^{14}C-glucose

[b] Assay 1 min

TABLE VIII

Effects of inhibitors on uptake[a] of ^{14}C-D-Glucose

	Inhibition (%)	
	Inocula	
Addition[b] (1.0 mM)	Log cell[c]	Spore[c]
Iodoacetic acid	68.5	0
Iodoacetamide	90	0
N-ethyl-maleimide[d]	92	0
Sodium fluoride	0	81

[a] cpm

[b] 5 min before ^{14}C-glucose

[c] Grown to mid-log phase

[d] 0.1 mM

TABLE IX

PEP phosphotransferase activities of decryptified cell preparations

		^{3}H-2-Deoxyglucose phosphorylated (nmoles)			
		Cell[a] protein (mg/ml)			
Assay Conditions		5.0	5.0[b]	2.0	2.0
Complete		278.3	259.6	16.6	11.4
Addition	Deletion				
ATP	PEP	77.7	172.2	54.6	38.3
none	PEP	29.4	16.6	16.4	13.2
ATP	none	149.0	ND	11.9	ND
NaF	PEP	ND	ND	14.4	10.4

[a]10 h, glucose grown cells from a log cell inoculum

[b]Cells not starved

ND No data

TABLE X

PEP phosphotransferase activities of decryptified cell preparations

Assay Conditions		^{14}C-Fructose* Phosphorylated (nmoles) Cell protein (mg/ml) 6.0[a]	6.0[a]	3.2[b]
Complete		80	101	9.3
Addition	Deletion			
ATP	PEP	55.5	55.3	74.5
	PEP	49.7	43.4	68.1

[a]10 h fructose grown starved cells from a log cell inoculum

[b]10 h glucose grown starved cells

*Specific activity 241 mC_i/mMol

TABLE XI

PEP phosphotransferase activities of decryptified cell preparations

Assay Conditions		^{3}H-2-Deoxy-glucose phosphorylated (nmoles) Cell protein (mg/ml) 8.0 [a]	5.0 [a]	12.4 [b]
Complete		8.4	8.3	427.9
Addition	Deletion			
ATP	PEP	8.2	7.2	411.0
	PEP	6.8	6.4	338.6
NaF	PEP	ND	5.8	ND

[a] 10 h glucose grown, starved cells from a spore inoculum

[b] Cells not starved

ND No Data

DISCUSSION

DISCUSSION

PART I

Glucose was essential for growth and development of *C. botulinum* MSp^+. Anaerobic bacteria carry out an incomplete fermentation of glucose, with the sugar serving as a source of energy required for life supporting processes such as the nutrient assimilation and biosynthesis of macromolecules. In order for these bacteria to utilise glucose, the cells beside being permeable must have the enzyme complement required for its degradation, and nutrients such as amino acids, vitamins, purines and pyrimidines must be supplied. (Levy *et al* 1973). In this study I have attempted to compare the systems of glucose transport occurring in cultures derived from log cell or spore inocula.

From the results obtained with the MSp^+ strain (Figs. 1-4), it would appear as though the amount of glucose used was a function of the growth rate only after mid-log phase. With log cells grown in TPG (Fig. 1), the onset of the stationary growth phase coincided with the depletion of glucose but may be a function of the drop in pH to 5.8. However in the cultures derived from spores glucose was depleted only 50% at pH 6.2, at the beginning of the stationary phase of growth which suggests that growth was more likely pH dependent (Fig. 2).

In cultures derived from spores, grown in a medium supplemented with yeast extract, the onset of stationary growth coincided with the depletion of glucose suggesting that enzyme complement for glucose utilization was stimulated (Fig. 3), compared to cultures without yeast extract. In studies with *C. thermohydrosulfuricum*, Weigel *et al*

(1979) showed that in the absence of glucose, little growth was obtained, but the yield was increased by the addition of yeast extract. They proposed that yeast extract did not serve as sole source of energy. Patni and Alexander (1971a) suggested that the effect of yeast extract on increased growth of *C. thermocellum* was due to increased production of glucokinase, perhaps activated by the additional source of water-soluble vitamins. It is likely that yeast extract had also a stimulatory effect on glucokinase activity in the test strain.

In spore-derived cultures grown in TPGY with a sublethal dose of rifampicin, the utilization of glucose was the same as in the untreated culture, although the rate of growth was significantly reduced (Fig. 4). These results suggest that growth and utilization of glucose are interdependent but separate processes and may be affected by environmental stimuli.

It is a general principle that sporulation occurs only when all glucose has been used up. In the log cell derived cultures, phase-bright endospores appeared when glucose was exhausted (Fig. 1) whereas in the spore derived cultures endospores were seen when as much as 50% glucose remained in the medium (Fig. 2). In view of the fact that 8 hours are required for the maturation of a spore, the results suggest that depletion of glucose is not an essential requirement for sporulation. Vinter (1969) reported that in some species of *Bacillus* and *Clostridium* under certain conditions, sporogenesis can occur when glucose is present. In *B. subtilis*, sporulation can be induced by the same nutrient deficiencies that also induce the synthesis of highly phosphorylated nucleotides; and synthesis of these nucleotides can be

inhibited by the same metabolites of glucose that also inhibited sporulation (Rhaese and Groscurth 1974 and 1976).

Catabolite repression of spore formation by glucose has been reported in *B. subtilis* (Schaeffer *et al* 1965) and in *Clostridium* but the amount of glucose in the medium required for the repression, varied with the different species (Zoha and Sodoff 1958). Many external factors present in the medium or released from the cells during growth are known to induce spore formation (Piggot and Coote 1976). In this study the decrease in pH (5.8-6.2) observed at the onset of sporulation may be attributed to the organic acids produced by fermentation of glucose in an unbuffered medium (Fig. 1-4). It has also been reported that weak organic acids can exist as undissociated molecules at low pH and as such can penetrate the cytoplasmic membrane exerting a toxic effect on the cytoplasm (Levy *et al* 1973). The study shows that phase-bright endospores appeared 8 hours after pH 6.5 (Table II) Since acetic acid is metabolized to provide energy for sporulation (Emeruwa and Hawirko 1973), it is possible that these undissociated molecules may promote induction of sporogenesis at pH 6.5. In *B. coagulans*, maximum sporulation occurred at pH 6.5, which was also in the range for growth (Ordaz 1957).

The effect of sulfhydryl group reactive agents on growth, as shown by the inhibition with iodoacetic acid, a known inhibitor of glyceraldehyde 3-P dehydrogenase in yeast, suggests a functional role of glycolysis (Lehninger 1970). Of the three reagents tested (Table IV), only NEM did not inhibit growth, possibly because the larger size of the molecule slowed the rate of penetration into cells (Goh 1975).

The versatility of the MSp^+ strain in the ability to utilise various carbohydrates is shown in Table I. The dissacharide maltose promoted the greatest amount of growth probably because it provides two molecules of glucose. The nonmetabolizable derivative of glucose, α-methylglucoside, promoted growth, perhaps by stimulating endogenous metabolism (Vary 1978). Similarly growth of *B. subtilis* was supported at one-third the rate of glucose by the sugar (Freese 1970).

PART II

Studies on the incorporation of ^{14}C-D-glucose, carried out by comparing log and spore inoculated cultures, showed that incorporation of glucose was proportional to the amount of growth in the cultures. Incorporation of ^{14}C-D-glucose after mid-log phase in the log-derived cultures was twice that of cultures derived from spores (Table III) suggesting that the difference between log-cell and spore-derived cultures might reflect a more efficient transport system in the log cell cultures (Fig. 1-2).

Glucose transport into species of the genus *Clostridium* has received little attention, probably because of the difficulties involved in growing anaerobes. The kinetic studies (Figs. 5-8) indicated that the transport system of the MSp^+ strain is a high affinity process. The spore-derived cell suspension exhibited a greater affinity for glucose but there is an indication from the graph (Fig. 8), that it is biphasic. At higher D-glucose concentration, the x and y axes intersected at the origin, implying a diffusion process without a K_m. A similar situation has been reported for *E. coli* in which the uptake of free α-methyl-glucoside into the internal pool occurred by passive diffusion, but the phosphorylation of the sugar had no relationship to the free pool (Kaback 1968).

From the results of the effect of carbon sources on growth (Table I) and transport (Table V), it was noted that mannose and sucrose while supporting growth, did not inhibit D-glucose transport even when present at 100-fold concentration. This could indicate that not all carbohydrates metaboized by the cells, were transported by the same mechanism. Since maltose was the only dissacharide that

reduced the rate of uptake of D-glucose, the specificity of the transport system may be determined not only by the size or number of rings but more likely by the configuration of the ring substituents. Based on the inhibition of D-glucose uptake, the structural requirements for the substrate of the accumulation process, were elucidated. It would appear as though carbons 2 and 4 were required to confer specificity for recognition by the transport system. The activity was reduced when substituents were added or when certain modifications in structure occurred. Epimerization of a C-2 hydroxyl group as in mannose and an addition of a substituent on C-2 as in N-acetyl-glucosamine had no inhibitory effect on D-glucose transport. Substitution of a C-4 hydroxyl group in the β configuration, as in lactose also did not impede the transport. The hydroxyl group on C-6 appeared to be essential in the log cell-derived suspensions, since 6 deoxy-glucose failed to inhibit D-glucose transport. These findings suggest that there may be more than one type of transport system in which interaction of D-glucose with a stereospecific site is required. However, the competitive studies need refining, by testing a wider range of concentration of substrates. It is possible that the amino acids used in the growth medium might also induce some transport processes.

It is generally agreed that transport of sugars and amino acids through biological membranes is mediated by protein carriers (Roseman 1972) and enzymes are known to be sensitive to pH and temperature changes. In the log cell-derived suspensions glucose uptake was pH and temperature dependent suggesting enzyme mediation

(Tables VI-VII). The pH profile of the spore-derived cell suspension, may reveal a nonspecific transport system. It may be that diffusion was also taking place at the D-glucose concentration used in the assay.

In log-cell derived cultures of the MSp^+ strain, the sulphydryl binding reagents inhibited transport, indicating that the -SH groups in proteins are essential for D-glucose transport (Table VIII). Postma and Roseman (1976) suggested that the PEP phosphorylating system in *E. coli* is extremely sensitive to sulphydryl group inhibitors such as NEM, causing chemical inactivation of the site of phosphorylation of E_{II} of the PTS. Gachelin (1970) has proposed that phosphorylation is not the only step occurring in the membrane. Another mechanism precedes it, suspected to ensure a facilitated access to E_{II}, which does not require the site of E_{II} responsible for phosphorylation. She also stated that glucose could be carried through the membrane, by a pH gradient to the site of E_{II}, where it is phosphorylated. Since an ATP-dependent pH gradient is now thought to be a general property of all procaryotes (Riebeling and Jungerman 1976), this may represent the system of transport in the log cell-derived culture of the MSp^+ strain and when E_{II} is inactivated, could represent the system in the spore-derived culture. The fact that sodium fluoride did not inhibit transport in the log cell-derived suspension, argues for the presence of a membrane potential and not a direct ATP involvement (Hoban 1977) whereas inhibition in the spore-derived cell suspension could support ATPase involvement, since sodium fluoride is known to inhibit many magnesium requiring enzymes.

The results of the phosphotransferase assays (Table IX) suggest

that perhaps the PTS present in the log cell-derived culture provides for a more efficient transport of D-glucose. Metabolites released from spores during outgrowth, could affect the structural integrity of membranes, thereby impairing transport and deranging glycolysis, which would lead to an increase in an unknown stimulator of sporulation in the spore-derived culture. Also if E_{II} responsible for rapid transport of glucose in the PTS, is inactivated by catabolite repression or by a mutation, a facilitated diffusion of D-glucose could occur; operating at a rate sufficient to allow growth of the bacteria (Gachelin 1970).

Although there are reports of PEP phosphotransferase activity in *Clostridium spp.*, these have been limited mainly to studies with fructose and mannitol (Patni and Alexander 1971a and b). Fructose phosphate specific activity of the MSp^+ strain (Table X) compares favourably with that given for *C. roseum* (Hugo and Gottschalk 1974). Although glucose PEP:phosphotransferase activity was not detected in *C. thermocellum* (Patni and Alexander 1971a), there is one report of such activity in *C. pasteurianum*, the data for which is unpublished (Booth and Morris 1975). It was therefore surprising to find glucose PEP:phosphotransferase activity in log cell-derived suspensions of the MSp^+ strain. Greater activity may be obtained however, by harvesting cells at mid log phase of growth rather than six hours later, as was done in these assays; and also by using the non-metabolizable derivative of D-glucose, ^{14}C-α-methyl D-glucoside instead of the analog ^{3}H-2-deoxy-D-glucose. Spectrophotometric assays (Kornberg and Reeves 1972) on toluenized cells of the MSp^+

strain, did not produce any detectable activity of the glucose PEP:PTS. A further study on the identity of the product of phosphorylation, by the extraction procedure and chromatographic method (Goh and LéJohn 1978) met with no success, probably because the amount of ^{3}H-2-deoxy D-glucose transported by the cells, was too small to be detected by the latter method.

The absence or low activity of the glucose PEP:PTS in the spore-derived culture, may also be associated with the level of endogenous reserves. In log cell-derived cultures, where the reserves may be low, the ability to utilise glucose efficiently becomes more important for the survival of the organism.

Many questions remain unanswered regarding the transport of D-glucose in the MSp^{+} strain, but must await *in vitro* studies using membrane preparations, to avoid some of the difficulties inherent in regulating PEP formation in intact cells. It may also be useful to refine the methods used in this study. Andrews and Lin (1976) stated that adequate identification of bacterial adaptive features requires an evaluation of transport activities under a variety of conditions, and a consideration of the biological framework in which the systems have to operate, is also needed.

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