GLUCOSE UPTAKE BY THE CELLULOLYTIC RUMEN ANAEROBE *BACTEROIDES SUCCINOGENES*

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Clifton Victor Franklund

In Partial Fulfillment of the Requirements
for the Degree of
Master of Science

Major Department: Bacteriology

July, 1986

Fargo, North Dakota

APPROVAL OF THESIS

This thesis is approved by:

ABSTRACT

Glucose uptake by the cellulolytic rumen anaerobe, Bacteroides succinogenes S85, was measured under conditions that maintained anaerobiosis and osmotic stability. This organism was found to possess a highly specific, active transport mechanism for glucose. Evidence for a phosphoenol-pyruvate:glucose phosphotransferase system was not detected. Compounds that inhibit electron transport systems (non-heme iron chelators, and sulfhydryl reagents) were effective inhibitors of glucose uptake. The strongest inhibitors were compounds (proton and metal ionophores) that interfere with maintenance of the proton motive force. Compounds which interfere with ATP synthesis also inhibited glucose uptake, but a role for ATP in energizing uptake could not be inferred from these results. Oxygen prevented glucose uptake (75% inhibition), reflecting possible active sulfhydryl centers (above) or autooxidation of electron transport components. The results suggest the fumarate reductase-coupled electron transport system of B. succinogenes can generate a proton motive force that is used to energize glucose uptake. Na^+ and Li^+, but not K^+, stimulated glucose uptake and may partly account for the growth requirement of B. succinogenes for Na^+. However, the data were insufficient to conclude that glucose uptake occurs by a Na^+ symport mechanism. Spheroplasts of B. succinogenes transported glucose as well as whole cells, indicating glucose uptake is not dependent on a periplasmic glucose binding protein. A variety of sugars including the

nonmetabolizable analog, ∝-methylglucoside, did not inhibit glucose uptake. Only cellobiose and 2-deoxyglucose were active and neither behaved as a competitive inhibitor. Metabolism of both sugars was probably responsible for the inhibition. Cellobiose-grown B. succinogenes showed a reduced ability to transport glucose compared to glucose-grown cells. This may indicate regulation of synthesis of the glucose carrier protein by cellobiose through a mechanism other than catabolite repression. Differences in the ability to transport glucose were detected between transition cells (transition from lag to log phase of growth) and log-phase cells. However, the differences were not due to different glucose transport mechanisms. Alterations in the structural integrity of the cell envelope, as reflected by osmotic- and cold-sensitivity features of transition and log cells, may have affected the glucose uptake abilities in these cell types.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION AND LITERATURE REVIEW

Many herbivorous animals obtain energy and nutrients for growth through an anaerobic, microbial digestion of plant tissue within a specialized forestomach known as the rumen. The rumen microbial ecosystem is composed of many interdependent populations of anaerobic protozoa and bacteria existing in a synergistic relationship with the host. The bacterial contributions to digestion within the rumen have been reviewed (27). Rumen bacteria belong to three broad physiological catagories: the polymer-hydrolyzing, the sugar-fermenting, and the methanogenic bacteria. Bacteria of the first group hydrolyze the high molecular weight polysaccharides in the ruminant diet (cellulose, hemicellulose, pectin, and starch) to their corresponding mono- and oligosaccharides. These soluble sugars are then fermented by bacteria of both the first and second groups to form acetate, propionate, butyrate, succinate, CO_2, and H_2 (53,58). Succinate is subsequently decarboxylated, by interspecies interactions, forming propionate and CO_2 (53). The final group, the methanogens, catalyze the oxidation of H_2 coupled to the reduction of CO_2, giving CH_4. This reaction promotes the formation of more oxidized products (acetate) at the expense of reduced products (ethanol and propionate) through a relationship known as interspecies H_2 transfer (64). Thus, the major end products of microbial carbohydrate fermentation in the rumen are acetate, propionate, butyrate, CO_2, and CH_4.

The primary carbon and energy source for the rumen bacteria is carbohydrate (27). Usually, carbohydrates are

supplied by the polysaccharides present in plant tissues. The walls of plant cells are comprised primarily of cellulose in association with varying amounts of hemicellulose, lignin, and pectin. Starch, which is located mainly in the seeds and tubers of plants, is not normally found in large quantities in the diet of forage-fed ruminants. Although ruminants depend upon cellulose as a carbon and energy source they do not produce a digestive cellulase and are, therefore, dependent on the cellulolytic rumen bacteria to degrade cellulose. For this reason, much work has been done to isolate and characterize the bacteria responsible for this reaction.

Presently, four cellulolytic bacterial species are recognized as ecologically significant in terms of their frequencies of isolation and rumen populations (27). These are Ruminococcus flavefaciens, Ruminococcus albus, Bacteroides succinogenes, and Butyrivibrio fibrisolvens. These bacteria have been studied to some extent regarding general microbiological features (8,27), nutrition (3), pathways of carbohydrate metabolism (30,31), and properties of their respective cellulases (20,48).

B. succinogenes initially attracted attention because it was the first cellulolytic rumen bacterium isolated in pure culture (26). Later, it was shown to be the only rumen bacterium capable of degrading both amorphous and crystalline forms of cellulose (22). In addition, original isolates possessed the ability to degrade hemicellulose (xylanase activity) (10,12,16), and pectin (27). However, strains maintained on synthetic media appear to have lost the latter

activity (11). These features, combined with the ability to adhere tightly to both pure cellulose and plant tissues (22,36), suggests it plays an important role in the initial degradation of plant tissues.

While B. succinogenes is an important organism of the bovine rumen, recent studies indicate it is also involved in cellulose digestion in the gut of other animals. Montgomery and Macy (44) determined that the predominant cellulolytic organism of the rat cecum is B. succinogenes. R. flavefaciens was detected in the cecum in lesser numbers. These organisms were also isolated from the pig large intestine, and again, the predominant cellulolytic organism present was B. succinogenes (61). Moreover, the relative population of B. succinogenes increased two-fold, compared to R. flavefaciens, when pigs were fed a high-fiber diet, further substantiating the role of this bacterium in fiber digestion.

Although the phenotypic characteristics of B. succinogenes originally conformed to the genus Bacteroides, recent phylogenetic analysis by 16S rRNA sequencing has shown the neotype strain, S85, is not related to other Bacteroides species (47). Furthermore, it does not cluster with any one of the other 10 eubacterial "phyla" described in this study. In light of these results, the present taxonomic status of this organism is uncertain.

The general nutritional requirements of B. succinogenes are similar to those of other rumen bacteria (3,5,60). All strains can use cellulose, cellobiose, and glucose as sole carbon and energy sources; however, strains vary in ability to

use starch, maltose, and trehalose (4,26). NH_4^+ serves as a sole nitrogen source and free amino acids are not efficiently used (3). CO_2 is required for growth since formation of the major fermentation end product, succinate, requires the carboxylation of phosphoenolpyruvate (PEP) as an intermediate step (42). A straight chain (n-valeric) and a branched-chain (isobutyric or 2-methylbutyric) volatile fatty acid (VFA) are growth factors for B. succinogenes. In many rumen bacteria, branched-chain VFAs are precursors for the biosynthesis of branched-chain amino acids, fatty acids and fatty aldehydes (3). B. succinogenes incorporates virtually all of its required VFAs into cellular lipid, largely in the form of plasmalogen (62). All strains require biotin for growth, while p-aminobenzoate may be stimulatory (3). Finally, B. succinogenes and other rumen bacteroides show an unusual requirement for high concentrations (20-100 mM) of Na^+ (5,6). Furthermore, Na^+ cannot be replaced by Rb^+, Li^+, or Cs^+. Except for marine organisms, alkalinophiles, and halophiles, no other bacteria, including nonrumen, intestinal bacteroides, show such a requirement. The basis for this is not known; however, the Na^+ concentration in the rumen is 65-130 mM (7).

Although B. succinogenes possesses cellulase activity, the term "cellulase" is a misnomer. No single enzyme is reponsible for degrading cellulose to its component glucose molecules. Rather, initial hydrolysis is thought to be catalyzed by an endo-β-1,4-glucanase(s) which randomly cleaves the glucan chains of cellulose (52). This increases the solubility of the glucan chains and the number of sites of attack for a second

enzyme, an exo-β-1,4-glucanase. Hydrolysis of the glucan chains from the nonreducing end by this enzyme yields either glucose or cellobiose. Finally, cellobiose is hydrolyzed by a cellobiase (β-1,4-glucosidase). Endo-β-1,4-glucanase activity can be measured using the water-soluble substrate, carboxy-methylcellulose; hence the enzyme is also referred to as carboxymethylcellulase (CMCase).

Despite the importance of B. succinogenes in ruminal cellulose digestion, only recently have studies been done on the properties of its cellulase system. Forsberg and associates (16,19) detected both CMCase and cellobiase activities in this organism; however, only very low exo-β-1,4-glucanase activity was found. CMCase activity was detected in both the soluble and particulate fractions of cell extracts. During cell growth, CMCase activity was also released into the culture supernatant. This release did not occur by simple excretion, but instead was associated with the formation of membranous vesicles ("blebs") derived from the outer membrane of the cell envelope (16,19). Bleb formation is not unique to B. succinogenes, but occurs in other gram-negative bacteria (25). With cellulose-grown cells, 70% of the total CMCase activity was found in the culture supernatant. Of this activity, 50-60% was associated with sedimentable (100,000 x g) membranous fragments (19). More recently, the sedimentable CMCase activity was resolved into four Triton X-100 solubilized, ionic fractions by DEAE-Sepharose chromatography (54). The soluble (culture supernatant) CMCase activity was separated into three molecular weight fractions on Sepharose 6B. Analysis of these seven

fractions by nondenaturing polyacrylamide gel electrophoresis (with Triton X-100) revealed a distinctive migration pattern for each fraction based on location of CMCase activity. Two of the seven fractions also showed some exo-β-1,4-glucanase activity. It is unclear how many different endoglucanase enzymes are present in these fractions; however, production of multiple endoglucanases is seen in other cellulolytic bacteria (48).

The release of outer membrane CMCase by bleb formation is not specific for this enzyme, because xylanse, aryl-β-xylosidase, and aryl-β-glucosidase activities are also associated with bleb vesicles (16). CMCase and xylosidase are probably located on the external surface of the vesicles, since trypsin treatment releases both activities (21). Contact with and adherence to cellulose appears to promote release of membranous fragments, since free, unattached cells show infrequent bleb formation (16). Likewise, the protein concentration in the culture supernatant from cellulose-grown cells is about 5 times greater than that from glucose- or cellobiose-grown cells (19). CMCase activity does not appear to be repressed by growth on glucose or cellobiose. Total CMCase activity of cellulose-grown cells is greater than that of glucose- or cellobiose-grown cultures, reflecting the increased release of CMCase into the culture supernatant as opposed to induction or derepression of enzyme synthesis. These results are consistent with other observations that neither glucose nor cellobiose directly inhibit the rate of cellulose degradation by B. succinogenes cultures (24).

Cellobiase activity is found in the soluble (25%) and particulate (75%) fractions of B. succinogenes cell extracts (16,19). However, unlike CMCase, little cellobiase activity is released into the culture supernatant during growth (16). This suggests the cellobiase is mostly associated with the cytoplasmic membrane. The specific activity of cellobiase is similar in glucose-, cellobiose-, or cellulose-grown cells, indicating that the enzyme is synthesized constitutively (19). Cellobiase, but not CMCase, is an O_2-labile enzyme with an essential SH-group(s) (17).

B. succinogenes ferments glucose with the production of succinate, acetate, and low amounts of formate (3,53). Enzymatic studies by Joyner and Baldwin (30) showed it uses the Embden-Meyerhoff-Parnas (EMP) pathway for glucose metabolism. A b-type cytochrome has been detected in this organism (9,50) and is presumably involved in electron transport coupled to the reduction of fumarate to succinate (33). Miller (42) reported cell extracts coupled the oxidation of pyruvate to the reduction of fumarate with flavin nucleotides (or a flavoprotein) acting as electron carriers. The fumarate reductase system was membrane-associated and the expected reductants, [NAD(P)H], did not serve as electron donors for the reaction. The NADH-dependent reduction of oxaloacetate to malate was the only reaction detected to account for the oxidation of NADH formed during glucose metabolism. Presumably, ATP is synthesized from reactions associated with the EMP pathway, GDP-dependent PEP carboxykinase, and the conversion of acetyl-CoA to acetate (30,42). In many anaerobes, the reduction of fumarate to

succinate is an energy-conserving process (33), but whether this occurs in B. succinogenes is not known.

Many basic physiological aspects of B. succinogenes, and the cellulolytic rumen bacteria in general, have not been studied. For example, although these bacteria are important in the carbohydrate metabolism of the rumen, nothing is known concerning their mechanisms of carbohydrate transport. This is despite the fact that transport processes are often subject to regulatory controls (13).

Dills et al. (13) and others (38,56) have published extensive reviews on the mechanisms and controls of bacterial carbohydrate transport. There are four general mechanisms of carbohydrate uptake. The simplest is facilitated diffusion, whereby movement of a substrate across the cell membrane is mediated by a protein that either forms a transmembrane pore or shuttles the substrate into the cell through a conformational change (13). This system does not require cellular energy and, thus, does not result in accumulation of substrate against a concentration gradient. Glycerol is the only carbohydrate usually transported by this mechanism (13). The remaining transport systems all involve the use of cellular energy to transport the substrate against electrochemical or concentration gradients and are, therefore, active transport systems. Many gram-positive and gram-negative bacteria transport sugars by the PEP:sugar phosphotransferase system (PTS) (13). This multi-enzyme mechanism uses PEP as a high-energy-phosphate donor to drive the simultaneous uptake and phosphorylation of hexoses and hexitols. Thus, sugars (outside) are accumulated

as sugar-6-phosphates (inside). The PEP:sugar PTS is the only transport system that chemically modifies the transported compound (group translocation). The final two transport mechanisms are "true" active transport systems, in that the substrates are not chemically modified during translocation (13). Both systems minimally involve the presence of a transport carrier protein (permease) integrated in the cell membrane which binds to and translocates the substrate. These mechanisms differ in whether additional proteins are involved in substrate transport. Transport of substrates (sugars, amino acids, ions, etc.) requiring only a membrane-bound permease for each compound occurs in both gram-positive and gram-negative bacteria (13,38). One such system carries out lactose transport in Escherichia coli (56). This permease co-transports a proton with each lactose molecule. Thus, lactose uptake in E. coli occurs by proton symport and is energized by the proton motive force (PMF) generated across the cell membrane. In gram-negative bacteria only, many substrates (sugars, amino acids, and ions) may be transported by systems which possess, in addition to a membrane-bound permease, other associated proteins including a substrate binding protein located in the periplasmic space (periplasmic binding proteins). Such a transport system can be characterized by the loss of activity associated with a cold, osmotic shock treatment of whole cells, or conversion of the cells to spheroplasts (13,38). In both cases, the transport ability is lost due to removal of the periplasmic binding proteins. Maltose and maltodextrins are transported by such a system in E. coli (56). Although maltose

uptake in this organism has been well characterized biochemically and genetically, the direct energy source for maltose transport is not clear (13,56). Evidence has been reviewed implicating either ATP or the PMF as the driving force.

The objective of the present study was to characterize the mechanism(s) of carbohydrate uptake in B. succinogenes. The transport systems of only two other rumen anaerobes have been studied. Stevenson (59) described some properties of amino acid active transport in Bacteroides ruminicola, while Dills et al. (14) demonstrated the presence of an inducible PEP:sugar PTS system for glucose and fructose in Megasphaera elsdenii. Although both of these bacteria metabolize carbohydrates in the rumen, neither is cellulolytic. With respect to nonrumen, cellulolytic anaerobes, carbohydrate transport systems have been described only for Clostridium thermocellum (46). In this bacterium, transport of glucose and cellobiose occurred through an energy-dependent process not involving the PEP:sugar PTS system.

MATERIALS AND METHODS

Growth of bacteria: B. succinogenes strain S85 was obtained from M. P. Bryant, Department of Dairy Science, University of Illinois. The Hungate anaerobic technique (28) as modified by Bryant (2) was used for the preparation of media and for growth of B. succinogenes. The medium (Table 1) was modified from Miller (42) by the deletion of Na_2S and the addition of $FeSO_4$ (5 mg/liter) and hemin (2 mg/liter). The medium (400 ml), prepared in 500-ml round-bottom flasks, was adjusted to pH 7.0, heated to boiling, and sealed under 100% CO_2 with a black butyl stopper before autoclaving.

Flasks were routinely inoculated with 0.5 ml of a 12 h culture and incubated at 37°C. Growth was followed by measuring culture turbidity at 660 nm using a Beckman model 24 spectrophotometer. Glucose utilization was determined by the glucose oxidase method (55).

Assay of glucose uptake: Cells were initially harvested either at transition from the lag to log phase (0.35 absorbance) or near mid-log phase (0.8 absorbance) by centrifugation (5,000 x g, 15 min, 10°C). Later, refrigeration was discontinued and the cells were harvested at room temperature (22°C). Anaerobiosis was maintained by gassing the centrifuge bottles with CO_2 whenever they were opened. The suspending buffer (50 mM sodium phosphate (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, and 0.5 M sucrose) was made anaerobic by boiling for 2-3 min, cooling under 100% N_2 to room temperature, and adding 1 mM dithiothreitol. The cells were suspended in a sufficient

Table 1. Culture medium for *B. succinogenes*

Component	Amount per liter
Basal salts solution # 1[a]	50 ml
Basal salts solution # 2[b]	50 ml
Trypticase (BBL Microbiology Systems)	5 g
Yeast extract (Difco Laboratories)	2 g
Glucose	5 g
n-Butyric acid	0.58 ml
Isobutyric acid	0.10 ml
n-Valeric acid	0.10 ml
Isovaleric acid	0.10 ml
2-Methylbutyric acid	0.10 ml
$FeSO_4$[c]	0.5 ml
Hemin[d]	20 mg
Resazurin	1.0 mg
Dithiothreitol	15.4 mg
Na_2CO_3[e]	4 g
Cysteine[e]	0.5 g

[a] K_2HPO_4 (4.8 g/liter)

[b] KH_2PO_4 (4.8 g/liter), $(NH_4)_2SO_4$ (20 g/liter), NaCl (9.6 g/liter), $MgSO_4$ (2.0 g/liter), and $CaCl_2$ (1.26 g/liter).

[c] A 1% solution dissolved in 1% HCl.

[d] Dissolved in 3 ml of 0.05 N KOH in 25% (v/v) ethanol.

[e] Na_2CO_3 (8% solution) and cysteine (10% solution) were autoclaved separately, and added after sterilizing the medium.

volume of buffer to give a reading of 130-160 Klett units (red filter) when diluted 1:10. This corresponded to 1.8 to 2.2 mg cell protein/ml undiluted cell suspension, as determined by the Lowry procedure with bovine serum albumin as the standard (40). Since the cells were not washed there was some carryover of glucose (<1 mM) from the medium. However, measurements indicated the glucose was consumed prior to initiating uptake assays. Reactions were carried out in 18 x 142 mm anaerobe culture tubes (Belco Glass, Inc., Vineland, NJ) fitted with black butyl rubber stoppers each containing an inlet and outlet (18 ga. needles) for continuous flushing with N_2. Unless otherwise indicated, the above buffer was used for all transport assays. The cell suspension was equilibrated anaerobically under N_2 at 37°C for 15 min. The standard assay (2.0 ml final volume) contained anaerobic buffer (0.8 ml), cell suspension (1.0 ml), and [U-^{14}C]-D-glucose (1.0 mM; 0.5 μCi/μmol)(0.2 ml) added to initiate the uptake. Samples (0.1 ml) were taken periodically, filtered through pre-washed membrane filters (0.45 μm pore diameter; Millipore Corp., Bedford, Mass.), and washed with 2.0 ml of 37°C buffer. Each filter was immediately placed in 10 ml of a Triton X-100 scintillation cocktail (49) and the amount of radioactivity determined.

Incorporation of glucose into cellular fractions: The glucose uptake assay was performed as above, except that at each time point an additional 0.1-ml sample was removed, added to 2.0 ml of ice-cold 10% trichloroacetic acid (TCA) and extracted for 30 min at 0°C (29). Each of these samples was

then filtered through a pre-washed membrane filter, washed with 2.0 ml of distilled water, and counted as above.

Effects of sugars on uptake: For these experiments, the standard assay was modified by decreasing the volume of anaerobic buffer (0.7 ml) to accommodate the sugar tested (0.1 ml; 10 mM final concentration). The sugars were either added 2 min after initiating uptake with glucose or allowed to preincubate with the cells in the assay for 10 min prior to initiating uptake.

Effects of metabolic inhibitors on uptake: The standard assay was modified as above to allow for the volume of the inhibitors tested. Assays containing sulfhydryl inhibitors were further modified by deleting dithiothreitol from the buffers. All inhibitors were preincubated with the cells for 15 min prior to initiating uptake. Inhibition was expressed as percent of control activity measured 5 min after initiating uptake.

Effects of monovalent cations on uptake: To perform these experiments, cells were harvested and the cell pellet was washed once with 20 ml of anaerobic suspending buffer modified to delete Na^+ (50 mM potassium phosphate, (pH 7.0), 5 mM $MgCl_2$, 0.5 M sucrose, and 1 mM dithiothreitol). Cells were then centrifuged (10,000 x g, 10 min, 22°C) under CO_2 using stainless steel tubes closed with O-ring seal covers. The cell pellet was then resuspended in the modified anaerobic buffer and adjusted to the desired turbidity as above. The buffer volume of the assays was modified to accommodate the volume of

the salt solutions tested. Na^+, Li^+, and K^+ were added as their chloride salts and preincubated with the cells for 10 min before adding glucose.

Formation of spheroplasts: Spheroplasts were prepared by adding whole cells (1.0 ml) to assays containing buffer (0.7 ml), 7 mM EDTA (0.2 ml), and 0.5 mg lysozyme (0.1 ml), and incubating at 37°C. Whole cell controls contained, in a final volume of 2.0 ml, cell suspension (1.0 ml), buffer (0.9 ml), and a sufficient quantity of $MgCl_2$ to give a final concentration of 20 mM. Spheroplast formation was followed by removing 0.1-ml samples from the spheroplast and whole-cell assays, diluting each sample with distilled water (0.9 ml), and recording the absorbance of the diluted cells at 660 nm.

Glucose uptake by spheroplasts: For these experiments, duplicate assays were prepared for whole-cell controls and spheroplasts. One set of assays was used to follow spheroplast formation (as above). In the second set of assays, the buffer volume was reduced (0.5 ml for spheroplasts; 0.7 ml for whole cells) to accomodate the addition of glucose (0.2 ml). After spheroplast formation had occurred (40 min) in the first set of assays, glucose uptake was initiated in the second pair of assay tubes.

Assay of PEP-dependent glucose phosphorylation: *B. succinogenes* was grown in 1 liter of medium in a 2-liter serum bottle (43). The medium was inoculated with 3 ml of a 12-h culture, and incubated at 37°C. *E. coli*, used as a positive control, was grown in 1 liter of Todd-Hewitt broth (BBL Micro-

biology Systems) containing 1.5% glucose. This medium was inoculated with 50 ml of an overnight culture, and incubated aerobically at 37°C. After the cultures reached the log phase of growth, the cells were harvested (5,000 x g, 15 min, 10°C), washed once in a minimal volume of 50 mM potassium phosphate buffer (pH 7.5) and recentrifuged (10,000 x g, 10 min, 10°C). The resulting cell pellets were resuspended in the same buffer, broken by two passages through a French pressure cell (12,000 lb/in^2), and the extracts treated with DNase and RNase (40 μg each). The broken cells were centrifuged (10,000 x g, 20 min, 10°C) to remove large, cell debris. The membrane-containing supernatants were collected and dialyzed overnight (4°C) against 1 liter of the same buffer. The PEP-dependent formation of glucose-6-phosphate was assayed using the glucose-6-phosphate dehydrogenase coupled method of Kundig and Roseman (34).

Chemicals: Carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. 2-Heptyl-4-hydroxy-quinoline-N-oxide (HOQNO), 2,4-dinitrophenol (2,4-DNP), carbonyl cyanide m-chlorophenylhydrazone (CCCP), p-chloromecuribenzoate (p-CMB), N,N-dicyclohexylcarbodiimide (DCCD), and all other organic metabolic inhibitors were obtained from Sigma Chemical Co., St. Lois, Mo. Egg white lysozyme and bovine serum albumin were also obtained from Sigma Chemical Co. [U-^{14}C]-D-glucose (346 mCi/mmol) was obtained from New England Nuclear, Boston, Mass. All other chemicals were reagent grade from various sources.

RESULTS

Cell growth: Cells for glucose uptake experiments were generally harvested at either 0.35 absorbance (approx. 12 h incubation) or 0.8 absorbance (approx. 15 h incubation). As Fig. 1 shows, these absorbances represent the transition from lag to log phase, and the log phase of growth, respectively. As indicated below, significant differences were found in the glucose uptake patterns of these cell types. These differences were not due to depletion of glucose from the medium (Fig. 1). For simplicity, the younger cultures will be referred to as "transition cells" and the older cultures as "log cells".

Glucose uptake by transition and log cells: The uptake of glucose by transition cells is illustrated in Fig. 2A and B. Cells were suspended in buffer containing 0.5 M sucrose as an osmotic stabilizer (Fig. 2A), or prepared in the absence of sucrose (Fig. 2B). In transition cells, sucrose had no effect on glucose uptake. Starvation of the cells for 1-2 h in suspending buffer decreased glucose uptake and this was not prevented by osmotic stabilization.

In contast to these results, log cells showed a considerably different uptake profile (Fig. 2C and D). Glucose uptake by log cells was two- to three-fold less than in transition cells (Fig. 2A and C). Moreover, log cells required osmotic stabilization by sucrose in order to detect glucose uptake (Fig. 2C and D). Unlike transition cells, glucose uptake by log cells was unaffected by starvation of the cell suspensions for 1-2 h (Fig. 2C). In most subsequent experiments, the

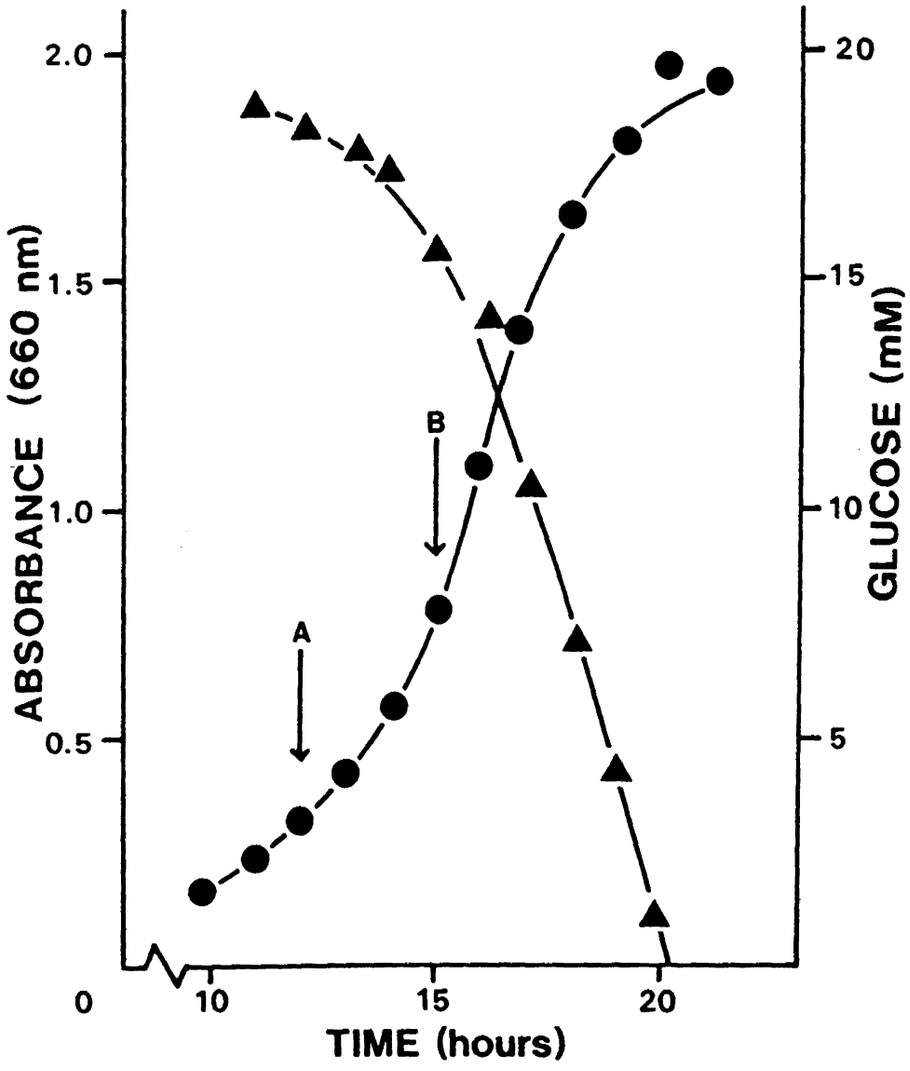


Fig. 1. Growth and glucose utilization by B. succinogenes. Culture turbidity, (●); and glucose utilization, (▲). Cells were routinely harvested during the transition from lag to log phase (A) or the log phase (B) of growth.

Fig. 2. Glucose uptake by B. succinogenes.

A. Transition cells suspended in buffer with 0.5 M sucrose.

Symbols: (●), glucose uptake at 0 h starvation.

(▲), glucose uptake at 1 h starvation.

(■), glucose uptake at 2 h starvation.

B. Transition cells suspended in buffer without sucrose

Symbols: (○), glucose uptake at 0 h starvation.

(△), glucose uptake at 1 h starvation.

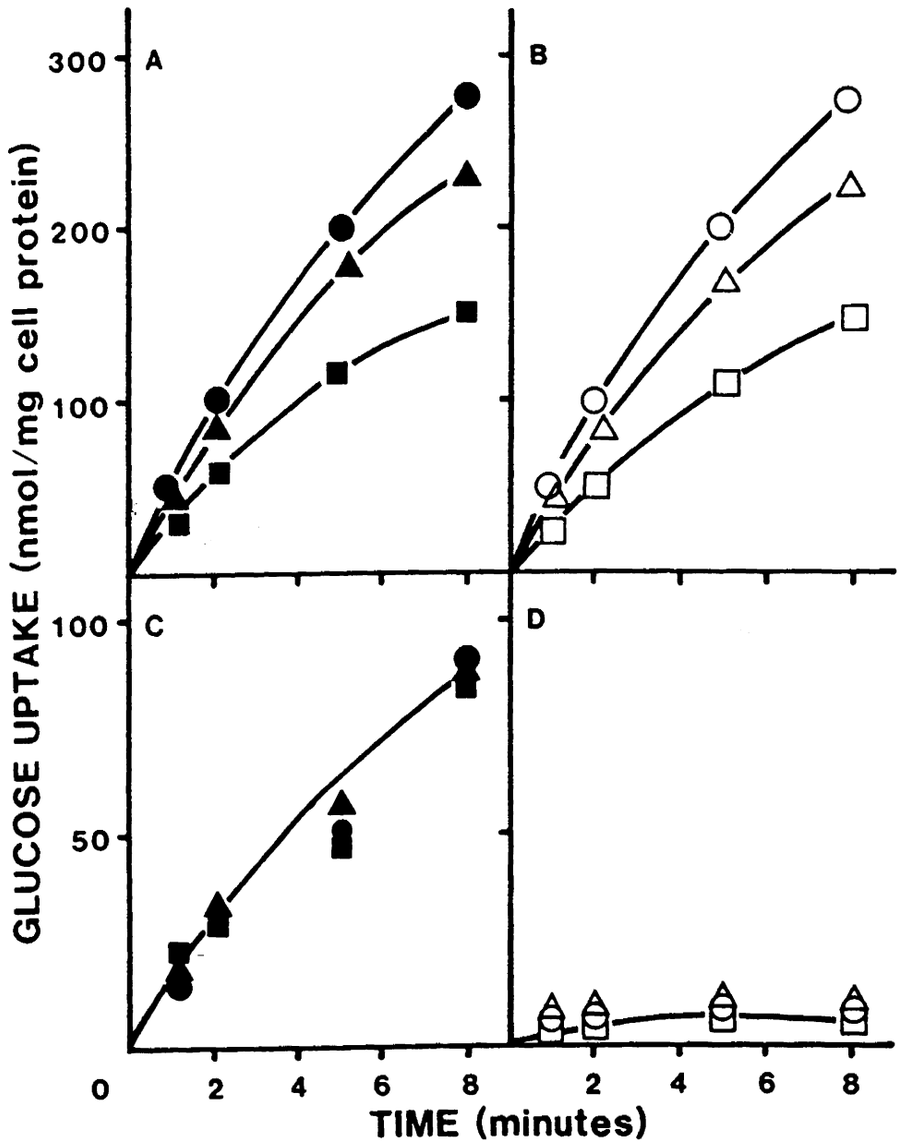
(□), glucose uptake at 2 h starvation.

C. Log cells suspended in buffer with 0.5 M sucrose.

Symbols: As in A.

D. Log cells suspended in buffer without sucrose.

Symbols: As in B.

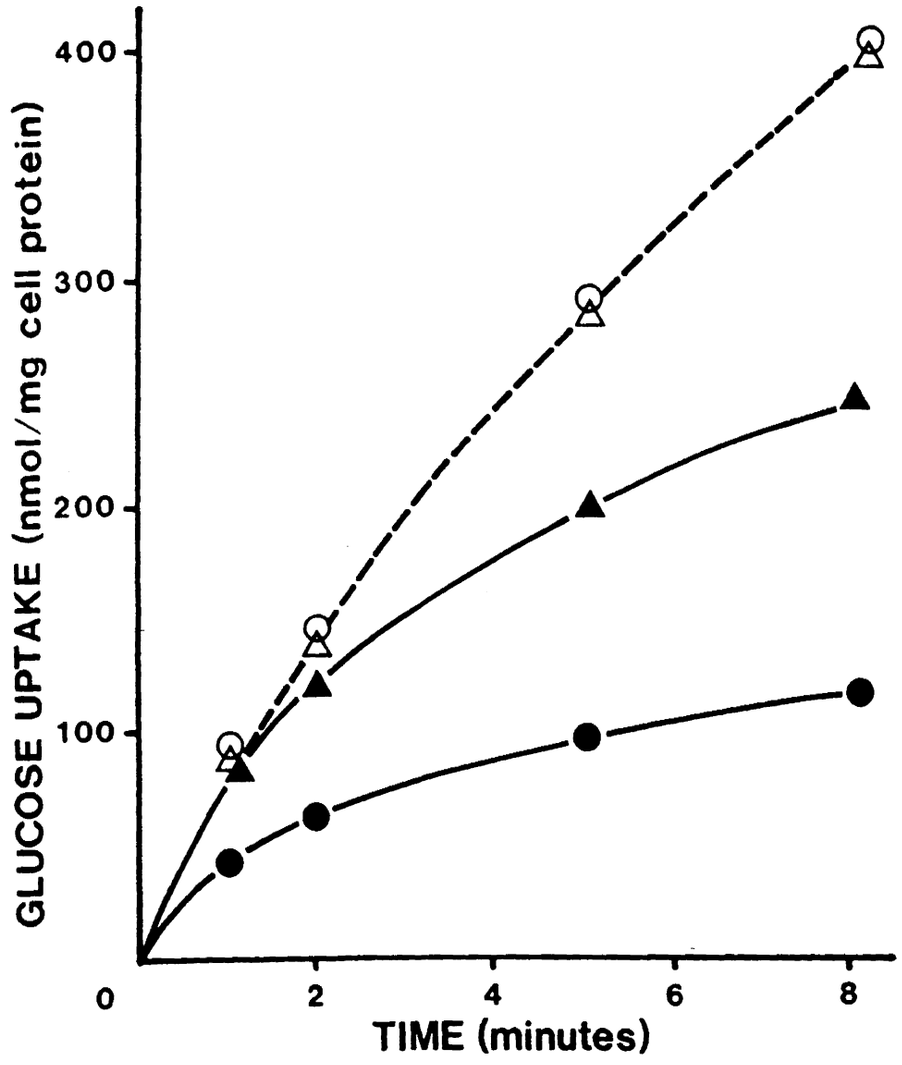


assays were generally completed within 1 h of preparing the cell suspensions.

Effect of harvest temperature on glucose uptake: The previous experiments were performed using cells that were refrigerated during harvesting (10°C). Additional experiments indicated that the difference in glucose uptake between transition and log cells was due partially to cold sensitivity of the latter during centrifugation. When log cells were harvested at room temperature (22°C), total glucose uptake was approximately twice that seen in cells harvested at 10°C (Fig. 3). In contrast, glucose uptake by transition cells was unaffected by harvest temperature. When the uptake patterns of both cell types harvested at 22°C were compared, it was clear that transition cells still showed greater glucose uptake than did log cells. All of the following experiments were performed using cells harvested at 22°C.

Glucose incorporation into cellular fractions: Glucose transported by bacteria may be fractionated into metabolic pool components and polymeric reserve material by extraction with cold 10% TCA (21). Both transition and log cells were capable of partitioning glucose radioactivity between TCA-soluble (pool) and -insoluble (reserve) fractions (Fig. 4). In transition cells, the metabolic pool was rapidly labeled (2 min) to saturation, after which net uptake reflected incorporation of radioactivity into the TCA-insoluble fraction. In log cells, the slower rate of glucose uptake extended the time required to

Fig. 3. Effect of harvest temperature on glucose uptake. Transition cells (open symbols) and log cells (closed symbols) were harvested either at 22°C (▲, △) or 10°C (●, ○).



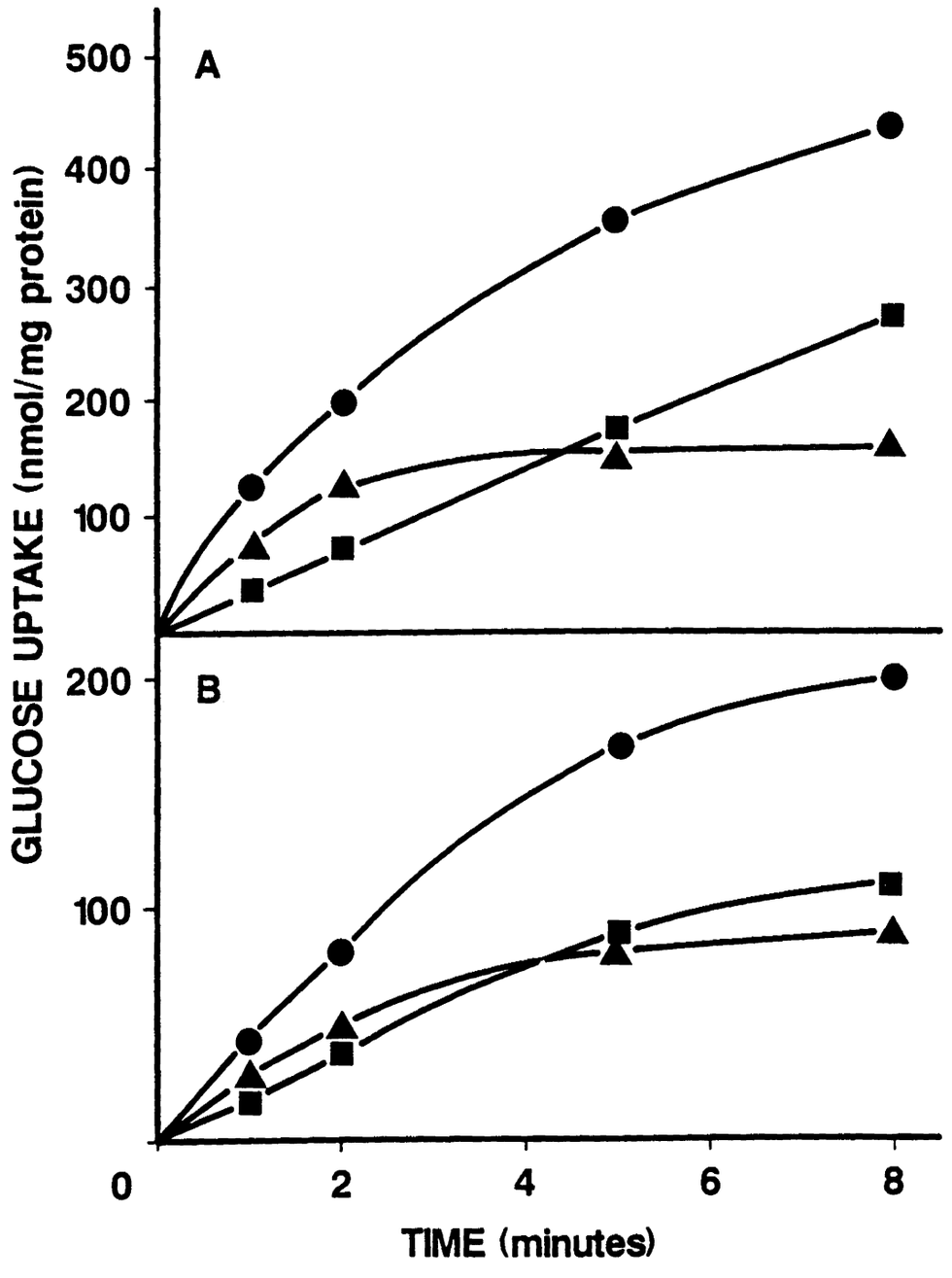


Fig. 4. Incorporation of glucose into cold TCA cellular fractions. Transition cells (A) and log cells (B) were examined. Total glucose uptake, (●); glucose incorporated into cold TCA-insoluble material, (■); glucose present in cold TCA-soluble pool (▲) was calculated as the difference between total glucose uptake and glucose present in the cold TCA-insoluble fraction.

saturate the metabolic pool (5 min) and was accompanied by a lower rate of glucose incorporation into the TCA-insoluble fraction. The TCA-soluble material was not identified, but glycogen has been demonstrated in another strain of B. succinogenes (60) and glucose is incorporated into a cold TCA insoluble polyglucose fraction by B. thetaiotaomicron (29).

Effects of sugars on glucose uptake: The specificity of the glucose uptake system of B. succinogenes was examined by measuring the ability of other sugars to inhibit transport activity. Maltose, mannose, galactose, fructose, α-methyl glucoside, and β-methylglucoside did not inhibit glucose uptake under the experimental conditions used. Only cellobiose and 2-deoxyglucose were inhibitory to glucose uptake by transition cells (Fig. 5). Cellobiose slightly inhibited glucose uptake when added at 2 min post-initiation. However, the inhibition was more pronounced when cellobiose was preincubated with the cells. 2-Deoxyglucose showed a similar profile, but the extent of inhibition was less than with cellobiose. Similar results were also obtained with log cells (data not shown).

Since glucose uptake by glucose-grown cells was inhibited by cellobiose, it was of interest to examine glucose uptake by cellobiose-grown cells. As illustrated in Fig. 6, glucose uptake by cellobiose-grown transition cells was reduced by nearly half. Similar results were obtained using log cells (data not shown).

Effects of metabolic inhibitors on glucose uptake: The

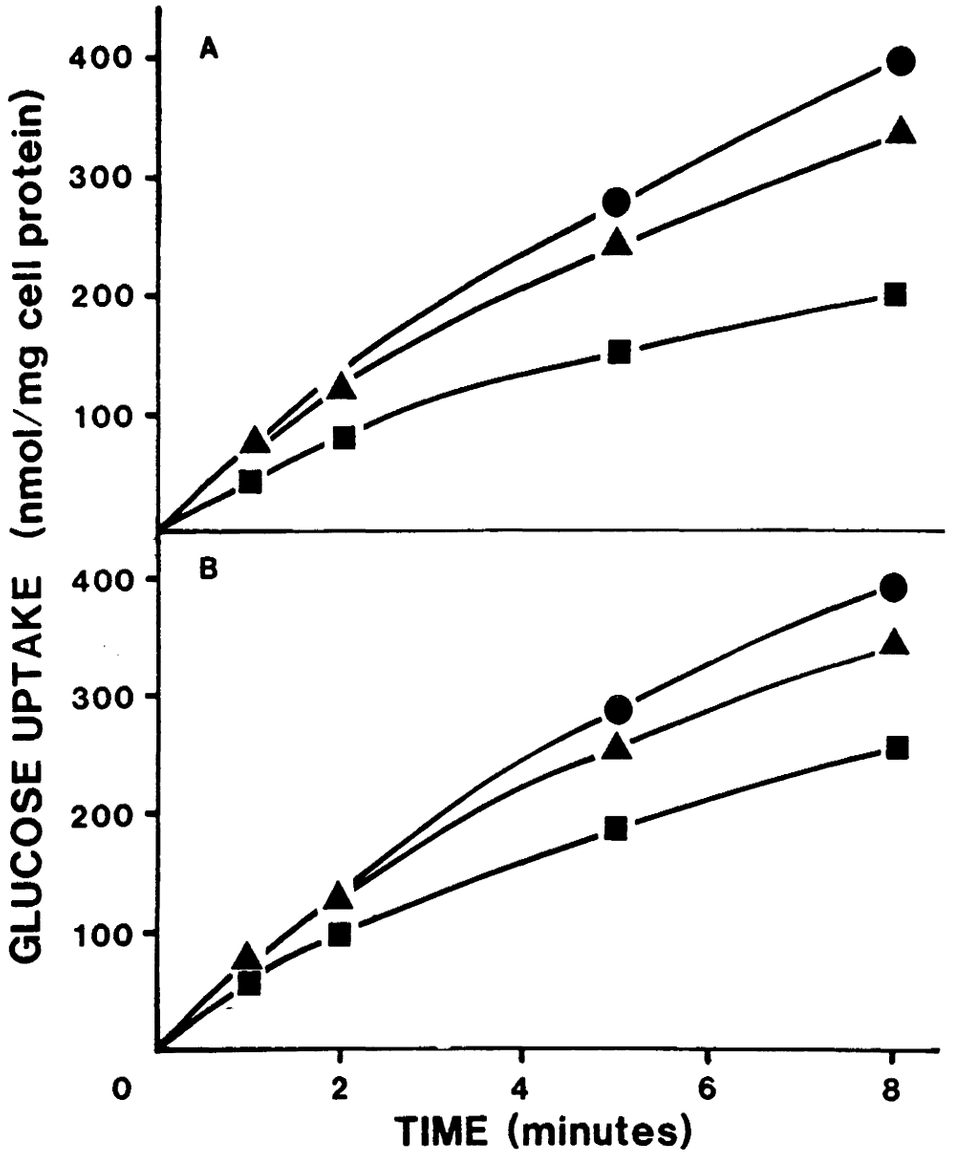


Fig. 5. Effect of cellobiose (A) and 2-deoxyglucose (B) on glucose uptake. Control, no additions, (●); sugar added 2 min post-initiation, (▲); sugar added 10 min prior to initiation of uptake, (■).

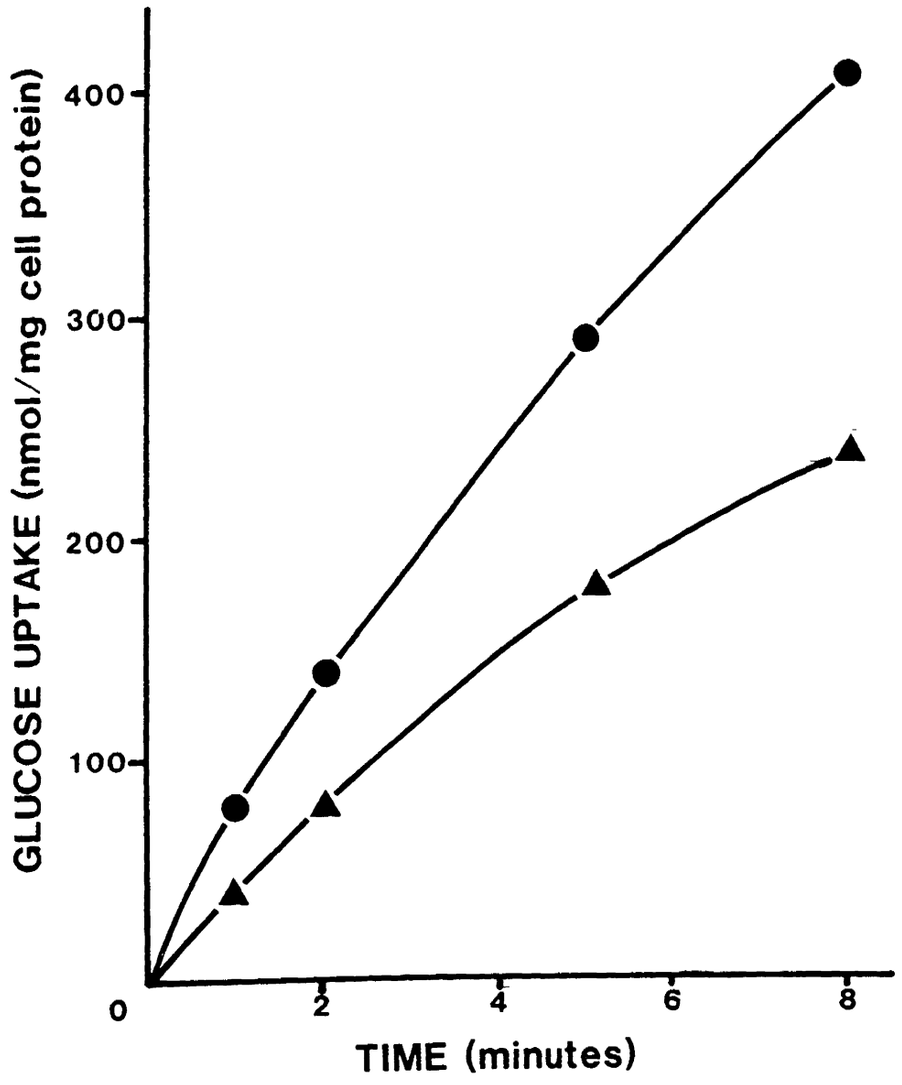


Fig. 6. Glucose uptake by cells grown on cellobiose. Glucose uptake by transition cells grown in glucose medium (●) or cellobiose (0.25%) medium (▲).

effects of various metabolic inhibitors on glucose uptake were compared between transition and log cells. In general, the inhibition patterns were the same between the cell types for all compounds tested, except for antimycin A (Table 2). This compound inhibited glucose uptake by 80% in log cells compared to 30% in transition cells. All of the water-insoluble inhibitors listed in Table 2 were dissolved in ethanol and compared to ethanol treated controls. Several compounds (HOQNO, CCCP, and pentachlorophenol) found to inhibit glucose uptake, were also tested with dimethylsulfoxide as the solvent and compared to similar assay controls. No differences were seen in the inhibitory effects of these compounds depending on the solvent used.

Allowing for the different concentrations tested, the most potent inhibitors of glucose uptake were the proton ionophores (FCCP, CCCP) and the metal ionophores (monensin, lasalocid). Of the two other proton ionophores, pentachlorophenol effectively inhibited glucose uptake (60-65%), while 2,4-DNP was only slightly inhibitory at a concentration eight-fold greater than the other proton conductors.

Several compounds, known to interfere with electron transport in bacteria, were also found to be strong inhibitors of glucose uptake in B. succinogenes. These included HOQNO, acriflavin, and antimycin A (log cells). Glucose uptake was relatively insensitive to dicumarol and resistant to menadione. The inhibition of glucose uptake by compounds that react with

Table 2. Effect of metabolic inhibitors on glucose uptake by *B. succinogenes*

Class	Inhibitor[a] (mM)	% Control activity[b]	
		Transition cells	Log cells
Controls	None	100	100
	Ethanol (5% v/v)	92	92
Iron-reactive compounds	KCN (10)+	14	17
	o-Phenanthroline (1.0)	7	17
	∝,∝-Dipyridyl (1.0)	25	25
Electron transport Inhibitors	HOQNO (0.05)	42	45
	Antimycin A (0.05)	69	17
	Menadione (0.1)	104	102
	Dicumerol (0.1)	71	61
	Acriflavin (1.0)	52	50
Proton ionophores	2,4-DNP (0.4)	84	80
	FCCP (0.05)	14	23
	CCCP (0.05)	12	21
	Pentachlorophenol (0.05)	34	40
Metal ionophores	Monensin (0.01)	18	20
	Lasalocid (0.01)	10	21
	Valinomycin (0.01)	69	70
Sulfhydryl inhibitors	$HgCl_2$ (0.5)+	<1	<1
	p-CMB (0.5)+	85	89
	N-ethylmaleimide (0.5)+	30	27
Miscellaneous	NaF (15)+	14	17
	DCCD (0.1)	6	18
	NaN_3 (10)+	12	11
	O_2 (Air)	23	24

[a] Inhibitors (+) were dissolved in water and compared to controls (no additions). All other inhibitors were dissolved in ethanol and compared to ethanol controls.

[b] The control (no additions) values were 315 and 200 nmols glucose/5 min/mg protein for the transition and log cells, repectively.

non-heme iron (α,α-dipyridyl; o-phenanthroline; KCN) is also consistent with the activity of an electron transport chain.

Sulfhydryl reagents were potent inhibitors of glucose uptake. The lack of inhibition by p-CMB compared to $HgCl_2$ indicates that reactive sites are either sterically protected or p-CMB does not effectively penetrate the outer membrane of this organism.

Of the miscellaneous compounds tested, inhibitors of ATP synthesis by substrate level phosphorylation (NaF) or oxidative phosphorylation (DCCD) blocked glucose uptake. Given the strictly anaerobic nature of B. succinogenes and the potential O_2-lability of electron transport systems in anaerobic bacteria (33), the inhibition of glucose uptake by O_2 was not suprising. Simple air exposure gave only slight inhibition (10-15%); however, gentle agitation of the assay resulted in the 75% inhibition listed in Table 2.

Effect of monovalent cations on glucose uptake: The inhibitory effects of monensin and lasalocid (Table 2) and the nutritional requirement of B. succinogenes for Na^+ prompted experiments to determine if monovalent cations affected glucose uptake. Initially, a Tris·HCl buffer lacking Na^+ and K^+, was used to suspend and wash the cells. However, transport activity was lost and could not be restored by the addition of monovalent cations. Therefore, the standard anaerobic buffer was modified by deleting KCl and substituting KH_2PO_4 for NaH_2PO_4. With this buffer and the protocol described, cells could be

washed once with retention of glucose uptake activity. An attempt to wash the cells twice resulted in loss of activity which was not regained by adding monovalent cations.

Figure 7 shows that total glucose uptake was specifically stimulated by Na^+ or Li^+ in a concentration-dependent manner up to 50 mM. Higher concentrations (100 mM) were only slightly more stimulatory. K^+ was slightly inhibitory to uptake demonstating that the Na^+ and Li^+ effects were specific and not due to increased ionic strength of the assay. Figure 8 illustrates the kinetics of the Na^+- and Li^+-stimulated glucose uptake. The effects of Na^+ and Li^+ were evident both on the initial rate and total amount of glucose transported. Glucose uptake by the control cells (washed once during processing) was less than that normally seen with log cells (Fig. 3). However, the addition of Na^+ or Li^+ restores this activity to near normal levels.

Glucose uptake by spheroplasts: To determine if glucose uptake was mediated by a periplasmic glucose binding protein, glucose transport was examined in spheroplasts prepared from transition and log cells. The treatment of cells with EDTA and lysozyme caused a time-dependent formation of osmotically sensitive cells (Fig. 9A, 9C). Formation of these spheroplasts was 90-95% complete after 40 min incubation. Phase-contrast microscopy suggested that while the peptidoglycan was breached by this time (Fig. 10B), the spheroplasts had not been extruded completely from their cell envelopes. Continued incubation of the cells up to 80 min yielded forms more closely resembling

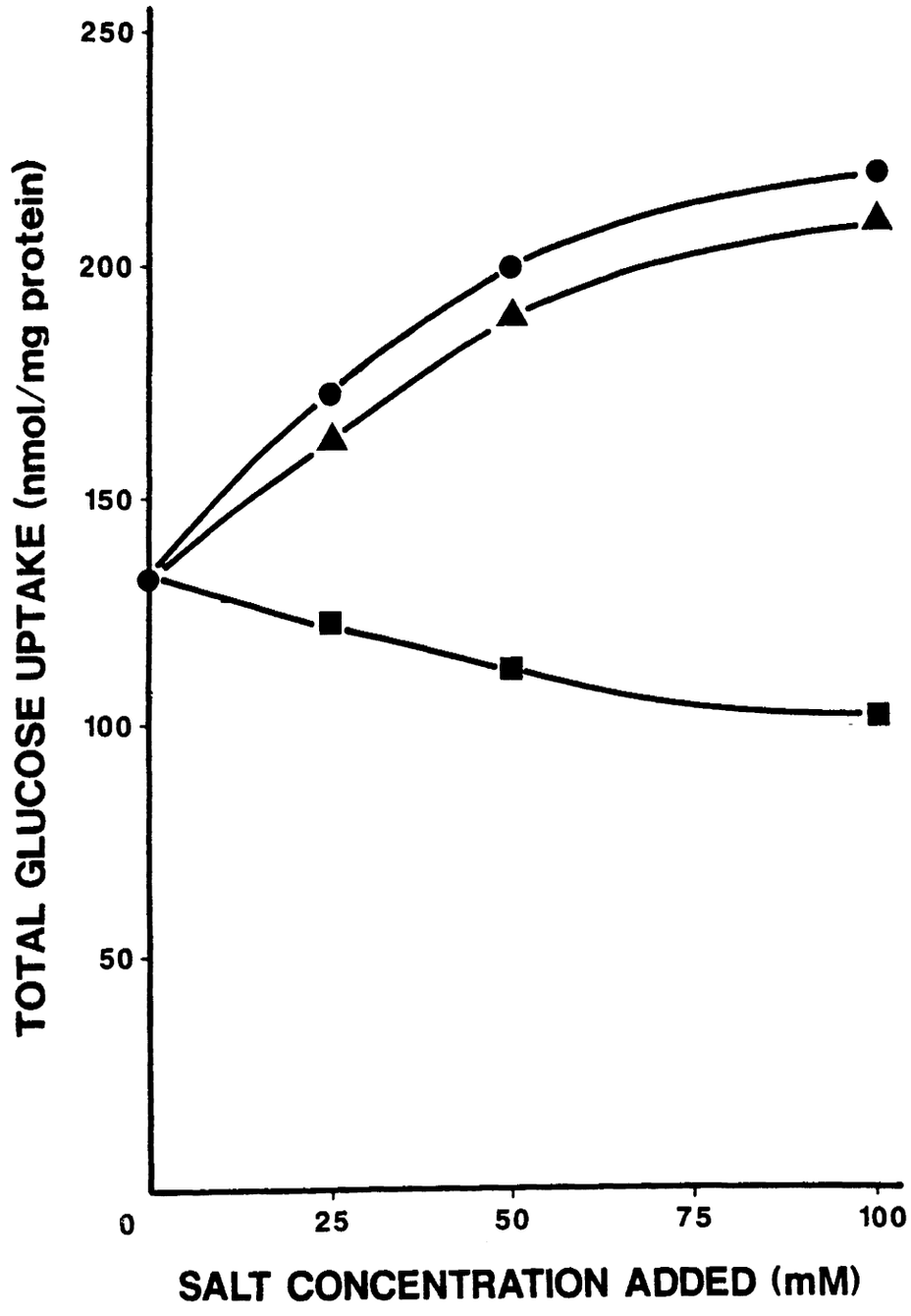


Fig. 7. Concentration dependence of Na^+ or Li^+ stimulation of glucose uptake. Glucose uptake by log cells was initiated after 10 min preincubation in the presence of NaCl (●), LiCl (▲), and KCl (■) at the concentrations indicated. The control (0 mM added salt) contained approx 50 mM K^+ from the buffer. Total glucose uptake was that measured 8 min after initiating the reaction.

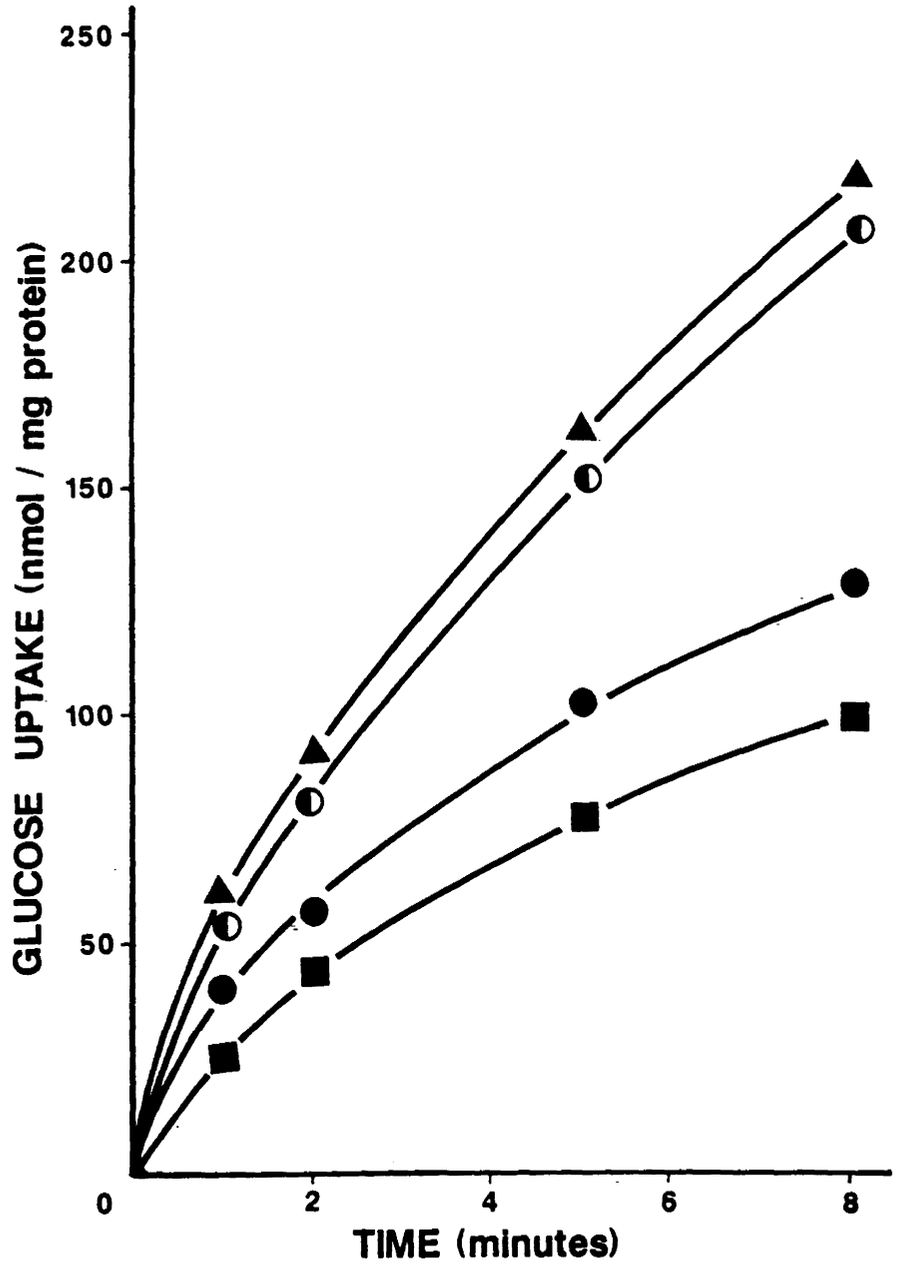


Fig. 8. Kinetics of Na^+- and Li^+-stimulated glucose uptake in log cells. Cells were preincubated for 10 min with 100 mM NaCl, (▲); LiCl, (◑); and KCl, (■) before initiating uptake. Control cells (●) received no additions.

Fig. 9. Glucose uptake by whole cells and spheroplasts. Spheroplast formation is shown by the increase in osmotic sensitivity (A and C) of cells treated with EDTA/lysozyme.

A. Formation of spheroplasts from transition cells.

Symbols: (●), whole cell control.

(○), spheroplasts.

B. Glucose uptake by whole cells and spheroplasts of transition cells.

Symbols: As in A.

C. Formation of spheroplasts from log cells.

Symbols: As in A.

D. Glucose uptake by whole cells and spheroplasts of log cells.

Symbols: As in A.

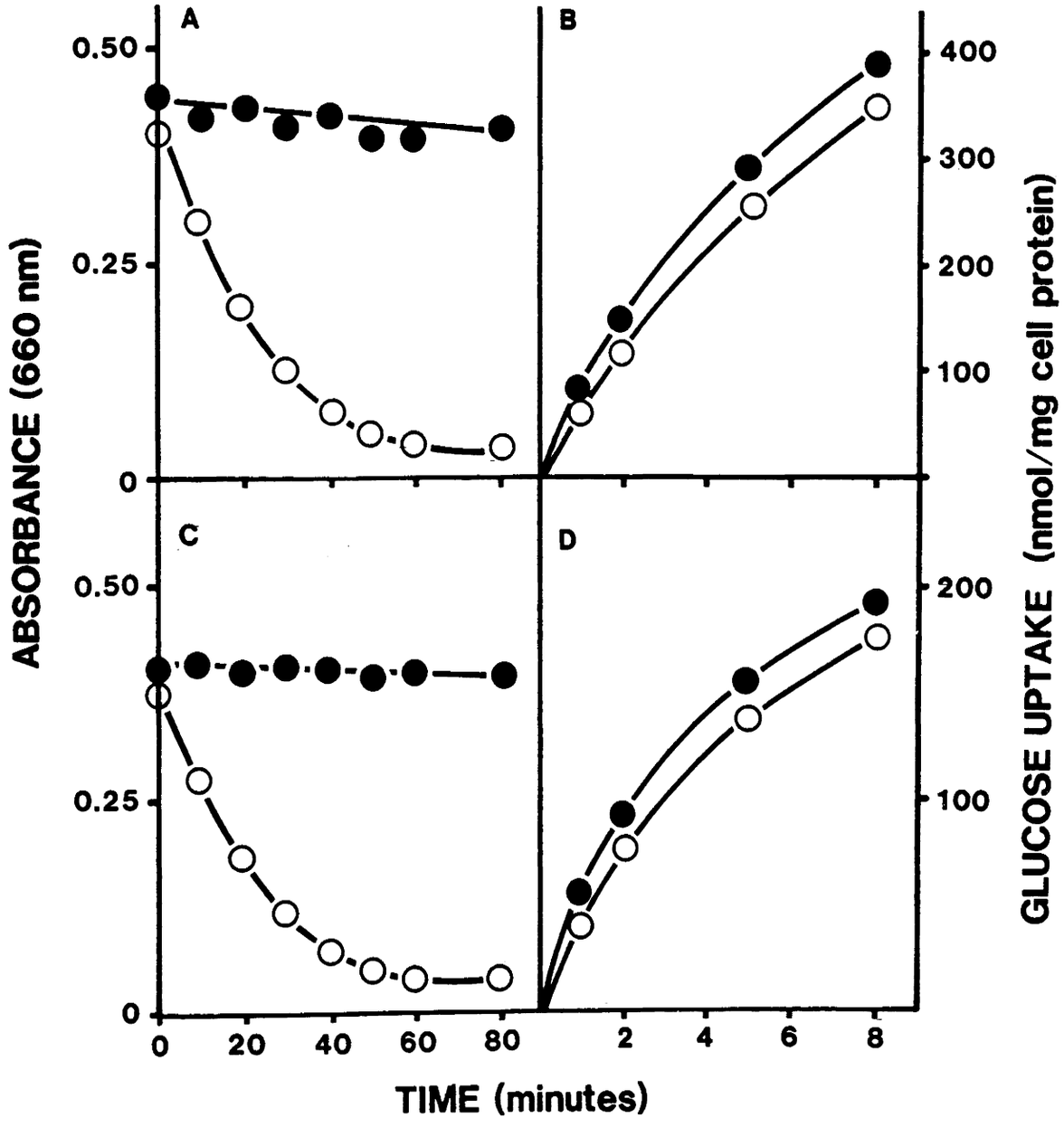
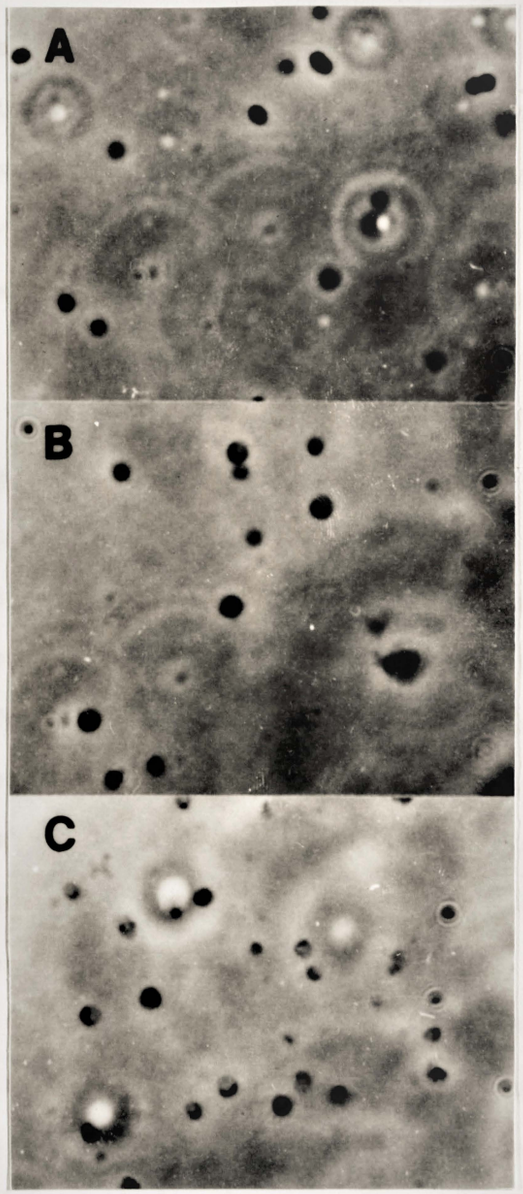


Fig. 10. Phase-contrast microscopy of spheroplast formation. Photographs (1,000 X) were taken 0, (A); 40, (B); and 80 min, (C) after initiating EDTA/lysozyme treatment.



spheroplasts, with the protoplast structure and attached remnant of the cell body (Fig. 10C). Figures 9B and 9D show the glucose uptake abilities of transition and log cell spheroplasts, respectively. Clearly, there was no appreciable loss of glucose uptake due to spheroplast formation in either cell type. Glucose uptake in spheroplasts obtained after 80 min of lysozyme/EDTA treatment was also measured. Control cells and spheroplasts both exhibited similar uptake patterns; however, transport activity had declined in both cell forms for reasons apparently unrelated to spheroplast formation.

Mg^{2+} stabilization of whole cells: During the initial work on spheroplast formation, Mg^{2+} was omitted from the cell suspension and assay buffers to maximize the effect of EDTA in disrupting the outer membrane and optimize lysozyme penetration. However, under this condition control cells underwent spontaneous autolysis which gave rise to osmotically fragile forms, presumably spheroplasts, that were stabilized by the 0.5 M sucrose in the buffer. In order to do the comparative experiment described above, it was necessary to stabilize the whole cells in the transport assay. Figure 11 shows this could be achieved by adding Mg^{2+} to the transport assay. A Mg^{2+} concentration of 20 mM was the minimum amount that prevented further autolysis in the system and was effective with both transition and log cells (data not shown).

Mg^{2+} not only prevented further autolysis of the cells, but also showed an apparent concentration-dependent effect

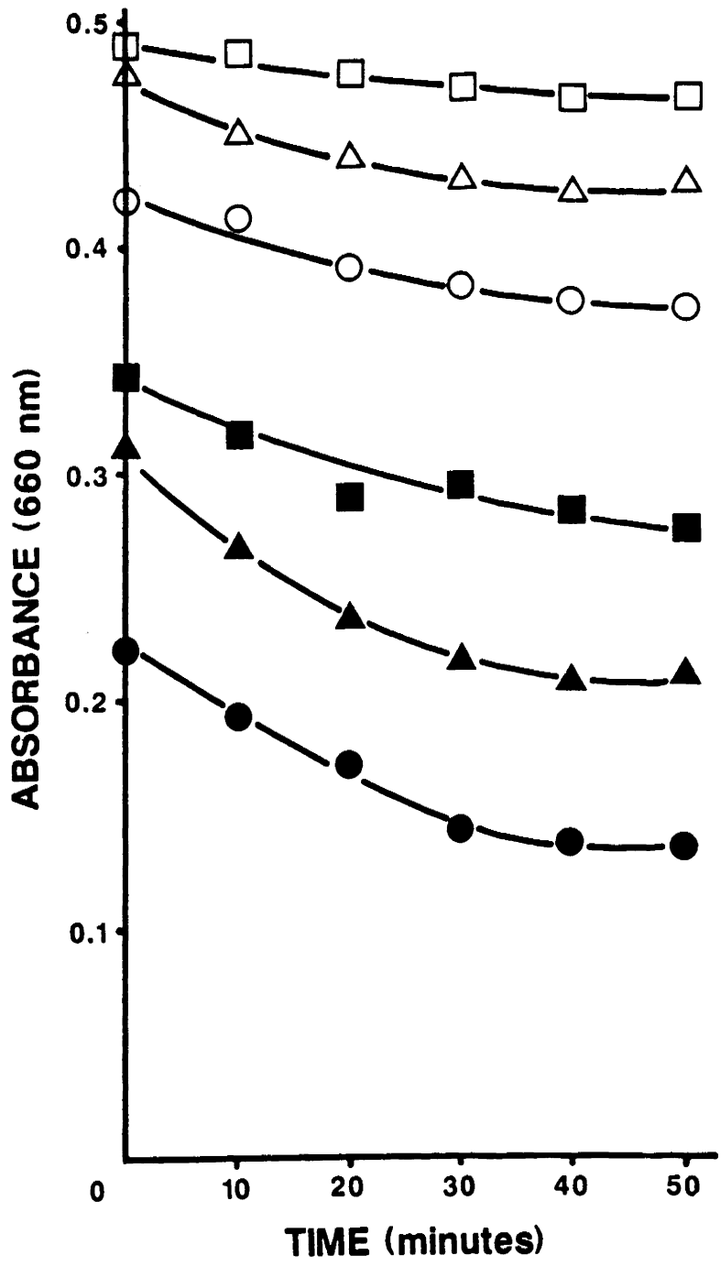


Fig. 11. Effect of Mg^{2+} on the osmotic stability of B. succinogenes. Cells were harvested and suspended in anaerobic buffer lacking Mg^{2+}. Cells were then added to assays containing 0, (●); 5, (▲); 10, (■); 20, (○); 30, (△); and 40, (□) mM $MgCl_2$. Periodically, 0.1 ml-samples were removed, diluted 1:10 with distilled water, and the absorbances recorded.

toward stabilizing already weakened cells that were formed prior to being added to the transport assay. This is seen by the increase in osmotic stability (absorbance) of the cells at the zero minute time point in Fig. 11.

While 20 mM Mg^{2+} appeared to stabilize already weakened control cells and prevent further autolysis, the lytic effect of lysozyme/EDTA against treated cells was not readily apparent. This was due to the extensive autolysis (spheroplast formation) which these cells had already undergone (0 mM Mg^{2+}, 0 min, Fig. 11). However, experiments showed that when 5 mM $MgCl_2$ was added back to the cell suspension buffer (i.e., when the standard anaerobic buffer was used), the cells to be treated with lysozyme/EDTA could be diluted into the transport assay with retention of cellular integrity. This is indicated by the zero time point absorbances in Fig. 9A and 9C. The presence of Mg^{2+} in the spheroplasting assays was overcome by a slight excess of EDTA (7 mM).

Based on these observations, it is likely that spheroplast formation in B. succinogenes was a combined result of endogenous autolytic activity and lysozyme/EDTA treatment.

Absence of PEP-dependent glucose phosphorylation: Dialyzed cell extracts of B. succinogenes were examined for the ability to form glucose-6-phosphate from glucose and PEP by coupling to NADP-linked glucose-6-phosphate dehydrogenase. Although activity could be detected in a cell extract of E. coli, none was seen with B. succinogenes.

DISCUSSION

The results of this study represent the first experimental measurements of carbohydrate uptake in a cellulolytic rumen bacterium. The data indicate the presence of a highly specific, active transport system for glucose in B. succinogenes. Furthermore, a periplasmic glucose binding protein does not mediate glucose uptake in this organism. Although the data are insufficient to distinguish the direct source of energy for uptake, an electrochemical gradient generated probably by the fumarate reductase-coupled electron transport system appears to be responsible for energizing uptake.

Many bacteria that metabolize sugars by the EMP pathway use the PEP:sugar phosphotransferase system for sugar transport (13). However, several lines of evidence suggest glucose is not transported by this mechanism in B. succinogenes. PEP-dependent glucose-6-phosphate formation could not be detected enzymatically in dialyzed cell extracts. Likewise, attempts to demonstrate PEP-dependent glucose phosphorylation using toluenized whole cells were also unsuccessful, although activity was found in other species of rumen bacteria (J. B. Russell, personal communication). Finally, the inhibition of glucose uptake by the proton and metal ionophores are inconsistent with the presence of a PEP:glucose phosphotransferase system (23).

The energy dependence of glucose transport in B. succinogenes is clearly indicated by the metabolic inhibitor data. Thus, glucose uptake is an active transport mechanism rather

than a facilitated diffusion. The effect of certain electron transport inhibitors, in particular HOQNO, antimycin A, and acriflavin, suggests the fumarate reductase-coupled electron transport system plays an important role in energizing glucose uptake. The electron transport system of B. succinogenes is not well characterized. Its activity is inferred from the presence of cytochrome b (50), the particulate nature of fumarate reductase (42), and properties of fumarate-dependent electron transport systems in other bacteria (33,45). HOQNO may inhibit electron transport at cytochrome b or menaquinone (33,45), while antimycin A probably acts at or below cytochrome b, and acriflavin is an antagonist of flavin (flavoprotein ?)-mediated reactions. The effect of acriflavin is interesting, since the oxidation of pyruvate coupled to fumarate reduction is flavin-dependent (42). The inhibition of glucose uptake by iron-reactive compounds (α,α-dipyridyl, o-phenanthroline, KCN) is consistent with the involvement of an electron transport system. However, the effect of these compounds is not specific for electron transport proteins and other iron-containing proteins involved with glucose metabolism may be the sites of inhibition. Similar considerations apply to the sulfhydryl inhibitors.

Fumarate-coupled electron transport systems in anaerobic and facultatively anaerobic bacteria conserve energy by generating a proton motive force (PMF) which can be coupled to ATP synthesis through a membrane-bound, proton-translocating, ATPase (32,33). Active transport systems may be coupled

directly to the PMF (e.g., H^+/lactose symport in E. coli) or to ATP (e.g., glutamine transport in E. coli) (32,51). Determining whether the transport mechanism for a particular solute is coupled directly to the proton gradient or to ATP is difficult in whole cells and generally requires the use of membrane vesicles (devoid of soluble cytoplasmic contents) and mutants that are defective in the proton-translocating ATPase (51).

The inhibition of glucose uptake in B. succinogenes by the proton-conducting ionophores (FCCP, CCCP, pentachlorophenol) together with the effects of electron transport inhibitors suggests a role for the PMF in energizing glucose uptake. The absence of inhibition by 2,4-DNP is not contrary to this conclusion, since Dawson et al. (9) showed the growth of B. succinogenes S85 was not inhibited by 2,4-DNP. However, the possibility that ATP (or a phosphorylated metabolic intermediate) and not the PMF energizes glucose uptake is not excluded by the inhibitor data. ATP pools can be depleted in cells that are treated with proton ionophores, since ATP may be consumed by the proton-translocating ATPase in a futile attempt to establish a PMF (51). Only two inhibitors (NaF and DCCD) were tested whose effects may have a bearing on this question. NaF prevents the formation of PEP by inhibiting enolase and thus blocks substrate level phosphorylation by the EMP pathway. PEP:sugar phosphotransferase systems are highly sensitive to F^- (18), but this mechanism is not present in B. succinogenes (above). The inhibition by F^- could indicate that ATP

energizes glucose uptake in B. succinogenes. However, Miller's data (42) show both the electron donor (pyruvate) and electron acceptor (fumarate) for the fumarate reductase system are derived from PEP. Thus, treatment with F^- could inhibit electron transport in B. succinogenes by depriving the system of its substrates and preventing formation of a PMF. DCCD is a well-known inhibitor of the proton-translocating ATPase. The inhibition of glucose uptake by DCCD might indicate that ATP (synthesized by oxidative phosphorylation) energizes glucose transport. However, at the concentrations tested (0.1 mM), DCCD has been shown to inhibit succinate and fumarate transport in an ATPase-negative E. coli strain (57). It was concluded that DCCD can inhibit transport systems by a mechanism(s) unrelated to inhibition of ATP synthesis. For this reason, the DCCD inhibition of glucose uptake in B. succinogenes cannot be equated unequivocally with a requirement for ATP. The inability to obtain transport activity with cells washed in Tris buffer precluded testing the effect of arsenate (an ATP synthesis inhibitor) on glucose uptake.

Despite the limitations on interpreting the metabolic inhibitor data, certain generalizations concerning the energy-coupling of transport mechanisms in gram-negative bacteria have been made (51). While exceptions have been found, sugar transport mechanisms that are dependent on a periplasmic substrate binding protein (osmotic shock sensitive) seem to be partially coupled to ATP or a phosphorylated metabolic intermediate. In contrast, transport mechanisms that are

independent of such proteins (osmotic shock insensitive) are energized by the PMF. Since B. succinogenes spheroplasts transport glucose as well as whole cells, a periplasmic glucose binding protein is not involved and energy-coupling to the PMF rather than ATP may be more likely.

The effects of monovalent cations and the metal ionophores on glucose uptake are of interest since Na^+ is required by B. succinogenes for growth. Certain transport mechanisms, notably those for melibiose in E. coli and Salmonella typhimurium and glutamate in E. coli (35,39), function as Na^+/solute symports. Solute uptake is coupled to an electrochemical Na^+ gradient and solutes are co-transported with Na^+ across the cell membrane. Maintenance of the Na^+ gradient requires a mechanism for removing Na^+ from the cell and may be accomplished by the Na^+/H^+ antiporter (35). This membrane protein catalyzes the exchange of cytoplasmic Na^+ for external H^+ and is driven by the PMF. Thus, the PMF, in addition to a Na^+ gradient, is required to energize uptake, as seen by the inhibition of melibiose/Na^+ symport by CCCP in E. coli (39). This may account for the observation that glucose uptake in B. succinogenes did not respond to stimulatory Na^+ concentrations (50-65 mM) present in assays performed with F^-, electron transport inhibitors, or proton ionophores.

Within the experimental limitations of the B. succinogenes system, it is not possible to conclude that glucose uptake occurs by a Na^+ symport mechanism. Monovalent cation dependence of transport systems may reflect: (a) effects of Na^+ (as

activators or cofactors) on the transport carriers themselves, or other transport components, (b) effects of the ions on the electrochemical potential across the membrane , or (c) true Na^+/solute symport (35). Stimulation of glucose uptake by Na^+ or Li^+ has been reported previously only in Micrococcus luteus (lysodeikticus), but the mechanism is unknown (1). Some Na^+/solute symporters do respond specifically to both Na^+ and Li^+, but not K^+ (35,39). The Na^+ concentrations that stimulated glucose uptake in B. succinogenes are in the range (25-100 mM) required by the bacterium for growth (5,6). However, only a Na^+ stimulation and not a direct dependence could be demonstrated with the whole cell assay. An alternative possibility for the Na^+ stimulation of glucose uptake is the observation that low concentrations of Na^+ stimulate the fumarate reductase activity in membrane preparations from Bacteroides amylophilus (63). Li^+ was not tested, but a similar effect in B. succinogenes might enhance glucose uptake by stimulating electron transport independent of a Na^+/solute symport mechanism. However, the electron transport system of B. amylophilus differs from that of B. succinogenes in lacking cytochrome b and menaquinone and using NADH as an electron donor. Furthermore, the Na^+ concentrations that stimulated fumarate reductase activity (K_m = 0.8 mM) were far less than those (20-90 mM) required by B. amylophilus for optimum growth.

The effects of the metal ionophores, monensin and lasalocid, must also be resolved with the energetics of glucose uptake. These compounds establish themselves in cell membranes

where they catalyze an exchange of H^+ for Na^+ (monensin) or K^+ (lasalocid) (23). Because both compounds respond to the PMF, the cells pump Na^+ or K^+ out coupled to an influx of H^+. Thus, in addition to dissipating Na^+ or K^+ cation gradients, the ΔpH component of the PMF is also discharged, but the $\Delta\Psi$ component is not. In membrane vesicles of E. coli, the uptake of glutamate by the glutamate/Na^+ symporter is abolished by monensin, but not by nigericin (a K^+/H^+ antiporter) (41). Since both monensin and lasalocid inhibit glucose uptake in B. succinogenes, this effect may be more closely related to dissipation of the ΔpH rather than collapse of a Na^+ gradient. However, in E. coli at pH 7.0, the ΔpH accounts for only 25% of the total PMF (32). Whether elimination of the ΔpH at pH 7.0 is sufficient by itself to account for the near total inhibition of glucose uptake will require further study.

The lack of inhibition of glucose uptake by a variety of carbohydrates indicates the glucose transport system is highly specific. This result was not suprising, given that glucose and cellobiose are the only soluble sugars supporting the growth of B. succinogenes. The only sugars that inhibited glucose uptake, cellobiose and 2-deoxyglucose, did not act as competitive inhibitors. Since preincubation of these sugars with the cells was required to detect significant inhibition, metabolism of these compounds is probably necessary to inhibit glucose uptake. The inhibition by cellobiose may be due to the presence of cellobiase, synthesized constitutively in B. succinogenes (19). Furthermore, since cellobiose-grown cells

can transport glucose, it is probable that glucose-grown cells can take up cellobiose. During preincubation, the internal (cytoplasmic) formation of glucose from cellobiase activity could dilute the amount of radiolabeled glucose taken up by the cells. Alternatively, the increased cytoplasmic pools of glucose (or sugar phosphates) may reduce glucose uptake in resting cells (13).

The mechanism of inhibition by 2-deoxyglucose is not clear. Unless it is a very weak competitive inhibitor (i.e., a weak substrate) of glucose uptake, 2-deoxyglucose may enter the cells by passive diffusion as in Pseudomonas aeruginosa (15). If 2-deoxyglucose were then phosphorylated by glucokinase, the cellular ATP pool might be depleted and the metabolism of glucose inhibited by blockage of the EMP pathway. Glucokinases generally do not phosphorylate 2-deoxyglucose; however, the enzyme from Selenomonas ruminantium was shown recently to catalyze this reaction (S. A. Martin and J. B. Russell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K178, p. 223). The ability of B. succinogenes glucokinase to phosphorylate 2-deoxyglucose has not been tested (J. B. Russell, personal communication).

Although cellobiose was not a competitive inhibitor of glucose uptake, it did appear to influence expression of the glucose uptake system. The 50% decrease in glucose uptake by cellobiose-grown cells was unexpected since both these sugars are derived from cellulose degradation. The effect of cellobiose was not due to catabolite repression, since the

cells were still competent for glucose uptake after growth on cellobiose. Perhaps, in these cells, synthesis of the glucose transport carrier is reduced, but not eliminated.

The effect of Mg^{2+} on the stability of B. succinogenes is of interest from the standpoint of future work involving preparation of membrane vesicles. Clearly, cells harvested and suspended in the absence of Mg^{2+} are subjected to sufficient autolytic activity to seriously weaken cell integrity. The ability of 20 mM Mg^{+2} to stabilize these cells and prevent further autolysis is interesting since the same concentration inhibits autolysis in E. coli (37). The inclusion of 5 mM Mg^{+2} in the cell suspension buffer may be an absolute minimum concentration to inhibit or slow autolysis. Possibly some of the difference in osmotic stability between transition and log cells may be due to differences in the sensitivity of their autolytic enzymes to the Mg^{+2} concentration of the suspension buffer.

Much of the research done was devoted to comparing glucose uptake in cells at two different stages of growth. Initial experiments showed interesting differences in uptake between transition and log phase-cells, that might have been due to different transport mechanisms. However, these differences were partially accounted for by the sensitivity of log cells to cold during harvesting. Attempts to determine the nature of the remaining difference were unsuccessful. Glucose transport in both cell types exhibited similar sugar specificity, effects of metal cations, lack of a periplasmic binding protein, and

inhibition patterns by nearly all metabolic inhibitors. The only inhibitor affecting uptake differently between the two cell types was antimycin A. This could be due to changes in the permeability of the cells to the compound with culture age. Thus, while differences in the uptake ability of transition and log-phase cells are present, they do not appear to be due to different transport mechanisms for glucose. Perhaps the osmotic- and cold- sensitivity properties of these cells may reflect changes in the structural integrity of the cell envelope of B. succinogenes during growth. Subsequent effects on the ability to transport glucose may be secondary to these changes.

Although B. succinogenes may not be a member of the genus Bacteroides (47), the only other work done on sugar uptake in a non-sporeforming anaerobe that contains a fumarate reductase system was with B. thetaiotaomicron (29). Significant differences in the glucose transport systems of these bacteria are evident from the results of the present study. Glucose uptake by B. thetaiotaomicron was relatively resistant to O_2 while that of B. succinogenes was extremely O_2-labile. Galactose and mannose (both growth substrates) were competitive inhibitors of glucose uptake by B. thetaiotaomicron. Neither sugar affected uptake in B. succinogenes. Menadione was a strong inhibitor of glucose uptake in B. thetaiotaomicron, but had no effect against that of B. succinogenes. In contrast, glucose uptake by B. thetaiotaomicron was resistant to NaN_3, KCN, and HOQNO all of which strongly inhibited the reaction in B. succin-

ogenes. However, the inhibitor experiments were all done aerobically with B. thetaiotaomicron and anaerobically with B. succinogenes. Glucose uptake by B. thetaiotaomicron was inhibited by NaF, but the activity was much less sensitive (55% inhibition at 50 mM) than in B. succinogenes (85% inhibition at 15 mM). Evidence for a PEP:sugar phosphotransferase system was not found in either organism. In general, the different properties noted are consistent with the proposition that B. succinogenes is not related to other Bacteroides (47).

The results of this study suggest several possible approaches for further study of glucose uptake in B. succinogenes. Since whole cell assays were inadequate to determine the actual energy source for glucose uptake, other experimental systems will be necessary to resolve this question. One possible system involves the use of membrane vesicles to measure glucose transport. These systems are best prepared by gentle lysis of bacterial spheroplasts. Membrane vesicles are advantageous in that glucose uptake is disassociated from metabolism (due to loss of soluble enzymes and cofactors during vesicle formation). Thus, glucose transport may be measured rather than transport plus metabolism, as in whole cells. The direct energy source for glucose uptake could then be determined by creating artificial transmembrane ion gradients and measuring glucose transport in vesicles. These experiments may clarify the function of Na^+ and H^+ gradients and the effects of metal ionophores on glucose transport. Membrane vesicles can also be used to confirm the involvement of the fumarate reductase

electron transport system in glucose transport. The involvement of a transmembrane proton gradient or ATP in energizing glucose transport could be further distinguished by obtaining a proton-translocating ATPase-negative mutant of B. succinogenes. Such strains might be obtained by selecting for DCCD-resistant coonies following chemical or transpositional mutagenesis. However, this would require the use of an anaerobic chamber and incubator for working with strict anaerobes. ATPase-negative mutants of E. coli have been used to determine whether ATP can directly energize different transport systems.

LITERATURE CITED

1. Ariel, M., and N. Grossowicz. 1974. Enhancement of transport in Micrococcus luteus by sodium ions. Biochim. Biophys. Acta 352: 122-126.

2. Bryant, M. P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. 25: 1324-1328.

3. Bryant, M. P. 1973. Nutritional requirements of the predominant rumen cellulolytic bacteria. Fed. Proc. Am. Soc. Exptl. Biol. 32: 1809-1813.

4. Bryant, M. P., and R. N. Doetich. 1954. A study of actively cellulolytic rod-shaped bacteria of the bovine rumen. J. Dairy Sci. 37: 1176-1183.

5. Bryant, M. P., I. M. Robinson, and H. Chu. 1959. Observations on the nutrition of Bacteroides succinogenes-a ruminal cellulolytic bacterium. J. Dairy Sci. 42: 1831-1847.

6. Caldwell, D. R., and C. Arcand. 1974. Inorganic and metal-organic growth requirements of the genus Bacteroides. J. Bacteriol. 120: 322-333.

7. Clarke, R. T. J. 1977. Methods for studying gut microbes, p. 1-33, In R. T. J. Clarke and T. Bauchop, (ed.), Microbial ecology of the gut. Academic Press Inc., New York.

8. Costerton, J. W., H. N. Damgaard, and K.-J. Cheng. 1974. Cell envelope morphology of rumen bacteria. J. Bacteriol. 118: 1132-1143.

9. Dawson, K. A., M. C. Preciosi, and D. R. Caldwell. 1979. Some effects of uncouplers and inhibitors on growth and electron transport in rumen bacteria. J. Bacteriol. 139: 384-392.

10. Dehority, B. A. 1965. Degradation and utilization of isolated hemicellulose by pure cultures of cellulolytic rumen bacteria. J. Bacteriol. 89: 1515-1520.

11. Dehority, B. A. 1969. Pectin-fermenting bacteria from the bovine rumen. J. Bacteriol. 99: 189-196.

12. Dehority, B. A., and H. W. Scott. 1967. Extent of cellulose and hemicellulose digestion in various forages by pure cultures of rumen bacteria. J. Dairy Sci. 50: 1136-1141.

13. Dills, S. S., A. Apperson, M. R. Schmidt, and M. H. Saier. 1980. Carbohydrate transport in bacteria. Microbiol. Rev. 44: 385-418.

14. Dills, S. S., C. A. Lee, and M. H. Saier. 1981. Phosphoenolpyruvate-dependent sugar phosphotransferase activity in Megasphera elsdenii. Can. J. Microbiol. 27: 949-952.

15. Eagon, R. G. 1971. 2-Deoxyglucose transportation via passive diffusion and its oxidation, not phosphorylation to 2-deoxygluconic acid by Pseudomonas aeruginosa. Can. J. Biochem. 49: 606-613.

16. Forsberg, C. W., T. J. Beveridge, and A. Hellstrom. 1981. Cellulase and xylanase from Bacteroides succinogenes and its importance in the rumen environment. Appl. Environ. Microbiol. 42: 886-896.

17. Forsberg, C. W., and D. Groleau. 1982. Stability of the endo-β-1,4-glucanase from Bacteroides succinogenes. Can. J. Microbiol. 28: 144-148.

18. Germaine, G. R., and L. M. Tellefson. 1986. Effect of endogenous phosphoenolpyruvate potential on fluoride inhibition of glucose uptake by Streptococcus mutans. Infect. Immun. 51: 119-124.

19. Groleau, D., and C. W. Forsberg. 1981. Cellulolytic activity of the rumen bacterium Bacteroides succinogenes. Can. J. Microbiol. 27: 517-530.

20. Groleau, D., and C. W. Forsberg. 1983. Partial characterization of the extracellular carboxymethylcellulase produced by the rumen bacterium Bacteroides succinogenes. Can. J. Microbiol. 29: 504-517.

21. Groleau, D., and C. W. Forsberg. 1983. Solubilization by trypsin of the extracellular membranous carboxymethylcellulase of Bacteroides succinogenes. Can. J. Microbiol. 29: 710-717.

22. Halliwell, G., and M. P. Bryant. 1963. The cellulolytic activity of pure strains of bacteria from the rumen of cattle. J. Gen. Microbiol. 32: 441-448.

23. Harold, F. M., K. H. Altendorf, and H. Hirata. 1974. Probing membrane transport mechanisms with ionophores. Ann. N. Y. Acad. Sci. 235: 149-160.

24. Hiltner, P., and B. A. Dehority. 1983. Effect of soluble carbohydrates on digestion of cellulose by pure cultures of rumen bacteria. Appl. Environ. Microbiol. 46: 642-648.

25. Hoekstra, D., J. W. van der Laan, L. de Leij, and B. Witholt. 1976. Release of outer membrane fragments from normally growing Escherichia coli. Biochim. Biophys. Acta 455: 889-899.

26. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. 14: 1-49.

27. Hungate, R. E. 1966. The rumen and its microbes. Academic Press Inc., New York.

28. Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes, p. 117-132, In J. R. Norris, and D. W. Ribbons (ed.), Methods in microbiology, vol 3. Academic Press Inc. New York.

29. Hylemon, P. B., J. L. Young, R. F. Roadcap, and P. V. Phibbs Jr. 1977. Uptake and incorporation of glucose and mannose by whole cells of Bacteroides thetaiotaomicron. Appl. Environ. Microbiol. 34: 488-494.

30. Joyner, A. E., and R. L. Baldwin. 1966. Enzymatic studies of pure cultures of rumen microorganisms. J. Bacteriol. 92: 1321-1330.

31. Kolze, J. P., and A. Kistner. 1974. Variations in enzyme activities of Butyrivibrio fibrisolvens and Ruminococcus albus grown in continuous culture. Can. J. Microbiol. 20: 861-869.

32. Konings, W. N., and P. A. M. Michels. 1980. Electron transfer-driven solute translocation across bacterial membranes, p. 33-86, In C. J. Knowles (ed.), Diversity of bacterial respiratory systems, vol. 1. CRC Press Inc., Boca Raton, Florida.

33. Kroger, A. 1980. Bacterial electron transport to fumarate, p. 1-17, In C. J. Knowles (ed), Diversity of bacterial repiratory systems, vol 2. CRC Press Inc., Boca Raton, Florida.

34. Kundig, W., and S. Roseman. 1971. Sugar transport: Characterization of constitutive membrane-bound enzyme II of the Escherichia coli phosphotransferase system. J. Biol. Chem. 246: 1407-1418.

35. Lanyi, J. K. 1979. The role of Na^+ in transport processes of bacterial membranes. Biochim. Biophys. Acta 559: 371-397.

36. Latham, M. J., B. E. Brooker, G. L. Pettifer, and P. J. Harris. 1978. Adhesion of Bacteroides succinogenes in pure culture and in the presence of Ruminococcus flavefaciens to cell walls in leaves of perennial rye grass (Lolium perenne). Appl. Environ. Microbiol. 35: 1166-1173.

37. Leduc, M., R. Kosra, and J. von Heijenoort. 1982. Induction and control of the autolytic system of Escherichia coli. J. Bacteriol. 152: 26-34.

38. Lo, T. C. Y. 1979. The molecular mechanism of substrate transport in gram negative bacteria. Can. J. Biochem. 57: 289-301.

39. Lopilato, J., T. Tsuchiya, and T. H. Wilson. 1978. Role of Na^+ and Li^+ in thiomethylgalactoside transport by the melibiose transport system of Escherichia coli. J. Bacteriol. 134: 147-156.

40. Lowry, O. H., N. J. Rosebrough, A. C. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

41. MacDonald, R. E., J. K. Lanyi, and R. V Greene. 1977. Sodium-stimulated glutamate uptake in membrane vesicles of Escherichia coli: The role of ion gradients. Proc. Natl. Acad. Sci. U.S.A. 74: 3167-3170.

42. Miller, T. L. 1978. The pathway of formation of acetate and succinate from pyruvate by Bacteroides succinogenes. Arch. Microbiol. 117: 145-152.

43. Miller, T. L., and M. J. Wolin. 1973. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. 27: 985-987.

44. Montgomery, L., and J. M. Macy. 1982. Characterization of rat cecum cellulolytic bacteria. Appl. Environ. Microbiol. 44: 1435-1443.

45. Mountfort, D. O., and A. M. Roberton. 1977. The role of menaquinone and b-type cytochrome in anaerobic reduction of fumarate by NADH in membrane preparations from Bacteroides ruminicola strain $B_1 4$. J. Gen. Microbiol. 100: 309-317.

46. Ng, T. K., and J. G. Zeikus. 1982. Differential metabolism of cellobiose and glucose by Clostridium thermocellum and Clostridium thermohydrosulfuricum. J. Bacteriol. 150: 1391-1399.

47. Paster, B. J., W. Ludwig, W. G. Weisburg, E. Stackebrant, K. O. Stetter, and C. R. Woese. 1985. A phylogenetic grouping of the bacteroides, cytophagas, and certain flavobacteria. Syst. Appl. Microbiol. 6: 34-42.

48. Pettipher, G. L., and M. J. Latham. 1979. Characteristics of enzymes produced by Ruminococcus flavefaciens which degrade plant cell walls. J. Gen. Microbiol. 110: 21-27.

49. Phibbs, P. V., and R. G. Eagon. 1970. Transport and phosphorylation of glucose, fructose, and mannitol by Pseudomonas aeruginosa. Arch. Biochem. Biophys. 138: 470-482.

50. Reddy, C. A., and M. P. Bryant. 1977. Deoxyribonucleic acid composition of certain species of the genus Bacteroides. Can. J. Microbiol. 23: 1252-1256.

51. Rosen, B. P., and E. R. Kashket. 1978. Energetics of active transport, p. 559-620. In B. P. Rosen (ed.), Bacterial transport. Marcel Dekker, Inc., New York.

52. Ryu, D. D., and M. Mandels. 1980. Cellulases: biosynthesis and applications. Enzyme Microb. Technol. 2: 91-102.

53. Scheifinger, C. C., and M. J. Wolin. 1973. Propionate formation from cellulose and soluble sugars by combined cultures of Bacteroides succinogenes and Selenomonas ruminantium. Appl. Microbiol. 26: 789-795.

54. Schellhorn, H. E., and C. W. Forsberg. 1984. Multiplicity of extracellular β-(1,4)-endoglucanases of Bacteroides succinogenes S85. Can. J. Microbiol. 30: 930-937.

55. Sigma Chemical Co. 1983. The enzymatic determination of glucose in whole blood, plasma, or serum at 425-475 nm. Sigma technical bulletin No. 510. Sigma Chemical Co., St. Louis.

56. Silhavy, T. S., T. Ferenci, and W. Boos. 1978. Sugar transport systems in Escherichia coli, p. 127-169, In, B. P. Rosen (ed.), Bacterial transport. Marcel Dekker, Inc., New York.

57. Singh, A. P., and P. D. Bragg. 1974. Effect of dicyclohexylcarbodiimide on growth and membrane-mediated processes in wild type and heptose-deficient mutants of Escherichia coli K-12. J. Bacteriol. 119: 129-137.

58. Stanton, T. B., and E. Canala-Parola. 1980. Treponema bryantii sp. nov., a rumen spirochete that interacts with cellulolytic bacteria. Arch. Microbiol. 127: 145-156.

59. Stevenson, R. M. W. 1979. Amino acid uptake systems in Bacteroides ruminocola. Can. J. Microbiol. 25: 1161-1168.

60. Stewart, C. S., C. Paniagua, D. Dinsdale, K.-J. Cheng, and S. H. Garrow. 1981. Selective isolation and characterictics of Bacteroides succinogenes from the rumen of a cow. Appl. Environ. Microbiol. 41: 504-510.

61. Varel, V. H., S. J. Fryda, and I. M. Robinson. 1984. Cellulolytic bacteria from pig large intestine. Appl. Environ. Microbiol. 47: 219-221.

62. Wegner, G. H., and E. M. Foster. 1963. Incorporation of isobutyrate and valarate into cellular plasmalogen by Bacteroides succinogenes. J. Bacteriol. 85: 53-61.

63. Wetzstein, H.-G., and G. Gottschalk. 1985. A sodium-stimulated membrane-bound fumarate reductase system in Bacteroides amylophilus. Arch. Microbiol. 143: 157-162.

64. Wolin, M. J. 1982. Hydrogen transfer in microbial communities, p. 323-356, In A. T. Bull, and J. H. Slater (ed.), Microbial interactions and communities, vol 1. Academic Press Inc., New York.

ACKNOWLEDGMENTS

I wish to extend my sincere thanks to my advisor, Dr. T. L. Glass, for all of his support and encouragement during my work on this project. Special thanks to Mrs. B. Baldwin and Dr. C. Paulson, both of whom sparked my interest in Bacteriology. Thanks also to the faculty and graduate students of the Bacteriology and Veterinary Science departments for their support. Finally, I would like to thank my wife, Carrie, for her constant love and support throughout the course of my studies.