Function of Limited Sorbitol Oxidation in *Gluconobacter oxydans*

by

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(ABSTRACT)

Bacteria of the genus *Gluconobacter* have very active, membrane-bound, NAD(P)-independent, polyol dehydrogenases which stoichiometrically produce the single-step oxidation product of polyols provided in the growth medium. These bacteria have a high respiratory quotient which is believed to result from oxidations by these dehydrogenases. These organisms grow and survive at pH values as low as 2.5 leading to speculation that their membrane-bound dehydrogenase activity provide the rapid electron flow necessary to purge cells of toxic levels of hydrogen ions. These dehydrogenases are also believed to be used for energy metabolism, and there is no clear understanding of their function in the cell metabolism. Oxidation of sorbitol in *Gluconobacter oxydans* ATTC 621 was studied to determine if the oxidations by the membrane bound sorbitol dehydrogenase (mSDH) were required for growth, and whether they functioned to protect the cells in low pH environments. *G. oxydans* required a high concentration of sorbitol in the medium, and a reduction in the concentration to 0.1% decreased the rate and

extent of growth. Using mutants with decreased levels of mSDH, we found that growth rates decreased as a result of this mutation, indicating that mSDH activity was needed for growth. No changes in the specific activity of mSDH in strain ATCC 621 occurred when the cells were grown at pH 7.0, 6.0, and 4.5. However, cytochrome levels were doubled in cells grown at pH 4.5 compared to pH 6.0 and 7.0. The increased cytochrome levels did not increase the oxygen uptake of the pH 4.5 grown cells on sorbitol. Cells grown at all pH values respired more rapidly when tested at pH 4.5, and respiration decreased as the pH increased. The higher activity at lower pH values may result from increased efficiency of mSDH, which has an in vitro pH optimum of 5.2. Magnesium and calcium increased the respiration of pH 6.0 grown cells but not pH 4.5 grown cells. Less cell mass per mg of sorbitol oxidized was obtained when cells were grown at pH 4.5 compared to pH 6.0 and 7.0. However, no differences were detected in the specific activity of any of the sorbitol oxidizing enzymes. The activity of mSDH in G. oxydans is necessary for the growth of this bacterium. The mSDH specific activity is not regulated by the growth pH, but increased levels of cytochromes and decreased cell yields indicate a change in the cell's oxidative system resulting from lowered growth pH values.

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PART I

INTRODUCTION

The genus Gluconobacter is composed of gram-negative rods which can be motile by means of polar flagella (29). These bacteria are found in environmental niches which contain high concentrations of sugars, polyols, or alcohol (3,16,19,53,74,89). The genus Gluconobacter is composed of strictly respiratory organisms, and the only known electron acceptor for respiration is oxygen (29). They also have extremely high respiratory quotients (20,93). Gluconobacters are of industrial importance, because they are able to stoichiometrically produce single-step oxidation products from polyols provided in the growth medium (9). They oxidize over 80 different polyols, and a number of the oxidation products are used by industry (75). These bacteria are also known for their ability to grow and survive in environments as acidic as pH 2.5 (29,71,72).

The gluconobacters have distinct metabolic characteristics. Despite their having a strictly respiratory metabolism, they do not have a complete tricarboxylic acid (TCA) cycle (42). They lack one of the enzymes of the TCA cycle (succinate dehydrogenase), and they use the rest of the enzymes only for biosynthesis (42).

Studies by Cheldelin (18) show that Gluconobacter species use the hexose monophosphate (HMP) pathway exclusively during the catabolism of sugars. Although Kersters and De Ley (47) demonstrated the presence of the key enzymes of the Entner-Doudoroff (ED) pathway, no one has shown that this pathway functions in Gluconobacter. Since these bacteria do not have a complete TCA cycle, it is advantageous for them to use the HMP instead of the ED pathway. The ED pathway nets 1 molecule of ATP, 4 of NADH, and 2 of acetyl-CoA, while the HMP pathway nets 1 molecule of ATP, 8 of NADH, and 1 of acetyl-CoA. Lacking a complete TCA cycle, Gluconobacter strains cannot further oxidize acetyl-CoA and cannot derive the reducing equivalents normally provided by this pathway. Thus, they obtain more reducing power by using the HMP pathway.

The gluconobacters are also known to have particulate dehydrogenases which are believed to carry out rapid single-step oxidations. Recently, investigators isolated and purified a number of these dehydrogenases (1,2,6,7,21,83,84). They find that these dehydrogenases are integral-membrane proteins which nearly always have a closely associated cytochrome. Their studies show these proteins to be NAD(P)-independent dehydrogenases. Initial reports indicated that these were flavoproteins (1). However, after the

discovery of a new class of proteins ("quinoproteins") containing pyrrolo-quinoline quinone (PQQ) (35,79), data were published which indicated that some of the earlier data were missinterpreted. Many of the gluconobacters dehydrogenases actually contain PQQ as their cofactor (5). Gluconobacter oxydans presumably have comparatively high numbers of these PQQ containing proteins, since cells of this bacterium are extracted to obtain quantities of PQQ for reconstitution experiments with apoenzymes from other bacteria (33).

Although the presence of these membrane-bound NAD(P)-independent dehydrogenases in the gluconobacters has been known for many years, and some of these enzymes have recently been purified and characterized, their function in the gluconobacters is not understood. It is assumed that these oxidative enzymes provide energy for the gluconobacters, because they are linked to cytochromes (48,49). Therefore, they could provide electrons to the electron transport chain for the formation of a proton gradient. This gradient could then be used to synthesize ATP (43). However, Klungsoyr, et al (52) have shown that the P/O ratio in gluconobacters is very low (0.09 to 0.4). This indicates that these bacteria are respiring considerably more than should be necessary for ATP synthesis. These low P/O ratios prompted some investigators to suggest that the energy de-

rived from the limited oxidation of polyols by these dehydrogenases is not linked to phosphate metabolism in the genus Gluconobacter (12,50).

A number of recent reports indicate that similar NAD(P)-independent dehydrogenases occur in a wide variety of bacteria: Acinetobacter (33,34), Serratia (82), Pseudomonas, (27,54,59), Escherichia (39,41,73), and Acetobacter (4,66). The role of these dehydrogenases in metabolism of these bacteria is also unclear, but these enzymes appear to be linked to electron transport systems which are involved in cellular energy production. These enzymes also catalyze single-step oxidations (apparently at the outer surface of the plasma membrane) with the oxidation product remaining outside the cell (27). Studies in Pseudomonas spp. and in Klebsiella aerogenes show that these membrane bound dehydrogenases are regulated in conjunction with transport proteins (27,69). For example, when the carbon source is present in high concentrations, the membrane-bound dehydrogenases are synthesized, but when the carbon source concentration in the medium is low, the cells synthesize more transport proteins rather than membrane-bound dehydrogenases, and the carbon source is then metabolized internally. To date, there are no published studies dealing with regulation of membrane-bound dehydrogenases and transport proteins in the gluconobacters.

Reports demonstrating that both synthesis and activity of these dehydrogenases increased when the cells entered the maximum stationary growth phase (20,93) led to questions concerning how the dehydrogenases functioned to provide energy for the gluconobacters. If these enzymes provide energy for growth, why did their activity increase when growth stopped?

An alternative hypothesis for the function of these dehydrogenases was suggested by Heefner (44). He discovered that G. oxydans cells quickly die in pH 3.2 buffer in the absence of an oxidizable polyol, but they survive if a polyol is present. He showed that cell death occurs after a rapid influx of hydrogen ions via a Na^+/H^+ antiport system. This influx is accompanied by a denaturing of the intracellular proteins and cell death. Heefner hypothesized that membrane-bound dehydrogenases oxidize the polyol and contribute electrons to the electron-transport chain, which then provides the energy to pump protons from the inside to the outside of the cell and prevents cell death. In other words, the membrane-bound dehydrogenases of the gluconobacters were at least partly responsible for their acid-tolerance.

The present study was based on the hypothesis that membrane-bound, NAD(P)-independent dehydrogenases in G. oxydans

might function for both energy metabolism and acid-tolerance of this bacterium. Previous investigations of membrane-bound dehydrogenases in the genus Gluconobacter have involved identification and isolation of the protein (1,6). With one exception (93), the effects of altering growth conditions on the specific activity of these enzymes have not been examined. Instead, the effects of changing growth conditions were studied by measuring growth rates and cell yields, and specific enzyme activities were not determined (88).

The present investigation was designed to overcome these deficiencies. Membrane-bound sorbitol dehydrogenase (mSDH) was chosen for this study, since it does not make an acid product. Therefore, pH levels in the medium could be more stringently controlled.

Specifically in this study the objectives were: i) to isolate mutants deficient in membrane-bound sorbitol dehydrogenase (mSDH) activity, ii) to utilize these mutants to determine if mSDH activity in G. oxydans is necessary for the growth of this bacterium, iii) to determine if mSDH activity is regulated and what conditions cause this regulation, iv) to determine if mSDH activity is used by these bacteria to protect them from acidic environments.

PART II

MATERIAL AND METHODS

ORGANISM, MAINTENANCE, AND CULTURE CONDITIONS.

Gluconobacter oxydans ATCC 621 was the organism predominately used in this study. For long-term storage, cultures were mixed with (w/v) 1% yeast extract, 1% peptone and 15% glycerol, sealed in glass vials, and placed in liquid nitrogen. For short term storage, cells were kept at -10 C in sorbitol stocks (93). Mutants isolated from strain ATCC 621 were also stored in liquid nitrogen and in sorbitol stock vials. In addition, they were maintained in the refrigerator on either 5% sorbitol or 5% sorbose agar slants.

Cells were grown in either 500-ml or 2000-ml Bellco Nephelometer flasks containing 10% (v/v) of medium. Physical parameters for growth were described by White (92). Growth was routinely followed by turbidimetric readings at 620 nm with a Bausch and Lomb Spectronic 20 spectrophotometer.

When a large cell mass was required, cells were grown in a 5 liter New Brunswick Microferm Fermentor Model MF-105. Cells were subcultured on the same medium and 500 ml of subculture was inoculated directly into the fermentor. The me-

dium temperature was maintained at 28 C, agitation was 320 rpm and aeration was 6 liter of air per minute. Optical density was followed by removing 10 ml samples from the inoculation port and reading in a spectrophotometer as described above.

MEDIA.

All media used in this study were made from a basal medium consisting of 1% yeast extract and 1% peptone. The unbuffered basal medium contained no other ingredients, but the pH was adjusted to 6.0 with HCl prior to autoclaving. Buffered basal media contained either 1.35% (w/v) cis, cis, cis, cis-1, 2, 3, 4-cyclopentanetetracarboxylic acid (cyclobuffer) or both 1.35% cyclobuffer and 1% (w/v) of 2-(N-morpholino) propanesulfonic acid (MOPS). The pH of this double buffered medium was adjusted with KOH. The MOPS buffer was not used in this study until it became necessary to maintain growth above pH 6.0. The addition of MOPS to the growth medium did not appear to change the growth characteristics, so it was routinely added to all buffered media thereafter. One drop of AF-90 antifoam (General Electric) was added to each flask, and 1 ml of antifoam was added to

the fermentor. Polyols were added to buffered or unbuffered basal media at a 5% (w/v) concentration unless specifically stated otherwise. Sorbitol was generally added before autoclaving, but other polyols were sterilized by filtration and added after autoclaving. Solid media were prepared by adding agar at a concentration of 1.5%. When preparing solid media having a pH of 4.5 or 3.5, double strength concentrations of the medium components and the agar were made up separately, and equal volumes were mixed after autoclaving. This was necessary to avoid acid hydrolysis of the agar during autoclaving.

MUTAGENESIS

These procedures were developed by combining information from several sources (15,61,62,97). Cells were grown on unbuffered sorbitol medium to an optical density of 0.2 at 620 nm. Cultures were harvested, washed once, and resuspended in either 0.05 M citrate at pH 6.0 or 0.05 M citrate plus 0.1% yeast extract and peptone. Cell concentrations in these "citrate" suspensions were determined using viable numbers obtained by plating onto 5% sorbitol medium plates. Viable numbers were approximately 10^9 cells/ml. Ethyl meth-

ane sulfonate (EMS) was prepared in 0.05 M citrate to a concentration of 0.4 M. Equal parts of cell suspension and EMS were mixed and placed on a roller drum apparatus (New Brunswick Scientific Co.) in a fume hood for 2 h. Following this mutagen treatment, cultures were diluted 10 fold into either 5% sorbitol or 5% sorbose unbuffered medium in 500-ml growth flasks. Viable numbers were determined again at this point. Survival rate was 70-100%. Cultures were incubated until growth was evident. Cultures were washed, and all were inoculated into unbuffered 5% sorbitol medium. Growth was allowed to proceed for approximately two generations. Samples were removed at this point for direct selection (procedures explained in the Results Section). The remainder of the cultures were treated with 200 micrograms/ml of cycloserine to kill the cells growing on sorbitol. Following lysis, cells were removed by centrifugation, washed twice, and a portion plated onto 5% sorbose plates. The remainder were put through a second counter selection identical to the first. Additional details about the counter selection procedures are given in the Results section.

CELL FRACTIONATION PROCEDURES

All fractionation procedures were carried out at 4 C. Cells were removed from cultures by centrifugation and washed three times. The buffer used for washing varied with how the cells were used. Specific buffers are listed elsewhere in the Materials and Methods, as a part of the Figure descriptions, or in the footnotes to the Tables. Washed cells were stored at -10 C as a frozen pellet until needed. Cells were thawed, resuspended in buffer, and disrupted using a French pressure cell as described by White (92). The resulting suspension was centrifuged for 10 min at 12,100 x g in a Sorvall refrigerated centrifuge (Model RC-2B). The supernate fraction from this centrifugation was called the crude cell extract. When crude cell extracts were centrifuged for 5 h at 120,000 x g in a Beckman Model L3- or L5-50 ultracentrifuge, the supernatant fluid was called the soluble fraction. If the cells had been grown in an antifoam-containing medium, a 1 mm milky layer formed on top of the soluble fraction. This milky layer was removed by aspiration and discarded. The pellet from this centrifugation was washed by resuspending in buffer using a Teflon tissue homogenizer and recentrifuging. This washed pellet was the particulate fraction. It was suspended as before and stored frozen at -12 C.

ENZYME ASSAYS

All enzyme assays were carried out using a Gilford spectrophotometer Model 250 equipped with a Gilford Thermo-set temperature controller and a thermostated cuvette holder.

Membrane-bound sorbitol dehydrogenase and NAD-linked soluble sorbitol dehydrogenase were assayed using the procedures of White and Claus (93). NADP-linked soluble sorbitol dehydrogenase was assayed using the same procedure as for the NAD-linked enzyme but with NADP substituted for NAD.

NADH-oxidase activity was measured by following the decrease in absorbance at 340 nm. The assay mixture contained per ml: 0.1 micromoles of $NADH+H^+$, 50 micromoles of pH 8.0 tris-succinate buffer, and 0.1 to 0.4 mg of protein. Protein used was the soluble fraction which was in a 0.01 M phosphate buffer at pH 7.0. When 2-heptyl-4-hydroxy quinolin-N-oxide (HQNO) was added, it was used at a final concentration of 0.05 micromoles per ml.

PROTEIN DETERMINATIONS

All samples used for protein analysis were first digested by boiling for 20 min with 0.1 M of KOH. Protein concentrations were then determined using the method of Lowry et al. (56). Bovine serum albumin, fraction V, was used as a standard.

TOTAL CELL NUMBERS

Total cell numbers were determined using a Petroff-Hauser counting chamber. The procedure used was that of White (92), except that cells were resuspended in a 0.01 M phosphate buffer and cell clumps were broken apart by two 30 second bursts of sonication. A Fisher Model 300 Sonic Dismembrator equipped with a microtip was used at an output of 35%.

DRY-WEIGHT DETERMINATIONS

Dry weights were measured using the procedures of White (92), except that the first weight determination was made after 48 h of drying (rather than 24 h). It was found that, by waiting for the additional 24 h, only two determinations were required to obtain accurate results. Four samples, two 1 ml aliquots and two 2 ml aliquots, were dried and weighed for each determination. Results reported are an average of these four samples.

Dry-weight measurements were attempted on cells washed and resuspended in buffered basal medium. The medium was very hygroscopic, and, even with cooling in a desiccator, the medium absorbed water during weighing. This was especially true during the summer months. Water absorbed was as much as 50% of the weight of the cells. This rapid uptake of water resulted in large standard deviations between replicates, and this method was discarded. Therefore, when experimental procedures required cells washed in basal medium, samples were taken from the cultures for dry weights at the same time. Both the experimental samples and the dry weight samples were subjected to similar washing and concentration procedures, except that distilled water was used for the dry-weight samples.

ELECTROPHORESIS

Electrophoresis was done on the octyl-glucoside- soluble portion of the particulate fraction (11). Samples were suspended at 7 parts protein to 3 parts sample buffer. Sample buffer was composed of 0.25 M Tris-HCl containing (w/v) 30% sucrose, 0.01% bromophenol blue, and 2% octyl glucoside. Electrophoresis buffers and other reagents were similar to those of Davis (26). Vertical slab polyacrylamide gels were made at a 7.5% concentration with 4% crosslinking. Stacking gels were 5% acrylamide. Both stacking and running gels also contained 1% octyl glucoside. Stacking gels were run at 10 mA per gel and running gels at 30 mA per gel. All gels were run at room temperature, but cold tap water was circulated in the inner chamber of the apparatus.

The protein stain was 0.1% (w/v) Serva blue in a solution of (v/v) 10% acetic acid, 25% isopropyl alcohol and 65% distilled water. Gels were stained for 30 min with gentle agitation. Destaining was done with the same solution minus the Serva blue. Gels were stored in 7% acetic acid.

Activity stains were attempted as described in the Results section. The stain contained (per 100 ml): 40 mg tetranitro blue tetrazolium, 14 mg phenazine methosulfate, 18 gm of sorbitol, 1 mg N-ethylmaleimide, and 5 mg potassium

cyanide in 0.1 M phosphate buffer at pH 5.0. The stain was made and degassed immediately prior to use. The stain was protected from light at all times. Gels were added to the stain, and the air above the stain was flushed out with nitrogen. Staining was done in the dark at 38 C. This procedure gave the darkest stain on the gel, but the stain lacked specificity for the enzyme extracted from strain ATCC 621 (see Appendix A).

CYTOCHROME ANALYSIS

Cytochrome analyses were done on octyl glucoside-soluble fractions prepared from either whole cells or particulate fractions. Whole cells were first mixed with octyl glucoside in 0.2 M phosphate buffer at pH 7.5. They were then passed twice through a French pressure cell at 18,000 to 22,000 psi. The broken-cell suspensions were stirred for three hours, then centrifuged for 90 min at 120,000 x g. The supernatant fluid was analyzed for cytochromes. Although some slight pink color remained in the pellets, re-extraction did not yield detectable levels of cytochromes. Particulate fractions were solubilized in the same manner as the whole cells, except they were not run through the French pressure cell.

Cytochromes were scanned in a double-beam Bausch and Lomb Spectronic 2000 spectrophotometer. Scans were made between 700 and 400 nm at room temperature. Samples were reduced with a few grains of sodium dithionite or with sorbitol in the presence of 0.2 M phosphate at pH 7.5. The dithionite used was newly purchased, placed in small vials, and stored frozen. A new vial was used each day that cytochromes were analyzed. Using fresh dithionite and having the sample in at least 0.2 M phosphate buffer helped to keep dithionite from forming a precipitate in the sample cuvette (Peter Jurtshuk, personal communication).

RESPIROMETER STUDIES

A Gilson Model G14 differential respirometer was used for all oxygen uptake experiments. The procedures used were those of White and Claus (93) except for experiments designed to simulate growth conditions. In these experiments, the following changes were made: i) succinate buffer in the reaction vessel was replaced with buffered basal medium, ii) cells were washed in buffered basal medium, iii) the final sorbitol concentration in the reaction chamber was 5% (w/v), and iv) temperature of the reaction was 28 C. Other changes made in procedures are described in the Results section.

SORBITOL DETERMINATIONS

Sorbitol concentrations remaining in the growth medium were determined using the methods developed by Feshami (36). Absorbance values measured in the standard curves were lower than those reported by Feshami. Carboxylic acids are known to interfere with the formation of phosphomolybdenum complexes (17). Since cyclobuffer is a tetracarboxylic acid, it was suspected that cyclobuffer was causing the interference detected. It was assumed that cyclobuffer was not precipitated in the deproteinization procedure. Therefore, standard curves were done in the double-buffered basal medium. Following deproteinization, all samples, both the unknowns and the standard curves, were diluted the same amount so they would all have the same amount of cyclobuffer in them. It was assumed that the concentration of cyclobuffer in the medium did not change during growth.

SORBOSE DETERMINATIONS

Sorbose was determined directly in the growth medium using an end point titration with Fehling's Solution. Increasing amounts of Fehling's solution were added to one ml samples of culture. Tubes were boiled for 10 min and centrifuged for one minute at 3400 x g. The end point was reached in the tube where supernatant from this centrifugation was light yellow. Above the endpoint, supernatant fluids were blue. Below the end point, they became increasingly darker yellow and eventually brown. Fehling's solution was made and used as described in Dawson et al. (28). A standard curve was made using known concentrations of sorbose.

CHEMICALS

All chemicals used in this study were reagent grade.

1-O-n-Octyl-β-D-glucopyranoside, phenazine methosulfate, NAD, NADP, NADH2, D-sorbitol, L-sorbose, D-glucose, D-mannitol, sodium carbonate, bovine-serum albumin (Fraction V), calcium chloride, ammonium persulfate, 2-heptyl-4- hydroxyquinolin-N-oxide, 2-(N-morpholino)propanesulfonic acid,

barium hydroxide, ascorbic acid, zinc sulfate, ammonium molybdate, magnesium sulfate, tetranitro blue tetrazolium, N-ethylmaleimide, ethyl methane sulfonate, citrate, cycloserine, ampicillin, succinic acid, Trizma base, Trizma hydrochloride, N,N,N',N'- teramethylethylenediamine, and sodium dithionite were purchased from Sigma Chemical Co., St Louis, MO.

Peptone, yeast extract, and Bacto agar were purchased from Difco Laboratories, Detroit, MI.

Sodium-potassium tartarate, sodium hydroxide, hydrochloric acid, sulfuric acid, glacial acetic acid, di- and mono-basic potassium phosphate, glycine, Folin reagent, potassium ferricyanide, and glycerol were obtained from Fisher Scientific Co., Pittsburgh, PA.

Acrylamide, N,N'-methylene bisacrylamide and Serva blue were purchased from Serva Fine Biochemicals, Inc., Garden City Park, NY.

Potassium hydroxide, potassium cyanide, magnesium chloride, and cupric sulfate were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ.

Cis-cis-cis-cis,1,2,3,4-cyclopentane tetracarboxylic acid was purchased from Aldrich Chemical Co., Milwaukee, WI.

GE-AF 90 Antifoam was obtained from General Electric Co., Waterford, NY.

PART III

RESULTS

ISOLATION OF MUTANTS IN THE MEMBRANE-BOUND SORBITOL DEHYDROGENASE

Results of selection procedures following EMS mutagenesis are summarized here to allow evaluation of the selection methods. Three different procedures were used; one counter-selection technique and two direct-selection procedures.

Counter selection

Before a counter selection could be used, Gluconobacter oxydans, ATCC 621, had to be tested for sensitivity to the antibiotics used in counter-selection procedures. Although strain ATCC 621 was sensitive to both ampicillin and cycloserine, it was more sensitive to cycloserine at concentrations normally used (62) (Table 1). In addition, no resistant colonies were observed in the zone of inhibition around the cycloserine discs, but many colonies were observed in the zone of inhibition around the ampicillin discs. Therefore, cycloserine was used.

TABLE 1

Sensitivity of *G. oxydans* ATCC 621 to Ampicillin and Cycloserine

Concentration of Antibiotic	Size of Inhibition Zone[1] (mm)	
	Ampicillin	Cycloserine
Full strength[2]	<1mm	8mm
Diluted 1:2	0	6mm
" 1:4	0	3mm
" 1:8	0	0

[1] Zones of inhibition were measured from the edge of the antibiotic disc to the point where normal density growth resumed on the plate.

[2] Full strength for ampicillin was 20 ug/ml and for cycloserine it was 200 ug/ml.

Counter selection was based on the idea that cells lacking membrane-bound sorbitol dehydrogenase should not be able to grow on sorbitol but could grow on sorbose. Sorbose (the single step oxidation product of sorbitol) was slelcted as the permissive growth compound. Available information did not indicate whether the membrane-bound dehydrogenases of G. oxydans were specific for a particular polyol. The oxidation product of the sorbitol dehydrogenase seemed less likely to be further oxidized by the same enzyme than similar polyols such as glucose or mannitol. The mutagenized culture was incubated in a medium containing sorbitol and cycloserine. Since strain ATCC 621 produces sorbose while growing on sorbitol, there was concern that mutants able to grow on sorbose might also be selected against. Strain ATCC 621 was grown on sorbose (data not shown), and cells grew at a rate aproximately 0.4 times that of cells growing on sorbitol. If the sorbose concentration was decreased to 1% (w/v) or lower, growth was even slower. Since sorbose would be present during counter selection at low concentrations, it was felt that the cells growing on sorbitol would be lysed by the cycloserine long before cells growing on sorbose would be affected.

The mutagenized cultures were plated after both single and double counter selections as detailed in the Materials and Methods. Approximately 30,000 colonies were screened by replica plating from sorbose onto sorbitol medium. From these, 20 colonies were selected. Ten of these did not grow on sorbitol plates, and the rest formed very small colonies on the sorbitol compared to the sorbose plates. These 20 colonies were further tested by growing them in broths containing either sorbitol or sorbose. All 10 colonies selected as having smaller colonies on sorbitol and five of those selected for no growth on sorbitol grew as well as the wild-type and were discarded. The remaining five colonies either did not grow or showed decreased growth on sorbitol compared to wild type. These five were examined for the specific activity of their mSDH. Only two had reduced activities (621-C3 and 621-C15). Mutant strain 621-C3 had only 10% of wild-type mSDH activity. This strain grew very poorly requiring three days to reach an optical density of 0.1. Frequently during growth, this culture would begin growing normally, and, when tested, normal mSDH activity had been restored in these cultures. Mutant 621-C15 had 20% of the wild type mSDH activity and appeared to be stable under the growth conditions used.

Direct Selection

Two direct selection techniques were devised. The first detected sorbose produced in the medium as the direct result of the oxidation of sorbitol by the membrane-bound sorbitol dehydrogenase. It was hoped to detect decreased mSDH activity by finding colonies with little or no sorbose excreted. Fehling's solution was used to detect sorbose, since sorbose is a reducing sugar and forms a yellow precipitate with Fehling's solution. In order to detect sorbose, plates had to be incubated for an additional 24 h after observing full sized colonies. In addition, the Fehling's solution was warmed before flooding the plates, and the plates were left undisturbed for 10 min after flooding. Using this technique, approximately 6,000 colonies were screened following EMS mutagenesis. Six colonies were selected as having no detectable sorbose excretion on sorbitol plates. All six were screened for their mSDH activity, and all had reduced levels of SDH activity - from 62% to 86% of the wild type. However, the growth characteristics of these mutants on sorbitol medium were not noticably affected.

The second method of direct selection was based on Heefner's hypothesis that the rapid, single-step oxidation of polyol in G. oxydans might function to protect the cells

from a low pH environment (44). Approximately 6000 colonies were replica-plated from sorbitol medium at pH 6.0 onto sorbitol medium at pH 3.5 and pH 4.5 following mutagenesis, as described in the Materials and Methods. Fourteen colonies were selected for their inability to grow at pH 3.5. Four of these also showed some growth inhibition on the pH 4.5 plates. When further tested by streaking onto plates at pH 3.5 and testing for growth in liquid media at pH 3.5 and 4.5, only eight of the 14 maintained their sensitivity to growth at the pH 3.5. These eight were tested for their mSDH activity, and only three had reduced levels ($\leq$60 %) of wild-type activity. These appeared quite similar and only one was kept, strain 621-M3. The growth rate of 621-M3 was less than that of wild-type cells on sorbitol medium. In further growth tests, strain 621-M3 was sometimes able to grow at pH 3.5. It was assumed that reversion was occurring, but the frequency of this reversion was not measured.

SOLUBILIZATION AND ATTEMPTS TO ISOLATE MEMBRANE-BOUND SORBITOL DEHYDROGENASE

Solubilization from the membrane

In order to characterize the mutants isolated and to determine if the mutation occurred in the mSDH itself, it was an important goal to isolate the mSDH. As an initial step in the isolation of mSDH from G. oxydans, the enzyme was solubilized from the membrane.

Reports on other membrane-bound dehydrogenases indicated that Triton X-100 might be an effective detergent for solubilization of sorbitol dehydrogenase (2,34,73). Using the particulate fraction, Triton X-100 concentrations from 0.1 to 1.0% (v/v) were tried. If the detergent concentration was high enough to solubilize protein from the membrane, then sorbitol dehydrogenase was inactiviated. Addition of 0.1 M KCl to facilitate solubilization (45) and 1.0 M sorbitol to stabilize the enzyme (87) had no effect on the Triton solubilization.

Other detergents were screened for their ability to solubilize sorbitol dehydrogenase. Deoxycholate solubilized some active enzyme, but this detergent precipitated at the pH (5.2) needed for optimum activity in the ferricyanide as-

say. Tween 80, Brij 35, Brij 58 and Zwittergent 3-14 did not solubilize active protein. However, the nonionic detergent, n-octyl-β-D-glucoside (octyl glucoside), yielded active soluble sorbitol dehydrogenase.

Solubilization was done by stirring the particulate fraction with octyl glucoside at 4 C for 3 h and then centrifuging 90 min at 120,000 x g. Sorbitol dehydrogenase found in the supernatant fluid was considered solubilized. Table 2 shows the effect of varying the octyl glucoside concentration on the amount of sorbitol dehydrogenase solubilized. A small amount of the enzyme was solubilized by this procedure without the addition of detergent. This probably resulted from the fraction being stored frozen and then thawed, thus releasing some proteins. As increasing amounts of detergent were added, more total protein and more active sorbitol dehydrogenase was solubilized. At 1% octyl-glucoside, the amount of total protein released varied, but about 60% of the enzyme activity was always released. At 1.5% detergent, the greatest amount of protein and virtually all of the sorbitol dehydrogenase activity was solubilized. Increasing detergent concentration beyond 1.5% had no further effect on solubilization.

TABLE 2

Solubilization of Sorbitol Dehydrogenase from Strain ATCC 621 with Octyl-Glucoside

Detergent Concentration (% w/v)	Percent Solubilized[1]	
	Protein (mg/ml)	Sorbitol Dehydrogenase Activity
0	6-10	1
1.0	10-22	63
1.5	23	90-100
2.0	22	90-100

[1] The amount of protein and sorbitol dehydrogenase activity remaining in the supernatant following centrifugation was divided by the total in the detergent treated sample before centrifugation and multiplied by 100 to give the percent solubilized. Results reported are an average of three experiments.

To facilitate protein removal during the treatment with 1.5% octyl glucoside, 0.1 M KCl was added. This caused a 4% increase in solubilized total protein (to 27%), with a slight decrease in specific activity of sorbitol dehydrogenase (to 88%). With the KCl added, the solubilized dehydrogenase was unstable, losing 50% activity when stored on ice overnight. Adding 0.1 M sorbitol during the extraction procedure had no effect on the amount of protein or activity solubilized.

The octyl glucoside solubilized protein was very stable. When tested at 0, 1, 8, 14, and 19 days after solubilization, the percentage of specific activity remaining was 100, 100, 84, 72, and 52 respectively.

Attempts to Isolate Active mSDH by Electrophoresis

Once the dehydrogenase was solubilized from the membrane in an active form, efforts were given to isolation by electrophoresis with identification on the gel being by an activity stain similar to those known to work with other dehydrogenases. Although many procedures were tried, an activity stain that was effective for the sorbitol dehydrogenase of strain ATCC 621 could not be developed. Although a

single band was seen following activity staining, the same band stained with or without sorbitol in the activity stain. The procedures used in attempts to develop an activity stain are summarized in Appendix I for use by future investigators.

LIMITED OXIDATIONS AS A GROWTH REQUIREMENT

A great deal needs to be learned about the function of mSDH. It was important, however, to keep in mind that the limited oxidation of sorbitol in *G. oxydans* involves not only the mSDH but also the associated cytochromes. In considering the possible regulation of limited sorbitol oxidation in *G. oxydans*, a number of possible changes in the system might need to be considered (Figure 1). If the schematic dehydrogenase cytochrome system (shown in the box in Figure 1) represented the components necessary for a specific activity of one unit, then the activity could increase as a result of any of the five changes described in Figure 1.

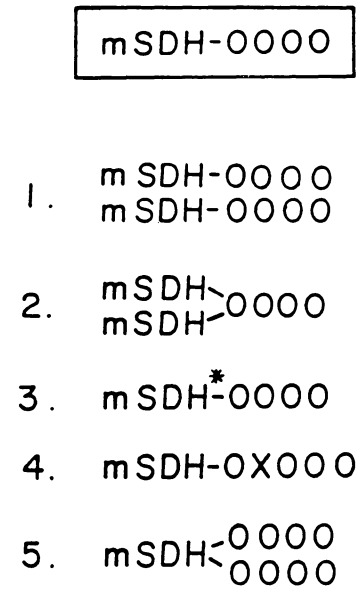

Figure 1. Hypothetical Ways to Increase the Specific Activity of mSDH in Strain ATCC 621.

"mSDH-OOOO" is a schematic representation of the membrane-bound sorbitol dehydrogenase and its associated cytochromes (O). The number of cytochromes depicted here is arbitrary and not intended to indicate a known quantity. Increases in activity are shown as: (1) a complete doubling of the system, (2) a doubling of only the mSDH, (3) an allosteric effect on the mSDH, (4) an altered cytochrome chain, and (5) a doubling of only the cytochromes.

Effect of Decreasing the Concentration of Sorbitol

Using Fehling's solution to measure the quantity of reducing sugar in the medium, it was found that ATCC 621 (grown on a 5% sorbitol medium at pH 6.0) produced 4.9% sorbose in the culture medium by early stationary phase. Since sorbose is the single-step oxidation product of sorbitol, this indicated that no more than 0.1% of the sorbitol was acting as a carbon source for growth. However, a reduction in the sorbitol concentration produced striking changes in growth (Figure 2). Reducing the sorbitol concentration to 1% decreased the extent (but not the rate) of growth. A reduction to 0.1% decreased both the extent and the rate of growth. If sorbitol was omitted from the medium, no growth occurred. Since all but 0.1% of the sorbitol in a 5% sorbitol medium is simply oxidized to sorbose and excreted, these results indicated that the limited sorbitol oxidation to sorbose served a vital function in the growth of these cells.

Effect of decreasing the mSDH activity

The rapid, single-step oxidation of sorbitol to sorbose in these cells is considered to be the result of action by mSDH (48). It was of interest to determine the rate of

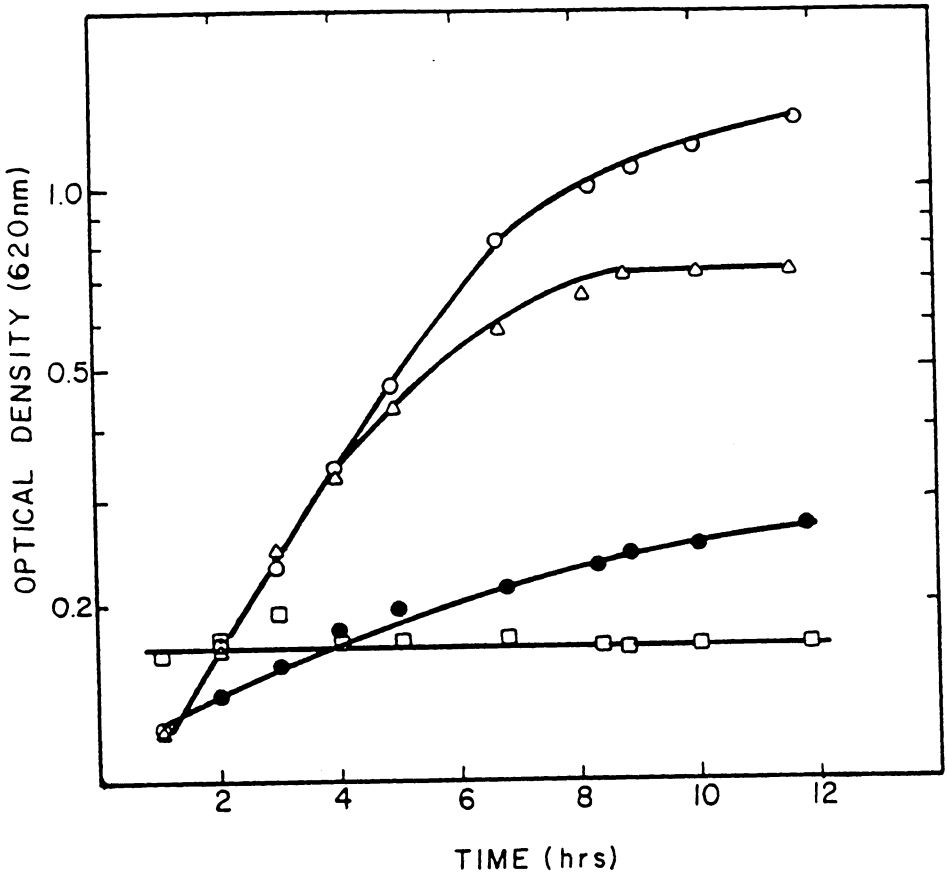

FIGURE 2. Growth of G. oxydans ATCC 621 on Buffered Sorbitol Medium with Varying Concentrations of Sorbitol.

Cells were subcultured on 5% buffered sorbitol medium to an optical density of 1.0 at 620 nm. They were harvested by centrifugation and washed three times with buffered basal medium before inoculation into the buffered media. Growth on (O) 5% sorbitol, (Δ) 1% sorbitol, (●) 0.1% sorbitol, and (□) no sorbitol added. Results presented here are representative of three separate experiments

growth of the mutant 621-C15, since this mutant had only 20% of the wild-type mSDH activity. The mutant had a slower rate of growth than the wild type (Figure 3). Mutant 621-C3, which had only 10% of the wild type mSDH activity, grew so poorly that generation time was measured in days, and cell concentration (as measured by optical density) did not exceed 0.1 (data not shown). These results are consistant with the single-step oxidation of sorbitol being necessary for the growth of this bacterium.

Although the above data show that the limited oxidations are necessary for growth, it does not define the function(s) of the limited oxidations. Since they are cytochrome-linked oxidations (8,31), they seemed likely to function to produce energy for the cells via the production of a proton gradient. This gradient could then be used for ATP synthesis. However, Klungsoyr and others (40,52) found that the P/O ratio in G. oxydans is very low (0.09 to 0.4). This suggests that either G. oxydans uses energy very inefficiently, or that these oxidations have a different or additional function.

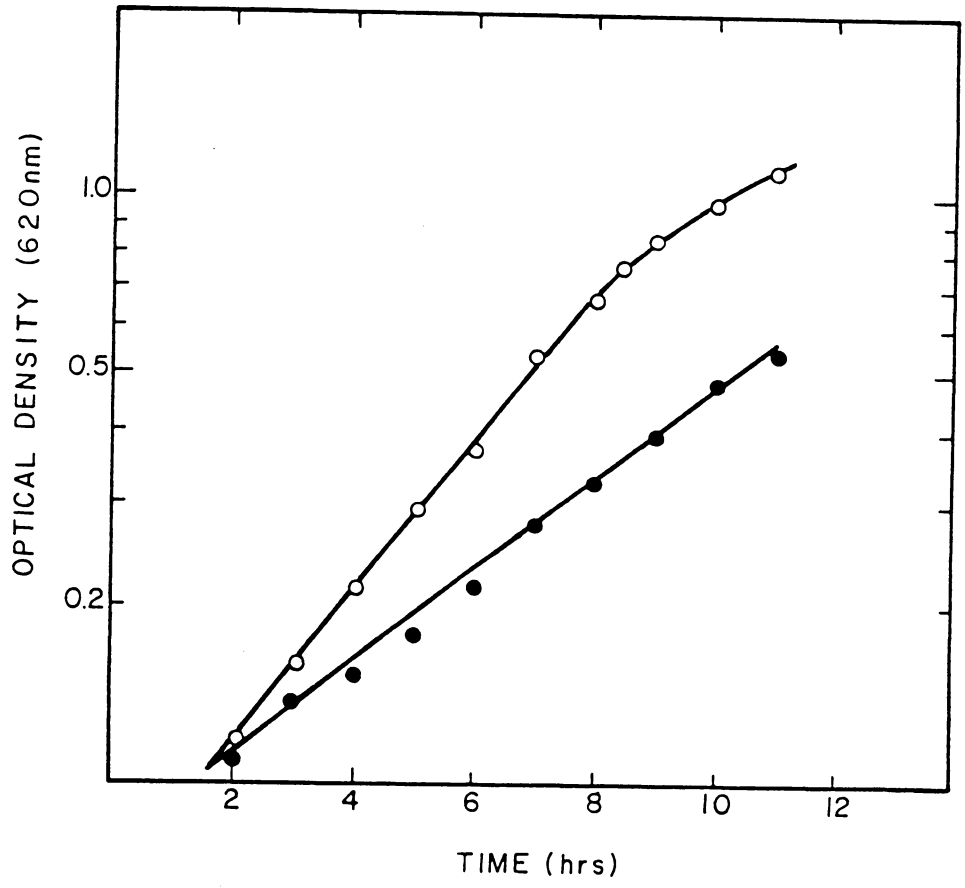

FIGURE 3. Growth of Wild-Type Strain ATCC 621 and Mutant Strain 621-C15 on Buffered Sorbitol Medium.

Cells were subcultured on 5% buffered sorbitol medium (pH 6.0) at an optical density of 1.0 at 620 nm. They were harvested by centrifugation and washed three times with buffered basal medium before being inoculated into the buffered medium. Growth of ATCC 621 (O), and 621-C15 (●). Results presented here are representative of five separate experiments.

REGULATION OF MEMBRANE-BOUND SORBITOL DEHYDROGENASE

Information on the function of a protein often comes from understanding how or when cells regulate the protein's activity. Possible regulatory effects of the growth phase of the culture, the energy source in the medium, and the pH of the growth medium on mSDH actvity were examined.

The effect of the phase of growth

White and Claus (93) found that G. oxydans increases its sorbitol oxidative capacity when the cells enter stationary growth phase. They determined that this increase resulted from an increase in the specific activity of the membrane-bound sorbitol dehydrogenase. The present study used a different medium. Therefore, this regulatory pattern was studied to see if it was the same under the present experimental conditions. Results showed that the specific activity of the mSDH increased in the maximum stationary phase and was 2.86 times higher than when cells were growing exponentially (Table 3).

TABLE 3

Effect of Growth Stage on the Specific Activity of Membrane-Bound Sorbitol Dehydrogenase

Stage of Growth	Specific Activity of Sorbitol Dehydrogenase[1]
Exponential[2]	1.49
Stationary[3]	4.26

[1] Specific activity was measured as micromoles of sorbose produced per minute per mg of protein.

[2] Exponential cells were harvested at 0.3 O.D. at 620nm.

[3] Stationary phase cells were harvested one hour into the stationary phase of growth at an optical density of of approximately 1.2 at 620nm.

The effect of alternate energy sources

When cells were grown on sorbose instead of sorbitol as the energy source, the specific activity of the mSDH in a crude extract was almost double that found in sorbitol grown cells (Table 4). However, when the crude extracts were separated into particulate and soluble fractions, the difference between the sorbose and sorbitol grown cells was no longer detected (Table 4). If the level of activity observed in the crude extract of sorbose grown cells represented normal mSDH activity, then particulate fractions should have a much higher specific activity. Such an increase was observed when crude fraction of sorbitol grown cells was separated into particulate and soluble fractions (Table 4).

G. oxydans cells contain soluble, NAD(P)-linked sorbitol dehydrogenases which are believed to be involved in catabolism of sorbitol as a carbon source (22,55). However, these enzymes are not active at pH 5.2 - the pH used for the mSDH assay. If a new soluble sorbitol dehydrogenase having activity at pH 5.2 was present in the sorbose grown cells, then its activity should have remained in the soluble fraction. Table 4 shows that there was essentially no pH 5.2 active protein in the soluble fraction. It is possible that

TABLE 4

Comparison of mSDH Activity in Strain ATCC 621 Grown in a Medium Containing either Sorbitol or Sorbose[1]

Cell Fraction[2]	Specific Activity of Sorbitol Dehydrogenase	
	Growth in Sorbitol	Growth in Sorbose
Crude Extract	2.8	4.7
Particulate	5.8	6.1
Soluble	0.06	0.09

[1] Cells were harvested in stationary phase of growth. Results reported are the average of three experiments.

[2] Cell fractionation procedures are described in the Materials and Methods Section.

the higher sorbitol dehydrogenase activity observed in the crude extract of sorbose grown cells is the result of a protein which is dependant upon a membrane-linked cofactor. This cofactor would be lost when the particulate fraction was removed.

Two other alternate energy sources were selected to test their effects on mSDH activity in G. oxydans. Glycerol was selected since it is a triose, and its metabolism differs signifigantly from sorbitol (9). Glucose was selected because of evidence in other bacteria that glucose causes catabolite repression.

Growth on glycerol did not cause a signifigant change in the specific activity of mSDH (Table 5). Growth on glucose, however, caused a two-fold increase in mSDH activtiy. This result is the reverse of the effect of glucose as a catabolite repressor. However, if the mSDH enzyme of G. oxydans also oxidized glucose, then the increase in specific activity could be an induction by the substrate, glucose.

Preliminary investigations into the growth of strain 621-C15 on other polyols indicated that the reduction in the growth rate of this mutant also occurred on glucose and mannitol (Figure 4) and on glycerol and fructose (data not

TABLE 5

Comparison of Specific Activity of mSDH in ATCC 621 Grown on Glucose, Glycerol, and Sorbitol

Polyol in the Medium	pH of Culture at Harvest[1]	Specific Activity of Sorbitol Dehydrogenase[2]
Sorbitol	5.9	1.89
Glycerol	5.7	1.50
Glucose	4.1	4.19

[1] Cells were harvested in late log phase of growth.

[2] Specific activities are representative of two experiments and are measured in particulate fractions as described in the Materials and Methods.

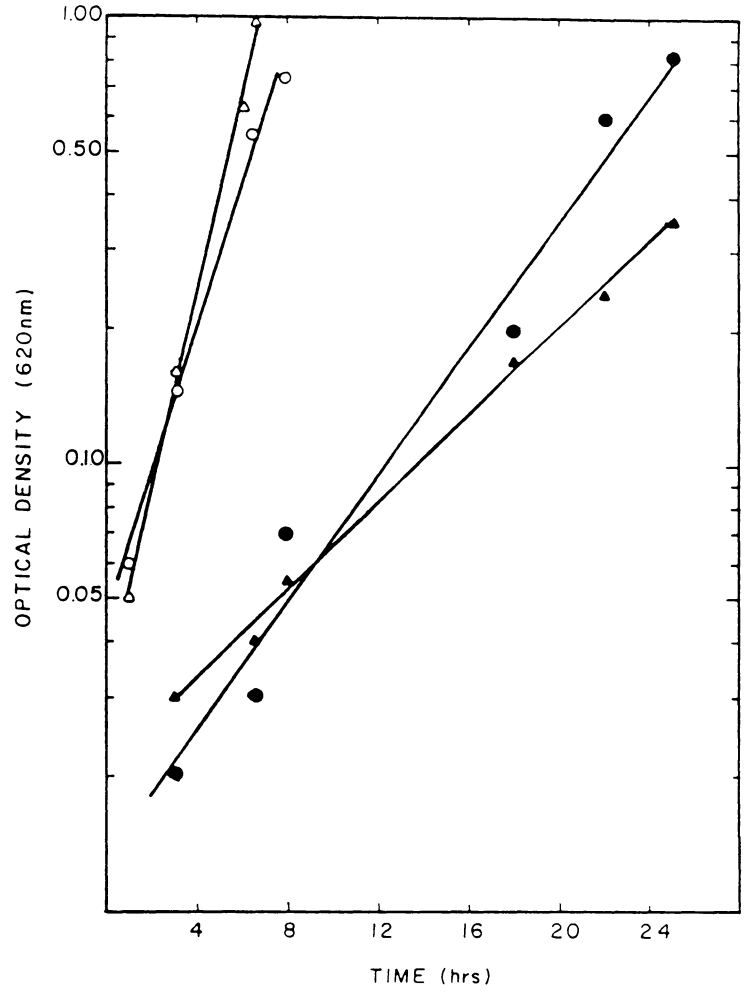

FIGURE 4. Comparison of Growth of Strain ATCC 621 and Strain 621-C15 in Buffered Medium Containing Mannitol or Glucose.

Cells were subcultured on 5% buffered sorbose medium to an optical density of 1.0 at 620 nm. They were harvested by centrifugation and washed three times with buffered basal medium before being inoculated into each growth medium. Growth of ATCC 621 on mannitol (△), ATCC 621 on glucose (○), 621-C15 on mannitol (▲), 621-C15 on glucose (●). Results are from one experiment and are considered preliminary.

shown). Although preliminary, these results indicate the possiblity that the oxidation of many different polyols in strain ATCC 621 is the result of action by one enzyme. It is also possible that all polyol oxidation enzymes have a unified control mechanism and are coordinately regulated.

It was also observed, however, that the pH of the cultures grown on glucose was much lower than either the sorbitol or glycerol grown cultures (Table 5), despite the medium being strongly buffered at pH 6.0. This is probably the result of the excretion of gluconic acid by the glucose grown cells (9). Heefner (44) showed that the oxidation of a carbon compound is necessary for the viability of G. oxydans under low pH conditions. Therefore, it was possible that the increased mSDH activity in glucose grown cells resulted from a response to the pH drop rather than the presence of the glucose.

Effect of growth pH

The optimum growth pH for G. oxydans is reported to be pH 6.0 (29). Therefore, values above and below pH 6.0 were tested for their effect on the specific activity of mSDH. A low pH of 4.5 was selected because lower values altered the

yeast extract concentration of the medium. Below pH 4.5 the yeast extract began to precipitate from the medium. A high pH of 7.0 was selected because higher values caused growth inhibition. The specific activity of the mSDH was the same for cells grown at pH 6.0 and 7.0 (Table 6). Growth at pH 4.5 resulted in a decrease in the specific activity of mSDH. As shown in Table 6, there was a corresponding decrease in total activity of mSDH at pH 4.5. Because this low pH had an effect opposite that expected, further experiments were conducted to verify this result.

Another method to measure specific activity is to measure the activity per cell. The results for this type of analysis (Table 7) show the same pattern of activity as seen in the total activity measurements in Table 6.

Effect of decreased mSDH activity on growth at pH 4.5, 6.0 and 7.0

Since no increase in mSDH activity was observed when wild-type cells were grown at pH 4.5, 6.0 and 7.0, the mutant strain 621-C15 was used to determine if a strain with 20% of wild-type mSDH activity would lose its acid toler-

TABLE 6

Effect of pH on the Activity of mSDH in Strain ATCC 621 Grown in Sorbitol Medium

pH of Growth[1]	Specific Activity of mSDH	Total Activity[2]
4.5	1.5	13
6.0	2.8	22
7.0	2.5	16

[1] Cells were harvested at an optical density of 0.5 at 620nm. The buffers in the medium were able to maintain the pH up to this amount of growth. Shortly thereafter the pH of the pH 6.0 and pH 7.0 cultures began to drop.

[2] Total activity is the specific activity multiplied by the total amount of protein in the sample. This is the capacity in micromoles per minute of the entire sample.

TABLE 7

Effect of pH on the Specific mSDH Activity per Cell in Strain ATCC 621 Grown on Sorbitol Medium

pH of Growth[1]	Total Number of Cells[2] (10^{10} cells/ml)	Specific Activity of mSDH per cell (x 10^{-10})
4.5	3.4	1.9
6.0	3.9	2.4
7.0	3.8	1.9

[1] See footnote 1 Table 6.

[2] Cells were counted in a Petroff-Hauser counting chamber. Results reported here are an average of three separate counts. Maximum error was $\pm 0.8 \times 10^{10}$.

ance. Wild-type and strain 621-C15 were both grown at all three pH values and their growth rates compared (Figure 5). The same pattern of growth is seen for the wild-type and 621-C15. If mSDH activity is responsible for acid tolerance in G. oxydans, then 20% of the wild-type activity must be sufficient to protect the cells.

During experminents measuring the affect of the growth pH on the mSDH activity, cell pellets harvested after growth at pH 4.5 appeared considerably pinker than those of cells grown at pH 6.0 or 7.0. It was assumed that this increase in color was due to increased cytochrome levels, corresponding to changes hypothesized in the fifth possibility in Figure 1. Therefore, experiments were designed to examine the cytochrome content and respiration levels of G. oxydans growing at different pH values.

THE EFFECT OF GROWTH PH ON SORBITOL OXIDATION SYSTEMS

Cytochromes

The types and quantity of cytochromes found in cells grown at pH 4.5, 6.0 and 7.0 were examined. The types of cytochromes present did not vary (Figure 6). Since equipment for ultralow temperature scans was not available, it

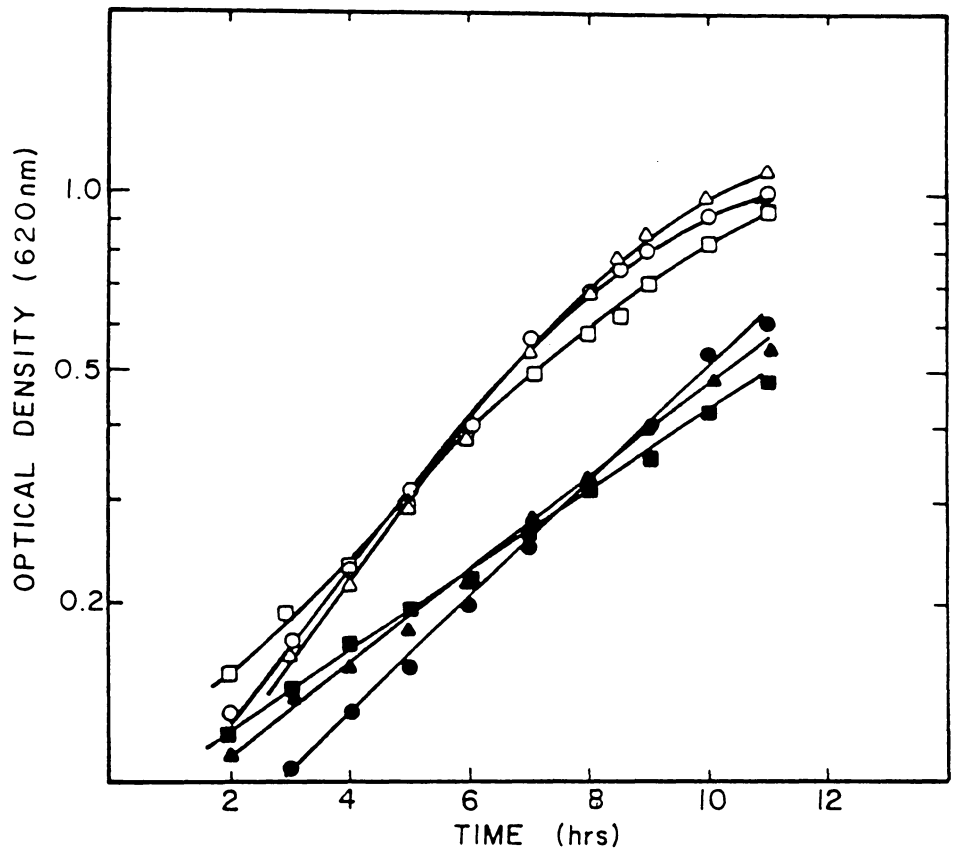

FIGURE 5. Effect of pH on the Growth of Strain ATCC 621 and Strain 621-C15 in Buffered Medium Containing Sorbitol.

Cells were subcultured on 5% unbuffered sorbitol medium. They were harvested by centrifugation and washed once with distilled water before being inoculated into the media. Growth of ATCC 621 at pH 4.5 (○), 6.0 (△), and 7.0 (□). Growth of 621-C15 at pH 4.5 (●), 6.0 (▲), and 7.0 (■). Curves are representative of three separate experiments.

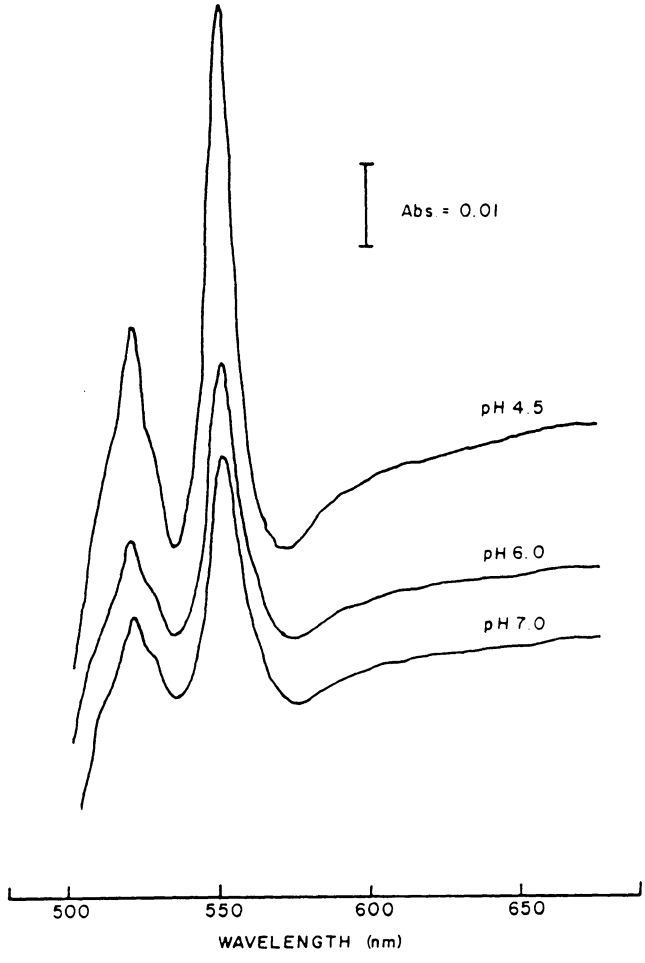

FIGURE 6. Effect of pH on the Cytochromes in Strain ATCC 621.

Scans were done on solubilized whole cells as described in the Materials and Methods. Sodium dithionite was used to reduce cytochromes. Protein concentration of each sample was adjusted to 8 mg/ml before scanning, so that a direct comparison of samples could be made. Cells were grown and samples prepared three times. Each time a sample was prepared it was scanned at least three times at two different protein concentrations. Data presented are representative curves from these samples.

was not possible to differentiate cytochromes with closely related absorbance patterns. The peaks found in the scans shown in Figure 6 correspond closely to the patterns seen by other investigators working with ATCC 621 (10,23,24,51,85). Samples reduced with sorbitol instead of sodium dithionite had the same peaks. However, the sorbitol peaks were much lower than the dithionite peaks. This may be due to reoxidation of the cytochromes, or it may indicate that only a portion of the cytochromes are linked to mSDH.

In some instances, an additional peak was observed at a wavelength of 630 nm in extracts of pH 4.5 grown cells. This corresponded to a d-type cytochrome. Unfortunately, this peak could not be consistently shown, even in the same sample. It might have been an artifact, or possibly the experimental conditions used did not keep it reduced or stable. Since d-type cytochromes act as terminal oxidases, this could indicate the presence of a branched cytochrome chain in G. oxydans. The presence of a d-type cytochrome in G. oxydans has not been previously reported so its presence in the pH 4.5 grown cells would certainly be of interest.

In addition to examining the types of cytochromes present, the amounts were quantified (Table 8). The cytochrome concentration in cells grown at pH 4.5 was double that found

TABLE 8

Effect of pH on the Cytochrome Concentration in Strain ATCC 621 Grown in Sorbitol Medium

pH of Growth Medium	Quantity of Cytochromes (change in Abs./mg protein)[1]	
	Whole Cell Extract[2]	Particulate Fraction Extract[2]
4.5	0.009	0.074
6.0	0.004	0.037
7.0	0.004	0.034

[1] Changes in the absorbance were measured between the peak and trough at wavelength pair 537-553 nm were used for all samples.

[2] Extracts were prepared by adding 1.5-2% w/v octyl glucoside to either the whole cells or a particulate fractions. After stirring at 4 C for 3 hours, samples were centrifuged at 120,000 x g for 90 minutes. The supernatant fluid from this centrifugation was the extract used in these experiments.

in cells grown at pH 6.0 or 7.0. Measurement of the levels of cytochromes in both crude extracts and the particulate fractions indicated that the cytochromes are enriched almost 10-fold in the particulate fraction compared to the rest of the cell at all growth pH values tested. This indicated that the additional cytochromes synthesized at pH 4.5 are being inserted into the membrane.

Since the cytochrome concentration increased in cells grown at pH 4.5, but the specific activity of the mSDH did not increase, it was of interest to examine the cytochrome levels of cells with altered mSDH activity to see if the regulation of mSDH and cytochromes are linked. The cytochromes of stationary phase cells (which had greater mSDH activity than exponential phase cells) and strain 621-C15 (which had less specific activity) mSDH were examined. The cytochrome level of stationary phase cells increased slightly (1.6 times that of exponential phase cells), but the increase in cytochromes was not proportional to the increase in mSDH activity (Table 9). The cytochrome concentration in the mutant was identical to that found in the wild-type, indicating that cytochrome levels did not decrease in conjuction with the mSDH (Table 9). No differences in the types of cytochromes were observed in either the mutant or the

TABLE 9

Comparison of Cytochrome Concentrations in ATCC 621 Exponential and Stationary Phase Cells and Strain 621-C15

Strain	Growth Phase	Quantity of Cytochromes (Change in Abs. per mg protein)[1]	Specific Activity mSDH[2]
621	Exponential	0.004	1.49
621	Stationary	0.006	4.26
621-C15	Exponential	0.004	0.31

[1] Cytochromes were measured as describe in Table 8. Samples used were whole cell extracts. Data presented is the average of five scans.

[2] Specific Activities measured in particulate fraction as micromoles of sorbose produced per min per mg of protein. Data taken from separate experiments not from samples used for cytochrome scans.

stationary phase cells. These data indicate that the cytochrome levels change independently of the mSDH activity

Since the quantity of cytochromes doubled in cells growing at pH 4.5, it was of interest to determine how this change affected the sorbitol oxidation in these cells.

Respiration on Sorbitol

The first experiment designed to measure the oxygen uptake during respiration on sorbitol yielded results exactly opposite from those expected (Figure 7). The pH 6.0 and 7.0 grown cells, which had fewer cytochromes, showed greater oxygen uptake. The pH 4.5 grown cells, which had the most cytochromes, showed the least oxygen uptake. These experiments were run under conditions developed by White (92). These conditions of temperature, shaking speeds, pH and buffer condition have been determined to be optimal for cells grown at a pH of 6.0. To determine if cells grown at different pH values had different optima, the experiment was redesigned to closely resemble the conditions occuring during growth. Each culture was harvested and washed in medium without sorbitol, but otherwise identical to the medium in which those cells grew. Each sample was then tested at the

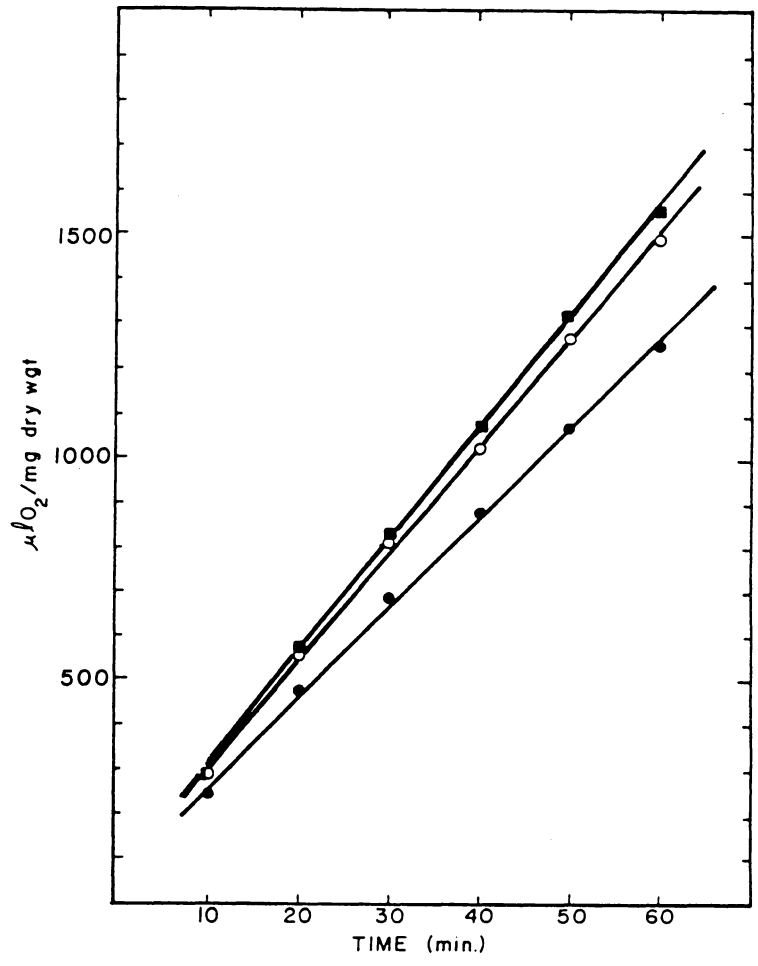

FIGURE 7. Effect of Growth pH on the Oxygen Uptake of Strain ATCC 621 Measured Using Conditions Optimal for pH 6.0 Grown Cells.

Cultures were harvested at an optical density of 0.5 at 620 nm. Respirometer experimental conditions were the same as that of White for optimal oxygen uptake (92). Oxygen uptake of pH 4.5 grown cells (●), pH 6.0 grown cells (O), and pH 7.0 grown cells (■). Each point represents and average of three separate determinations. Endogenous activity was zero for all samples.

pH and temperature at which it was grown. This resulted in a reversal of the results, with the pH 4.5 grown cells showing the greatest oxygen uptake (Figure 8). This indicated that the extra cytochromes might function to increase respiration. However, each of the samples was run under its own growth conditions. It was possible the differences observed resulted from differences in the pH at which the experiments were run and not from the increased cytochrome levels of the pH 4.5 grown cells.

Experiments were designed to determine if the difference in oxygen uptake resulted from a phenotypic difference in the cells and not from differences in the pH at which the samples were tested. Cells grown at each pH were harvested as before, but each sample was tested for oxygen uptake at pH 4.5, 6.0 and 7.0. The test run at pH 4.5 (Figure 9) showed the differences in oxygen uptake were not due to phenotypic differences in the cells but were a result of the experimental design. The pH 7.0 grown cells had a slightly lower oxygen uptake, but their rate of uptake is almost identical to the pH 6.0 and 4.5 grown cells. Similar results (data not shown) were seen for the respirometer experiments done at pH 6.0 and 7.0.

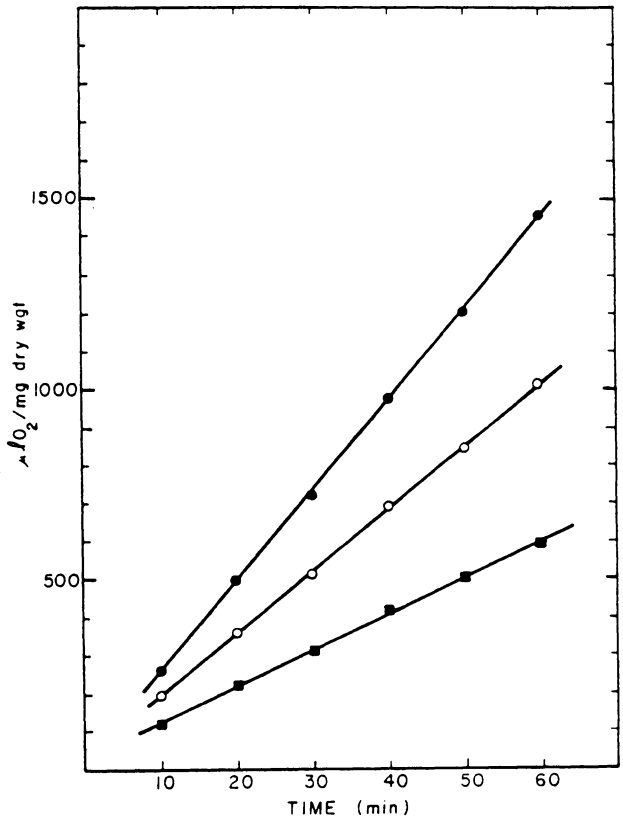

FIGURE 8. Effect of Growth pH on the Oxygen Uptake of Strain ATCC 621 Measured Using Conditions Simulating Growth.

Cells were harvested at 0.5 optical density at 620 nm. They were centrifuged and washed three times with buffered basal medium with the pH adjusted to growth pH of the cells. Respirometer flasks contained 2.1 ml of basal medium buffered at the growth pH of the cells and 0.4 ml of washed cells. The sidearm contained 0.5 ml of 30% (w/v) sorbitol. The reaction was started by tipping the sorbitol into the reaction chamber. The water bath temperature was 28 C. All other conditions were the same as in Figure 6. Oxygen uptake of pH 4.5 grown cells (●), pH 6.0 grown cells (O), and pH 7.0 grown cells (■). Endogenous activities were subtracted from each point before plotting the data. Each point is the average of 3 respirometer flasks. Data points are the average of three respirometer flasks. Curves are representative of three separate experiments.

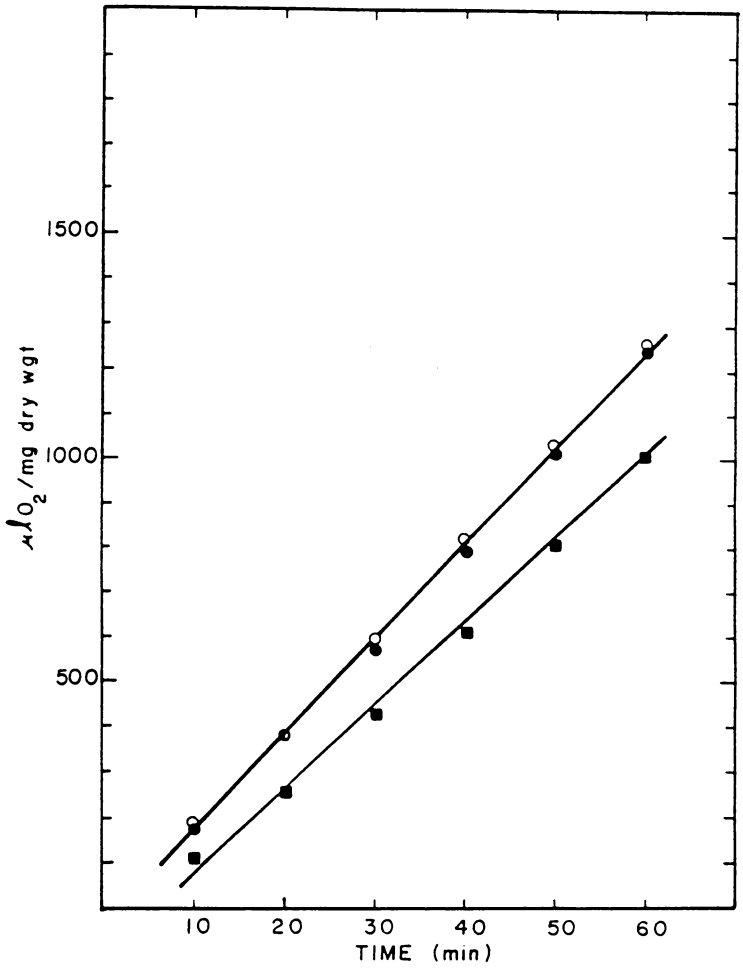

FIGURE 9. Effect of Growth pH on the Oxygen Uptake of Strain ATCC 621 Measured at pH 4.5.

Conditions are the same as in Figure 8 except that all the respirometer flasks contained buffered basal medium at pH 4.5. Oxygen uptake of pH 4.5 grown cells (●), pH 6.0 grown cells (○), and pH 7.0 grown cells (■). Data points are the average of three separate respirometer flasks. Curves are representative of two experiments.

It was noted that the respiration rate on sorbitol increased as the pH of the experiment decreased, probably due to the acidic pH optimum of the mSDH enzyme (pH 5.2). This acidic pH optimum may be a form of regulation of this membrane-bound protein, especially if it is exposed to the outer surface of the plasma membrane (where it could be directly affected by the environmental pH).

This does not explain why cells grown at pH 4.5 - which had twice the cytochrome concentration of cells grown at pH 6.0 or 7.0 - did not have increased ability to oxidize and respire on sorbitol. A number of variations in these oxygen uptake experiments were tried. Although no explanation was found for the increased cytochrome level not being detected as an increase in respiration, differences in the oxygen uptake on sorbitol in pH 4.5 grown cells were discovered.

If cells are harvested and washed in a complex medium without substrate and their respiration measured, they respired much more rapidly than the identical cells washed instead with distilled water (Figure 10). King and Cheldelin (49) have also noted that cells of ATCC 621 washed in distilled water lose some oxidative capacity. They attributed this to a loss of phosphate and were able to restore some of the oxidative capacity by washing the cells in phosphate

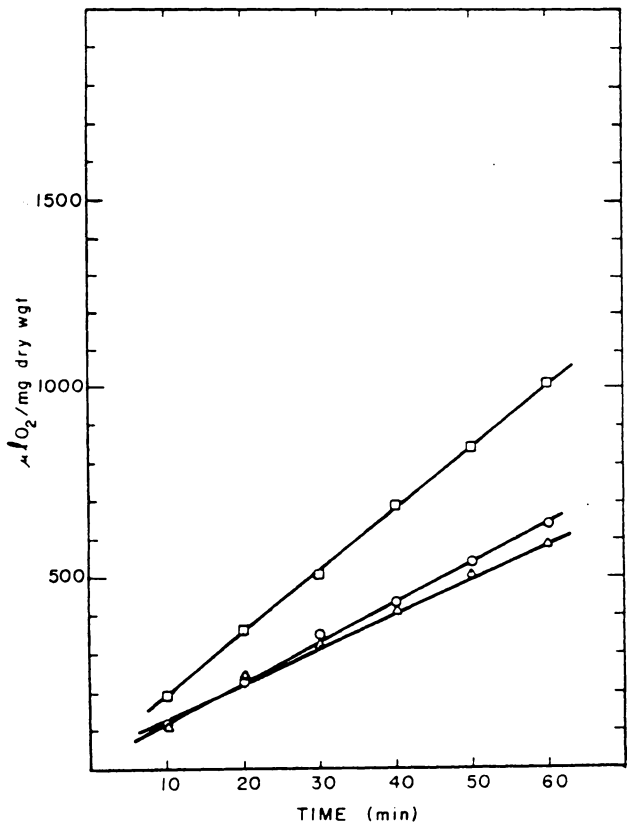

FIGURE 10. Comparison of Oxygen Uptake in Cells Washed in Medium, Distilled Water or Phosphate Buffer.

Cells were grown on buffered sorbitol medium at pH 6.0 and harvested at 0.5 optical density at 620 nm. Cell pellets were resuspended the first time in buffered basal medium at pH 6.0. They were then divided into three parts. One part was washed three times with 0.01 M phosphate at pH 6.0; one part was washed three times with distilled water; and, one part was washed twice with the buffered basal medium. All respirometer flasks contained 2.1 ml of basal buffered medium at pH 6.0 and 0.4 ml of washed cells. The reaction was started by tipping in 0.5 ml of 30% sorbitol from the side-arm. The temperature of the water bath was 28 C. Oxygen uptake of cells washed in buffered basal medium (□), distilled water (O), or phosphate buffer (Δ). Results are the average of three separate respirometer flasks.

buffer. As shown in Figure 10, washing the cells in a phosphate buffer did not restore any of the oxidative capacity to cells in these experiments.

An attempt to measure the oxygen uptake of G. oxydans using an oxygen electrode provided a clue to what substance was removed from the cells washed in distilled water or phosphate. No respiration occurred on sorbitol unless calcium and magnesium were added to the sample (data not shown). Subsequently, the affects of calcium and magnesium addition on oxygen uptake of cells in the respirometer were determined. The addition of calcium and magnesium restored full oxidative capacity to the distilled-water washed, pH 6.0 grown cells (Figure 11). The effect of these additions was greater when the measurements were made at 38 C than at 28 C. However, when cells grown at a pH of 4.5 were tested, the effects of calcium and magnesium were almost undetectable (Figure 12).

Because calcium and magnesium are required for optimum activity of the mSDH (8,92), and these compounds did not affect respiration in the pH 4.5 grown cells, it was speculated that the increase in cytochromes in the pH 4.5 grown cells did not function in the limited oxidation of sorbitol to sorbose by mSDH. Perhaps these extra cytochromes func-

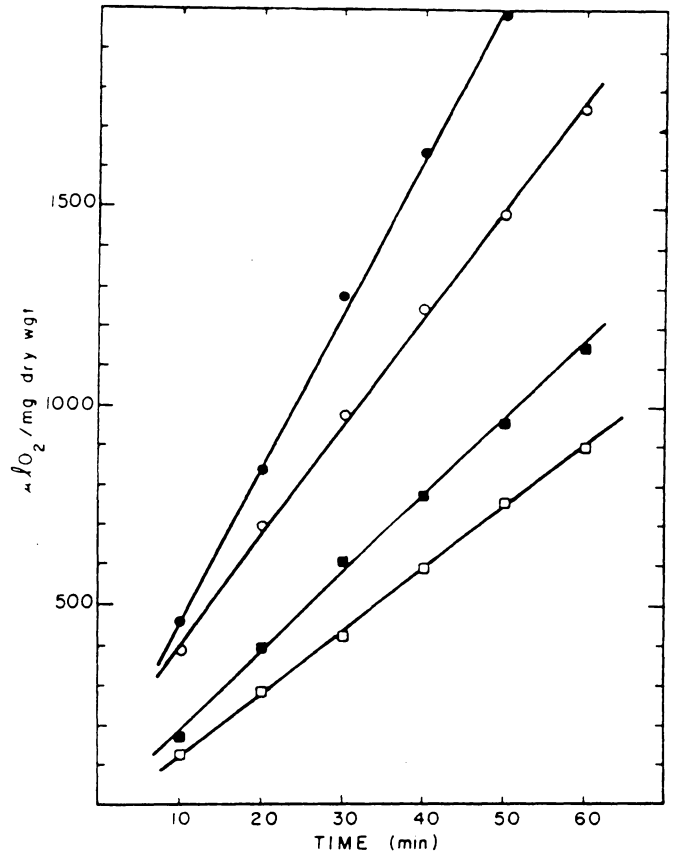

FIGURE 11. Effect of Calcium and Magnesium Ions on the Oxygen Uptake of pH 6.0 Grown Cells.

Cells were grown on buffered sorbitol medium at pH 6.0 and harvested at 0.5 optical density at 620 nm. Cells were washed three times with distilled water before being used in the respirometer. Buffer, cell concentration, shaking speed, and sorbitol concentration used for this experiment are those shown by White (1977) to be optimal for pH 6.0 grown cells. Respirometer flasks that contained calcium chloride and magnesium chloride had 15 and 30 micromoles respectively. Oxygen uptake was determined at either 28 C or 38 C. Oxygen uptake without Ca^{++} or Mg^{++} at 28 C (□) or at 38 C (○), oxygen uptake with Ca^{++} and Mg^{++} added at 28 C (■) or at 38 C (●). Data points are the average of three respirometer flasks. Curves are representative of two separate experiments.

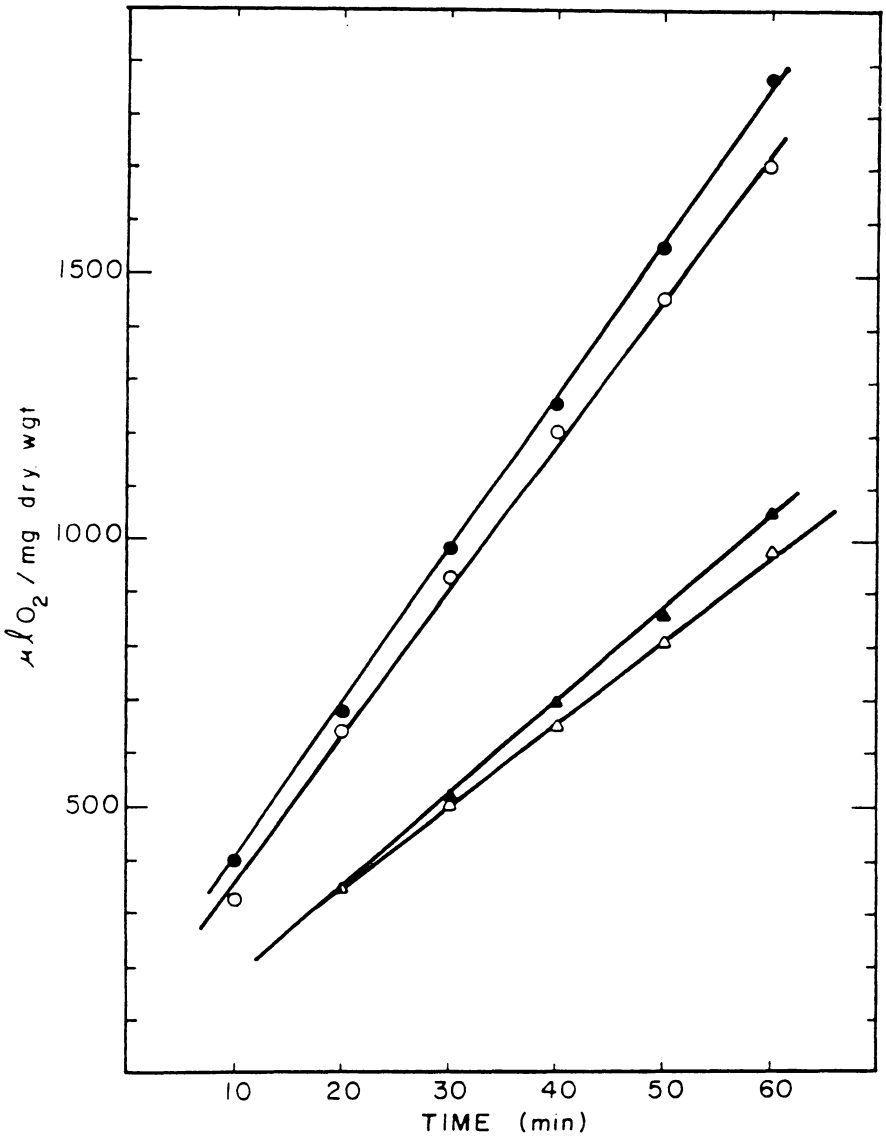

FIGURE 12. Effect of Calcium and Magnesium Ions on the Oxygen Uptake of pH 4.5 Grown Cells.

Experimental conditions were the same as those shown in Figure 11, except that the cells were grown at pH 4.5 instead of pH 6.0. Oxygen uptake without Ca^{++} or Mg^{++} at 28 C (△) or at 38 C (○), oxygen uptake with Ca^{++} and Mg^{++} added at 28 C (▲) or at 38 C (●). Data points are the average of three respirometer flasks. Curves are representative of tw separate experiments.

tion in sorbitol oxidation involving NAD- or NADP-linked soluble sorbitol dehydrogenases. Information on how the activity of these soluble enzymes are affected by changes in growth pH might help explain the function of the increased cytochrome levels in pH 4.5 grown cells.

Soluble Sorbitol Dehydrogenase Activity

Specific activities of both NAD- and NADP-linked sorbitol dehydrogenases were measured for cells grown at pH 4.5, 6.0 and 7.0 (Table 10). No differences were detected. The levels of NADH oxidase for cells grown at each pH were also examined to insure that the NADH produced by the NAD-linked sorbitol dehydrogenase was not being re-oxidized at different rates in the cells grown at different pH values. If the NADH-oxidase levels varied, then the values could vary independently of the actual sorbitol oxidation. The level of NADH-oxidase activity increased as the pH of growth increased (Table 10). Therefore, the soluble dehydrogenase assays were re-run with HQNO added to the assay mixture to inhibit the NADH oxidase activity (25,42). This did not change the specific activities of the soluble sorbitol dehydrogenase (data not shown). No change in the specicfic ac-

TABLE 10

Effect of pH Soluble Sorbitol Dehydrogenase and NADH-Oxidase Activity in Strain ATCC 621

pH of Growth Medium	Specific Activities[1]		
	NAD-Linked SDH	NADP-Linked SDH	NADH Oxidase
4.5	0.509 ±.026	0.081 ±.017	0.757 ±.131
6.0	0.519 ±.066	0.074 ±.021	1.222 ±.082
7.0	0.500 ±.024	0.096 ±.019	1.804 ±.119

[1] All cultures were harvested at the mid-log phase of growth. All activities were measured in soluble fractions as described in the Materials and Methods. All activities are measured as micromoles of product per minute per mg of protein.

tivity of the soluble sorbitol dehydrogenases was detected which corrresponded to the increase in cytochrome levels.

EFFECT OF GROWTH PH ON GROWTH YIELDS

Several types of data indicated that sorbitol oxidation in pH 4.5 grown cells was different from that of cells grown at pH 6.0 or 7.0. For example, the differences observed in the respirometer upon the addition of calcium and magnesium to pH 6.0 compared to pH 4.5 grown cells (Figures 11 and 12), the increased cytochrome content of pH 4.5 grown cells compared to pH 6.0 and 7.0 grown cells, and the increased sorbitol respiration of all the cells when measured at decreased pH values, all indicated that sorbitol oxidation of pH 4.5 grown cells was different.

Therefore, the growth yields for cells growing on sorbitol at pH 4.5, 6.0 and 7.0 were examined to see if there were differences in the efficiency with which the cells used sorbitol (Figure 13). At all points tested - from early to late exponential phase - it took more sorbitol to produce an equivalent weight of cells at a growth pH of 4.5 than at pH 6.0 or 7.0. This indicates that cells growing at pH 4.5 are

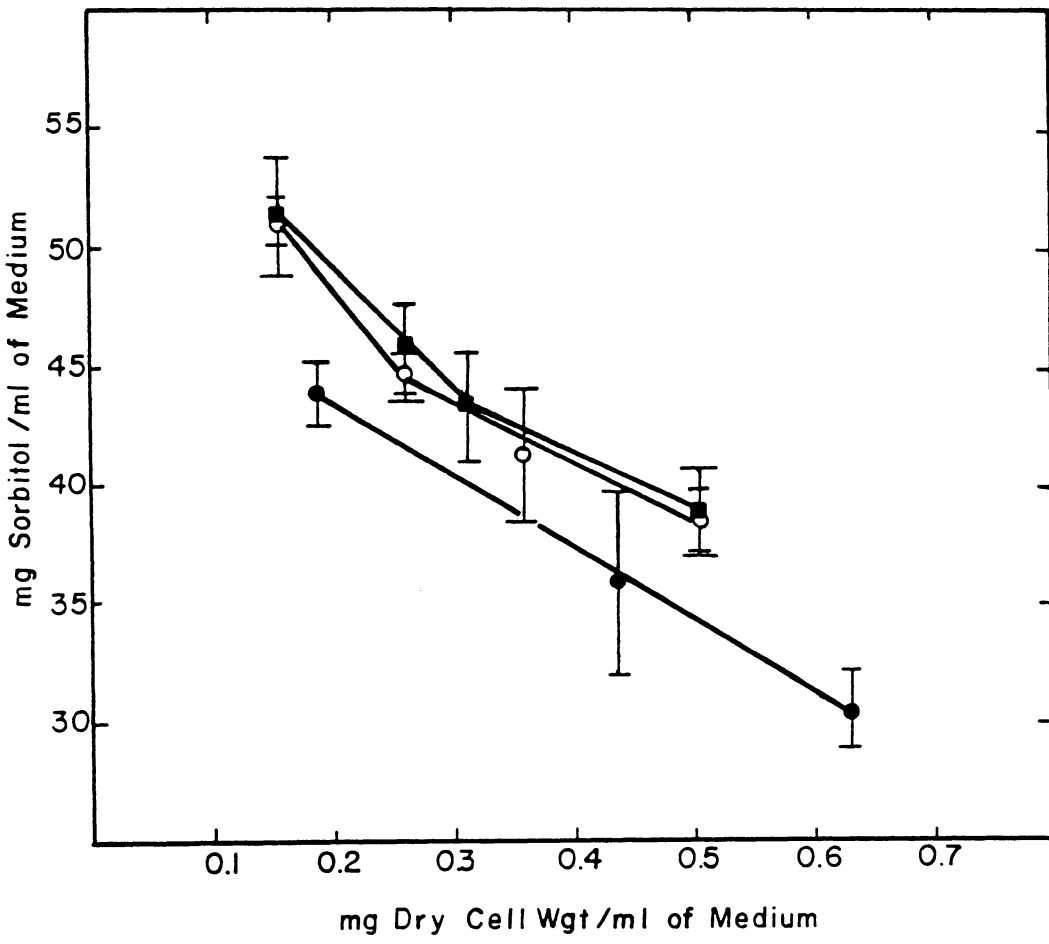

FIGURE 13. Effect of Growth pH on the Cell Yield on Sorbitol

Cells were inoculated from sorbitol stock vials into buffered sorbitol medium. Zero time was arbitrarily assigned to be when the cultures reached 0.2 optical density. Samples were taken for dry weights and to measure the amount of sorbitol remaining in the medium between optical densities of 0.2 and 0.8. Dry weight samples were processed as described in the Materials and Methods. Sorbitol was determined as described in the Material and Methods. Data for pH 4.5 (●), pH 6.0 (○), and pH 7.0 (■). Results are the average of two separate experiments.

less efficient in their use of sorbitol than cells growing at pH 6.0 or 7.0.

PART IV

DISCUSSION

MUTANT ISOLATIONS

Although considerable time has been spent trying to understand the physiology of the gluconobacters, very few investigators have used mutants as an experimental approach. This may be due to the total lack of information on the genetics of the gluconobacters which makes characterization of mutants more difficult.

In this investigation, it was thought best to determine the function of limited polyol oxidations in Gluconobacter oxydans by obtaining mutants affected in this function. Mutant isolation is achieved using information or hypotheses concerning the function of the enzyme to be studied. This investigator hypothesized that: i) cells excreting less sorbose would have decreased mSDH activity; ii) mSDH was essential for growth on sorbitol and; iii) mSDH activity was necessary for the cells to grow at low pH values. The mutants isolated in this study confirmed the first two of these hypotheses on the mSDH activity of G. oxydans.

The first hypothesis was based on the assumption that NAD(P)-independent sorbitol dehydrogenase was membrane-bound, and that the sorbose released from the cells was the product of this mSDH. We found that every isolate selected for decreased sorbose excretion had a decrease in the specific activity of mSDH.

This was especially interesting, since the gluconobacters also have a NADP-linked soluble enzyme which is known to convert sorbitol to sorbose (22,55). Many investigators have hypothesized that NAD- and NADP-linked, soluble polyol dehydrogenases in the gluconobacters function in more complete oxidation of polyols (9,22,30,31,49,50). If this is true, then one would not expect excretion of sorbose produced by the soluble enzymes. The mutants isolated appear to confirm this. Future investigators might examine these mutants for their levels of the soluble NADP-linked sorbitol dehydrogenases. If the only dehydrogenase activity affected was the activity of mSDH, this would strengthen the argument that the mSDH is solely responsible for the release of sorbose from the cells.

The second hypothesis (on which the counter selection was based) was that cells lacking mSDH would be unable to grow on sorbitol. However, Gluconobacter oxydans also has both NAD- and a NADP-linked soluble sorbitol dehydrogenases

(8,22,30,55), and these soluble enzymes might have been sufficient to support growth on sorbitol. In that case, all three sorbitol dehydrogenase activities would have to be lost for this counter selection to succeed.

Of the 30,000 colonies screened following the counter selection, two had significantly reduced levels of mSDH activity. No mutants were isolated which completely lacked mSDH activity, indicating that a total loss of mSDH activity is a lethal event. However, this assumes that the counter selection procedures were adequate. For example, if sorbitol and sorbose metabolism are inter-connected, then $mSDH^-$ mutants would not be able to grow on sorbose either, and would be lost. This interconnection seemed unlikely, since Sato *et al.* (80) showed that there is a sorbitol dehydrogenase distinct from the sorbose dehydrogenase in *G. suboxydans*. However, it is possible that the mutants isolated in the present study were regulatory mutants, and that sorbitol and sorbose oxidations are co-regulated. The following characteristics of the two mutants isolated by counter selection (621-C15 and 621-C3) make this seem likely. First, both mutants had reduced rates of growth on both sorbitol and sorbose. Second, one of the mutants (621-C3) spontaneously regained wild-type mSDH activity and then grew normally on both sorbitol and sorbose. Therefore, 621-C3 appears

to be a single mutation affecting growth on both sorbitol and sorbose. The mutant 621-C15 was stable, and it is not known whether it is a single mutation. It also had reduced growth rates on a number of other polyols (Figure 3). Thus, it is possible that both of these mutants were regulatory mutants in a mechanism that affected a number of aspects of polyol oxidations. However, these data could also be interpreted as meaning that the limited oxidation of many polyols is the result of action by a single membrane-bound enzyme.

In this regard, it is unfortunate that the separation of mSDH on polyacrylamide gels was unsuccessful. If electrophoretic characterization of the protein had been accomplished, it should have been possible to determine if the mutation was in the structural gene of the mSDH protein or if a decreased amount of normal protein was present (possible regulatory mutant).

The third hypothesis used in the isolation of mutants was that the membrane-bound polyol dehydrogenase might function to protect cells from a low pH environment. This idea is the result of work by Heefner (44), which indicated that G. oxydans survives in a low pH enviroment only if an oxidizable polyol was present. Interestingly, in the present

study, only three mutants were isolated which were found to be both acid sensitive and to have reduced mSDH activity. When characterized for growth rates and mSDH levels, all three mutants appeared similar. Therefore, it is likely that these mutants were clones, and, in essence, only one acid-sensitive mSDH mutant was found. This mutant sometimes grew at low pH values. Therefore, it could not be rigorously tested to confirm the original hypothesis - that mSDH activity was required for acid tolerance.

Data presented in this study showed: i) wild-type cells grown at low pH do not have increased mSDH activity (Table 6 and 7) and ii) that mutant strain 621-C15 (which has only 20% of the mSDH activity of wild type cells) grows as well at pH 4.5 as at pH 6.0 (Figure 5). If mSDH does function to protect cells in low pH environments, it need not be present at high levels of activity to do so. Since this is true, why was an acid sensitive mutant with low mSDH activity isolated? Particularly since the mutant was not stable indicating that the phenotype may have resulted from a single mutation.

Yamada et al. (97) suggested that membrane lipid composition might affect the acid tolerance of Gluconobacter cerinus. They isolated acid-sensitive mutants but did not

characterize them beyond examining growth at pH 4.0. The idea that a membrane mutation might affect acid-tolerance is interesting, since a membrane mutation might simultaneously affect the activity of an integral membrane protein like mSDH.

SOLUBILIZATION OF MSDH

The solubilization of membrane-bound sorbitol dehydrogenase of G. oxydans led to some interesting discoveries. After the octyl glucoside solubilization procedure used in this study was established, Shinagawa et al. (84) reported a Triton X-100 method for isolation of mSDH from another strain of G. oxydans, IFO 3254. We found that strain IFO 3254 does have a Triton-soluble mSDH (11). However, it was a distinctly different enzyme from the mSDH of strain ATCC 621. The mSDH of strain ATCC 621 had much higher specific activities and was very stable compared to that of the IFO strain. This successful solubilization of mSDH from strain ATCC 621 makes a highly active and very stable enzyme available for studies on immobilized enzyme production of L-sorbose for vitamin C synthesis.

FUNCTION OF LIMITED OXIDATIONS IN THE GROWTH OF G. OXYDANS

Products of the single-step oxidations of polyols by the gluconbacters have been used for centuries (75). The function of these oxidations for the gluconobacters, however, is still not understood. Suggestions in the literature about possible functions range from these oxidations having no energy or growth function (88) to their being the major route for energy production and carbohydrate metabolism (81). The data presented in this dissertation show that limited oxidation of sorbitol (and presumably other polyols) is required for growth, but these data do not define the precise function of these limited oxidations.

Understanding the potential function(s) of membrane-bound NAD(P)-independent dehydrogenases in the metabolism of the strictly aerobic genus Gluconobacter has been aided by the discovery of similar pathways in other genera. One of the best studied is glucose metabolism in Pseudomonas aeruginosa (27,60,63,78,94). These investigators suggest that a single-step oxidation of glucose occurs at the surface of the plasma membrane with gluconate being released into the growth medium. They found that these single step oxidations occur only at concentrations of glucose exceeding 15 mM. Below those concentrations, a transport protein is induced,

and glucose is metabolized inside the cells via the Entner-Doudoroff and pentose phosphate pathways. Oxygen limitation also inhibits these "extracellular" glucose oxidations. Dawes et al. (27) speculated that the oxidation of glucose to gluconate at high glucose concentrations enables P. aeruginosa to sequester glucose in the environment as gluconic acid which is a less "available" form for other microorganisms. Neijssel et al. (68,69) studied a similar system for the assimilation of glycerol in Klebsiella aerogenes. Similar systems are also found in P. fluorescens (57,58,76) and P. cepacia (54).

Another interesting example of dual pathways for carbon utilization was shown in Pseudomonas oxalaticus OX1 (32). In this study, it was argued that a membrane-bound, NAD-independent formate dehydrogenase and an NAD-linked soluble formate dehydrogenase compete for formate. This study suggests that the membrane-bound enzyme provides electrons for the energization of the membrane needed for ATP synthesis, transport, and other energy-requiring membrane functions, while the soluble enzyme provides reducing power for biosynthesis.

Information available from other organisms, combined with the well documented evidence for dual pathways for polyol assimilation in the gluconobacters

(13,14,48,49,50,71,72), suggests the possibility that both membrane-bound and soluble polyol dehydrogenases serve similar functions in G. oxydans. In other bacteria, however, the dual pathways for utilization were well regulated. In these other organisms, single-step oxidations occur only at high concentrations of substrate and oxygen, or (as in the case of P. oxalaticus) neither system predominates. In G. oxydans, on the other hand, single-step oxidations are one of the most outstanding characteristics of its metabolism, and large quantities of single-step oxidation products are always excreted into the surrounding medium (91). Work done by White (92) and that given in the present study (Tables 4 and 5) indicate that many membrane-bound polyol dehydrogenases activities are constituitively synthesized by G. oxydans.

Many other respiring bacteria can support rapid and prolific growth with 0.1% carbon source in the medium (27,69). However, in the present study, the growth rate of G. oxydans dropped significantly when the polyol concentration was lowered to 0.1% (Figure 1). The difference might be caused by the gluconobacters' inability to turn off the "extracellular" metabolism of polyol. It is known that G. oxydans does use some of the polyol in the growth medium as a source of carbon (36). If 98% of all polyol present is

converted and released as the single-step oxidation product (regardless of the original concentration), then lowered polyol levels would drastically limit use of the polyol as a carbon source. The cells would, in effect, be starving themselves for carbon.

Gluconobacters are found in environments where high sugar concentrations occur (such as on the surface of fruits and in honey). They might have become limited to these environments, because they have lost the ability to control the "extracellular" metabolism of carbon sources. However, mutant strain 621-M3 (with only 10% of wild-type mSDH activity) barely grew at all, and strain 621-M15 (with 20% of the wild-type activity) grew at a reduced rate even at a 5% polyol concentration (Figure 2). It would appear then, despite the "waste" of carbon during the limited oxidations, that these oxidations contribute significantly to the growth of the cells.

The exact nature of this contribution, however, is still not clear. In looking for an energy contribution, others have used dinitrophenol to uncouple respiration from ATP synthesis. Their results indicate that membrane-bound dehydrogenases provide energy in a manner that is not linked to phosphate metabolism (12,50). It is possible that the

concentrations of dinitrophenol used in these studies was insufficient to cause uncoupling. These investigators were using concentrations of dinitrophenol which uncouple respiration in other organisms. However, G. oxydans has very high levels of membrane-bound dehydrogenases and a very high respiratory quotient. It is known that the P/O ratio in G. oxydans is quite low - 0.09 to 0.5 (40,52). This indicates an excessive amount of oxidation compared to that needed or used for ATP synthesis. Future investigators should repeat these dinitrophenol experiments using higher levels of dinitrophenol or other more efficient uncouplers. It might also be helpful to limit oxygen or to utilize the mutant strain 621-C15. This mutant might be more susceptible to dinitrophenol, since it has decreased mSDH activity.

FUNCTION OF LIMITED POLYOL OXIDATIONS IN LOW PH ENVIRONMENTS

Decreased growth rates by mutants with lowered mSDH activity indicated that the mSDH activity of G. oxydans growing on sorbitol contributed to the growth of the cells. However, evidence presented in this study (Table 3) and others (20,37,67,93) indicates that limited oxidations increase when cell growth stops in maximum stationary phase. This -

combined with the low P/O ratio and high respiratory quotients of these bacteria - leaves this investigator curious about other potential functions for these rapid limited oxidations.

In this study, the possiblity was tested that these membrane-bound dehydrogeanses contribute to the acid tolerance of this genus. We found no evidence that the mSDH specific activity is increased in G. oxydans grown at pH 4.5 compared to pH 6 (Table 6). In addition, the loss of 80% of mSDH activity in the mutant strain 621-C15 did not alter its ability to grow at pH 4.5 compared to pH 6.0 (Figure 5). These data suggest that high level of rapid oxidations by mSDH are not required for the acid tolerance of G. oxydans growing on sorbitol at pH 4.5.

The present study did show that cells grown at lowered pH values had double the cytochrome concentration of cells grown at pH 6.0 (Table 8 and Figure 6) and that the mSDH activity changed its requirement for calcium and magnesium ions (Figure 11 and 12). Both of these changes involve enzymes required for single-step sorbitol oxidation. However, none of the experiments done in this study revealed a respiratory difference in the cells having these changes.

An increase in the respiration of all cells on sorbitol was found when they were tested at pH 4.5 compared to pH 6.0

or 7.0. From this, we can conclude that the mSDH is more active at lower pH values. Therefore, even though no new enzyme is synthesized, the cells have more mSDH activity at lower pH values. It not known whether this increase is connected with a need for increased protection from an acid environment.

Possibly, only membrane-bound dehydrogenases which make acid products are synthesized at higher levels when cells grow in low pH environments. For example, Ohmori et al. (70) reported that a strain of a Acetobacter aceti simultaneously lost its acetic acid tolerance and its ability for limited ethanol oxidation. However, in Ohmori's study, ethanol-oxidizing ability was not measured by the specific activity of ethanol dehydrogenase. Instead, the production of acid in the medium was measured. Therefore, it is difficult to say whether the loss of acid tolerance is due to a loss of ethanol dehydrogenase.

Yamada et al. (97) isolated acid pH-sensitive mutants of a strain of G. cerinus. These mutants were unable to grow at pH 4.5 in glucose. They reported that these mutants had "activities" of glucose dehydrogenase, alcohol dehydrogenase, and mannitol dehydrogenase which "coincided" with the parental strain without quantifying these activities.

No other studies were found in the literature which specifically examined the enzymes of the limited oxidation system of cells growing at different hydrogen ion concentrations. Several investigators detected increases in limited polyol oxidation when the pH of the growth medium was lowered (37,71,72,86). This agrees with data presented here (Figure 8 and 9) showing increased respiration in cells when respirometer experiments were conducted in pH 4.5 buffered sorbitol medium compared to pH 6.0 medium. It is possible that the increased respiration of sorbitol at lower pH values is partly due to the pH optimum of mSDH being pH 5.2 (8,93). However, this simple explanation does not account for the increased cytochrome levels in the cells grown at low pH values, nor for the change in magnesium and calcium dependence during respiration on sorbitol, shown in the present study. None of the other investigators measured the specific activities of membrane-bound dehydrogenases or examined cytochrome levels in their bacteria.

The reason for the doubling of the cytochrome concentration in G. oxydans grown at pH 4.5 compared to cells grown at pH 6.0 remains a mystery. Cytochromes should be expensive proteins for cells to synthesize. They contain iron, a mineral of low solubility for which many cells synthesize a special transport protein (64). Also, cytochromes

contain a complex porphyrin-ring structure which must be synthesized by the cells. Therefore, it does not seem advantagous for cells to synthesize large quantities of cytochromes if they have no beneficial function. Future investigators might examine the pH sensitive mutants isolated in this study to determine if any of them are incapable of increasing cytochrome content when grown at lower pH. If the increased cytochrome content has a function for cells grown in the lower pH environments, then some of these mutants should not be able to synthesize the extra cytochromes.

Enzyme analyses and respiration data do not directly show that cells growing at pH 4.5 have a greater limited sorbitol oxidation capacity than cells grown at pH 6.0 or 7.0. However, cell-yield data at these growth pH values show a difference in the way sorbitol is being used (Figure 13). More sorbitol was used to obtain the same cell mass for cells grown pH 4.5 than at pH 6.0 or 7.0.

These data appear to agree with data of Olijve and Kok (71,72). They found that cultures of G. oxydans growing in a chemostat on a complex glucose medium at pH 5.5 produced 15 times more cells per mole of glucose than cells grown at pH 2.5. Unfortunately, in addition to the pH difference of their media, they also had 5 times more glucose in their pH 2.5 medium than in their pH 5.0 medium. This is unfortu-

nate, because other investigators have shown that increasing concentrations of polyol in the medium decreases the cell yield per mole of substrate utilized (38,65,67,88). Therefore, the decrease in glucose concentration in Olijve and Kok's pH 5.0 medium may account for much of the reported increase in cell yield in that medium. It might also explain the smaller differences observed in the present study (Figure 13) compared to Olijve and Kok's.

Olijve and Kok concluded from their experiments at different growth pH values that there was regulation of the pentose phosphate pathway in G. oxydans (71). They believe that all of the glucose is being oxidized to gluconate when cells are grown at pH 2.5, and that there is no pentose phosphate cycle activity in these cells. They tested for the pentose phosphate pathway activity by measuring carbon dioxide evolution and found that cells grown at pH 2.5 had negligible activity. Unfortunately, prior to determining carbon dioxide evolution from glucose, they incubated the pH 2.5-grown cells in a buffer at pH 2.5 without an oxidizable carbon source. According to the experiments of Heefner (44), this means that they were testing nonviable cells with denatured cytoplasmic enzymes. Therefore, lack of detectable pentose phosphate pathway activity does not indicate it was absent in the viable growing cells.

The data presented in this study (Figure 13), which showed a decrease in cell yield for cells grown at lower pH values, is the first evidence for regulation of carbon utilization pathways in G. oxydans as a result of the growth pH. The present study did not detect evidence that this regulation resulted from changes in the mSDH. Therefore, future investigators might look for changes in the pentose phosphate pathway of cells grown at different pH values.

PART V

CONCLUSIONS

1. Growth studies using mutants with 10 and 20% of wild-type activity of mSDH showed that high activity of these membrane bound polyol dehydrogenases is essential for normal growth of G. oxydans.

2. Preliminary evidence on the regulation of mSDH when G. oxydans is grown on other polyols and the effect of the mutation in strain 621-C15 on growth of that strain on other polyols yields two possible conclusions: i) the mSDH of G. oxydans is a multifunctional enzyme and is responsible for the oxidation of many polyols, or ii) polyol oxidation systems in G. oxydans are coordinately regulated and the mutant 621-C15 is a regulatory mutant.

3. None of the sorbitol oxidizing enzymes increased when the growth pH was lowered from 7.0 to 4.5. This leads to the conclusion that no increase in the amount of sorbitol oxidizing enzymes is required to protect cells in environments with pH values as low as pH 4.5.

4. Respiration on sorbitol increased 1.8 fold when respirometer experiments were carried out at pH 4.5 compared

to pH 6.0. This supports the hypothesis that the limited polyol oxidation is part of the acid tolerance mechanism of these bacteria, as suggested by Heefner. However, growth of mutant 621-C15 was not adversely affected by lowering the pH to 4.5 indicating that the primary function of high polyol oxidation is not for acid tolerance.

5. A doubling in the cytochrome concentration in cells grown at pH 4.5 had no effect on the ability of these cells to respire on sorbitol.

6. Although mSDH activity is cytochrome-linked, the mSDH and cytochrome levels are regulated independently of one another.

7. When grown at pH 4.5 compared to pH 6.0, the cell yield on sorbitol decreased. From this it can be concluded that there is a decrease in the efficiency of sorbitol usage in cells grown at the lower pH.

PART VI

LITERATURE CITED

1. Adachi, O., K. Tayama, E. Shinagawa, K. Matsushita, and M. Ameyama. 1978. Purification and characterization of particulate alcohol dehydrogenase from Gluconobacter suboxydans. Agric. Biol. Chem. 42:2045-2056.

2. Adachi, O., K. Tayama, E. Shinagawa, K. Matsushita, and M. Ameyama. 1980. Purification and characterization of membrane-bound aldehyde dehydrogenases from Gluconobacter suboxydans. Agric. Biol. Chem. 44:503-515.

3. Ameyama, M. 1975. Gluconobacter oxydans subsp. sphaericus, a new subspecies isolated from grapes. Int. J. Syst. Bacteriol. 25:365-370.

4. Ameyama, M., K. Osada, E. Shinagawa, K. Matsushita, and O. Adachi. 1981a. Purification and characterization of aldehyde dehydrogenase of Acetobacter aceti. Agric. Biol. Chem. 45:1889-1890.

5. Ameyama, M., K. Matsushita, Y. Ohno, E. Shinagawa, and O. Adachi. 1981b. Existance of a novel prosthetic group, PQQ, in membrane-bound electron transport chain-linked, primary dehydrogenases of oxidative bacteria. FEBS Letters 130:179-183.

6. Ameyama, M., E. Shinagawa, K. Matsushita, and O. Adachi. 1981c. D-glucose dehydrogenase of Gluconobacter suboxydans: Solubilization, purification, and characterization. Agric. Biol. Chem. 45:851-861.

7. Ameyama, M., E. Shinagawa, K. Matsushita, and O. Adachi. 1981d. D-fructose dehydrogenase of Gluconobacter industrius: Purification, characterization, and application to enzymatic microdetermination of D-fructose. J. Bacteriol. 145:814-823.

8. Arcus, A. C. and N. L. Edson. 1956. Polyol dehydrogenases 2. The polyol dehydrogenases of Acetobacter suboxydans and Candida utilis. Biochem. J. 64:385-394.

9. Asai, T. 1968. Acetic acid bacteria: classification and biochemical activities. University of Tokyo Press. Tokyo. 343 pp.

10. Bachi, B. and L. Ettlinger. 1974. Cytochrome difference spectra of acetic acid bacteria. Int. J. Syst. Bacteriol. 24:215-220.

11. Baker, C. A. and G. W. Claus. 1983. Solubilization of NAD(P)-independent sorbitol dehydrogenase of Gluconobacter oxydans subspecies suboxydans using octylglucoside. FEMS Microbiol. Lett. 18:123-125.

12. Batzing, B. L. and R. Dellork. 1983. The effect of 2,4-dinitrophenol on glycerol-limited, biphasic growth of Gluconobacter oxydans. Can. J. Microbiol. 29:288-290.

13. Butlin, K. R. 1936. Aerobic breakdown of glucose by Bact. suboxydans. Biochem. J. 30:1870-1877.

14. Butlin, K. R. 1938. Enzyme system of Bact. suboxydans-II. Effect of acids and pH. Biochem. J. 32:1185-1190.

15. Carlton, B. C. and B. J. Brown. 1981. Gene mutation. p.222-242. In P. Gerhardt (ed.), Manual of Methods for General Microbiology. American Society for Microbiology, Washington, D. C.

16. Carr, J. G. and G. C. Whiting. 1971. Microbiological aspects of production and spoilage of cider. J. Appl. Bacteriol. 34:81-93.

17. Chalmers, R. A. and A. G. Sinclair. 1967. Organic molybdate complexes. J. Inorganic nucl. Chem. 29:2065-2089.

18. Cheldelin, V. H. 1961. Metabolic Pathways in Microorganisms. John Wiley and Sons, New York. 91 p.

19. Cho, J. J., A. C. Hayward, and K. G. Rohrbach. 1980. Nutritional requirements and biochemical activities of pineapple pink disease strains from Hawaii. Antonie van Leewenhoek J. Microbiol and Serol. 46: 191-204.

20. Claus, G. W., B. L. Batzing, C. A. Baker and E. M. Goebel. 1975. Intracytoplasmic membrane formation and increased oxidation of glycerol during growth of Gluconobacter oxydans. J. Bacteriol. 123: 1169-1183.

21. Criddle, W. J., J. C. Fry, and M. M. Keaney. 1974. Myo-inositol dehydrogenase from Acetomonas oxydans. Optimization of conditions for solubilization of membrane-bound enzyme. Biochem. J. 137: 449-452.

22. Cummins, J. T., T. E. King., and V. H. Cheldelin. 1957. The biological oxidation of sorbitol. J. Biol. Chem. 224:323-329.

23. Daniel, R. M. 1970. The electron transport system of Acetobacter suboxydans with particular reference to cytochrome o. Biochem. Biophys. Acta 216:328-341.

24. Daniel, R. M. and S. K. Erickson. 1969. The effect of glucose on the electron-transport systems of the aerobic bacteria Azotobacter vinelandii and Acetobacter suoxydans. Biochem. Biophys. Acta 180:63-67.

25. Daniel, R. M. and E. R. Redfearn. 1968. The reduced nicotinamide adenine dinucleotide oxidase system of Acetobacter suboxydans. Biochem. J. 106:49P-50P.

26. Davis, B.J. 1964. Disc electrophoresis-II. method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121:404-427.

27. Dawes, E. A., M. Midgley, and P. H. Whiting. 1976. Control of transport systems for glucose, gluconate, 2 oxo-gluconate, and of glucose metabalism in Pseudomonas aeruginosa. p.197-207. In: Continuous culture 6: Applications and New Fields. A. C. R. Dean, D. C. Ellwood, G. G. T. Evans, and J. Melliny., eds. Ellis Horwood, Ltd. Publ. Chichester, England.

28. Dawson, R. M. C., D. Elliott, W. H. Elliott, and K. M. Jones. (eds.). 1968. Data for Biochemical Research. Oxford University Press. N.Y.

29. De Ley, J. and J. Frateur. 1974. p.251-253. In: Bergey's Manual of Determinative Bacteriology, 8th Ed. (R. E. Buchanan and N. E. Gibbons, eds.) Williams and Wilkins, Baltimore.

30. De Ley, J. and K. Kersters. 1964. Oxidation of aliphatic glycols by acetic acid bacteria. Bacteriol. Rev. 28:164-180.

31. De Ley, J. and Stouthamer, A. J. 1959. The mechanism and localization of hexonate metabolism in Acetobacter suboxydans and Acetobacter melanogenum. Biochem. Biophys. Acta 34:171-183.

32. Dijkhuizen, L., J. W. C. Timmerman, and W. Harder. 1979. A pyridine nucleotide-independent membrane-bound formate dehydrogenase in Pseudomonas oxalaticus OX1. FEMS Microbiol. Lett. 6:53-56.

33. Duine, J. A., J. Frank,Jzn., and J. K. Van Zeeland. 1979. Glucose dehydrogenase from Acinetobacter calcoaceticus. (A quinoprotein'). FEBS Lett. 108:443-446.

34. Duine, J. A. and J. Frank,Jzn. 1981a. Quinoprotein alcohol Dehydrogenase from a non-methylotroph, Acinetobacter calcoaceticus J. Gen. Microbiol. 122:201-209.

35. Duine, J. A. and J. Frank,Jzn. 1981b. Quinoproteins: a novel class of dehydrogenases. Trends. Biochem. Sci. 6:278-280.

36. Feshami, B. H. 1983. Factors affecting use of sorbital in a carbon source by exponentially growing Gluconobactor oxydans ATCC 19357. Masters Thesis. Virginia Polytechnic Institute and State University.

37. Fewster, J. A. 1958. Growth of Acetobacter suboxydans and the oxidation of aldoses, related carboxylic acids, and aldehydes. Biochem. J. 69:582-595.

38. Flickinger, M. C. and D. Perlman. 1977. Application of oxygen-enriched aeration in the conversion of glycerol to dihydroxyacetone by Gluconobacter melanogenus IFO 3293. Appl. Environ. Microbiol. 33:706-712.

39. Franklin, F. C. H. and W. A. Venables. 1976. Biochemical, genetic, and regulatory studies of alanine catabolism in E. coli K12. Mol. gen. Genet. 149:229-237.

40. Glatti, H. and L. Ettlinger. 1970. Substrate-energy relationships in Acetomonas oxydans. Arch. Mikrobiol. 74:273-282.

41. Graham, A. and D. H. Boxer. 1981. The organization of formate dehydrogenase in the cytoplasmic membrane of Escherichia coli. Biochem. J. 195:627-637.

42. Greenfield, S. and G. W. Claus. 1969. Isocitrate dehydrogenase and glutamate synthesis in Acetobacter suboxydans. J. Bacteriol. 100:1264-1270.

43. Harold, F. M. 1972. Conservation and transformation of energy by bacterial membranes. Bacteriol. Rev. 36:172-230.

44. Heefner, D. L. 1977. Mechanism of acid tolerance of the gluconic acid bacterium Gluconobacter oxydans. Ph.D. Dissertation. Virginia Polytechnic Institute and State University.

45. Helenius, A. and K. Simons. 1975. Solubilization of membranes by detergents. Biochem. Biophys. Acta 415:29-79.

46. Hodes, M.E. and J. E. Petz. 1981. A positive zymogen for distinguishing among RNase and phosphodiesterase. Anal. Biochem. 100:150-152

47. Kersters, K. and J. DeLey. 1968. The occurrence of the Entner-Doudoroff pathway in bacteria. Antonie van Leeuwenhoek J. Microbiol and Serol. 34:393-408.

48. Kersters, K., W. A. Wood, and J. DeLey. 1965. Polyol dehydrogenases of Gluconobacter oxydans. J. Biol. Chem. 240:964-974.

49. King, T. E. and Cheldelin, V. H. 1952. Phosphorylative and non-phosphorylative oxidation in Acetobacter suboxydans. J. Biol. Chem. 198:135-141.

50. King, T. E. and Cheldelin, V. H. 1953. Sources of energy and the dinitrophenol effect in the growth of Acetobacter suboxydans. J. Bacteriol. 66:581-584.

51. King, T. E. and V. H. Cheldelin. 1957. Glucose oxidation and cytochrome in solubilized particulate fractions of Acetobacter suboxydans. J. Biol. Chem. 224:579-590.

52. Klungsoyr, L., T. E. King, and V. H. Cheldelin. 1957. Oxidative phosphorylation in Acetobacter suboxydans. J. Biol. Chem. 227:135-149.

53. Lambert, B., K. Kersters, F. Gossele, J. Swings, and J. DeLey. 1981. Gluconobacters from honey bees. Antonie van Leeuwenhoek J. Microbiol and Serol. 47:147-157.

54. Lessie, T. G., T. Berka, and S. Zamanigian. 1979. Pseudomonas cepacia mutants blocked in the direct oxidative pathway of glucose degradation. J. Bacteriol. 139(1):323-325.

55. Liber, E. E., A. I. Dorozhko, and N. V. Pomortseva. 1978. Coenzyme-induced slow transformations of NADP sorbitol dehydrogenase from Gluconobacter oxydans. Biochemistry (trans. f. Russian) 43(6):845-854.

56. Lowry, O., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

57. Lynch, W. H. and M. Franklin. 1978. Effect of temperature on diauxic growth with glucose and organic acids in Pseudomonas fluorescens. Arch. Microbiol. 118:133-140.

58. Lynch, W. H. 1982. The role of some membrane dehydrogenase in Pseudomonas fluorescens. Can. J. Microbiol. 28:907-914.

59. Matsushita, K., J. Ohno, E. Shinagawa, O. Adachi, and M. Ameyama. 1982. Membrane-bound, electron transport-linked, D-glucose dehydrogenase of Pseudomonas fluorescens. Interaction of the purified enzyme with ubiquinone or phospholipid. Agric. Biol. Chem. 46:1007-1011.

60. McCowen, S. M., P. U. Phibbs,Jr., and T. W. Feary. 1981. Glycerol catabolism in wild-type and mutant strains of Pseudomonas aeruginosa. Curr. Microbiol. 5:191-196.

61. Meynell, G. G. and E. Meynell. 1970. Theory and practice in experimental bacteriology, 2nd ed. Cambridge University Press. 347 p.

62. Miller, J. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. p. 138.

63. Mitchell, C. G. and E. A. Dawes. 1982. The role of oxygen in the regulation of glucose metabolism, transport, and the tricarboxylic acid cycle in Pseudomonas aeruginosa. J. Gen. Microbiol. 128:49-49.

64. Moat, A. G. 1979. Microbial Physiology. John Wiley and Sons, New York.

65. Muller, J. 1966. Untersuchungen uber die sorbosegarung in stationarer und kontinuier Kultur. Zentralbl. Bakeriol. Parasitenkel. Infectionakr. Hyg. II. |120|:356-377.

66. Muraoka, H., Y. Watabe, N. Ogasawara, and H. Takahashi. 1982. Purification and properties of coenzyme-independent alcohol dehydrogenase from the membrane fraction of Acetobacter aceti. J. Ferm. Technol. 60:41-50.

67. Nabe, K., S. Izuo, S. Yamada, and I. Chibata. 1979. Conversion of glycerol to dihydroxyacetone by immobilized whole cells of Acetobacter xylinum. Appl. Environ. Microbiol. 38:1056-1060.

68. Neijssel, O. M. and D. W. Tempest. 1975. Production of gluconic acid and 2-ketogluconic acid by Klebsiella aerogenes NCTC 418. Arch. Microbiol. 105:183-185.

69. Neijssel, O. M., S. Huetings, K. J. Crabbendam, and D. W. Tempest. 1975. Dual pathways of glycerol assimilation in Klebsiella aerogenes NCIB 418. Arch. Microbiol. 104:83-87.

70. Ohmori, S., T. Uozumi, and T. Beppu. 1982. Loss of acetic acid resistance and ethanol oxidizing ability in an Acetobacter strain. Agric. Biol. Chem. 46:381-389.

71. Olijve, W. and J. J. Kok. 1979a. Analysis of growth of Gluconobacter oxydans in glucose containing media. Arch. Microbiol. 121:283-290.

72. Olijve, W. and J. J. Kok. 1979b. An analysis of the growth of Gluconobacter oxydans in chemostat cultures. Arch. Microbiol. 121:291-297.

73. Olsiewski, P. J., G. J. Kaczorski, and C. Walsh. 1980. Purification and properties of D-amino acid dehydrogenases, an inducible, membrane-bound, iron-sulfur flavoenzyme from E. coli B. J. Biol. Chem. 255:4487-4494.

74. Passmore, S. M. and J. G. Carr. 1975. The ecology of the acetic acid bacteria with particular reference to cider manufacture. J. Bacteriol. 38:151-158.

75. Prescott, S. C. and C. G. Dunn. 1959. "Industrial Microbiology", 3rd ed. McGraw-Hill Book Co., Inc. New York.

76. Quay, S. C., S. B. Friedman, and R. C. Eisenberg. 1972. Gluconate regulation of glucose catabolism in Pseudomonas fluorescens. J. Bacteriol. 112:291-298.

77. Racker, E. 1955. Mechanism of action and properties of pyridine nucleotide-linked enzymes. Physiol. Rev. |35|:1-56.

78. Roberts, B. K., M. Midgley, and E. A. Dawes. 1973. The metabolism of 2-oxogluconate by Pseudomonas aeruginosa. J. Gen. Microbiol. 78:319-329.

79. Salisbury, S. A., H. S. Forrest, W. B. T. Cruse, and O. Kennard. 1979. A novel coenzyme from bacterial primary alcohol dehydrogenases. Nature 280:843-844.

80. Sato, K., Y. Yamada, K. Aida, and T. Uemera. 1969. Enzymatic studies on the oxidation of sugars and sugar alcohol VIII. Particle-bound L-sorbose dehydrogenase from Gluconobacter suboxydans. J. Biochem. 66:521-527.

81. Shinagawa, E., T. Chiyonobu, K. Matsushita, O. Adachi, and M. Ameyama. 1978. Distribution of gluconate dehydrogenase and ketogluconate reductases in aerobic bacteria. Agric. Biol. Chem. 42:1055-1057.

82. Shinagawa, E., K. Matsushita, O. Adachi, and M. Ameyama. 1978. Membrane-bound D-gluconate dehydrogenase

of Seratia marcesens: purification and properties. Agric. Biol. Chem. 42:2355-2361.

83. Shinagawa, E., K. Matsushita, O. Adachi, and M. Ameyama. 1981. Purification and characterization of 2-keto-D-gluconate dehydrogenase from Gluconobacter melanogenus. Agric. Biol. Chem. 45:1079-1085.

84. Shinagawa, E., K. Matsushita, O. Adachi, and M. Ameyama. 1982. Purification and characterization of D-sorbitol dehydrogenase from membrane of Gluconobacter suboxydans var. alpha. Agric. Biol. Chem. 46:135-141.

85. Smith, L. 1954. Bacterial cytochromes. Bacteriol. Rev. 18: 106-130.

86. Stadler-Szoke, A., L. Nyeste, and J. Hollo. 1980. Studies on the factors affecting gluconic acid and the 5-ketogluconic acid formation by Acetobacter. Acta Alimentaria 8:155-172.

87. Sussman, M. L. and J. B. Hays. 1977. Irreversible inactivation of the membrane-bound enzyme II-lac of the lactose phosphotransferase system of Staphylococcus aureus by Triton X-100 and protection by substrates. Biochem. Biophys. Acta 465:559-570.

88. Uspenskaya, S. N. and M. S. Loitsyanskaya. 1979. Effectiveness of the utilization of glucose by Gluconobacter oxydans. Mikrobiologiya 48:400-405.

89. Van Keer, C., P. Vanden Abeele, J. Swings, F. Gosselle, and J. DeLey. 1981. Acetic acid bacteria as causal agents of browning and rot of apples and pears. Zentralbl. Bakteriol. Parasitenkl. Infektionskr. I. Abt. Orig. |C2|:197-204.

90. Venugopal, K. S. S. and P. R. Adiga. 1980. Artifactual staining of proteins on polyacrylamide gels by nitroblueterrazolium chloride and phenazine methosulfate. Anal. Biochem. 101:215-220.

91. Wallen, L. C., F. H. Stodsola, and R. W. Jackson. 1959. Type reactions in fermentation chemistry. ARS-71-13 Agricultural Res. Service. U.S. Dept. of Agriculture. 496p.

92. White, S. A. 1975. Relationship of intracytoplasmic membrane development in Gluconobacter oxydans to rates of sorbital oxidation. Master thesis. Virginia Polytechnic Institute and State University.

93. White, S. A. and G. W. Claus. 1982. Effect of intracytoplasmic membrane development on oxidation of sorbitol and other polyols by Gluconobacter oxydans. J. Bacteriol. 150:934-943.

94. Whiting, P. H., M. Midgley, and E. A. Dawes. 1976. The role of glucose limitation in the regulation of the transport of glucose, gluconate, and 2-oxogluconate and of glucose metabolism in Pseudomonas aeruginosa. J. Gen. Microbiol. 92:304-310.

95. Worsfold, M., M. J. Marshall, and E. B. Ellis. 1977. Enzyme detection using phenazine methosulphate and tetrazolium salts: interference by oxygen. Anal. Biochem. 79:152-156.

96. Yagi, T., H. Kagamiyama, and M. Nozak. 1981. A sensitive method for the detection of aspartate: 2-oxoglutarate aminotransferase activity on polyacrylamide gels. Anal. Biochem. 110:146-149.

97. Yamada, Y., Y. Tahara, and K. Kondo. 1976. Isolation of "acidic pH-sensitive mutants" in Gluconobacter cerinus. J. Gen. Appl. Microbiol. 22:277-279.

PART VII

APPENDIX A

ATTEMPTS TO DEVELOP AN ACTIVITY STAIN FOR MEMBRANE-BOUND SORBITOL DEHYDROGENASE

The following summarizes what was learned from attempts to develop an activity stain for the mSDH of G. oxydans, strain ATCC 621.

1. Octyl-glucoside must be present in the running gel, the stacking gel, and the sample buffer at levels above the critical micelle concentration of 0.7%. This was necessary to get the majority of the sample proteins to enter the gel. Reverse polarity and acid gels were tried to see if the mSDH had not been entering the gel under the standard conditions used. No activity stained bands were detected except under the electrophoretic conditions described in the Materials and Methods.

2. Variations in the activity stain were tested on the crude extract prior to electrophoresis. From these experiments, the following things were determined: a) Octyl-glucoside, or perhaps free glucose present in the detergent, acts as a substrate at pH 6.0 and 7.0, but this interference is eliminated if the pH is lowered to

5.0. b) Sample buffers must be made with sucrose, since the glycerol normally used also acts as a substrate for some proteins in the extract. c) The reaction proceeds with the following artificial electron acceptors: nitroblue tetrazolium (NBT), tetranitro blue tetrazolium(TNBT) and 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide: thiazolyl blue (MTT). However, the strongest reaction is with TNBT. When used on samples following electrophosis however, none of the techniques listed above improved the specificity of the activity stain.

3. Since the gels had a band following activity staining whether or not there was sorbitol in the stain, it was presumed this band had "nothing dehydrogenase" activity (46, 77). Techniques known to eliminate these "ghost" bands were tried. Eliminating phenazine methylsulfate breakdown products by scrupulously avoiding light (96) and use of sulfhydryl reagents to block spontaneous reductions in the stains by proteins containing sulfhydryl groups (90) were tried. However, these resulted in an intensification rather than a lessening of the band. Methods to strengthen any mSDH reaction such as addition of cyanide, degassing the gel and stain, and

staining under a nitrogen atmosphere (95),or washing the gel in three changes of 0.01 M phosphate buffer at pH 5.2 to get the gel to the optimum pH for the enzyme and the addition of Ca^{++} and Mg^{++} (required in the ferricyanide assay for the sorbitol dehydrogenase of this strain) were also tried. With the exception of the Ca^{++} and Mg^{++}, all of these techniques noticably increased the darkness of the stain but did not increase the specificity for the substrate.

4. The activity stain was tested with the sorbitol dehydrogenase of a different strain of G. oxydans, IFO 3254. The enzyme was prepared and handled exactly as that from strain ATCC 621. The activity stain worked for the sorbitol dehydrogenase of the IFO strain. A single band of protein was detected and was only seen when sorbitol was present in the stain. The IFO strain has a different membrane-bound sorbitol dehydrogenase than strain ATCC 621 (11); however, it served to show that the activity stain was able to stain specifically and, therefore, the problems lie with the extract.

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