

**The Nature of Sorbitol (A Primary) and Sorbose (A Secondary)**

**Dehydrogenases of *Gluconobacter* species**

by

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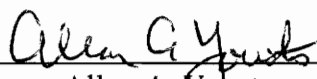
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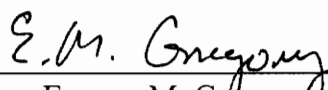
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# **The Nature of Sorbitol (A Primary) and Sorbose (A Secondary)**

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Department of Biology

### **(ABSTRACT)**

The genus *Gluconobacter* is known to carry out limited oxidations using the NAD(P)-independent membrane-bound dehydrogenases in which the products are released back to the medium. Reports of further limited oxidations of these primary oxidation products by *Gluconobacter* in single step or sequential oxidations by secondary dehydrogenases are also published. The objective of this project was to evaluate the nature of one primary (sorbitol) dehydrogenase and one secondary (sorbose) dehydrogenase because of their importance in Vitamin C production. My hypotheses were that sorbitol (the primary) dehydrogenase is constitutive, while sorbose (the secondary) dehydrogenase is inducible. Six *Gluconobacter* strains from three different species grew on plates containing 5% sorbose, indicating their ability to oxidize sorbose thus possessing a secondary dehydrogenase. When four strains were tested for their

ability to carry out the sequential oxidation of sorbitol and then sorbose on media containing growth-limiting sorbitol concentrations, three strains showed possible biphasic growth. However, thin layer chromatography of culture media did not support sequential sorbitol and sorbose oxidation. Ferricyanide assays for sorbitol and sorbose dehydrogenases from membrane fractions isolated from cells grown on glycerol, sorbitol, or sorbose showed that sorbitol dehydrogenase activity in all four strains (three species) tested was always present (constitutive) and its specific activity was always enhanced by growth on sorbose. Membrane fractions showed no or very low constitutive sorbose dehydrogenase activity and no evidence that this secondary dehydrogenase was induced.

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## Table of Contents

<b>List of Figures</b> .....	<b>viii</b>
<b>List of Tables</b> .....	<b>ix</b>
<b>List of Appendices</b> .....	<b>x</b>
<b>INTRODUCTION</b> .....	<b>1</b>
Gluconobacter morphology and habitat.....	1
Oxidative ability & metabolism .....	2
Membrane-bound dehydrogenases (MBDH).....	6
Evidence for constitutive or inducible nature of MBDH.....	7
Purpose of this investigation .....	10
<b>MATERIALS AND METHODS</b> .....	<b>12</b>
Reagents.....	12
Organisms .....	13
Basal Medium.....	13
Growth in broth.....	13
Sorbitol culture maintenance.....	14
Preparing washed inocula.....	15
Growth on 5% sorbose plates .....	15
Growth in broth with varying concentration of sorbitol .....	15
Thin Layer Chromatography (TLC).....	16
Preparation of membrane fractions.....	19

Potassium ferricyanide assay of dehydrogenase activity .....	21
<b>RESULTS.....</b>	<b>25</b>
Growth on 5% sorbose plates .....	25
Constitutive and / or inducible nature of sorbitol and sorbose dehydrogenases from <i>G. frateurii</i> IFO strain 3254.....	27
<i>Biphasic growth</i> .....	27
<i>Ferricyanide assay of dehydrogenase activity</i> .....	30
Constitutive and / or inducible nature of sorbitol and sorbose dehydrogenases from <i>G. oxydans</i> ATCC strain 621 .....	32
<i>Biphasic growth</i> .....	32
<i>Ferricyanide assay of dehydrogenase activity</i> .....	35
Constitutive and / or inducible nature of sorbitol and sorbose dehydrogenases from <i>G. oxydans</i> IFO strain 3293.....	37
<i>Biphasic growth</i> .....	37
<i>Ferricyanide assay of dehydrogenase activity</i> .....	39
Constitutive and / or inducible nature of sorbitol and sorbose dehydrogenases from <i>G. asaii</i> ATCC strain 43781.....	39
<i>Biphasic growth</i> .....	39
<i>Ferricyanide assay of dehydrogenase activity</i> .....	41
Comparisons of sorbitol and sorbose dehydrogenase activities among four <i>Gluconobacter</i> strains.....	44
<b>DISCUSSION.....</b>	<b>46</b>
<i>G. frateurii</i> IFO strain 3254.....	46
<i>G. oxydans</i> ATCC strain 621 and IFO strain 3293.....	50
<i>G. asaii</i> ATCC strain 43781.....	56
<b>CONCLUSIONS .....</b>	<b>58</b>
<b>SUGGESTIONS .....</b>	<b>60</b>
<b>REFERENCES.....</b>	<b>62</b>

**APPENDICES.....67**

**CURRICULUM VITAE .....87**

## List of Figures

- Figure 1. Determination of growth characteristics (a) and concentrations of sorbitol and sorbose (b) using cultures of *G. frateurii* IFO strain 3254 29
- Figure 2. Rates of sorbitol and sorbose oxidation by membrane fraction of *G. frateurii* IFO strain 3254 grown on basal medium plus 5% of either glycerol, sorbitol, or sorbose 31
- Figure 3. Determination of growth characteristics (a) and concentrations of sorbitol and sorbose (b) using cultures of *G. oxydans* ATCC strain 621 34
- Figure 4. Rates of sorbitol and sorbose oxidation by membrane fraction of *G. oxydans* ATCC strain 621 grown on basal medium plus 5% of either glycerol, sorbitol, or sorbose 36
- Figure 5. Determination of growth characteristics (a) and concentrations of sorbitol and sorbose (b) using cultures of *G. oxydans* IFO strain 3293 38
- Figure 6. Rates of sorbitol and sorbose oxidation by membrane fraction of *G. oxydans* IFO strain 3293 grown on basal medium plus 5% of either glycerol, sorbitol, or sorbose 40
- Figure 7. Determination of growth characteristics (a) and concentrations of sorbitol and sorbose (b) using cultures of *G. asaii* ATCC strain 43781 42
- Figure 8. Rates of sorbitol and sorbose oxidation by membrane fraction of *G. asaii* ATCC strain 43781 grown on basal medium plus 5% of either glycerol, sorbitol, or sorbose 43

## List of Tables

Table 1. Growth of <i>Gluconobacter</i> species and strains on plates containing 5% sorbose	26
Table 2. Comparison of rates of sorbitol and sorbose oxidation among gluconobacter species and strains grown on basal medium containing 5% glycerol, sorbitol, or sorbose	45

## List of Appendices

- |  |    |
|--|----|
| Appendix A. Assay of dehydrogenase activity using whole cells and the oxygen uptake assay        | 67 |
| Appendix B. Effect of membrane quantity on sorbitol dehydrogenase (ferricyanide reduction) assay | 86 |

# INTRODUCTION

## *Gluconobacter morphology and habitat*

The genera *Gluconobacter* and *Acetobacter* are called acetic acid bacteria because their species commonly produce acetic acid from ethanol (3). The genus *Gluconobacter* consists of gram negative short rods that are obligately aerobic and characteristically carry out limited oxidations (10). In nature, the gluconobacters are usually found in sugary (and acidic) environments such as flowers and fruits, as opposed to the acetobacters which are found in alcohol-enriched (and acidic) environments such as wines, beer, and vinegar (15). The gluconobacters are also responsible for some spoilage of pineapple, wine, and beer (43). In industries, these bacteria are used during the production Vitamin C, because they can inexpensively oxidize sorbitol to sorbose, a precursor of vitamin C (1, 6, 35, 46).

## ***Oxidative ability & metabolism***

The name *Gluconobacter* was derived from the ability of this genus to partially oxidize glucose to gluconic acid in great quantities (see page 27, Ref. 3). The gluconobacters characteristically accomplish the partial oxidation of sugars and many other aliphatic compounds having 3 or more hydroxyl groups (polyols) (see page 142, Ref. 3). This unique ability is one characteristic which separates *Gluconobacter* from *Acetobacter* because the acetobacters can more completely oxidize polyols.

Limited polyol oxidation serves as the main mechanism of energy production for the gluconobacters (21). These limited oxidations are primarily performed by NAD(P)-independent membrane-bound dehydrogenases that are linked to the electron-transport system (ETS) in the plasma membrane. This process is generally called direct single-step (37) or limited oxidation (31). Oxygen serves as a sole terminal acceptor for the electrons removed by these membrane-bound dehydrogenases and transported through the electron transport system. Products of these limited oxidations are quantitatively released back into the growth medium (3). In fact, Levering et al. (25) and Feshami and Claus (13) reported that these limited oxidations are responsible for 90% conversion of substrates to the oxidation products in these bacteria. Thus, accumulation of the oxidation products in the media and rapid rate of oxygen uptake accompany the oxidation process (19).

Polyol oxidation can also take place inside the gluconobacter cell. Olijve and Kok (31) found that the gluconobacters can transport the primary substrate (glucose) into the

cell when grown in media containing low concentrations of glucose (below 5 mM). A soluble (cytosolic) NAD(P)-*dependent* dehydrogenase then removes a hydrite and a proton and transfers the hydrite to NAD(P)<sup>+</sup>, which in turn is utilized by the electron transport system. Arcus and Edson (2) and Matsushita (27) found that the NAD(P)-*dependent* soluble dehydrogenase have an optimum pH of 7-9, whereas particulate (membrane-bound) enzymes are NAD(P)-*independent* and have an optimum activity at pH of 4-6. The activity of these soluble dehydrogenases, however, is very small when compared to that of the membrane-bound enzymes (27). The products of the soluble dehydrogenases in the gluconobacters are excreted to the medium or can be further oxidized in the cytoplasm, but not completely to CO<sub>2</sub>.

When cytosolic (soluble) dehydrogenases are used for internal polyol oxidation, the hexose monophosphate (HMP) pathway is thought by some to further oxidize the oxidation product to glyceraldehyde-3-phosphate (33). On the other hand, the presence of 2-keto-5-deoxy-6-phosphogluconate (KDPG) aldolase, which is unique to the Entner Doudoroff (ED) pathway, was reported by Kersters & De Ley (20) who suggested that these bacteria use the ED pathway for catabolism. Glyceraldehyde-3-phosphate resulting from either pathway is probably further oxidized to pyruvate using the Embden Meyernhoff Parnas (EMP) pathway. In most cases, the glucobacters do not completely oxidize pyruvate to CO<sub>2</sub> and H<sub>2</sub>O but probably oxidize pyruvate only to acetic acid which is then transported out of the cell.

It is the absence of succinate dehydrogenase in the gluconobacters that renders the TCA cycle to be incomplete and prevents the complete oxidation of energy source to CO<sub>2</sub> and H<sub>2</sub>O (14). Probably, these bacteria use enzymes that are typically associated with the TCA cycle for biosynthesis such as production of glutamate from pyruvate. This incomplete TCA cycle is characteristic of the genus *Gluconobacter* but not genus *Acetobacter* (3, 10).

In addition to the direct single-step oxidations, the gluconobacters may (under specific conditions) carry out a sequence of primary, secondary, and tertiary oxidations of polyols catalyzed by membrane-bound dehydrogenases. During these sequential oxidations, the gluconobacters not only oxidize the primary substrate using primary dehydrogenase, but they further oxidize the primary oxidation product to secondary and perhaps tertiary products using what are called secondary and tertiary dehydrogenases.

Batzing and Claus (5) reported that *Gluconobacter oxydans* in a low concentration of glycerol (0.25%) shows biphasic growth when glycerol is oxidized to dihydroxyacetone. Chemical analysis of the media revealed that during the first phase of exponential growth, glycerol depletion was accompanied by accumulation of the dihydroxyacetone. There was a short lag following the first phase of exponential growth followed by dihydroxyacetone depletion during the second phase of exponential growth. This phenomenon suggested that the initial oxidation of glycerol (a primary substrate) by the glycerol dehydrogenase (a primary MBDH) was followed by a subsequent oxidation

of dihydroxyacetone by a secondary MBDH (dihydroxyacetone dehydrogenase). The lag phase in between the two phases of exponential growth might be caused by induction of the dihydroxyacetone (a secondary) dehydrogenase.

Sequential oxidation of glucose by *G. oxydans* ATCC strain 621-H at pH 5.5 was reported by Weenk et al. (45). They demonstrated that during the primary oxidation, glucose was first oxidized to gluconic acid which accumulated in the medium and resulted in a pH decrease to 3.0. Only when the pH in the medium was maintained at 5.5 could the gluconic acid be further oxidized to ketogluconates (2-ketogluconate and 5-ketogluconate). They also found that ten other strains of *G. oxydans* show this sequential oxidation, indicating that this characteristic is not specific only to ATCC strain 621-H. Two strains from *G. oxydans* subsp. *melanogenes* were shown to further oxidize the ketogluconates to 2,5 diketogluconate. Olijve (31) reported the presence of triphasic growth during this glucose oxidation. These findings suggest the inducibility of the gluconic acid (a secondary) dehydrogenase and ketogluconate (a tertiary) dehydrogenase.

All of these sequential oxidations show that these bacteria are capable of further oxidizing the primary oxidation products and suggest induction of secondary and perhaps tertiary membrane-bound dehydrogenases. However, if the secondary or tertiary dehydrogenases are constitutive, then why do the products of primary or secondary oxidations accumulate in the growth medium? Are these primary, secondary, and tertiary

dehydrogenases the same enzyme and produced constitutively, or are they different enzymes which are induced by the primary or the secondary oxidation products?

### ***Membrane-bound dehydrogenases (MBDH)***

Characterization studies of the NAD(P)-independent membrane-bound dehydrogenases in the gluconobacters revealed that these enzymes are a complex containing either quinoproteins with pyrroloquinoline quinone (PQQ), or flavoproteins with flavin adenine dinucleotide (FAD) as a prosthetic group (27). In single-step oxidations, each of these components plays a role in the transfer of electrons from the substrate to the electron transport system. Purified D-sorbitol (a primary) dehydrogenase of *Gluconobacter suboxydans* subspecies a has three subunits: a flavoprotein (63 kDa), a cytochrome c (51 kDa), and a 17 kDa protein of unknown function (36). Purified L-sorbose (a secondary) dehydrogenase of *Gluconobacter melanogenus* contains only a single polypeptide (58 kDa) with covalently-bound flavin as a prosthetic group (27, 39). Different membrane-bound dehydrogenases were also reported to have different optimum pH (27). These suggest that these dehydrogenases are probably different enzymes.

On the other hand, gluconobacters reportedly oxidize over 100 different compounds (18, 30). Edwards (12) demonstrated that all 40 substrates tested from 9 different chemical categories were oxidized by a membrane fraction isolated from *G.*

*oxydans* ATCC strain 621 grown in glycerol. Because it is inefficient for the *Gluconobacters* to produce 40 different primary membrane-bound dehydrogenases, she suggested that there are only a few types of primary dehydrogenases which are constitutively produced by the gluconobacters. She also suggested that these enzymes may recognize and bind to specific part of the substrate. Sorbitol dehydrogenase, isolated by Van Lare from the membrane fraction of sorbitol-grown *Gluconobacter oxydans* ATCC strain 621 (44), exhibits oxidation of mannitol, glycerol, and other polyols. All of these findings suggest that there is one primary membrane-bound dehydrogenase in *Gluconobacter* for each general class of substrate such as the aliphatic polyols.

It is still not known whether the primary and secondary membrane-bound dehydrogenases in one strain of *Gluconobacter* are the same enzyme or whether it is (they are) constitutive or induced. More convincing evidence for the constitutive and / or inducible nature of these dehydrogenases may come from studies showing the presence or the absence of specific activities from membranes of cells grown using different primary or secondary substrates.

### ***Evidence for constitutive or inducible nature of MBDH***

De Ley and Dochy (11) were the first to report the constitutive nature of particulate (membrane-bound) dehydrogenases (MBDH) from cells grown on glucose, galactose, xylose, and mannitol. They found that particulate (membrane) fractions,

isolated from *G. oxydans* ATCC strain 621 cells grown on one primary substrate, oxidize glucose, gluconate, galactose, L-arabinose, xylose, mannitol, inositol, and *meso*-erythritol with similar rates as cells grown on other substrates. Because all of these are primary substrates, except gluconate, this study suggests that the primary dehydrogenases in this *Gluconobacter* strain are constitutive. However, these authors did not study how growth on secondary substrates might affect the presence of primary or secondary dehydrogenase activity.

Stephen White (47) found that cells grown on sorbitol oxidize several primary and secondary substrates at rates similar to cells grown on sorbose. Since membrane fractions were not used in this study, it is not known if soluble (cytosolic) dehydrogenases contributed to the total oxidation activity.

Buchert and Viikari (8) reported that membrane fractions from *G. oxydans* ATCC strain 621 grown on either of two primary substrates (xylose or glucose) were able to oxidize xylose and glucose even though the specific activities were different.

Furthermore, Edwards (12) showed that four *Gluconobacter* strains grown on one primary substrate (glycerol) did not demonstrate a measurable lag period when shifted to a medium containing another primary substrate (either mannitol, erythritol, adonitol, or glucose). This again supports the constitutive nature of a primary polyol dehydrogenases in *G. oxydans* ATCC strain 621.

Sequential oxidation of some primary substrates suggests the inducible nature of membrane-bound dehydrogenases. However, there are other types of evidence that also support this hypothesis. The first type was reported by Tsukada and Perlman (41), who worked with sorbose (a secondary substrate) oxidation in *G. melanogenus* (*G. oxydans*) IFO strain 3293. They found that these cells maximally convert sorbose to 2-keto-L-gulonic acid only when the cells are grown on sorbose. They used either D- glucose, D- galactose, glycerol, D-fructose, or D-sorbitol as alternative growth substrates.

Kitamura & Perlman (23), showed that sorbose (a secondary) dehydrogenase activity is high when *G. melanogenus* (*oxydans*) IFO strain 3293 cells are grown on sorbitol, fructose, or sorbose (primary and secondary substrates), but low when grown on glycerol, glucose, or galactose (all primary substrates). Hence, they concluded that membrane-bound sorbose dehydrogenase is induced by sorbitol, sorbose, and fructose. Since sorbitol is always converted to sorbose, they proposed that it is actually sorbose that induces the sorbose dehydrogenase. This hypothesis seems legitimate, because fructose can also be converted to sorbose by *G. melanogenus* IFO strain 3292 with 5-ketofructose as an intermediate (see page 202, Ref. 3).

Sugisawa et al. (39) were unable to obtain sorbose dehydrogenase activity from *G. melanogenus* IFO strain 3293 as reported by Kitamura (22). Therefore, Sugisawa et al. purified the membrane-bound L-sorbose dehydrogenase from *Gluconobacter melanogenus* (*G. oxydans*) UV 10 for further study. The UV 10 strain is a mutant

derived from IFO strain 3293. They then demonstrated that sorbose dehydrogenase activity is induced by sorbitol or sorbose in the growth medium. The 100% activity found in sorbitol- or sorbose-grown cells was lowered to 12 % when cells were grown on mannitol, glycerol, or fructose, even though the cells grow to the same optical density on all of these growth substrates. This again supports the hypothesis that sorbose (a secondary) dehydrogenase is inducible.

In summary, some evidence from different *Gluconobacter* strains support the constitutive nature of the primary dehydrogenase and the inducibility of the secondary dehydrogenases. Whether these characteristics can be found in the same *Gluconobacter* strain yet remains a question.

### ***Purpose of this investigation***

The **primary purpose** of my research is to learn more about sorbitol (a primary) dehydrogenase and sorbose (a secondary) dehydrogenase in more than one strain of *Gluconobacter*. Based upon the literature available, my hypotheses were (i) the membrane-bound sorbitol (primary) dehydrogenase in gluconobacters is constitutive, and (ii) the membrane-bound sorbose (secondary) dehydrogenase in *Gluconobacters* is inducible.

There is inconsistency in the literature about the use of the terms *constitutive* and *inducible*. Therefore, I will define an enzyme as constitutive when enzyme activity is

always present in detectable amount regardless the conditions used to grow cells. I will define an enzyme as inducible when it has undetectable activity when cells are grown under one set of conditions and easily detectable activity when the cells are grown under other conditions. This definition is logical, because in order to be constitutive, there must be constant expression of the gene (24). Hence, some dehydrogenase activity can always be observed. On the other hand, if it is inducible, the enzyme will not be produced (except at undetectable levels) unless intracellular conditions are present for induction (derepression).

## MATERIALS AND METHODS

*Reagents.* Bacto peptone, granulated agar, and yeast extract were obtained from Difco Laboratories, Detroit, MI. Bovine serum albumin, Folin & Ciocalteu's phenol reagent, mannitol, sodium carbonate, sodium hydroxide, sorbitol, sorbose, and succinic acid were purchased from Sigma Chemical Co., St. Louis, MO. In addition, cupric sulfate and sodium citrate were obtained from J. T. Baker Chemical Co., Phillipsburg, NJ. Ethanol was obtained from Aaper Alcohol & Chemical, Shellbyville, KY, while ethyl acetate was purchased from Mallinckrodt Chemical Inc., Paris, KY. Acetic acid, acetone, ammonium hydroxide, calcium chloride, glycerol, magnesium chloride, potassium ferricyanide, pyridine, and silver nitrate were purchased from Fisher Scientific, Co., Fair Lawn, NJ.

**Organisms.** The organisms used in this study were *Gluconobacter oxydans* (ATCC strain 621 and IFO strain 3244), *Gluconobacter frateurii* (IFO strain 3254 and IFO strain 3271), and *Gluconobacter asaii* (ATCC strain 43781 and IFO strain 3297a). All of these strains were shown to have high to intermediate oxidation rates for different polyhydroxy compounds (7). In addition, *Gluconobacter oxydans* IFO strain 3293, which reportedly has the ability to oxidize sorbose (40, 41), was also used. These organisms were obtained as described by Micales et al. (29).

**Basal medium.** *Gluconobacter* cultures were routinely grown on a basal medium with varying amounts of one growth substrate (such as glycerol, mannitol, sorbitol, or sorbose). The basal medium was made from (w/v) 1% peptone and 1% yeast extract in ultrapure water (13). Agar plates were prepared with the same basal medium plus growth substrate and 2% of granulated agar.

**Growth in broth.** Cells were grown in 500 ml Bellco Nephelometer flasks containing 50 ml of autoclaved 5% sorbitol, sorbose, or glycerol medium. I found that sorbose caramelized when autoclaved, thus the basal medium plus 5% sorbose was prepared differently. Sorbose was first dissolved separately from the peptone and yeast extract solution, each in half of the final volume of ultrapure H<sub>2</sub>O. This dissolved double-strength sorbose solution was filter sterilized and poured into autoclaved 500 ml Bellco

flasks, that contained the sterile double-strength peptone and yeast extract solution, so that the final volume was 50 ml. When a large cell mass was needed, 2 L Bellco Nephelometer flasks containing 200 ml of broth were prepared in a similar way.

The cultures were incubated at 28°C in a New Brunswick Psychrotherm model R-20 incubator with shaking at 200 reciprocations per minute. Growth was measured with Bausch and Lomb Spectronic 20 spectrophotometer at 620 nm, and cells were harvested in the late exponential phase when the optical density ( $OD_{620}$ ) reached about 0.95.

Broth cultures were tested for purity by inoculating onto plates containing basal medium plus 2.5% mannitol, and 2% granulated agar. After inoculation, the plates were incubated at 28°C for 48 hours.

***Sorbitol culture maintenance.*** All gluconobacter strains were maintained in sorbitol-stock vials according to the method published by White & Claus (46). One ml of broth culture grown in basal medium plus 5% sorbitol was transferred to sterile 5 ml vials containing 2 ml of 6.0 M sterile sorbitol. Vials were then vortexed until all contents were uniformly mixed. These sorbitol-stock cultures were stored in the freezer at -8°C, and the high sorbitol concentration prevented these cell suspensions from freezing. Unfortunately, I found that the sorbitol in these stock cultures very often crystallized. Therefore, a new stock was prepared about every two months. A 0.2 ml volume of stock culture was used to inoculate each 50 ml of broth.

***Preparing washed inocula.*** Washed inocula were prepared in the following way. Cells were grown in basal medium plus 5% of the substrate to be used for preparing membrane fractions. Cells from these subcultures were harvested by 10 minute-centrifugation in a table top centrifuge when the OD<sub>620</sub> reached about 0.95. The cell pellet was washed twice with sterile basal medium (no growth substrate), resuspended in the same basal medium (4 times more concentrated), and then chilled on ice and refrigerated before use. This washing was important for two reasons: the first was to remove the substrate that might remain with the cells, and the second was to starve the cells so that any internalized substrate might be used up during the washing process.

***Growth on 5% sorbose plates.*** This experiment was done to select for strains that can oxidize sorbose well, and therefore might have a higher rate of sorbose oxidation. Cell suspensions from six *Gluconobacter* strains were washed as described, then used to stripe-inoculate sorbose plates. Each plate contained three separate 1.25 inch long-strips from different strains. Plates were incubated at 28°C for 48 hours, and growth characteristics were then assessed based on the amount of growth on each stripe.

***Growth in broth with varying concentration of sorbitol.*** To examine the possibility of biphasic growth, cells were grown in basal medium containing varying amounts of sorbitol as growth substrate. Theoretically, if all of sorbitol in the medium

were oxidized to sorbose, then cells might induce sorbose dehydrogenase and shift to the oxidation of sorbose. Secondary growth on sorbose would then indicate their ability to induce sorbose dehydrogenase. Biphasic growth would only be observed if the two growth rates on sorbitol and sorbose are different (5).

Washed inocula were prepared from cell cultures grown in basal medium plus 5% sorbitol as described previously. A 0.8 ml sample of the washed cell suspension was used to inoculate 200 ml of basal medium containing either 0%, 0.3 %, or 0.5% sorbitol in 2 L Bellco flasks. A 1 ml sample was taken from the flask every hour after inoculation for about 20 hours, and the optical density (OD) of the 10x diluted sample was measured at 620 nm and then multiplied by the dilution factor. The OD<sub>620</sub> values from one type of medium (e.g. 0.3%) were taken for the first 10 hours in one day. On the next day, the OD values for the second 10 hours were determined using another flask of the same medium that had already grown for 10 hours. The OD<sub>620</sub> values from both flasks were then displayed on the same graph to see if the cells show a characteristic of biphasic growth.

***Thin Layer Chromatography (TLC).*** Substrate and oxidation product from broth culture containing 0.3% sorbitol were detected by thin layer chromatography. If broth cultures were undergoing biphasic growth, TLC should show sorbitol depletion and sorbose accumulation during the first (primary) growth phase followed by sorbose depletion during the second growth phase.

One ml samples were taken from the growth flasks at designated times and placed in sterile 1.5 ml microcentrifuge tubes. These samples were centrifuged for 10 minutes in a table top microcentrifuge to remove whole cells. The resulting supernatant fluid was then transferred to another sterile microcentrifuge tube and frozen at  $-8^{\circ}\text{C}$  until all samples could be chromatographed.

Prior to use, TLC plates (glass-backed microcrystalline cellulose thin layer chromatograms [Avicel plates], 20x20 cm, 250  $\mu$  thick, 30  $\mu$  pore size, Analtech Inc., Newark, DE) were activated by heating at  $105^{\circ}\text{C}$  for 10 minutes. Silica gel on the TLC plate served as the stationary phase, and the mobile phase that I used contained a 5:5:1:3 ratio of pyridine : ethyl acetate : water : acetic acid (4, 17).

A developing jar was filled with the mobile phase to a depth of between 0.5 to 1 cm. To saturate the atmosphere quickly during the early chromatography period, the inside surface of the developing jar was lined with 3MM Whatman chromatography paper moistened with the same solvent used in the mobile phase (4). To remove possible impurities, TLC plate was predeveloped by placing it in the developing jar at room temperature until the solvent reached the top of plate. This predevelopment generally took about 2 hours, then the predeveloped TLC plate was dried in a hood for at least 6 hours at room temperature.

Five  $\mu\text{l}$  samples were applied to the predeveloped TLC plate using a micropipettor, so that the diameter of the spot was not more than 4 mm. These spots

were placed 2 cm from the bottom of the plate, and exactly 4.5 cm from other samples. Five  $\mu\text{l}$  aqueous solutions of 0.5% authentic sorbitol or sorbose were also spotted onto the plate. The spots were air-dried, and the plate was developed, as described in the predevelopment, until the solvent reached its top. After air-drying at room temperature for at least 6 hours, the plate was developed in the second dimension ( $90^\circ$  from the first direction) using the same solvent, and then dried again at room temperature. Usually, only 3 samples in one chromatogram could be effectively separated in two dimensions.

Sorbitol and sorbose spots were visualized by the following treatment. Saturated  $\text{AgNO}_3$  solution (0.1 ml) was diluted with acetone to a final volume of 20 ml, then water was added dropwise with stirring until all silver nitrate was redissolved. TLC plates were then dipped into this silver nitrate-acetone solution. After all the silver nitrate solution had evaporated from the dipped TLC plates, the plates were lightly sprayed with a solution of 0.5 N NaOH in ethanol until brown spots were obtained on a white background (42). Too much spray with NaOH solution resulted in a yellow background. To remove excess silver oxide from the background, and also to fix the spots on the TLC plates (4), the plates were sprayed with 6N aqueous ammonium hydroxide until saturated. The plates were then immediately rinsed under a slowly running stream of water for 1 to 4 hours. Rinsing was done very gently, since the highly basic ammonium hydroxide solution could dissolve the calcium sulfate binder and cause the silica gel layer

to become detached from the glass plate. As soon as the background was fully cleared, TLC plates were dried by heating at 95°C for 10 minutes.

At equilibration between the stationary and mobile phase, sorbitol and sorbose in the sample were retained at different location in the TLC plates. The retention occurred such that the ratio between the migration distance traveled by sorbitol and sorbose and the total distance traveled by the leading edge of the mobile phase, which is also called  $R_f$  values, were 0.41 and 0.44, respectively (17). Because these values were very close to each other, I did not get good separation between sorbitol and sorbose in one dimension. For this reason, the TLC plates were developed in two dimensions.

Isono (17) also reported that a solvent containing water-saturated phenol gave better separations for sorbitol and sorbose with  $R_f$  values of 0.55 and 0.45, respectively. However, I found that development in this solvent resulted in big smears instead of clear round spots and with gray instead of white background.

***Preparation of membrane fractions.*** Membrane fractions were prepared for use in the ferricyanide assay. Washed cell suspensions were prepared as described for washed inocula, except that the final cell pellet was resuspended so that cells were 20 times more concentrated than in the original culture. A 0.2 ml sample of these suspensions was used to inoculate 2 L flasks containing 200 ml of basal medium plus 5% (w/v) of appropriate growth substrate.

Cultures for membrane preparation were grown to an optical density (620 nm) of approximately 0.95, and harvested by centrifugation at 13,500 x g for 20 minutes at 4°C in a Beckman Centrifuge (model J2-21) with a JA-14 rotor. Cell pellets resulting from centrifugation were washed twice with 0.07 M of cold succinate buffer (pH 5.2) then resuspended in 40 ml of this buffer. This suspension was chilled on ice, held in the refrigerator until the next day, and called the washed whole cell suspension.

The washed whole cell suspension was passed through a pre-cooled 40K French Pressure cell (SLM AMINCO, Urbana, IL) three times to break open the cells. A pressure of 20,000 pound per square inch (psi) was maintained by the French Pressure press (SLM AMINCO, Urbana, IL). The resulting suspension was then centrifuged twice at 30,000 x g for 20 minutes at 4°C in a Beckman Centrifuge (model J2-21) with a JA-20 rotor to remove all unbroken cells. The supernatant fluid from the second centrifugation now contained both the membrane fraction and the soluble fraction. To separate these fractions, the supernatant fluid was transferred to nitrocellulose ultracentrifuge tubes (Beckman) and centrifuged at 120,000 x g in a Beckman ultracentrifuge (model J8-80) for 2 hours at 4°C with an SW 41 Ti swinging bucket rotor. The resulting supernatant fluid was called the soluble fraction, and pellet, which contained the membrane fraction, was resuspended in 0.07 M succinate buffer (pH 5.2) using a tissue grinder and ultracentrifuged as before. The resulting pellet was mixed with about 1.5 ml of 0.07 M succinate buffer (pH 5.2), and thoroughly homogenized with a tissue

grinder. This homogenized pellet was called the membrane fraction. Before use, the membrane fraction was chilled on ice and stored at 4°C (12).

***Potassium ferricyanide assay of dehydrogenase activity.*** Potassium ferricyanide ( $K_3Fe(CN)_6$ ) is an artificial electron acceptor that loses color upon reduction. In the enzyme assay, the rate of color loss is proportional to the rate of substrate oxidation. Therefore, the rate of enzyme activity can be measured spectrophotometrically at 301 nm, the wavelength at which  $K_3Fe(CN)_6$  absorbs maximally.

Reaction mixtures in a 1 ml quartz cuvette contained 10  $\mu$ moles of  $MgCl_2$ , 5  $\mu$ moles of  $CaCl_2$ , 1  $\mu$ mole of potassium ferricyanide ( $K_3Fe(CN)_6$ ), and 250  $\mu$ moles of substrate, all dissolved in 0.8 ml of 0.07 M succinate buffer (pH 5.2). The volume of succinate buffer was adjusted so that, after the addition of membrane fraction, the final volume of the reaction mixture was 1.0 ml. The reaction was started by adding 5 to 20  $\mu$ l (0.02 - 0.06 mg) of membrane fraction with a Hamilton syringe. All reagents except the membrane fraction were kept at room temperature before use. The membrane fraction was kept on ice and refrigerated, and it was homogenized well just prior to its addition. A reaction mixture lacking potassium ferricyanide was used as a blank to adjust the spectrophotometer to 100% transmission.

Immediately after the addition of membrane fraction, the cuvette was inverted three times and placed in the Milton Roy Spectronic spectrophotometer (model 1201). The rate of ferricyanide reduction (substrate oxidation) was measured as described by Edwards (12), except that the absorbance change in the reaction mixture was measured every three seconds instead of every thirty seconds. This change was made in order to measure the *initial* enzymatic reaction (see page 104, Ref. 28). Absorbance readings were taken for a total of 150 seconds at a wavelength of 301 nm. I confirmed the findings of Edwards (12) who demonstrated that potassium ferricyanide was absorbed maximally at 301 nm (data not shown). A standard curve using known amounts of potassium ferricyanide dissolved in 0.07M succinate buffer (pH 5.2) was prepared at 301 nm for determining the quantities of ferricyanide reduced by membrane fractions (12).

I ran all reactions at room temperature. Ideally, the reactions should have been done at 38°C, which is the optimum temperature for some membrane-bound dehydrogenases (12, 46). However, I was interested in the *relative* dehydrogenase activities among cells grown with different substrates, and I reasoned that the results would be comparable, because all experiments were done in the same way.

Assays for each membrane fraction and each substrate (sorbitol and sorbose) were performed in triplicate, and the rate of change in absorbance was plotted for each replicate reaction. A best fit regression line was drawn through each curve, and oxidation rate was

calculated over a 90-second linearity that was closest to the initial measurement. A linear rate was observed after 3 to 15 seconds. The total change in absorbance over this 90-second period of linear reaction was then quantitatively determined using the standard curve. Then the rate was expressed in  $\mu\text{mole}$  of substrate oxidized per minute.

The Lowry assay was done to calculate how much protein was present in each membrane fraction, and the final rate of oxidation was expressed as nmole of substrate oxidized per minute per mg of membrane protein.

Three controls were performed to determine the amount of ferricyanide reduction attributed to the membrane-bound dehydrogenases. The first two controls lacked either membrane fraction or substrate, and the third control contained boiled membrane fraction. These controls were also used by Edwards (12). I found that the membrane fraction from the gluconobacters was not fully destroyed until after 1.5 to 2 hours boiling or 15 minutes of autoclaving (data not shown). The amount of ferricyanide reduction from controls lacking substrate was subtracted from the reduction observed in the presence of substrate (12). In addition to what was done by Edwards, I also subtracted the amount of ferricyanide reduced in reaction mixtures lacking membrane fraction. I found that this was important because some substrates (especially sorbose) seemed to cause a slight amount of ferricyanide reduction.

When the assays were done with 5, 10, 15 or 20  $\mu$ l of membrane fractions, activity rates for 10, 15, and 20  $\mu$ l samples demonstrated close to twice, three and four times the rates of ferricyanide reduction observed with 5  $\mu$ l samples, respectively (Figure B1, appendix B). These results demonstrate that potassium ferricyanide reduction is caused by enzymes, and not other materials in these membrane fractions.

## RESULTS

### *Growth on 5% sorbose plates*

Different gluconobacter strains were grown on sorbose plates to determine which might best grow on sorbose, and therefore, be most likely to induce the greatest amount or most active sorbose dehydrogenase. All of the six strains tested grew on sorbose plates, but the amount of growth was different (Table 1). Both *Gluconobacter frateurii* strains grew best, while strains of *G. oxydans* and *G. asaii* showed less growth. Based upon these results, the following three strains, representing three different *Gluconobacter* species and three different levels of growth, were selected for the subsequent experiments: *G. oxydans* ATCC strain 621 (+), *G. frateurii* IFO strain 3254 (+++), and

Table 1. Growth of *Gluconobacter* species and strains on plates containing 5% sorbose <sup>a</sup>

Species	Strain	Growth on 5% sorbose plates <sup>b</sup>
<i>Gluconobacter oxydans</i>	ATCC 621	+
	IFO 3244	++
<i>G. frateurii</i>	IFO 3254	+++
	IFO 3271	+++
<i>G. asaii</i>	ATCC 43781	++
	ATCC 3297a	+

<sup>a</sup> Plates containing 5% sorbose were prepared and stripe-inoculated with a washed cell suspension as described in the Materials and Methods.

<sup>b</sup> Growth was assigned a plus value relative to the amount of growth on the plate. A + indicates the least growth and a +++ the most growth.

*G. asaii* ATCC strain 43781 (++)). In addition, *G. oxydans* IFO strain 3293 was included in the following studies because that strain was known to oxidize sorbose (16, 40, 41).

### ***Constitutive and/or inducible nature of sorbitol and sorbose dehydrogenases from G. frateurii IFO strain 3254***

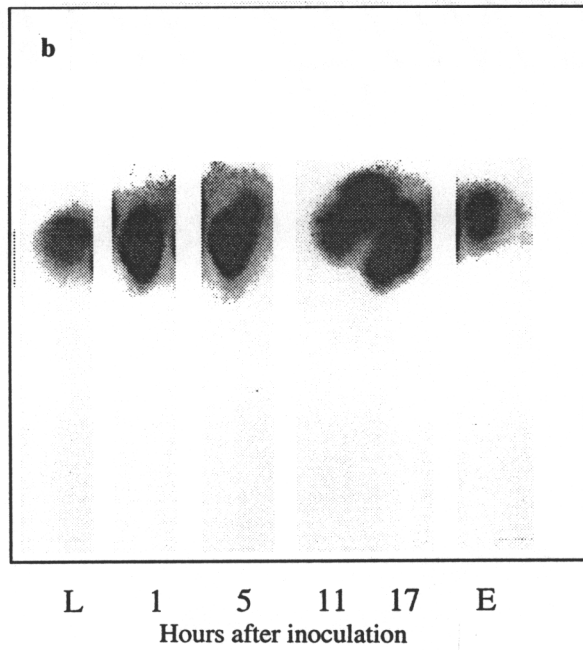
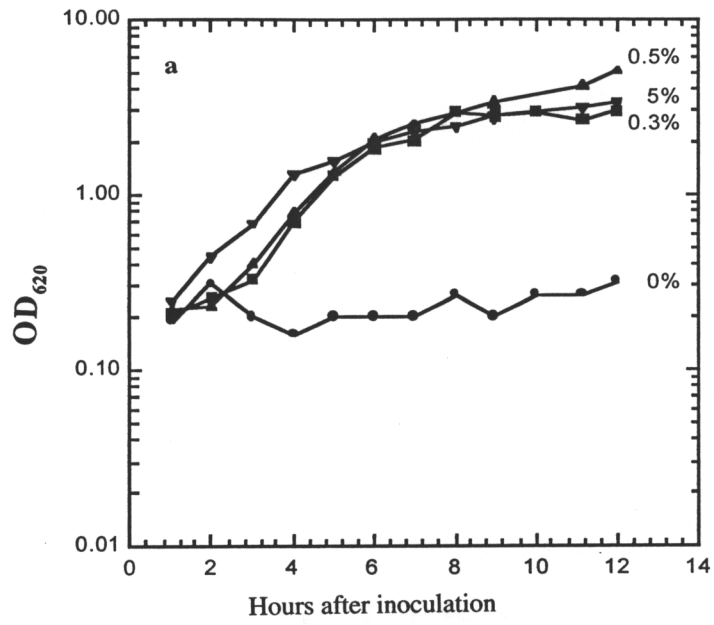
**Biphasic growth.** I examined *G. frateurii* IFO strain 3254 first, because this strain was one of two strains that showed the best growth on 5% sorbose plates (Table 1). Therefore, I assumed that this strain could induce the most active or the greatest amount of sorbose dehydrogenase. I used substrate concentration similar to that used by Batzing & Claus (5) for biphasic growth in limiting concentrations of glycerol. Therefore, I expected to see biphasic growth when cells were grown on growth-limiting concentration (0.3%) of sorbitol, assuming that these cells had different growth rates on sorbitol and sorbose.

The results, however, did not support my hypothesis. *Gluconobacter frateurii* IFO strain 3254 showed very similar rates of growth on 0.3%, 0.5%, and 5% sorbitol media (Figure 1a), and no evidence of biphasic growth was found at these substrate concentrations.

The thin layer chromatographic analysis of the culture medium containing 0.3% sorbitol was performed to look at substrate consumption by these cells. The results (Figure 1b) do not support the hypothesis of biphasic growth. A spot corresponding to

Figure 1. Determination of growth characteristics (a) and concentrations of sorbitol and sorbose (b) using cultures of *G. frateurii* IFO strain 3254. Growth of cells in basic medium containing 0.3% sorbitol is shown in (a) along with growth on the basic medium containing 0%, 0.5%, and 5% sorbitol as comparison. A composite of thin layer chromatograms in (b) shows the disappearance of sorbitol and the accumulation of sorbose in the medium during the cells growth on 0.3% sorbitol. Cell cultures were sampled at designated hours and spotted on thin layer chromatograms after removal of cells. A known amount of authentic sorbitol (L) and sorbose (E) were also spotted. Chromatograms were developed in two dimensions using pyridine solution as described in materials and methods. The chromatographed results from multiple spots on two separate chromatograms were cut and put together in one diagram.

Figure 1. (continued)



authentic sorbitol appears at hour one, and this spot, together with a spot corresponding to authentic sorbose appear at hour five. This indicates that sorbitol was being converted to sorbose during the first 5 hours of incubation. At 11 and 17 hours after incubation, the sorbitol is still present and the size of sorbose spots did not decrease. These results indicate that these cells grew on 0.3% sorbitol throughout this 17 hours of incubation. Apparently, 0.3% sorbitol was not a growth-limiting concentration for *G. frateurii* IFO strain 3254, and this condition was not adequate for inducing detectable quantities of sorbose dehydrogenase.

**Ferricyanide assay of dehydrogenase activity.** Membrane fractions were prepared from *G. frateurii* IFO strain 3254 grown on 5% of either glycerol, sorbitol, or sorbose and then assayed for their rates of sorbitol and sorbose oxidations. I hypothesized that if a membrane-bound dehydrogenase is constitutive, activity will be shown regardless of the substrate used for cell growth, but if the dehydrogenase is inducible, then activity will be present only when the inducer (derepressor) is present in the growth medium.

Since *G. frateurii* IFO strain 3254 grew well on sorbose plates, I expected to see a high sorbose dehydrogenase activity in membrane from sorbose-grown cells. The results (Figure 2), however, show that cells grown on all three growth substrates had no significant sorbose dehydrogenase activity. Glycerol-, sorbitol-, and sorbose-grown cells

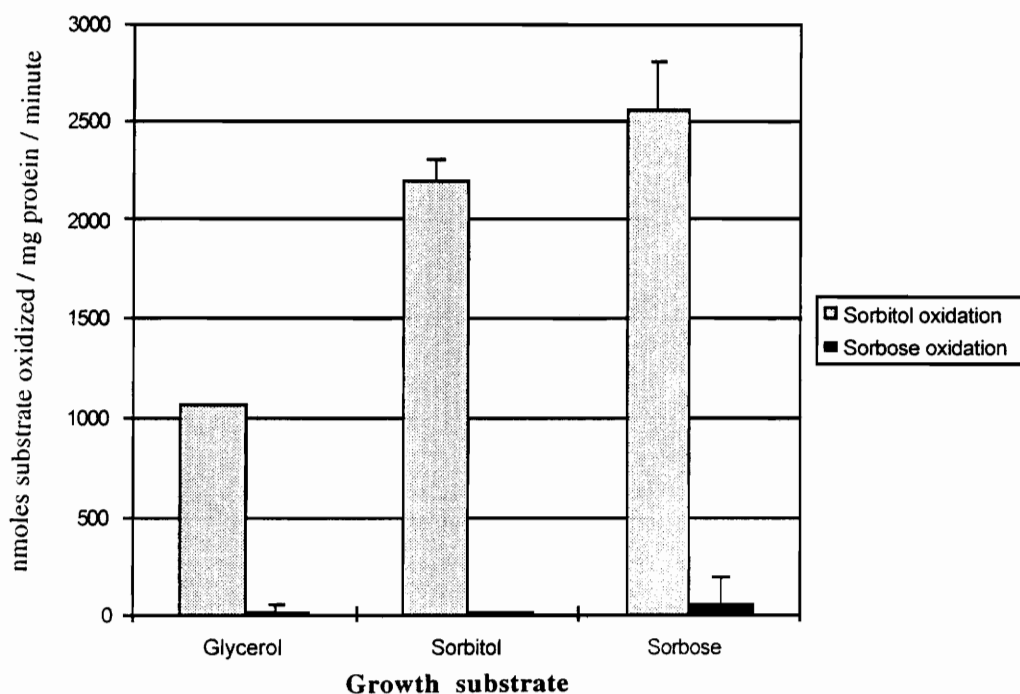


Figure 2. Rates of sorbitol and sorbose oxidation by membrane fraction of *G. frateurii* IFO strain 3254 grown on basal medium plus 5% of either glycerol, sorbitol, or sorbose. Oxidation rates were determined spectrophotometrically from the rate of disappearance of oxidized potassium ferricyanide as described in materials and methods. Oxidation rates shown here were obtained from the average of at least three replicates from one batch of cells minus substrate auto-oxidation and the membrane endogenous oxidation. Standard deviations were calculated from replicate values, and the plus value for each is shown on top of each bar.

show sorbose dehydrogenase activities of  $14 \pm 32$ ,  $11 \pm 6$ , and  $58 \pm 131$  nmoles of substrate oxidized / mg protein / minute, respectively. Even though the activity seemed higher in sorbose-grown cells, the high standard deviation showed that these numbers were not significant. Therefore, sorbose dehydrogenase was not detected under the conditions used in this study.

On the other hand, sorbitol dehydrogenase seemed to be constitutive. Its activity was always present in cells grown on glycerol, sorbitol, and sorbose. The glycerol grown cells show an oxidation rate of  $1,100 \pm 12$  nmoles of substrate oxidized / mg protein / minute. This activity was enhanced by sorbitol ( $2,200 \pm 11$  nmoles / mg protein / minute) and sorbose ( $2,600 \pm 240$  nmoles / mg protein / minute).

### ***Constitutive and/or inducible nature of sorbitol and sorbose dehydrogenases from *G. oxydans* ATCC strain 621***

**Biphasic growth.** Since *G. frateurii* IFO strain 3254 showed no biphasic growth on basal medium plus 0.3% sorbitol, I tested *G. oxydans* ATCC strain 621. This was the strain that exhibits biphasic growth when grown on 0.25% glycerol (5). In contrast to *G. frateurii* IFO strain 3254, *G. oxydans* ATCC strain 621 showed the least growth on sorbose medium (Table 1). I reasoned that since *G. oxydans* ATCC strain 621 does not grow well on sorbose, it might have a lower growth rate on sorbose than on sorbitol, and may demonstrate biphasic growth if sorbose dehydrogenase were induced.

The rate of growth of ATCC strain 621 cells on 0.3% and 0.5% sorbitol medium decreased when the optical density reached about 0.7 and 1.0, respectively (Figure 3a). On the other hand, this strain continued growing to 4.0 OD when placed in media containing 5% sorbitol. Therefore, 0.3% and 0.5% sorbitol concentrations were *limiting* cell growth in this basal medium.

Based on the shape of the curves shown in Figure 3a, *G. oxydans* ATCC strain 621 showed a possible biphasic-type of growth on medium containing 0.3% sorbitol. The primary growth phase appeared to take place between 1 and 5 hours after incubation, while the secondary phase, with an apparent lower growth rate, took place between 5 and 11 hours after inoculation. If this were true, then cells might be growing on sorbitol during the primary phase and on sorbose during the secondary phase.

However, chromatography of the growth medium, did not confirm biphasic growth on 0.3% sorbitol (Figure 3b). Only some of the sorbitol was shown to be converted to sorbose by the end of the apparent primary phase (hour 5). Sorbitol was not fully depleted until sometime between hour 11 and 17. Therefore, growth on sorbose actually took place between hour 1 and sometime after hour 11, instead of between hours 1 and 5. In addition, the cells that were thought to undergo the secondary phase and grow on sorbose between hour 5 and 11, did not seem to consume any sorbose through hour 17. Instead, the sorbose concentration appears to be increasing between hours 5 and 11, and reached an apparent maximum at 17 hours. As a result, the chromatographic data did

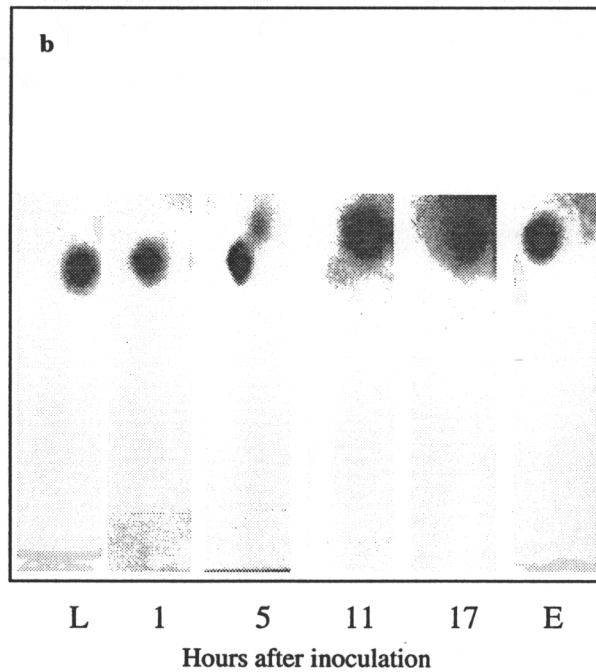
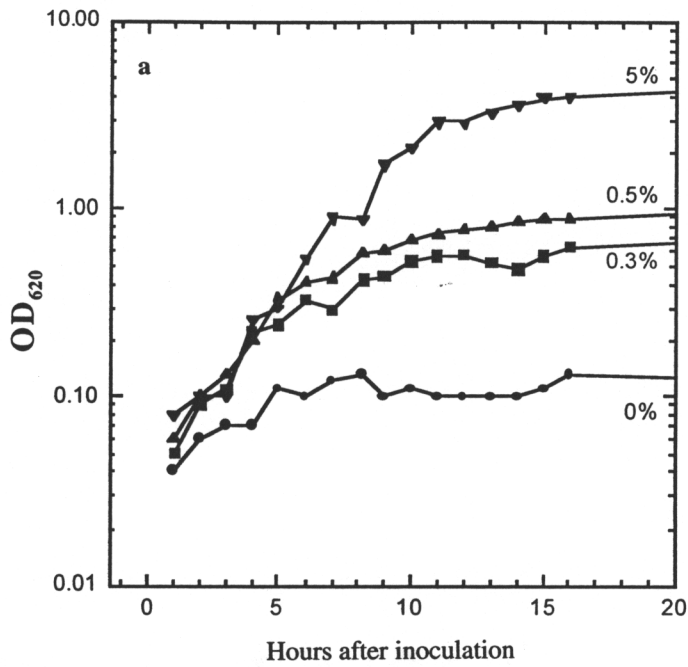


Figure 3. Determination of growth characteristics (a) and concentrations of sorbitol and sorbose (b) using cultures of *G. oxydans* ATCC strain 621. Experiments were done as described in Figure 1.

## RESULTS

not support the presence of biphasic growth on media containing limiting sorbitol concentrations, nor did they support the existence of an active sorbose dehydrogenase.

**Ferricyanide assay of dehydrogenase activity.** Sorbitol dehydrogenase activities were shown by membrane fractions from *G. oxydans* ATCC strain 621 grown on glycerol (3,000 nmoles of sorbitol oxidized / mg protein / minute), sorbitol (4,800 nmoles / mg protein / minute), and sorbose (11,000 nmoles / mg protein / minute) (Figure 4). Similar to that in *G. frateurii* IFO strain 3254 (Figure 2), sorbitol dehydrogenase activity was higher in sorbose-grown cells.

Low sorbose dehydrogenase activities were found in cells grown on glycerol (120 nmoles / mg protein / minute), sorbitol (36 nmoles / mg protein / minute), and sorbose (51 nmoles / mg protein / minute). Since only one experiment was done, the significance of these low values is not known.

These results again support the constitutive nature of sorbitol dehydrogenase, because activity was present regardless of the substrate used to support cell growth, and the noninducible nature of sorbose dehydrogenase.

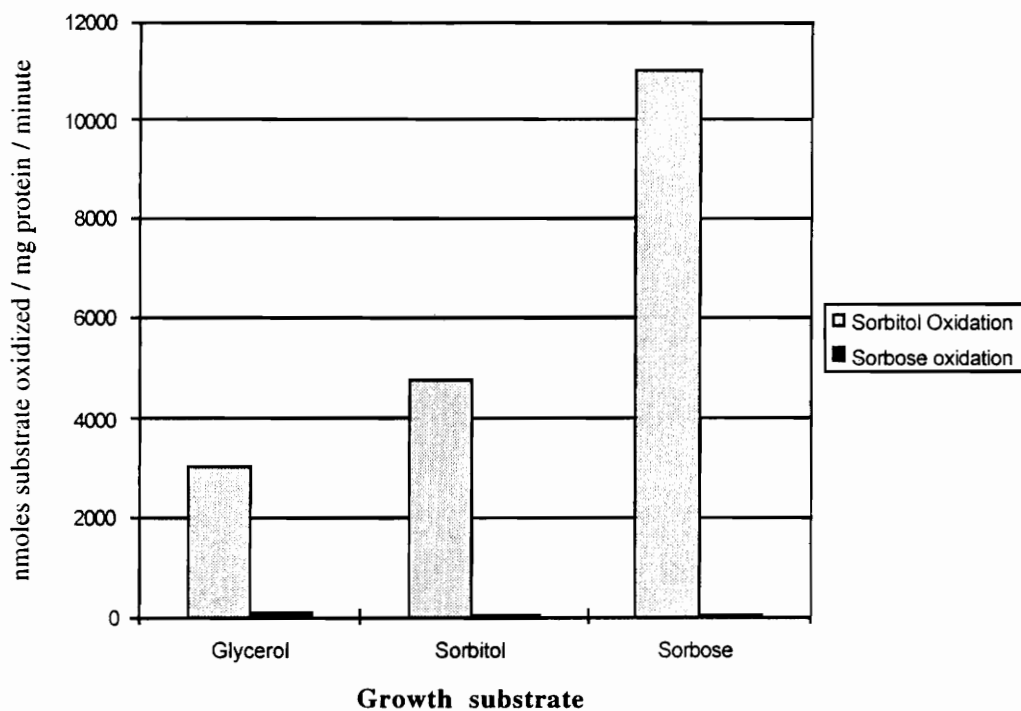


Figure 4. Rates of sorbitol and sorbose oxidation by membrane fraction of *G. oxydans* ATCC strain 621 grown on basal medium plus 5% of either glycerol, sorbitol, or sorbose. Rates of sorbitol and sorbose oxidation were measured as described in Figure 2, except that the rates were obtained from only one replicate.

### ***Constitutive and/or inducible nature of sorbitol and sorbose dehydrogenases from G. oxydans IFO strain 3293***

**Biphasic growth.** Since I had no evidence of sorbose dehydrogenase activity in either *G. frateurii* IFO strain 3254 or *G. oxydans* ATCC strain 621, I then chose *G. oxydans* IFO strain 3293 that was used in sorbose oxidation experiments of Tsukada et al. (40, 41). Others have found that IFO strain 3293 was found to oxidize sorbose to precursors of vitamin C such as sorbosone (22, 39) or 2-keto-L-gulonic acid (16, 38). Since this strain can oxidize sorbose, I proposed that it would show biphasic growth on limiting sorbitol concentration and exhibit sorbose dehydrogenase activity using the potassium ferricyanide assay.

Growth on basic medium plus 0.3% sorbitol appeared biphasic (Figure 5a). Primary growth seemed to take place between hours 1 and 5, and secondary growth between hours 5 and 13.

Thin layer chromatography of growth medium (Figure 5b) showed that during the primary phase, there was an active sorbitol dehydrogenase which converted sorbitol to sorbose. However, during the suspected secondary phase (hours 5 to 13) there was no detectable sorbose consumption by the cells. Therefore, TLC detection of sorbitol and sorbose concentration did not support the presence of biphasic growth nor the induction of sorbose dehydrogenase.

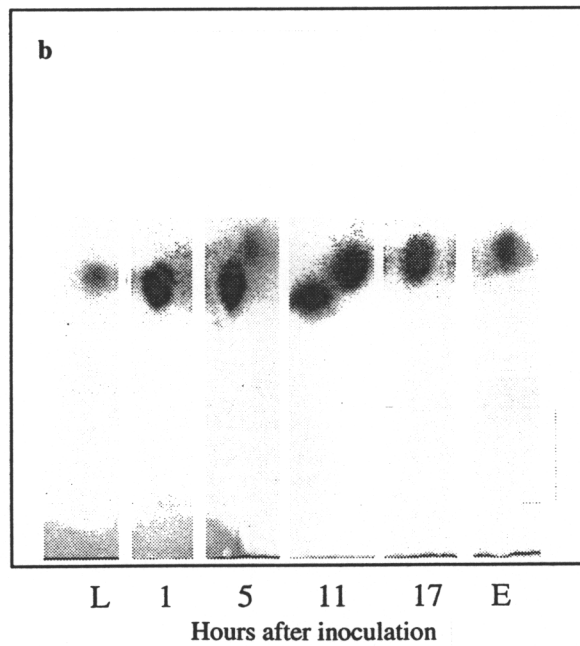
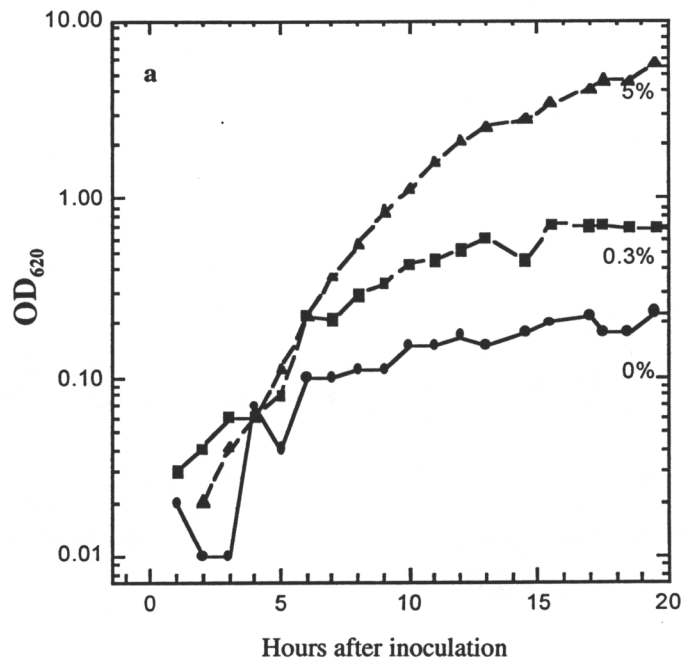


Figure 5. Determination of growth characteristics (a) and concentrations of sorbitol and sorbose (b) using cultures of *G. oxydans* IFO strain 3293. Experiments were done as described in Figure 1.

RESULTS

**Ferricyanide assay of dehydrogenase activity.** Figure 6 shows that membranes from cells grown on sorbitol or glycerol demonstrated no significant difference in sorbitol dehydrogenase specific activity ( $630 \pm 201$  and  $530 \pm 102$  nmoles / mg protein / minute, respectively). This activity was increased to  $2,400 \pm 163$  nmoles / mg protein / minute when sorbose was used as the growth substrate. Therefore, it appears that sorbitol dehydrogenase is constitutive in membranes of *G. oxydans* IFO strain 3293, but enhanced by growth on sorbose.

In contrast to the previous two strains, a low but significant sorbose dehydrogenase activity was observed in cells grown on glycerol, sorbitol, or sorbose ( $230 \pm 117$  and  $240 \pm 137$ , and  $120 \pm 42$  nmoles of sorbose oxidized / mg protein / minute, respectively). Considering the higher standard deviations for sorbose dehydrogenase activities by glycerol- and sorbitol-grown cells, I believe that this sorbose dehydrogenase activity by cells grown on three different growth substrates was very similar. These results show that both sorbitol and sorbose dehydrogenase from *G. oxydans* IFO strain 3293 are constitutive.

### ***Constitutive and/or inducible nature of sorbitol and sorbose dehydrogenases from G. asaii ATCC strain 43781***

**Biphasic growth.** I next examined *G. asaii* ATCC strain 43781 which showed moderate growth on sorbose plates. The growth of this strain was limited by 0.3%

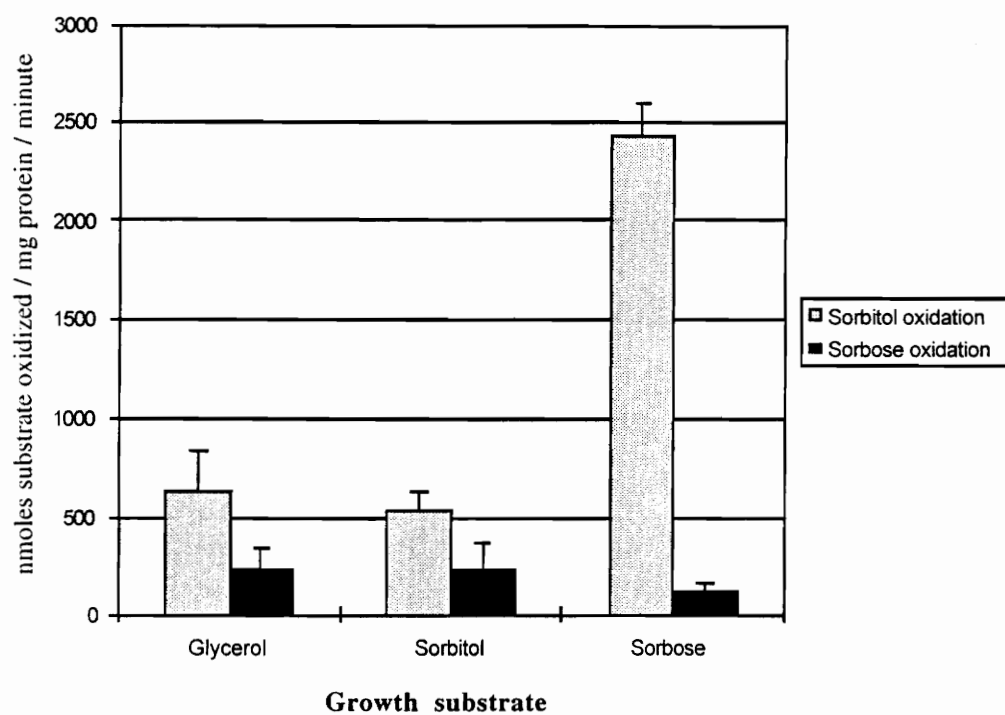


Figure 6. Rates of sorbitol and sorbose oxidation by membrane fraction of *G. oxydans* IFO strain 3293 grown on basal medium plus 5% of either glycerol, sorbitol, or sorbose. Rates of sorbitol and sorbose oxidation were measured as described in Figure 2.

sorbitol and appeared to be biphasic, although the two phases were not very distinct (Figure 7a). The apparent primary phase took place between hours 1 and 5, where the rate of growth was the same as that in 5% sorbitol medium. The apparent secondary phase took place between hours 5 and 13. However, these assumptions were not supported by evidence from the chromatograms, which again showed only sorbitol consumption during the primary phase and no sorbose consumption during the secondary phase (Figure 7b). Therefore, it appeared that measurable sorbose dehydrogenase was not induced by growth of *G. asaii* ATCC strain 43781 on limiting concentrations of sorbitol.

**Ferricyanide assay of dehydrogenase activity.** *G. asaii* ATCC strain 43781 (Figure 8) showed a very similar oxidative characteristics with *G. oxydans* IFO 3293 (Figure 6). Sorbitol dehydrogenase activity was found in *G. asaii* ATCC strain 43781 cells when grown on glycerol, sorbitol, or sorbose medium. In glycerol- and sorbitol-grown cells, the specific activities were  $560 \pm 87$  and  $350 \pm 20$  nmoles of sorbitol oxidized / mg protein / minute. The activity of sorbose-grown cells was  $1,100 \pm 44$  nmoles / mg protein / minute.

Low but significant amount of sorbose dehydrogenase activity was also found in glycerol-, sorbitol-, and sorbose-grown cells (specific activities of  $170 \pm 21$ ,  $160 \pm 93$ , and  $180 \pm 28$  nmoles of sorbose oxidized / mg protein / minute, respectively).

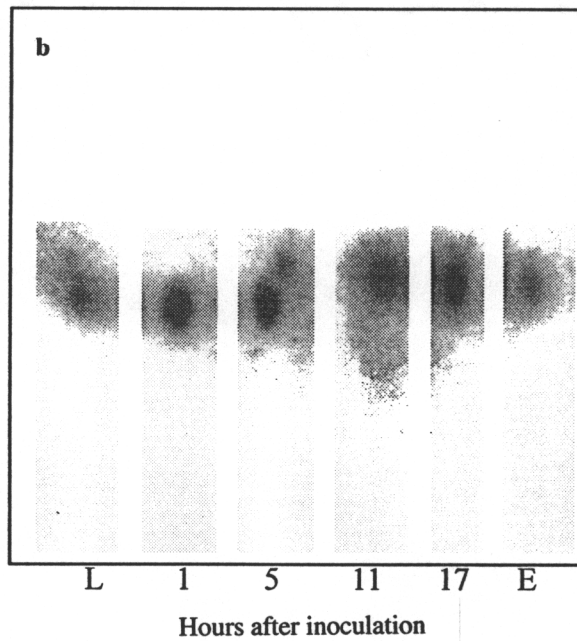
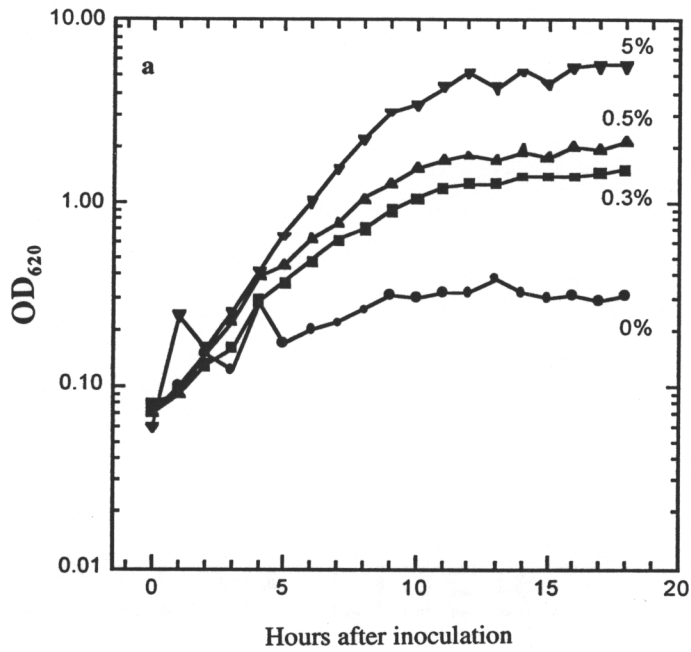


Figure 7. Determination of growth characteristics (a) and concentrations of sorbitol and sorbose (b) using cultures of *G. asaii* ATCC strain 43781. Experiments were done as described in Figure 1.

## RESULTS

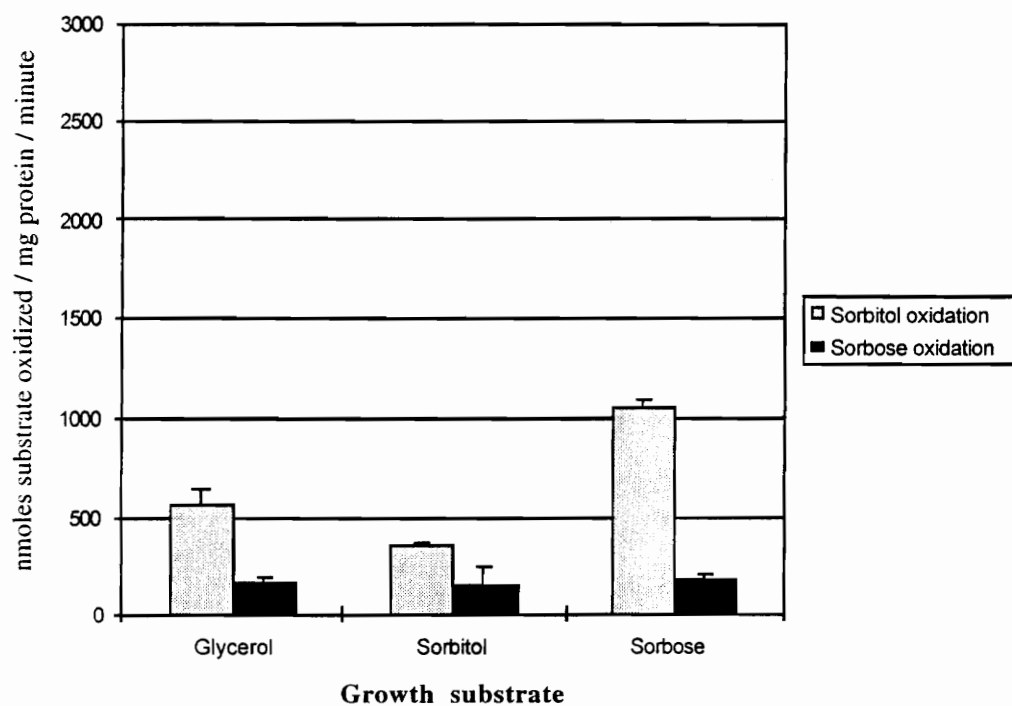


Figure 8. Rates of sorbitol and sorbose oxidation by membrane fraction of *G. asaii* ATCC strain 43781 grown on basal medium plus 5% of either glycerol, sorbitol, or sorbose. Rates of sorbitol and sorbose oxidation were measured as described in Figure 2.

These data support the constitutive nature of both sorbitol and sorbose dehydrogenases in *G. asaii* ATCC strain 43781.

### ***Comparisons of sorbitol and sorbose dehydrogenase activities among four Gluconobacter strains***

Sorbitol dehydrogenase in gluconobacters, as represented by three gluconobacter species, is a constitutive enzyme (Table 2). Even though the specific activity of the sorbitol dehydrogenase varies greatly among gluconobacter strains, it seems always enhanced by growth on sorbose.

In addition, under the assay conditions used in this study, low but significant amounts of constitutively produced sorbose dehydrogenase were detected in only two out of the four strains studied. However, none of the growth conditions used in this study caused *induction* of sorbose dehydrogenase in the four strains from three different *Gluconobacter* species.

Table 2. Comparison of sorbitol and sorbose oxidation rates among gluconobacter species and strains grown on basal medium plus 5% of either glycerol, sorbitol, or sorbose <sup>a</sup>

Species	Strain	Growth Substrate	Rate of substrate oxidation (nmoles / mg protein / min)	
			Sorbitol	Sorbose
<i>Gluconobacter frateurii</i>	IFO 3254	Glycerol	1,100 ± 14	14 ± 32
		Sorbitol	2,200 ± 116	11 ± 6
		Sorbose	2,600 ± 240	58 ± 132
<i>G. oxydans</i>	ATCC 621 <sup>b</sup>	Glycerol	3,000	120
		Sorbitol	4,800	36
		Sorbose	11,000	51
	IFO 3293	Glycerol	630 ± 202	230 ± 117
		Sorbitol	530 ± 102	240 ± 137
		Sorbose	2,400 ± 163	120 ± 42
<i>G. asaii</i>	ATCC 43781	Glycerol	560 ± 87	170 ± 21
		Sorbitol	350 ± 20	160 ± 93
		Sorbose	1,100 ± 44	180 ± 28

<sup>a</sup> Rates of sorbitol and sorbose oxidation were measured by the reduction of potassium ferricyanide in the assay reaction mixture in the presence of sorbitol or sorbose as described in materials and methods. Each number represents an average of three replicates from one batch of cells ± standard deviations.

<sup>b</sup> Data obtained from one replicate.

## DISCUSSION

***Gluconobacter frateurii* IFO strain 3254.** Since both *G. frateurii* strains grew well on plates containing 5% sorbose (Table 1), these strains either have constitutive sorbose dehydrogenase or are able to induce this enzyme. If they induce sorbose dehydrogenase, they should show biphasic growth characteristics on medium containing growth-limiting amounts of sorbitol (0.3%). The results in Figure 1, however, show that *G. frateurii* strain IFO 3254 has similar growth characteristics on basal media containing either 0.3%, 0.5%, or 5% sorbitol. There are two possible explanations for this. First, after the cells in 0.3% sorbitol medium ran out of sorbitol, they might grow on sorbose at the *same* growth rate as on sorbitol. As a result, the two growth phases cannot be

distinguished. Second, 0.3% sorbitol may not be a growth-limiting concentration, thus these cells may reach maximum stationary phase during growth on 0.3% sorbitol.

Chromatographic results did not support the first explanation, because the size of sorbose spots did not appear to decrease during 17 hours incubation (Figure 1). This suggests that cells grew only on sorbitol during these 17 hours. Therefore, I found that 0.3% sorbitol does not limit the growth of *G. frateurii* IFO strain 3254 and, as a result, this medium did not provide the conditions for sorbose dehydrogenase induction.

My laboratory observations show that *G. frateurii* strains always grow fast. For example, strain IFO strain 3271 also showed similar growth rates on basal medium containing 0.3%, 0.5%, and 5% sorbitol (data not shown). Likewise, during growth of IFO strain 3254 for preparation of membrane fractions, I noticed that the cells grew at almost the same rate on basal medium plus 5% of either glycerol, sorbitol, or sorbose (data not shown). Therefore, fast growth seems to be a species characteristic. This observation is supported by the phenotypic studies of Mason & Claus (26). Their results show that all three *G. frateurii* strains tested grew to more than 2.8 OD on glucose and xylitol media after 24 hours, while only one out of three strains from *G. oxydans* or *G. asaii* grew to that optical density on these substrates. Furthermore, all ten *G. frateurii* strains tested by Mason and Claus grew to 2.3 OD or above on ribitol, while only one out of 20 strains from the other two species grew to that density. I suspect that the

ability to grow well on different substrates was probably caused by the high efficiency of the dehydrogenases in this species.

Ferricyanide reduction assays reveal that cells grown on glycerol, sorbitol, or sorbose always had sorbitol dehydrogenase activity (Figure 2). These data support the hypothesized constitutive nature of sorbitol dehydrogenase. The specific activity is about doubled by growth on sorbose or sorbitol compared to glycerol. Therefore, it seems that the specific activity of sorbitol dehydrogenase differs under different growth conditions.

The doubling of sorbitol dehydrogenase activity by growth on sorbitol and sorbose (Figure 2) was also shown by the oxygen uptake assays (Figure A3, Appendix A). However, these dehydrogenase characteristics were shown only when cells from sorbitol-stock culture were first grown as a subculture on the polyol in question then cultured in medium containing that same polyol. Different sorbitol dehydrogenase activities were observed when the cells were not subcultured (Figure A2, Appendix A). These results show that subculture is important, because addition of some sorbitol from inoculum in growth medium might affect the dehydrogenase activity on cells grown in a medium containing another substrate such as glycerol.

The difference in sorbitol dehydrogenase activity under different growth conditions might due to the different amount of some components of electron transport

system. The ferricyanide-reduction assay depends not only on the presence of the dehydrogenases but also on components of the electron transport system (27). It is possible that some of these components were limiting when cells are grown on different conditions. A quantitative cytochrome assay would determine if this were a problem in comparing membrane fractions of cells grown with different substrates.

No significant sorbose dehydrogenase activity was shown by membrane fractions from *G. frateurii* IFO strain 3254 (Table 2). I used the same assay conditions to detect both sorbose dehydrogenase and sorbitol dehydrogenase. I now realized that these assay conditions were not optimal for sorbose dehydrogenase. Kitamura et al. (22) found that when phenazine methosulfate (PMS) alone was used instead of potassium ferricyanide as electron acceptor, cell free extracts from *G. melanogenus* demonstrated three times higher sorbose dehydrogenase activity. Others have shown that the optimum pH for sorbose dehydrogenase is 6.5 to 7 (22, 27, 39, 40), unlike sorbitol dehydrogenase which is optimum at pH 4.5 (27). Furthermore, Sugisawa (39) demonstrates that purified membrane-bound sorbose dehydrogenase shows 4 times higher activity at 48°C than at 25°C. Sugisawa (39) also found that additional of metal ions such as  $\text{Co}^{2+}$  and  $\text{Fe}^{3+}$  enhances sorbose dehydrogenase activity. The assay conditions that I used in my study were shown to be optimum for some primary dehydrogenases such as sorbitol dehydrogenase (46) and glycerol dehydrogenase (12).

Kitamura (23) demonstrated that the activity of sorbose dehydrogenase can be induced by some growth substrates. My data do not support similar phenomenon as shown by Kitamura, because I did not detect significant sorbose dehydrogenase activity from membrane fraction of IFO strain 3254.

Sorbose dehydrogenase activity of *G. frateurii* IFO strain 3254 was indirectly shown only by the ability by this strain to grow on sorbose plates in 48 hours, but not by chromatography of growth media containing sorbitol or by the ferricyanide assay. I suspect that the length of time for the assay or for cell growth in broth was not enough to allow induction of sorbose dehydrogenase. It would be desirable to chromatograph the culture medium containing 0.3% sorbitol grown for more than 20 hours to see if biphasic growth in this species occurred during a later growth stage or if sorbose depletion could be demonstrated.

Because sorbitol and sorbose dehydrogenase showed different specific activities under the same assay conditions, it is possible that they are actually two different membrane-bound dehydrogenases. Further purification of both enzymes and enzyme kinetics tests would help determine which of these possibilities is correct.

***Gluconobacter oxydans* ATCC strain 621 and IFO strain 3293.** The growth of both of these strains seemed limited in basal medium containing 0.3% sorbitol (Figures 3

and 5), and the characteristics of both strains were similar to biphasic growth on limiting glycerol concentrations (5).

The primary and secondary phases previously observed by Batzing & Claus (5) using 0.25 % glycerol medium, however, differed from my results with 0.3% sorbitol (Figures 3 and 5). In glycerol medium, the primary and secondary phases took place between hours 11 to 15 and 15 to 37, respectively, while in sorbitol medium they took place between hours 1 to 5 and 5 to 15. Using washed inoculum of ATCC strain 621, I found that cells in basal medium containing 0.3% sorbitol grew exponentially from 0.05 OD (undiluted culture medium) at hour 0 to 0.53 at hour 15 (data not shown). The optical density was increased slightly to 0.58 at hour 38 and was unchanged when measured at hours 45 and 63. Hence, the slow growth between hours 15 and 38 might be the secondary phase.

Chromatography of basal medium plus 0.3% sorbitol during growth of both ATCC strain 621 and IFO strain 3293 (Figures 3b and 4b) did not support biphasic growth during the first 17 hours of incubation. There was only *sorbitol* consumption but no apparent *sorbose* consumption by the cells during what appeared to be secondary growth (between hours 5 to 15). It is likely that what was thought to be primary and secondary phases (hours 1 to 17) was actually only the primary phase, such as shown during growth in 0.25% glycerol (5). The secondary phase in medium containing 0.25%

glycerol does not start until after 15 hours of growth. Therefore, the induction of the secondary (dihydroxyacetone or sorbose) dehydrogenase probably takes place after about 15 hours of growth. Kitamura (22) reported that active conversion of sorbose to sorbosone by sorbose dehydrogenase in whole cells of *G. oxydans* IFO strain 3293 occurs after 4 day incubation. In future studies, I suggest that the presence of the secondary phase during growth on limiting sorbitol should be tested in chromatograms after 20 hours of cell growth, and it might be better observed using a method that could detect sorbose depletion as well as accumulation of sorbose oxidation products.

The constitutive nature of the sorbitol dehydrogenase from both *G. oxydans* strains was shown by the ferricyanide assay which demonstrate that membrane fractions from cells grown on glycerol, sorbitol, and sorbose always exhibit sorbitol dehydrogenase activity (Figures 4 and 6). The activity in both strains was enhanced to the greatest extent by growth on sorbose. I also found that the activity of sorbitol dehydrogenase from ATCC strain 621 grown on three different substrates was much higher than that in any other strains tested (Table 2).

When White and Claus (46) used the ferricyanide assay performed at 38°C to measure sorbitol dehydrogenase activity by membrane fraction of sorbitol-grown *G. oxydans* ATCC strain 621 harvested in early stationary phase, they obtained a specific activity of 800 nmoles of sorbitol oxidized / mg protein / minute. When Edwards (12)

used membrane fraction from glycerol-grown cells harvested at late exponential phase, with the same assay conditions as was done by White, she found specific activities for sorbitol dehydrogenase to be 370 and 740 nmoles of sorbitol oxidized / mg protein / minute in two separate experiments. For the most part, the activities that I obtained from the assay performed at room temperature (25°C), using membrane fractions from cells harvested at late stationary phase, were much higher (Table 2).

Because sorbitol dehydrogenase from ATCC 621, IFO 3293, and IFO strain 3254 show different specific activities (Table 2), it seems that sorbitol dehydrogenases from different *Gluconobacter* species and different strains are different. Choi (9) found that sorbitol dehydrogenases from ATCC strain 621 and IFO 3254 even have different properties when solubilized with different detergents.

My preliminary data using membranes from ATCC strain 621 suggest low sorbose dehydrogenase activity when cells were grown on glycerol, sorbitol, and sorbose (Table 2). Because the data were obtained from only one experiment, the significance of these low numbers is unknown. However, these numbers are in similar range as sorbose dehydrogenase activity found in other strains (Table 2).

Preliminary results that show high sorbitol dehydrogenase activity and low sorbose dehydrogenase activity from *G. oxydans* ATCC strain 621 might confirm why a strain that is very close to this strain is used in Reichstein method for the production of

vitamin C (1). In this production method, *Gluconobacter oxydans* is used for sorbitol oxidation because it gives a high yield of sorbose. However, further oxidation of sorbose to 2-keto-gulonic acid, a direct precursor of vitamin C, is done chemically (1), or with another *Gluconobacter* strain because of the low yield from *G. oxydans* (1, 35, 38). Yet, I do not rule out the possibility that the low sorbose dehydrogenase activity in my results was due to the assay conditions that were not optimum for sorbose dehydrogenase.

*G. oxydans* IFO strain 3293, which is known to oxidize sorbose (16, 22, 23, 38, 39), shows a significant amount of low but constitutive sorbose dehydrogenase activity. This is contrary to the previous results which showed that the activity of sorbose dehydrogenase from the wild type (23) and a mutant (39) of IFO strain 3293 was induced by both sorbitol and sorbose. However, in these previous studies, the assays were done under optimum conditions.

The presence of sorbose dehydrogenase activity in these membrane fractions (Table 2) is in accordance to the report that this enzyme is present in the plasma membrane of *G. melanogenus (oxydans)* IFO strain 3293 (16, 22, 23). In the study of Kitamura (22), it was shown that the activity of this enzyme can be detected with electron acceptors such as phenazine methosulfate, methylene blue, potassium ferricyanide, but not  $\text{NAD}^+$  or  $\text{NADP}^+$ , indicating that sorbose dehydrogenase is present in the plasma membrane. However, this presence of sorbose dehydrogenase activity from

membrane fractions of IFO strain 3293 (Table 2) conflicts with the report of Sugisawa et al. (39) who were not able to obtain sorbose dehydrogenase activity from this strain. But these investigators were able to isolate sorbose dehydrogenase from a mutant strain of IFO strain 3293.

My results show that the sorbose dehydrogenase activity in IFO strain 3293 was the highest among the four strains that I tested (Table 2). Even though this activity was quite low compared to that of sorbitol dehydrogenase (Table 2), I found that sorbose dehydrogenase activity was similar to that reported by Hoshino (16). In the study by Hoshino, sorbose dehydrogenase activity of the membrane fractions from mutants of IFO strain 3293 ranged from 160 to 420 nmoles of substrate oxidized / minute / mg protein. In future investigation, the activity of sorbose dehydrogenase could probably be improved by changing the growth conditions. For example, Kitamura (22) showed that calcium ions added to growth medium stimulates sorbose dehydrogenase to convert sorbose to sorbosone.

In summary, my data demonstrate that sorbitol dehydrogenase in *G. oxydans* ATCC strain 621 and IFO 3293 was constitutive. In addition, sorbose dehydrogenase in IFO strain 3293 was constitutive. Based on preliminary evidence, it appears that sorbose dehydrogenase in ATCC strain 621 is noninducible.

***Gluconobacter asaii* ATCC strain 43781.** Growth of *G. asaii* ATCC strain 43781 on medium containing 0.3% sorbitol appeared to be to be biphasic (Figure 7a). However, the chromatography results show only sorbitol consumption with no detectable sorbose consumption during both apparent primary and secondary phases (Figure 7b). These results appear to demonstrate an active sorbitol dehydrogenase and no sorbose dehydrogenase activity under these growth conditions.

However, results from the ferricyanide assay show that both sorbitol and sorbose dehydrogenases were constitutive (Table 2). It is possible that the thin layer chromatography was not sensitive enough to detect some sorbose consumption by the cells. The pyridine solvent used to develop the chromatogram can detect sorbitol, sorbose (4), and sorbosone (22). Therefore, under these growth conditions, either sorbose was not oxidized, or detectable quantities of sorbosone were not produced, or other compounds such as 2-keto-L-gulonic acid were formed and not detected.

There are not many reports about ATCC strain 43781 in the literature. A tetrazolium assay using whole cells demonstrated that this strain can oxidize sorbitol, along with several other polyol compounds (7). Phenotypic studies by Mason and Claus (26) show that this strain (called strain RS 203b in that publication), were able to grow to less than 0.4 OD on ribitol or arabitol in 24 hours. Results from both of these studies suggest that the primary dehydrogenase in this strain is probably constitutive. My study

is the first to report that sorbose (a secondary) dehydrogenase in *G. asaii* ATCC strain 43781 is also constitutive.

## CONCLUSIONS

1. No correlation between growth on sorbose plates and ability of membrane fraction from *Gluconobacter* species to oxidize sorbose was found in this study.
2. Concentration of 0.3% sorbitol in basal medium was limiting for two *Gluconobacter* species, *G. oxydans* and *G. asaii*. Even though these two species showed possible biphasic growth, chromatography did not support the presence of two distinct growth phases supported by sorbitol and sorbose during the first 20 hours of growth.
3. Sorbitol dehydrogenase appeared to be a constitutive enzyme in the gluconobacters, although different strains show different specific activity when grown on basal medium containing 5% of either glycerol, sorbitol, or sorbose. The specific activity of sorbitol dehydrogenase in all strains was enhanced by growth on sorbose.
4. No evidence was found for constitutive sorbose dehydrogenase in the four *Gluconobacter* strains tested. Slight but constitutive sorbose dehydrogenase activity

was shown in two out of four gluconobacter strains tested. This low or undetectable activity might be due to the assay conditions used in the study.

## **SUGGESTIONS FOR FUTURE INVESTIGATORS**

1. Perform thin layer chromatography on culture samples that were taken after 24 to 48 hours of growth in medium containing 0.3% sorbitol to see if biphasic growth and sorbose depletion occurs later than 17 hours.
2. Perform another type of chromatographic assay that can detect sorbose as well as products of sorbose oxidation to see if there is some sorbose oxidation during growth on 0.3% sorbitol.
3. Perform the ferricyanide assay with freshly made membrane fractions that are prepared in the same way for every batch of cells to get the highest dehydrogenase activity.
4. Perform ferricyanide assay for sorbose dehydrogenase activity under conditions optimum for this dehydrogenase (22, 39).

5. Assay the soluble fractions for dehydrogenase activity to see if sorbose dehydrogenase is present in the cytosol.
6. Perform quantitative assays for cytochromes in membrane fractions from cells grown in basal medium plus either 5% glycerol, sorbitol, or sorbose to determine if the cytochrome is the limiting factor in dehydrogenase assay.
7. Test the rate of sorbitol and sorbose dehydrogenase activity with oxygen uptake by using subcultured cells to see if similar characteristics of sorbitol and sorbose dehydrogenase as shown by ferricyanide assay could be obtained.
8. Test the enzyme kinetics of isolated sorbitol and sorbose dehydrogenases to see if they are different enzymes.

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## APPENDIX A

### *Assay of Dehydrogenase Activity Using Whole Cells and the Oxygen Uptake Assay*

#### ***Introduction***

This assay provided indirect evidence for the membrane-bound dehydrogenase activity (32), since most of the dehydrogenase activity by whole cells is contributed by membrane-bound dehydrogenases, not the cytoplasmic dehydrogenase. The activity of dehydrogenases in the gluconobacters was assayed by measuring the rate of oxygen consumption by whole cells during substrate oxidations. The rate of oxygen uptake was proportional to the rate of substrate oxidation since oxygen serves as the sole terminal acceptor for electrons removed from the oxidized substrate.

## *Apparatus*

The following were used in measuring the oxygen uptake by whole cells: a YSI model 53 biological monitor and a YSI model 5331 oxygen probe (Yellow Spring Instrument Co., Inc.), a Gilson Clark cell with a capillary cap, a Haake heating bath and circulator model FE2, a Corning magnetic stirrer model PC-351, a Pulsar One aquarium pump, and an Omniscrite recorder model D-5000 (Fisher).

The Clark cell contained a chamber which held a maximum of 1.6 ml reaction mixture. A capillary cap sealed the chamber from the atmospheric air. Outside and surrounding the chamber was a container with two openings where water from the heating bath circulated to maintain temperature at 38°C (optimum temperature for the assay) (46). An oxygen electrode covered with a Teflon membrane (Yellow Spring Instrument Co., Inc.) was inserted in the chamber. A solution of KCl electrolyte was applied between the electrode and the membrane to maintain electroneutrality. The membrane was placed very carefully to exclude air bubbles from underneath the membrane. To maintain a uniform oxygen concentration, the reaction mixture was constantly mixed with a 8 mm x 1.5 mm flea stir bar (Fisher) and a magnetic stirrer with a constant speed of 4 (on scale of 5). The recorder was set to run at a speed of 10 divisions of paper chart (model S-72167, Graphic Controls Co., Buffalo, NY) per minute.

## ***Reagents***

All reagents were prepared as described in Materials and Methods. In addition, the following reagents were also used: potassium phosphate monobasic and dibasic, which were obtained from Fisher Scientific Co., Fair Lawn, NJ. Also, phenazine methosulfate (PMS), solid catalase, and disodium salt  $\beta$ -NADH were purchased from Sigma Chemical Co., St. Louis, MO.

## ***Methods***

**Calibration of the oxygen uptake apparatus.** This calibration was done to measure the number of  $\mu$ l of dissolved oxygen per ml of buffer under my experimental conditions. It was done by using a reaction that consumed a known amount of oxygen (for detail, see pages 15-16 Ref. 12).

The procedure in Edwards' thesis (12) was followed with some modifications according to the methods of Robinson & Cooper (34). The reaction mixture contained 10.667  $\mu$ g of phenazine methosulfate (PMS) and 213.333  $\mu$ g of catalase (3200 units/mg) in 1.6 ml of 0.1M phosphate buffer adjusted to pH 7.4. Phosphate buffer was prepared by dissolving 2.82 g of  $K_2HPO_4$  and 0.518 g of  $KH_2PO_4$  in 200 ml of ultrapure water. A stock of NADH solution containing 10 mg of disodium salt  $\beta$ -NADH in 1 ml buffer was prepared separately. These solutions, except for the buffer, were prepared fresh daily,

since the reagents were unstable. The PMS is very light sensitive, therefore, the mixture was always protected from light.

Since the amount of oxygen consumed in the reaction mixture was proportional to the amount of NADH oxidized, I first needed to determine the exact concentration of NADH that was added to the reaction mixture. Twenty  $\mu\text{l}$  of the NADH solution was diluted 200 times with phosphate buffer. Using the buffer as a blank, the absorbance of the diluted NADH solution was determined with Spectronic 1201 spectrophotometer at 338 nm. The concentration of NADH in the original solution was calculated using Beer's law ( $A = \epsilon bc$ ), where  $A$  is the absorbance of NADH at 338 nm multiplied by the dilution factor (200),  $\epsilon$  is the extinction coefficient of NADH (6200),  $b$  is the light path length (1 cm), and  $c$  is the NADH concentration.

The reaction mixture (total volume of 1.6 ml) containing 10.667  $\mu\text{g}$  of PMS and 213.333  $\mu\text{g}$  of catalase (3200 units/mg) in 0.1M phosphate buffer was added to the Clark cell chamber. Air was then pumped from the aquarium pump for 10 minutes with stirring to allow for air saturation and temperature adjustment of the reaction mixture to 38°C. Next, the capillary cap was placed on the Clark cell and the biological monitor and the recorder were set to read 100%. The reaction mixture was further stirred for 2 minutes to allow the electrode to stabilize. If needed, both the monitor and the recorder were readjusted to 100% until the reading was stable. A machine drift with a rate of approximately 0.1 to 0.3 divisions per minute was usually obtained, and the drift was

subtracted from the change in deflection by NADH. The reaction was started by injecting 10  $\mu\text{l}$  of NADH solution (10 mg/ml buffer) using a Hamilton syringe with a 3.5 inch needle through the capillary cap.

Immediately after adding NADH, the monitor and recorder showed some deflection in oxygen concentration. After the reading became stable, another 10  $\mu\text{l}$  NADH solution was injected. This was done repeatedly until the oxygen concentration reached very close to zero (Figure A1). During the experiment, caution was taken to prevent direct sunlight to the reaction mixture which could cause autooxidation of PMS (34).

Then, the concentration of oxygen in the air-saturated reaction mixture ( $c$ ) was calculated using the following formula (34) :

$$c = \frac{100}{2x} \cdot \frac{n}{v} \quad \mu\text{mole / ml}$$

where  $n$  is the  $\mu\text{mole}$  of NADH added to a volume ( $v$ ) of reaction mixture, and  $x$  is the recorder deflection resulted from the addition of a known concentration of NADH. In the calculation,  $x$  is multiplied by 2 since two moles of NADH are needed to consume one mole of oxygen used in the reaction. To obtain the amount of  $\text{O}_2$  consumed in  $\mu\text{l}$ , the resulted number was multiplied by 22.4  $\mu\text{l} / \mu\text{mole}$ , since the volume of 1  $\mu\text{mole}$  oxygen is 22.4  $\mu\text{l}$ .

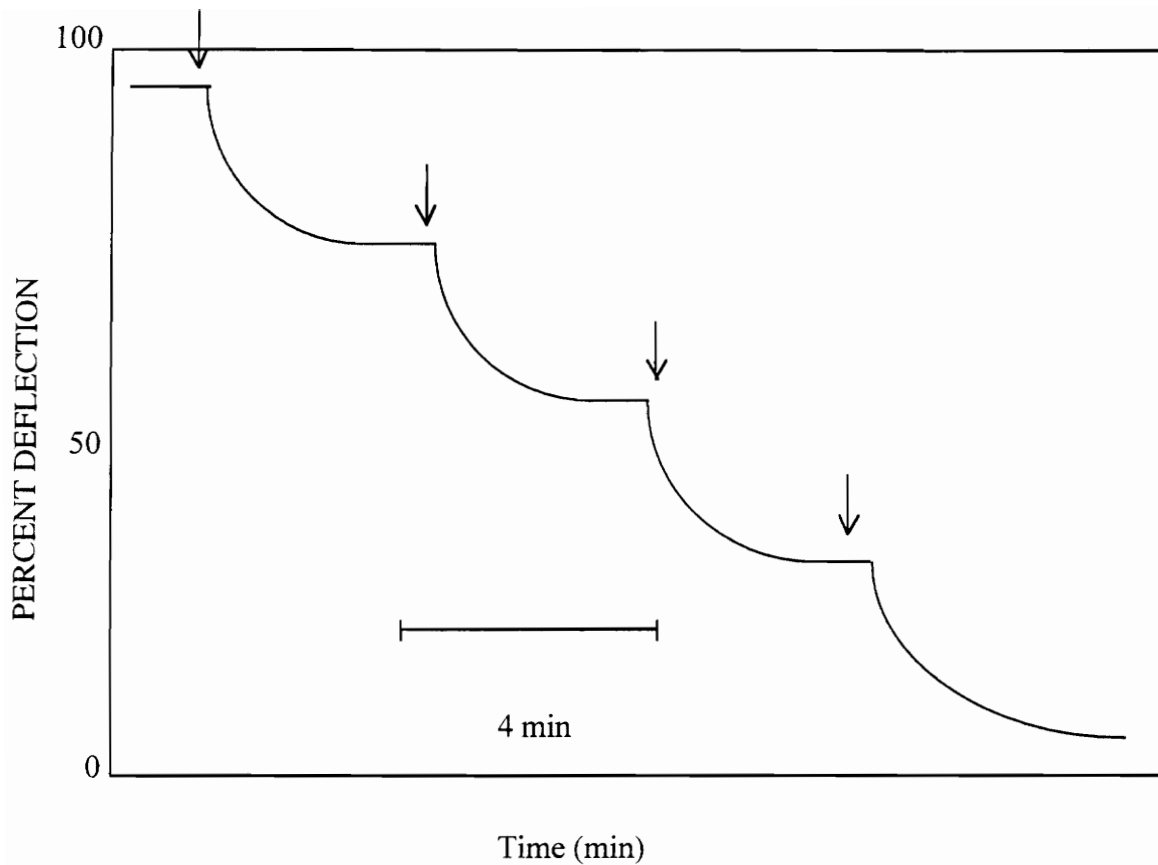


Figure A1. Typical results of recorder deflection when 10 µl NADH solution was injected (arrows) to the reaction mixture containing 10.667 µg PMS and 213.333 µg catalase (3200 units/mg) in 0.1M phosphate buffer in a total of 1.6 ml. Experiment was done at 38°C.

Four 10  $\mu$ l injections could result in the consumption of all oxygen in the reaction mixture. Therefore, only the first four deflections were used in the calculations. The average of oxygen concentration in  $\mu$ l per ml in air-saturated reaction mixture from three separate experiments were obtained (Table A1). When this number was divided by 100 division in the chart, the  $\mu$ l of oxygen consumed per chart division was obtained.

**Methods used for measuring oxygen uptake by whole cells.** Cells from either sorbitol-stock culture or washed inoculum (only done with IFO 3254) were grown in medium containing 5% glycerol, sorbitol, or sorbose as described in materials and methods. The cells were harvested when they reached 0.95 OD (620 nm), and then washed twice with 0.07 M succinate buffer pH 5.2. The cell pellet was resuspended in the same buffer so that it was 20 x more concentrated than the original culture. This was necessary to have enough cells for measurable oxygen uptake (12).

Reaction mixture containing 0.07 M of succinate buffer adjusted to pH 5.2 and 0.7 M of either sorbitol or sorbose dissolved in the same buffer were added to the Clark cell. The volume of succinate buffer was adjusted such that the final volume in the chamber, after addition of different volumes of cell suspension, became 1.6 ml. Air was pumped into the mixture, and the monitor was set to 100 % as described previously. Usually the electrode drift was higher when sorbose was used as a substrate. The capillary cap was placed on the Clark cell, and, after the monitor was stable, the reaction

Table A1 . Determining the quantity of oxygen in saturated reaction mixtures

1/30/1996 Determination			1/31/1996 Determination			2/13/1996 Determination		
Experiment	Replicates	$\mu\text{l O}_2/\text{ml}$ reaction mixture <sup>a</sup>	Experiment	Replicates	$\mu\text{l O}_2/\text{ml}$ reaction mixture	Experiment	Replicates	$\mu\text{l O}_2/\text{ml}$ reaction mixture
1	a	4.954	1	a	4.632	1	a	4.313
	b	4.954		b	4.904		b	4.887
	c	5.750		c	5.000		c	5.096
	d	5.854		d	5.192		d	5.545
			2	a	5.189	2	a	4.77
				b	5.309		b	4.988
				c	5.297		c	5.135
				d	5.300		d	5.560
			3	a	5.051	3	a	5.400
				b	5.051		b	5.752
				c	5.256		c	6.067
				d	5.215		d	6.547
						4	a	5.469
							b	5.560
							c	6.362
							d	6.182

<sup>a</sup>Oxygen concentration in air-saturated reaction mixture was calculated with the following formula:

$$c = \frac{100}{2x} \cdot \frac{n}{v} \mu\text{mole} / \text{ml}$$

where  $c$  is the concentration of oxygen consumed per ml of reaction mixture,  $n$  is the  $\mu\text{mole}$  of NADH added to the reaction mixture,  $x$  is the percent recorder deflection, and  $v$  is the total volume of reaction mixture in ml. The concentration of oxygen in  $\mu\text{mole} / \text{ml}$  was multiplied by  $22.4 \mu\text{l} / \mu\text{mole}$  to obtain the volume of oxygen consumed per ml reaction mixture.

was started by injecting 10 to 40  $\mu\text{l}$  of cell suspension. Cell suspension was added last to prevent foaming of the mixture when the air is pumped into the mixture. Finally, Lowry assay was done to quantitate the amount of protein in the cell suspension.

### **Calculation of oxygen quantity represented by one chart paper division.**

Figure A1 shows the typical response of the recorder in the chart paper when 10  $\mu\text{l}$  NADH was added consequently to the mixture containing PMS and catalase in 0.07 M succinate buffer (pH 5.2). The results of the calibration of the oxygen uptake apparatus from three determinations are shown in Table A1. The average results of  $\mu\text{l O}_2$  consumed per ml reaction mixture in air-saturated solution were calculated to be 5.329  $\mu\text{l/ml}$ . Therefore, the amount of  $\text{O}_2$  consumed per chart paper division was 0.05329  $\mu\text{l}$  per ml reaction mixture.

The rate of oxygen consumption in scale divisions per minute, which reflects the rate of substrate oxidation, was calculated from the fastest and the most linear deflection curves in the chart paper. Using the  $\mu\text{l O}_2$  per ml that was obtained from the calibration as a conversion factor, the oxidation rate in  $\mu\text{l O}_2$  per ml reaction mixture per minute was obtained. The resulting number was divided by the amount of protein in whole cell suspension that was added to the reaction mixture. After multiplied by 60 minute (1 hour) the final oxidation rate was then expressed as  $\text{QO}_2$  ( $\mu\text{l O}_2$  consumed per mg protein per hour). A sample for calculation is shown in Table A2. The rates of oxygen

Table A2. Rate of oxygen uptake by whole cells of *G. oxydans* ATCC 621.

Growth substrate	mg protein (in 20 $\mu$ l cell suspension)	Substrate	Deflections/min	$\mu$ lO <sub>2</sub> /min <sup>a</sup>	$\mu$ lO <sub>2</sub> /mg protein /min <sup>b</sup>	QO <sub>2</sub> ( $\mu$ lO <sub>2</sub> /mg protein /hr) <sup>c</sup>
Glycerol	0.120	Sorbitol	1.0	0.053	0.446	27
Sorbitol	0.124	Sorbitol	1.3	0.067	0.538	32
Sorbose	0.115	Sorbitol	23.0	1.226	10.628	640
Glycerol	0.120	Sorbose	0.6	0.029	0.245	15
Sorbitol	0.124	Sorbose	0.4	0.023	0.185	11
Sorbose	0.115	Sorbose	0.4	0.019	0.162	10

<sup>a</sup>  $\mu$ lO<sub>2</sub> consumed by the cells per minute was obtained by multiplying the number of chart paper divisions (deflection) per minute with the amount of O<sub>2</sub> consumed per chart paper division (0.05329  $\mu$ l per ml reaction mixture)

<sup>b</sup>  $\mu$ l O<sub>2</sub> / mg protein / minute was calculated by dividing the number of  $\mu$ lO<sub>2</sub> consumed by the cells per minute by mg protein in the cell suspension that was added to the reaction mixture

<sup>c</sup> QO<sub>2</sub> was obtained by multiplying the number of  $\mu$ l O<sub>2</sub> / mg protein / minute with 60 minute / hour

consumption during sorbitol and sorbose oxidation by four *Gluconobacter* strains were calculated and are shown in Table A3 and in Figures A2 to A6.

### ***Results and discussion***

**Oxygen uptake by *G. frateurii* IFO strain 3254.** The results show that this strain has constitutive sorbitol dehydrogenase activity because the cells show sorbitol oxidation regardless the growth substrates (Figure A2). However, the activity of glycerol-grown cells was decreased to about half when the cells were first subcultured on glycerol (Figure A3). This shows that sorbitol from the sorbitol stock, that might have been present when cells were not first subcultured on glycerol, might have an affect on the sorbitol dehydrogenase activity.

In contrast, I found no significant activity for sorbose dehydrogenase when cells were grown on glycerol, sorbitol, or sorbose (Figure A2). Therefore, the characteristics of sorbitol and sorbose oxidation (oxygen uptake) by IFO strain 3254 (Figure A3) seem very similar to the results obtained when the dehydrogenase enzymes were assayed with ferricyanide (Figure 2). Low oxygen uptake with sorbose was probably due to the oxygen uptake assay conditions in my study that was probably not optimum for sorbose dehydrogenase (22, 39), even though these conditions were shown to be optimum for oxygen uptake with sorbitol (46).

Table A3 . Effect of growth substrate on oxidation of sorbitol and sorbose by whole cells of the *Gluconobacters*<sup>a</sup>.

Species	Strains	Growth Substrate <sup>a</sup>	Rate of oxidation ( $\mu\text{l O}_2 / \text{mg protein} / \text{hr}$ ) <sup>b</sup>	
			Sorbitol	Sorbose
<i>G. oxydans</i>	ATCC 621	Glycerol	28 ± 6	12 ± 2
		Sorbitol	25 ± 5	11 ± 2
		Sorbose	690 ± 53	13 ± 4
	IFO 3293	Glycerol	120 ± 78	9 ± 6
		Sorbitol	310 ± 127	5 ± 3
		Sorbose	1200 ± 91	6 ± 3
<i>G. frateurii</i>	IFO 3254	Glycerol	2000 ± 285	3 ± 1
		Sorbitol	2000 ± 71	4 ± 1
		Sorbose	1900 ± 88	3 ± 1
<i>G. asaii</i>	ATCC 43781	Glycerol	1200 ± 412	48 ± 24
		Sorbitol	1300 ± 194	67 ± 14
		Sorbose	990 ± 91	48 ± 10
<i>G. frateurii</i> <sup>c</sup>	IFO 3254	Glycerol	950 ± 272	4 ± 2
		Sorbitol	2200 ± 362	4 ± 0
		Sorbose	2100 ± 33	4 ± 0

<sup>a</sup> Cells from sorbitol-stock culture were grown in basal medium containing 5% of either glycerol, sorbitol, or sorbose and washed twice as described in Materials and Methods.

<sup>b</sup> Oxidation rate was measured from the rate of oxygen uptake in the reaction mixture containing 0.7M substrate in 0.07M succinate buffer (pH 5.2), and 10 to 40  $\mu\text{l}$  cell suspension (approximately 0.05 - 0.30 mg protein). Each number represents the average rate of sorbitol or sorbose oxidations from 3 separate experiments from at least two batches of cells  $\pm$  standard deviation.

<sup>c</sup> Cells were first subcultured

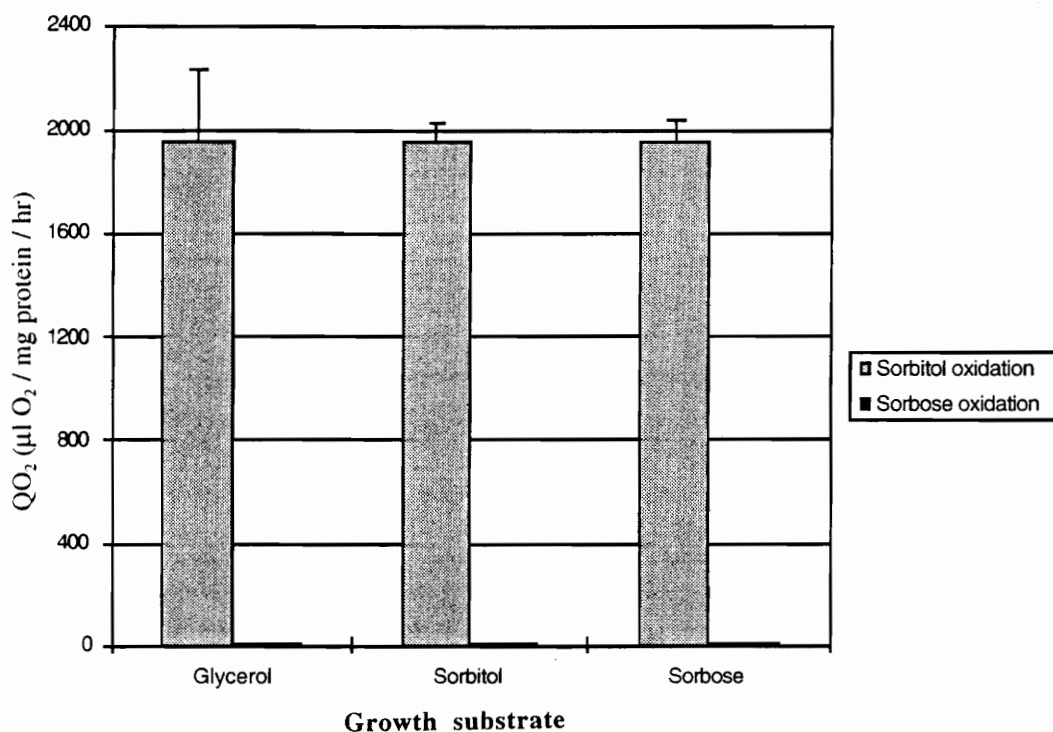


Figure A2. Rates of sorbitol and sorbose oxidation by whole cells of *G. frateurii* IFO strain 3254 culture grown on medium containing 5% glycerol, sorbitol, or sorbose. Sorbitol-stock culture was used as inoculum and washed cell suspension was prepared as described in materials and methods. Oxidation rates were measured from the rate of oxygen uptake in reaction mixture containing 0.7 M sorbitol or sorbose in 0.07 M succinate buffer (pH 5.2), and 10 to 40 µl cell suspension (approximately 0.05 to 0.30 mg protein) at 38°C. Each bar represents an average of three experiments from two batches of cells minus the rate of oxygen consumption in the absence of cells. No endogenous reaction occurred in the absence of substrates.

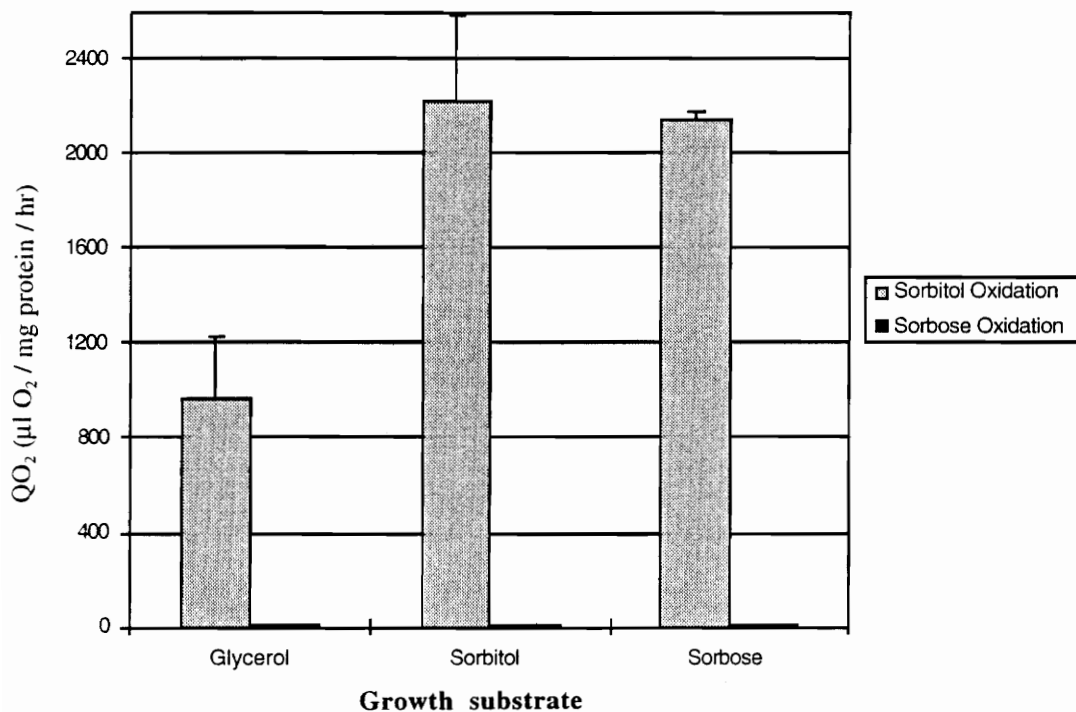


Figure A3. Rates of sorbitol and sorbose oxidation by whole cells of *G. frateurii* IFO strain 3254 grown on medium containing 5% glycerol, sorbitol, or sorbose. Experiments were done as described in Figure A2, except that cells were first subcultured as described in Materials and Methods.

**Oxygen uptake by *G. oxydans* ATCC strain 621 and IFO strain 3293.** Both ATCC strain 621 and IFO strain 3293 showed some oxygen uptake in the presence of sorbitol when cells were grown on glycerol and sorbitol, and this activity was significantly increased when cells were grown on sorbose (Figure A4 and A5). This indicates that the sorbitol dehydrogenase is constitutive but its activity is improved by sorbose in the medium. However, it is strange that the sorbitol dehydrogenase activity of cells grown on *sorbitol* was not increased as well, because sorbitol in the medium was always converted to sorbose (Figure 3b and 5b). It may be that larger quantities is needed by these cells in order to enhance the activity of sorbitol dehydrogenase.

Under the conditions that I used for my study, the activity of sorbose dehydrogenase by IFO strain 3293 was almost undetectable. In ATCC strain 621, however, some very low but consistent activity was shown by cells grown on the three different growth substrates.

**Oxygen uptake by *G. asaii* ATCC strain 43781.** Oxygen uptake activity in the presence of sorbitol was shown by cells grown on glycerol, sorbitol, or sorbose (Figure A6). This suggests that the sorbitol dehydrogenase in this strain was constitutive. This strain was shown to have the highest sorbose dehydrogenase activity among the four *Gluconobacter* strains tested (Table A3). Because this activity was found in cells grown on three different substrates, this suggests that sorbose dehydrogenase in this strain is

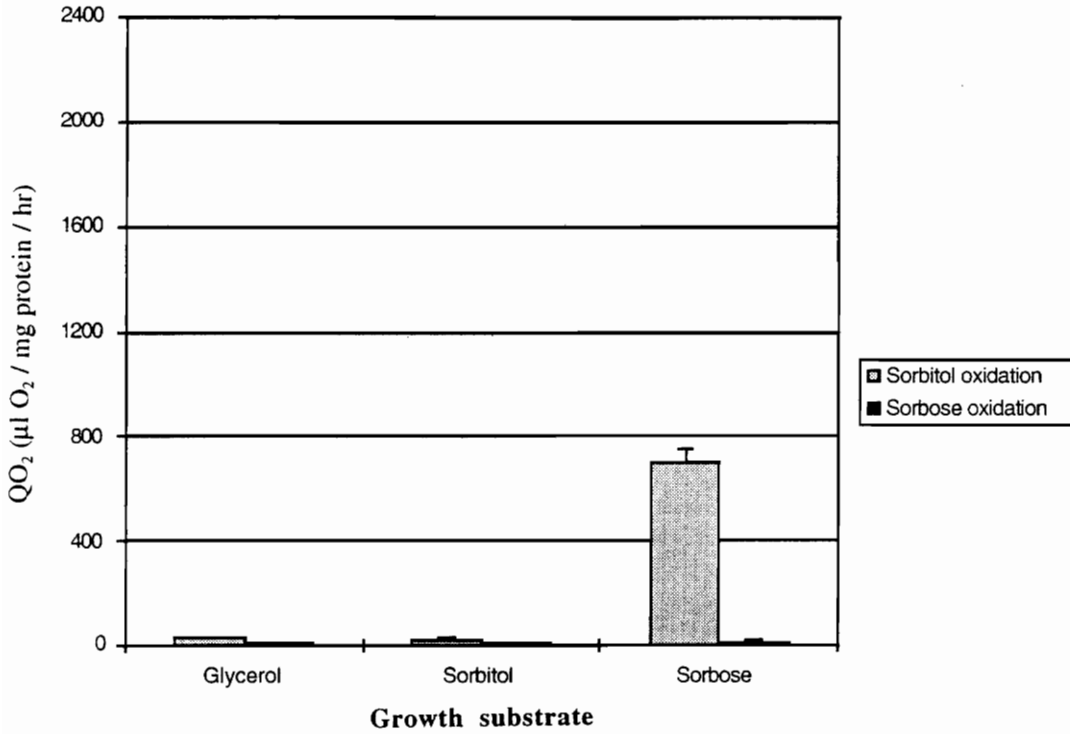


Figure A4. Rates of sorbitol and sorbose oxidation by whole cells of *G. oxydans* ATCC strain 621 grown on medium containing 5% glycerol, sorbitol, or sorbose. Experiments were done as described in Figure A2.

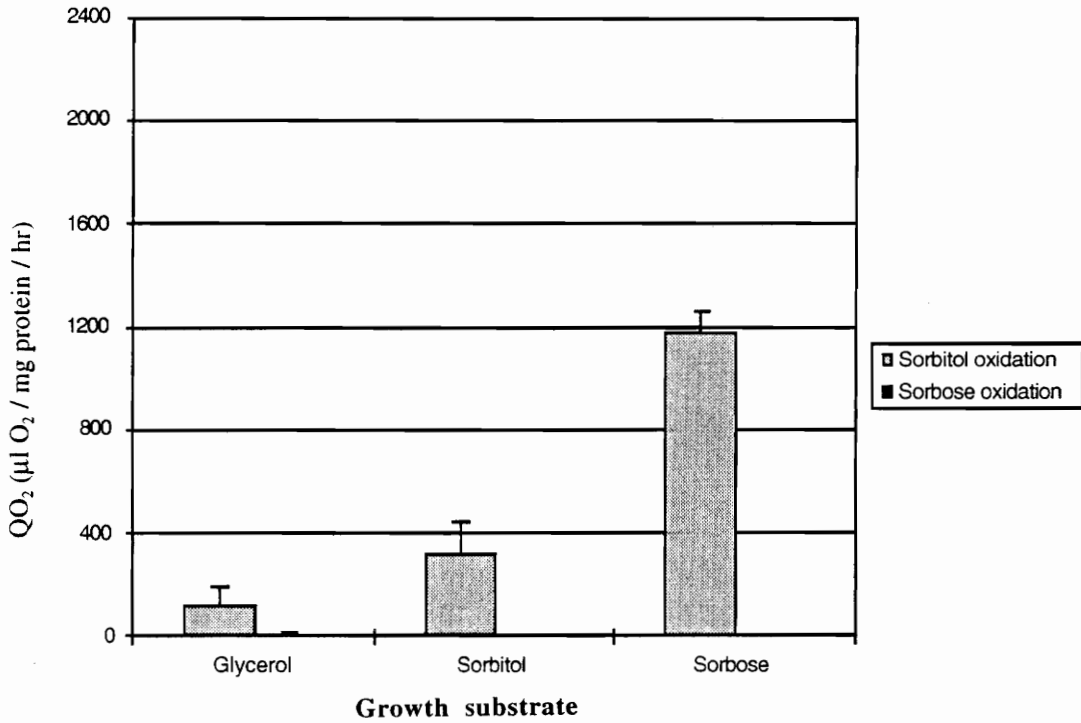


Figure A5. Rates of sorbitol and sorbose oxidation by whole cells of *G. oxydans* IFO strain 3293 grown on medium containing 5% glycerol, sorbitol, or sorbose. Experiments were done as described in Figure A2.

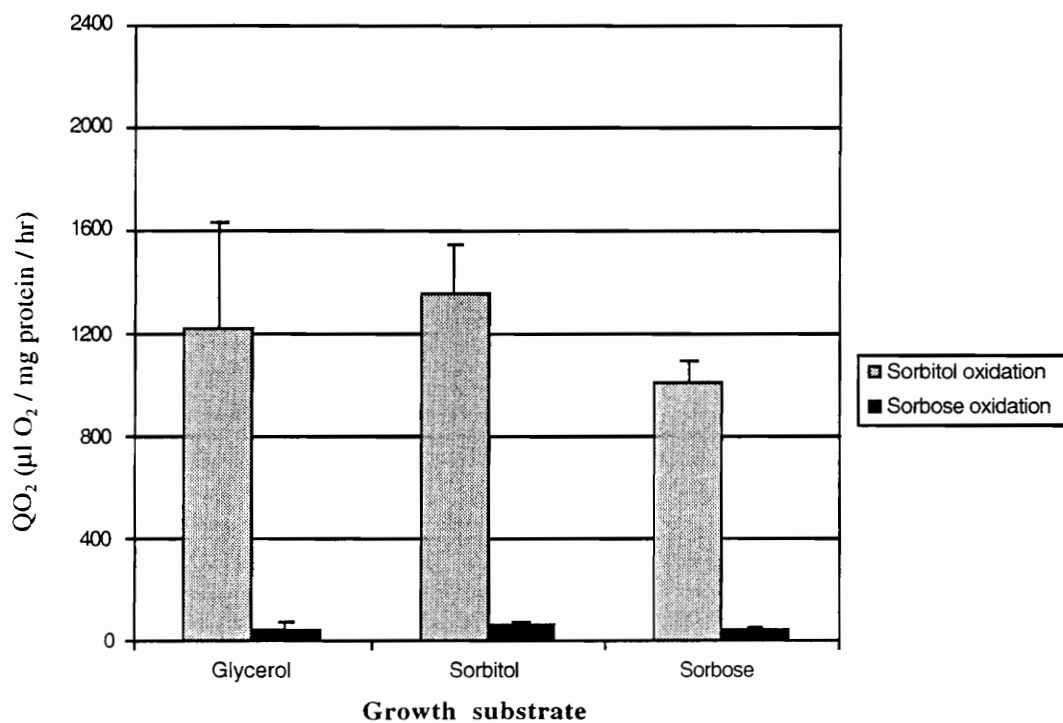


Figure A6. Rates of sorbitol and sorbose oxidation by whole cells of *G. asaii* ATCC strain 43781 grown on medium containing 5% glycerol, sorbitol, or sorbose. Experiments were done as described in Figure A2.

constitutive. Therefore, this result is in accordance with the results from sorbose dehydrogenase shown by ferricyanide assay (Figure 8).

### ***Conclusions***

Oxygen uptake shown in the presence of sorbitol was shown by all four *Gluconobacter* strains (from three different species) tested regardless the growth substrates, suggesting that the sorbitol dehydrogenase in *Gluconobacters* was constitutive. The amount of oxygen uptake was enhanced by sorbose as growth substrate. Therefore, these results were similar to the results obtained by ferricyanide assay. In contrast, low and constitutive activity was detected only in one out of four strains tested, and no evidence for sorbose dehydrogenase induction under the assay conditions that I used. It is possible that the oxygen uptake assay conditions were not optimum for sorbose dehydrogenase.

When cells were first subcultured, the oxygen uptake in the presence of sorbitol by glycerol-grown *G. frateurii* IFO strain 3254 cells was decreased. Therefore, it seems that sorbitol from sorbitol-stock culture might have an effect on sorbitol dehydrogenase. For future studies, I suggest that the whole cells assayed with oxygen uptake should first be subcultured.

## APPENDIX B

### *Effect of membrane quantity on sorbitol dehydrogenase (ferricyanide reduction) assay*

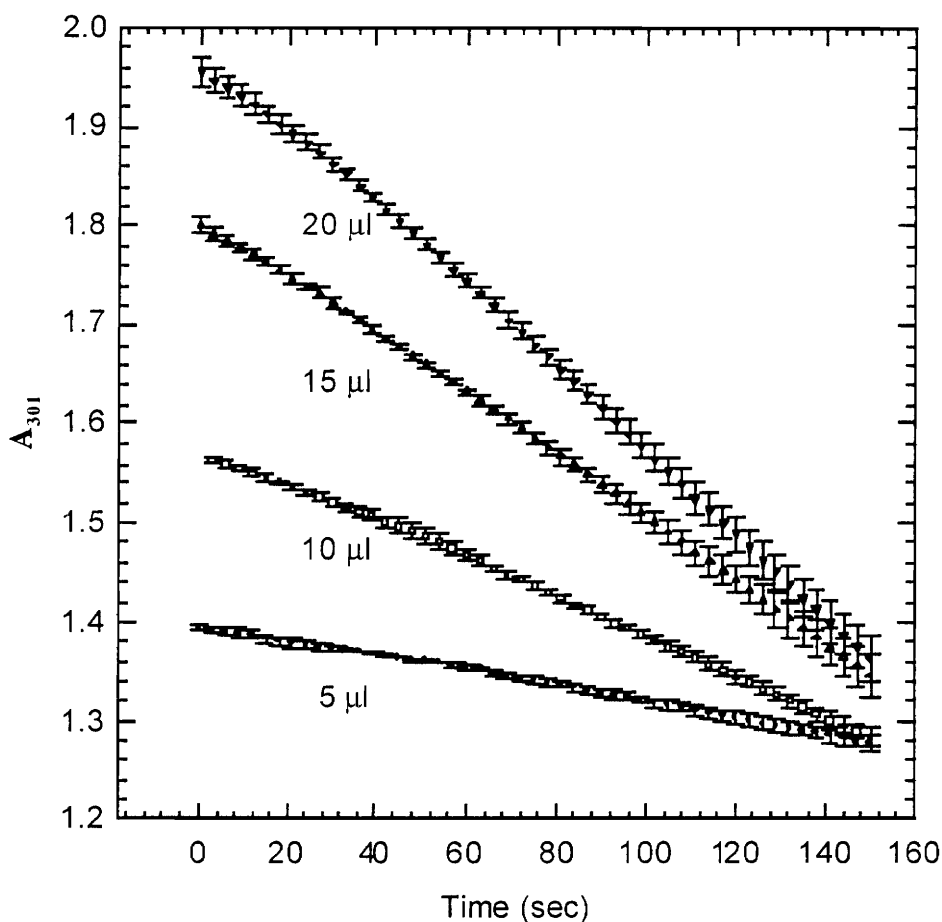


Figure B1. Sample rates of ferricyanide reduction (sorbitol oxidation) in response to adding various amounts (5, 10, 15, and 20  $\mu$ l) of membrane fractions from sorbose-grown *G. oxydans* IFO strain 3293 to the ferricyanide assay reaction mixture. Membrane fractions and ferricyanide reaction mixture were prepared as described in materials and methods. Each data point represents an average from three samples from one batch of cells. Standard deviations were calculated and are shown as bars across each data point.

# *CURRICULUM VITAE*

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## **Current Graduate QCA :**

3.95

**Research Experience :**

- |                   |   |
|-------------------|---|
| Fall 1993         | Undergraduate Research : " <i>Bacteroides fragilis</i> aerotolerance"   |
| Summer 1994       | Undergraduate Research : "Enzyme regulation by the gluconobacters"  |
| Fall 1994-present | Graduate Research : "The nature of sorbitol (a primary) dehydrogenase and sorbose (a secondary) dehydrogenases by <i>Gluconobacter</i> species" |

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- Spring 1995 : General Biology Laboratory
- Fall 1995 : General Biology Laboratory
- Spring 1996 : General Microbiology Lecture

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- Magna cum laude graduate
- Who's Who among American Students 1994
- Dean's List (three semesters)
- Undergraduate Scholarship from Indonesian Government

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- Garnet and Gold Women's Honor Society
- Gamma Beta Phi Honor Society
- Phi Beta Kappa National Honor Society

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- Virginia Branch-American Society for Microbiology
- Biology Graduate Student Association

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Indonesian Student Association  
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Member, 1991-present

**Research Grant Submitted :**

"Investigation of membrane-bound sorbitol-and sorbose dehydrogenases of the genus *Gluconobacter* to determine if their synthesis or activity is regulated"  
Sigma Xi Grant-In-Aid of Research, submitted on February 1995 (not funded)

**Meeting Attended :**

Annual Meeting, Virginia Branch-American Society for Microbiology  
Richmond, VA, 3-4 December 1994

The 95th General Meeting of the American Society for Microbiology  
Washington, DC, 21-25 May 1995

Annual Meeting, Virginia Branch-American Society for Microbiology  
Charlottesville, VA, 1-2 December 1995

**Seminars Given :**

"Determining the regulation of membrane-bound dehydrogenases of *Gluconobacter* species"  
Microbiology Seminar, Spring 1996

"The formation of biofilm by bacteria"  
Five minute talk in Undergraduate Microbiology Seminar, Fall 1992

