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MEMBRANE BOUND DEHYDROGENASE ENZYMES
AND RELATED ELECTRON TRANSPORT SYSTEMS
IN ACETOBACTER ACETI VAR. LIQUEFACIENS:
THEIR INDUCTION AND PLEIOTROPIC NATURE

by

John C. Rakoczy

A Thesis
Submitted to the
Faculty of the School of Graduate
Studies in partial fulfillment
of the
Degree of Master of Arts

Western Michigan University
Kalamazoo, Michigan
October 1968
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The author wishes to express his gratitude to Dr. Stephen B. Friedman for his patience, guidance, and encouragement throughout the present study, and to Dr. Robert C. Eisenberg for his kindly advice and assistance. Sincere appreciation is extended to the many persons, too numerous to mention, who have given their time, advice and encouragement to the enterprise.

John C. Rakoczy
MASTER'S THESIS

RAKOCZY, John C.
MEMBRANE BOUND DEHYDROGENASE ENZYMES AND RELATED ELECTRON TRANSPORT SYSTEMS IN ACETOBACTER ACETI VAR. LIQUEFACIENS; THEIR INDUCTION AND PLEIOTROPIC NATURE.

Western Michigan University, M.A., 1969
Biology-Genetics

University Microfilms, Inc., Ann Arbor, Michigan
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INTRODUCTION

Under the cell wall of bacterial organisms lies a distinct, vitally important and delicate membrane, first isolated from the cell as an independent entity by Weibull (1) from *Bacillus megaterium*, and referred to as the cytoplasmic membrane. Since Weibull's isolation, a number of investigations of the composition of bacterial membranes have been reported, including those by Salton and Freer (2) and Shockman et al. (3). These studies have established that the overall composition of bacterial membranes is similar to membranes isolated from other sources. They are made up primarily of protein and lipid. The amount of protein ranges from 55 to 75% of the weight of the membrane while the lipids account for about 20 to 30%.

In thin sections, observed in electron micrographs, the bacterial membrane is morphologically similar to the limiting membranes of other cells and organelles, viz., it appears as a 'unit' membrane (4). In this connection Abram (5) found spherical structural units, 65 to 85 Å long. The units appeared to be attached to only one side of the membrane, that facing the cytoplasm. This work by Abram was first done on *Bacillus stereothermophilus* and similar work was successfully carried out on several mesophilic organisms, four *Bacillus* species and three
gram-negative organisms: *Escherichia coli* C, *Proteus vulgaris*, and *Shigella dysenteriae* Y6R. With such evidence at hand, it still remains to be shown that this fine structure is common to all microorganisms.

The structural units reported by Abram are very similar to those found on the inner membrane of mitochondria of eucaryotic organisms (6). Concomitant with the structural similarities are the functional similarities of bacterial membranes and mitochondria. The mitochondria have long been known as the site of respiratory enzymes (7). The cytoplasmic membrane of bacteria, on the other hand, was first established as the site of respiratory enzymes and cytochromes by the studies of Weibull in 1953 (1) and Storck and Wachsman in 1957 (8) on highly purified ghost preparations from *B. megaterium*. Since then, the localization of such enzymes has been found in many other aerobic organisms (9). Particulate fractions from mechanically broken cells have been shown to have the same enzymatic constitution (9,10).

In addition to the respiratory enzymes, other enzymes have been shown to occur in/on the cytoplasmic membrane. They include such enzymes as succinic dehydrogenase (11, 8), ATPase (12), nicotinic acid and other aromatic hydroxylases (12), and polyol dehydrogenases which occur exclusively in microorganisms (9). A galactitol dehydrogenase induced by sorbitol and dulcitol, a less stable
enzyme, D-iditol dehydrogenase, induced by dulcitol, and a labile mannitol dehydrogenase from sorbitol-grown cells has been found by Shaw (13) in a species of Pseudomonas.

A number of membrane-bound enzymes have been described in Acetobacter (10,14,15,16,17,18,19). They include dehydrogenases for glucose, galactose, L-arabinose, ribose, gluconate, mannitol, mannose, xylose, D-erythritol, glycerol, ethanol and numerous glycols. It was observed by De Ley (14) that an induction period occurred when testing the ability of Acetobacter to oxidize glycerol. It was felt this was due to the formation of an inducible enzyme to the enzyme chain (possibly glycerokinase). It was also shown by De Ley (15) that the enzymes on the cytoplasmic membranes were very tightly bound. The Acetobacter membrane also supports a cytochrome system allowing for the transport of electrons from the substrate to oxygen.

Of the many enzymes identified in various organisms, all display a certain degree of specificity. This specificity can be such as that found in urease which will catalyze only the hydrolysis of urea (20,21). A lesser degree of specificity is found in many enzymes, e.g., pepsin which catalyzes the hydrolysis of peptide linkages involving amino acids having either aromatic or carboxylated side chains (21). This lack of specificity or the ability of a single enzyme to catalyze the reaction of
a number of different substrates may be referred to as pleiotropism.

When one considers the size of a bacterial cell and the number of enzymatic capabilities of that cell, it becomes evident that pleiotropic enzymes could mean fewer enzymes and a greater efficiency for the cell. Such pleiotropism has been suggested by Kersters and De Ley (16) for the membrane-bound polyol dehydrogenase of *Acetobacter aceti* var. *liquefaciens*. Preliminary evidence has indicated that one dehydrogenase is responsible for the oxidation of galactose and glucose (22) in this organism. This investigation was carried out to further characterize the membrane bound dehydrogenases and related electron transport systems of *A. aceti* var. *liquefaciens*, with particular emphasis on their degree of pleiotropism.
METHODS AND MATERIALS

Organisms

Acetobacter aceti var. liquefaciens strain 20 was used in all studies.

Media and Cultivation of Organisms

The bacteria were routinely grown in a broth medium containing 0.04M KH$_2$PO$_4$, 0.025M Na$_2$HPO$_4$, 0.002M MgSO$_4$, 1.0% yeast extract, and 1.0% substrate. The substrates employed are described under the section dealing with the selection of mutants or in the results. The above medium, when supplemented with 1.5% agar, was used to check the purity of the cultures and in the selection of polyol dehydrogenase mutants. For the selection of hexose and pentose dehydrogenase mutants, the following modified EMB agar was used: 0.02% eosin-y, 0.0033% methylene blue, 1.0% yeast extract, 1.0% substrate, and 1.5% agar. Stock cultures were maintained on slants containing 3.0% calcium carbonate, 1.0% yeast extract, 5.0% glucose, and 1.5% agar. The same medium was used for starter cultures and for cell suspensions used in oxygen uptake studies. The cells were grown at 30°C. Broth cultures were incubated in a New Brunswick gyratory shaker incubator.
Isolation of Mutants Lacking Membrane Bound Hexose, Pentose, and Polyol Dehydrogenases:

Prior to the selection of mutants, mutagenesis was carried out as follows: Cells of the parent strain were grown in 250 ml sidearm flasks containing 25 ml of broth medium, supplemented with the substrate for which mutants were to be selected, until they were in the log phase. Ten ml of cells were then collected by centrifugation, washed twice with 0.2M acetate buffer (pH 5.0), and resuspended in 4.0 ml of the same buffer. To the cell suspension, 1.0 ml of the buffer containing 10,000 µg of N-methyl-N-nitroso-N'-nitroguanidine (NTG) was added and allowed to stand for 60 minutes at 30°C., with occasional shaking. The cells were then washed in 0.02M phosphate buffer (pH 6.2), placed in fresh broth containing an alternate substrate, and allowed to attain log phase growth.

Selection of mutants:

The selection of glucose mutants was made by plating the appropriate dilution of NTG treated cells on modified EMB plates containing glucose. The resultant colonies were then screened for glucose dehydrogenase negative mutants by their inability to produce acid. Non-acid formers produced light pink colonies, while the acid formers produced dark purple colonies.
In the selection of galactose mutants, the penicillin method was employed. From mutagenized cells, which were grown on glucose to the log phase, an aliquot of cells was removed, washed with phosphate buffer, and used to inoculate a broth medium containing galactose. The cells were allowed to grow for 12 hours to oxidize any contaminating glucose. Penicillin-G was then added to a final concentration of 100,000 units/ml, and the cells allowed to grow an additional 12 hours. A 10 ml sample was then removed from the culture, washed with phosphate buffer and used to inoculate a broth medium containing glucose. Following growth, serial dilutions were made, and the diluted cells plated on modified EMB plates containing galactose. Colonies unable to produce acid were selected. Galactose mutants were also selected by their inability to grow on galactose containing plates following replication from glycerol plates.

Arabinose and xylose mutants were selected in a manner similar to that for glucose on modified EMB plates containing arabinose or xylose.

Potential polyol mutants were selected by a modification of the oxydogram method of De Ley (23). Appropriate dilutions of NTG treated cells were grown on plates containing either sorbitol or glycerol. The resulting colonies were replicated onto fresh sorbitol or glycerol plates, and the original plates then flooded
with 0.033% 2,4-dinitrophenylhydrazine in 0.66N HCl for 15 minutes. Forty percent KOH was then added and the plates read immediately. Colonies lacking a yellow ring (inability to oxidize the polyols to the appropriate ketones) were isolated from the replicated plates.

Oxygen Uptake Studies

All potential mutants were screened for a reduction in oxygen uptake on a GME oxygraph model KM using a YSI Clark oxygen electrode with a 0.001 inch Teflon membrane. The reaction was carried out at 30°C using a jacketed cell.

Cells to be tested were grown on CaCO₃-glucose slants. The cells were suspended in 0.02M phosphate buffer (pH 6.2), washed twice, and resuspended to a Klett reading of 40 at 660 mp. The cells were then preincubated at 30°C in a Metabolyte water bath shaker prior to testing.

The reaction mixture consisted of 1.4 ml of cell suspension and 0.14 ml of the substrate. The concentrations of the substrates used were 0.33M glucose, 1.1M galactose, 1.5M arabinose, 1.1M sorbitol, and 2.0M glycerol. Those mutants which showed an oxygen uptake rate of half or less than half with respect to the wild-type rate were retained for direct testing of the membrane-bound dehydrogenases.
Preparation of the Particulate Fraction

Particles (oxidosomes) \(^\text{(24)}\) were prepared from cells grown in the basal medium to a Klett of 180-200 at 660 \(\text{mu}\). All the following steps were carried out at 4°C. The cells were collected by centrifugation in a Sorvall RC2-B centrifuge and washed twice with 0.02M phosphate (pH 6.2). The cells were resuspended in the same buffer (approximately 1 gram wet weight/10 ml) and disrupted with three 25 second intervals of sonic oscillation, using a Branson Sonifier Cell Disrupter model W-140. Large pieces of debris and intact cells were removed by centrifugation at 14,500 \(\times\) g for 15 minutes. The supernatant was then centrifuged for 1 1/2 hours at 150,000 \(\times\) g in a Beckman preparative ultracentrifuge model L-2. The gelatinous, red-brown pellet of membrane particles resulting from the centrifugation was washed twice with buffer in the above manner.

Enzyme Assay

Assay of the particle-linked dehydrogenases was carried out on a Gilford 2000 recording spectrophotometer, using the procedure of De Ley (24) with the following modification: 1.1 \(\times\) 10\(^{-4}\)M 2,6-dichlorophenol-indophenol, 3.3 ml; 1.873 \(\times\) 10\(^{-1}\)M NaCN, 0.2 ml; 200 mg/ml of substrate (except as stated in the results), 0.25 ml; and
membrane particles adjusted to a Klett of 150 at 660 m\(\mu\), 0.5 ml. The velocity (v) is expressed in \(\Delta O.D./\text{min}\).

**Protein Determination**

The determination of protein was done by the method of Lowry (25).

**Cytochrome Determination**

The particles, used in the determination of the cytochromes, were prepared as given above for the enzyme assay. The spectral scans were made on a Gilford 2000 recording spectrophotometer.

**Purification of Substrates**

Galactose was freed of contaminating glucose by oxidizing the glucose to gluconate with glucose oxidase and then separating the gluconate and galactose by ion exchange chromatography. \(\alpha\)-Methylglucoside was purified in a similar manner.

**Chemicals**

N-Methyl-N' -nitro-N-nitrosoguanidine (NTG) was purchased from K & K Laboratories, Inc. Penicillin-G (potassium salt) was purchased from Sigma Chemical Co. as was the glucose oxidase used in the purification of galactose. The \(\alpha\)-methylglucoside was bought from Mann
Research Laboratories, Inc. 2,6-Dichlorophenol-indophenol was obtained from Matheson, Coleman, and Bell Laboratories.

All sugars were of the D-configuration except for L-arabinose.
RESULTS

To characterize the degree of pleiotropism or the absolute types of particulate dehydrogenases of Acetobacter aceti var. liquefaciens, mutants with reduced particulate dehydrogenase activity for individual substrates were isolated. These mutants were then examined for a concomitant loss of particulate dehydrogenase activity with other substrates. Approximately 100 such mutants were isolated and characterized. A representative cross section is presented in Tables 1 and 2. From these tables it can be seen that the number of such dehydrogenases is limited. Table 1 illustrates that in every case, a mutational event which affected the dehydrogenase activity for glucose also affected the activity for galactose and L-arabinose. Similarly, mutants selected for reduced dehydrogenase activity for galactose showed an accompanying loss of activity for glucose and L-arabinose, and mutants selected for reduced dehydrogenase activity for L-arabinose showed a corresponding loss of activity for glucose and galactose.

To confirm the mutational data obtained for the dehydrogenase of glucose, galactose, and L-arabinose, competitive inhibition tests were carried out. α-Methyl-D-glucoside, a glucose analogue, was used as the inhibitor.
The tests were unsuccessful, for the organism in our possession is able to oxidize the analogue. β-Methyl-D-xylopyranose was also tried, but was ineffective as an inhibitor of the 'pyranose' dehydrogenase.

While only a limited number of polyol mutants were obtained, it does appear from Table 1 that glycerol and sorbitol are oxidized by a single enzyme. Where there is a complete loss of dehydrogenase activity (Gly-963) for glycerol, there is an accompanying loss for sorbitol. In general, the level of enzyme activity within each mutant is of the same order of magnitude for glycerol and sorbitol. It was observed (not shown in Table 1) that all mutants which were grown on glycerol showed an induction or increase in glycerol and sorbitol dehydrogenase activity when compared with glucose-grown cells.

Table 3 shows that in the wild type the oxidation of L-erythritol is also induced with glycerol and that glycerol dehydrogenase activity is enhanced in sorbitol-grown cells.

The particulate dehydrogenase for ribose appears to be independent of the other dehydrogenases. As can be seen from Table 1, reduced activity of the polyol or pyranose dehydrogenase, with several exceptions, is accompanied by little or no reduction in the activity of the ribose dehydrogenase.

In those mutants which were tested for gluconate
Table 1

Mutants with Impaired Pyranose Dehydrogenase

Percent Activity

<table>
<thead>
<tr>
<th>Mutant&lt;sup&gt;a&lt;/sup&gt;</th>
<th>D-Glu</th>
<th>D-Gal</th>
<th>L-Ara</th>
<th>D-Rib</th>
<th>Gly</th>
<th>Sorb</th>
<th>Gluconate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Glu-855&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38</td>
<td>35</td>
<td>52</td>
<td>83</td>
<td>28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>83&lt;sup&gt;d&lt;/sup&gt;</td>
<td>75</td>
</tr>
<tr>
<td>Glu-828&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75</td>
<td>68</td>
<td>68</td>
<td>113</td>
<td>71&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100&lt;sup&gt;d&lt;/sup&gt;</td>
<td>84</td>
</tr>
<tr>
<td>Glu-840</td>
<td>44</td>
<td>43</td>
<td>60</td>
<td>111</td>
<td>116</td>
<td>120</td>
<td>25</td>
</tr>
<tr>
<td>Glu-8106</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>42</td>
<td>79</td>
<td>105</td>
<td>2</td>
</tr>
<tr>
<td>Glu-614</td>
<td>31</td>
<td>18</td>
<td>31</td>
<td>45</td>
<td>82</td>
<td>61</td>
<td>9</td>
</tr>
<tr>
<td>Gal-5-9</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>26</td>
<td>95</td>
<td>126</td>
<td>---</td>
</tr>
<tr>
<td>Ara-727&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55</td>
<td>61</td>
<td>58</td>
<td>195</td>
<td>116</td>
<td>147</td>
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</tr>
<tr>
<td>Ara-721&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>43</td>
<td>39</td>
<td>50</td>
<td>---</td>
</tr>
<tr>
<td>Gly-963&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
<td>9</td>
<td>7</td>
<td>39</td>
<td>0</td>
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<td>---</td>
</tr>
<tr>
<td>Gal-2-4</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>58</td>
<td>124</td>
<td>112</td>
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<tr>
<td>Gal-2-7</td>
<td>0</td>
<td>6</td>
<td>11</td>
<td>43</td>
<td>106</td>
<td>106</td>
<td>---</td>
</tr>
</tbody>
</table>
Legend for Table 1

(a) The following abbreviations are used: Glu, Gal, Ara, Rib, Gly, and Sorb indicate the substrates α-D-Glucose, α-D-Galactose, β-L-Arabinose, D-Ribose, Glycerol, and Sorbitol, and are also used to indicate the particulate dehydrogenase for which the mutant was selected.

(b) Glucose-grown cells. All others grown on glycerol.

(c) The activity is given in terms of % activity of the wild-type #20 grown on glycerol.

(d) The activity is given in terms of % activity of the wild-type #20 grown on glucose.


<table>
<thead>
<tr>
<th>Mutant</th>
<th>Substrate</th>
<th>D-Glu</th>
<th>D-Gal</th>
<th>L-Ara</th>
<th>D-Rib</th>
<th>Gly</th>
<th>Sorb</th>
<th>Gluconate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Glu-852</td>
<td></td>
<td>10</td>
<td>12</td>
<td>11</td>
<td>---</td>
<td>14</td>
<td>---</td>
<td>26</td>
</tr>
<tr>
<td>Glu-812</td>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>---</td>
<td>1</td>
<td>---</td>
<td>10</td>
</tr>
<tr>
<td>Glu-806</td>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>---</td>
<td>0</td>
<td>---</td>
<td>4</td>
</tr>
<tr>
<td>Glu-818</td>
<td></td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>20</td>
<td>0</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Gly-610b</td>
<td></td>
<td>22</td>
<td>18</td>
<td>14</td>
<td>39</td>
<td>21</td>
<td>50</td>
<td>---</td>
</tr>
<tr>
<td>Glu-834</td>
<td></td>
<td>40</td>
<td>41</td>
<td>41</td>
<td>60</td>
<td>4</td>
<td>20</td>
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</tr>
<tr>
<td>Gal-1-2</td>
<td></td>
<td>4</td>
<td>3</td>
<td>6</td>
<td>43</td>
<td>16</td>
<td>18</td>
<td>---</td>
</tr>
</tbody>
</table>
Legend for Table 2

(a) The following abbreviations are used: Glu, Gal, Ara, Rib, Gly, and Sorb indicate the substrates α-D-Glucose, α-D-Galactose, β-L-Arabinose, D-Ribose, Glycerol, and Sorbitol, and are also used to indicate the particulate dehydrogenase for which the mutant was selected.

(b) Glucose grown cells. All others grown on glycerol.

(c) The activity is given in terms of % activity of the wild-type #20 grown on glycerol.

(d) The activity is given in terms of % activity of the wild-type #20 grown on glucose.
dehydrogenase activity, reduction of pyranose dehydrogenase activity was followed by a similar loss of activity by the gluconate dehydrogenase (Table 1). It is clearly seen that the degree to which the activity was reduced for the gluconate dehydrogenase was not of the same magnitude as for the pyranose dehydrogenase. Thus, the gluconate dehydrogenase could be a separate enzyme.

The data in Table 1 also indicates that the gluconate dehydrogenase is an inducible enzyme. When mutants Glu-840 and Glu-614 were grown on glycerol (where gluconate was not present either exogenously or endogenously) the level of gluconate dehydrogenase activity was much lower than with other glucose-grown mutants. The inducibility of the gluconate dehydrogenase was confirmed by determining the enzyme activity following growth of the wild-type organism on glucose, glycerol, and gluconate (see Table 4).

Finally, it can be seen from Table 2 that a number of mutants were deficient in dehydrogenase activity for all classes of substrates. These were labelled integrational or membrane mutants.

Since few polyol dehydrogenase mutants were isolated, the mixed substrate method for the determination of $K_m$ was employed (26), to substantiate the limited mutational data. In this method, when two substrates are used in equimolar amounts the following relationship is true:
where a and b are the substrates; $K$, the $K_m$ of that substrate; $V$, the maximal velocity and $V_m$ the maximal velocity of the equimolar mixture. This method is accurate only if $V_a$ differs considerably from $V_b$, and $K_a$ does not differ greatly from $K_b$. Also, if $V_a - V_m$ or $V_m - V_b$ or both are very small, then the relationship is not accurate. The above conditions appeared to be fulfilled when using glycerol and sorbitol, and glycerol and arabinose (see Figures 1, 2, 3, 4 & 5 for the $K_m$ determinations for D-glucose, D-galactose, L-arabinose, glycerol, and sorbitol dehydrogenases respectively). In the mixed substrate method, if the two compounds in question are substrates for the same enzyme, the observed activity for the mixture should lie between the activities obtained for each substrate separately. Such results were obtained with glycerol and sorbitol, as shown in Figure 6. If the two compounds are substrates for two different enzymes, respectively, then the observed activity for the mixture should be additive. As can be seen in Figure 7, the mixture of glycerol and L-arabinose resulted in a lower $K_m$ than for either substrate alone. The kinetic data thus substantiates the mutant data, indicating the same dehydrogenase for glycerol and sorbitol.
During the polyol dehydrogenase induction studies, it was observed that glucose grown cells produced more of a rich red-brown pigment than polyol grown cells. The particulate pellet from sorbitol-grown cells was relatively pale looking, while that from glucose-grown cells was quite red, implying a possible induction of cytochromes with glucose.

A spectral scan showed that cytochromes, reduced with glucose, are present in lesser quantities on the particles of polyol-grown cells (Figure 8). From the spectra it can be seen that the absorption maxima occur at the same wavelengths, 523, and 554 μ, but the area under the peaks is reduced for glycerol-grown cells. Similar results were obtained with particles reduced with Na₂S₂O₄.
Table 3
Percent activity of the polyol dehydrogenase of the wild-type when grown on glucose, glycerol, and sorbitol.

<table>
<thead>
<tr>
<th></th>
<th>Glycerol</th>
<th>Sorbitol</th>
<th>i-Erythritol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose grown</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Glycerol grown</td>
<td>176</td>
<td>233</td>
<td>525</td>
</tr>
<tr>
<td>Sorbitol grown</td>
<td>586</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

The activity of the glucose-grown cells is taken to be 100%.
Table 4

Specific activity of the gluconate dehydrogenase of the wild-type when grown on glucose, gluconate, and glycerol.

<table>
<thead>
<tr>
<th>Glucose- grown</th>
<th>Gluconate- grown</th>
<th>Glycerol- grown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific Activity*</td>
<td>6.90x10^{-2}</td>
<td>6.0x10^{-3}</td>
</tr>
</tbody>
</table>

*The specific activity is given in mmoles reduced 2,6-Dichlorophenol-indophenol/min./mg. protein.
Figure 1

Lineweaver-Burk plot of the activity of the pyranose dehydrogenase with glucose as substrate. The Least Squares method was employed in determining the line that best fitted the points. The $K_m$ for glucose is $1.4 \times 10^{-3}$ M, and the $V_m$ is $6.8 \times 10^{-1}$. 
Figure 2

Lineweaver-Burk plot of the activity of the pyranose dehydrogenase with galactose as substrate. The $K_m$ is $4.9 \times 10^{-2} M$ and the $V_m$ is $8.1 \times 10^{-1}$. The Least Squares method was employed in determining the line that best fitted the points.
Figure 3

Lineweaver-Burk plot of the activity of the pyranose dehydrogenase with L-arabinose as substrate. The $K_m$ is $7.0 \times 10^{-2} M$ and the $V_m$ is $3.7 \times 10^{-1}$. The Least Squares method was employed in determining the line that best fitted the points.
Figure 4

Lineweaver-Burk plot of the activity of the polyol dehydrogenase with glycerol as substrate. The $K_m$ is $1.1 \times 10^{-2}$ M and the $V_m$ is $8.8 \times 10^{-2}$. The Least Squares method was employed in determining the line that best fitted the points.
Figure 5

Lineweaver-Burk plot of the activity of the polyol dehydrogenase with sorbitol as substrate. The $K_m$ is $5.3 \times 10^{-2} \text{M}$ and the $V_m$ is $1.8 \times 10^{-1}$. The Least Squares method was employed in determining the line that best fitted the points.
Figure 6

Lineweaver-Burk plots of dehydrogenase activity with glycerol, sorbitol, and equimolar amounts of glycerol and sorbitol in the mixed substrate method of determining pleiotropism. The $K_m$ of the equimolar mixture is $2.5 \times 10^{-2} M$. The Least Squares method was employed in determining the line that best fitted the points.
Figure 7

Lineweaver-Burk plots of dehydrogenase activity with glycerol, arabinose, and equimolar amounts of glycerol and arabinose in the mixed substrate method of determining pleiotropism. $K_m$ of the equimolar mixture is $6.6 \times 10^{-3} \text{M}$. The Least Squares method was employed in determining the line that best fitted the points.
Figure 8

Absorption spectra of the cytochromes on membrane particles of *A. aceti* var. *liquefaciens* reduced with glucose. Absorption maxima occur at 520 and 555.

- indicates glucose-grown cells.
- indicates glycerol-grown cells.
Figure 9

Structural formulas of D-glucose, D-galactose, D-ribose, and L-Arabinose.
D-Glucopyranose

D-Ribofuranose

D-Galactopyranose

L-Arabinopyranose

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DISCUSSION

Pleiotropism

The results presented confirm the previous evidence by Kersters and De Ley (16) and Friedman (22) that membrane-bound dehydrogenases of Acetobacter aceti var. liquefaciens are pleiotropic, in that a single enzyme is responsible for the oxidation of more than one substrate. It was not anticipated that the dehydrogenase for glucose and galactose, both hexoses, would also be responsible for the oxidation of L-arabinose, a pentose. In all cases of enzymes showing group specificity, the substrates involved possess common configurational characteristics. In this instance, the first observable feature is that all three sugars are of the pyranose configuration (Figure 9). Also, the constituents about carbons 1, 2, and 3 are identical, which may be of importance for the possible formation of the three non-covalent bonds at the enzyme's active site (33). The ability of this enzyme to oxidize other substrates with the same configurational similarities remains to be determined.

From a combination of mutation and induction data and the mixed substrate method of $K_m$ determination, a single enzyme also appears responsible for glycerol, sorbitol, and i-erythritol oxidation. The multiple induction could be attributable to a non-specific inducer.
were it not for the kinetic data. The degree to which the polyol enzyme is induced appears to be dependent upon the number of carbon atoms in the polyol substrate, viz., the longer the carbon chain the greater the inducing power. However, more data is necessary to confirm this finding.

The question arises as to why there was an appreciable loss of activity of the 'pyranose' dehydrogenase when the polyol dehydrogenase activity was reduced through mutation. One could speculate that the membrane-bound enzymes are found in closely packed clusters on the cytoplasmic membrane. Under such conditions a mutation of the polyol enzyme might change the structure of the membrane which in turn could affect the bound enzyme adjacent to it; the overall effect being a mutational event in the polyol enzyme which is partially expressed in neighboring enzymes. This might be said to be a semi-integrational mutation.

Although the data for the ribose dehydrogenase shows only that it is independent of the other dehydrogenases tested, it is possible that this enzyme is also pleiotropic, and functioning as a 'furanose' dehydrogenase (Figure 9). The lower ribose dehydrogenase activity of some of the 'pyranose' mutants could be attributable to the loss of a small degree of non-specificity by the 'pyranose' enzyme.
While there is little doubt that the gluconate dehydrogenase is an inducible enzyme, the data in Table 4 can be interpreted in two ways. First, the prime inducer is glucose, which actually induces the synthesis of all enzymes in the entire oxidative sequence. Second, the gluconate dehydrogenase is induced by gluconate. This fact, together with the evidence for inducible cytochromes (discussed below), suggests the possibility of an internal sequential induction of the membrane-bound enzymes involved with the catabolism of glucose. The decreased specific activity of the gluconate dehydrogenase from gluconate-grown cells may indicate a poor transport system for gluconate.

Integrational Mutants

Through the use of electron microscopy, evidence has been obtained which suggests the presence of regular repeating structures in the planes of unit membranes. Pease (27) has found a pattern of dense beads repeating on the outer mitochondrial membranes and Robertson (28) has observed beading in transverse sections of synaptic discs which appeared as a hexagonal array of closely packed polygonal facets in frontal views. In bacteria, precise information is lacking concerning the subunits of bacterial membrane systems. However, subunit material has been shown to be present (29). Several
mutants found in this study may indirectly show the presence of a repeating pattern of the structural protein(s) in the cytoplasmic membrane in Acetobacter. These mutants showed a uniform reduction in activity for all substrates tested. Thus, the mutants appear to be integrational mutants. Such mutants would have an alteration of the structure of a membrane protein at a specific intracellular site which could lead to the malfunction of the enzyme(s) bound to that protein (30). The assumption is also made that all the dehydrogenases tested are bound to or near the same site.

Cytochromes

Some of the membrane-bound dehydrogenases of Acetobacter are cytochrome-linked (17). The cytochromes of bacteria may be classified on the basis of their absorption spectra as cytochromes a, b, and c, but these designations do not necessarily connote relationships to those found in animal mitochondria. The bacterial cytochromes are quite flexible as is apparent from the many varieties of the three 'basic' forms. Work has been done which shows that the cytochrome complement of an organism often changes with growth conditions. An example of this is when certain facultative organisms are grown aerobically and then anaerobically. Bacillus cereus when grown anaerobically loses cytochromes a and
c and much of its b (31). *E. coli* and related organisms, under anaerobic conditions, will form a type of cytochrome c which is soluble (32). These organisms depend on oxygen or a lack of it for induction. This investigation has produced preliminary evidence for an inducible cytochrome system in *Acetobacter*. This finding is rather unique, in that *Acetobacter* is a strict aerobe and the inducer is glucose.

Such a finding may be explained by the manner in which this species, *A. aceti* var. *liquefaciens*, carries out its metabolic functions. In this organism, the early enzymatic steps in the oxidation of glucose are more rapid than subsequent steps. Under such conditions, incompletely oxidized products of intermediary metabolism accumulate. In glucose metabolism the early steps consist of the rapid oxidation of glucose to gluconate which is then oxidized with even greater rapidity to the 2-keto acid. These two reactions liberate four moles of hydrogen for every mole of glucose. This influx of hydrogen must be removed as rapidly as it is produced, and an increase in the amount of cytochromes appears to be the means by which this organism achieves the removal of this hydrogen.

Compared with the activity of the 'pyranose' dehydrogenase, oxidation of the polyols occurs at a relatively slow rate (about 1/10 that of glucose). There is
less need for cytochromes and they are thus found in smaller quantities.
CONCLUSIONS

1. A single membrane-bound dehydrogenase is responsible for the oxidation of glucose, galactose, and L-arabinose.

2. A single membrane-bound dehydrogenase appears responsible for the oxidation of glycerol, sorbitol, and i-erythritol.

3. The synthesis of membrane-bound cytochromes appear to be induced in glucose-grown cells.

4. The membrane-bound polyol and gluconate dehydrogenases are induced by their respective substrates.
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