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Regulation of Dextranucrase Secretion by Proton Motive Force in Leuconostoc Mesenteroides.

David Roland Otts
Louisiana State University and Agricultural & Mechanical College

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Regulation of dextran sucrose secretion by proton motive force in *Leuconostoc mesenteroides*

Otts, David Roland, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1987
Regulation of Dextranase Secretion by Proton Motive Force
in
Leuconostoc mesenteroides

A Dissertation
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in
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by
David Roland Otts
B.S., University of Wisconsin, 1983
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The relationship between proton motive force and the secretion of the enzyme dextranucrase (E.C. 2.4.1.5) in Leuconostoc mesenteroides was investigated. The transmembrane pH gradient was determined by measurement of the uptake of radiolabeled benzoate or methylamine while the membrane potential was determined by measurement of the uptake of radiolabeled tetraphenylphosphonium bromide. Leuconostoc mesenteroides was able to maintain a constant proton motive force of -130 mV when grown in fermenters at constant pH while a value of -140 mV was determined from concentrated cell suspensions. The contribution of the membrane potential and transmembrane pH gradient to the proton motive force varied depending on the cation concentration and pH of the medium. The differential rate of dextranucrase secretion was relatively constant at 1040 ΔmU/Δmg dry weight when cells were grown at pH 6.0 to pH 6.7. At this pH range, the cell interior was alkaline with respect to the growth medium. Above pH 7.0, dextranucrase secretion was severely inhibited. This inhibition was accompanied by an inversion of the pH gradient across the cytoplasmic membrane as the cell interior became acidic with respect to the growth medium. Treatment of cells with the lipophilic cation methyltriphenylphosphonium bromide had no effect on the rate of dextranucrase secretion at pH 5.5 but inhibited secretion by 95% at pH 7.0. The inhibition of secretion by methyltriphenylphosphonium bromide was correlated to the dissipation of the proton motive force by this compound. Values of proton motive force greater than -90 mV were required for maximal rates of dextranucrase secretion. The
results of this study strongly indicate that dextranucrase secretion
*Leuconostoc mesenteroides* is dependent on the presence of a proton
gradient across the cytoplasmic membrane. The results further suggest
that dextranucrase secretion is coupled to proton influx into the
cell.
INTRODUCTION

The production of dextran by the bacterium *Leuconostoc mesenteroides* has been a nuisance to the sugar cane industry for decades (23,54). Processing problems associated with dextran include increased juice viscosity, poor clarification, and crystal elongation. The sugar refineries have recently instituted monetary penalties to the producers of raw sugar for the dextran content in their raw sugar. This policy has made the control of dextran formation in the raw sugar factories a priority.

Dextran is produced by the action of dextran sucrase (E.C. 2.4.1.5) on sucrose (124). This enzyme has been extensively studied over the past forty years (79,80,81,100,101,124,125) yet relatively little is known about the regulation of its production (90,149). The research that has been done on the regulation of dextran sucrase production has centered on the optimization of dextran formation, rather than the control of it. A study on the regulation of dextran sucrase production with a focus on the inhibition of its production is thus lacking.

Dextran sucrase is an extracellular enzyme (100,101,149). The cell must have an efficient mechanism to transport this protein across the cytoplasmic membrane if it is going to act on its substrate, sucrose. Over the past seven years, the involvement of proton motive force in the process of protein secretion has been investigated in *Escherichia coli* (5,19,25,26,162). Several proteins that are secreted into the periplasmic space of *Escherichia coli* were demonstrated to be dependent on the presence of the proton motive force. A study on the
energetic requirements of β-lactamase secretion demonstrated that a specific level of proton motive force was required for optimal secretion (5). It was of interest, then, to ask whether proton motive force plays a role in the regulation of dextranucrase secretion in Leuconostoc mesenteroides.
LITERATURE REVIEW

I. Sucrose Metabolism in Leuconostoc mesenteroides

*Leuconostoc mesenteroides* produces several enzymes that act on sucrose (64,81). In addition to the extracellular enzymes dextran-sucrase and levansucrase (81,125), sucrose phosphorylase is also produced (151,156). This enzyme catalyzes the hydrolysis of sucrose by the addition of inorganic phosphate to the glucose moiety of the sucrose molecule. The products of the reaction are fructose and glucose-1-phosphate (156). Sucrose phosphorylase is a constitutive enzyme and is thought to be located intracellularly (151).

In addition to fructose and glucose-1-phosphate, the metabolism of sucrose entails the production of dextran and fructose by dextran-sucrase (100) and the production of levan and glucose by levansucrase (125). The organism lacks a phosphoenolpyruvate:hexose phosphotransferase system (127) and instead phosphorylates glucose and fructose via ATP-dependent enzymes (127,130). Glucose is fermented by the phosphoketolase pathway, generating 1 ATP under anaerobic conditions (28). The fermentation products of this pathway are ethanol, lactic acid, and CO₂ in equal molar quantities (28). The growth of several strains of *L. mesenteroides* has been demonstrated to be improved in the presence of oxygen (62,96). During aerobic growth, ethanol production is reduced while acetate becomes a major end-product of glucose metabolism (163). In the pathway to ethanol formation, acetyl-phosphate is formed by the action of phosphoketolase on xyulose-5-phosphate (163). Acetate is then formed from acetyl-phosphate by the action of acetate kinase. This enzymatic reaction
generates the production of 1 ATP (163). Thus, the ATP yield under aerobic conditions is twice that under anaerobic conditions for the catabolism of glucose.

When glucose is the sole carbon source under aerobic growth conditions, the production of acetate at the expense of ethanol presents a redox problem for the cell as it needs to regenerate NAD$^+$ for catabolic functions (57). It has been demonstrated that the regeneration of NAD$^+$ is accomplished by NADH oxidase (96). This enzyme utilizes oxygen as an electron acceptor to oxidize NADH (96). The production of this enzyme has been shown to be stimulated when L. mesenteroides is grown in the presence of oxygen while alcohol dehydrogenase is repressed (57, 96). Not all strains of L. mesenteroides produce NADH oxidase (96). These strains do not produce acetate from acetyl-phosphate and thus do not generate an additional ATP from glucose fermentation.

In L. mesenteroides, sucrose fermentation differs from glucose utilization in that a major end product of sucrose fermentation is mannitol (151). Mannitol is produced by the reduction of fructose by mannitol dehydrogenase (151). This enzyme is intracellular and is NADH-dependent (151). The mannitol that is produced is excreted by the cell (151). The production of mannitol allows the cell to maintain its redox balance and thus not all acetyl-phosphate is reduced to ethanol. Additional ATP is then available for the cell under both aerobic and anaerobic conditions when grown in sucrose medium. The reaction scheme for fructose utilization is:

\[
3 \text{Fructose} \rightarrow 1 \text{lactic acid} + 1 \text{acetic acid} + 1 \text{CO}_2 + 2 \text{mannitol}
\]
Although *L. mesenteroides* will utilize oxygen as an electron acceptor, it does not carry out oxidative phosphorylation as it lacks a cytochrome system (96). This characteristic is exploited in selective media for *Leuconostoc*. The addition of sodium azide to media will make them selective for *Leuconostoc* and other organisms that lack electron transport chains (62).

Dextranucrase (E.C. 2.4.1.5) is produced by species of *Leuconostoc*, *Streptococcus*, and *Lactobacillus* (59,81). This enzyme polymerizes the glucosyl moiety of sucrose to form dextran, an α-(1->6)-linked glucan with various degrees of branching (79). Dextranucrase has been purified from several *L. mesenteroides* strains (79,81,100). The dextranucrase from strain NRRL B-512F has been extensively studied (100,101,108,124,125). This enzyme has been purified by a variety of techniques but probably the most novel was the use of aqueous-phase partitioning (116). The use of a polyethylene glycol-dextran phase partition system allowed the purification of dextranucrase to be achieved with high recovery and high specific activity (116). The phase system removes contaminating levansucrase from the enzyme preparation (116).

There has been some discrepancy on the molecular weight of B-512F dextranucrase determined by various groups (81,101). Molecular weights of 64-65,000 daltons as well as 177,000 daltons have been reported (81,100,101). The dextranucrase of this strain has been shown to aggregate (81). This may affect the size determinations depending on the conditions of purification (81,100) and on the amount of contaminating dextran present (81,100,116). The enzyme has a pI of
4.1 (100) and has been demonstrated to contain carbohydrate other than dextran (81). Acid hydrolysis, followed by paper chromatography, showed that the major carbohydrate was mannose (125). The pH optimum of the enzyme is between pH 5.0 to pH 5.5 (81). The reported values of the Km of the enzyme for sucrose range from 15 to 20 mM (81, 125). When carbohydrates other than sucrose are present in the reaction mixture, the D-glucosyl group of sucrose is diverted from the synthesis of dextran and is added to the carbohydrate (124). When these carbohydrates, or acceptors, are of low molecular weight, the products are also short, low molecular weight oligosaccharides (124). When sucrose or dextran concentrations are low, a hydrolysis reaction predominates (90).

There have been few studies on the regulation of extracellular dextran sucrase production in Leuconostoc mesenteroides. Dextran sucrose is an inducible enzyme that is only produced when the organism is grown in the presence of sucrose (108). The production of dextran sucrose is not subject to catabolite repression as enzyme is produced in glucose medium with added sucrose. Continuous culture studies also failed to demonstrate dextran sucrose production under conditions of glucose limited growth (90). The production of dextran sucrose by L. mesenteroides differs from that of the oral streptococci as in the latter the enzyme is constitutive (61, 157).

Tsuchiya et al. (149) examined the effect of various medium components on the production of extracellular dextran sucrose. They found that the level of extracellular enzyme obtained was dependent on the sucrose concentration in the medium. Additionally, they found
that ammonium ions had a detrimental effect on enzyme production. Enzyme production was found to be optimal at pH 6.7. In cultures where the pH of the medium was not controlled, enzyme production declined once the medium pH fell below 6.0 (149).

The dependence of dextranase production on the sucrose concentration in the growth medium has been taken advantage of by various workers to produce large quantities of dextranase. Use of a semicontinuous fermentor with sucrose as the batch feed allowed for the production of 8 U/ml of dextranase as compared to 2 U/ml with a normal batch fermentor (104).

Dextranase synthesis has been demonstrated to take place on membrane-bound and free ribosomes in *L. mesenteroides* (83,84). Intracellular, membrane-bound, and extracellular dextranase were isolated in this study (83). Addition of colchicine to cells inhibited the transport of dextranase and caused accumulation of the intracellular form (84).

Magnetism has also been demonstrated to affect dextranase secretion (92). When 70-120 mT of magnetic force was applied to *L. mesenteroides*, higher levels of dextranase were found in the culture medium. The pH of the cultures subjected to the magnetic force was higher than that of cultures without the force. Penicillin further enhanced the effect of magnetism (92).

The regulation of dextranase production in the oral streptococci has been more thoroughly studied than in *L. mesenteroides*. Membrane lipid composition has been postulated to effect glucosyltransferase secretion in *Streptococcus salivarius*. 
Tween 80 markedly stimulated the production of extracellular glucosyltransferase and also increased the degree of unsaturation of the membrane lipid fatty acids (59, 60). The production of glucosyltransferase has also been demonstrated to be stimulated by K⁺ in the growth medium (74, 98). Increasing the K⁺ concentration in the medium increased the degree of unsaturation of the membrane lipid fatty acids (98).

The secretion of glucosyltransferase was demonstrated to be inhibited by Na⁺ in *Streptococcus sanguis* (74). Inhibition of secretion could also be obtained by treating the cells with gramicidin (74). It was postulated that proton motive force (Δp) was involved in the process of secretion, though no actual measurements of Δp were made (74, 158).

II. Chemiosmotic Theory.

According to the chemiosmotic theory (42, 102), the cytoplasmic membrane forms topologically closed structures that are essentially impermeable to ions, specifically H⁺. Protons that are vectorially pumped outside the cell by various metabolic pathways establish a gradient of protons across the membrane. The transport of charge associated with the proton across the membrane generates an electrical potential across the membrane with the cytoplasm being negative. The transport of protons also generates a pH gradient across the membrane with the cytoplasm alkaline with respect to the exterior. Both gradients contribute to the electrochemical potential of protons, ΔμH⁺, which is the force that tends to pull protons back across the membrane into the cell. The free energy change (ΔG) as protons flow...
down these two gradients is equal to:

\[ \Delta G = \Delta \mu_{H^+} = F\Delta \Psi - 2.3RT(\log \frac{[H^+]_{in}}{[H^+]_{out}}) \]  

(1)

In this equation, \( \Delta \mu_{H^+} \) is defined as the difference in electrochemical potential of protons across the cytoplasmic membrane and has the unit of kcal \( \text{(102)} \). The membrane potential is represented by the symbol \( \Delta \Psi \). The gas constant is represented by \( R \) while \( T \) is equal to the temperature in degrees Kelvin. To convert this expression to units of electrical potential, all terms in the equation are divided by Faraday's constant \( (F) \). Equation 1 then becomes:

\[ \frac{\Delta \mu_{H^+}}{F} = \Delta p = \Delta \Psi - \frac{2.3RT}{F} \log \frac{[H^+]_{in}}{[H^+]_{out}} \]  

(2)

The logarithmic factor is equivalent to the pH difference across the cell membrane, \( \Delta p\text{H} = \text{pH}_{\text{out}} - \text{pH}_{\text{in}} \). Commonly, \( 2.3RT/F \) is given the abbreviation \( Z \) and at 25°C is equivalent to 59 mV. Making these substitutions equation 2 becomes:

\[ \Delta p = \Delta \Psi - 59\Delta p\text{H} \]  

(3)

The proton motive force \( (\Delta p) \) has the unit of mV.

The study of proton motive force and how it affects cell processes has been advanced with the use of ionophores. Ionophores are compounds that increase the ionic permeability of membranes \((39,120)\). Two general classes of ionophores are mobile carriers and channel formers. Mobile carriers are hydrophobic compounds that can bind specific ions and actively transport them across cell membranes.
Valinomycin is a mobile carrier ionophore which catalyzes the transport of $\text{K}^+$, $\text{Rb}^+$, $\text{NH}_4^+$ and $\text{Cs}^+$ (120). Valinomycin is an antibiotic produced by species of *Streptomyces* and consists of alternating hydroxy- and amino acids (120). Due to its structure, the affinity of valinomycin for $\text{Na}^+$ is approximately $10^4$ less than for $\text{K}^+$ (120). Valinomycin is uncharged but it acquires the charge of the complexed ion (39). Valinomycin is commonly used to dissipate $\Delta \Psi$ due to its transport of charge across the membrane (39,120). Cells treated with valinomycin have been demonstrated to increase $\Delta p\text{H}$ to partially compensate for the decrease in $\Delta \Psi$ in a number of systems (1,75,150).

A mobile carrier that is commonly utilized to dissipate $\Delta p\text{H}$ is nigericin. Like valinomycin, the active form of nigericin is a cyclic compound but differs in that nigericin loses a proton when it binds a cation (120). Nigericin forms a neutral complex with an ion and generally catalyzes the exchange of $\text{K}^+$ for $\text{H}^+$ (39). Other ionophores which catalyze similar electroneutral exchanges are monensin and dianemycin (39,120). The usual result of nigericin treatment of bacterial cells is the equilibration of $\text{K}^+$ and $\text{H}^+$ gradients across the cytoplasmic membrane (120).

Uncouplers are chemical agents that uncouple ATP synthesis from respiration (39,120). Examples include dinitrophenol, carbonylcyanide-m-chlorophenylhydrazone (CCCP), and salicylanilide (39,120). These compounds are acidic, lipid soluble substances that can pass through the lipid bilayer of the membrane when combined with protons. The transport of protons across the membrane thus dissipates $\Delta p$. When treated with uncouplers, cells increase their rate of respiration with
no ATP synthesis (120). Uncouplers are commonly used to deenergize cells (25,26,33,63).

The accurate determination of cytoplasmic volume is crucial for correct determinations of $\Delta \psi$ and $\Delta p$H (58,70,123). Most techniques employ the use of membrane permeable and impermeable radiolabeled probes to estimate the cytoplasmic volume and contaminating extracellular fluid (58,70). Cells are incubated with $^3$H$_2$O and a $^{14}$C-probe that is generally a polymer such as dextran (58), inulin (51), or polyethylene glycol (58). Alternatively, low molecular weight nontransportable solutes, such as sucrose, are sometimes employed (58). After sufficient incubation time to allow for equilibration, cells are separated from the extracellular fluid by either centrifugation through silicone oils (8) or by membrane filtration (1,63). Membrane filtration offers the advantage of being rapid and the disadvantage of large volumes of contaminating extracellular fluid (58). Silicone oil centrifugation minimizes extracellular fluid contamination but can be disadvantageous for use with strictly aerobic organisms due to poor diffusion of oxygen through the silicone oil (58).

Determination of $\Delta \psi$ in bacteria relies on indirect rather than on direct methods (58,70,123). The use of microelectrodes has been reported with giant E. coli cells (70), but for practical purposes bacterial cells are too small for this type of measurement at physiological conditions. Valinomycin plus $K^+$ has been utilized to determine the membrane potential (71). At equilibrium, the concentration gradient of $K^+$ across the membrane is equal to $\Delta \psi$. The
ΔΨ can be calculated from the distribution of radiolabeled K⁺ or Rb⁺ ions through use of the Nernst equation (58,70). This method is valid only if bacteria are depleted of energy-yielding compounds (70). For example, K⁺ may be accumulated by ATP-dependent transport proteins in *E. coli* (34).

The most commonly used probes for ΔΨ are radiolabeled lipophilic cations (70,123). These include DDA⁺ (N,N-dibenzyl N,N-dimethylammonium) (70), TPP⁺ (tetraphenylphosphonium) (58,70,123) and TPMP⁺ (triphenylmethylphosphonium) (38,123). These cations are able to diffuse through phospholipid bilayers due to their dispersed positive charge (123). In theory, these cations should distribute themselves across cell membranes according to ΔΨ. Thus, at equilibrium, the potential of the ion should equal that of ΔΨ and the Nernst equation can be utilized to calculate ΔΨ. This is applicable provided the cations are not actively transported or metabolized by the cell (123). In addition, the concentration of the probe selected should not be high enough to dissipate ΔΨ (70,123). Lipophilic cations have been demonstrated to equilibrate rapidly across biological membranes. Equilibration times range from less than 1 minute in mitochondria (123) to 5-10 minutes in *Streptococcus* (4,72).

The major setback to the use of these probes is the significant amount of binding to cellular components (94,123). Commonly, the amount of radiolabeled probe accumulated by cells after deenergization (51) or treatment with n-butanol (8,72) is taken to equal the amount of probe that is bound to cellular components. The difference between this value and that obtained from energized cells is then used to calculate ΔΨ (72,94).
Determination of ΔpH is accomplished by measuring the accumulation of weak acids such as benzoate (8,12) or weak bases such as methylamine (12,70). Weak acids are utilized when the interior compartment is alkaline with respect to the media while weak bases are used when it is acidic compared to the medium. Care must be taken to use low concentrations of these compounds (in the micromolar range) as dissipatory effects are seen at higher concentrations (129). As with the ΔΨ probes, the weak acids or bases should not be actively transported or metabolized by the cell (12,70). Another recently developed technique is the use of $^{31}$P-NMR to measure intracellular pH (147). The determination of ΔpH by these two techniques was shown to be in good agreement (70,147).

III. Generation of Proton Motive Force in Bacteria.

The generation of a proton motive force can be accomplished through the expulsion of protons by an electron transport chain (102) or by the action of ATPase (35,78). Under anaerobic conditions, facultative anaerobes generate Δp essentially by the latter mechanism. Consequently, the Δp generated under anaerobic conditions is less than that generated under aerobic conditions (66,67). Staphylococcus aureus maintains a Δp ranging from -250 mV to -160 mV under aerobic conditions at pH 6.0 to pH 7.5 respectively (67). The same organism grown under anaerobic conditions maintains a Δp 50 to 100 mV less than under aerobic conditions (67). In general, fermentative bacteria maintain a Δp less than -150 mV (70). Examples include -120 mV for Streptococcus cremoris (144), -143 mV for Streptococcus lactis (72),
-120 mV for *Clostridium thermoaceticum* (8), -150 mV for *Streptococcus faecalis* (4,78), and -106 mV for *Clostridium acetobutylicum* (51).

The central tenet to Mitchell's chemiosmotic theory was that cells had a mechanism to convert the energy stored in the potential difference of protons across a membrane to chemical energy. This link has been shown to be the ATP synthase, or ATPase. This enzyme can either act to hydrolyze ATP or synthesize ATP, depending on the direction of proton flow through the enzyme (35,103). The ATPase is also referred to as a $H^+-$ATPase because of this coupling of proton flow to its action on ATP.

The $H^+-$ATPase is a complex that consists of two main portions, $F_1$ and $F_0$. The catalytic portion of the complex is $F_1$ which is an extrinsic membrane protein and consists of five different subunits (35,103). The $F_0$ portion is a transmembrane complex that acts as the "proton pore" through the membrane (35,103).

Several studies have linked the $H^+-$ATPase to $\Delta p$. These have included work with chloroplasts (160), chromatophores (91), and submitochondrial particles (145). Purification of the $H^+-$ATPase complex from PS3, a thermophilic bacterium, and incorporation into proteoliposomes demonstrated the link between $\Delta p$ and ATP synthesis (138). ATP was synthesized only when a $\Delta pH$ was applied to the liposomes in the presence of $K^+$ and valinomycin (138) or when an electric field was applied in a voltage-pulse experiment (126). Proton motive force dependent ATP synthesis was also achieved in *Streptococcus lactis*, even though under normal physiological conditions the organism is unable to do so (97). This was achieved through the imposition of a proton motive force across the membranes.
of vesicles prepared from the organism (97).

The $H^+/ATP$ stoichiometry, (i.e. the number of protons necessary to synthesize 1 molecule of ATP), can be calculated from a comparison of $\Delta p$ and the phosphorylation potential (42,102). Reported values include 2 or 3 for mitochondria (102), 3 for chloroplasts (103), 3 for Paracoccus denitrificans (42), and 3 for E. coli (68).

Mutations within the genes coding for the $H^+-ATPase$ have been classified as unc mutations (35). Cells with these mutations are uncoupled and cannot synthesize ATP via a proton gradient. E. coli strains that carry these mutations are unable to grow on organic acids such as lactate and succinate, but will grow fermentatively on glucose (13).

Although the major purpose of the $H^+-ATPase$ in respiratory organisms is the synthesis of ATP, it has recently been proposed that the major function of the enzyme in fermentative bacteria is to maintain the intracellular pH by the active extrusion of protons (12,78). Kobayashi et al. (78) has demonstrated that the activity of $H^+-ATPase$, as well as its synthesis, is stimulated under conditions that acidify the cytoplasm.

The compound N,N'-dicyclohexylcarbodiimide (DCCD) has been used extensively to inhibit the $H^+-ATPase$ in the study of proton motive force (18,19,51). This compound binds to the hydrophobic part of the $H^+-ATPase$ and blocks the transmembrane movement of protons, thus inhibiting its catalytic function (107).

Another mechanism has been proposed for the generation of proton motive force by bacteria grown under fermentative conditions (99).
The energy-recycling model postulates that metabolic end-products can be excreted with protons out of the cell. As a result of the translocation of protons, a proton motive force is generated. Using the fermentative bacterium *Streptococcus cremoris* as the model system (134), the fate of lactate translocation out of the cell has been examined. The metabolic scheme of this organism was simplified to:

\[
\text{Glucose} + 2\text{ADP} + 2\text{Pi} + 2(n-1)H^+_{\text{in}} \rightleftharpoons 2 \text{lactate}^-_{\text{out}} + 2 \text{ATP} + 2nH^+_{\text{out}} + 2H_2O
\]

As a consequence of this equation, if \( n=1 \) there will be no generation of membrane potential as a result of lactate efflux. However, if \( n=2 \), the efflux process results in the translocation of 4 protons and thus the translocation of 2 positive charges per 2 molecules of lactate excreted. As a result of this, both \( \Delta \Psi \) and \( \Delta p \)H can be generated by lactate excretion and less ATP has to be hydrolyzed to generate \( \Delta p \). Lactate efflux has been demonstrated to drive the uptake of leucine in deenergized cells (112). The uptake was shown to be sensitive to protonophores but not DCCD, an inhibitor of the ATPase. This indicated that ATP was not the driving force for leucine transport (112). Additionally, uptake of tetraphenylphosphonium demonstrated that lactate efflux increased \( \Delta \Psi \) by 51 mV, indicative that lactate is translocated across the membrane with more than one proton (112).

Lactate translocation has also been studied in membrane vesicles from anaerobically grown *E. coli* (143). Alteration of the components of \( \Delta p \) through variation of the external pH in the presence of valinomycin or nigericin demonstrated that at pH 5.5 lactate uptake was driven by \( \Delta p \)H while at pH 8.0 \( \Delta \Psi \) was the main driving force.
Thus, at alkaline pH lactate transport is electrogenic while at pH 5.5 it is electroneutral (143). These results indicate a variable H⁺/stoichiometry. This has also been demonstrated in *Streptococcus faecalis* where the ratio of proton influx to lactate influx increased from about 1 at pH 6.5 to about 2 at pH 7.5 (133,144). It has been postulated that the pH effect on stoichiometry is caused by a change in the dissociation state of the lactate carrier (144).

IV. Ion transport and its relationship to Proton Motive Force.

Virtually all bacteria expend energy to expel intracellular sodium. The reasons for this are unclear, though it is believed that Na⁺ competes with K⁺, the major cytoplasmic cation (4,30), for use in various cell processes (43). Aerobic bacteria, such as *E. coli*, utilize Na⁺/H⁺ antiporters which are dependent on Δp, not ATP, for their driving force (9,128). West and Mitchell (159) found that sodium increased the proton permeability of *E. coli* cytoplasmic membranes and first postulated the presence of a bacterial Na⁺/H⁺ antiporter. Work in various laboratories has demonstrated this antiporter activity through the use of inverted (106) and right-side out (9) membrane vesicles. The energetics of sodium efflux has also been carried out *in vivo* in *E. coli* (14). This study confirmed the results obtained with vesicles; sodium efflux was dependent on the electrochemical proton gradient rather than ATP (14,15,128).

Na⁺ extrusion by *Streptococcus faecalis* differs from that in *E. coli* due to the use of an ATP-driven pump by the former (47). In initial studies, net Na⁺ movement and ²²Na⁺/Na⁺ exchange were observed
only in cells capable of generating ATP (47). Cells were demonstrated to extrude Na⁺ against a 100-fold concentration gradient in the presence of dissipators of both ΔpH and ΔΨ (47). The production of inverted membrane vesicles has allowed for the isolation of a sodium-stimulated ATPase that is distinct from F₁Fₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐ¢ (65). It was demonstrated that elevated activities of both Na⁺ extrusion and Na⁺-stimulated ATPase could be detected when cells were grown in the absence of Δp (65). Further work has demonstrated that the Na⁺-ATPase is actually a secondary K⁺ transport system named KtrII (65,77). Mutants that lack the Na⁺-ATPase also lack KtrII. KtrII and the Na⁺-ATPase are induced in parallel when cells are grown in high sodium media, particularly under conditions that limit the generation of Δp (65). The uptake of K⁺ in exchange for Na⁺ is thought to be an electroneutral process (43,65).

The effects of K⁺ on Δp in bacteria are well documented (4,6,71). In K⁺-depleted cells of Streptococcus faecalis, the addition of K⁺ ions depolarized the membrane by 60 mV (6). This decrease in ΔΨ was compensated by an increase in ΔpH leaving Δp unchanged (6). Bakker and Harold (4) showed that K⁺ accumulation only occurred in metabolizing cells. The accumulation of K⁺ could be prevented by reagents that short circuited the proton circulation. It was noted that the gradient of accumulation was too steep (i.e. 50,000 fold) to be sufficiently accounted for by ΔΨ (4). They concluded that K⁺ accumulation in S. faecalis involves both ATP and ΔΨ and is regulated by Δp, or by a membrane carrier that translocates K⁺ inward with H⁺ (4). K⁺/H⁺ antiporters have been identified in both E. coli (34) and S. faecalis (73). The K⁺/H⁺ antiporter in E. coli has been implicated
S. faecalis (73). The K⁺/H⁺ antiporter in E. coli has been implicated in the mechanism for internal pH regulation in alkaline environments (12,69).

The pH of the growth medium also greatly affects the relative contribution of ΔΨ and ΔpH to Δp. In growing S. lactis cells, ΔΨ decreased from -108 mV to -83 mV during a pH change from 6.8 to 5.1 while -ZΔpH increased from -25 mV to -60 mV over the same pH range (72). The Δp was kept relatively constant over this pH range. The interconversion of ΔΨ and -ZΔpH has also been demonstrated in a variety of other fermentative and respiring bacteria (1,8,51,75,132). The decrease in ΔΨ is thought to occur via the electrogenic import of cations, in particular K⁺, to buffer Δp due to the increased export of protons at lower external pH values (4,6,30).

V. Utilization of Proton Motive Force by Bacteria.

Proton motive force has been demonstrated to affect the transport of lactose and proline by altering the redox state of dithiols in the respective carrier proteins (32). Oxidation of a dithiol to a disulfide increased the Km for both carriers. The addition of dithiothreitol restored the full activity of the carriers (85). Evidence for the involvement of dithiols located at the cytoplasmic side of the membrane was obtained from studies with inside-out membrane vesicles (32). The imposition of a proton motive force protected the L-proline carrier against inactivation by glutathione hexane maleimide (32). The results indicated that two redox-sensitive dithiol groups are involved in the transport of proline. The establishment of a proton motive force by whole cells ensures the
dithiols located on the cytoplasmic side of the membrane, thus making that side a low affinity form of the protein.

Proton motive force has been suggested to be critical in DNA transformation of Bacillus subtilis (150). In this study, the effects of ionophores, weak acids, and lipophilic cations on the components of the proton motive force and on the entry of transforming DNA into the cells were investigated. DNA entry into the cells was demonstrated to be severely inhibited under conditions where Δp was small and also under conditions where ΔpH was small and ΔΨ was high. This suggested that Δp, in particular ΔpH, functions as the driving force for DNA uptake in transformation (150).

One of the better studied systems that has been examined for proton motive force involvement is bacterial motility (49,75,132). Translational swimming of starved B. subtilis cells was induced by an artificially created Δp (75). Similar results were also obtained with a motile Streptococcus sp. (141) and Rhodospirillum rubrum (141). Two groups have examined the amount of Δp required for motility in B. subtilis (75,132). Titration of ΔΨ with valinomycin plus K+ demonstrated that at least -30 mV of Δp was required for flagellar rotation (75). At approximately -100 mV, no further increase in rotation rate could be demonstrated. This indicated that saturation of the system had been achieved. The alkalophilic bacterium, Bacillus strain YN-1, has been demonstrated to utilize Na+ rather than protons to drive its flagellar motors (49).

Although the generation of a proton motive force is vital for various cell processes (30,43), it has been suggested that it is not
obligatory for cell growth (44, 76). Treatment of *Streptococcus faecalis* with gramicidin D dissipated Δψ but still allowed for cell growth to occur (44). Growth was dependent on the presence of a complex medium supplemented with a high concentration of K⁺. Growth was also pH dependent as alkaline pH values (ca. pH 8.0) supported better growth than lower pH values (44). Treatment of *E. coli* with the protonophore CCCP dissipated both Δψ and ΔpH, however cell growth still occurred at pH 7.5 in the presence of glucose (76). Substitution of succinate or lactate for glucose in the growth medium inhibited the growth of *E. coli* in the presence of CCCP. This indicated that oxidative phosphorylation was inhibited by CCCP treatment (76).

VI. Protein Export in Bacteria.

The export of proteins is an important aspect of bacterial physiology. Extracellular enzymes facilitate the utilization of macromolecular nutrients by degrading polymers to low-molecular weight products that can be assimilated by the bacterial cell. Examples of enzymes that act in this manner include the pectinase and cellulase of *Erwinia chrysanthemi* (2). Conversely, extracellular enzymes are also utilized by the bacterial cell to synthesize polymers. Examples include the dextranucrases of *Leuconostoc* (81) and the oral *Streptococci* (61) and the levansucrase of *Bacillus subtilis* (36).

Other functions of extracellular proteins in bacteria include those that provide defense mechanisms such as the beta-lactamase of *Bacillus licheniformis* (46) and *E. coli* (5). Cell to cell communication is also achieved through the use of extracellular
proteins. The sex pheromone produced by *Streptococcus faecalis* (121) is secreted by donor cells to facilitate conjugation to recipient cells (121).

In bacteria, most exported proteins are synthesized as precursors containing an amino-terminal sequence which is not found in the mature protein (154). These sequences have been detected by comparing the native protein with the products of *in vitro* translation under conditions where processing is prevented (154). They have also been determined from comparing the amino-terminal end of the mature protein with the nucleotide sequence of the structural gene (154). Many of these amino-terminal sequences, or signal peptides, have been compared and although there is not strict sequence homology, there are some general features that are shared by most. The signal peptide is anywhere from 20 to 40 amino acid residues in length. The amino-terminus of the peptide contains 1-3 positively charged amino acid residues, usually consisting of either arginine or lysine. The amino-terminus is followed by a stretch of 14-20 primarily hydrophobic amino acids. It has been postulated that the function of the positively charged residues is to make the initial contact with the membrane via interaction with negative charges, possibly those on membrane phospholipids (111). The hydrophobic core is postulated to form the first membrane transversing portion of the protein. Alteration of the hydrophobic portion of the signal peptide has inhibited the export of proteins in a variety of bacterial systems (22,46,119). Accumulation of precursors in the cytoplasmic membrane have been shown via these alterations.
The processing of signal peptides in *E. coli* is accomplished by the action of two enzymes; leader peptidase, or signal peptidase I, and lipoprotein leader peptidase, signal peptidase II (24, 29). Signal peptidase I has been purified to homogeneity and consists of a single polypeptide chain of 36,000 daltons (161). Its 323 amino acids span the membrane with the carboxy-terminus exposed to the periplasm. Signal peptidase I lacks a cleavable leader sequence (24). The purified enzyme in the presence of nonionic detergents can efficiently process precursors of M13 coat protein, leucine-binding protein, OmpA and OmpF proteins, as well as the LamB protein (155). This enzyme has been demonstrated to cleave precursors of eukaryotic as well as prokaryotic origin (117). Signal peptidase I has been demonstrated to act on precursor protein molecules after translocation into the membrane has commenced (24). Dalbey et al. (24) constructed an *E. coli* strain in which the synthesis of signal peptidase I was under the control of the arabinose B promoter. Under conditions where the leader synthesis was repressed, precursors to maltose binding protein as well as OmpA protein accumulated in the cytoplasmic membrane. The precursors were demonstrated to be anchored at the outer surface of the membrane by their susceptibility to digestion with proteinase K (24). The results suggested that signal peptidase I acts on precursors on the periplasmic side of the *E. coli* cytoplasmic membrane.

Signal peptidase II action is restricted to precursors that contain a cysteine residue after the cleavage site that is covalently modified with a glyceride thioether group. A consensus sequence has been compiled for the target site of signal peptidase II (121). It
been compiled for the target site of signal peptidase II (121). It consists of Leu-Leu-Ala-Gly-Cys, with cleavage occurring between the glycine and cysteine residues. Modification of the cysteine residue is absolutely required for correct processing to occur (46,52,53). Proteins processed by this enzyme include the penicillinase of B. licheniformis and the Braun lipoprotein in E. coli. Signal peptidase II is inhibited by the antibiotic globomycin, which contains a structural analog of the cleavage site on the target signal peptide (29). This antibiotic has been utilized to demonstrate accumulation of precursors in the above described systems (55). The purified enzyme consists of a single polypeptide chain of 18,000 daltons (148). In gram-negative cells, the enzyme is located exclusively in the inner membrane (148).

Although a proper signal sequence has been found to be mandatory for efficient export, evidence has accumulated that export-specific information exists within the mature protein (24,86,87). Through the use of stepwise deletions, Dalbey and Wickner (24) demonstrated that a region carboxy-terminal to the membrane-spanning domain of signal peptidase I was necessary for efficient translocation. Similar studies have been done with chain-terminating mutants in the β-lactamase gene (86,87) and in the gene coding for arginine-binding protein (16).

Protein export in bacteria can occur both cotranslationally and posttranslationally (122,136). Using membrane impermeable labeling agents, polypeptide chains of exported proteins have been labeled as they cross the cytoplasmic membrane (134,135,136). Randall (122) has demonstrated that certain secreted proteins are translocated
posttranslationally. Translocation of the maltose binding protein occurred only after 80% of the protein had been synthesized while ribose-binding protein was translocated only after its synthesis was complete. The data suggested that entire domains of polypeptides are transferred after their synthesis (122).

There is accumulating evidence for other protein complexes involved in bacterial protein export. Genetic studies have been utilized in *E. coli* to identify these components. Temperature-sensitive mutants in two genes, prlA (secY) and secA, have been isolated (3,121). These mutations result in the accumulation of unsecreted cytoplasmic precursors (3,121). The prlA (secY) gene encodes a 49,000 dalton hydrophobic protein that is in the cytoplasmic membrane (3). This protein has been demonstrated to act posttranslationally to enhance the translocation of pro-OmpA across the cytoplasmic membrane (3).

VII. **Energetic Requirements of Protein Secretion.**

The first demonstration of a requirement for proton motive force during export of protein by bacteria was done with the insertion of phage M13 coat protein into the cytoplasmic membrane of *E. coli* (26). Treatment of cells with arsenate to deplete cellular ATP levels had no effect on the conversion of the precursor to the mature transmembrane form. Treatment of cells with uncouplers such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) caused the precursor to be bound to the cytoplasmic side of the membrane (26). This indicated a role for proton motive force in the integration of the coat protein into the membrane.
Uncouplers have been used to examine the role of proton motive force in the secretion of a variety of proteins in *E. coli*. Daniels *et al.* (25) demonstrated inhibition of processing of leucine-specific binding protein by CCCP as well as with valinomycin. They postulated a specific requirement for membrane potential in export. The use of a uncA mutant that was deficient in membrane $\text{H}^+\text{-ATPase}$ demonstrated that the export of the OmpF and LamB proteins was dependent on proton motive force and not due to depletion of cellular ATP (33).

The first examination of the actual levels of proton motive force necessary for protein export was done on the β-lactamase in *E. coli* (5). In this study, the magnitude of the membrane potential was manipulated by varying the concentrations of $\text{K}^+$ in the presence of valinomycin in the medium. The pH gradient was manipulated by varying the external pH of the experiment. The export of β-lactamase was inhibited over a wide range of values for either $\Delta \Psi$ or $-Z\Delta \text{pH}$. However, the level of total proton motive force where half-maximal inhibition was observed was constant at approximately $-150 \text{ mV}$. Similar experiments using a mutant strain defective in the membrane $\text{H}^+\text{-ATPase}$ demonstrated that cellular ATP was not depleted as a result of dissipation of the proton motive force.

By using both *in vitro* and *in vivo* assays, it has been shown that conditions which prevent the generation of proton motive force across the mitochondrial membrane block the uptake of mitochondrial precursors (41,110,118). Valinomycin plus $\text{K}^+$ prevented the import of the ADP/ATP translocator as well as ATPase subunit precursors into *Neurospora crassa* mitochondria (131,142). The addition of nigericin
mitochondria (131). These results suggested that membrane potential alone is required for protein import. This is supported by evidence showing that import of the ADP/ATP translocator protein could be driven by a diffusion potential generated by valinomycin plus $K^+$, even under conditions where the membrane was made permeable to protons through the use of CCCP (118).

The energetic requirements of export in gram-positive bacteria have not been as extensively studied as in E. coli. The secretion of an alpha-amylase by Bacillus amyloliquefaciens is dependent on proton motive force (104a). This was demonstrated through the use of uncouplers or valinomycin plus $K^+$. When treated with these compounds, a precursor form of the enzyme accumulated in the cytoplasmic membrane. No actual measurements of proton motive force were made (104a).

Unlike translocation across bacterial membranes, proton motive force has been shown to have no involvement in export in yeast microsomal membranes (153). Through the use of an in vitro, cell-free translocation system, it was shown that translocation was totally dependent on the availability of ATP (153). Ionophores such as valinomycin and monensin, as well as the uncoupler CCCP, had no effect on translocation.

The development of an in vitro protein translocation system in E. coli (17) has allowed for a closer examination of the energy requirements of protein export. Alkaline phosphatase and OmpA protein were demonstrated to require ATP rather than a proton motive force for efficient translocation (18). ATP could support translocation, though
less efficiently, in the presence of uncouplers or with membranes prepared from mutants defective in the membrane H^+-ATPase (19). Further work demonstrated that the H^+-ATPase is not involved in ATP-dependent translocation (19,20). Strain CK1801 membrane vesicles, devoid of H^+-ATPase, could use ATP for protein translocation in the presence of magnesium almost as efficiently as membrane vesicles with a functioning H^+-ATPase. The addition of D-lactate, which generates Δp via oxidative respiration (13), stimulated the ATP-dependent translocation of OmpA at low ATP concentrations. These results suggested that Δp acts only in a contributory fashion in translocation (18,19). Examination of the effects of various nucleotide analogs demonstrated the requirement for ATP involved the specific recognition of both the adenosine and phosphate moieties of ATP (20).

One drawback to the above described studies was the absence of determinations of Δp under the various experimental conditions utilized. In a separate study, measurement of ΔΨ was done under different experimental conditions through the use of fluorescence quenching (37). Using membrane vesicles devoid of H^+-ATPase, ATP or NADH could only support 20% of the translocation of OmpA observed when both were present. The data indicated that both ΔΨ and ATP were necessary for optimal pro-OmpA assembly into membrane vesicles (37). Stripping membranes of the F1 subunit of the H^+-ATPase rendered membranes incapable of both generating ΔΨ and translocating OmpA (37).

A study on the translocation of a chimeric ompF-lpp protein has provided additional evidence for proton motive force involvement in protein export (162). The precursor protein is translocated post-translationally in an in vitro translocation system. Concurring with
other studies (18,19,20), translocation was equally efficient with membrane vesicles from a strain that had its genes for the H^+-ATPase deleted or with wildtype cells (17). The uncoupler CCCP severely inhibited the translocation of the chimeric protein. This inhibition was not suppressed by the addition of ATP to the in vitro system (162). Similar results were obtained with the ionophores nigericin and valinomycin. ATP was shown, however, to have a significant role in translocation as the addition of glucose and hexokinase, an ATP-consuming system, almost totally inhibited the translocation of the chimeric protein.

The studies described above demonstrate there is no universal mechanism for driving protein export in all organisms. Prokaryotes have been demonstrated to rely on proton motive force and/or ATP to drive protein export. Unfortunately, studies on the energetic requirements of protein export in bacteria have centered on only two organisms, *E. coli* and *B. amyloliquefaciens*. Both of these organisms rely on respiratory pathways to generate an electrochemical potential and synthesize ATP (69,70,75,132). Thus, the energetic requirements of protein export in organisms with other modes of metabolism remain to be elucidated.
MATERIALS AND METHODS

I. Organism.

Leuconostoc mesenteroides ATCC 10830 was purchased from the American Type Culture Collection, Rockville, MD. It was transferred daily in All Purpose Tween Broth (APT) (Difco Co., Detroit, MI) and biweekly on APT agar plates.

II. Preparation of packed cells.

Leuconostoc mesenteroides ATCC 10830 was maintained on APT agar and broth. To prepare packed cell suspensions, cells were grown overnight in standing culture at 30°C in APT broth and then transferred (5% vol/vol) into 100 ml of fresh APT broth. This culture was incubated, without shaking, at 30°C until the turbidity at 660 nm \( (A_{660}) \) reached approximately 0.5. The cells were harvested by centrifugation and washed twice with the medium of interest (Table 1). The buffer MES (2[N-morpholino]ethanesulfonic acid) was included at a concentration of 200 mM to prevent rapid acidification of the growth medium by the packed cell suspension. The cells were resuspended to 9 ml in 10/9 strength medium in a 25 ml screw capped tube (Corex). The cells were allowed to equilibrate in a 30°C water bath for 5 minutes after which 1 ml of a 50 % (wt/vol) sucrose solution was added to initiate growth and dextranucrase synthesis. The cell concentration at the start of each experiment gave an \( A_{660} \) between 4.5 to 5.0.
Table 1. Media composition. (a)

<table>
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<th>COMPONENT</th>
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<tr>
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<tr>
<td>Yeast Extract</td>
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<table>
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<th>MEDIUM</th>
<th>COMPONENTS</th>
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<td>LM + 50 mM $K_2HPO_4$</td>
</tr>
<tr>
<td>LMK250</td>
<td>LM + 50 mM $K_2HPO_4 + 150$ mM KCl</td>
</tr>
<tr>
<td>LMNa100</td>
<td>LM + 50 mM $Na_2HPO_4$</td>
</tr>
<tr>
<td>LMNa250</td>
<td>LM + 50 mM $Na_2HPO_4 + 150$ mM NaCl</td>
</tr>
</tbody>
</table>

(a) The pH of the media varied according to the experimental conditions.

(b) Autoclaved separately as a 50 % (wt/vol) solution.

(c) 2[N-morpholino]ethanesulfonic acid was included only in media for experiments with packed cells.
III. Determination of cellular dry weight.

Cellular dry weight was determined by the measurement of the $A_{660}$ of appropriate culture dilutions and comparison to the values obtained from a standard curve. To generate the standard curve, cells were grown in LMK250 medium and harvested by centrifugation. The cell pellets were washed twice with distilled water and resuspended to various densities in 10 ml volumes. Aliquots were transferred to aluminum weigh boats that had been heated at 110°C for 24 hours. The turbidity of these aliquots was determined at 660 nm. The weigh boats containing the cell suspensions were heated at 110°C for 24 hours. After this time, the weigh boats were removed from the oven and transferred to dessicators where they were allowed to cool. The weigh boats were weighed on an analytical balance and then returned to the oven. The samples were again heated for 24 hours and reweighed. The cellular dry weight was determined from the difference of the weight of the weigh boat with the dried cell paste and the weigh boat. The turbidity values were then plotted against the corresponding weights determined by this method. An $A_{660}$ of 5.0 was equal to 1.85 mg/ml dry weight cells.

IV. Growth of cells in batch fermenters at constant pH.

*Leuconostoc mesenteroides* ATCC 10830 was maintained on APT agar and broth (Difco Laboratories, Detroit Mich.). For all experiments, cells were grown overnight for 16 hr in APT broth. A 5% (vol/vol) inoculum from this culture was transferred to a 500 ml fermenter (Model C30 Bioflo, New Brunswick scientific Co., Edison, New Jersey) that contained LMK100 medium lacking MES buffer (see table 1).
Sucrose was prepared as a 50% stock solution in water that was adjusted to pH 7.0 and autoclaved separately from the medium. Sterile sucrose (50 ml) was added to 450 ml of LMK100 medium that was prepared in 10/9 strength. The medium pH was maintained by the addition of 5 N KOH by a Model pH-40 Automatic pH Controller and Pump Module (New Brunswick Scientific Co., Edison, New Jersey). All fermentation experiments were carried out at 30°C with an agitation rate of 100 rpm and no aeration. Cell concentrations were determined as dry weights from measurements of the turbidity at 660 nm from appropriate culture dilutions.

V. Extraction and assay of extracellular dextranase.

Dextranase was separated from interfering enzymes through the use of an aqueous two-phase partition technique (116). For each sample, 0.35 ml of culture supernatant was added to a 1.5 ml microfuge tube containing 0.35 ml of a 10% Dextran T-500 solution. The tube's contents were mixed and then 0.7 ml of a 20% polyethylene glycol (PEG) solution (m.w. 3350) were added. The contents were again mixed and then centrifuged in a microfuge for 3 minutes. The upper phase, which consisted primarily of PEG, was removed with a pasteur pipette and discarded while the lower dextran-rich phase was brought to a volume of 0.5 ml with distilled water. Then, 0.5 ml of the PEG solution was added, mixed, and centrifuged. This was repeated twice with the final dextran phase diluted to 0.5 ml with 0.1 M sodium acetate pH 5.0. Dextranase activity was measured by following the rate of production of reducing sugars using either the Nelson-Somogyi (109,137) or the 3,5-dinitrosalicylic acid method (139). Fructose was
used as the standard. Final concentrations in the assay were: sucrose, 150 mM; CaCl₂, 1 mM; sodium acetate pH 5.0, 100 mM; and dextran T-40, 1 mg/ml. One unit is defined as the amount of enzyme that catalyzes the formation of 1 μmole of fructose per minute at 30°C and pH 5.0. Protein was determined by the method of Lowry et al. (95). Levansucrase activity was measured by following the rate of reducing sugar production from raffinose. The buffer for the assay of dextranulose was used, with the substitution of 150 mM raffinose for sucrose and the exemption of dextran T-40.

The differential rate of dextranulose secretion was determined from the slope of an extracellular dextranulose activity vs cellular dry weight graph. The unit for the specific production of dextranulose was expressed as the increase of extracellular dextranulose per increase of cellular dry weight (ΔmU/ Δmg dry weight).

Kinetic constants were determined from the method of Lineweaver and Burk (93) using enzyme that had been purified by the aqueous two-phase partition technique described above.

VI. Determination of the pH stability of dextranulose.

To determine the pH stability of dextranulose, culture supernatant of an 11 hour, LMK100 grown, culture was separated into 50 ml fractions and the pH was adjusted to various values between pH 5.0 to pH 8.0 with KOH or HCl. Immediately after adjusting the pH of the fractions, dextranulose was purified by the aqueous two-phase partition technique described above and assayed for dextranulose.
activity. To mimic the conditions in the fermenter, the 50 ml fractions were held at 30°C for 7 hours. At this point, dextranulose was purified and assayed as described above.

VII. **Determination of extracellular polysaccharide.**

Total extracellular polysaccharide was defined as the total carbohydrate precipitated from culture supernatant by 67% (vol/vol) ethanol. To 0.5 ml of culture supernatant, 1.0 ml of absolute ethanol was added. Precipitated polysaccharide was recovered by centrifugation for 2 minutes in a microfuge. This precipitate was washed three times with 67% (vol/vol) ethanol, dried, and then resuspended to 0.5 ml with distilled water. Total carbohydrate was determined with the phenol-sulfuric assay (31) using glucose as the standard.

VIII. **Preparation and use of ionophores.**

With the exception of valinomycin, stock ionophore solutions were prepared in ethanol at the following concentrations: nigericin 5 mg/ml, gramicidin D 2 mg/ml and methyltriphenylphosphonium bromide (MPTP\(^+\)) 200 mM. Valinomycin was prepared in acetone at a concentration of 5 mg/ml. For all experiments, ionophores were added such that the final concentration of ethanol, or acetone, did not exceed 1.0%. A control culture was included in each experiment that had appropriate amounts of ethanol or acetone added. In experiments utilizing packed cells, ionophores were added at 45 minutes after sucrose induction unless otherwise specified. In studies employing cultures grown at a constant pH in batch fermenters, ionophores were added at 6 hours after inoculation unless otherwise specified.
IX. Determination of cytoplasmic and extracellular water spaces.

Cytoplasmic and extracellular water spaces were determined by incubating cells in the presence of $^{14}$C]inulin and $^3$H$_2$O (66). One ml of growing cells was transferred to a 15 x 100 mm capped culture tube, the radiolabeled probes were added, and the tube was incubated in a 30°C water bath for 5 minutes. Then, 0.4 ml of this mixture was centrifuged through 0.4 ml of silicone oil ((79-82%) Dimethyl-(18-21%)-diphenylsiloxane copolymer) for 1 minute at 10,000 rpm in an Eppendorf microfuge. The upper aqueous layer and the silicone oil were removed by vacuum with a Pasteur pipette. The cellular pellets were resuspended and quantitatively transferred to 20 ml scintillation vials and dissolved with 10 ml of scintillation fluid. All samples were counted for $^{14}$C and $^3$H on a Packard TRI-Carb scintillation counter and corrected for quench via a quench curve. The counts obtained for each radiolabeled probe were divided by its specific activity. This value was taken to equal the amount of probe present in the sample.

X. Determination of ΔpH.

The ΔpH values were calculated from the uptake of $^{14}$C]benzoate (6.4 μM, 43 Ci/mol) or $^{14}$C]methylamine (2.1 μM, 49 Ci/mol) (12,66). One ml of growing cells was transferred to a 15 x 100 mm capped culture tube and the ΔpH probe was added. After 5 minutes of incubation at 30°C, 0.4 ml of this suspension was centrifuged through silicone oil as described in the determination of cytoplasmic volumes. After centrifugation, 25 μl of the upper aqueous layer was removed and transferred to a scintillation vial. This sample represented the
concentration of the probe in the extracellular medium (i.e. $[\text{benzoate}]_{\text{out}}$). The remainder of the upper aqueous layer and the silicone oil were removed by vacuum with a Pasteur pipette. The cell pellet was resuspended with distilled water and quantitatively transferred to a scintillation vial. This sample represented the concentration of the probe inside the cells (i.e. $[\text{benzoate}]_{\text{in}}$) as well as the contribution of the probe from the contaminating extracellular fluid. The equations used to determine $\Delta \text{pH}$ were:

1. $\Delta \text{pH} = \log \frac{[\text{benzoate}]_{\text{in}}}{[\text{benzoate}]_{\text{out}}} \left(1 + 10^{\text{pKa} - \text{pH}_{\text{out}}}\right) - 10^{\text{pKa} - \text{pH}_{\text{out}}}$

2. $\Delta \text{pH} = \log \frac{[\text{methylamine}]_{\text{in}}}{[\text{methylamine}]_{\text{out}}} \left(1 + 10^{\text{pH}_{\text{out}} - \text{pKa}}\right) - 10^{\text{pH}_{\text{out}} - \text{pKa}}$

Equation 1 was used to calculate $\Delta \text{pH}$ when benzoic acid was the $\Delta \text{pH}$ probe while equation 2 was used when methylamine was the probe. The pH gradient was converted to millivolts by multiplying $\Delta \text{pH}$ by $2.3 \frac{RT}{F}$ where $R$ is equal to the gas constant, $F$ is Faraday's constant, and $T$ is the temperature of the assay in degrees Kelvin. This multiplication factor, which commonly is represented by $Z$, is equal to 60 at 30°C.

XI. Determination of $\Delta \psi$.

The membrane potential was determined by measuring the uptake of $[^3\text{H}]\text{tetraphenylphosphonium bromide (TPP}^+) (54,66)$. One ml of growing cells was transferred to a 15 x 100 mm culture tube, TPP$^+$ was added, and the suspension was incubated at 30°C for 5 minutes. After this time, 0.4 ml was centrifuged as described above. The extracellular
concentration of TPP$^+$ was determined by counting 25 μl of the upper aqueous layer after centrifugation. Non-specific binding of the probe to cellular components was corrected for by treating a separate tube with 5% (vol/vol) n-butanol for 30 minutes prior to addition of the isotope. The value obtained from liquid scintillation counting for the butanol-treated cells was subtracted from that of the untreated culture. The membrane potential was calculated according to the Nernst equation (123) as follows:

$$\Delta \Psi = \frac{-RT}{zF} \frac{2.3 \log [TPP^+]_{\text{in}}}{[TPP^+]_{\text{out}}}$$

where $R$ is the gas constant, $F$ is Faraday's constant, and $T$ is the temperature in degrees Kelvin. The letter $z$ represents the valency of the probe. Substitution of the values of the respective terms in this expression reduces this equation to:

$$\Delta \Psi = 60.05 \frac{\log [TPP^+]_{\text{in}}}{[TPP^+]_{\text{out}}}$$

XII. **Calculation of the proton motive force.**

The proton motive force was calculated according to the following equation:

$$\Delta p = \Delta \Psi - Z\Delta pH$$

where $\Delta \Psi$ is the membrane potential, $\Delta pH$ is the transmembrane pH gradient, and $\Delta p$ is the proton motive force which has the unit of millivolts (mV).
XIII. Isolation and assay of membrane ATPase.

L. mesenteroides ATCC 10830 protoplasts were prepared as previously described (113). Briefly, cells were cultivated in APT Broth overnight at 30°C. Then, 500 ml of fresh APT Broth was inoculated with 50 ml of this culture and incubation was continued at 30°C until the A_{660} reached approximately 0.5. The cells were harvested, washed with protoplast buffer, and resuspended to 80 ml with the same buffer. Lysozyme was added at a concentration of 2 mg/ml and incubation was carried out at 37°C for 1 hour. Protoplasts were lysed with the addition of NaCl to give a final concentration of 1.5 M. The suspension was incubated at room temperature for 10 minutes and then centrifuged at 3000 x g for 10 minutes to remove unlysed cells. The resulting supernatant was then centrifuged at 30,000 x g for 30 minutes. The pellet was washed twice with 50 mM Tris, 10 mM MgSO_4, pH 7.5 and was stored at -20°C until assayed. Membrane ATPase was assayed by following the production of inorganic phosphate from ATP hydrolysis (11). Assay buffer consisted of 5 mM ATP, 5 mM MgCl_2, and 100 mM Tris-HCl for pH above 7.5.

XIV. Statistics.

All determinations were done in triplicate unless otherwise indicated. The sampling size is represented by the letter n in these circumstances. The values are reported as the sampling mean and where indicated are ± the standard deviation.
XV. **Chemicals.**

All radioactive compounds were obtained from New England Nuclear, Medford, Mass. Silicone oil was obtained from Petrarch Systems, Bristol, PA. All other chemicals were of reagent grade and are available commercially.
RESULTS

I. Use of an aqueous-phase system to purify dextransucrase.

The study of dextransucrase regulation in L. mesenteroides required a method that would accurately measure the levels of extracellular dextransucrase under various conditions. One assay that is commonly used to measure dextransucrase activity, the measurement of reducing sugar production, is not feasible under normal growth conditions due to the large background of reducing sugar found in the medium. Additionally, the organism also produces extracellular levansucrase which will also produce reducing sugar from sucrose. These problems were circumvented by the use of an aqueous two-phase partition technique (116). The procedure outlined in the Materials and Methods section recovered dextransucrase at 95% yield with a specific activity of 30 U/mg protein. The preparation lacked levansucrase activity as judged by its inability to cleave raffinose, a substrate for the levansucrase but not the dextransucrase from this organism (125). The $K_m$ of the enzyme for sucrose was determined to be 20 mM from a Lineweaver-Burke plot. This is within the range of $K_m$ reported in the literature for this enzyme (80,100). The $V_{max}$ was calculated to be 3.31 µmol/min/mg protein under the assay conditions utilized.

II. Effect of ionophores on the growth of packed cells.

Ionophores are a valuable tool in examining the energetic requirements of various systems (39,120). In order to determine
whether or not proton motive force was involved in the regulation of dextransucrase secretion, the effects of various ionophores were examined using packed cells. Extracellular dextransucrase could be determined after short periods of growth using this method. *Leuconostoc mesenteroides* ATCC 10830 was grown in various complex media that was supplemented with 200 mM MES to prevent rapid acidification of the media. The organism grew equally well in LMK100, LMK250, and LMK100 media (see Materials and Methods). The mean generation time in each medium was 80 ± 5 minutes (n=5). The cells lowered the culture pH from 6.7 to 5.8 in approximately three hours, regardless of medium composition. The addition of valinomycin to LMK250 medium at a concentration of 10 µg/ml at pH 6.6 essentially doubled the mean generation time (154 ± 5 minutes, (n=3). When added to cultures at pH values below 6.0, there was no effect on cell growth. Methyltriphenylphosphonium bromide (MPTP⁺), a lipophilic cation that is also a dissipator of membrane potential, affected growth in a manner similar to valinomycin when added at a pH of 6.6. The mean generation time of such treated cells was 158 ± 6 minutes (n=3). Cells treated with gramicidin D, a channel forming ionophore that equilibrates ions and protons across cytoplasmic membranes (120), required K⁺ in the growth medium to sustain growth (Figure 1). The mean generation times in LMK250 and LMK100 media were essentially the same as values of 100 ± 5 minutes (n=3) and 102 ± 6 minutes (n=3) were obtained respectively. Substitution of Na⁺ for K⁺ as the predominant cation in the growth medium prevented growth of ATCC 10830 in the presence of gramicidin D.
Figure 1. Effect of gramicidin D on the growth of *L. mesenteroides* ATCC 10830. Gramicidin D was added at a concentration of 10 μg/ml at \( t = 45 \) min to cultures in either LMK250 or LMNa100 media. Symbols: ©, LMK250; O, LMK250 + gramicidin D; △, LMNa100; ♦, LMNa100 + gramicidin D.
Interestingly, cells treated with gramicidin D were able to lower the culture pH as effectively as control cultures (Figure 2). This is in contrast to the results obtained with monensin and nigericin, both equilibrators of protons across membranes. In the presence of either of these ionophores, growth and acid production were severely inhibited once the medium pH fell below 6.2 (Data not shown). This indicated that these ionophores were having different mechanistic effects on the cells as compared to gramidicin D.

III. Effect of ionophores on the secretion of dextransucrase.

The effects of ionophores on extracellular polysaccharide production by *L. mesenteroides* ATCC 10830 are shown in Table 2. The addition of nigericin and valinomycin, which should dissipate both $\Delta\Psi$ and $\Delta\mathrm{pH}$, inhibited extracellular polysaccharide production. The addition of dissipators of $\Delta\mathrm{pH}$ inhibited polysaccharide production over five fold in $\mathrm{K}^+$ and $\mathrm{Na}^+$ media.

The effects of the addition of nigericin, valinomycin, or a combination of the two ionophores on the export of dextransucrase are shown in Figure 3. Initially, at a culture pH of 6.33, the amount of extracellular dextransucrase obtained from valinomycin treated cells was depressed as compared to nigericin or control treated cells. As the pH of the medium decreased, valinomycin treated cells exported dextransucrase at a higher rate. Conversely, nigericin treated cells were inhibited in their ability to export dextransucrase as the culture pH decreased. Cells treated with monensin in $\mathrm{Na}^+$ containing medium (LMNa100) gave results essentially identical to those obtained with nigericin treated cells in potassium medium (data not shown).
Figure 2. Culture pH of cells treated with gramicidin D or nigericin. Gramicidin D or nigericin were added to cultures in LMK250 or LMNa100 media at t = 45 min. Symbols: ◇, LMK250 and LMNa100; ○, LMK250 + 10 μg/ml gramicidin D; □, LMNa100 + 10 μg/ml gramicidin D; △, LMK250 + 10 μg/ml nigericin.
Table 2. Effect of ionophores on the production of extracellular polysaccharide.(a)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Ionophore</th>
<th>Polysaccharide&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMK250</td>
<td>None</td>
<td>1050</td>
</tr>
<tr>
<td></td>
<td>Valinomycin (10 µg/ml)</td>
<td>750</td>
</tr>
<tr>
<td></td>
<td>Gramicidin D (10 µg/ml)</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>Nigericin (1 µM)</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>Valinomycin (10 µg/ml) +</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nigericin (1 µM)</td>
<td></td>
</tr>
<tr>
<td>LMNa100</td>
<td>None</td>
<td>660</td>
</tr>
<tr>
<td></td>
<td>Monensin (5 µg/ml)</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>Gramicidin D (10 µg/ml)</td>
<td>0</td>
</tr>
</tbody>
</table>

(a) Ionophores were added to cultures at a pH of 6.6. Cells were allowed to incubate for 150 minutes and then extracellular polysaccharide was determined as described in Materials and Methods.

(b) Extracellular polysaccharide = µg/mg dry weight cells.
Figure 3. Effects of nigericin and valinomycin on the production of extracellular dextranase. Ionophores were added to cultures at \( t = 45 \) min at the following concentrations: control, (○); nigericin, 10 \( \mu \)g/ml (■); valinomycin, 10 \( \mu \)g/ml (▲); valinomycin, 10 \( \mu \)g/ml and nigericin, 10 \( \mu \)g/ml (◇). Dextranase was purified and assayed as described in Materials and Methods.
Cells treated with a combination of valinomycin and nigericin were unable to produce extracellular dextranucrase. The effect of valinomycin on dextranucrase export was dependent on the medium pH (Table 3). As the medium pH decreased, the ability of valinomycin to inhibit dextranucrase export also decreased. Valinomycin showed virtually no effect on dextranucrase export when it was added to cells at a pH of 5.5, suggesting that $\Delta \Psi$ was not critical for efficient dextranucrase secretion at this pH.

In order to determine whether valinomycin was inhibiting dextranucrase export by dissipating $\Delta \Psi$ or by dissipating a $K^+$ gradient, export in the presence of MPTP$^+$ was examined. When MPTP$^+$ was added at a concentration of 1 mM to cells at pH 6.6, the effect on dextranucrase export was essentially identical to that of valinomycin (Figure 4). This suggested that $\Delta \Psi$ was specifically affected. Cultures treated with gramicidin D were deficient in the export of dextranucrase in LMK250 medium (Figure 5). This inhibition also paralleled the effects of valinomycin and MPTP$^+$. This indicates that gramicidin D does not act as a dissipator of $\Delta p$ in this system but may only be dissipating $\Delta \Psi$.

To determine whether the ionophores altered dextranucrase activity, they were added at the same concentrations used in the above described experiments to a dextranucrase preparation that had been aqueous-phase partitioned from an exponentially growing culture. There was no significant effect of the ionophores on dextranucrase activity (data not shown). To eliminate the possibility that the ionophore effects on extracellular dextranucrase levels were due to the result of growth inhibition rather than secretion, cells were
Table 3. Effect of pH on valinomycin mediated inhibition of dextranucrase secretion. (a)

<table>
<thead>
<tr>
<th>Medium pH</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>70</td>
</tr>
<tr>
<td>5.9</td>
<td>55</td>
</tr>
<tr>
<td>5.7</td>
<td>45</td>
</tr>
<tr>
<td>5.5</td>
<td>0</td>
</tr>
<tr>
<td>5.3</td>
<td>0</td>
</tr>
</tbody>
</table>

(a) Cells were incubated in LMK250 medium containing 5% sucrose with valinomycin added at a concentration of 10 μg/ml at the appropriate culture pH. The percentage inhibition was determined as the % decrease of dextranucrase secreted (U/mg dry weight) as compared to a control culture with no valinomycin added.
Figure 4. Effect of methyltriphenylphosphonium bromide (MPTP\(^+\)) on dextranase secretion. MPTP\(^+\) was added to a concentration of 1 mM at \(t = 45\) min (▲). Control, (●); valinomycin, 10 \(μg/ml\) (■).
Figure 5. Effect of gramicidin D on dextranucrase export in LMK250 medium. Gramicidin D was added at a concentration of 10 µg/ml at t = 45 min. Symbols: LMK250, (○); LMK250 + gramicidin D, (□).
incubated for two hours in the presence of sucrose prior to the addition of ionophores. Sufficient dextran sucrose would then have been exported to provide ample fructose for cell growth. The results of this experiment are shown in Figure 6. When ionophores were added at culture pH values of 6.17, only nigericin significantly affected the growth of L. mesenteroides. The addition of MPTP+ produced a slight lag followed by normal growth whereas valinomycin and gramicidin had no affect on cell growth. The export of dextran sucrose, however, was severely inhibited by all the ionophores examined. This suggests that the secretion of dextran sucrase, not the production of the enzyme, was specifically affected.

These results suggested that proton motive force was involved in dextran sucrose secretion. In order to further examine this hypothesis, it was necessary to measure the components of the proton motive force under various conditions. Packed cells were again chosen for these studies.

IV. Determination of Δp in packed cells.

The Δp of growing cells of Leuconostoc mesenteroides is shown in Figure 7. Equilibration of all the probes utilized was rapid and complete within five minutes. The cytoplasmic volume was calculated to be 1.64 ± 0.10 μl/mg dry weight cells (n=30). The organism was able to increase -ZΔpH from -10 mV to -50 mV as the external pH fell from 6.6 to 5.0. This increase came at the expense of the membrane potential as ΔW decreased from -130 mV to -90 mV over this pH range. This gave a relatively constant value of Δp of -140 mV over a wide range of external pH. As the pH of the medium was raised above 7, Δp
Figure 6. Effect of the addition of ionophores after allowing dextransucrase export. Cells were allowed to incubate in the presence of 5% sucrose in LMK250 medium for 120 minutes to allow abundant dextransucrase production. At this point, ionophores were added at the following concentrations: valinomycin, 10 µg/ml (□); MPTF⁺, 100 µM (▲); gramicidin D, 10 µg/ml (△); nigericin, 10 µg/ml (□). A separate culture containing no ionophores (●) acted as the control.
Figure 7. Proton motive force of *L. mesenteroides* ATCC 10830 in LMK100 medium. Cells were grown and assayed as described in Materials and Methods. The data presented are the averages calculated from three separate experiments. Symbols: (△) Δp, (□) Δψ, (⊗) -Z ΔpH.
steadily decreased due to the increased positivity of $-\Delta pH$. *L. mesenteroides* was unable to maintain a constant internal pH (Figure 8). Only over a narrow range of external pH from 6.6 to 7.0, was the internal pH held constant. Although the contribution of $\Delta pH$ to $\Delta p$ increased with decreasing external pH, $\Delta \Psi$ was always the predominant component. The addition of MES buffer to the culture medium had no effect on the generation of $\Delta pH$ (Data not shown). This buffer has been demonstrated to dissipate $\Delta pH$ in other fermentative bacteria (8).

V. **Effect of cations on the generation of $\Delta pH$.**

The effect of $Na^+$ or $K^+$ on $\Delta pH$ is shown in Figure 9. The stimulation of $\Delta pH$ generation by the presence of $K^+$ increased as the external pH decreased, but was prevalent over a wide range of external pH. Increasing the $Na^+$ content in the growth medium significantly inhibited the ability of the cells to generate $\Delta pH$. This was true over a wide range of external pH values. Substitution of 250 mM $Na^+$ for 250 mM $K^+$ decreased $-\Delta pH$ by approximately 20 mV. The concentrations of cations utilized had no effect on the cytoplasmic volume indicating that the effect on $\Delta pH$ was not due to changes in the internal volume of the cell (table 4).

VI. **Effect of cations on $\Delta \Psi$.**

The effect of $Na^+$ or $K^+$ on the generation of $\Delta \Psi$ is shown in Figure 10. The decrease in $\Delta \Psi$ as a function of increasing $K^+$ was constant over a wide range of external pH as increasing the added $K^+$ from 100 to 250 mM decreased $\Delta \Psi$ by approximately 20 mV. Substitution of $Na^+$ for $K^+$ as the primary medium cation had no significant effect
Figure 8. Effect of external pH on internal pH in \textit{L. mesenteroides}. The intracellular pH was determined by measuring the accumulation of [\textsuperscript{14}C] benzoic acid or [\textsuperscript{14}C] methylamine as described in Materials and Methods. The data represents averages obtained from three experiments.
Figure 9. Effect of K⁺ or Na⁺ on the generation of ΔpH by L. mesenteroides. The data represent averages from three experiments. Symbols: (•) LMK100, (□) LMK250, (○) LMNa100, (□) LMNa250.
Table 4. Effect of K⁺ or Na⁺ on the cytoplasmic volume\(^{(a)}\).

<table>
<thead>
<tr>
<th>MEDIUM</th>
<th>VOLUME(^{(b)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM</td>
<td>1.63 ± 0.10</td>
</tr>
<tr>
<td>LMK100</td>
<td>1.64 ± 0.10</td>
</tr>
<tr>
<td>LMK250</td>
<td>1.58 ± 0.08</td>
</tr>
<tr>
<td>LMNa100</td>
<td>1.59 ± 0.09</td>
</tr>
<tr>
<td>LMNa250</td>
<td>1.70 ± 0.10</td>
</tr>
</tbody>
</table>

\(^{(a)}\) The data represent the mean of three determinations.
\(^{(b)}\) Volume = μl/mg dry weight cells (± standard deviation)
Figure 10. Effect of K$^+$ or Na$^+$ on the generation of $\Delta \Psi$ by *L. mesenteroides*. The data represent averages obtained from three experiments. Symbols: (●) LMK100, (■) LMK250, (○) LMNa100, (□) LMNa250.
on $\Delta \psi$. These results suggest that internal Na$^+$ is extruded from *L. mesenteroides* by electroneutral exchange with external protons.

VII. Effect of potassium on $\Delta p$.

The effect of increasing extracellular [K$^+$] on the generation of $\Delta p$ is shown in Figure 11. At an external pH of 5.5, an increase the extracellular [K$^+$] increased $-Z\Delta p\xi$ at the expense of $\Delta \psi$. An increase in the external K$^+$ concentration by 500 mM increased $-Z\Delta p\xi$ by 24 mV. The membrane potential decreased by 18 mV over this range of K$^+$. Thus, $\Delta p$ slightly increased from $-135$ mV to $-141$ over the range of external K$^+$ examined. The organism, thus, can maintain a relatively constant $\Delta p$ over a wide range of external K$^+$.

VIII. Activity of the membrane ATPase.

The pH activity curve of H$^+$-ATPase in isolated membranes from *L. mesenteroides* is shown in Figure 12. The pH optimum was between pH 5.0 to 5.5. Below pH 5.0, ATPase activity sharply declined as no activity could be observed at pH 4.5. The H$^+$-ATPase activity was depressed at alkaline pH. To examine the role of the H$^+$-ATPase in the generation of $\Delta p$ in *L. mesenteroides*, packed cells were treated with DCCD, a known inhibitor of membrane ATPases in bacteria (107) (Table 5). The addition of 250 $\mu$M DCCD to growing cells totally inhibited the generation of $\Delta p$. This suggests that the membrane H$^+$-ATPase is solely involved in generating $\Delta p$ at the pH examined.
Figure 11. Effect of $K^+$ on $\Delta p$ at pH 5.5. Cells were grown in media containing different $K^+$ concentrations and were allowed to metabolize until the medium pH was lowered to pH 5.5. They were then assayed as described in Materials and Methods. Symbols: ($\Delta$) $\Delta p$, (□) $\Delta \psi$, (○) $-Z\Delta pH$. 
Figure 12. The pH activity curve of the $H^+$-ATPase activity from isolated membranes of \textit{L. mesenteroides}. 
Table 5. Effect of DCCD on the generation of Δp.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>-ZΔpH</th>
<th>Δψ</th>
<th>Δp</th>
<th>Doubling Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-37</td>
<td>-109</td>
<td>-146</td>
<td>56</td>
</tr>
<tr>
<td>DCCD</td>
<td>13</td>
<td>3</td>
<td>16</td>
<td>548</td>
</tr>
</tbody>
</table>
IX. Inhibition of dextranucrase secretion by sodium.

It was noted that the level of dextran determined from supernatant of cells grown in Na\textsuperscript{+} medium was lower than that in K\textsuperscript{+} medium (Table 2). This occurred despite the lack of growth effects between the different media (Figure 1). Dextranucrase was isolated from culture supernatants of cells grown in LMK250 and LMNa100 media. Dextranucrase from LMK250 grown cells reached 47 mU/mg dry wt at 110 minutes as compared to 28.1 mU/mg dry wt from LMNa100 grown cells. This represented a 40% inhibition of dextranucrase secretion. Thus, the presence of sodium in the growth medium inhibits the production of extracellular dextranucrase as well as dissipates the transmembrane pH gradient.

X. Dextranucrase secretion by cells grown in batch fermenters at constant pH.

The use of packed cells presented a disadvantage in that the medium pH continually decreased, presumably due to organic acid production by the organism. Thus, manipulation of the pH gradient, vital to the study of its involvement in dextranucrase secretion, was impossible. This problem was overcome by studying the secretion of dextranucrase in cultures grown at constant pH in batch fermenters. The pH gradient could be manipulated by simply varying the medium pH at which the fermentation was run while \( \Delta \psi \) could be altered by an ionophore such as valinomycin or methyltriphenylphosphonium bromide.

The optimum pH for growth was between 6.0-7.0 (table 6). Within this pH range the mean doubling time was 31 ± 2 min (n=4). The mean doubling time increased when the medium pH was above or below this pH.
Table 6. Cell growth as a function of medium pH.

<table>
<thead>
<tr>
<th>Medium pH</th>
<th>Mean Doubling Time</th>
<th>Cell yield (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>60</td>
<td>0.548</td>
</tr>
<tr>
<td>5.5</td>
<td>50</td>
<td>0.858</td>
</tr>
<tr>
<td>6.0</td>
<td>32</td>
<td>1.547</td>
</tr>
<tr>
<td>6.3</td>
<td>34</td>
<td>1.448</td>
</tr>
<tr>
<td>6.7</td>
<td>29</td>
<td>1.500</td>
</tr>
<tr>
<td>7.0</td>
<td>31</td>
<td>1.360</td>
</tr>
<tr>
<td>7.5</td>
<td>54</td>
<td>0.991</td>
</tr>
<tr>
<td>8.0</td>
<td>67</td>
<td>0.531</td>
</tr>
</tbody>
</table>

(a) Mg/ml dry weight at t = 6 hours
range. At pH 8.0, the mean doubling time increased to 67 min while at pH 5.0 it increased to 60 min. _Leuconostoc mesenteroides_ was able to secrete dextranucrase over a wide pH range (Figure 13). An optimal rate of 1240 ΔmU/Δmg dry wt. was determined at pH 7.0. Decreasing the medium pH had little effect on the rate of dextranucrase secretion. Above pH 7.0, the rate of dextranucrase secretion was severely inhibited. At pH 8.0, no dextranucrase could be detected in culture supernatants. To determine whether the depressed levels of dextranucrase were a result of pH inactivation, the pH stability of dextranucrase was determined (Figure 14). Dextranucrase activity decreased 40% when incubated at pH 7.5 for 7 hours whereas it was totally inactivated at pH 8.0. Thus, at pH 7.5 the low levels of dextranucrase observed cannot be totally accounted for by the inactivation of the enzyme. To further examine this, a culture grown at pH 6.7 for 6 hours was shifted to pH 7.5 and extracellular dextranucrase was determined at 10 minute intervals for 1 hour. This shift decreased dextranucrase secretion to 107 ΔmU/Δmg dry wt. and had no effect on cell growth. After 1 hour at pH 7.5, the pH of the medium was lowered back to 6.7. Dextranucrase secretion increased to 1130 ΔmU/Δmg dry wt. This increase in the rate of dextranucrase secretion was detected 10 minutes after the shift in culture pH to pH 6.7. These results indicated that the low levels of dextranucrase found in cultures at pH 7.5 were due to inhibition of secretion rather than pH inactivation of the enzyme.
Figure 13. Extracellular dextran sucrase production as a function of the medium pH. Culture supernatants were extracted and assayed for dextran sucrase as described in Materials and Methods between t=6 and t=7 hours.
Figure 14. Effect of pH on the stability of dextranucrase. Dextranucrase was pH adjusted to various pH values and held at 30 °C for 7 hours. Symbols: (■) immediately after pH adjustment, (□) held at 30 °C for 7 hours.
XI. Determination of \( \Delta p \) in cells grown at a constant pH.

The components of the proton motive force were determined at various pH (Figure 15). Cells maintained a constant proton motive force of \(-130\) mV from pH 7.0 to pH 5.8. Below pH 5.8 \( \Delta p \) decreased and reached a level of \(-112\) mV at pH 5.3. The contribution of \(-Z\Delta p_H\) to the proton motive force increased from 0 mV at pH 7.0 to \(-50\) mV at pH 5.3. The membrane potential decreased from \(-130\) mV to \(-62\) mV over this pH range. The organism was able to maintain a relatively constant internal pH of 7.0 when grown within a pH range of 6.6 to 7.5 (Figure 16). Thus, above pH 7.0 \( \Delta p_H \) inverted (i.e. the interior was acidic with respect to the medium). The inverted \( \Delta p_H \) increased with increasing external pH until at a culture pH of 7.9 the internal pH was 7.18. This inversion of \( \Delta p_H \) caused a decrease in \( \Delta p \) even though \( \Delta \Psi \) increased to \(-140\) mV. As demonstrated previously through the use of packed cells (figure 8), the organism was unable to maintain a constant internal pH when the external pH was lowered below pH 6.6. This decrease in internal pH, did not allow the organism to compensate for the decrease in \( \Delta \Psi \) and thus caused a decrease in \( \Delta p \) at lower pH values.

XII. Effect of methyltriphenylphosphonium bromide on \( \Delta p \) and dextranucrase secretion.

The lipophilic cation methyltriphenylphosphonium (MPTP\(^+\)) bromide was used to manipulate the membrane potential of growing cells while the transmembrane pH gradient was manipulated by varying the pH of the medium. The titration of \( \Delta p \) with MPTP\(^+\) is shown in Table 7. The effects of MPTP\(^+\) were not specific for \( \Delta \Psi \) as \( \Delta p_H \) was slightly
Figure 15. Effect of medium pH on the generation of $\Delta_{p}$ by *L. mesenteroides*. Cells were grown at different pH values for 6 hours and were assayed for $\Delta_{p}$ and $\Delta_{\psi}$ as described in Materials and Methods. Symbols: (▲) $\Delta_{p}$, (■) $\Delta_{\psi}$, (⊗) $-Z \Delta_{p}$.
Figure 16. Effect of medium pH on the internal pH of *L. mesenteroides*. The internal pH was determined from measurements of the accumulation of $^{14}$C benzoic acid or $^{14}$C methylamine as described in Materials and Methods.
Table 7. Effect of MPTP\(^+\) on the generation of Δp at pH 6.0.

<table>
<thead>
<tr>
<th>[MPTP(^+)] (mM)</th>
<th>-ZΔpH</th>
<th>Δψ</th>
<th>Δp</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-35</td>
<td>-95</td>
<td>-130</td>
</tr>
<tr>
<td>0.1</td>
<td>-31</td>
<td>-85</td>
<td>-116</td>
</tr>
<tr>
<td>0.5</td>
<td>-31</td>
<td>-61</td>
<td>-92</td>
</tr>
<tr>
<td>1.0</td>
<td>-27</td>
<td>-54</td>
<td>-81</td>
</tr>
</tbody>
</table>
dissipated. Between pH 5.5 to 6.0, the addition of 1 mM MPTP\(^+\) inhibited \(\Delta pH\) by 15\%. Addition of MPTP\(^+\) at a concentration of 1 mM decreased the membrane potential by approximately 40 \% regardless of the medium pH (data not shown). The addition of MPTP\(^+\) to the cells inhibited dextransucrase secretion in a pH dependent manner (Figure 17). At a culture pH of 5.5, MPTP\(^+\) had no effect on the rate of dextransucrase secretion. Dextransucrase secretion decreased as a function on increasing culture pH until at pH 7.0 it was inhibited by 95 \% as compared to cells not treated with the lipophilic cation. When added to cells at pH 5.5, MPTP\(^+\) increased the doubling time of the cells from 96 to 146 minutes. At pH 7.0, MPTP\(^+\) increased the doubling time of the cells from 113 to 134 minutes. Thus, the pH dependent effects of MPTP\(^+\) on dextransucrase secretion were not the result of growth effects. The rate of dextransucrase secretion was demonstrated to be dependent on the proton motive force (Figure 18). Dextransucrase secretion was severely inhibited at values of \(\Delta p\) less than -80 mV. Between -80 to -90 mV, there was a rapid rise in the rate of extracellular dextransucrase production. Above -90 mV there was no significant increase in the rate of dextransucrase export indicating that a saturation level had been reached. The ability of \textit{L. mesenteroides} to secrete dextransucrase after treatment with MPTP\(^+\) was directly related to the presence of a pH gradient (inside alkaline) across the cell membrane (Figure 19). These results suggest that proton influx is required for efficient dextransucrase secretion.
Figure 17. Dependence of MPTP\(^+\) mediated inhibition of dextranucrase secretion on medium pH. Symbols: (□) cultures treated with 1 mM MPTP\(^+\), (■) untreated control.
Figure 18. Dependence of extracellular dextransucrase production on Δp. Cells were treated with 1 mM MPTP+ at different pH values between 5.5-7.0 and the rate of dextransucrase secretion and Δp was determined as described in Materials and Methods. Symbols: (□) culture treated with 1 mM MPTP+, (■) untreated cultures.
Figure 19. Dependence of dextranase secretion on the transmembrane pH gradient after treatment with 1 mM MPTP\. Procedures are as described in the legend to figure 5.
XIII. Effect of nigericin on dextransucrase secretion.

At pH 7.5 extracellular dextransucrase production was severely inhibited (Figure 13). The proton motive force of _L. mesenteroides_ at this pH was higher than the level of Δp required for optimal dextransucrase secretion as determined by MPTP\(^+\) treatment (Figure 18). At a culture pH of 7.5, ΔpH was equal to 0.48 (interior acidic). Cellular processes dependent on proton influx would not be expected to function well at pH 7.5 due to the large amount of energy required to bring protons into the cell against the concentration gradient. To examine whether dextransucrase secretion was affected in this manner, nigericin was added to cells grown at pH 7.5 (Table 8). This ionophore is commonly used to dissipate pH gradients across biological membranes (39). Addition of nigericin to cells at pH 7.5 stimulated dextransucrase secretion four-fold, from 124 to 494 ΔmU/Δmg dry wt. The pH gradient (interior acidic) was partially collapsed from 29 to 24 mV and Δp increased from -110 to -119 mV. Thus, partial dissipation of the inverted pH gradient stimulated the production of extracellular dextransucrase. When nigericin was added to cells at pH 6.7, the generation of ΔpH was severely, but not totally, inhibited with a corresponding increase in ΔΨ. This increase in ΔΨ kept Δp relatively constant. The doubling time of the cells increased from 116 to 254 minutes but there was no effect on the specific production of extracellular dextransucrase. This provides evidence that dextransucrase secretion is independent of general growth effects caused by the ionophore. Addition of nigericin at pH 6.3 totally dissipated ΔpH and increased ΔΨ. However, growth and dextransucrase secretion were severely inhibited indicating the importance of the
Table 8. Effect of nigericin on $\Delta p$ and dextranucrase secretion.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>$-Z\Delta pH$</th>
<th>$\Delta \psi$</th>
<th>$\Delta p$</th>
<th>Growth$^a$</th>
<th>Dextranucrase$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.3</td>
<td>-34</td>
<td>-99</td>
<td>-133</td>
<td>116</td>
<td>1038</td>
</tr>
<tr>
<td>Nigericin</td>
<td>1</td>
<td>-129</td>
<td>-128</td>
<td>553</td>
<td>328</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.7</td>
<td>-26</td>
<td>-115</td>
<td>-141</td>
<td>116</td>
<td>1038</td>
</tr>
<tr>
<td>Nigericin</td>
<td>-6</td>
<td>-135</td>
<td>-141</td>
<td>254</td>
<td>1078</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.5</td>
<td>29</td>
<td>-140</td>
<td>-111</td>
<td>107</td>
<td>124</td>
</tr>
<tr>
<td>Nigericin</td>
<td>24</td>
<td>-143</td>
<td>-119</td>
<td>105</td>
<td>494</td>
<td></td>
</tr>
</tbody>
</table>

(a) Doubling time between $t= 6-7$ hrs.

(b) $\Delta mU/\Delta mg$ dry wt.
transmembrane pH gradient at pH 6.3.
DISCUSSION

Most of the studies on the energetic requirements of protein secretion in bacteria have utilized *E. coli* as the model system (5,19,21,37). Although the use of this organism offers the investigator the advantage of a very well defined system, it offers the disadvantage of too narrow a focus. What is true in *E. coli* is not true for all organisms. Thus, any theory that is espoused for all bacteria from work done in only one organism must be tested in others. The study of protein secretion by *Leuconostoc mesenteroides* offered several advantages. This organism is very proficient at secreting dextranucrase, as well as other enzymes. Additionally, this organism lacks electron transport chains. It uses a strictly fermentative metabolism and thus offers an energetic system distinct from that found in *E. coli*. Dextranucrase production by *L. mesenteroides* is also very costly to the sugar industry due to the processing problems caused by dextran (23,54). This project, then, was of interest as both a basic and applied research problem.

The dependence of dextranucrase secretion on the protonic potential has been demonstrated by manipulation of the components of \( \Delta p \) with ionophores. The results shown in figure 18 demonstrate that, between pH 5.5 to pH 7.0, dextranucrase export is dependent on proton motive force. Optimal rates of dextranucrase secretion require \( \Delta p \) to be greater than \(-90\) mV. Above this level of \( \Delta p \), there was no significant increase in dextranucrase secretion. This "saturation" of the dextranucrase secretion system by \( \Delta p \) is similar to the findings of \( \Delta p \) involvement in involvement in flagellar motility in *B.*
subtilis (75,132). The maximal rate of flagellar rotation was achieved at values of Δp greater than -100 mV (132).

Interestingly, the proton motive force of L. mesenteroides when grown above pH 7.0 was above the level of Δp required for optimal dextranase secretion but there was no enzyme detectable in culture supernatants. From experiments measuring the stability of dextranase as a function of pH (Figure 14), it was clear that significant inactivation of enzyme occurs at pH 8.0. However, at pH 7.5, the turnover of dextranase due to pH was only 40%. Extracellular enzyme levels were depressed by greater than 90% as compared to optimum levels obtained at 7.0. The proton motive force at pH 7.5 was -112 mV, which was also above the saturation level of Δp required for optimal dextranase secretion. The proton gradient, however, was inverted (i.e. the interior of the cell was acidic with respect to the exterior) as compared to cells grown below pH 7.0. Treatment of cells with nigericin at pH 7.5 partially dissipated the inverted gradient (Table 8) and stimulated dextranase by approximately four-fold. The result of shifting the medium pH from 6.7 to 7.5 and then back to 6.7 demonstrated that secretion of dextranase, and not cell growth, was specifically affected. The internal pH remained relatively constant at pH 7.0 when the external pH was varied from 6.7 to 7.5 (figure 16). This ruled out internal pH as a control factor in dextranase secretion. Thus, dextranase is dependent on the presence of a proton gradient that is oriented in the proper direction (i.e. where the interior is alkaline with respect to the exterior).

The idea of regulation of dextranase secretion by the proton gradient is supported by the results obtained with cells grown in
sodium containing media. Virtually all bacteria expend energy to extrude internal sodium. In most bacteria, this task is entrusted to Na⁺/H⁺ antiporters that are dependent on the protonic potential for their driving force. The extrusion of sodium in the fermentative bacterium *Streptococcus faecalis* is accomplished by the action of a Na⁺-K⁺-ATPase (43,47). The results of this study suggest that *L. mesenteroides* utilizes the protonic potential to drive the expulsion of sodium from the cell. The dissipation of ΔpH but not ΔΨ suggests that Na⁺ is exchanged for H⁺ in an electroneutral manner. The dissipation of ΔpH by Na⁺ correlated with a decrease in the production of extracellular dextran and dextranulase by *L. mesenteroides* (Table 2). This further supports the hypothesis that dextranulase secretion requires a protonic potential for efficient secretion. The inhibition of glucosyltransferase secretion by Na⁺ has also been noted in the oral streptococci (74,140). However, Na⁺ has been demonstrated to decrease ΔΨ in that system (73). Thus, the oral streptococci utilize an electrogenic mechanism for the expulsion of internal Na⁺ rather than a electroneutral one demonstrated in *L. mesenteroides*.

Apparently a value of proton motive force for *L. mesenteroides* is not equivalent at acidic and alkaline pH. The total dissipation of ΔΨ and ΔpH by DCCD suggests that the H⁺-translocating ATPase is solely responsible for the generation of Δp in *L. mesenteroides* (Table 5). It has been proposed that lactate efflux contributes to the generation of potential energy in fermentative bacteria (99,143). The results of this study suggest that this process does not contribute to the
generation of $\Delta p$ in *L. mesenteroides* under the experimental conditions examined. Because of the large inverted pH gradient, the large membrane potential above pH 7.0 must be generated by additional pathways other than simply proton efflux via membrane $H^+$-ATPases. Thus, the term "proton motive force" may not be applicable to the membrane energetics of *L. mesenteroides* above pH 7.0. The organism may utilize other ions to drive solute transport systems such as is the case with alkalophilic bacteria (30, 40). These organisms utilize $Na^+$ to drive many transport systems (30) as well as flagellar movement (49). Conversely, it is also possible that protons are held in a "localized" fashion that allow proton driven solute transport possible even under conditions where the bulk phase to phase proton gradient is not directed in the correct orientation (82, 105). It has been postulated that alkalophilic bacteria accomplish ATP synthesis by localized movement of protons across the cell membrane (40). This localized movement would not be detected by the methods currently used to estimate $\Delta pH$. Apparently, dextranucrase is not driven in this manner. The secretion of dextranucrase at alkaline pH is suppressed as compared to pH values at or below pH 7.0. This does not rule out the possibility that localized proton flow could drive other transport systems in *L. mesenteroides*.

ATP cannot be ruled out as an energy source for dextranucrase secretion as no measurements of the intracellular ATP levels of cells treated with various ionophores were made in this study. However, measurements of the bulk intracellular ATP concentration do not reflect the involvement of ATP in secretion. *E. coli* mutants defective in the proton translocating ATPase are still able to utilize
ATP to drive protein export (19,20,21). Additionally, MPTP\(^+\) has been documented in *Bacillus subtilis* to actually increase the intracellular ATP concentration (63). Lipophilic cations induced cell lysis of *B. subtilis* at the concentrations used in this study (63,164). Although the growth of *L. mesenteroides* was slowed by the addition of MPTP\(^+\), the effects were not as severe as were noted in *B. subtilis*.

Lipophilic cations have been demonstrated to bind to cellular components (94,123). Thus, any biological effects caused by these compounds must be carefully examined. Unfortunately, radiolabeled MPTP\(^+\) was not available for this research. However, the pH dependency of MPTP\(^+\)-mediated inhibition of dextranucrase secretion (figure 17) was very similar to the action of valinomycin on packed cells (table 3). This indicated that the two compounds were exerting their effects on dextranucrase secretion by the same mechanism, namely the dissipation of the membrane potential.

The energetic requirements of protein export in bacteria have been examined predominantly in *Escherichia coli* (5,18,19,20,26,37). This organism has been demonstrated to use both ATP (18,19,20) and proton motive force to drive protein export (5,37,162). The proton motive force in *E. coli* is typically -175 to -200 mV (1,66). It has been demonstrated that a decrease in the proton motive force to -150 mV decreased the export of β-lactamase by 50 % (5). This value of proton motive force is larger than that obtained by *L. mesenteroides* under optimal growth conditions. The level of proton motive force where dextranucrase export was inhibited by 50 % can be calculated to be approximately -85 mV. This represents a decrease in Δp by 35 % as
compared to approximately 25% for *E. coli* (5). The results of this study suggest that a certain percentage of the proton motive force is required for efficient protein secretion. Thus, a fermentative organism such as *L. mesenteroides* requires a smaller absolute value of Δp for protein secretion than *E. coli*, but the relative percentage of the normal Δp required is fairly similar for both organisms. Certainly, more systems need to be examined in order to determine if this relationship holds true for all bacteria.

Protein export in several gram-positive bacteria has been suggested to be dependent on Δp. The export of α-amylase in *Bacillus amyloliquefaciens* was demonstrated to be inhibited by CCCP or valinomycin plus K⁺ (104a) while gramicidin was shown to inhibit the export of a glucosyltransferase in *Streptococcus sanguis* (74). Neither of these studies addressed the question of whether protein export was driven solely by ΔΨ, ΔpH, or by Δp. This study, therefore, represents the first examination of the actual relationship between Δp and protein secretion in gram-positive bacteria.

It is not yet clear what the exact role of proton motive force is in the process of protein secretion in bacteria. The most simplistic model would be the direct coupling of proton movement to protein translocation through the membrane. It is difficult to conceive how this would occur. This model would require a proteinaceous membrane complex that would act as both a proton pore and as a secretory apparatus. Alternatively, this protein export complex might require proton motive force for the maintenance of a proper conformation within the membrane as described by Bakker and Randall (5). This type of model has also been ascribed to the role of proton motive force in
peptidoglycan and teichoic acid synthesis in *Bacillus subtilis* (45). Interestingly, Lancaster *et al.* (89) have postulated two different types of extracellular proteins in *Bacillus licheniformis*. Penicillinase was secreted maximally during phosphate limited growth and was unaffected by the presence of Na⁺ in the growth medium while the secretion of α-amylase was severely inhibited under both of the growth conditions. During phosphate limited growth, *B. licheniformis* produces teichuronic rather than teichoic acids as the major cell wall anionic polymer (89). Thus, the secretion of α-amylase was correlated to the presence of teichoic acids in the cell wall. Inhibition of Δp in *B. subtilis* also inhibits teichoic acid synthesis (45). It would be interesting to speculate that the secretion of dextranulcraze is dependent on teichoic acid synthesis. There is evidence that the extracellular glucosyltransferase of *S. mutans* is associated with teichoic and lipoteichoic acids (88). It is possible that the dependence of dextranulcraze secretion in *L. mesenteroides* on Δp is actually due to the dependence of cell wall polymer synthesis on Δp. No measurements of this type were made in this study, however, so proof of this hypothesis is still lacking.

Membrane lipid composition has been postulated to play an important role in glucosyltransferase secretion in *Streptococcus* (60,98). An increase in the K⁺ concentration of the growth medium stimulated glucosyltransferase secretion and an increase in the amount of unsaturated membrane lipid fatty acids (98). Recently, Hope and Cullis (50) demonstrated that the presence of a pH gradient across large unilamellar vesicle membranes affected the transbilayer
distribution of the membrane lipids. Oleic and stearic acid were localized to the inner side of the membrane when the vesicle interior was basic. When the vesicle interior was acidic, stearylamine and sphingosine localized to the inner side. It is possible that the direction of the pH gradient could affect the localization of membrane lipids in \textit{L. mesenteroides} and thus affect dextranucrase secretion. Studies on the effect of \( \Delta p \) on the membrane lipid composition would be useful in the further elucidation of the role of \( \Delta p \) in dextranucrase secretion.

\textit{L. mesenteroides} maintains a level of proton motive force that is typical for organisms that utilize a strictly fermentative metabolism (8,72,78). Like other fermentative organisms, the contribution of the membrane potential and the pH gradient to the proton motive force varies according to the external pH. Generally, as the external pH decreases the membrane potential also decreases. The decrease in the membrane potential is compensated by an increase in the contribution of the pH gradient to the proton motive force. This is also common in other bacteria (5,8,72,78). This interconversion is thought to prevent the large buildup of charge across the membrane caused by the expulsion of protons from the cell (30,42). If too large a charge ratio is generated across the membrane, then the expulsion of protons from the cell would not be thermodynamically feasible. In most bacteria, the decrease in membrane potential is usually accomplished through the uptake of cations, in particular \( K^+ \) (6,71). The results of this study suggest that \textit{L. mesenteroides} also utilizes cation transport to buffer \( \Delta p \). An increase in the \( K^+ \) concentration of the medium decreased \( \Delta \Psi \) with a corresponding increase in \( -Z \Delta \rho H \) (Figure
The results suggest that *L. mesenteroides* may utilize $K^+$ as the buffering cation for $\Delta p$.

Evidence has accumulated that a major role of the $H^+$-translocating ATPase in fermentative bacteria is the regulation of internal pH (12,69,78). The data presented in figures 8 and 16 show that *L. mesenteroides* can only regulate its internal pH over a narrow range of external pH. Cells grown in pH-controlled fermenters were able to maintain an internal pH of 7.0 over an external pH range of 6.6 to 7.5. Conversely, packed cells inverted its pH gradient at pH 6.7 (figure 8). The discrepancy in internal pH regulation between the two growth conditions is not immediately obvious. In both packed cells and pH-controlled grown cells, the internal pH decreased as a function of decreasing external pH once the external pH dropped below approximately 6.5. The cells were able to increase $\Delta pH$, but not enough to compensate for the decrease in external pH. The response of the $H^+$-ATPase to a change in pH demonstrates the integral role of the enzyme in the extrusion of protons from the cell (figure 12). A change in pH from 7.0 to 6.0 resulted in a 50% increase in ATPase activity. Thus, the ATPase can help to regulate the cell's internal pH. This type of regulation system is common among fermentative bacteria (12,78). The activity of the $H^+$-ATPase greatly decreased over pH 7.0. These results indicate that as the cell's internal pH is raised the activity of the $H^+$-ATPase is inhibited. This inhibition would then keep more protons in the cytoplasm and an inverted pH gradient could be established. Obviously, other factors are involved in the generation of the inverted pH gradient due to its occurrence in
a pH range where the internal pH is kept constant. These other factors are not apparent from the results obtained in this study.

The inhibitory action of gramicidin D on the growth of *L. mesenteroides* in Na⁺-containing media is consistent with the findings of Harold and Van Brunt in *Streptococcus faecalis* (44). Interestingly, cells grown in medium containing high concentrations of K⁺ were able to lower the medium pH as effectively as control cultures without the ionophore, even to acidic pH values (figure 2). Gramicidin D is a channel forming ionophore that equilibrates ions and protons across biological membranes (120) and thus should dissipate both ΔΨ and ΔpH. Treatment of cells with nigericin (figure 2, table 7) suggests that growth is inhibited once the pH falls below 6.3. This suggests that gramicidin D is not acting as an equilibrator of protons in this system. If it was, then the growth effects seen should be similar to those obtained with nigericin. The inhibition of dextransucrase secretion by gramicidin D follows the same pattern as that of valinomycin and MPTP⁺ (figures 4,5) and thus may be acting as a dissipator of ΔΨ but not ΔpH in this system.

The production of dextran by *L. mesenteroides* represents a major loss of profits for the sugar cane industry. The results of this study indicate two major avenues for the control of dextran formation by this organism. The factor that first comes to mind is the control of pH. The results of this study demonstrate the inability of the organism to secrete dextransucrase above pH 7.0. Although the enzyme was found to be relatively stable at pH 7.5, all activity was lost at pH 8.0. The pH of raw sugar cane juice is generally between 5.0-5.5, ideal for the production of dextransucrase and dextran. In the sugar
factory, the addition of lime in the liming tanks raises the pH sufficiently high enough to inactivate the enzyme and inhibit the production of dextranucrase by the organism. However, this is under ideal conditions. The product flow in a sugar factory is quite rapid and times do exist in the liming tank where the pH is not sufficiently high enough to inhibit production of dextran. The possibility of washing the cane as it comes in on the conveyor belts with a basic solution could be explored. It is interesting to note that Texas does not have the dextran problems that Louisiana has. One of the major differences between the two states is the high degree of potash in the soil of Texas as compared to Louisiana. It would be attractive to speculate that the low dextran content in Texan sugar is due to the high cation concentration of the soil.

Many of the preservatives that are presently on the food market are weak acids that inhibit bacterial growth by acidification of the cytoplasm (129). Since dextranucrase secretion is highly dependent on the presence of a proton gradient, these types of compounds would be highly effective in inhibiting extracellular dextranucrase production as well as growth of the organism. Examples of these weak acids include benzoate and sorbate (129).
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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Microbiology

Title of Dissertation: Regulation of Dextranase Secretion by Proton Motive Force in Leuconostoc mesenteroides.

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Major Professor and Chairman

Dean of the Graduate School

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Date of Examination:

Monday, June 29, 1987