MONOSACCHARIDE TRANSPORT IN PS. FLUORESCENS.

AMEERA. AL-JOBORE

University of Windsor

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS RÉCEUE
MONOSACCHARIDE TRANSPORT
IN
PS. FLUORESCENS

BY

AMMERA AI-JOBORE

A DISSERTATION
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy at the
University of Windsor

Windsor, Ontario
1978
Ameera Al-Jobore' Doctor of Philosophy 1978
ABSTRACT

The transport of sugars in bacterial whole cells and isolated membrane vesicles is reviewed.

The kinetics of transport of D-glucose, glucose analogues and D-gluconate by whole cells of \textit{Ps. fluorescens} A.3.12., grown on glucose and succinate, and by isolated membrane vesicles from glucose- and succinate-grown cells is reported.

Transport of Sugar in Membrane Vesicles

1. Transport of D-glucose, 3-deoxy-3-fluoro-D-glucose and D-gluconic acid in isolated membrane vesicles from glucose-grown cells occurs actively by specific transport systems which are linked to the electron transport chain. The transport of these substrates by membrane vesicles is stimulated by electron donors (reduced PMS or L-malate-PAD); in the presence of an electron donor, the substrates or their oxidized forms are concentrated several-fold inside the membrane vesicles; and inhibitors of the electron transport chain markedly inhibit this electron-donor-mediated transport.

2. The kinetics of transport of D-glucose, 2-deoxy-D-glucose, 3-deoxy-3-fluoro-D-glucose, D-gluconic acid methyl \(\alpha\)-D-glucopyranoside and L-glucose by isolated membrane vesicles from glucose-grown cells gives \(K_m\) values of 167 \(\mu\)M, 286 \(\mu\)M, 400 \(\mu\)M, 65.5 \(\mu\)M, \(\infty\), and \(\infty\)
respectively.

(3) In isolated membrane vesicles from succinate-grown cells, the transport of D-glucose, 3-deoxy-3-fluoro-D-glucose and 2-deoxy-D-glucose were nonspecific with Km values, \( \infty \). Gluconic acid was transported with a Km of 62.5 \( \mu M \).

(4) Membrane vesicles from glucose-grown cells oxidize D-glucose, 3-deoxy-3-fluoro-D-glucose and D-gluconic acid to an extent of two \( \delta \) atoms of oxygen/molecule of glucose, one \( \delta \) atom of oxygen/molecule of 3FG or D-gluconic acid respectively. The kinetics of oxidation show that glucose and 3-deoxy-3-fluoro-D-glucose are oxidized by membrane vesicles with Km values of 833 \( \mu M \) and \( 25 \times 10^3 \) \( \mu M \) respectively and that D-gluconic acid and 3-deoxy-3-fluoro-D-gluconic acid are oxidized by gluconate dehydrogenase (EC 1.1.99.3), with Km values of 714 \( \mu M \) and \( 12.5 \times 10^3 \) \( \mu M \) respectively.

(5) Membrane vesicles from succinate-grown cells oxidize D-glucose and 3-deoxy-3-fluoro-D-glucose to the extent one \( \delta \) atom oxygen/molecule of substrate and D-gluconic acid is not oxidized.

(6) Inhibition studies with sulphydryl group inhibitors show no significant effect on the transport or oxidation.
Transport of Sugar in Whole Cells

(1) Glucose is transported in whole cells grown on glucose with low and high Km of 1.7 μM and 79 μM respectively. In succinate-grown cells, the low Km component is present only.

(2) 2-Deoxy-D-glucose, 3-deoxy-3-fluoro-D-glucose and methyl α-D-glucopyranoside are transported actively by whole cells grown on glucose but not by succinate-grown cells with Km values of 167 μM, 64.5 μM and 43.5 μM respectively.

(3) Gluconic acid is accumulated by glucose and succinate-grown cells with Km values of 16.7 μM and 52.6 μM respectively.

(4) D-glucose inhibited the uptake of 2-deoxy-D-glucose and 3-deoxy-3-fluoro-D-glucose respectively.

(5) Cytochalasin A and Cytochalasin B have no significant effect on the growth and transport of hexoses in glucose-grown cells.
STATEMENT

The work described in this thesis was carried out by the author in the Biochemical Laboratory of the University of Windsor between January 1975 and July 1978, under the supervision of Dr. E. F. Taylor.

Unless otherwise stated it is the work of the author and has neither been presented nor is it currently being presented for any other degree.

Signed

A.A.H. Al-Jobore

July 1978
ACKNOWLEDGEMENTS

I wish to express my sincere thanks to my supervisor, Dr. C. F. Taylor, for his constant encouragement and valuable guidance throughout this work.

I would also like to acknowledge the financial support provided by the University of Windsor and University of Baghdad.

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<tr>
<td>G</td>
<td>D-glucose</td>
</tr>
<tr>
<td>GA</td>
<td>D-gluconic acid</td>
</tr>
<tr>
<td>3PG</td>
<td>3-deoxy-3-fluoro-D-glucose</td>
</tr>
<tr>
<td>3PGA</td>
<td>3-deoxy-3-fluoro-D-gluconic acid</td>
</tr>
<tr>
<td>2KGA</td>
<td>2-keto-D-gluconic acid</td>
</tr>
<tr>
<td>2K3PGA</td>
<td>2-keto-3-deoxy-3-fluoro-D-gluconic acid</td>
</tr>
<tr>
<td>2DOG</td>
<td>2-deoxy-D-glucose</td>
</tr>
<tr>
<td>α-LG</td>
<td>methyl α-D-glucopyranoside</td>
</tr>
<tr>
<td>U-(^{14})C-D-G</td>
<td>U-(^{14})C-D-glucose</td>
</tr>
<tr>
<td>U-(^{14})C-D-GA</td>
<td>U-(^{14})C-D-gluconic acid</td>
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<tr>
<td>(^3)H-(C(_3))3FG</td>
<td>(^3)H-(C(_3))-3-deoxy-3-fluoro-D-glucose</td>
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<td>1(^4)C-(C(_1))-2DOG</td>
<td>1(^4)C-(C(_1))-2-deoxy-D-glucose</td>
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<tr>
<td>U-(^{14})C-α-MG</td>
<td>methyl U-(^{14})C-α-D-glucopyranoside</td>
</tr>
<tr>
<td>GOX</td>
<td>glucose oxidase</td>
</tr>
<tr>
<td>GADH</td>
<td>gluconate dehydrogenase</td>
</tr>
<tr>
<td>L-mal.DH</td>
<td>L-malate dehydrogenase</td>
</tr>
<tr>
<td>L-mal.</td>
<td>L-malate</td>
</tr>
<tr>
<td>OAA</td>
<td>oxaloacetic acid</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>NAD(^+)</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>PES</td>
<td>phenazine methosulphate</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitrophenol</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
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PHMB  p-hydroxymercuribenzoate
DGCD  dicyclohexylcarbodiimide
CN⁻  cyanide ion
CA  cytochalasin A
CB  cytochalasin B
nmole  nanomole
µmole  micromole
µM  micromolar
mM  millimolar
M  molar
µg  microgram
mg  milligram
g  gram
ml  millilitre
µm  micrometer
cm  centimeter
s  second
min  minute
c.p.m  counts/minute
temp.  temperature
O.D.  optical density
d.p.m  disintegration/minute
TMG  methyl-1-thio-β-D-galactopyranoside
HPr  histidine-containing phosphocarrier protein of the PTS
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CHAPTER I
INTRODUCTION

Part I
Section A. Active Transport of Carbohydrates in Bacterial Whole Cells

There are two basically different mechanisms of carbohydrate transport in bacteria: 1 (1) indirect coupling and (2) solute modification. In the former, the solute is transported actively across the membrane without being changed. This mechanism requires a carrier molecule in the membrane which changes its affinity during the translocation process: carrier outside has high affinity for the substrate, carrier inside has low affinity. The affinity change of the carrier during the transport process requires energy which is derived from cellular energy via an indirect coupling mechanism such as the production of an ion or proton gradient or a membrane potential. In the latter, the solute is also transported actively across the membrane, but during the transport process the substrate is chemically modified, for instance, the sugar is phosphorylated. This mechanism has been called group translocation. 2,3 However, a more appropriate designation is
vectorial phosphorylation,⁴,⁵ which means that the transport process is achieved by a vectorial chemical reaction whereby the solute is phosphorylated into the cell.

Substrates other than carbohydrates are also known to be transported in bacteria via solute modification. For example, adenine is glycosylated to adenosine monophosphate in a vectorial reaction catalyzed by a membrane-bound enzyme.⁶,⁷ A second example is that of fatty acids, which are converted to acyl-CoA during the transport process in which the acyl-CoA synthetase in E. coli serves as catalyst.⁸

There are two quite distinct mechanisms of indirect coupling of substrate translocation found among microorganisms. A wide range of sugars, organic acids, amino acids and small peptides are transported in some Gram-negative bacteria by a mechanism involving the participation of soluble binding proteins. These are released from the periplasmic space by osmotic shock, or during the formation of spheroplasts or membrane vesicles so that such structures lack these binding protein transport reactions. From studies with uncouplers, and inhibitors and mutations affecting respiration and adenosine triphosphatase (ATPase) activity, it has been concluded that the energy source for these binding protein systems is either ATP itself or a closely related source of covalent bond energy.⁹-¹³

The most extensively investigated sugar binding protein is probably the galactose binding protein of E. coli. Its involvement in galactose transport has been
reported by using a mutant strain defective in this binding protein. The transport of galactose by *E. coli* appears to be mediated by up to six possible systems. The highest affinity system is that for \(\beta\)-methyl galactoside which incorporates a binding protein. Other sugar binding proteins of *E. coli* which were obtained in homogeneous form are the arabinose binding protein and the ribose binding protein. Recently the three dimensional structure of the L-arabinose binding protein has been published. The maltose binding protein has been purified to homogeneity. The release of these binding proteins from the *E. coli* cells, by osmotic shock, resulted in a decreased transport activity of the sugar, which is bound by the released protein. There are also claims that glucose transport in *Ps. aeruginosa* may require a shock-releasable binding protein and that in Brucella abortus the uptake of D-glucose may involve ATP directly. It has been reported that both ATP and the energised membrane state are required for the uptake of binding-protein-dependent substrates.

Unfortunately, studies that show periplasmic binding protein mediated transport in membrane vesicles are not possible due to the loss of binding proteins during vesicle preparation. The role of these binding proteins is still unclear in facilitating the transport of materials across the hydrophobic barrier of the cytoplasmic membrane.
These binding proteins have been shown to play an integral role in certain chemotactic responses. 23

The other broad class of substrate translocating mechanisms requires only membrane-associated components. The coupling of metabolic energy operates through what has been described as the energised state of the membrane.

The lactose transport system in E. coli consists of a carrier which is able to equilibrate lactose into a cell, a catalytic step demonstrating Michaelis-Menten Kinetics. 24 Under physiological conditions, the transport system is able to concentrate lactose or certain $\beta$-D-galactosides against a concentration gradient. If uncouplers of oxidative phosphorylation are added, the system is poisoned, but the carrier function per se can now be studied.

The isolation of the lactose carrier protein was reported in 1965 by Fox and Kennedy. 25 This was obtained by protecting a reactive sulphydryl group of the carrier against N-ethylmaleimide (NEM) with the substrate analogue $\beta$-D-thiodigalactoside (TDG). Upon removal of the TDG this reactive sulphydryl group was labeled with radioactive NEM, so that lactose carrier (M-protein) could then be extracted with detergent.

The lactose carrier protein behaves like a typically integral membrane protein. 26, 27 The integration of the M-protein into the membrane after induction of the E. coli lac-operon 25 required phospholipid biosynthesis, since a
fatty acid auxotroph of *E. coli* with defective biosynthesis of oleate was no longer able to integrate the functional lactose carrier into the membrane if grown in the absence of oleate. When supplied with oleate, the mutants produced functional M-protein upon induction. The experiments indicate that lipid biosynthesis is necessary to integrate the biologically active lactose carrier protein into the cytoplasmic membrane. It has now been established that lipoprotein interaction (annular lipids) is necessary for the activity of a number of membrane-bound enzymes and carrier proteins.

Around 1970 there was still great confusion about the energy-coupling mechanism of the lactose transport system. Scarborough suggested that adenosine triphosphate (ATP) alone was involved in the lactose accumulation process. However, Pavlasova and Harold (1969) had evidence that in anaerobically grown cells of *E. coli* TMG transport could be blocked by uncouplers of oxidative phosphorylation without significantly altering ATP levels. Even more confusion was produced by the suggestion of Kundig et al. that components of the phosphoenol pyruvate—PEP—dependent phosphotransferase system (PTS) might be involved in galactoside transport of *E. coli*.

Involvement of the membrane-bound ATPase in TMG uptake has been described in detail. It has been observed that potassium cyanide-poisoned cells are still able to accumulate TMG, although with half the efficiency of the
unpoisoned cells. Various ATPase mutants accumulate TMG at a very low rate compared to the wild-type strain. Then Kaback\textsuperscript{32} showed very clearly that D-lactate is able to serve as an energy source during active galactoside transport in \textit{E. coli} membrane vesicles (see Part II of this Chapter).

As already pointed out, there is another mechanism of carbohydrate transport in bacteria which is called vectorial phosphorylation. The most characteristic feature of this transport system is the fact that extracellular sugar is phosphorylated during passage through the membrane and appears as a phosphorylated derivative in the cytoplasm. This phosphorylation of the sugar is catalyzed by the PEP-dependent phosphotransferase system (PTS) which was discovered in \textit{E. coli} by Kundig and co-workers.\textsuperscript{33} Genetic and biochemical evidence of PTS function in sugar transport has been discussed.\textsuperscript{4,5,34}

The following reactions are considered to occur during vectorial phosphorylation of carbohydrates (Fig. 1).

The PTS proteins, Enzyme I and HPr, are synthesized constitutively by the bacterial cells. They serve as phospho-donors for several sugars. A genetic analysis of these components has been presented using \textit{E. coli} and \textit{Salmonella}.\textsuperscript{35,36}

Enzyme II-B is sugar specific and membrane-bound. A family of Enzyme II-B exists which is recognized by the
FIGURE 1

Reaction Scheme of The PEP-dependent Phosphotransferase System

Key:

PEP - phosphoenolpyruvate
Sugar-P - sugar phosphate
HPr - histidine-containing phosphocarrier protein of the PTS.
FIGURE 1

Reaction Scheme of The PEP-dependent Phosphotransferase System

The overall reaction is:

PEP + Sugar $\rightarrow$ Sugar-P + Pyruvate
Enzyme II-A or the sugar specific Factor III components. Enzyme II-B is the component responsible for the translocation of the sugar.

Initially it was believed that the PTS was quite separate structurally and functionally from any substrate translocation mechanism, but this conclusion seems to be no longer tenable. Free galactose can enter *E. coli* and *Salmonella typhimurium* by facilitated diffusion on Enzyme II, without the involvement of Enzyme I or HPr.

In *Anthrobacter pyridinolis* there are two pathways for the uptake of fructose: a PTS, giving intracellular fructose-1-phosphate, and a substrate translocation of free fructose (see Part II of this Chapter).

Studies of microbial membrane transport have been dominated by the controversy regarding the merits of the permease, respiration-linked and chemiosmotic models that have been put forward to describe the mechanisms involved. Each of these models assumes some form of membrane-associated carrier which specifically facilitates the permeation of the translocated substrate across the hydrophobic barrier of the phospholipid membrane. The argument for and against these models have already been discussed extensively, in the literature.

Since the acceptance by Kaback of Mitchell's chemiosmotic hypothesis and the abandonment of his own respiration-linked model of membrane transport only the chemiosmotic model will be discussed here.
The chemiosmotic hypothesis postulates that the cytoplasmic membrane is essentially impermeable to most ions and in particular to OH⁻ and H⁺. The respiratory chain is an alternating sequence of hydrogen and electron carriers, arranged across the membrane in loops. The oxidation of a substrate results in the translocation of protons from one side of the membrane to the other, in any one loop, two protons pass across. Translocation of protons is equivalent to the movement of OH⁻ in the opposite direction so that oxidation of a substrate results in the distribution of H⁺ and OH⁻ on opposite sides of the membrane. Both a pH gradient and an electrical potential are therefore established across the membrane, and the sum of these forces constitutes the proton-motive force (PMF). The proton-motive force (PMF) generated by the respiratory chain reverses the direction of ATPase so as to bring about net synthesis of ATP. On the other hand, ATPase itself can function as a proton translocotor, and the hydrolysis of intracellular ATP leads to the efflux of protons into the medium and consequently establishes a proton-motive force (PMF).

According to the chemiosmotic coupling model, the proton-motive force is the driving force for active transport of substrates. Neutral substrates, such as lactose, will be transported via a coupled movement with protons. It is postulated that the transport proteins, the carriers, have affinity for both the substrate and the protons; the
pH gradient and the electrical potential will drive the movement of protons and consequently the active transport and accumulation of substrate (i.e. symport).

The so-called β-galactoside permease of *E. coli* is the classical active substrate translocation system. The membrane carrier or "M-protein" is coded for by the y-gene in the lac-operon. Galactoside uptake in both *E. coli* and *Streptococcus lactis* is accompanied by proton movement; in *E. coli* the stoichiometry is one proton per mole of galactoside. In an *E. coli* mutant with impaired coupling of energy to galactoside transport, the stoichiometry of protons:galactoside is reduced to well below one (i.e. facilitated diffusion). There is no evidence for the involvement of a periplasmic binding protein, and transport activity can be demonstrated and studied in subcellular membrane vesicle preparations. Strong evidence for the role of an electrical potential difference comes from studies in intact cells of two species of *Streptococcus* and *E. coli* vesicles. When loaded with K⁺ and suspended in K⁺-free media, addition of valinomycin leads to high specific electrogenic K⁺ permeability and does not involve protons (i.e. uniport), resulting in a large transient diffusion potential interior negative which is dissipated by other ion movements allow K⁺ to leave. A transient accumulation of amino acids or galactoside was seen, coincident with the duration of the electrical potential differences as measured by accumulation of a
lipid soluble cation, dimethyldibenzyl ammonium ion (DDA⁺). In S. lactis imposition of a sudden pH gradient led to a transient accumulation of galactosides, again suggesting that a PMF can drive transport. A PMF of 79 mV with glucose as a fermentable energy source has been measured and 39 mV with arginine. In both cases the potential was around 37 mV, with a pH gradient of 0.75 with glucose and 0.0 with arginine. It has been shown that proton uptake was associated with the transport of galactose, fucose and arabinose in an appropriately induced strain of E. coli. The anionic glucose-6-phosphate is taken up by E. coli with a minimum proton stoichiometry of 1:1. Proton fluxes associated with hexose uptake in Chlorella vulgaris, Neurospora crassa and Rhodotorula gracilis have also been recorded. In whole-cells studies with Clostridium pasteurianum the accumulation of galactose by an electrogenic mechanism driven by a membrane potential and/or transmembrane pH gradient has been demonstrated. Gluconate on the other hand is taken up electroneutrally in response to a pH gradient only. Lactate flux across the Streptococcus faecalis cell membrane is also electroneutral and is driven by the pH gradient.

The attractive feature of the chemiosmotic coupling model is that the proton-motive force (PMF) is visualized as the common factor for the synthesis of ATP, transport and other energy-linked functions of the membrane. In addition, this model offers an explanation for the
inhibitory action of uncoupling agents on transport. It is proposed that these compounds are soluble in the membrane and act as circulating carriers, conducting protons across the membrane, thereby short-circuiting the PMF.

Studies in whole cells of microorganisms, as well as in membrane vesicles, have supplied evidence in favour of a chemiosmotic type of energy coupling, and it appears to be beyond dispute that the PMF plays an essential role in the mechanism of transport under both aerobic and anaerobic conditions. Recently the chemiosmotic hypothesis and energy transduction has been reviewed.129

Section B. Substrate Uptake Systems in Pseudomonas Species

Although the pseudomonads are biochemically very versatile, few species are able to metabolize many of the sugars and related compounds. Ps. acidovorans and Ps. testosteroni do not even utilize glucose which is a growth substrate for most of the other Pseudomonas species. However, various strains have been described which can grow with glucose, gluconate, galactose, mannose, fructose, arabinose, ribose and xylose. The species that has been studied most extensively is Ps. saccharophila.

Most of the hexoses and their derivatives are metabolized by the reactions of the Entner-Doudoroff pathway which was first established in Ps. saccharophila.60 The enzymes of the Entner-Doudoroff pathway are induced
by growth with glucose, gluconate or glycerol, but not with citrate, succinate or pyruvate. In *Ps. fluorescens*, a membrane-bound glucose oxidase was found to be present in glucose-grown cells but the activity was very low in gluconate-grown cells. It has been found that gluconate rather than glucose is the inducer of the Entner-Doudoroff pathway enzymes in this strain. The particulate gluconate dehydrogenase is inducible too.

*Ps. fluorescens*, *Ps. putida* and *Ps. aeruginosa* have been grouped together on the basis of common physiological characters, as comprising the aerobic fluorescent pseudomonads. All of these bacteria are known to produce gluconate (1) and 2-ketogluconate (2), via particulate glucose and gluconate oxidases (Fig. 2). These oxidized glucose products are then metabolized to 6-phosphogluconate, which is degraded via the Entner-Doudoroff pathway and pentose phosphate pathway. A reported lack of glucokinase activity in *Ps. fluorescens* and *Ps. putida* has resulted in separation of these organisms from *Ps. aeruginosa* on the basis of glucose utilization. According to Eisenberg, however, *Ps. fluorescens* metabolizes glucose to glucose-6-phosphate and does have cytoplasmic glucokinase activity. He tried various combinations of assay reaction mixtures to detect glucokinase activity in crude extracts of *Ps. fluorescens*. These experiments revealed that by simply raising the pH of the enzyme assay reaction mixtures used previously, he could detect
Entner-Doudoroff Pathway in *Pseudomonas* Sp.

Scheme for glucose utilization in the aerobic fluorescent *Pseudomonas* species.


*The first step of oxidation of D-glucose by *Ps. fluorescens* is the conversion of D-glucose into D-glucono-δ-lactone. This lactone is hydrolysed to D-gluconate by lactonase, which is found in the soluble fraction of cell-free extracts of the bacterium.* 145
glucokinase activity in crude extracts of *Ps. fluorescens*. The distribution of kinase activity in supernatant and particulate fractions of crude cell-free extracts is consistent with this kinase activity being a soluble cytoplasmic enzyme in *Ps. fluorescens*. Uridine triphosphate, cytidine triphosphate, or guanosine triphosphate did not substitute for ATP as phosphorylating agents for glucose-6-phosphate formation in this assay reaction mixture.

A general hypothetical scheme for oxidation and metabolism of glucose in the aerobic fluorescent pseudomonads has been proposed$^{65}$ (Fig. 2). A similar scheme has also been recently presented from *Ps. aeruginosa* $^{66,70}$ and is consistent with glucose and gluconate transport in *Ps. aeruginosa* as described by Roberts et al. $^{66}$ The glucose uptake in *Ps. fluorescens* occurs via a glucose inducible active transport mechanism. Particulate glucose oxidase is not an obligatory first step of glucose uptake, since a glucose oxidase-deficient mutant of this organism also accumulated glucose and rapidly metabolized glucose to glucose-6-phosphate and 6-phosphogluconate. $^{69}$ Glucose-uptake process has been induced by glucose, and not gluconate. This leads to the conclusion that although gluconate induces the Entner-Doudoroff pathway in this organism, $^{62}$ glucose uptake is regulated separately. The relative contributions of the various alternatives for glucose permeation in the aerobic fluorescent pseudomonads
is not known. *P. putida* has been reported to utilize glucose primarily via oxidation to 2-keto-gluconate. 64,65,67

Independent regulation of glucose transport and glucose catabolism has been established in *Ps. aeruginosa*. 68,71

In a series of investigations, 72-75 the uptake of amino acids by *Ps. aeruginosa* has been studied. It has been found that while energy-dependent transport systems for 18 of the 20 common amino acids were present in glucose-grown cells, it was possible to increase the activity of transport systems for particular amino acids by growing the bacteria in the presence of the amino acid. The advantage in having low constitutive levels of transport systems for potential growth substrates is that when the compound is present in the environment it can be taken into the cells and then induce synthesis of the metabolic enzymes and of its own transport system.

It has now been shown that the phosphoenol pyruvate phosphorylating system (PTS) for sugar uptake 33 does not participate in the uptake of glucose by *Ps. aeruginosa*. 76,77 After a general survey, of uptake of sugars by bacteria, 77 it has been concluded that the PTS was operative in facultative anaerobes in which substrate level phosphorylation plays a major energetic role, but was not present in the strict aerobes which derive most of their ATP by oxidative phosphorylation. Phibbs and Eagon 76 showed that the hexose uptake system in *Ps. aeruginosa* is energy dependent and inducible. They
suggested that the transported hexoses were trapped as phosphate derivatives formed by intracellular kinases. A different conclusion was reached by Midgeley and Dawes who found that the analogue α-methylglucoside was transported but not phosphorylated, by cultures of *Ps. aeruginosa*.

This transport system is temperature dependent and inhibited by uncouplers. Evidence has been presented in support of an alternate, more complex mechanism for the transport of glucose by *Ps. aeruginosa*. This mechanism involves conversion of glucose to gluconate via membrane-bound oxidase followed by transport of gluconate through the cell membrane. This data has been supported by work on membrane vesicles (see Part II of this Chapter). Glucose analogues, 2-deoxyglucose and α-methylglucoside have also been used to study the transport system in *Ps. aeruginosa*.

The enzymology and metabolism of fluoro-sugars in *Ps. fluorescens* has been studied and it has been demonstrated that these compounds can act as pseudosubstrates.
Part II
Transport Studies With Bacterial Membrane Vesicles

Isolated bacterial cytoplasmic membrane vesicles have proved to be a particularly useful model system for studies of active transport. These vesicles are devoid of the cytoplasmic constituents of the intact cell, and their metabolic activities are restricted to those provided by the enzymes of the membrane itself. This constitutes a great advantage over intact cells in the study of transport mechanisms, since transport of various solutes by membrane vesicles is practically nil in the absence of the appropriate exogenous energy source. Thus the energy source for transport of the particular substrate can be determined by studying which substrates stimulate solute accumulation. Moreover, metabolic conversion of the transported substrate and the energy source is minimal, allowing clear definition of the reactions involved in transport. With intact bacteria, transported solutes are usually rapidly utilized by virtue of the cell's endogenous metabolism, and addition of an external energy source has little or no effect. Moreover, extreme measures are often required to deplete these endogenous energy reserves to the point where added energy sources stimulate transport. Even when this can be accomplished, the results may be
difficult to interpret in view of the cell's ability to metabolize the energy source, the transported solute, or both.

Preparation and Properties of Bacterial Membrane Vesicles

The outer membrane, present in Gram-negative bacteria only, is the lipopolysaccharide layer of the cell wall; the inner is the plasma membrane. Located between these two membranes, known as the periplasmic space, is the peptidylglycan layer of the cell wall. The rigid peptidylglycan layer is partially responsible for the shape and stability of bacteria cells which allows the concentration of solutes against the large concentration of gradients, and prevents the bacterium from bursting in hypotonic environments. 81 Within the plasma membrane are the ribosomes, nucleoplasm, and most of the "soluble" components of the cell.

When a bacterium is growing in the presence of penicillin or treated with enzymes that attack the rigid layer of the cell wall, such as lysozyme, or lysostaphin with staphylococci, the rigid peptidylglycan layer is outgrown or degraded, respectively. As a result, the cell becomes sensitive to changes in osmolarity and will burst in sufficiently hypotonic media. This manipulation is the basis for the preparation of bacterial membrane vesicles (Fig. 3a).
FIGURE 3a

Scheme for The Isolation of Bacterial Membrane Vesicles

Key:

LPS indicates lipopolysaccharide layer
CW indicates cell wall
CM indicates cytoplasmic membrane

After Konings\textsuperscript{146}.
FIGURE 3a

GRAM-NEGATIVE CELL

lysozyme-EDTA treatment in hypotonic medium

SPHEROPLAST

lysis in hypotonic medium

VESICLES
The purity and homogeneity of the vesicles have been established by a variety of criteria. In summary they retain only minute quantities of soluble cytoplasmic components, as well as at least 70 percent of the phospholipids of the cells from which they are prepared. Less than 10 percent of the diaminopimelic acid of the osmotically sensitized form is found in the vesicles, indicating that very little peptidylglycan is left in the preparations. Expressed as a function of dry weight, the vesicles are approximately 60 to 70 percent protein, 30 to 40 percent phospholipid, and about 1.0 percent carbohydrate.

An essential property of a system that is to be used to study transport is that it must have a continuous surface. Although electron micrographs of sections through membrane vesicles suggest that the vesicles are closed structures, other techniques must be utilized to substantiate this impression. The electron micrographs were obtained with positive staining or freeze etching so that the surface of the vesicles can be observed. More convincing evidence for membrane continuity is the demonstration that the vesicles are osmotically intact. They shrink and swell appropriately when the osmolarity of the medium is altered, as shown by light scattering or by measurement of the dextran-impermeable intramembranal space.

A number of observations indicate that the vesicle membrane does not become inverted during lysis. The most
direct evidence comes from freeze etch microscopy.\textsuperscript{87,88} Finally a high proportion of the vesicles catalyze active transport.\textsuperscript{89}

Transport studies with membrane vesicles\textsuperscript{90} are carried out by incubating vesicles with a radioactive transport substrate in the presence of a specific energy source. At a given time the reaction mixtures are diluted to terminate the uptake reaction and the vesicles are separated from the medium by means of rapid millipore filtration.

As early as 1960, evidence was presented which suggested that bacterial membrane vesicles might provide a useful model system for the study of active transport and other membrane-related phenomena.\textsuperscript{91} Subsequent studies have demonstrated that the vesicles catalyze the active transport of a wide variety of metabolites in the presence of appropriate energy sources.\textsuperscript{5,83} Initial rates of transport of many of these substances are comparable to those of the intact cell\textsuperscript{92} and the vesicles accumulate these solutes to concentrations many times in excess of that in the external medium. Isolated membrane vesicles have also provided an interesting system for studying certain aspects of phospholipid biosynthesis.\textsuperscript{93}

The energy source for transport in isolated membrane vesicles vary with the organism and the substance transported. In \textit{E. coli} membrane vesicles,\textsuperscript{83} the uptake of \(\alpha\)-methylglucoside, glucose, fructose and mannose occurs by
vectorial phosphorylation via the PTS. This phosphotransferase system, which was described initially in 1964, catalyzes the transfer of phosphate from phosphoenoilpyruvate to certain carbohydrates, which has already been discussed in Part I.

Further information about the lactose binding site was obtained from studies of isolated membrane vesicles which contained the lactose carrier. By observing changes in the fluorescence emission spectrum it was shown that vesicles bound the fluorescent lactose analogs 2-(N-dansyl)aminoethyl-1-thio-β-D-galactopyranoside (DG$_2$)(1) and 6'-(N-dansyl)aminoethyl-1-thio-β-D-galactopyranoside (DG$_6$)(2). This binding required energised membrane vesicles or lactose efflux from the vesicles and resulted in an increased intensity of fluorescence for the bound

![Chemical Structure](image)

**Fluorescent β-Galactosides**

1. DG$_2$: $R = S(CH_2)_2$
2. DG$_6$: $R = S(CH_2)_6$
DG₂ or DG₆ molecules. Recently these studies of binding have been repeated, but this time using tritium labeled DG₆. Flow dialysis experiments confirmed the observation that the binding of DG₆ required energised membrane vesicles.⁹⁹,¹⁰⁰

It has been shown very clearly⁴² that D-lactate is able to serve as an energy source during active galactoside transport in E. coli membrane vesicles. In this experiment, the vesicles oxidized D-lactate to pyruvate, which required the existence of a membrane-bound D-lactate dehydrogenase (D-LDH). This membrane-bound D-LDH of E. coli has since been solubilized and purified to homogeneity.¹⁰¹ In the presence of the purified D-LDH, vesicles derived from a mutant strain defective in D-LDH could be reconstituted.¹⁰² In contrast to wild-type membrane vesicles which are insensitive to antibody against D-LDH, the D-LDH-reconstituted vesicles are inhibited by D-LDH antiserum.¹⁰³ This suggests that in native wild-type vesicles D-LDH is bound at the inner surface of the membrane vesicles, whereas in reconstituted vesicles the enzyme is bound at the outer surface.

A suicide substrate 2-hydroxy-3-butyroate (HBA) has been used to probe the functional role of D and L-LDH's in D-lactate-dependent active transport in isolated E. coli membrane vesicles. This acetylenic hydroxy acid¹⁰⁴,¹⁰⁵ is an irreversible inactivator of a number of flavine-linked hydroxy acid oxidizing enzymes. Serving in each
case as a covalent titrant for the bound flavine coenzyme.\textsuperscript{106} The compound is a substrate for the membrane-bound, flavine-linked D-LDH, which undergoes 15 to 30 turnovers prior to inactivation. The following reaction sequence has been proposed\textsuperscript{104} (Scheme I).

Inactivation of D and L-LDH and D-lactate-dependent transport by HBA is highly specific. Other membrane-bound dehydrogenases, flavoprotein dehydrogenase, NADH dehydrogenase and succinate dehydrogenase remain fully active and transport of substrates in the presence of ascorbate-phenazine methosulfate is not affected. Furthermore $\alpha$-glycerolphosphate-dependent transport of amino acids in Staphylococcus aureus vesicles is not inactivated by the acetylenic hydroxy acid.\textsuperscript{41}

Both the $\alpha$-hydroxy group and acetylenic functionality are crucial to covalent modification of flavine coenzymes by HBA. Thus, butynoic acid has no effect, and the alkenoic analog, 2-hydroxy-3-butenoate (vinylglycolate or VG), is a noninactivating substrate for either D- or L-lactic dehydrogenase in E. coli membrane vesicles but can function as an electron donor for solute uptake. However, rapid oxidation of vinylglycolate (VG) produces thousands of reactive 2-keto-3-butenoate product molecules (Scheme II).\textsuperscript{104} This ketoacid has an $\alpha,\beta$-unsaturated carbonyl system, is susceptible to rapid 3,4 attack by protein nucleophilic groups, and ought to be a reactive alkylating agent in the vesicles. Indeed, vinylglycolate
**Scheme I**

\[
\text{HC} \equiv \text{C} - \text{C} - \text{COOH} \xrightarrow{\text{D-LDH}} \text{HC} \equiv \text{C} - \text{C} - \text{COOH}
\]

(HBA)

\[
\text{HC} \equiv \text{C} - \text{C} - \text{COOH} \xrightarrow{\text{D-LDH}} \text{HC} \equiv \text{C} - \text{C} - \text{COOH}
\]

\[
\text{H}_2\text{C} = \text{C} = \text{C} - \text{COOH}
\]

\[
\text{H}_3\text{C} - \text{C} = \text{C} - \text{COOH}
\]

\[
\text{H}_2\text{C} = \text{CH} - \text{C} - \text{COOH}
\]

**Scheme II**

\[
\text{H}_2\text{C} = \text{CH} - \text{C} - \text{COOH} \xrightarrow{\text{LDH}} \text{H}_2\text{C} = \text{CH} - \text{C} - \text{COOH}
\]

(VG)

\[
\text{H}_2\text{C} = \text{CH} - \text{C} - \text{COOH}
\]

\[
\text{H}_2\text{C} = \text{CH} - \text{C} - \text{COOH}
\]

2-keto-3-butyroate

\[
\text{RX.C} = \text{CH} - \text{C} - \text{COOH}
\]

RX-H

2-keto-3-butyroate
addition to either E. coli whole cells or isolated membrane vesicles generates rapid and irreversible inactivation of the second major type of bacterial transport system, the PTS for hexose transport and phosphorylation. Active transport of glucose, fructose, mannose, and α-methylglucoside is thus specifically abolished, while D-lactate dehydrogenase coupled transport of proline, lactose and rubidium remains intact. It was demonstrated that 2-keto-3-butenoate, generated from VG oxidation, inactivates Enzyme I of the PTS, and by this means blocks vectorial phosphorylation in whole cells and membrane vesicles of E. coli. The relative lack of potency of hydroxybutyrate is due to its covalent attachment and inactivation of D- and L-LDH's.

An interesting model, which integrated the lactose carrier into the electron transport chain, was proposed by Kaback. In the model he postulated that the lactose carrier performed its cycle of conformational changes via a redox process occurring at the level of the carrier.

The capacity of membrane vesicles to demonstrate the characteristic transport reactions of whole cells is important in two respects. Firstly, the vesicles clearly differentiate between transport systems which are positive in this assay and those requiring the participation of a periplasmic binding protein. Secondly, the membrane vesicle represents a significant attempt to produce a partially purified and viable membrane-bound transport.
protein. For this second reason membrane vesicles have been very widely used in transport studies since their potentialities were first demonstrated by Kaback. In fact, the use of vesicles led directly to the experimental results on which Kaback built his respiration-linked model, and it was the eventual demonstration of proton-motive force in these structures that led Kaback to abandon his own hypothesis in favour of Mitchell's chemiosmotic model. This rise and fall of the respiration-linked transport model has been one of the most significant features of the last few years in the field of microbial transport.

The respiration-linked model of transport proposed a direct coupling of the carrier to specific sites of the respiratory chain. According to this model, the transport proteins, the carriers, possess high affinity for their transport substrates only in the oxidized (disulfide) form, whereas the reduced (sulfhydryl) form has low substrate affinity. Active uptake of a particular sugar or amino acid is associated with the reduction of the appropriate carrier by the electron donor. Upon reduction, the high affinity form of the carrier undergoes a conformational change that results in the translocation of the bound substrate from the outer surface of the membrane to the inner surface. The resulting low-affinity (sulfhydryl) form of the carrier then releases the substrate, and the carrier is reoxidized. By alternative oxidation and
reduction of the carrier, substrate is transferred from
the outside to the inside against a concentration gradient
until the internal concentration is sufficient to saturate
the reduced form of the carrier. At that point the rate
of efflux will equal the rate of influx, and a steady
state will be achieved. In the reduced state, the carrier
is mobile in the membrane and can mediate facilitated
diffusion. This model postulates that carriers of dif-
erent substrate specificity are coupled to specific sites
in the electron transfer chain, thereby conferring func-
tional heterogeneity on otherwise identical electron
transfer chains (Fig. 3b).

This model does not require any special characteristics
of the membrane other than it functions as a diffusion
barrier. The model explains the absence of any correlation
between the rates of oxidation of various electron donors
and their relative effects on transport. However, the
model fails to adequately explain the behaviour of electron
transfer-coupling mutants of E. coli and Salmonella
typhimurium\textsuperscript{109,110} that exhibit normal electron transfer
properties, but are defective in D-lactate-dependent
transport. The model also does not account for the
inhibitory action of uncouplers of electron flow.

This model has been called into question since
Hirata\textsuperscript{51,111} showed that active lactose transport can be
driven by an electrical potential across the vesicular
membrane. This potential can be generated by the efflux
Coupling of Transport to D-Lactic Acid Dehydrogenase

**Key:**
- D-LAC, D-lactate; PYR, pyruvate; fp, flavoprotein;
- Cy. $b_\perp$, cytochrome $b_\perp$; OX, oxidized; RED, reduced; OUT, signifies the outside surface of the membrane; IN, signifies the inside surface of the membrane. The hemisphere located between fp and Cy. $b_\perp$ represents the "carrier": ( ), a high-affinity binding site; and ( ), a low-affinity binding site. The remainder of cytochrome chain from cytochrome $b_\perp$ to oxygen has been omitted. According to Kaback and Barnes\textsuperscript{107}. 
of $K^+$ ions from preloaded vesicles with valinomycin or by
the generation of a pH gradient which can be monitored
by the uptake of a lipophilic organic cation such as the
dibenzyl dimethyl ammonium ($DDA^+$) ion.$^{112,113}$

Experimental evidence is still accumulating that the
lactose pump of $E.~coli$ is indeed a proton symport, as
was reported earlier by Mitchell.$^{48}$ As a logical conse-
quence of the validity of the Mitchell hypothesis about
energy coupling of bacterial sugar transport, the involve-
ment of the membrane-bound ATPase in the energy coupling
of proline transport in $E.~coli$ could be demonstrated.
Membrane vesicles prepared from an ATPase mutant (DL-54)$^{110}$
did not accumulate proline, rubidium (in the presence of
valinomycin), or the lipidsoluble $DDA^+$ during D-lactate
oxidation. All these functions were restored by treating
the vesicles with $N,N'$-dicyclohexyl carbodiimide (DCCD).
Unlike vesicles of the wild-type, those of DL-54 cannot
generate an electrical potential across the membrane
during respiration. Certain lesions in the ATPase complex
secondarily render the vesicle membrane permeable to
protons and thus result in dissipation of the membrane
potential or energised state. The molecular nature of
the lesion in vesicles of DL-54 and of its repair by DCCD
remain uncertain. However, it is known that inhibition
of ATPase by DCCD results from covalent reaction with a
site in the membrane distinct from the ATPase proper$^{114}$
and it is a reasonable presumption that the recoupling
effects described involve binding of DCCD to the same site. It thus appears that, at least under certain conditions, the DCCD-binding site exhibits the properties of a proton-conducting channel. This conclusion is, of course, entirely compatible with Mitchell's proposal that the ATPase complex functions as a system for the translocation of protons across the membrane.

In order to explain the above observation, in the context of the chemiosmotic model of energy-coupling, it has been suggested that the membrane potential is in equilibrium with the redox state of the respiratory chain at that site between D-lactate dehydrogenase and cytochrome b₁ which generates the membrane potential. Inhibition of electron flow in a manner which leads to oxidation of the energy-coupling site does not result in a collapse of the potential. Such an explanation reconciles aspects of the chemiosmotic model and the direct coupling model. It emphasizes that the site of the respiratory chain between D-lactate dehydrogenase and cytochrome b₁ plays a special role in the generation of the membrane potential.

Although vesicles could oxidize a number of electron donors, the capacity to drive transport was very much more restricted. Membrane vesicles from E. coli oxidize the electron donors D-lactate, succinate and NADH at a high rate. The oxidation of D-lactate stimulated markedly the transport of sugars and amino acids. Other electron donors in E. coli such as succinate, L-lactate, NADH and
D,L-hydroxybutyrate, also could energise transport, but these electron donors were less effective energy sources for active transport than D-lactate.118

A similar effect of electron donors on transport of solutes is observed in membrane vesicles from many other Gram-negative as well as many Gram-positive bacteria. Vesicles from the Gram-positive *B. subtilis* accumulate amino acids in the presence of NADH and, to a lesser extent, with NADPH.119,120 In vesicles from *Staph. aureus*, amino-acid transport is energised by L-α-glycérol phosphate or L-lactate.121,122 In a number of membrane vesicles, transport of several solutes was also energised by a nonphysiological electron donor system, namely ascorbate plus phenazine methosulphate (PMS).119,120,123,124

Accumulation of solutes in membrane vesicles is only observed in the presence of electron donors. No other intermediate metabolites or cofactors, like ATP, phosphoenolpyruvate, glucose, hexose phosphates and many others, energised transport in membrane vesicles to any significant extent.117,120 These observations strongly point to a coupling of active transport to the respiratory chain in membrane vesicles from aerobically-grown bacteria. This contention is supported by the observation that all substrates that are oxidized by membrane vesicles from *E. coli*, *B. subtilis* and *Staph. aureus* reduce the same cytochromes as dithionite.118,120 The same observation is made for the nonphysiological electron donor ascorbate-PMS.
Conclusive evidence for the involvement of the respiratory chain in the transport processes in membrane vesicles from aerobically-grown microorganisms has been obtained from studies with respiratory chain inhibitors. D-lactate-dependent lactose transport by *E. coli* membrane vesicles is strongly inhibited by anaerobiosis, and by the respiratory chain inhibitors cyanide, 2-heptyl-4-quinolione-N-oxide (HQNO) and amytal, and all of these inhibitors effectively block oxidation of D-lactate. The effect of D-lactate on the uptake of β-D-galactosides, that are transported, was investigated in *E. coli* membrane vesicles. In membrane vesicles from cells containing the N-protein (the product of the y-gene of the lac-operon), D-lactate markedly stimulated the initial rate of transport of lactose and other β-D-galactosides. At steady-state levels of accumulation, internal concentrations were reached which were 100-fold or more the concentration in the external medium. These galactosides are not metabolized by the membrane preparations and can be recovered from the vesicles in an unmodified form after transport. The effects of the different electron donors which are oxidized by *E. coli* membrane vesicles were analogous to the effects on amino-acid transport; the highest initial rates are obtained with D-lactate and NADH.

Transport of arabinose, glucuronate, gluconate and glucose-6-phosphate, in *E. coli* membrane vesicles, is coupled to oxidation of D-lactate in a similar manner to
that described for amino acids and β-D-galactosides. The transport of these sugars is inhibited by the same conditions that affect amino-acid transport and β-D-galactoside transport. Evidence has been presented that transport of these sugars does not involve the PTS, and that induction of the parent cells for these transport systems is required. A gluconate-transport system is also present in membrane vesicles from Ps. aeruginosa which concentrates free gluconate with high affinity (K<sub>app</sub> 20 μM) in the presence of ascorbate-PMS. 20,125 Gluconate transport in these vesicles is coupled to the L-malate oxidation by a FAD-dependent L-malate dehydrogenase. However, Ps. aeruginosa membrane vesicles did not retain the ability to transport glucose. This suggests that some component of the glucose transport system was lost during the preparation of the vesicles. Evidence that this component may be a periplasmic binding protein has been presented. 20 Thus membrane vesicles of Ps. aeruginosa oxidized glucose to gluconate which was then taken up. This may constitute an alternate mechanism of transport of glucose as has been suggested. 68 Glucose oxidase however, was not a necessary component for glucose transport by intact cells since strain PAO 57, which lacks glucose oxidase was able to transport glucose at a rate equal to that of the wild type. 20

D-galactose is transported in the presence of D-lactate by membrane vesicles prepared from galactose-induced E.
coli. The galactose-transport system in the membrane vesicles does not require the galactose-binding protein in the periplasmic space, and this protein is absent from the vesicles. These findings, together with the observation that the membrane vesicles fail to transport β-methylgalactoside, indicate that the galactose-transport system retained by the vesicles is the so-called "galpermease" system. 126

Studies on whole cells and membrane vesicles from Arthrobacter pyridinolis demonstrated that this organism accumulates D-fructose and L-rhamnose via a PTS and a respiratory chain-coupled transport system. The respiratory chain-coupled system is stimulated by the addition of L-malate in membrane vesicles. Vesicles from D-fructose-grown cells carry out malate-dependent uptake of D-fructose but not of D-glucose or L-rhamnose indicating that there is a sugar-specific component to the respiration-coupled transport system. 38 Vesicles from fructose-grown cells of a PTS-negative strain (AP100) exhibited malate-dependent D-fructose uptake, while PTS-dependent uptake was reduced to a small fraction of that seen with vesicles from wild-type cells. Inhibitors of electron transport, carbonyl cyanide m-chlorophenyl-hydrazone, 2,4-dinitrophenol and N-ethylmaleimide caused marked inhibition of malate-dependent D-fructose uptake while exerting little or no effect on PTS-dependent transport of the sugar in vesicles from wild-type cells. A mutant which is deficient in the
D-fructose-specific component of the respiration-coupled system can grow on L-rhamnose using the PTS, but cannot grow on D-fructose at all. This mutant fails to produce the inducible D-fructose-specific PTS component, Enzyme II and Factor III, when grown in the presence of D-fructose. These results indicate a requirement for a functional respiration-coupled transport system for the induction of the PTS. The results further suggest that sufficient free D-fructose, or D-fructose-6-phosphate derived from it, must be present inside the cell in order for induction of the PTS to occur. The entry of sufficient fructose to cause induction of the PTS cannot occur by facilitated diffusion in the absence of energy coupling.\(^{39}\)

Membrane vesicles from *Azotobacter vinelandii* catalyse the active transport of D-glucose via an inducible glucose-transport system.\(^{127}\) The best electron donors for energising the glucose transport are L-malate and tetramethyl-phenylenediamine (TMPD) reduced by ascorbate. Other electron donors such as NADH, NADPH and D-lactate are oxidized at a high rate by these membrane vesicles, but are far less efficient in energising glucose transport. Evidence based on the inhibitory effect of respiratory-chain inhibitors on the oxidation rate of the electron donors, and their effect on transport, has been presented which indicates that glucose transport is linked to two distinct sites of the respiratory chain.\(^{128}\)
Objectives of the Study

1. To prepare vesicles from glucose and succinate-grown cells of *Ps. fluorescens*.

2. To ascertain whether D-glucose and D-gluconate transport in vesicles and whole cells of *Ps. fluorescens* has a carrier system which is energy dependent.

3. Attempt to analyze and separate the membrane-bound enzymes from the D-glucose and D-gluconate carrier proteins by induction/repression experiments.

4. On the basis of the above results a mechanism is proposed for the transport of D-glucose and D-gluconate by *Ps. fluorescens*. 
CHAPTER II
MATERIALS AND METHODS

Characterization and Cultivation of
Pseudomonas fluorescens A.3.12

The organism used in these studies was Pseudomonas fluorescens, strain A.3.12, (ATCC 12633), first isolated by Stainer,\textsuperscript{130} and subsequently proposed by him as the neotype strain for Pseudomonas putida, biotype A.\textsuperscript{131} This particular strain has been used over the past twenty years in many different biochemical and physiological studies, and there is consequently a large body of information about its metabolism and physiology. The organism was obtained as a freeze-dried sample from the American Type Culture Collection (ATCC), and was periodically tested for purity by a variety of morphological and biochemical criteria. Table 1 lists the biochemical criteria used in the periodic characterization of the organism.
TABLE 1

Characteristic Biochemical Properties
of Ps. fluorescens A.3.12

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of growth in Hugh and Leifson's medium (1.0 % glucose)</td>
<td>Acid production only at top of aerobic tube</td>
</tr>
<tr>
<td>Methyl red</td>
<td>-</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>-</td>
</tr>
<tr>
<td>Indole production</td>
<td>-</td>
</tr>
<tr>
<td>Ammonia from arginine</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Growth in Koser's citrate medium</td>
<td>+</td>
</tr>
<tr>
<td>Growth on Milk Agar* 132</td>
<td>Growth, no hydrolysis, no pigment</td>
</tr>
</tbody>
</table>

* Used to distinguish between Ps. fluorescens and Ps. aeruginosa.

For the cultivation of the bacterium, the following semi-defined medium of David and Kingioli133 was used throughout the studies:
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_2HPO_4$</td>
<td>7.0</td>
</tr>
<tr>
<td>$KH_2PO_4$</td>
<td>3.0</td>
</tr>
<tr>
<td>$(NH_4)_2SO_4$</td>
<td>1.0</td>
</tr>
<tr>
<td>$MgSO_4 \cdot 7H_2O$</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast Extract (Difco)</td>
<td>0.2</td>
</tr>
<tr>
<td>Trace elements solution</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Carbon source (glucose or succinate)</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Solution of trace elements (Barnett and Ingram). $^{134}$

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>$\mu g/100\text{.ml}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$FeSO_4 \cdot 4H_2O$</td>
<td>40</td>
</tr>
<tr>
<td>$MnSO_4 \cdot 4H_2O$</td>
<td>40</td>
</tr>
<tr>
<td>$ZnSO_4 \cdot 7H_2O$</td>
<td>20</td>
</tr>
<tr>
<td>$CuSO_4 \cdot 5H_2O$</td>
<td>4</td>
</tr>
<tr>
<td>$CoCl_2 \cdot 6H_2O$</td>
<td>4</td>
</tr>
<tr>
<td>KI</td>
<td>30</td>
</tr>
<tr>
<td>$Na_2MoO_4 \cdot 2H_2O$</td>
<td>5</td>
</tr>
<tr>
<td>$CaCl_2 \cdot (\text{anhyd.})$</td>
<td>500</td>
</tr>
<tr>
<td>NaCl</td>
<td>1000</td>
</tr>
</tbody>
</table>

The ingredients were dissolved in distilled water and sterilized by autoclaving at $120^\circ C$ per 15 minutes, without the carbon source, a 3.0 percent (w/v) solution of which was sterilized by autoclaving separately at $120^\circ C$ per 15 minutes to prevent caramelisation, and was
aseptically added to the sterilized salts medium to give a final concentration of 0.2 percent carbon source. For the routine maintenance of the organisms, the glucose/mineral salts medium was solidified by the addition of 2.0 percent (w/v) agar. Alternatively, slopes of nutrient agar (Difco 0001) were used. All the chemicals used were of AR grade.

Culture Techniques:

For the transport study of washed whole cell suspensions, the necessary cell yields were achieved by growth of *Pseudomonas fluorescens A.3.12* in 1.0 litre Erlenmyer flasks, each containing 225 ml of glucose or succinate/mineral salts medium. Inocula for these cultures were prepared from 24 hour slope cultures, by washing with 10 ml of sterile distilled water and aseptically transferring an appropriate aliquot to each Erlenmyer flask. Succinate-grown cells were obtained from cultures trained by at least six subcultures through the succinate/mineral salts medium. Cultures were incubated at 30°C in an orbital rotary shaker setting at 4 (Lab-Line Instruments, Inc., Melrose Park, Illinois). Using the above procedure, cell yields in the region of 0.5 to 1.0 mg dry weight of cells/ml of medium were obtained after 16 hours incubations, at which time the cells were in the late exponential phase.
Cells were harvested in a Sorvall superspeed RC2-B centrifuge (Ivan Sorvall Inc., Newtown, Connecticut, U.S.A.) at 23,000 xg for 5 minutes, washed twice at 30°C with 0.067 M of sodium-potassium phosphate buffer pH 7.1, containing 0.1 percent (w/v) MgSO₄·7H₂O, referred to as Mg²⁺-phosphate buffer. The cells were resuspended to the desired concentration in this same buffer and held at 30°C on a Dobnoff Metabolic shaking incubator (Precision Scientific Co., Chicago, Illinois, U.S.A.) for 10 minutes before the addition of the radioactive substrate.

Unless otherwise indicated, the filtration of whole cells and isolated membrane vesicles in the uptake assays were performed in a filter assembly provided with membrane filter HA 24 mm diameter and 0.45 μm pore size (Millipore Corp., Bedford, Massachusetts, U.S.A.).

Preparation of Vesicles:

Ps. fluorescens A·3·12 was grown in 1.0 litre Erlenmyer flasks, each containing 225 ml of glucose or succinate/mineral salts medium. Inocula for these cultures were prepared from 24 hour slope cultures, by washing with 10 ml of sterile distilled water and aseptically transferring 1.0 ml aliquot to each Erlenmyer flask. Cultures were incubated at 30°C in an orbital rotary shaker setting at 4 for 12 hours, at which time the cells were in the mid exponential phase. The cells were harvested by
centrifuging in a Sorvall superspeed RC2-B centrifuge at 10,000 xg for 10 minutes at 30°C. The pellet was suspended in 2/3 the original culture volume in a solution containing, in final concentration, 2.5 percent LiCl, 0.75 M of sucrose, 10 mM of potassium phosphate pH 7.0, 10 mM of MgSO₄, and 500 µg of lysozyme/ml. This suspension was incubated for 1 hour at 30°C on a rotary shaker. The osmotically fragile rods were then harvested by centrifuging at 10,000 xg for 30 minutes at 4°C. The pellet was suspended with a Waring blender in the smallest volume possible of 2.5 percent LiCl in 0.75 M of sucrose at 4°C, then added rapidly to 50 volumes of ice-cold 10 mM of potassium phosphate buffer pH 6.6 containing 1.0 mM of MgSO₄ and blended for 10 seconds. Deoxyribonuclease I (DNase) and ribonuclease A (RNase) were added to a final concentration each of 20 µg/ml and the mixture was incubated at 25°C with gentle stirring for 30 minutes. The suspension was centrifuged at 40,000 xg for 30 minutes at 4°C, in an IEC International Preparative Ultracentrifuge Model B-60 (300 Seconds Avenue, Needham Heights, Massachusetts). The pellet was suspended by means of a loose-fitting glass homogenizer (ACE Glass Inc., Vineland, New Jersey) in a 1:4 (v/v) ratio in ice-cold 100 mM of potassium phosphate buffer pH 6.6 containing 10 mM of MgSO₄. The final pellet, which was composed of membrane vesicles, was suspended in the same buffer system to a final concentration of 10 mg of protein/ml sealed in glass vials and stored in liquid nitrogen.
It has been found that vesicles prepared from aged bacteria (more than six months old) loose their transport activity.

Samples of the membrane vesicles suspension were negatively stained with 1.0 percent phosphotungstate pH 7.2 and examined using a Philips EM 201C Highresolution Electron Microscope (Eindhoven, The Netherlands) (Fig. 4). The diameter of these vesicles vary from 0.69 to 2.0 μm.

**Manometry:**

The oxidation of exogenous substrates by isolated membrane vesicle suspensions was followed by the manometric method using a Gilson Differential Respirometer with fifteen calibrated reaction flasks (Gilson Medical Electronics, Middleton, Wisconsin) containing 2.0 ml reaction volume. A solution of 0.2 ml 20 percent KOH, absorbed on a folded paper wick, was placed in each centre well to absorb CO₂. The incubation temperature was 30°C.

**Oxygen Electrode:**

The Km values for oxidation by isolated membrane vesicles were determined with a Clark-type oxygen electrode (YSI, Model 53, Yellow Springs Instruments Co., Yellow Springs, Ohio, U.S.A.) connected to a Beckman 10" recorder model 1005 (Beckman Instrument Inc., Scientific and Process Instruments, Div., Fullerton, California). The instrument
Electron Micrograph of *Ps. fluorescens* Membrane Vesicles

A suspension of membrane vesicles was negatively stained with 1% phosphotungstic acid (PTA), pH 7.2, on a formvar grid and examined using a Phillips EM 201C electron microscope.

**PLATE 1.**

The arrows indicate membrane vesicles of diameters 0.69 μm.

**PLATE 2.**

The arrow indicates a membrane vesicle of diameter 2.0 μm.
was adjusted so that full scale deflection of the recorder was equivalent to the uptake of 0.63 µmole of O₂ from a reaction mixture having a total volume of 3.0 ml. All measurements were made in a water-jacketed cell at 25°C for a period of 2-5 minutes. Oxidation rates of various substrates were determined in a reaction mixture containing in a total volume of 3.0 ml, 50 mM of potassium phosphate buffer pH 6.6, 12 mM of MgSO₄, 1.0 mg of membrane vesicle protein, and the appropriate substrate. Oxygen uptake was calculated from the slopes of the recorder traces, which were linear over the period of measurement.

Chromatography:

Paper chromatography was performed using Whatman No. 1 chromatography paper and the descending technique. The solvent system used was ethyl acetate:pyridine:acetic acid: water (5:5:1:3, v/v).

Marker standard sugars were detected by spraying the chromatogram with 0.6 percent AgNO₃ in acetone, dried and sprayed with 0.5 M of alcoholic KOH. The control chromatogram values were then employed to establish the location of individual ¹⁴C-sugars and ³H-sugars, obtained by cutting experimental chromatograms into sections, by determining the labeled content via liquid scintillation measurements as described in the next section.
**Estimations:**

1. Quantitative method of the assay for dry weight of whole cells: known volume (1.0 ml) of the cell suspension was filtered onto a previously dried and weighed millipore filter, washed free of suspending buffer and the filter dried to constant weight. The filtration was performed on a Gelman borosilicate filter funnel assembly (Gelman Instrument Company, Ann Arbor, Michigan).

2. Protein was determined by the Biuret method. Bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri) was used to construct a calibration curve. Optical density (O.D) readings were taken at 450 mm on a Beckman DB-GT spectrophotometer (Beckman Instrument LTD., Glenrothes FIFE, Scotland).

3. The uptake of radioactive material was determined by counting with a Beckman LS-3100 Series liquid scintillation spectrometer (Beckman Instruments, Inc., Scientific Instruments Division, Irvine, California). After subtracting background radiation, the data were reported as nmole/mg protein.

4. Lineweaver-Burk plots were estimated by the least square method.

5. All the experiments of uptake and kinetic studies in membrane vesicles and whole cells were repeated at least five times and the results are represented as the average of the nearest four to five determinations.
Electron Microscopy:

Samples of membrane vesicles suspension (1 drop) were put on copper grids (200-mesh); Formvar-coated, carbon-reinforced, and negatively stained with 1.0 percent phosphotungstate pH 7.2 (PTA) by putting 1 drop on the grid for 1 minute; excess solution was removed by filter paper; and negatively stained for 30 seconds. Samples were examined using a Phillips EMHigh resolution Electron Microscope (Eindhoven, The Netherlands).

Chemicals:

$^{14}C$-D-glucose (14 and 13.9 nCi/nmole) and $^{14}C$-L-glucose (51.2 nCi/nmole) and liquifLOUR were purchased from New England Nuclear Corp., Boston, Massachusetts. $^{14}C$-D-gluconic acid (3.91 nCi/nmole), methyl $^{14}C$-α-D-glucopyranoside (184 nCi/nmole), $^{14}C$-(C$_1$)-2-deoxy-D-glucose (58.5 nCi/nmole) and inulin-$^{14}C$-carboxylic acid (18.9 nCi/nmole) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England; $^3$H-(C$_3$)-3-deoxy-3-fluoro-D-glucose was synthesized in this laboratory by Dr. D. Lopes. $^{137}$ 3-deoxy-3-fluoro-D-gluconic acid was kindly provided by Dr. N. F. Taylor.

Unless otherwise stated, chemicals were obtained from Fisher Scientific Company, Fair Lawn, New Jersey; Sigma Chemical Company, St. Louis, Missouri; and Aldrich Chemical Company Inc., Wisconsin, U.S.A.
The enzymes lysozyme, deoxyribonuclease I and ribonuclease A were purchased from Sigma Chemical Company, St. Louis, Missouri.
CHAPTER III
EXPERIMENTAL RESULTS

The Uptake of Sugar by Isolated Membrane Vesicles of Pseudomonas fluorescens

Initial experiments were designed to determine whether Ps. fluorescens membrane vesicles, prepared from glucose-grown cells, could transport and accumulate D-glucose, glucose analogues and D-gluconate in the presence and absence of electron donors.

Experiment 1: D-Glucose and D-Gluconate Uptake by Isolated Membrane Vesicles from Glucose and Succinate-Grown Cells.

The accumulation of radioactivity was determined at 30°C on a reciprocal shaking water bath. The incubation mixture contained 50 μmoles of potassium phosphate buffer (pH 6.6), 1.0 mg of membrane vesicle protein, 74 nmoles of U-14C-D-gluconate (3.91 nCi/nmole) or 70 nmoles of U-14C-D-glucose (14 nCi/nmole), 12 μmoles of MgSO4, and unless otherwise indicated, 20 μmoles of ascorbic acid and 0.1 μmole of PMS in a total volume of 1.0 ml in a 10 ml Erlenmeyer flask. Each vessel was continuously gassed with oxygen. The reactions were started by the addition of
vesicles. At time intervals, 0.1 ml samples were withdrawn, diluted into 20 volumes of 0.1 M LiCl, 25°C, overlaying a Millipore filter HA membrane, filtered under vacuum, and immediately washed with another 20 volumes of 0.1 M LiCl. The filtration time was generally 15 to 20 seconds. The filters were then transferred to scintillation vials containing scintillation fluid of the following composition: 42 ml of liquifluor [0.625 g of 1,4-bis-2' (5'-phenyl-oxazolyl) benzene (POPOP) and 80 g of 2,5-diphenyloxazole (PPO) per 500 ml of toluene], 1.0 litre of toluene and 600 ml of ethylene glycol monomethyl ether. This cocktail is referred to as (TEG).

The results in Fig. 5 show the uptake of D-glucose and D-gluconate in the presence or absence of an electron donor.

**Experiment 2:** Sugar Transport in the Presence of Various Electron Donors.

The uptake of D-glucose and D-gluconate by isolated membrane vesicles, from glucose-grown cells, were performed as described in Expt. 1.

Each flask contained 1.0 mg of membrane vesicle protein; 50 μmoles of potassium phosphate buffer (pH 6.6), 12 μmoles of MgSO₄ and 70 nmoles of U-¹⁴C-D-glucose (14 nCi/nmole) or 74 nmoles of U-¹⁴C-D-gluconate (3.91 nCi/nmole) and the electron donor, in a total volume of 1.0 ml.
FIGURE 5

Time Course of The Uptake of D-Glucose and D-Gluconate by Membrane Vesicles From Glucose-Grown *Ps. fluorescens* in The Presence and Absence of Electron Donor

Key:

- **○** 70 μM U-14C-D-glucose
- **●** 70 μM U-14C-D-glucose + 20 mM ascorbate + 0.1 mM FMS
- **▲** 74 μM U-14C-D-gluconate
- **▲** 74 μM U-14C-D-gluconate + 20 mM ascorbate + 0.1 mM FMS
Figure 5

Time Course of the Uptake of D-Glucose and D-Gluconate by Membrane Vesicles from Glucose-Grown Ps. fluorescens in the Presence and Absence of Electron Donor.
Each reaction was continuously gassed with oxygen. The results are shown in Table 2.

**Experiment 3:** The Uptake of 3FG by Membrane Vesicles in the Presence or Absence of Electron Donor.

The uptake of $^3$H-(C$_3$)-3FG was performed as described in Expt. 1.

The accumulation of radioactivity, in vesicles from glucose-grown cells, was determined at 30°C on a reciprocal shaking water bath. The incubation mixture contained 50 μmoles of potassium phosphate buffer (pH 6.6), 12 μmoles of MgSO$_4$, 1.0 mg of membrane vesicle protein, 70 nmole of $^3$H-(C$_3$)-3FG (15.2 nCi/nmole) and unless otherwise indicated 10 mM L-malate and 50 μM FAD in a total volume of 1.0 ml. Each vessel was continuously gassed with oxygen. The results are shown in Fig. 6.

**Experiment 4:** The Uptake of 3FG in the Presence and Absence of D-Glucose.

The effect of glucose on the uptake of 3FG by membrane vesicles from glucose-grown cells, was performed as described in Expt. 1.

The accumulation of radioactivity was determined at 30°C on a reciprocal shaking water bath. The incubation mixture contained 50 μmoles of potassium phosphate (pH 6.6), 12 μmoles of MgSO$_4$, 70 nmole of $^3$H-(C$_3$)-3FG (15.2 nCi/nmole), 70 nmole of D-glucose and 1.0 mg of membrane
### TABLE 2

Effect of Various Electron Donors on Glucose and Gluconate Uptake by *Ps. fluorescens* Membrane Vesicles Prepared from Glucose-Grown Cells

<table>
<thead>
<tr>
<th>ADDITIONS (conc.)</th>
<th>UPTAKE (nmoles/mg protein/5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLUCOSE (70 μM)</td>
</tr>
<tr>
<td>None</td>
<td>0.182</td>
</tr>
<tr>
<td>L-Malate, 20 mM; FAD, 10 μM</td>
<td>0.633</td>
</tr>
<tr>
<td>L-Malate, 20 mM; FAD, 50 μM</td>
<td>0.584</td>
</tr>
<tr>
<td>L-Malate, 10 mM; FAD, 50 μM</td>
<td>0.151</td>
</tr>
<tr>
<td>L-Malate, 20 mM</td>
<td>0.628</td>
</tr>
<tr>
<td>FAD, 50 μM</td>
<td>0.190</td>
</tr>
<tr>
<td>L-Malate, 20 mM; FAD, 50 μM</td>
<td>1.028</td>
</tr>
<tr>
<td>Mg$$^2+$$, 2 mM (final conc.)</td>
<td></td>
</tr>
<tr>
<td>Gluconate, 10 mM</td>
<td>0.180</td>
</tr>
<tr>
<td>2-Deoxy-D-Glucose, 10 mM</td>
<td>0.433</td>
</tr>
<tr>
<td>Ascorbate, 20 mM; PMS, 0.1 mM</td>
<td>0.640</td>
</tr>
<tr>
<td>D-Malate, 10 mM; FAD, 50 μM</td>
<td>0.301</td>
</tr>
<tr>
<td>D-Lactate, 10 mM; FAD, 50 μM</td>
<td>0.240</td>
</tr>
<tr>
<td>Succinate, 10 mM; FAD, 50 μM</td>
<td>0.200</td>
</tr>
<tr>
<td>Pyruvate, 10 mM</td>
<td>0.230</td>
</tr>
<tr>
<td>Oxaloacetate, 10 mM</td>
<td>0.061</td>
</tr>
<tr>
<td>D-Glucose, 10 mM</td>
<td>—</td>
</tr>
<tr>
<td>ATP, 10 mM</td>
<td>0.184</td>
</tr>
<tr>
<td>L-Malate, 10 mM; NAD$$^+$$, 50 μM</td>
<td>0.640</td>
</tr>
</tbody>
</table>
FIGURE 6

Time Course of the Uptake of 3FG by Membrane Vesicles from Glucose-Grown *Ps. fluorescens* in the Presence and Absence of D-Glucose or Electron Donor

Key:

- 70 μM $^3$H-(C$_3$)-3FG + 10 mM L-mal. + 50 μM FAD
- 70 μM $^3$H-(C$_3$)-3FG
- 70 μM $^3$H-(C$_3$)-3FG + 70 μM D-glucose
FIGURE 6

Time Course of the Uptake of 3PG by Membrane Vesicles From Glucose-Grown Pa. fluorescens in the Presence and Absence of D-Glucose or Electron Donor

Uptake of H-3PG (nmol/mg protein)

Time (min.)

0 1 2 3 4 5 6 7
vesicle protein in a total volume of 1.0 ml. Each flask was continuously gassed with oxygen. The reactions were started by the addition of vesicles. The results are shown in Fig. 6.

Experiment 5: The Uptake of D-Glucose in the Presence or Absence of 3FG.

The effect of 3FG on the uptake of glucose by membrane vesicles from glucose-grown cells, was performed as described in Expt. 1.

The accumulation of radioactivity in vesicles from glucose-grown cells was performed at 30°C on a reciprocal shaking water bath. The incubation mixture contained, 50 μmoles of potassium phosphate buffer (pH 6.6), 12 μmoles of MgSO₄, 70 nmoles of U-¹⁴C-D-glucose (13.9 nCi/nmole), 20 μmoles of 3FG and 1.0 mg of membrane vesicle protein. Each flask was continuously gassed with oxygen. The reactions were started by the addition of vesicles. The results are shown in Fig. 7.

Experiment 6: Paper Chromatography on the Uptake of Glucose and 3FG.

Membrane vesicles from glucose-grown cells, were incubated at 30°C with phosphate buffer and substrate (either 70 nmoles of ³H-(C₃)-3FG or 70 nmoles of U-¹⁴C-D-glucose) as described in Expt. 1, at time intervals samples were withdrawn and filtered. The filters bearing the
**FIGURE 7**

Time Course of the Uptake of D-Glucose by Membrane Vesicles from Glucose-Grown *Ps. fluorescens* in the Presence and Absence of 3FG

**Key:**

- ▲ ▲

- ▼ ▼

- ▼ ▼ 70 µM U-14C-D-glucose

- ▼ ▼ 70 µM U-14C-D-glucose + 20 mM 3FG
FIGURE 7

Time Course of The Uptake of D-Glucose by Membrane Vesicles From Glucose-Grown Ps. fluorescens in The Presence and Absence of 3PG

Uptake of $^{14}$C-glucose (nmol/mg protein)

Time (min.)
vesicles were extracted with 15 ml of hot water at 85°C for 15 minutes, and the filtrates were collected and lyophilized. In order to obtain sufficient products to detect and identify, 30 filter membranes bearing vesicles were used. The extract was centrifuged at 40,000 xg for 30 minutes. The supernatant fluid was lyophilized to dryness, reconstituted with 0.1 ml of water then applied to Whatman No. 1 chromatography paper, 23 x 57 cm. Descending chromatography was performed for 10 hours using a solvent of ethylacetate:pyridine:acetic acid:water (5:5:1:3). The paper was dried at room temperature, cut into 1.0 sq. cm and counted in vials containing 10 ml of TEG. The results (Fig. 8a) show the presence of 3FGA extravesicularly after 3 minutes. Fig. 8b shows the presence of 3FG and 3FGA intravesicularly.

The results (Fig. 8c) of glucose uptake by membrane vesicles show the presence of a main component which is identified as 2-keto-gluconic acid (2KGA) by comparing its Rf value with a marker standard (2KGA), and to a less extent a presence of two components identified as glucose and gluconic acid. Fig. 8d shows the presence of traces of 2KGA extravesicularly which leaks out as an end product of the oxidation.
FIGURE 8

Paper Chromatogram of Extra- and Intravesicular Material From The Uptake of \(^3\)H and D-Glucose by Membrane Vesicles

Membrane vesicles from glucose-grown Ps. fluorescens were incubated with the labeled substrate and at time intervals samples were withdrawn as described in the experimental section.

**Fig. 8a and 8b** Paper Chromatogram of Materials From The Incubation of Membrane Vesicles With 70 \(\mu\)M \(^3\)H-(C\(_3\))-3FG

**Fig. 8a**

The suspending medium in which membrane vesicles were incubated with 70 \(\mu\)M \(^3\)H-(C\(_3\))-3FG and 30s. to 7 min. products were detected as described. 3FGA was detected after 3 min. incubation.

**Fig. 8b**

Represents a hot-water extract of membrane vesicles after incubation with \(^3\)H-(C\(_3\))-3FG for 30s. to 7 min. 3FGA and 3FG were detected as shown in the figure after 3 min.
Figs. 8c and 8d  Paper Chromatogram of Materials From 
The Incubation of Membrane Vesicles 
With 70 μM U-14C-D-Glucose

Fig. 8c

As in Fig. 8b, only GA, 2KGA and G were detected
intravesicularly after 30 s.

Fig. 8d

As in Fig. 8a, only 2KGA was detected in the
suspending medium in which membrane vesicles were incubated
with 70 μM U-14C-D-glucose. 2KGA was detected after 5 min.
in the suspending medium.
Inhibition Studies

Several inhibitors of the electron-transport chain were used to study their effect on the electron-donor mediated transport of D-glucose and D-gluconate by isolated membrane vesicles of \textit{Ps. fluorescens} grown on glucose.

**Experiment 7: The Effect of Electron-Transport-Chain Inhibitors on the Uptake of Glucose, 3FG and Gluconate.**

The accumulation of radioactivity was determined at 30°C on a reciprocal shaking water bath. The incubation mixture contained 50 μmoles of potassium phosphate buffer (pH 6.6), 1.0 mg of membrane vesicle protein, either 70 nmoles of \textit{U}^{14\text{C}}\text{-D-glucose} (14 nCi/nmole), 70 nmoles of \textit{3H-(C$_3$)}-3FG (15.2 nCi/nmole) or 74 nmoles of \textit{U}^{14\text{C}}\text{-D-gluconate} (3491 nCi/nmole), 12 μmoles of MgSO$_4$, 10 mM of L-malate and 50 μM of PAD, and the inhibitor; 1.0 mM of rotenone, 1.0 mM of antimycin A, 10 mM of potassium cyanide or 0.1 mM of 2,4-dinitrophenol(DNP), was added. The vesicles were incubated with each inhibitor for 2 minutes at 30°C before adding the substrate. The experiment was performed as described in Expt. 1.

The results of the uptake of glucose and gluconate in the presence and absence of DNP and cyanide are shown in Table 3, Fig. 9 and Fig. 10 respectively. Rotenone
<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>ADDITION</th>
<th>INHIBITOR</th>
<th>INITIAL RATE OF TRANSPORT (n mole/mg. prot./min.)</th>
<th>INHIBITION OF SUBSTRATE TRANSPORT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (70 μM)</td>
<td>None</td>
<td>None</td>
<td>0.22</td>
<td>—</td>
</tr>
<tr>
<td>L-Malate (10 mM) + FAD (50 μM)</td>
<td>None</td>
<td>DNP (0.1 M)</td>
<td>0.20</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN⁻ (10 mM)</td>
<td>0.24</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN⁻ (10 mM) + ATP (10 mM)</td>
<td>0.22</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rotenone (1.0 mM)</td>
<td>0.40</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antimycin (1.0 mM)</td>
<td>0.42</td>
<td>—</td>
</tr>
<tr>
<td>Gluconate (70 μM)</td>
<td>None</td>
<td>None</td>
<td>0.43</td>
<td>—</td>
</tr>
<tr>
<td>L-Malate (10 mM) + FAD (50 μM)</td>
<td>None</td>
<td>DNP (0.1 mM)</td>
<td>0.29</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN⁻ (10 mM)</td>
<td>0.50</td>
<td>32</td>
</tr>
<tr>
<td>3FG (70 μM)</td>
<td>None</td>
<td>None</td>
<td>0.16</td>
<td>—</td>
</tr>
<tr>
<td>L-Malate (10 mM) + FAD (50 μM)</td>
<td>None</td>
<td>DNP (0.1 mM)</td>
<td>0.15</td>
<td>79</td>
</tr>
</tbody>
</table>
FIGURE 9

The Effect of Electron Transport Chain Inhibitors on The Electron-Donor-Mediated Transport of D-Glucose by Membrane Vesicles of Ps. fluorescens Grown on Glucose

Key:

- 70 μM U-\textsuperscript{14}C-D-glucose + 10 mM L-mal. + 50 μM FAD
- 70 μM U-\textsuperscript{14}C-D-glucose + 10 mM L-mal. + 50 μM FAD + 0.1 mM DNP
- 70 μM U-\textsuperscript{14}C-D-glucose + 10 mM L-mal. + 50 μM FAD + 10 mM CN\textsuperscript{-}
- 70 μM U-\textsuperscript{14}C-D-glucose
FIGURE 9

The Effect of Electron Transport Chain Inhibitors on The Electron-Donor-Mediated Transport of D-Glucose by Membrane Vesicles of Ps. fluorescens Grown on Glucose
FIGURE 10

The Effect of Electron Transport Chain Inhibitors on The Electron-Donor-Mediated Transport of Gluconate by Membrane Vesicles of *Ps. fluorescens* Grown on Glucose

**Key:**

1. 74 μM U-^{14}C-D-gluconate + 10 mM L-mal. + 50 μM FAD
2. 74 μM U-^{14}C-D-gluconate + 10 mM L-mal. + 50 μM FAD + 0.1 mM DNP
3. 74 μM U-^{14}C-D-gluconate + 10 mM L-mal. + 50 μM FAD + 10 mM CN⁻
FIGURE 10

The Effect of Electron Transport Chain Inhibitors on The Electron-Donor-Mediated Transport of Gluconate by Membrane Vesicles of *P. fluorescens* Grown on Glucose.
and antimycin A have no effect on the uptake of glucose in the presence of electron donor.

Experiment 8: The Effect of -SH Group Inhibitors on the Uptake of Glucose.

The effect of some sulphydryl-group sensitive reagents on the uptake of glucose by membrane vesicles was studied.

The inhibitors used were 0.1 mM of CA, 0.1 mM of CB, 0.5 mM of NEM or 0.5 mM of PHMB. The membrane vesicles from glucose-grown cells were incubated with the inhibitor, in the same reaction mixture used in Expt. 1, for 10 minutes prior to the addition of 70 nmoles of U-14C-D-glucose (14 nCi/nmole).

The results from these experiments show no significant inhibition of the uptake of glucose by membrane vesicles from glucose-grown cells.

Kinetics of Sugar Uptake

The kinetic studies of transport of D-glucose, 3FG, 2DOG, α-MG, L-glucose and D-gluconic acid by membrane vesicles from glucose and succinate-grown cells of Ps. fluorescens, were designed and performed as described in Expt. 1. The initial rates were measured in duplicate 30 seconds after the addition of labeled substrate at 30°C.
Experiment 9: Kinetics of D-Glucose, 3FG, 2DOG, α-MG, and L-Glucose Uptake.

A series of 10 ml Erlenmeyer flasks contained in a total volume of 1.0 ml: 50 μmoles of potassium phosphate buffer (pH 6.6), 12 μmoles of MgSO₄, 1.0 mg of membrane vesicle protein, from glucose or succinate-grown cells, and substrate to a final concentration as indicated in Tables 4 to 8. Each vessel was continuously gassed with oxygen. The reactions were started by the addition of vesicles. After 30 seconds of incubation, a sample was taken and filtered, washed and counted as described in Expt. 1. Specific activities of labeled sugars are:

\[ \text{U}^{14}\text{C-D-glucose (14 nCi/nmole), } \text{³H-(C}_3\text{)-3FG (15.2 nCi/nmole), } \text{¹⁴C-(C}_1\text{)-2DOG (5.58 nCi/nmole), } \text{U}^{14}\text{C-α-MG (18.4 nCi/nmole) and U}^{14}\text{C-L-glucose (14 nCi/nmole).} \]

A Lineweaver-Burk plot of sugar uptake by membrane vesicles from glucose-grown cells is shown in Fig. 11 and Fig. 12.

A Lineweaver-Burk plot of sugar uptake by membrane vesicles from succinate-grown cells is shown in Fig. 12.

Experiment 10: Kinetics of D-Gluconic Acid Uptake.

A series of 10 ml Erlenmeyer flasks contained in a total volume of 1.0 ml: 50 μmoles of potassium phosphate buffer (pH 6.6), 12 μmoles of MgSO₄, 1.0 mg of membrane vesicle protein, from glucose or succinate-grown cells,
### TABLE 4

Transport of U-\(^{14}\)C-D-Glucose by Membrane Vesicles of *Ps. fluorescens* Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>CONCENTRATION GLUCOSE ((\mu)M)</th>
<th>INITIAL RATE OF TRANSPORT (n mole/mg protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOLE CARBON SOURCE FOR GROWTH</td>
</tr>
<tr>
<td></td>
<td>GLUCOSE</td>
</tr>
<tr>
<td>35</td>
<td>0.125</td>
</tr>
<tr>
<td>50</td>
<td>0.170</td>
</tr>
<tr>
<td>70</td>
<td>0.220</td>
</tr>
<tr>
<td>100</td>
<td>0.262</td>
</tr>
<tr>
<td>150</td>
<td>0.360</td>
</tr>
<tr>
<td>200</td>
<td>0.420</td>
</tr>
<tr>
<td>500</td>
<td>n.d.</td>
</tr>
<tr>
<td>1000</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. -- Not Done
**TABLE 5**

Transport of $^{3}$H-(C$_3$)-3FG by Membrane Vesicles of *Ps. fluorescens* Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>CONCENTRATION 3FG (µM)</th>
<th>INITIAL RATE OF TRANSPORT (nmole/mg protein/min.)</th>
<th>SOLE CARBON SOURCE FOR GROWTH</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GLUCOSE</td>
<td>SUCCINATE</td>
</tr>
<tr>
<td>35</td>
<td>0.18</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.24</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>0.33</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>0.35</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.40</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>0.50</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>210</td>
<td>1.60</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>420</td>
<td>n.d.</td>
<td>1.60</td>
<td></td>
</tr>
</tbody>
</table>

n.d.—Not Done
<table>
<thead>
<tr>
<th>CONCENTRATION 2DOG (µM)</th>
<th>INITIAL RATE OF TRANSPORT (nmole/mg protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOLE CARBON SOURCE FOR GROWTH</td>
</tr>
<tr>
<td></td>
<td>GLUCOSE</td>
</tr>
<tr>
<td>40</td>
<td>0.20</td>
</tr>
<tr>
<td>100</td>
<td>0.42</td>
</tr>
<tr>
<td>200</td>
<td>0.62</td>
</tr>
<tr>
<td>300</td>
<td>0.76</td>
</tr>
<tr>
<td>400</td>
<td>1.00</td>
</tr>
<tr>
<td>500</td>
<td>1.08</td>
</tr>
</tbody>
</table>
TABLE 7

Transport of Methyl U-\(^{14}\)C-\(\alpha\)-D-Glucopyranoside
by Membrane Vesicles of *Ps. fluorescens*
Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>CONCENTRATION (\alpha)-MG ((\mu)M)</th>
<th>INITIAL RATE OF TRANSPORT (nmole/mg protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOLE CARBON SOURCE FOR GROWTH GLUCOSE Succinate</td>
</tr>
<tr>
<td>40</td>
<td>0.075</td>
</tr>
<tr>
<td>70</td>
<td>0.13</td>
</tr>
<tr>
<td>100</td>
<td>0.20</td>
</tr>
<tr>
<td>140</td>
<td>0.28</td>
</tr>
<tr>
<td>200</td>
<td>0.42</td>
</tr>
</tbody>
</table>
TABLE 8
Transport of U-\(^{14}\)C-L-Glucose by Membrane Vesicles of \textit{Ps. fluorescens} Grown on Glucose

<table>
<thead>
<tr>
<th>Concentration L-Glucose ((\mu)M)</th>
<th>Initial Rate of Transport (nmol(\cdot)mg protein/min.) for Sole Carbon Source for Growth Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>0.06</td>
</tr>
<tr>
<td>50</td>
<td>0.08</td>
</tr>
<tr>
<td>70</td>
<td>0.12</td>
</tr>
<tr>
<td>85</td>
<td>0.14</td>
</tr>
<tr>
<td>100</td>
<td>0.16</td>
</tr>
<tr>
<td>120</td>
<td>0.19</td>
</tr>
<tr>
<td>160</td>
<td>0.26</td>
</tr>
</tbody>
</table>
Lineweaver-Burk Plot of The Initial Rates of D-Glucose, 2DOG and 3FG Transport by Membrane Vesicles of *Ps. fluorescens* Grown on Glucose

**Key:**

- $^{14}$C-D-Glucose
  - $K_m = 167 \, \mu M$
  - $V_{max} = 0.79 \, \text{n mole/mg protein/min.}$

- $^{14}$C-(C$_1$)-2DOG
  - $K_m = 286 \, \mu M$
  - $V_{max} = 1.56 \, \text{n mole/mg protein/min.}$

- $^3$H-(C$_3$)-3FG
  - $K_m = 400 \, \mu M$
  - $V_{max} = 2.70 \, \text{n mole/mg protein/min.}$
FIGURE 11

Lineweaver-Burk Plot of The Initial Rates of D-Glucose, 2DOG and JFG Transport by Membrane Vesicles of Pa.
fluorescens Grown on Glucose

\[
\frac{1}{V_i} (\text{mol/mg protein per min.})
\]

\[
\frac{1}{[S]} \times 10^{-3} \mu M^{-1}
\]
FIGURE 12

Lineweaver-Burk Plot of the Initial Rates of Sugar Uptake by Membrane Vesicles of *Pseu.
fluorescens* Grown on Glucose or Succinate

Key:

- membrane vesicles from glucose-grown cells
  - U-\(^{14}\)C-\(\alpha\)-MG
  - U-\(^{14}\)C-L-glucose

- membrane vesicles from succinate-grown cells
  - U-\(^{14}\)C-D-glucose
  - \(^{14}\)C-(C\(_1\))-2DOG
  - \(^3\)H-(C\(_3\))-3FG
FIGURE 12

Lineweaver-Burk Plot of The Initial Rates of Sugars Uptake by Membrane Vesicles of Ps. fluorescens Grown on Glucose or Succinate
and U-14C-D-gluconate (3.91 nCi/nmole) to final concentrations as indicated in Table 9. Each vessel was continuously gassed with oxygen. The reactions were started by the addition of vesicles. After 30 seconds of incubation, a sample was taken and filtered, washed and counted as described in Expt. 1.

A Lineweaver-Burk plot of gluconic acid uptake by membrane vesicles from glucose and succinate-grown cells is shown in Fig. 13.

Oxidation Studies by Membrane Vesicles of \textit{Ps. fluorescens}

Since glucose, 3FG and gluconic acid were transported by membrane vesicles from glucose-grown cells and succinate-grown cells of \textit{Ps. fluorescens}, experiments were designed using the Gilson Differential Respirometer to determine if these sugars could be oxidized by membrane vesicles and to what extent.

In all experiments performed, the concentration of all sugars was 6.0 mM, unless otherwise indicated.

**Experiment 11:** Oxidation of Glucose, 3FG and Gluconic Acid by Membrane Vesicles.

In Gilson flasks, membrane vesicles, from glucose or succinate-grown cells, were incubated with D-glucose, 3FG or gluconic acid in a reaction mixture consisting of: 50 mM of potassium phosphate buffer (pH 6.6), 12 mM of
**TABLE 9**

Transport of U-$^{14}$C-D-Gluconic Acid, by Membrane Vesicles of *Ps. fluorescens* Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>CONCENTRATION GLUCONIC ACID (μM)</th>
<th>INITIAL RATE OF TRANSPORT (n mole/mg protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>SOLE CARBON SOURCE FOR GROWTH</strong></td>
</tr>
<tr>
<td>20</td>
<td>0.25</td>
</tr>
<tr>
<td>30</td>
<td>0.34</td>
</tr>
<tr>
<td>60</td>
<td>0.54</td>
</tr>
<tr>
<td>90</td>
<td>0.65</td>
</tr>
<tr>
<td>120</td>
<td>0.69</td>
</tr>
<tr>
<td>200</td>
<td>0.80</td>
</tr>
</tbody>
</table>

n.d.—Not Done
Lineweaver-Burk Plot of The Initial Rates of D-Gluconate Transport by Membrane Vesicles of *Ps. fluorescens* Grown on Glucose or Succinate

U-$^{14}$C-D-gluconate rate of uptake was measured as described in the experimental section.

**Key:**

- membrane vesicles from glucose-grown cells
  
  $K_m = 66.7 \, \mu M$
  
  $V_{max} = 1.11 \, \text{nmole/mg protein/min.}$

- membrane vesicles from succinate-grown cells
  
  $K_m = 62.5 \, \mu M$
  
  $V_{max} = 0.58 \, \text{nmole/mg protein/min.}$
FIGURE 13

Lineweaver-Burk Plot of The Initial Rates of D-Gluconate Transport by Membrane Vesicles of Ps. fluorescens Grown on Glucose or Succinate

\[
\frac{1}{V_i} \text{ (nmol/mg protein per min.)}
\]

\[
\frac{1}{[S]} \times 10^{-3} \mu M^{-1}
\]
MgSO₄ in a total volume of 2.0 ml. The flasks were incubated at 30°C with shaking for 10 hours. The endogenous respiration (no substrate was added) was negligible in membrane vesicles from the glucose and succinate-grown cells. The rates and extent of oxidation of these sugars is shown in Fig. 14 and Table 10.

**Experiment 12:** The Effect of Respiratory-Chain Inhibitors on the Oxidation of D-Glucose and D-Gluconate.

The effect of respiratory-chain inhibitors, CN⁻ and the uncoupler DNP, on the oxidation of D-glucose and D-gluconic acid by membrane vesicles from glucose-grown cells were studied.

The vesicles were incubated with the inhibitor for 10 minutes at 30°C by shaking before the addition of the substrate. The reaction was initiated by adding 12 μmoles (6 mM) of glucose or gluconic acid to the reaction mixture. The experiment was performed as described in Expt. 11.

The results are shown in Fig. 15a and Fig. 15b.

**Experiment 13:** Kinetics of Sugar Oxidation.

Initial rates of oxidation of D-glucose, 3FG, D-gluconic acid and 3FGA were measured using the oxygen electrode. Membrane vesicles from glucose-grown cells were incubated with varying concentrations of substrate in a final reaction volume of 3.0 ml. The initial rate of oxidation
FIGURE 14

Oxidation of D-Glucose, 3FG and D-Gluconate by Membrane Vesicles From Glucose and Succinate-Grown Ps. fluorescens

Glycol respiration conditions: temp. 30°C, reaction volume 2.0 ml, gas phase = air.
Each flask contained: main compartment: 1 mg membrane vesicle protein, 50 mM phosphate buffer pH 6.6 and 12 mM MgSO₄ to 1.5 ml.
side arm: 0.5 ml substrate (12 μmoles).
center well: 0.3 ml 20% HOH+paper wick.
The reaction was initiated by tipping contents from side arm.

Key:
open symbols, membrane vesicles from glucose-grown cells
closed symbols, membrane vesicles from succinate-grown cells

6 mM D-glucose
6 mM D-gluconate
6 mM 3FG
endogenous
FIGURE 14

Oxidation of D-Glucose, 3FG and D-Gluconate by Membrane Vesicles From Glucose and Succinate-Grown Ps. fluorescens

Time (min.)

μM α2 / mg protein
### Table 10

Oxidation of Various Substrates by Membrane Vesicles of *Ps. fluorescens* Grown on Glucose and Succinate, in the Presence or Absence of Electron Donors

<table>
<thead>
<tr>
<th>Substrate Oxidized</th>
<th>Net O$_2$ Consumption (µl)</th>
<th>Atoms O$_2$/mole of Substrate Oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>12 µmoles glucose</td>
<td>275</td>
<td>132</td>
</tr>
<tr>
<td>12 µmoles glucose</td>
<td>693</td>
<td>288</td>
</tr>
<tr>
<td>10 mM L-Mal./</td>
<td>300</td>
<td>291</td>
</tr>
<tr>
<td>50 µM FAD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µmoles L-Mal./</td>
<td>270</td>
<td>151</td>
</tr>
<tr>
<td>50 µM FAD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 µmoles glucose</td>
<td>124</td>
<td>0</td>
</tr>
<tr>
<td>0.1 mM PMS/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mM ascorbate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 µmoles gluconate</td>
<td>n.d.</td>
<td>169</td>
</tr>
<tr>
<td>10 mM L-Mal./</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µM FAD</td>
<td>n.d.</td>
<td>119</td>
</tr>
<tr>
<td>12 µmoles 3PG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.d. — Not Done
**FIGURE 15**

Oxidation of D-Glucose and D-Gluconate by Membrane Vesicles From Glucose-Grown *Ps. fluorescens* in The Presence and Absence of Electron Transport Chain Inhibitors

The vesicles were incubated with the inhibitor DNP or CH⁻ for 10 minutes before the addition of substrate.

Gilson respirometer conditions: temp. 30°C, reaction volume 2.0 ml, gas phase = air.

Each flask contained: main compartment: 1 mg membrane vesicle protein, 50 mM phosphate buffer pH 6.6 and 12 mM MgSO₄ with or without inhibitor to 1.5 ml.

side arm: 0.5 ml substrate (12 μmoles).

center well: 0.2 ml 20% KOH + paper wick.

The reaction was initiated by tipping contents from side arm.

**Fig. 15a** Oxidation of D-Glucose

**Key:**

- 6 mM D-glucose
- 6 mM D-glucose + 10 mM CH⁻
- 6 mM D-glucose + 0.1 mM DNP
- endogenous
FIGURE 15a

Oxidation of D-Glucose

\[ \text{\( \mu L O_2 / mg \) protein} \]

Time (min.)
Fig. 15b Oxidation of D-Gluconate

Key:

- - - - - - - - 6 mM D-gluconate

- - - - - - - - 6 mM D-gluconate + 10 mM CN⁻

- - - - - - - - 6 mM D-gluconate + 0.1 mM DNP

- - - - - - - - endogenous
FIGURE 15b

Oxidation of D-Gluconate
was expressed as nmole O₂/mg protein/minute. The reaction mixture composed of 1.0 mg of membrane vesicle protein, substrate, 50 mM of potassium phosphate buffer (pH 6.6) and 12 mM of MgSO₄ in a final volume of 3.0 ml. Reaction was initiated by the addition of membrane vesicles to the reaction vessel in a water bath at 25°C. Endogenous oxidation was negligible. The initial rate of oxidation was obtained by measuring the initial slope of the trace produced by the chart recorder. The results of the initial rates of oxidation of glucose, 3FG, gluconic acid and 3FGA by membrane vesicles are shown in Table 11 and Table 12.

A Lineweaver-Burk plots of the data are shown in Figs. 16a, 16b, 16c and 16d.

**Estimation of Vesicle Volume**

Intravesicular water is determined from the ratio of wet weight to dry weight of a packed vesicles water. The correction was determined as described by Konings and Freese.¹²⁰

**Experiment 14:**

To 1.0 ml of membrane vesicles (7.0 mg protein/ml) obtained from glucose-grown *Ps. fluorescens*, 50 μl of carboxy-¹⁴C-inulin (2.5 μCi; sp. act.=1.89 nCi/nmole) were added. After centrifugation at 12,000 xg for
<table>
<thead>
<tr>
<th>SUBSTRATE OXIDIZED (mM)</th>
<th>INITIAL RATE OF SUBSTRATE OXIDATION (nmole O₂/mg protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLUCOSE</td>
</tr>
<tr>
<td>0.05</td>
<td>11.0</td>
</tr>
<tr>
<td>0.10</td>
<td>18.2</td>
</tr>
<tr>
<td>0.25</td>
<td>37.8</td>
</tr>
<tr>
<td>0.50</td>
<td>69.3</td>
</tr>
<tr>
<td>1.00</td>
<td>116.6</td>
</tr>
<tr>
<td>2.00</td>
<td>138.6</td>
</tr>
<tr>
<td>—</td>
<td>63.00</td>
</tr>
</tbody>
</table>
TABLE 12

Rate of Oxidation of Gluconic Acid and 3PGA by Membrane Vesicles of *Ps. fluorescens* Grown on Glucose

<table>
<thead>
<tr>
<th>SUBSTRATE OXIDIZED (mM)</th>
<th>INITIAL RATE OF OXIDATION (n mole O₂/mg protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUCONATE</td>
<td>3PGA</td>
</tr>
<tr>
<td>0.10</td>
<td>1</td>
</tr>
<tr>
<td>0.25</td>
<td>5</td>
</tr>
<tr>
<td>0.50</td>
<td>10</td>
</tr>
<tr>
<td>1.00</td>
<td>20</td>
</tr>
<tr>
<td>2.00</td>
<td>30</td>
</tr>
<tr>
<td>2.50</td>
<td>40</td>
</tr>
</tbody>
</table>
FIGURE 16

Lineweaver-Burk Plot of The Initial Rates of Sugars Oxidation by Membrane Vesicles of Ps. fluorescens Grown on Glucose

**Oxygen electrode conditions:** temp. 25°C, reaction volume 3.0 ml.

Final reaction volume contained: 50 mM phosphate buffer pH 6.6, 12 mM MgSO₄, 1 mg membrane vesicle protein and substrate to 3.0 ml.

Reaction was initiated by the addition of the membrane vesicle suspension to the reaction vessel.

Endogenous oxidation (no added substrate) was negligible. The initial rate of oxidation was obtained by measuring the initial slope of the trace produced by the chart recorder.

**Fig. 16a Lineweaver-Burk Plot of The Initial Rates of D-Glucose Oxidation.**

\[ K_m = 833 \mu M \]

\[ V_{max} = 150 \text{ nmole oxygen/}mg \text{ protein/min.} \]
FIGURE 16a

Lineweaver-Burk Plot of The Initial Rates of D-Glucose Oxidation

\[ \frac{1}{V_i} \text{(nmol/mg protein per min.)}^{-1} \]

\[ \frac{1}{[S]} \times 10^{-1} \text{ M}^{-1} \]
Fig. 16b  Lineweaver-Burk Plot of The Initial Rates of 3FG Oxidation

\[ K_m = 25 \times 10^3 \text{ mM} \]

\[ V_{\text{max}} = 105 \text{ mmole oxygen/mg protein/min} \]
FIGURE 16b

Lineweaver-Burk Plot of The Initial Rates of 3FG Oxidation

\[
\frac{1}{V_i} \text{ (nmmol/mg protein per min.)}^{-1}
\]

\[
\frac{1}{[S]} \times 10^{-2} \text{ mM}^{-1}
\]
Fig. 16c  Lineweaver-Burk Plot of The Initial Rates of
D-Gluconate Oxidation

\[ K_m = 714 \, \text{µM} \]
\[ V_{\text{max}} = 86 \, \text{nmol} \, \text{oxygen/mg protein/min}. \]
Lineweaver-Burk Plot of The Initial Rates of D-Gluconate Oxidation

\[ \frac{1}{V} \times \frac{10^{-1}}{\text{mM}} \times [S]^{-1} \]
Fig. 16d  Lineweaver-Burk Plot of the Initial Rates of 3PGA* Oxidation

\[ K_m = 12.5 \times 10^{-3} \text{ M} \]

\[ V_{\text{max}} = 27.0 \text{ nmole oxygen/mg protein/min} \]

* calcium 3-deoxy-3-fluoro-D-gluconate was converted to the free acid by passage through a Dowex 50-H\(^+\) ion exchange column.
Lineweaver-Burk Plot of The Initial Rates of 3FGA Oxidation

\[ \frac{1}{V_i} \times 10^{-1} \text{ (nmol/mg protein per min.)} \]

\[ \frac{1}{[S]} \times 10^{-2} \text{ mM} \]
30 minutes the total water volume in the pellet was determined from the difference of the wet weight and the dry weight (dried 3 hours at 110°C). The interstitial volume between the vesicles was determined by dissolving the pellet in 0.5 ml of 10 percent sodium dodecyl sulfate and counting the radioactivity of the remaining inulin in vials containing 10 ml of TEG using the liquid scintillation counter. The total water minus the interstitial volume gave the volume inside the vesicles. There were 3.9 µl water/mg of vesicle protein.

The accumulation of various substrates was calculated, the results are shown in Table 13.

Kinetic Constants for Transport and Oxidation in Membrane Vesicles

Table 14 gives Km values for the transport of D-glucose, gluconic acid and glucose analogues by membrane vesicles from glucose-grown cells. The Km values for transport of the various substrates were between 66 to 400 µM.

As a further check on the role of membrane-bound oxidase and dehydrogenase in transport, the Km values for oxidation of glucose, 3FG, gluconic acid and 3FGA by membrane vesicles of Pseudomonas fluorescens grown on glucose were determined.
<table>
<thead>
<tr>
<th>TABLE 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of Various Substrates in Membrane Vesicles of <em>Ps. fluorescens</em> Grown on Glucose and Succinate</td>
</tr>
</tbody>
</table>

Concentration of the various substrates inside the membrane vesicles was calculated from the maximal uptake values shown in Figs. 5, 6, 9 and 10; 1.0 mg of vesicle protein corresponds to an internal vesicle volume of 3.91 µl was previously determined. Values in parentheses give the ratio between substrate concentration in membrane vesicles at the time of maximum uptake and the initial substrate concentration in the extravesicular incubation mixture (given in the legend to Figs. 5, 6, 9 and 10).
### TABLE 13

Concentration of Various Substrates in Membrane Vesicles of *Ps. fluorescens* Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>MAXIMUM UPTAKE (nmole/mg protein)</th>
<th>CONCENTRATION IN MEMBRANE VESICLES (µM)</th>
<th>SOLE CARBON SOURCE FOR GROWTH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GLUCOSE</td>
</tr>
<tr>
<td>70 µM D-Glucose</td>
<td>0.67</td>
<td>172</td>
<td>(2.5)</td>
</tr>
<tr>
<td>70 µM D-Glucose + PMS-Ascorbate</td>
<td>0.90</td>
<td>231</td>
<td>(3.3)</td>
</tr>
<tr>
<td>70 µM D-Glucose + L-Mal.-FAD</td>
<td>1.40</td>
<td>359</td>
<td>(5.1)</td>
</tr>
<tr>
<td>70 µM D-Glucose + 20 mM 3FG</td>
<td>0.75</td>
<td>192</td>
<td>(2.7)</td>
</tr>
<tr>
<td>62 µM Gluconate</td>
<td></td>
<td>82</td>
<td>(1.4)</td>
</tr>
<tr>
<td>74 µM Gluconate</td>
<td>1.05</td>
<td>269</td>
<td>(3.6)</td>
</tr>
<tr>
<td>74 µM Gluconate + PMS-Ascorbate</td>
<td>1.50</td>
<td>385</td>
<td>(5.2)</td>
</tr>
<tr>
<td>74 µM Gluconate + L-Mal.-FAD</td>
<td>4.00</td>
<td>1025</td>
<td>(13.9)</td>
</tr>
<tr>
<td>70 µM 3FG</td>
<td>0.40</td>
<td>103</td>
<td>(1.5)</td>
</tr>
<tr>
<td>70 µM 3FG + L-Mal.-FAD</td>
<td>0.72</td>
<td>185</td>
<td>(2.6)</td>
</tr>
<tr>
<td>100 µM α-MG</td>
<td>0.20</td>
<td>51</td>
<td>(0.51)</td>
</tr>
<tr>
<td>100 µM 2DOG</td>
<td>0.29</td>
<td>74</td>
<td>(0.74)</td>
</tr>
</tbody>
</table>
TABLE 14

Kinetic Parameters for Oxidation and Transport of Various Substrates by Membrane Vesicles of P. fluorescens Grown on Glucose

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>PROCESS</th>
<th>Km VALUES (µM)</th>
<th>Vmax (nmole/mg protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose*</td>
<td>Transport</td>
<td>167.0</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>833.0</td>
<td>160</td>
</tr>
<tr>
<td>Gluconic Acid**</td>
<td>Transport</td>
<td>66.7</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>714.0</td>
<td>86</td>
</tr>
<tr>
<td>3FG*</td>
<td>Transport</td>
<td>400.0</td>
<td>2.70</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>$25 \times 10^3$</td>
<td>105</td>
</tr>
<tr>
<td>3FGA</td>
<td>Transport</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>$12.5 \times 10^3$</td>
<td>27</td>
</tr>
<tr>
<td>2DOG*</td>
<td>Transport</td>
<td>286.0</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* Km values for transport of D-glucose, 3FG and 2DOG by membrane vesicles of succinate-grown cells are oo.

** Km value for transport of gluconic acid by membrane vesicles of succinate-grown cells is 62.5 µM and Vmax=0.55 nmole/mg protein/min.

n.d.--Not Done
Transport Studies by Whole Cells of *Ps. fluorescens* Grown on Glucose or Succinate

Experiments were designed to study the kinetics of transport of glucose, glucose analogues and gluconate by whole cells grown on glucose and succinate.

**Experiment 15: Kinetics of D-Glucose Uptake.**

225 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of *Ps. fluorescens* and incubated with aeration at 30°C for 16 hours, at which time they were in late exponential phase. Cells were harvested by centrifugation at 23,000 xg for 5 minutes and washed twice at 30°C with 0.067 M of sodium-potassium phosphate buffer (pH 7.1), containing 0.1 percent (w/v) MgSO₄·7H₂O, referred to as Mg²⁺-phosphate buffer. The cells were resuspended in the same buffer to 2.4 mg protein/ml, and held at 30°C on a shaking water bath for 10 minutes before the addition of the radioactive substrate. The incubation mixture contained 0.2 ml of chloramphenicol (25 mg/ml), 1.0 ml of Mg²⁺-phosphate buffer, 0.2 ml of bacterial suspension and various concentrations of U-¹⁴C-D-glucose (1.39 nCi/nmole), in a total volume of 2.5 ml in 25 ml Erlenmeyer flask. The final cell density was between 66 μg and 480 μg protein/ml, depending upon the experimental requirement. 0.5 ml
samples were removed at time intervals and transferred into 2.0 mM of Mg\(^{2+}\)-phosphate buffer, pH 7.1, supplemented with 1.0 percent (w/v) NaCl, referred to henceforth as NaCl buffer, which was at room temperature and contained in the filter assembly, filtered on a Millipore filter HA (pore size 0.45 µM and 24 mm in diameter) under vacuum and washed with 1.0 ml portions of NaCl buffer, the filtration time was generally less than 30 seconds. The filters were transferred to scintillation vials containing 10 ml of scintillation fluid (TEG). The filters were dissolved by vigorous shaking. Samples were counted as described in Expt1 & the background was subtracted. The uptake was expressed as nmole substrate/mg protein/minute.

Table 15 shows the data of initial rates of uptake of glucose by whole cells grown on glucose or succinate (Fig. 17). These rates were measured in duplicate, 30 seconds after the addition of the substrate. For concentrations up to and including 5.0 µM the bacterial density was 66 µg protein/ml, above this concentration, 480 µg protein/ml was employed.

A Lineweaver-Burk plot of the data for the system at low substrate concentration is shown in Fig. 18 and the data for the system saturated at high substrate concentration is shown in Fig. 19.

Similar experiments were performed with succinate-grown cells. The results are shown in Fig. 17. A Lineweaver-Burk plot of the data is shown in Fig. 20.
**TABLE 15**

Transport of $^{14}$C-D-Glucose by *Ps. fluorescens* Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>CONCENTRATION GLUCOSE ($\mu$M)</th>
<th>INITIAL RATE OF TRANSPORT (n mole/mg protein/min.)</th>
<th>SOLE CARBON SOURCE FOR GROWTH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GLUCOSE</td>
</tr>
<tr>
<td>0.25</td>
<td>0.74</td>
<td>n.d.</td>
</tr>
<tr>
<td>0.50</td>
<td>1.26</td>
<td>n.d.</td>
</tr>
<tr>
<td>1.00</td>
<td>1.78</td>
<td>n.d.</td>
</tr>
<tr>
<td>2.00</td>
<td>2.92</td>
<td>n.d.</td>
</tr>
<tr>
<td>3.00</td>
<td>3.40</td>
<td>n.d.</td>
</tr>
<tr>
<td>5.00</td>
<td>4.35</td>
<td>1.39</td>
</tr>
<tr>
<td>10.00</td>
<td>5.00</td>
<td>1.74</td>
</tr>
<tr>
<td>20.00</td>
<td>5.30</td>
<td>n.d.</td>
</tr>
<tr>
<td>25.00</td>
<td>n.d.</td>
<td>1.96</td>
</tr>
<tr>
<td>40.00</td>
<td>6.20</td>
<td>n.d.</td>
</tr>
<tr>
<td>60.00</td>
<td>6.40</td>
<td>n.d.</td>
</tr>
<tr>
<td>75.00</td>
<td>n.d.</td>
<td>2.30</td>
</tr>
<tr>
<td>80.00</td>
<td>6.13</td>
<td>n.d.</td>
</tr>
<tr>
<td>100.00</td>
<td>6.35</td>
<td>2.40</td>
</tr>
<tr>
<td>140.00</td>
<td>n.d.</td>
<td>3.00</td>
</tr>
<tr>
<td>200.00</td>
<td>8.10</td>
<td>n.d.</td>
</tr>
<tr>
<td>300.00</td>
<td>8.94</td>
<td>n.d.</td>
</tr>
<tr>
<td>400.00</td>
<td>9.50</td>
<td>n.d.</td>
</tr>
<tr>
<td>600.00</td>
<td>10.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>1000.00</td>
<td>10.06</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. = Not Done
FIGURE 17

Plot of Initial Rates of Transport of Various Substrates by Whole Cells of *Ps. fluorescens* Grown on Glucose and Succinate

Key:

**glucose-grown cells**
- U-\(^{14}\)C-D-glucose
- \(^3\)H-(C\(_3\))-3PG
- U-\(^{14}\)C-\(\alpha\)-MG
- \(^{14}\)C-(C\(_1\))-2DOG
- U-\(^{14}\)C-L-glucose

**succinate-grown cells**
- O—O U-\(^{14}\)C-D-glucose
- \(^3\)H-(C\(_3\))-3PG
- U-\(^{14}\)C-\(\alpha\)-MG
- \(^{14}\)C-(C\(_1\))-2DOG
Plot of Initial Rates of Transport of Various Substrates by Whole Cells of \textit{Ps. fluorescens} Grown on Glucose and Succinate
Lineweaver-Burk Plot of The Initial Rates of D-Glucose Uptake of "low-Km" by Glucose-Grown Cells of *P. fluorescens*

U-$^{14}$C-D-Glucose rate of uptake was measured as described in the experimental section. The system was saturated at low substrate concentration and gave a $K_m$ value of 1.7 $\mu$M and $V_{max} = 5.15$ nmole/mg protein/min. This is, henceforth, referred to as the "low-$K_m$" transport system. The bacterial density was 66 $\mu$g protein/ml. The range of substrate concentration values plotted are between 0.25 $\mu$M and 20 $\mu$M.
FIGURE 18

Lineweaver-Burk Plot of The Initial Rates of D-Glucose Uptake of "low-Km" by Glucose-Grown Cells of *Ps. fluorescens*

\[
\frac{1}{V_i} \times 10^{-1}
\]

(rmol/mg protein per min.)

\[
\frac{1}{[S]} \times 10^{-2} \mu M^{-1}
\]
FIGURE 19

Lineweaver-Burk Plot of The Initial Rates of D-Glucose Uptake of "high-Km" by Glucose-Grown Cells of *Ps. fluorescens*.

U-\(^{14}\)C-D-glucose rate of uptake was measured as described in the experimental section. The system was saturated at high substrate concentration. The Km value is estimated at approximately 76.9 \(\mu\)M and Vmax is 11 nmole/mg protein/min. The bacterial density was 480 \(\mu\)g protein/ml. The substrate concentration values plotted are between 30 \(\mu\)M and 1 mM.
FIGURE 19

Lineweaver-Burk Plot of the Initial Rates of D-Glucose Uptake of "high-Km" by Glucose-Grown Cells of Ps. fluorescens

\[ 1/\frac{V}{V_i} \text{ (nmol/mg protein per min.)} \]

\[ 1/[S] \times 10^{-3} \mu M^{-1} \]
FIGURE 20

Lineweaver-Burk Plot of The Initial Rates of D-Glucose Transport by Succinate-Grown Cells of *Ps.* *fluorescens*

$U^{14}C$-D-glucose rate of uptake by whole cells was measured as described in the experimental section. The bacterial density was 32 μg protein/ml. The system appeared to have one site with a $K_m$ value of 3.9 μM and $V_{max}$ of 2.4 μmole/mg protein/min.
FIGURE 20

Lineweaver-Burk Plot of The Initial Rates of D-Glucose Transport by Succinate-Grown Cells of <i>Ps. fluorescens</i>

\[ \frac{1}{V} \times 10^{-1} \text{ (nmol/mg protein per min.)} \]

\[ \frac{1}{[S]} \times 10^{-2} \text{ M}^{-1} \]
Experiment 16: Kinetics of Glucose Analogue Uptake.

The uptake of glucose analogues, 3FG, 2DOG, α-MG, and L-glucose by whole cells grown on glucose or succinate were performed as described in Expt. 15.

The initial rate of uptake was measured in duplicate, 30 seconds after the addition of the substrate.

In glucose-grown cells, different bacterial densities were used for different substrates: for \(^3\text{H}-\text{(C}_3\text{)}-3\text{FG}\) (1.68 nCi/nmole) the bacterial density was 280 µg protein/ml for the concentrations 10 µM to 210 µM. For \(^{14}\text{C}-\text{(C}_1\text{)}-2\text{DOG}\) (1.12 nCi/nmole) the bacterial density was 330 µg protein/ml for the concentration 9.0 µM to 500 µM. For methyl \(^{14}\text{C}-\alpha\text{-D-glucopyranoside}\) (1.5 nCi/nmole) the bacterial density was 280 µg protein/ml, for the concentrations 10 µM to 200 µM. For \(^{14}\text{C}-\text{L-glucose}\) (0.5 nCi/nmole), the bacterial density was 360 µg protein/ml.

The data of initial rates of uptake of 3FG, 2DOG, α-MG and L-glucose are shown in Tables 16 to 19 and Fig. 17. A Lineweaver-Burk plots of these data are shown in Figs. 21 to 24.

In succinate-grown cells, the bacterial density for 3FG, 2DOG, α-MG and L-glucose uptake, was 320 µg protein/ml, 320 µg protein/ml, 500 µg protein/ml and 360 µg protein/ml respectively. The results of Lineweaver-Burk plots are shown in Fig. 25, and the data of initial rates of uptake are shown in Tables 16 to 19.
TABLE 16

Transport of $^3$H-(C$_3$)-3FG by *Ps. fluorescens*  
Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>CONCENTRATION 3FG (μM)</th>
<th>INITIAL RATE OF TRANSPORT (nmole/mg protein/min.)</th>
<th>SOLE CARBON SOURCE FOR GROWTH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GLUCOSE</td>
</tr>
<tr>
<td>10.5</td>
<td>n.d.</td>
<td>0.14</td>
</tr>
<tr>
<td>21.0</td>
<td>0.65</td>
<td>0.24</td>
</tr>
<tr>
<td>42.0</td>
<td>n.d.</td>
<td>0.42</td>
</tr>
<tr>
<td>52.5</td>
<td>1.20</td>
<td>n.d.</td>
</tr>
<tr>
<td>63.0</td>
<td>n.d.</td>
<td>0.44</td>
</tr>
<tr>
<td>80.0</td>
<td>1.30</td>
<td>n.d.</td>
</tr>
<tr>
<td>105.0</td>
<td>1.38</td>
<td>0.83</td>
</tr>
<tr>
<td>157.5</td>
<td>2.22</td>
<td>n.d.</td>
</tr>
<tr>
<td>210.0</td>
<td>1.94</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. = Not Done
TABLE 17

Transport of $^{14}$C-$(C_1)$-2-Deoxy-D-Glucose by *Pseudomonas fluorescens* Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>CONCENTRATION 2DOG (µM)</th>
<th>INITIAL RATE OF TRANSPORT (nmole/mg protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOLE CARBON SOURCE FOR GROWTH</td>
</tr>
<tr>
<td></td>
<td>GLUCOSE</td>
</tr>
<tr>
<td>4.44</td>
<td>n.d.</td>
</tr>
<tr>
<td>9.00</td>
<td>0.136</td>
</tr>
<tr>
<td>11.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>18.00</td>
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</tr>
<tr>
<td>22.00</td>
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</tr>
<tr>
<td>27.00</td>
<td>0.392</td>
</tr>
<tr>
<td>33.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>36.00</td>
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</tr>
<tr>
<td>44.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>50.00</td>
<td>0.710</td>
</tr>
<tr>
<td>100.00</td>
<td>1.100</td>
</tr>
<tr>
<td>111.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>150.00</td>
<td>1.180</td>
</tr>
<tr>
<td>200.00</td>
<td>1.520</td>
</tr>
<tr>
<td>250.00</td>
<td>1.790</td>
</tr>
<tr>
<td>500.00</td>
<td>3.400</td>
</tr>
</tbody>
</table>

n.d. -- Not Done
<table>
<thead>
<tr>
<th>CONCENTRATION $\alpha$-MG (µM)</th>
<th>INITIAL RATE OF TRANSPORT (nmole/mg protein/min.)</th>
<th>SOLE CARBON SOURCE FOR GROWTH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GLUCOSE</td>
</tr>
<tr>
<td>10</td>
<td>0.56</td>
<td>0.06</td>
</tr>
<tr>
<td>25</td>
<td>1.04</td>
<td>0.15</td>
</tr>
<tr>
<td>50</td>
<td>1.50</td>
<td>0.29</td>
</tr>
<tr>
<td>100</td>
<td>2.55</td>
<td>0.54</td>
</tr>
<tr>
<td>150</td>
<td>3.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>200</td>
<td>3.00</td>
<td>1.10</td>
</tr>
</tbody>
</table>

n.d. — Not Done
### Table 19

Transport of U-\(^{14}\)C-L-Glucose by *Ps. fluorescens*
Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>Concentration L-Glucose ((\mu)M)</th>
<th>Initial Rate of Transport (nmole/mg protein/min.)</th>
<th>Sole Carbon Source for Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>50</td>
<td>0.122</td>
<td>0.120</td>
</tr>
<tr>
<td>100</td>
<td>0.280</td>
<td>0.275</td>
</tr>
<tr>
<td>200</td>
<td>0.600</td>
<td>0.604</td>
</tr>
<tr>
<td>300</td>
<td>0.970</td>
<td>0.950</td>
</tr>
<tr>
<td>400</td>
<td>1.220</td>
<td>1.200</td>
</tr>
</tbody>
</table>
Lineweaver-Burk Plot of The Initial Rates of 3FG Transport by Glucose-Grown Cells of *P.s.fluorescens*

Transport of $\text{H}^3-(\text{C}_3)$-3FG was measured as described in the experimental section. The bacterial density was 280 μg protein/ml.

$K_m = 64.5 \mu M$

$V_{max} = 2.6 \text{ nmole/mg protein/min.}$
FIGURE 21

Lineweaver–Burk Plot of The Initial Rates of 3FG Transport by Glucose-Grown Cells of Ps. fluorescens

\[
1/V_1 \times 10^{-1} (\text{mol/mg protein per min})^{-1}
\]

\[
1/[S] \times 10^{-3} \text{ \mu M}
\]
FIGURE 22

Lineweaver-Burk Plot of The Initial Rates of 2DOG Transport by Glucose-Grown Cells of *Ps. fluorescens*

$^{14}C-(C_1)-2DOG$ rate of uptake by whole cells was measured as described in the experimental section. The bacterial density was 360 $\mu$g protein/ml.

$K_m = 167 \mu$M

$V_{max} = 3.29$ nmole/mg protein/min.
FIGURE 22

Lineweaver-Burk Plot of The Initial Rates of 2DOG Transport by Glucose-Grown Cells of *Ps. fluorescens*

\[
\frac{1}{V_i} \times 10^4 \text{ (umol/mg protein per min.)}^{-1}
\]

\[
\frac{1}{[S]} \times 10^{-3} \mu M^{-1}
\]

Graph showing the relationship between the initial rate of 2DOG transport and substrate concentration.
Lineweaver-Burk Plot of The Initial Rates of \( \alpha \)-MG Transport by Glucose-Grown Cells of \textit{Ps. fluorescens}.

U\(^{14}\text{C}-\alpha \)-MG rate of uptake by whole cells was measured as described in the experimental section. The bacterial density was 280 \( \mu \text{g protein/ml} \).

\[ \text{K}_m = 43.5 \ \mu\text{M} \]
\[ \text{V}_{\text{max}} = 3.08 \ \text{mmole/mg protein/min} \]
FIGURE 23

Lineweaver-Burk Plot of The Initial Rates of α-MG Transport by Glucose-Grown Cells of Ps. fluorescens

1/V_i \times 10^{-1} (\text{mmol/mg protein/min})^{-1}

1/[S] \times 10^{-3}
FIGURE 24

Lineweaver-Burk Plot of The Initial Rates of L-Glucose Transport by Glucose-Grown Cells of Ps. fluorescens

U-$^{14}$C-L-glucose rate of uptake by whole cells was measured as described in the experimental section. The bacterial density was 360 μg protein/ml.

\[ \text{Km} = \infty \]
\[ \text{Vmax} = \infty \]
Lineweaver-Burk Plot of The Initial Rates of L-Glucose Transport by Glucose-Grown Cells of \textit{Ps. fluorescens}

\[ \frac{1}{V_i} \times 10^{-1} \text{ (n mole/mg protein/min.)}^{-1} \]

\[ \frac{1}{[S]} \times 10^{-3} \text{ \(\mu\)M} \]
FIGURE 25

Lineweaver-Burk Plot of The Initial Rates of Glucose Analogues Transport by Succinate-Grown Cells of Ps. fluorescens

Km = ∞
Vmax = ∞

Key:

- U-^{14}C-L-glucose
- ^{14}C-(C_{1})-2DOG
- U-^{14}C-α-MG
- ^{3}H-(C_{3})-3FG
Lineweaver-Burk Plot of The Initial Rates of Glucose Analogues Transport by Succinate-Grown Cells of *Ps. fluorescens*
Experiment 17: Kinetics of Gluonic Acid Uptake.

The uptake of U-14C-D-gluonic acid (0.391 nCi/nmole) by whole cells grown on glucose and succinate was performed as described in Expt. 15. The initial rates of uptake were measured in duplicate 30 seconds after the addition of the substrate.

In glucose-grown cells, the bacterial density used in the uptake was 360 μg protein/ml and the concentrations of U-14C-D-gluonic acid (0.391 nCi/nmole) was 20 μM to 250 μM. The results of initial rates are shown in Table 20. A Lineweaver-Burk plot of the data is shown in Fig. 26.

In succinate-grown cells, the bacterial density used in the uptake was 500 μg protein/ml. The data of the initial rates are shown in Table 20 and a Lineweaver-Burk plot is shown in Fig. 27.

The results from the kinetics study on glucose, glucose analogues and gluonic acid are shown in Table 21.

**Specificity of D-Glucose Transport by Glucose-Grown Whole Cells of Ps. fluorescens**

It has been shown previously that the glucose analogues, 3FG, 2DOG and α-MG are transported by whole cells grown on glucose by a saturable process. The effect of unlabeled D-glucose on the accumulation of glucose analogues is measured.
### TABLE 20

Transport of U-\(^{14}\)C-D-Gluconic Acid by *Ps. fluorescens* Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>Concentration Gluconic Acid (µM)</th>
<th>Initial Rate of Transport (nmole/mg protein/min.)</th>
<th>Sole Carbon Source for Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>20</td>
<td>n.d.</td>
<td>1.27</td>
</tr>
<tr>
<td>30</td>
<td>19.24</td>
<td>n.d.</td>
</tr>
<tr>
<td>40</td>
<td>22.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>50</td>
<td>21.20</td>
<td>2.66</td>
</tr>
<tr>
<td>100</td>
<td>25.50</td>
<td>2.81</td>
</tr>
<tr>
<td>150</td>
<td>26.40</td>
<td>n.d.</td>
</tr>
<tr>
<td>200</td>
<td>27.80</td>
<td>3.28</td>
</tr>
<tr>
<td>250</td>
<td>29.80</td>
<td>4.00</td>
</tr>
</tbody>
</table>

n.d. — Not Done
FIGURE 26

Lineweaver-Burk Plot of The Initial Rates of D-Glucanate Transport by Glucose-Grown Cells of *Ps. fluorescens*

U-\(^{14}\)C-D-glucanate rate of uptake by whole cells was measured as described in the experimental section. The bacterial density was 360 μg protein/ml.

\[ K_m = 16.7 \mu M \]
\[ V_{max} = 30.1 \mu \text{mole/mg protein/min} \]
Transport by Glucose-Grown Cells of P. fluorescens

Lineeweaver-Burk Plot of the Initial Rates of D-Glucurate

Figure 26
Lineweaver-Burk Plot of The Initial Rates of D-Gluconate Transport by Succinate-Grown Cells of \textit{Ps. fluorescens}

U-14C-D-gluconate rate of uptake by whole cells was measured as described in the experimental section. The bacterial density was 500 \( \mu \text{g protein/ml} \).

\[ \text{Km} = 52.6 \ \mu\text{M} \]

\[ \text{Vmax} = 4.59 \ \text{n mole/mg protein/min} \].
Lineweaver-Burk Plot of The Initial Rates of D-Gluconate Transport by Succinate-Grown Cells of Ps. fluorescens
### TABLE 21

Kinetics Parameters for Transport of Various Substrates by Whole Cells of *Ps. fluorescens* grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>Km (µM)</th>
<th>Vmax (nmole/mg protein/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose (&lt;br&gt;glucose-grown)</td>
<td>1.7</td>
<td>5.15</td>
</tr>
<tr>
<td>(&lt;br&gt;succinate-grown)</td>
<td>77.0</td>
<td>11.00</td>
</tr>
<tr>
<td>D-Gluconate (&lt;br&gt;glucose-grown)</td>
<td>16.7</td>
<td>30.10</td>
</tr>
<tr>
<td>(&lt;br&gt;succinate-grown)</td>
<td>52.6</td>
<td>4.59</td>
</tr>
<tr>
<td>3PG (&lt;br&gt;glucose-grown)</td>
<td>64.5</td>
<td>2.60</td>
</tr>
<tr>
<td>2DOG (&lt;br&gt;glucose-grown)</td>
<td>167.0</td>
<td>3.29</td>
</tr>
<tr>
<td>α-MG (&lt;br&gt;glucose-grown)</td>
<td>43.5</td>
<td>3.08</td>
</tr>
</tbody>
</table>
Experiment 18: The Uptake of 3FG in the Presence and Absence of D-Glucose.

The uptake of \( ^3\text{H}-(C_3)-3\text{FG} \) by glucose-grown cells was performed as described in Expt. 15.

In a total volume of 5.0 ml, the reaction mixture contained 0.4 ml of chloramphenicol (2.5 mg/ml), 2.0 ml of \( \text{Mg}^{2+} \)-phosphate buffer pH 7.1, 0.5 ml of bacterial suspension; to a final concentration of 330 \( \mu \)g protein/ml, 100 \( \mu \)M \( ^3\text{H}-(C_3)-3\text{FG} \) (1.68 nCi/nmole) and unless otherwise indicated, 10 mM of D-glucose. The reaction was initiated by the addition of bacteria. Samples (0.5 ml) were removed at time intervals, filtered, washed and counted in 10 ml of TEG as previously described.

The results are shown in Fig. 28.

Experiment 19: Study of The Uptake of 2DOG in the Presence and Absence of D-Glucose.

The uptake of \( ^{14}\text{C}-(C_1)-2\text{DOG} \) by glucose-grown cells in the presence and absence of 10 mM of D-glucose was performed as described in Expt. 15.

In a total volume of 5.0 ml, the reaction mixture contained 0.4 ml of chloramphenicol (2.5 mg/ml), 2.0 ml of \( \text{Mg}^{2+} \)-phosphate buffer pH 7.1, 0.5 ml of bacterial suspension and 90 \( \mu \)M of \( ^{14}\text{C}-(C_1)-2\text{DOG} \) (1.12 nCi/nmole) and unless otherwise indicated 10 mM of D-glucose. The bacterial cells were added to a final concentration of
FIGURE 28

Time Course of The Uptake of 3FG in The Presence and Absence of D-Glucose by Glucose-Grown Cells of Ps. fluorescens

Key:

100 μM $^3$H-(C$_3$)-3FG

100 μM $^3$H-(C$_3$)-3FG + 10 mM D-glucose
FIGURE 28

Time Course of The Uptake of $^3$H$_2$O in The Presence and Absence of D-Glucose by Glucose-Grown Cells of Ps. fluorescens.
330 μg protein/ml. The reaction was initiated by the addition of bacteria as described in Expt. 18. The results are shown in Fig. 29.

Experiment 20: The Uptake of α-MG in the Presence and Absence of D-Glucose.

The uptake of α-MG was performed as described in Expt. 15 and Expt. 18. The reaction mixture contained 10 μM of U-14C-α-MG (1.5 nCi/nmole) and, unless otherwise stated, 10 mM of glucose. The bacterial density was 500 μg protein/ml. The results from this experiment show that D-glucose has no significant effect on the uptake of α-MG.

Determination of Intracellular Water in Ps. fluorescens

Intracellular cell water was determined from the ratio of wet weight to dry weight of a packed pellet of water. The correction was determined as described by Winkler and Wilson140 except that carboxy-14C-inulin was employed instead of 14C-inulin.

Experiment 21:

<225 ml of glucose/mineral salts medium was inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of Ps. fluorescens and incubated with aeration at 30°C for 16 hours at which
Time Course of The Uptake of 2DOG in The Presence and Absence of D-Glucose by Glucose-Grown Cells of *P. fluorescens*

Key:

- **△—△** 90 μM $^{14}$C-$\left(C_1\right)$-2DOG
- **△—△** 90 μM $^{14}$C-$\left(C_1\right)$-2DOG + 10 mM D-glucose
Time Course of The Uptake of 2DOG in The Presence and Absence of D-Glucose by Glucose-Grown Cells of *Ps. fluorescens*
time they were in late exponential phase and gave an optical density of 0.7. The cells were harvested by centrifugation at 23,000 xg for 5 minutes and washed twice with the above medium without glucose and resuspended in 20 ml of media to a concentration of 2.4 mg protein/ml.

To 5.0 ml of cell suspension (i.e. 12 mg of protein), 100 µl of carboxy-$^{14}$C-inulin (.5.0 µCi; 1.98 nCi/nmole) were added. The cells were centrifuged at 12,000 xg for 5 minutes, the total water volume in the cell was determined from the difference of the wet weight and the dry weight (dried 3 hours at 110°C). The interstitial volume between the cells was determined by dissolving the cells in 0.5 ml of 10 percent sodium dodecyl sulfate (SDS) and counting the radioactivity of the remaining inulin in the liquid scintillation counter in vials containing 10 ml of TEG. The total water minus the interstitial volume gave the volume inside the cells. There were 9.5 to 10 µl of cell water/mg protein.

The internal concentration of various substrates was calculated and the results are shown in Table 22.

**Experiment 22:** The Effect of CA and CB on the Uptake of Glucose by Whole Cells Grown on Glucose.

The uptake of 50 µM $^{14}$C-D-glucose (1.39 nCi/nmole) in the presence and absence of 0.1 mM of CB or CA was performed as described in Expt. 15. No inhibition was observed.
TABLE 22

Concentration of Various Substrates in Whole Cells of *Ps. fluorescens* Grown on Glucose and Succinate

Concentration of the various substrates in cells was calculated from the maximal uptake values; 1.0 mg of cell protein corresponds to an internal cell volume of 9.75 μl was determined. Values in parentheses give the ratio between the substrate concentration in cells at the time of maximum uptake and the initial substrate concentration in the reaction mixture.
TABLE 22

Concentration of Various Substrates in Whole Cells of Ps. fluorescens Grown on Glucose and Succinate

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>MAXIMUM UPTAKE (nmole/mg protein)</th>
<th>CONCENTRATION IN CELLS (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SOLE CARBON SOURCE FOR GROWTH</td>
</tr>
<tr>
<td></td>
<td>GLUCOSE</td>
<td>SUCCINATE</td>
</tr>
<tr>
<td>50 µM D-Glucose</td>
<td>21.20</td>
<td>—</td>
</tr>
<tr>
<td>5 µM D-Glucose</td>
<td>—</td>
<td>4.50</td>
</tr>
<tr>
<td>100 µM Gluconic Acid</td>
<td>50.00</td>
<td>—</td>
</tr>
<tr>
<td>20 µM Gluconic Acid</td>
<td>—</td>
<td>2.10</td>
</tr>
<tr>
<td>100 µM 2DOG</td>
<td>1.30</td>
<td>—</td>
</tr>
<tr>
<td>11 µM 2DOG</td>
<td>—</td>
<td>0.07</td>
</tr>
<tr>
<td>50 µM α-MG</td>
<td>4.70</td>
<td>—</td>
</tr>
<tr>
<td>100 µM α-MG</td>
<td>—</td>
<td>0.63</td>
</tr>
<tr>
<td>105 µM 3FG</td>
<td>1.38</td>
<td>—</td>
</tr>
<tr>
<td>21 µM 3FG</td>
<td>—</td>
<td>0.14</td>
</tr>
<tr>
<td>100 µM L-Glucose</td>
<td>—</td>
<td>0.40</td>
</tr>
<tr>
<td>10 µM L-Glucose</td>
<td>—</td>
<td>0.03</td>
</tr>
</tbody>
</table>
The bacterial density was 330 µg protein/ml. The cells were incubated with the inhibitor for 10 minutes before the addition of the substrate.

The results show no significant inhibition of the uptake of glucose.

**Experiment 23:** The Uptake of Glucose in the Presence and Absence of Electron Donors by Glucose-Grown Whole Cells.

The uptake of 50 µM of U-¹⁴C-D-glucose (1.39 nCi/nmole) in the presence and absence of 10 mM of L-malate-50 µM of FAD and 20 mM of ascorbate-0.1 mM of PMS, was performed as described in Expt. 15. The bacterial density was 360 µg protein/ml, the reaction was initiated by the addition of cells. The results show a significant inhibition with L-malate and PMS (Fig. 30).
FIGURE 30

Time Course of the Uptake of D-Glucose in the Presence and Absence of Electron Donors by Glucose-Grown Cells of Ps. fluorescens

Key:

- 50 μM U-14C-D-glucose.
- 50 μM U-14C-D-glucose + 10 mM L-mal. + 50 μM FAD.
- 50 μM U-14C-D-glucose + 20 mM ascorbate + 0.1 mM FAD.
FIGURE 30

Time Course of the Uptake of Glucose in the Presence and Absence of Electron Donors by Glucose-Grown Cells of *Ps. fluorescens*
CHAPTER IV
DISCUSSION

The biochemical characterization of transport systems is difficult unless the specific act of transport can be placed apart from the process of substrate metabolism. Otherwise it is impossible to determine which phenomenon is rate limiting. Separation of these processes is usually achieved by (1) isolation of mutant microorganisms that may transport substrates but are no longer able to catabolize them, (2) the use of nonmetabolizable substrate analogs that are suitable substrates for the transport system, and (3) the use of isolated membrane vesicles that are devoid of metabolic enzymes but which contain the transport system under study.

Membrane vesicles of *Ps. fluorescens A-3-12* are employed to investigate the transport of glucose, glucose analogues and gluconate. The disadvantage of this system is the loss of the periplasmic binding protein in the preparation of isolated membrane vesicles. For this reason and to gain more information on sugar transport systems in *Ps. fluorescens*, whole cells, grown on glucose and succinate are employed for this study.
The transport of sugars in whole cells is more complicated, since this involves the uptake and catabolism, but the advantage of using whole cells is to obtain information about the periplasmic proteins and their possible involvement in sugar transport systems.

**Transport of Sugar by Membrane Vesicles**

Transport of D-glucose and D-gluconate by isolated membrane vesicles from glucose-grown cells is stimulated by electron donors, capable of entering the electron transport chain either directly, i.e. reduced PMS (Fig. 5) or via the membrane-bound dehydrogenases, i.e. L-malate (Table 2). Various physiological electron donors are used, the relative efficiencies of these electron donors in energising transport of glucose and gluconate vary for the same membrane vesicles. From Table 2 maximal stimulation of glucose and gluconate transport required 10 mM of L-malate and 50 μM of FAD. This would suggest that the transport of glucose and gluconate is coupled to a membrane-bound FAD-linked L-malate dehydrogenase. Glucose and gluconate on the other hand, did not inhibit the transport of one another. This would suggest that both sugars are transported independently using separate transport systems. However D-malate, D-lactate, succinate, pyruvate, oxaloacetate and ATP have little or no effect on glucose and gluconate transport. These findings indicate
that transport systems for the various substrates are coupled with different efficiency to the various primary dehydrogenases. An analogous situation has been described for transport systems for various amino acids in \textit{E. coli}, \textsuperscript{92} glucose in \textit{Azotobacter vinelandii} \textsuperscript{127} and gluconate in \textit{P. aeruginosa}. \textsuperscript{125}

The transport of the glucose analogue, 3FG is stimulated by the electron donor L-malate (Fig. 6). L-malate has no effect on glucose and 3FG uptake by membrane vesicles from succinate-grown cells. However, gluconate uptake by the same type of membrane vesicles is stimulated by L-malate.

Inhibition studies show that inhibitors of the electron transport chain, such as cyanide (CN\textsuperscript{-}), which inhibits electron flow from cytochrome aa\textsubscript{3} to oxygen, and DNP, an uncoupler of oxidative phosphorylation and ionophore inhibitor, caused marked inhibition of this electron-donor-mediated transport of glucose, 3FG and gluconate by membrane vesicles from glucose-grown cells (Fig. 9 and Fig. 10). The energy generated by transfer of electrons is clearly required for the transport systems reported here, and it may be concluded that these transports occur actively. Similar active electron-donor-mediated transport of many substrates have been reported in \textit{E. coli}, \textsuperscript{83} \textit{B. subtilis} \textsuperscript{120} and marine pseudomonad. \textsuperscript{142} Apart from energy dependence, another criterion for active transport is the accumulation of substrates against a
concentration gradient. Table 13 shows that membrane vesicles from glucose-grown cells accumulate glucose 3 to 5 fold and gluconate 5 to 13 fold higher than the extra- vesicular concentrations, in the presence of electron donors. It was difficult to estimate the maximum uptake of glucose analogue, 3FG because, this sugar leaks out 3 minutes after the uptake process. This will be discussed later.

3FG is readily taken up by membrane vesicles from glucose-grown cells, radioactive material leaked out 3 minutes after the uptake had commenced (Fig. 6). Descending paper chromatography on intravesicular and extravesicular contents was performed against a marker standard chemically synthesized 3FG and 3FGA. The latter is known to be the product of 3FG oxidation by the membrane-bound glucose oxidase:

\[ \text{3FG} + \frac{1}{2} \text{O}_2 \xrightarrow{\text{GOX}} \text{3FGA} \]

3FG and 3FGA were the only intravesicular products that were detected in vesicles from glucose-grown cells of Ps. fluorescens (Fig. 8b). 3FGA was also identified in the supernatant fluid of the suspension medium (Fig. 8a) 3 minutes after the uptake had commenced. These results indicate that not only did 3FG diffuse freely into the membrane vesicles, but that 3FGA also diffused freely out of the membrane vesicles.
Specificity studies on the uptake of glucose by membrane vesicles from glucose-grown cells showed that unlabeled 3FG in concentrations 1 to 300 fold higher than the concentration of glucose stimulated labeled glucose uptake (Fig. 7). This is suggestive evidence that energy may be provided for the carbohydrate transport system in membrane vesicles by coupling the carrier protein to the membrane-bound glucose oxidase via an electron transport chain. This would be analogous to the transport roles played by membrane-bound D-lactic dehydrogenase and β-galactosides in *E. coli*. The effect of unlabeled glucose on the uptake of labeled 3FG by membrane vesicles from glucose-grown cells was studied. The results (Fig. 6) show that the addition of glucose brought about leakage of the accumulated labeled 3FG or its product 3FGA, one minute after the uptake had commenced. This indicates that glucose is an inhibitor of 3FG uptake.

Kinetics of Sugar Transport in Membrane Vesicles

The uptake of D-glucose, 2DOG and 3FG by membrane vesicles from glucose-grown cells, show saturation kinetics with respect to substrate concentration, with an apparent Km values of 167 μM, 286 μM and 400 μM respectively. Lineweaver-Burk plots of the uptake of glucose and glucose analogues shown in Fig. 11, are linear. The difference in the Km values for the transport of glucose and of
glucose analogues, however is 1 or 2 fold smaller. This indicates that the carrier has a high affinity for glucose and a lower affinity for 2DOG and 3FG. 2DOG and 3FG are metabolized to 2-deoxy-D-gluconic acid and 3FGA respectively. The oxidation of 2DOG by the membrane-bound glucose oxidase of \textit{Ps. aeruginosa} has been reported.\textsuperscript{143}

The uptake of \textalpha-MG and L-glucose by membrane vesicles of glucose-grown cells is also a linear function of initial rates and concentrations (Fig. 12) but the intersect of the x and y axes occurs at the origin, implying a nonsaturable process without a \textit{Km} value. It appears, therefore, that the uptake of \textalpha-MG and L-glucose into the internal pool occurs by passive diffusion.

Membrane vesicles prepared from succinate-grown cells accumulate radioactivity passively from D-glucose, 2DOG or 3FG. Lineweaver-Burk plots, however, of the uptake of these substrates show a linear function of initial rates and concentrations (Fig. 12) without \textit{Km} values, implying a nonsaturable process. This phenomena could be interpreted to be due to the repression of a glucose transport protein by growing the cells on succinate. Similarly the glucose transport system of \textit{Ps. aeruginosa} is inducible, and is subject to strong repression by a number of organic acids including acetate, citrate, succinate and malate.\textsuperscript{78}

The transport of gluconate by membrane vesicles prepared from glucose or succinate-grown cells of \textit{Ps.
fluorescens show saturation kinetics. A plot of the data in the Lineweaver-Burk form (Fig. 13) revealed an apparent Km values of 66.7 μM and 62.5 μM respectively. The Vmax of the former type of vesicles is 1.11 nmole/mg protein/minute and the latter type of membrane vesicles is 0.58 nmole/mg protein/minute. This similarity in Km values but the difference in Vmax imply the presence of a saturable transport process which is constitutive for gluconate, and the repression of gluconate dehydrogenase. In order to confirm this point the respiration of vesicle preparations was examined.

Oxidation of Sugars by Membrane Vesicles

Membrane vesicles prepared from glucose-grown cells of Ps. fluorescens oxidized D-glucose and D-gluconate immediately and without a lag (Fig. 14). The oxidation proceeded to the extent of 2.0 g atoms of oxygen/molecule of glucose and 1.0 g atoms of oxygen/molecule of gluconate. These membrane vesicles did not oxidize 3PG rapidly until after a period of lag. The oxidation proceeded to an extent of 1.0 g atoms of oxygen/molecule of 3PG.

Membrane vesicles prepared from succinate-grown cells, however, oxidized glucose and 3PG to an extent of 1.0 g atoms of oxygen/molecule of substrate (Fig. 14). Moreover, these membrane vesicles were not capable of oxidizing gluconate to any significant extent (Table 10).
The endogenous respiration in membrane vesicles from glucose and succinate-grown cells is negligible. These results may be interpreted as follows:

In membrane vesicles prepared from glucose-grown cells, glucose is oxidized two steps by the membrane-bound glucose oxidase and gluconate dehydrogenase:

\[
\begin{align*}
\text{D-glucose} & \xrightarrow{\frac{3}{2} O_2} \text{GA} & \frac{3}{2} O_2 & \xrightarrow{\text{GADH}} & 2\text{KGA}
\end{align*}
\]

The presence of 2KGA as a final product in membrane vesicles is confirmed by paper chromatography as it is detected intravesicularly (Fig. 8c and 8d). Gluconate and 3FG are oxidized one step only by membrane vesicles from glucose-grown cells presumably by gluconate dehydrogenase and glucose oxidase respectively:

\[
\begin{align*}
3\text{FG} & \xrightarrow{\frac{3}{2} O_2} 3\text{FGA} \\
\text{D-gluconate} & \xrightarrow{\frac{3}{2} O_2} 2\text{KGA}
\end{align*}
\]

3FGA was detected by paper chromatography in the supernatant suspension apparently it leaks out after 3 minutes as it is not capable of being metabolized any further (Fig. 8a and 8b). The oxidation of 3FG to 3FGA by whole cells of Ps. fluorescens has been previously reported. In the case of cell-free extracts, however, 3FG is oxidized to 2K3FGA. An explanation for this has not yet been provided.
In membrane vesicles prepared from succinate-grown cells, D-glucose and 3FG are oxidized one step only, clearly by the constitutive glucose oxidase to gluconate and 3FGA respectively. However, gluconate is not oxidized to any significant extent (Table 10). This is evidence that gluconate dehydrogenase is repressed in succinate-grown cells. For this reason the Vmax of the uptake of gluconate by this type of membrane vesicles was about 50 percent of that of glucose-grown cells, since only the transport protein remains intact. In membrane vesicles from glucose-grown cells the total rate of transport of gluconate, i.e. uptake and metabolism, could be measured, while in membrane vesicles from succinate-grown cells, only the rate of uptake could be measured by the gluconate carrier.

In membrane vesicles from glucose-grown cells, the addition of the physiological electron donor L-malate-FAD to D-glucose increased the consumption of oxygen to 3.5 g atom/molecule of substrate (Table 10). Glucose is oxidized to an extent of 2.0 g atoms of oxygen/molecule of glucose, this leaves approximately 1.45 g atoms of oxygen/molecule of L-malate. It is obvious that these extra g atoms of oxygen were consumed to oxidize L-malate to oxaloacetic acid (OAA) by the membrane-bound FAD-linked L-malate dehydrogenase.
Moreover, the nonphysiological electron donor PMS-ascorbate has no significant effect on the oxygen consumed by D-glucose in membrane vesicles (Table 10).

In membrane vesicles from succinate-grown cells, the addition of L-malate to glucose increases the extent of oxidation to 1.7 g atoms of oxygen/molecule of substrate and glucose is oxidized to an extent of 0.98 g atoms of oxygen/molecule of glucose, therefore, approximately 0.7 g atoms of oxygen/molecule of L-malate were consumed. Furthermore, the oxidation of D-gluconate in the presence of L-malate gives similar results (Table 10). 0.75 g atoms of oxygen/molecule of L-malate are consumed, since gluconate is not oxidized to any significant extent by membrane vesicles from succinate-grown cells.

Membrane vesicles from glucose-grown cells failed to oxidize D-glucose in the presence of the uncoupler DNP, while gluconate oxidation was slightly inhibited (Fig. 15a and 15b). ON⁻, slightly inhibited the oxidation of D-glucose and D-gluconate by membrane vesicles from glucose-grown cells. It has been reported that glucose oxidase in Ps. aeruginosa is sensitive to DNP.¹³⁹
As a further check on the role of membrane-bound glucose oxidase and gluconate dehydrogenase in transport, the Km values for oxidation of D-glucose, 3FG, gluconate and 3FGA by membrane vesicles of *Ps. fluorescens* grown on glucose were also determined, using the substrate concentrations range specified in Tables 11 and 12. Oxidation of these sugars exhibit a saturable process from which the Km values for glucose, 3FG, gluconate and 3FGA are 833 μM, 25 x 10^3 μM, 714 μM and 12.8 x 10^3 μM respectively (Figs. 16a to 16b). From these data it may be concluded that D-glucose and gluconate affinities for their enzymes are greater than the affinity of 3FG and 3FGA.

The results in Table 14 suggest that the Km for the transport of D-glucose, 3FG and gluconate is 4 to 60 fold lower than the Km for their oxidation. If the oxidation of these substrates accompanies transport, then the Km for their transport cannot be lower than the Km for their oxidation. It may be concluded that the affinity of transport is higher than the affinity of oxidation.

Comparison of the rates of D-glucose oxidation and of glucose transport by membrane vesicles from glucose-grown cells revealed that the former exceeded the latter. The Vmax of the uptake and oxidation measured are respectively 0.79 and 160 nmole/mg protein/minute. Similar observations are made with 3FG and gluconate; their conversion into 3FGA and 2KGA respectively, exceeding the rate of uptake of labeled substrate into membrane vesicles from
glucose-grown cells. It may be concluded that oxidation of glucose and gluconate by the membrane-bound glucose oxidase and gluconate dehydrogenase respectively, is not a condition for their transport by the membrane vesicles. Similar results on whole cells have been reported by Eisenberg and co-workers. In a subsequent preliminary paper, however, it is argued that glucose oxidase is serving a role for energising active transport of glucose in Ps.fluorescens whole cells.  

Transport of Sugars by Whole Cells

The initial rates of uptake of glucose by whole cells of Ps. fluorescens grown on glucose, increased in response to increasing external substrate concentration (Table 15 and Fig. 17). A Lineweaver-Burk plot of the data for the system saturated at low substrate concentration gave a Km value of 1.7 μM (Fig. 18). This is henceforth referred to as the "low-Km" transport system. The Km for the second process capable of being saturated with glucose is estimated at approximately 76.9 μM (Fig. 19), clearly the involvement of two components is suggested. Similar experiments were performed with succinate-grown cells. There was no uptake of 14C-glucose by the high-Km component that could be detected but, the uptake was mediated by a process with Km of approximately 3.9 μM in this type of cell (Fig. 20 and Table 24).
The correlation between these results and the absence of gluconate dehydrogenase in succinate-grown cells suggested that the glucose entry by the high Km component involved in some way the activity of this enzyme, and the Km of 3.9 \( \mu \text{M} \) is due either to the activity of the constitutive glucose oxidase and/or the presence of trace amount of the repressed glucose protein carrier, although, the cells were trained by growing them on succinate for six generations.

The uptake of 3FG, 2DOG and \( \alpha \)-MG are saturable processes (Fig. 17) with Km values of 64.5 \( \mu \text{M} \), 167 \( \mu \text{M} \) and 43.5 \( \mu \text{M} \) respectively in glucose-grown cells (Figs. 21, 22 and 23), whilst L-glucose is transported by an unsaturable process (Fig. 24). 2DOG and \( \alpha \)-MG have been reported to be transported actively in \textit{Ps. aeruginosa} whole cells.\(^{20}\)

In succinate-grown cells 3FG, 2DOG, \( \alpha \)-MG and L-glucose are transported nonspecifically (Fig. 25).

Some of these results are consistent with those obtained from membrane vesicles i.e. glucose, 3FG and 2DOG are transported actively in both membrane vesicles and whole cells grown on glucose, while these sugars, except D-glucose, are transported nonspecifically by membrane vesicles and whole cells grown on succinate. However, \( \alpha \)-MG is not transported by membrane vesicles actively (Fig. 12) but it is in whole cells grown on glucose (Figs. 17
and 23). These results suggested that a component of α-MG transport system has been lost in membrane vesicles preparation.

Gluconate was taken up by glucose and succinate-grown cells actively with Km values of 16.7 μM and 52.6 μM respectively. These results are consistent with the results obtained by membrane vesicles. Km values for gluconate uptake by membrane vesicles from succinate-grown cells is 62.5 μM and this is close to Km of 52.6 μM in whole cells (Figs. 26 and 27 and Table 21).

Specificity studies by whole cells grown on glucose show that the addition of unlabeled D-glucose at 100 fold concentration to labeled 3FG inhibits the initial rate of 3FG uptake (Fig. 28). This suggests that both sugars used the same transport system in Ps. fluorescens. Similar experiments were done to study the effect of unlabeled glucose on the uptake of labeled 2DOG. The results (Fig. 29) suggest that a 100 fold concentration of glucose inhibited the initial rate and extent of 2DOG uptake and that 2DOG is using the same glucose transport system. On the other hand glucose has no effect on the uptake of α-MG when the concentration of glucose is 1000 fold higher than the concentration of α-MG. The results support the suggestion made previously, that α-MG is transported independently by a separate system which is lost in the membrane vesicles preparation.
From the above studies, therefore, the results suggest that glucose entry into *P. fluorescens* is mediated by an active transport system of rather broad specificity since it is also capable of transporting 3FG and 2DOG. 3FG and 2DOG were apparently transported non-specifically by succinate-grown cells whilst glucose has some residual saturable transport characteristics (Fig. 20) with a $K_m$, 3.9 $\mu$M and $V_{max}$, 2.4 nmol/mg protein, although the extent of uptake is rather poor. The highest concentration used in the kinetic studies of 3FG and 2DOG was 110 $\mu$M (Table 16 and 17). The concentrations are considerably below those obtained for the saturation of the glucose carrier protein in membrane vesicles from glucose-grown cells. It is also possible, however, that a periplasmic glucose carrier protein is present in succinate-grown whole cells which is lost in vesicle preparations.

Whole cells grown on glucose and succinate accumulated glucose and gluconic acid several fold higher than the external concentration (Table 22). It was difficult to estimate the maximal uptake of 3FG because it leaks out 1 minute after the uptake had commenced as has been discussed previously. 2DOG uptake by glucose-grown cells is in a ratio of 1.3:1.0, with the external concentration, while it is below 1.0 in succinate-grown cells (Table 22). Perhaps 2DOG is leaking out of the cells as 2-deoxy-D-gluconate. $\alpha$-MG was accumulated several fold higher
than the external $\alpha$-MG concentration, in glucose-grown cells, while its concentration in succinate-grown cells was below 1.0. This supports the suggestion made previously that $\alpha$-MG is transported actively in whole cells and the binding protein is repressed in succinate-grown cells in which $\alpha$-MG is transported passively. L-glucose uptake and accumulation in glucose and succinate-grown cells is below 1.0; this suggests that whole cells grown on glucose and succinate do not accumulate L-glucose against the concentration gradient.

Inhibitors of the sulfhydryl group such as NEM, CA and PHMB have no effect on the uptake of glucose in membrane vesicles, from glucose-grown cells. Similarly, CB, CA, NEM and DCCD have no effect on the growth and respiration of whole cells grown on glucose. These results indicate that the sulfhydryl group is not involved in the active site of the glucose transport system.

Finally, the uptake of glucose by glucose-grown cells was studied in the presence and absence of L-malate-FAD (Fig. 30) and the results show an inhibition of glucose uptake by this electron donor. The same effect is observed when PMS-ascorbate is used. At present there is no definitive explanation for these results. It will be recalled that these electron donors enhance glucose and gluconate transport in vesicles. Possibly in whole cells L-malate and ascorbate are preferentially transported
and metabolized and that this function is lost during the preparation of vesicles.

On the basis of these results, a mechanism is proposed in Fig. 31 for D-glucose and D-gluconate transport system in *Ps. fluorescens*. In the proposed model, the electron donor L-malate release electrons which energize the high affinity carrier protein to form a carrier-substrate complex, the substrate is glucose in Fig. 31. The electrons then channeled through the electron transport chain. Consequently, there is a net efflux of protons which generates a membrane potential. The carrier-glucose complex, therefore, changes conformation and glucose is released intracellularly.
Proposed Model for The Energy-Coupled Active Transport System in *Ps. fluorescens*

Key:
- L-mal., L-malate; OAA, oxaloacetic acid; C, D-glucose carrier protein; RED, reduced; OX, oxidized; cy.b₁, c, cytochrome b₁ and c; C'....C, carrier protein-glucose complex of low affinity; IN, inside surface of the membrane; OUT, outside surface of the membrane; M, membrane.
Proposed Model for The Energy-Coupled Active Transport System in *Ps. fluorescens*
APPENDIX

Calculation of Radioactive Substrate Accumulated by
Membrane Vesicles or Whole Cells

A plot of ESCR (external standard channel ratio) against
efficiency for standard tritium and $^{14}C$-samples are shown
in Figs. 32 and 33.

Efficiency ($E$) = \( \frac{c.p.m}{d.p.m} \)

The efficiency for $^3H-(C_3)-3FG$ or $^{14}C$-sugars was calculated
from Figs. 32 and 33. To convert d.p.m to nCi, the following
factor was used:

1 nCi = \( 2.2 x 10^3 \) d.p.m

Example

The uptake of $^3H-(C_3)-3FG$ (15.2 nCi/nmole) by 1 mg membrane
vesicle protein, in a total volume of 1 ml, was 209 c.p.m/
0.1 ml of the reaction mixture and ESCR = 0.322.

From Fig. 32, \( E = 0.26 \)

and from equation (1), d.p.m/ml = 8040

Therefore, from equation (2), 8040 d.p.m = 3.6 nCi

The uptake of $^3H-(C_3)-3FG$ = \( \frac{3.6}{15.2 x 1 \text{ mg protein}} \)
$^{3}\text{H}$ Quench Curve

$^{3}\text{H}$-Toluene quenched standard was used of 163,000 d.p.m.
$^{14}\text{C}$ Quench Curve

$^{14}\text{C}$-Toluene quenched standard was used of 190,000 d.p.m.
Calculation of Oxygen Consumption by Membrane Vesicles

Example

In Table 10, 12 µmoles of glucose consumed 275 µl of oxygen after 8 hours. The extent of substrate oxidation, expressed as g atom O₂/molecule of glucose, was calculated as follows:

2 µg atoms of O₂ occupy 27.4 µl, therefore,
24.6 µg atoms of O₂ occupy 275 µl, and
24.6 µg atoms of O₂ was consumed by 12 µmoles of glucose.
Therefore, 2.05 µg atoms of O₂ was consumed/molecule of glucose.

Calculation of Internal Vesicle and Cell Volume

Example

Internal cell volume was estimated as follows:
5.0 ml of cell suspension (12 mg protein) contain 5.0 nCi of carboxy-14C-inulin.
The wet weight of the pellet = 141.2 mg
The dry weight of the pellet = 27.1 mg
Therefore, the weight of water inside the cell and the interstitial water = 114.1 mg
The interstitial space = 46.35 nCi = 0.2 mg
Therefore the intracellular water = 113.9 mg 113.9 µl/12 mg protein. The volume inside the cell = 9.5 µl/mg protein.

This experiment was repeated and there were 10 µl of cell water/mg protein. The average of cell water is 9.75 µl/mg protein.
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