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Defluorination of 4-deoxy-4-fluoro-D-glucose in Pseudomonas putida.

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DEFLUORINATION OF 4-DEOXY-4-FLUORO-D-GLUCOSE

IN *PSEUDOMONAS PUTIDA*

by

MAX LUIS TEJADA

A THESIS

Submitted to the Faculty of Graduate Studies through the

Department of Chemistry and Biochemistry in

Partial Fulfillment of the Requirements for the

Degree of Master of Science at the

University of Windsor

Windsor, Ontario, Canada

1992

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in Biochemistry:

NAME:	Mr. Max Tejada
DATE:	Wednesday, July 15, 1992
TIME:	10:30 a.m.
PLACE:	Room 273, Essex Hall Chemistry Conference Room

DISSERTATION TITLE

Defluorination of

4-Fluoro-D-glucose in *Pseudomonas putida*

Examination Committee:

N.F. Taylor, Advisor
R.J. Thibert
D.A. Cotter, Biological Sciences

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"God, give me the grace to accept with serenity the things that cannot be changed, courage to change the things which should be changed , and the wisdom to distinguish one from the other."

R. Niebuhr

ABSTRACT

DEFLUORINATION OF 4-DEOXY-4-FLUORO-D-GLUCOSE

IN *PSEUDOMONAS PUTIDA*

by

Max Luis Tejada

Growth of *P. putida* using gluconate as a carbon source effects a considerable decrease in the rate of 4-deoxy-4-fluoro-D-glucose (4FG) metabolism to 2,3-dideoxy-D-*glycero*-pentonic acid (2,3-DDRA). Borate column analysis of supernatants from these incubations revealed the presence of 3 defluorinated intermediate metabolites distinct from 2,3-DDRA. One of these, the Peak h metabolite (R_f= 0.74), was tentatively identified by ^{13}C-NMR spectroscopy and a colourimetric spectrophotometric analysis as 3,4-dideoxy-2-keto-D-*glycero*-hexanoic acid.

In vivo ^{19}F-NMR spectroscopic analyses of 4FG metabolism indicate that fluoride release is the initial step preceeding 4FG metabolism to 2,3-DDRA. The products of membrane-bound glucose oxidase and gluconate dehydrogenase, namely 4-deoxy-4-fluoro-D-gluconic acid (4FGA) and 4-deoxy-4-fluoro-2-keto-D-gluconic acid (4F2KGA), are also shown to be good substrates for defluorination.

Osmotically shocked cells defluorinate normally. Additionally, crude periplasmic extracts produced by osmotic shock lack fluoride release activity. This suggests that defluorination is not dependent upon the initial binding of a periplasmic protein to 4FG.

SDS-PAGE analysis of periplasmic extracts indicates the existence of a glucose-inducible, gluconate- and succinate-repressible protein band with an apparent molecular weight of 44 kDa.

Unlike the binding protein of *P. aeruginosa*, the binding protein of *P. putida* lacks a binding affinity for 4FG as indicated by the absence of significant chemical shifting observed by ^{19}F-NMR in incubations of 4FG with periplasmic extracts.

French pressure generated cytoplasmic membrane preparations exhibit significant fluoride release activity only in the presence of the electron donors L-malate/FAD and phenazine methosulphate/ascorbate. SDS-PAGE analysis indicates the presence of a glucose-inducible, gluconate- and succinate-repressible protein band with an apparent molecular weight of 65.4 kDa. This putative defluorinating protein is resistant to heat modification in the presence of reducing agents. However, in the absence of reducing agents it undergoes auto-oxidation to an inactive conjugated form.

Fluoride release activity is reconstituted into proteoliposomes. In the presence of electron donors approximately 20-25% of the initial fluoride substituent of 4FG is released as fluoride ion. Reconstitution of optimal defluorinating activity necessitates the addition of exogenous phospholipid in the solubilization and reconstitution procedures. Glucose elicits a total inhibition of fluoride release indicating the greater affinity of the defluorinating protein for glucose than 4FG. The lack of fluoride release inhibition observed in the presence of D-galactose indicates that binding of this protein to carbon 4 is stereospecific for a hydroxyl group.

DEDICATION

To the three most important people in my life without whom none of this would have been possible. To my mother and father, Emiliana and Maximo Tejada, whose hard work, sacrifices and belief that this day would come, helped to make my life possible, I will forever be trying to deserve all that you have given me. To my beautiful fiancée Lorena, the promise of a lifetime of love and a happiness beyond this world, ti amo con tutto il mio cuore per sempre.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

CHAPTER III

CHAPTER IV

APPENDIX I

APPENDIX II

APPENDIX III

APPENDIX IV

APPENDIX V

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

NMR	Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement Polarization Transfer
PETT	Positron Emission Transaxial Tomography
GMR	Glucose Metabolic Rates
2FG	2-deoxy-2-fluoro-D-glucose
3FG	3-deoxy-3-fluoro-D-glucose
3FS	3-deoxy-fluoro-D-sorbitol
3FF	3-deoxy-3-fluoro-D-fructose
3FGA	3-deoxy-3-fluoro-D-gluconic acid
3F2KGA	3-deoxy-3-fluoro-2-keto-D-gluconic acid
4FG	4-deoxy-4-fluoro-D-glucose
4FGA	4-deoxy-4-fluoro-D-gluconic acid
4F2KGA	4-deoxy-4-fluoro-2-keto-D-gluconic acid
PG	peptidoglycan
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
EDTA	ethylenediaminetetraacetic acid
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
Ala	alanine
Glu	glutamic acid
m-DAP	*meso*-diaminopimelic acid
ED	Entner-Doudoroff Pathway
EM	Embden-Meyerhof Pathway

ATP	adenosine-5'-triphosphate
ADP	adenosine-5'-diphosphate
Pi	inorganic phosphate
NEM	N-ethylmaleimide
K_m	Michaelis-Menten Constant
V_{max}	maximal rate
2,3-DDRA	2,3-dideoxy-D-*glycero*-pentonic acid or 2,3-dideoxyribonic acid
HPLC	High Pressure Liquid Chromatography
DNase 1	deoxyribonuclease 1
RNase A	ribonuclease A
PMS	phenazine methosulfate
FAD	flavin adenine dinucleotide
BSA	bovine serum albumin (fraction V)
PMSF	phenylmethylsulfonyl fluoride
TEMED	N,N,N',N'-tetramethylethylenediamine
DMSO	dimethylsulfoxide
PPO	2,5-diphenyloxazole
2,4-DNP	2,4-dinitrophenol
ATCC	American Type Culture Collection
°C	degrees celsius
nm	namometers
pm	picometers
g	grams
mg	milligrams

ng	nanograms
L	litres
mL	millilitres
μL	microlitres
cm	centimeters
mM	millimolar
μM	micromolar
rpm	revolutions per minute
psi	pounds per square inch
TCA	trichloroacetic acid
(w/v)	grams per millilitre
(v/v)	milllitre per millilitre
mA	milliamperes
mV	millivolts
V	volts
D_2O	deuterium oxide
TLC	thin-layer chromatography
Hz	hertz
MHz	Megahertz
ppm	parts per million
SR	spectral reference
-	approximately
dpm	disintegrations per minute

CHAPTER 1
INTRODUCTION

The Carbon-Fluorine Bond

The attraction of an atom for the electrons in a covalent bond connecting it to another atom may be described in terms of the electronegativity of said atom. This electronegativity can cause disruption of the electron cloud that bonds any two atoms distorting it towards one side of the bond or the other depending on which atom (nucleus plus electrons) maintains the greater attraction for the cloud. Electronegativity is greatest for atoms in the upper-right hand corner of the periodic table and lowest for atoms in the lower left-hand corner.

Numerous attempts have been made to set up quantitative tables of electronegativity to give some indication as to the direction and extent of electron-cloud distortion for a bond between any pair of atoms. The most popular of these, the Pauling scale, is based on bond energies of diatomic molecules (1). The rationale being if a molecule A-B has symmetrical electron distribution (with respect to the plane that perpendicularly bisects the bond), then the bond energy would be the mean of the bond energies in A-A and B-B since in these cases the electron cloud must be undistorted. If the actual bond energy of A-B is greater than this mean value, which is normally the case, it is the result of partial charges which attract each other creating a stronger bond that requires more energy to break. This scale is a comparative one in which other electronegativity values are calculated with respect to a value assigned to a single element arbitrarily. This element is fluorine which is given the value of four. Electronegativity is denoted by a symbol ,X, and is proportional to the covalent radius r, the effective distance from the centre of a nucleus to the outer valence shell of that atom in a covalent bond. The electronegativity of another element is obtained from the difference, Δ, between the actual energy of A-B and the mean values of A-A and B-B using the formula

$$X_A - X_B = \sqrt{\frac{\Delta}{23.06}}$$

1

where X_A and X_B are the electronegativities of the known and unknown atoms and 23.06 is an arbitrary constant (Table 1) (1).

In terms of atomic size, fluorine is slightly smaller than oxygen. It has an r value of 64 picometers (pm) compared to 66 pm (for the σ-bond) for oxygen and a van der Waals radius (r') of 135 pm as compared to a value of 140 pm for oxygen. The latter radius is the internuclear distance of closest approach of one atom to another without bonding (2). The values of r and r' respectively for some other pertinent elements are 37 and 120 pm for H, 77 and 185 pm for C, 99 and 181 pm for Cl, 114 and 195 pm for Br and 133 and 215 for I (3).

The C-F bond-energy is 485 KJ mol^{-1} (116 Kcal mol^{-1}) as calculated from the bond energies of carbon tetrafluoride. In comparison to the given values for other bond energies including C-Cl, 330; C-Br, 275; C-I, 220; C-H, 410; C-C, 350; and C-O, 370 KJ mol^{-1} this is an extremely high value. Indeed this high C-F bond energy may be the result of the high overlap in the hybridized sp^3 orbital of the carbon atom and the p orbital of the fluorine atom.

The lower (C-X) bond energies of the other halogens may be attributable to a decrease in the overlap of carbon-halogen atoms which are reflections of the broad p-orbitals of the X atoms, for example, Cl has an electronic structure of $1s^2\, 2s^2\, 2p^6\, 3s^2\, 3p^5$ (3).

It is the high electron density of fluorine polarizing the C-F bond coupled with its relatively small atomic size and the high C-F bond energy with its lack of active hydrogen, that makes fluorine a unique element among other halogens and gives rise to some of the special characteristics observed in fluorine containing sugars (3).

These characteristic properties are reflected by the fact that the fluoride ion is a hard base (1) with low nucleophilicity. Thus the introduction of a fluorine atom by nucleophilic displacement is generally not easily done on account of the relatively weak nucleophilic character of the fluorine ion (4,5). The poor nucleophilicity of fluorine along with problems such as the

TABLE 1

Electronegativities of Some Atoms on the Pauling Scale

Element	Electronegativity
F	4.0
O	3.5
Cl	3.0
N	3.0
Br	2.8
S	2.5
I	2.5
C	2.5
H	2.1
P	2.1
B	2.0
Si	1.8
Mg .	1.2
Na	0.9
Cs	0.7

solvent dependence of the reactions and the availability of suitably stable blocked sugars made initial attempts to synthesize secondary 2-, 3-, or 4-deoxyfluorosugars unsuccessful. These difficulties were progressively overcome by careful systematic studies of the reaction conditions and by the initial introduction of powerful fluorinating agents such as tetrabutylammonium fluoride (Bu_4NF) by Henbest and Jackson (6). Use of this reagent under extremely anhydrous conditions permitted the synthesis of 3-deoxy-3-fluoro-D-glucose (7) which was subsequently followed by the synthesis of other secondary fluorosugars such as 4-deoxy-4-fluoro-D-glucose especially as the use of O-methyl and O-benzyl groups came to be used for protecting OH centers (7,8). Efficiencies of these synthetic operations were further increased by Middleton's signal discovery (9) of the powerful fluorinating agents diethylaminosulphur trifluoride (Et_2NSF_3) also known as **DAST** and bis(diethylamino)sulphur difluoride [$(Et_2N)_2SF_2$] as easily usable reagents for replacing hydroxyl groups by fluorine directly under mild conditions such that even O-acetates provided adequate protection of the starting sugar. This type of approach was further extended by the introduction of tris(trimethylamino)sulphonium difluorotrimethylsilicate (TSAF) (10) another fluorinating agent for OH compounds. The introduction of improved leaving groups such as $CF_3SO_3^-$ (triflate) led to the synthesis of 1-deoxy-1-fluoro-D-fructose (11) and a range of other secondary fluoromonosaccharides. Reactions such as these occur rapidly and unambiguosly under mild anhydrous conditions. An extensive review of fluorinated compounds, has recently been published (3).

Fluorine containing mono-, di- and oligo-saccharides have been prepared with various intentions but especially to clarify the binding mechanisms between target enzymes and their natural substrates. Sometimes these substrate analogues are observed to be useful inhibitors for their target enzymes.

It had been suggested in early studies (12) that replacement of a hydroxyl group in saccharides by a fluorine atom would cause a minimal disturbance of the conformation to the parent saccharides in terms of atomic size (O and F) and electronegativity (Table 2) (12). Evidence to support this contention was provided by the similar comparative x-ray crystallographic data obtained for 2-deoxy-2-fluoro-($\pm$)-erythritol (I) and erythritol (II) (13).

$$
\begin{array}{cc}
CH_2OH & CH_2OH \\
| & | \\
H-C-F & H-C-OH \\
| & | \\
H-C-OH & H-C-OH \\
| & | \\
CH_2OH & CH_2OH \\
(I) & (II)
\end{array}
$$
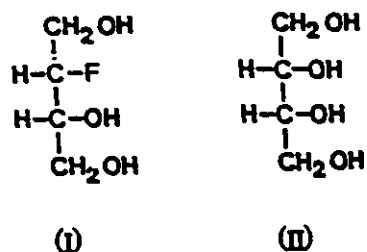
More recently, similar supporting data has been obtained for methyl-α-D-glucopyranoside and methyl 4-deoxy-4-fluoro-α-D-glucopyranosides (14).

This replacement would also allow the removal of the active hydrogen in the targeted OH group of the saccharide (15). Since the hydroxyl group may participate both as a donor and acceptor of hydrogen bonding while the deoxyfluoro substituted analogue is only capable of acting as the acceptor of a hydrogen bond, the replacement of the active hydrogen by fluorine confers subtle differences in the chemical and biochemical reactivities of the parent saccharide (16). This biochemical reactivity may sometimes give rise to either a stimulatory or an inhibitory biological effect which differs dramatically from that of the natural compound. In these cases, fluorinated analogues of this kind can be useful in medicine as specific therapeutic agents. Biochemical reactivity normally involves the specific binding interaction between a substrate molecule and a protein such as an enzyme, membrane transport or receptor protein, or antibody. Hence fluorinated analogues may also be valuable as sensors or probes which can provide information about specific structural and stereochemical features of a molecule required for that interaction

TABLE 2

Comparison of the Size and Electronegativity of Some Elements

Element	Bond length (CH_3)-X) (Å)	Van der Waals radius (Å)	Total (Å)	Electronegativity
F	1.09	1.20	2.29	4.0
H	1.39	1.35	2.74	2.1
O (in OH)	1.43	1.40	2.83	3.5
Cl	1.77	1.80	3.57	3.0
S (in SH)	1.82	1.85	3.67	2.5

Elements with an electronegativity of about 3.0, or greater, form the hydrogen bond. Adapted from Taylor *et al.* (17).

and ultimately information about the stereochemistry of the protein at its binding site (17). In the case of enzymes, the information provided may give insights into reaction mechanisms involved in certain kinds of enzymatic catalysis (18). Additionally, the sensitivity of the fluorine nucleus to nuclear magnetic resonance (NMR) spectroscopy makes it an excellent probe permitting the study of substrate-protein interactions as well as the localization of the specific fluorosubstrate (19,20). In living human patients external detection of radionuclide emissions by β-decay (^{14}C and ^3H) poses problems due to the relatively short range of electron travel. Alternatively, compounds labeled with nuclides of positron emitters (^{11}C, ^{13}N, ^{15}O, or ^{18}F) produce penetrating γ-rays which permit the principle developed by Sokoloff (21-23) to be applied to living humans. However, these must be used selectively as the half-lives ($T_{\frac{1}{2}}$) of some may be too short to be of any significant use (^{11}C: $T_{\frac{1}{2}}$= 20.4 min, ^{13}N: 10.0 min, ^{15}O: 120 s, and ^{18}F: 111 min) (24). Thus deoxyfluoro sugars labeled with fluorine-18 have found important uses as imaging agents in positron emission transaxial tomography (PETT). This technique permits the *in vivo* use of these fluorinated sugars as non-invasive probes of metabolic flux in animals and humans (25,26). For example, 2FG was observed to be a good substrate (K_m= 0.19 $\pm$ 0.03 mM, V_{max}= 0.50) for yeast hexokinase as compared to D-glucose (K_m= 0.17 mM, V_{max}= 1.0). However the resultant 2FG-6-phosphate is a poor substrate for phosphohexose isomerase or D-glucose-6-phosphate dehydrogenase (27-29) and remains trapped in the cells. These and other studies suggested that 2-deoxy-2-[^{18}F]-fluoro-D-glucose (^{18}F-2FG) may be used effectively as a diagnostic tool for the evaluation of initial D-glucose flux in tissues. Hence, 2FG has been used in conjunction with PETT to measure changes in glucose metabolic rates (GMR) in subjects performing complex visuospacial motor tasks (30,31). These studies show a decrease of GMR as the subjects became more efficient in their performance of these tasks. Results of these studies indicate that learning may result in the decreased use of extraneous or inefficient brain areas.

Clinical case studies involving the use of 2FG in conjunction with PETT describe regional cerebral glucose metabolism in newborn infants suffering from seizures or hypotonia (32). Studies of this type are used to develop typical pattern profiles of regional glucose metabolism for infants that have suffered perinatal asphyxia leading to hypoxic-ischemic encephalopathy or cerebral palsy. It is hoped that long term neurologic follow-up of these case studies may be used to determine the prognostic significance of exhibited patterns of cerebral glucose consumption.

2-Deoxy-2-fluoro-hexoses have been used extensively in studies of glycoprotein biosynthesis and function in chick embryo cells (33,34). These inhibitors of protein glycosylation were discovered as a result of their observed antiviral activities. They inhibit the multiplication of a variety of enveloped viruses by interfering with the glycosylation of virus-coded glycoproteins which are constituents of the viral envelope (35).

3FG has been demonstrated to be a specific competetive inhibitor of mammalian glucose transport (16). 3FG has been observed to be transported into tissues, including brain tissue at rates similar to those of glucose (36,37) making 3FG a strong candidate for glucose tracer studies of other carbohydrate metabolic pathways. More recent studies on 3FG metabolism and distribution in isolated cell lines and in rabbit tissues in vivo in conjunction with ^{19}F NMR and gas chromatography-mass spectrometry reveal that production of 3-deoxy-3-fluoro-D-sorbitol (3FS) correlates well with the known distribution of aldose reductase in the systems studied (38). Moreover, the subsequent metabolism of of 3FS to 3-deoxy-3-fluoro-D-fructose (3FF) catalyzed by sorbitol dehydrogenase was verified to occur in brain tissue as had been previously reported (39). These studies have proven that direct oxidation and reduction are the major metabolic routes of 3FG, and not metabolism through glycolysis or the pentose phosphate shunt (38). As a result, 3FG metabolism coupled with ^{19}F NMR may be useful for monitoring aldose reductase and glucose dehydrogenase activities in vivo. Subsequent studies by this group (40) compared

the kinetics of 3FG as a substrate for aldose reductase from extracts of canine eye lens with the production rates of 3FS from 3FG in the intact eye lens as monitored by ^{19}F NMR. Results indicate that there is an excellent correlation between the determined rates of 3FS production in the *in vitro* situation as determined by conventional enzyme assays of aldose reductase activity and the *in situ* situation as monitored by ^{19}F NMR. Observed increases in activity of aldose reductase in the eye lens are used as early clinical symptoms for the diagnosis of the development of mature onset diabetes. Hence 3FG in conjunction with ^{19}F-NMR may have potential use as a non-invasive probe for the early diagnosis of diabetes.

In *Locusta migratoria* (41,42), 3FG became incorporated into trehalose and glycogen in flight muscle and fat body irreversibly inhibiting glycolysis and resulting in the death of the insect. In glucose-grown cells of *P. putida* (43,44), 3FG was oxidized by membrane-bound glucose and gluconate dehydrogenases to 3-deoxy-3-fluoro-D-gluconic acid (3FGA) and 2-keto-3-deoxy-3-fluoro-D-gluconic acid (3F2KGA) with retention of the C-F bond. There was no evidence of phosphorylation; however, both 3FGA and 3F2KGA were competitive inhibitors of the cytosolic glucokinase (45). In contrast, glucose-grown cells of *P. putida* incubated in the presence of 1 mM concentrations of 4FG exhibited a dramatic and extensive release of fluoride ion with no observable respiration. A more detailed consideration of 4FG metabolism in bacteria will be dealt with later.

The Gram-Negative Bacterial Cell Envelope

Bacteria may generally be classified as either gram-positive or gram-negative according to a differential staining technique, the Gram stain, introduced by a Danish bacteriologist, Christian Gram in 1884 (46). This differential staining makes use of the fundamental differences in the composition and structure of gram-positive and gram-negative cell envelopes. Bacteria with the exception of mycoplasmas produce cell walls of which a component common to eubacteria

is the murein or peptidoglycan, which confers mechanical rigidity to the cell. Gram-negative bacteria possess a peptidoglycan layer that is less substantial and not as closely associated with the cytoplasmic membrane as in gram-positive bacteria. The cell wall of gram-positive bacteria contain teichoic acids which are linear polymers of glycerol or ribitol phosphate, covalently linked to a thick peptidoglycan layer. However, the main distinguishing feature common to all gram-negative bacteria is the presence of an additional layer to the cell wall structure that is referred to as the outer membrane (Figure 1).

The Outer Membrane

The outer membrane of gram-negative bacteria provides the ultimate surface contact with the environment. It provides a size-selective permeability barrier that allows relatively hydrophilic solutes, including various nutrients such as sugars amino acids, and ions to diffuse freely via small water-filled, hydrophilic channels or pores formed by a group of outer membrane proteins referred to as 'porins' (47).

In stark contrast to this, the outer membrane provides protection against host defence systems by making the bacteria impermeable to lysozyme, β-lysin, as well as conferring resistance to host immunological defence mechanisms that are activated in response to infection (e.g. phagocytosis by leucocytes and the bactericidal action of serum complement). In addition to these capabilities, the outer membrane also makes the bacteria impermeable to many antibiotics including γ-lactones, penicillins, novobiocins, rifamycins, and others (48). The unique composition and structure of the outer membrane of gram-negative bacteria also allows them to thrive in such harsh environmental conditions as those found in the digestive tracts of animals. Under these conditions, the outer membrane provides resistance to disruptive agents including bile salts, fatty acids, glycerides and digestive enzymes like proteases, lipases, and glycosidases (49).

FIGURE 1

Model of the Cell Envelope of Gram-Negative Bacteria

Legend

The cytoplasm is enclosed by three layers and one space, (1) the outer membrane (OM), (2) the periplasmic space (PS) which divides the outer membrane from (3) the cytoplasmic membrane (CM) which is associated with (4) the peptidoglycan (PG) or murein layer. The lipopolysaccharide (LPS) is usually composed of three moieities, Lipid A (LA) the hydrophobic component, the core (C) attached to lipid A through KDO and the o-antigen (o-Ag) side chain which is the serologically dominant part of the molecule. LPS and Phospholipid (PL) are distributed assymetrically throughout the outer membrane. Three types of proteins are included in the outer membrane and these are: a trimeric porin protein (PP), a transmembrane protein (TP) and the major lipoprotein (LP) which is anchored to the peptidoglycan. The CM is a phospholipid bilayer containing a variety of proteins (CP) which function in energy production, active and fascilitated transport of nutrients and export of toxic metabolites, and in enzymatic synthesis and translocation of cell-envelope components. The PS separating the OM and CM contains proteins which may function in the catabolism of various nutrients, detoxification of antimicrobial agents or in binding (BP) of nutrients for transport within the cell. Adapted from reference 47.

FIGURE 1
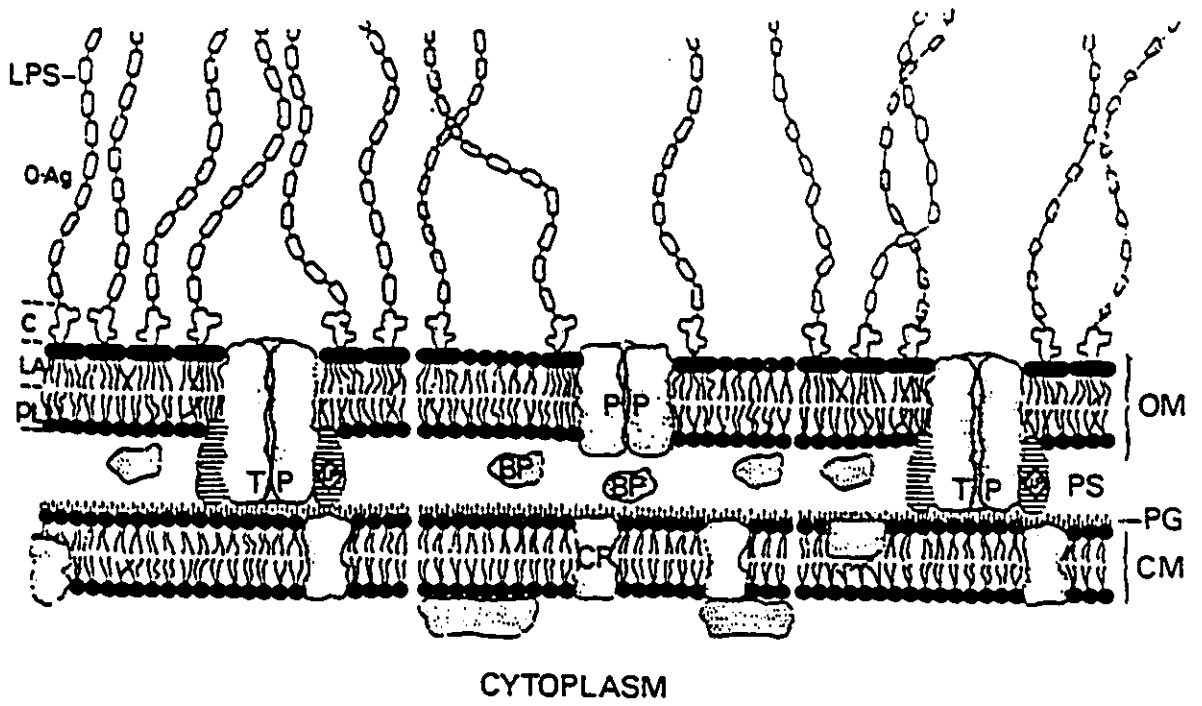
Analytical and structural studies showed that the outer membrane consisted of three components, lipopolysaccharide (LPS), proteins and phospholipid. A characteristic of the outer membrane is the asymmetric arrangement of lipid and lipopolysaccharide. The inner layer of the outer membrane bilayer is composed of phospholipids (48,50). These cover approximately half of the inner surface of the outer membrane in enteric bacteria and are predominantly phosphatidylethanolamine (50,51). The outer surface of enteric bacteria is covered by about 40% lipopolysaccharide (LPS), and 60% protein making it extremely hydrophilic (51). The characteristic properties of the outer membrane can be attributed to the LPS.

In addition to providing the permeability barrier responsible for resistance to phagocytosis, serum complement, as well as different hydrophobic antibiotics, LPS also serves as a receptor for the absorption of some bacteriophages (52). The LPS structure in pseudomonas which is similar to that of the enterobacteriaceae, is composed of three covalently linked regions (53).

1) **Lipid A,** the hydrophobic component of LPS which is imbedded in the phospholipid bilayer of the outer membrane anchoring LPS. The basic structure of lipid A contains a D-glucosaminyl-β-(1-6)-D-glucosamine backbone to which five to seven short (10-16 carbon atoms) hydroxy fatty acid (usually β-hydroxy fatty acids) residues are linked via ester (at the carbon-3 position) or amide (at the carbon-2 position). This differs from the phospholipid structure which has only two fatty acid chains connected to a glyceryl backbone. Also unlike the phospholipids, all of the fatty acid chains found in LPS are saturated, some even having 3-hydroxy fatty acids. In addition some fatty acid residues are linked to the carbon-3 hydroxyl group of another fatty acid resulting in the characteristic 3-acyl-oxy-acyl structure (48). The glucosamine disaccharide is also substituted at carbon-1 and carbon-4 with phosphate groups (Figure 2A) (54).

2) **The core,** is a short oligosaccharide which is attached to lipid A through 3-deoxy-D-

manno-2-octulosonic acid (formerly known as 2-keto-3-deoxy-octulosonic acid or KDO) (Figure 2B).

3) the o-side chain is the serologically dominant part of the molecule. Both the core oligosaccharide and this o-antigen polysaccharide contain sugars such as deoxyhexoses and amino sugars which are not only unique to gram-negative bacteria but whose configuration are both species and strain specific (Figure 2C) (54,56).

As a result of the phosphate and acidic groups on the lipid A and adjacent polysaccharide chains, LPS possesses a substantial net negative charge. It has been suggested that binding of divalent cations such as calcium and magnesium cross-link LPS molecules through divalent cation cross-bridging contributing to the overall stability and integrity of the outer membrane (59,60). The importance of these divalent cation cross-bridges is readily shown by the loss of LPS and concomitant increase in outer membrane permeability upon treatment of cells with ethylenediaminetetraacetic acid (EDTA), a divalent metal-ion chelator (59,61,62).

Outer membranes of gram-negative bacteria that are analyzed for their protein composition by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), exhibit profiles that are less complex than those found in the cytoplasmic membrane. Normally outer membrane proteins of a given species have a distinct pattern of bands corresponding to a limited number of major proteins (63-66). As a result of the aggregation behaviour of these proteins, clear and reproducible patterns of banding on SDS-PAGE of outer membrane proteins necessitate the boiling of samples for at least several minutes in 1% SDS (50,67,68). Covalently bound lipids associated with these proteins are post-translationally added. In some cases the lipid provides a 'hydrophobic anchor' by which the protein becomes attached to the membrane. This is seen in lipoprotein-deficient mutants which show a significant loss of outer

FIGURE 2

Structure of Lipopolysaccharide and the Lipid A Core of

Gram-Negative Bacteria

Legend

A. The detailed structure of Lipid A from *E. coli* K-12 (ref. 48).

B. Portions of various oligosaccharide core structures from *P. aeruginosa*. Covalently bound phosphate groups are designated by p, glucose by Glc, rhamnose by Rha, galactosamine (2-acetamido-2-deoxygalactose) is GalN, L-alanine is L-Ala, heptose is Hep, and KDO is 3-deoxy-D-*manno*-2-octulosonic acid.

C. Various o-antigenic structures of *P. aeruginosa*. The various structures are designated as follows: **DL-FulNac** is 2-acetamido-2,6-dideoxy-(DL)-galactopyranose (N-acetylfucosamine), **D-GalNacAn** is 2-acetamino-2-deoxy-D-galacturonamide, **D-GalNFmA** is 2-formamido-2-deoxy-D-galacturonic acid, **D-QuiNac** is 2-acetamino-2,6-dideoxy-D-glucopyranoside (N-acetylquinovosamine) and **BacNac₂** is 2,4-diacetamino-2,4,6-trideoxyglucose (N,N'-deacetylbacillosamine). References are found in brackets.

FIGURE 2

A

$$R = \overset{O}{\underset{\|}{C}} - (CH_2)_n - CH_3 \text{ or } H$$
$$n = 10,14$$

$$R' = \overset{O}{\underset{\|}{C}} - CH_2 - \overset{H \text{ or } OH}{\underset{\cdot}{CH}} - (CH_2)_{11} - CH_3 \text{ or } H$$

B

```
            Glcp(α1→6)Glcp
                 ⋮
Glcp(β1→2)Rhap(α1→6)Glcp ⋯ GalN(⋯3)Hepp(1→4/5)KDO→Lipid A   (55)
                 ⋮
               L-Ala
```

```
            Rha
             α
             1
             ↓
             6
Glc(α1→6)Glc(β1→3)GalN(α1→?)Hep(1→4/5)KDO(β2→4/5)KDO→Lipid A  (56)
             2
             ↑
           L-Ala
```

C

[→3)L-FucNac(α1→3)D-FucNac(β1→2)D-Glc(β1→]$_2$ (59)

[→4)D-GalNacAn(α1→6)D-GalNFmA(α1→3)D-QuiNac(β1→3)L-Rha(α1→]$_2$ (58)

[→3)Rhap(β1→6)GlcpNAc(α1→3)BapNac2(α→]$_2$ (57)
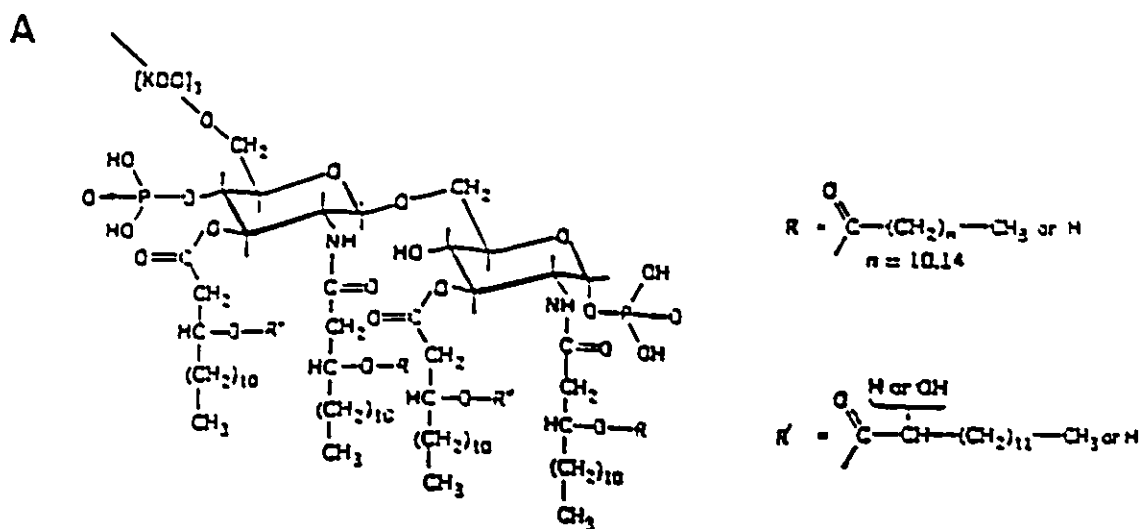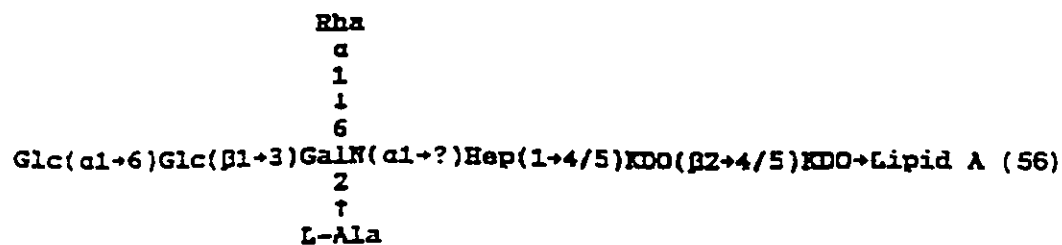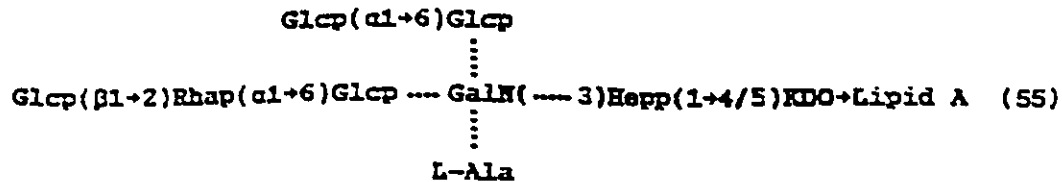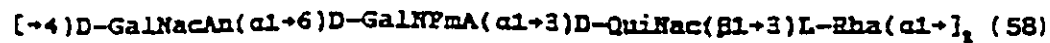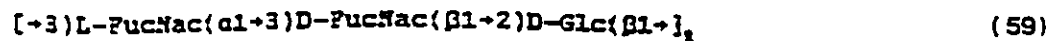
membrane in the form of vesicles. Those mutants display surface vesicles or 'blebs' on electron microscope examination, are hypersensitive to EDTA and detergents and have a tendency to leak periplasmic proteins into their surroundings (48,59,61). In other cases, the roles that the lipid moieties play are less clear and may possibly involve the directing of the protein to the appropriate sub-cellular location or in the case of enveloped viral proteins, facilitating membrane fusion (69).

Perhaps the best characterized example of an outer membrane protein is Braun's lipoprotein (70,71) the major lipoprotein from *Escherichia coli* (Figure 3). It is a small (7.2 kDa) protein that exists in a large number of copies (7×10^5) per cell representing about 6% of total cell protein (48). It occurs in two forms; about a third of the lipoprotein is covalently bound to the peptidoglycan, the remainder existing freely in the outer membrane. The N-terminal portion of the mature form of this lipoprotein consists of glycerylcysteine [S-(propane-2',3'-diol)-3-thioaminopropionic acid] to which two fatty acids are attached by ester linkages and one fatty acid is attached by an amide linkage (see Figure 3). In the peptidoglycan-linked form the C-terminal lysine of the lipoprotein is covalently linked through the ϵ-amino group to the L-carboxyl group of every tenth to twelfth meso-diaminopimelic acid linked to muramic residue of peptidoglycan. The non peptidoglycan-bound lipoprotein contains a free lysine residue at the C-terminal. Unlike most of the other outer membrane proteins, lipoprotein does not serve as a binding site for any bacteriophages; neither can antibodies prepared against isolated lipoprotein, be shown to bind to intact cells. These observations suggest that the majority of the protein molecule is embedded in the cell envelope. Treatment with trypsin leads to detachment of the outer membrane from peptidoglycan suggesting that one function of the peptidoglycan-linked lipoprotein is to anchor the outer membrane to the cell wall (54).

Mizuno and Kageyama (72) have reported the presence of a lipoprotein I (8 kDa) in

Pseudomonas aeruginosa that is analogous to the Braun's lipoprotein in *E. coli*. Lipoprotein I, like its counterpart, has covalently bound fatty acids (72). Some evidence suggests that it may not be covalently linked to the peptidoglycan as Braun's lipoprotein; however, both proteins share an identical sequence at the site of attachment.

Another major outer membrane protein found in *Escherichia coli* is the outer membrane protein A (**Omp A**). The Omp A protein is a 35 kDa transmembrane protein. Omp A is exposed at the outer surface of the cell since it serves as a phage receptor (53,73), and has also been found to be cross-linked to the underlying peptidoglycan layer indicating some sort of structural function (74). Indeed mutants lacking the Omp A protein are observed to produce unstable outer membrane and are defective in conjugation functions (75). Apparently, Omp A is required in the outer membrane of the F cell to allow the stabilization of mating contacts with F plasmid-carrying donor cells. It is thought to exert these stabilizing effects through an association with lipoprotein and via interaction with peptidoglycan by ionic bonds (61). In addition to these major proteins other so-called 'minor' outer membrane proteins exist. Many of these other outer membrane proteins are involved in substrate transport through the permeability barrier of the outer membrane. The entry of these essential substrates and minerals through the outer membrane into the peripiasm of gram-negative cells is accomodated in two ways:

1) Through the passive entry of low molecular weight, hydrophilic solutes (< 600 daltons) present at relatively high concentrations (10^{-3} M). This type of entry is facilitated by a group of special pore-forming proteins called porins, which span the outer membrane (76). 2) Through the specific binding of the solute or a solute-chelator complex to an outer membrane receptor protein. This facilitates entry of essential solutes that are too large to pass through the porins and which are present at very low concentrations (down to 10^{20} M). Examples of the receptor-mediated

18

FIGURE 3

The Complete Chemical Structure of the Bound and Free Forms of

the Braun's Lipoprotein (70)

Legend

The lipoprotein consists of 58 amino acid residues lacking histidine, tryptophan, glycine, proline and phenylalanine. It is linked via the ϵ-amino group of its C-terminal lysine (Lys) to the carboxyl group of every tenth to twelfth *meso*-diaminopimelic acid (*m*-Dap) residue of the peptidoglycan (PG). Its N-terminus consists of a glycerylcysteine [s-(propane-2',3'-diol)-3-thioaminopropionic acid] to which two fatty acids (R and R') are attached by ester linkages and one fatty acid (R") is attached by an amide linkage. Adapted from reference 54.

FIGURE 3

Pseudomonas aeruginosa that is analogous to the Braun's lipoprotein in *E. coli*. Lipoprotein I, like its counterpart, has covalently bound fatty acids (72). Some evidence suggests that it may not be covalently linked to the peptidoglycan as Braun's lipoprotein; however, both proteins share an identical sequence at the site of attachment.

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1) Through the passive entry of low molecular weight, hydrophilic solutes (< 600 daltons) present at relatively high concentrations (10^{-6}M). This type of entry is facilitated by a group of special pore-forming proteins called porins, which span the outer membrane (76). 2) Through the specific binding of the solute or a solute-chelator complex to an outer membrane receptor protein. This facilitates entry of essential solutes that are too large to pass through the porins and which are present at very low concentrations (down to 10^{-20} M). Examples of the receptor-mediated

uptake processes are the BtuB- mediated uptake of vitamin B and the Cir-, Ton A-, Fec-, and Fep A- mediated uptake of ferric ion chelator complexes (47,77).

Porins of gram-negative bacteria fall into two functional classes. **General diffusion porins** are chemically nonspecific acting as molecular sieves that allow the selective permeation of solutes through the outer membrane according to size (48,50,78,79). **Specific porins** differ in that they contain saturable binding for specific classes of solutes, such as LamB and Tsx of *E. coli* and OprP of *P. aeruginosa* (50). Consistent with the pore structure these proteins are transmembrane and are non-covalently associated with peptidoglycan and lipopolysaccharide (80-82). Gram-negative bacterial outer membranes contain up to 10^5 copies of the different porins per cell (83,84).

Over 40 different porins from gram-negative bacteria have been examined under various growth conditions (Table 3). These include porins from *E. coli* (Omp C, Omp F, Pho E, maltoporin, Tsx, protein K, and Nmp C) (61), *Salmonella typhimurium* (Omp F, Omp C, Omp D) (85), *P. aeruginosa* (Opr B, Opr D, Opr E, Opr F, Opr L, Opr I, Opr H, Opr G, Opr P) (69,86-88), *Neisseria gonorrhoeae* (89) and *Brucella* species (90) as well as *Chlamydia trachomatis* (91) to name a few.

In all cases studied porins are found to be approximately two-thirds β-pleated sheet with little α-helical structure and range in size from 30-50 kDa (50,69,92-95). Furthermore, there are indications that the β-strands are antiparallel, oriented perpendicular to the plane of the membrane and have an average length of 10-12 residues, which is sufficient to cross the non-polar region of the bilayer. Models suggest that the β-strands may arrange themselves to form a β-barrel with polar and charged amino residues forming the lining of this water-filled channel (96). Indeed most but not all porins have been shown to arrange themselves in the outer membrane as trimers of three identical subunits (97-100).

TABLE 3

Properties of Porin Proteins of Gram-Negative Bacteria

Porin Protein	Species	Molecular weight (monomer)	Native Oligomeric State	SDS Stable Oligomers	β-Sheet Structure	Peptidoglycan Associated	LPS Associated	Conditions Favouring Production
OprF	P. aeruginosa	39,000	Trimer	Yes/No	Yes	Yes	Yes	
OprP	P. aeruginosa	48,000	Trimer	Yes	Yes	No	Yes	Low Phosphate
OprB	P. aeruginosa	46,000	Trimer	No		No	Yes	Glucose as C-source
OprD	P. aeruginosa	45,500						Certain C-sources
OmpF	E. coli	32,705	Trimer	Yes	Yes	Yes	Yes	Low osmolarity
OmpC	E. coli	36,000	Trimer	Yes	Yes	Yes	Yes	High osmolarity
LamB	E. coli	47,932	Trimer	Yes	Yes	Yes	Yes	Maltose as C-source
PhoE	E. coli	36,782	Trimer	Yes	Yes	Yes	Yes	Low phosphate
OmpF	S. typhimurium	39,300	Trimer	Yes	Yes	Yes	Yes	Low osmolarity
OmpC	S. typhimurium	39,800	Trimer	Yes	Yes	Yes	Yes	High osmolarity
OmpD	S. typhimurium	38,000	Trimer	Yes	Yes	Yes	Yes	
PhoE	S. typhimurium	34,000				Yes		Low phosphate
LamB	S. typhimurium	46,000				Yes		Maltose as C-source
—	N. gonorrhoeae	34,000	Trimer	No		Yes		
—	N. meningitis	37,000		Yes/No				
Por A		45,000						
E	Y. pestis	33,000			Yes			

Porins are tightly associated with lipopolysaccharide and peptidoglycan and can be distinguished from non-peptidoglycan associated proteins which are removed by extraction of cell envelope with 2% SDS at 35°C (101). Others (80) have used non-ionic detergents such as Triton X-100 to extract these non-peptidoglycan associated proteins. A combination of Triton X-100 (2%) and ethylenediaminetetraacetic acid (EDTA, 10mM), has also been used to extract porin proteins (80,81,85). Alternative uses of ionic detergents such as cholic acid (2%) or lithium dodecyl sulfate (LDS, 2%) have also been proven themselves amenable to porin extraction (98); however, these methods preclude the use of ion-exchange chromatography for subsequent purification. Ionic detergent extracts have been purified by gel permeation chromatography in the respective detergent (98). This methodology has subsequently been used along with various modifications to purify gram-negative porins. These methods include extraction with heating in varying temperatures up to 60°C (102,103).

Porins of *Pseudomonadaceae*

There are several known outer membrane proteins of *P. aeruginosa* which have been isolated and characterized (Table 4). *Pseudomonas putida* is a member of the *Pseudomonas fluorescens* branch (group I) of the family Pseudomonadaceae (104,105) that includes *P. aeruginosa*. Recently a glucose-inducible outer membrane protein Opr B (previously protein D1) from *Pseudomonas putida* has been purified and characterized (106).

OprB

In *P. aeruginosa* OprB is induced by growth on D-glucose and also on metabolizable inducers such as D-galactose and 2-deoxy-D-glucose. It is repressed by growth on citrate, succinate, and D-gluconate (63,85). This pattern of induction and repression is also shared by the high affinity glucose transport system (108,109) and the periplasmic glucose-binding protein in *P. aeruginosa* (110,111). OprB is an SDS-labile trimer in the outer membrane (98), and is

heat modifiable on SDS-PAGE from an apparent molecular weight of 35.5 kDa at 37°C to 46 kDa at 65°C or over (68,85). Liposome-swelling studies suggest a preference of the channel for glucose and xylose over other sugars of similar size (88).

More recently the OprB of *P. putida* has been purified (106). This OprB was identified as a 43,000 molecular weight glucose inducible, organic acid repressible protein. Circular dichroism spectra indicated a high content of β-sheet that compared with CD spectra for OprF and OprP of *P. aeruginosa* (93). The amino acid composition of *P. putida* was comparable not only to OprB of *P. aeruginosa* but also to the maltose-inducible LamB of *E. coli*. In preliminary studies the two proteins were immunologically cross-reactive as has also been shown for a wide variety of outer membrane protein in pseudomonads (Table 4).

Previous studies on *P. aeruginosa* indicated OprB was permeable to sugars with sizes as large as disaccharides (86,88) and that there was an apparent preference of the channel for glucose and related monosaccharides (88). In contrast to these results, the studies carried out on *P. putida* OprB demonstrated a low permeability to small ions indicative of a rather narrow channel which was confirmed by subsequent observations that even monosaccharides substantially blocked ion conductance though the channel presumably by occupying the glucose-binding site within the channel.

Single-channel conductance experiments of this OprB and of LamB revealed similar linear relationships between salt concentration and conductance which implied that the channels were water-filled and lacked strong ion-binding sites. The strong selectivity for cations revealed the existence of anionic amino acids surrounding the mouths of the channels for LamB and OprB (106). Significant differences between OprB and LamB were also observed. These included the lower ion conductance of OprB which has been interpreted in several ways (106).

The most profound difference between LamB and OprB indicated by these studies was

TABLE 4

Identification of Related Outer Membrane Protein

Equivalent P. aeruginosa protein	P. putida	P. syringae	P. fluorescens	P. chlororaphis	P. aureofaciens	P. stutzeri	P. anguilliseptica	A. vinelandii
IROMP	B	B	B	B		NI		B
Opr B	B[A]							
OprP	I,B,M	NP	I,B,M	I,B,M	I,B,M	NP		
OprF	I,B,M	I,B,M	B,M	M		M	M	B,M
OprH	B	NP	B			B		
OprL	I,B	I,B	I,B	I,B	I,B	I,B	I,B	I,B
Fbp	I		I			I		

I = immunological cross-reactivity

B = biochemical similarities

M = cross-hybridization with OprP and OprF gene probes

NP = no protein observed but hybridizing DNA was present

NI = no information available

A = reference (106)

in the sugar binding characteristics of each. Macroscopic conductance inhibition experiments performed on OprB revealed that with the exception of glucose, the binding affinities of sugars to OprB were generally lower than they were for LamB. Similar studies using LamB demonstrate that binding affinities increase as the size of the maltodextrin increases from 2 to 5 glucose units and then remains constant for maltodextrins of 6 or 7 glucose units. However, although OprB bound glucose and maltose with affinities similar to LamB (K_s = 110 and 30 mM respectively), it was found to bind maltotriose poorly (K_s = 60 mM) and maltotetraose ($K_s \geq$ 1M) extremely poorly. These results have been interpreted to imply an elongated binding site for maltodextrins which may accommodate 5 glucose units (106). The binding site for OprB is believed to be much shorter, with a binding site corresponding to 2 glucose molecules (approximately 1.2 nm long).

The Periplasmic Space

The region denoted by the outer membrane and the cytoplasmic membrane boundaries is referred to as the periplasmic space or periplasm. It constitutes 20 to 40% of the total cell volume. The periplasm contains a unique series of proteins that are prevented from escaping into the environment by the restrictive permeability of the outer membrane. A wide variety of essential solutes such as organic acids, amino acids, and sugars exist in the aqueous environment outside the cell. These are permitted entry into the cell via the outer membrane porin proteins and sequestered in the cell by the action of a number of soluble substrate-specific periplasmic proteins. Generally the transport mechanisms of these proteins necessitate their close association with other protein components on the outer leaflet of the cytoplasmic membrane. There are also periplasmic enzymes which are involved in the degradation of metabolizable compounds that are too large or too highly charged to pass through the cytoplasmic membrane (e.g., alkaline phosphatase). These can be described as scavenger hydrolytic periplasmic enzymes. Another

group of periplasmic enzymes catalyzes the destruction of some antibacterial agents able to penetrate the outer membrane. This β-lactamase enzyme is a good example of these types of enzymes. It degrades penicillins and cephalosporins and is common in both gram-positive and gram-negative bacteria. Containment of these 'detoxifing enzymes' within the periplasm ensures that inactivating enzyme concentration is greatest where it is needed the most, to prevent entry of toxic solutes into the cell. This type of compartmentalization confers an advantage to gram-negative bacteria since, unlike gram-positive bacteria, they do not need to produce large quantities of drug inactivating enzymes that are constantly being lost to the environment (54). Periplasmic contents are released into the medium by mild osmotic shock (112). Bacterial cells are plasmolized in a hypertonic sucrose/EDTA solution followed by a rapid dilution in cold distilled water. The bacteria remain viable, though unable to perform any function that is dependent upon the lost periplasmic protein (48). Transport systems may be categorized according to a variety of principles (113) but only those systems classified as osmotic shock-sensitive or osmotic shock-resistant permeases will be dealt with here. A recent review by Ames (114) warns that the term 'periplasmic protein' should be taken as more of an operational definition rather than as an indicator of a physical location. The review notes that proteins such as EF-Tu (elongation factor Tu), which functions well within the confines of the cell is released by a treatment similar to osmotic shock while a number of phosphatases which are typically periplasmic enzymes are poorly released by osmotic shock (115). Shock-sensitive or periplasmic permeases are inactivated during osmotic shock due to the loss of an essential protein component (referred to as the periplasmic component) that binds the transported solute with high affinity. The first of these periplasmic transport proteins shown to be released by osmotic shock was the sulfate-binding protein component of a sulfate permease in *Salmonella typhimurium* (116). Shock-resistant permeases are those that retain all of their activity [(and therefore their component(s)] upon

osmotic shock. A classic representative of this class is the β-galactoside permease which has been well studied (117,118).

The nature of the mechanism of energy coupling has been used as an additional characteristic to distinguish between these two classes of permeases. Shock-resistant permeases are powered by the proton-motive force (PMF) (117,118), while energy coupling in shock-sensitive permeases has been postulated to depend on substrate level phosphorylation (119,120). Berger and Heppel (120) presented evidence that the energy donors for the two classes of transport systems in *E.coli* were fundamentally different. Arsenate, which was previously shown to cause a drastic depletion of intracellular ATP levels (121) via deprivation of glycolytic sources of high energy phosphate, abolished the activity of shock sensitive permeases. The activities of shock-resistant permeases on the other hand, were not affected by arsenate treatment but were strongly inhibited by the uncoupler 2,4-dinitrophenol (2,4-DNP). Shock-sensitive permeases are far less affected by 2,4-DNP (120).

The Peptidoglycan Layer

The structure which allows the bacterial cell to contain its cytoplasm against the osmotic pressures pushing against the cell envelope is the cell wall. This cell wall owes its great strength to the peptidoglycan which comprises the bulk of its mass. Peptidoglycan defines a linear network of polysaccharide chains or glycan strands of alternating units of N-acetylglucosamine β-(1-4) linked to N-acetylmuramic acid (Figure 4) (47,55). These glycan strands may be up to 200 units in length and make up to 10 to 20% of the gram-negative cell wall (54). Glycan strands are cross-linked to each other by a tetrapeptide covalently bound to the carboxyl group of a muramic acid residue. In *E. coli* the tetrapeptide has the sequence L-alanine, D-glutamate, meso-diaminopimelic acid, and D-alanine (46,54). Like N-acetylmuramic acid, diaminopimelic acid is unique to the bacterial cell wall. In this way, the cross-linked peptidoglycan strands form

FIGURE 4

The Structure of Murein or Peptidoglycan Layer from Gram-Negative Bacteria

Legend

Schematic of a basic unit containing two amino sugars N-Acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) and a tetrapeptide containing the peptidoglycan-specific amino acid *meso*-diaminopimelic acid (*m*-Dap). Also shown is the formation of the peptide cross-links between adjacent glycan strands. The linkage is formed between the ε-amino group of diaminopimelic acid on CHAIN 1 and the carboxyl group of the terminal D-alanine on CHAIN 2. Adapted from reference 54.

FIGURE 4

CHAIN 1   CHAIN 2
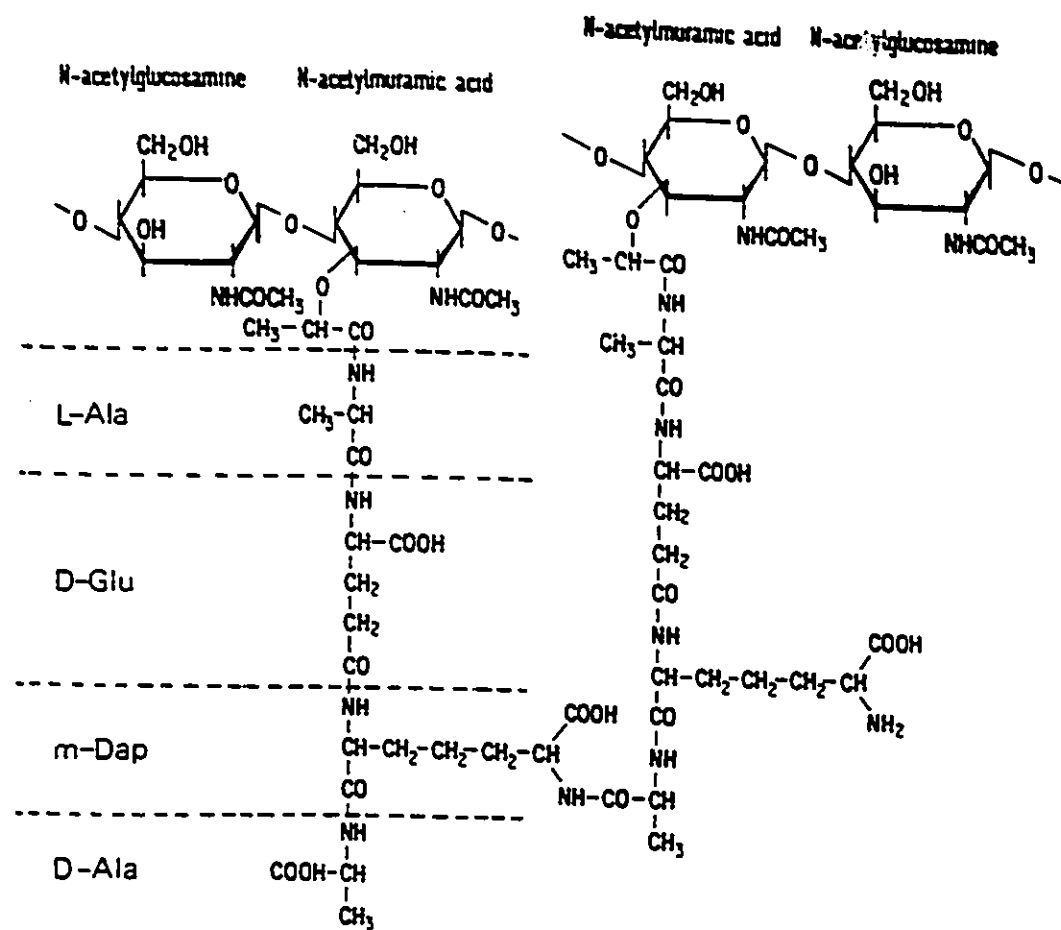
a large macromolecular structure; the sacculus, which serves as the delimiting structure of the bacterium (54,92). The peptidoglycan is covalently linked to the outer membrane lipoprotein (73). Additionally, several other outer membrane proteins, particularly porins, are non-covalently associated with the cell wall (54,80,122). It is due to this close association between the outer membrane and peptidoglycan that has led to the outer membrane being considered a part of the cell wall complex.

The Cytoplasmic Membrane

The cytoplasmic inner membrane is the other main permeability barrier of the bacterium. In conjunction with the peptidoglycan (murein), it provides an osmotic barrier that retains metabolites and excludes external compounds. It is composed of protein (60-70%), lipid (20-30%, mainly phosphatides) and carbohydrates (46). The cytoplasmic membrane serves as the site of important and divergent biological functions. It contains the enzyme components of the electron transport chain (cytochromes, ubiquinones, carotenoids, respiratory enzymes), oxidative phosphorylation as well as the ATP synthase and ATPase systems. It provides a foundation for the protein "machinery" that allows for the various transport systems which include mineral, ion, sugar and amino acid transport. Although each kind of transport system is specific for a given compound or class of compounds, many have the added capacity for active transport. Additionally, there are transport systems for the necessary excretion of waste products. The cytoplasmic membrane also houses the synthetic apparatus necessary for the production and transport of the larger building blocks that are incorporated into exterior layers (e.g., lipid biosynthesis, peptydoglycan biosynthesis, phospholipid biosynthesis).

In contrast to the situation in outer membrane proteins it is very difficult to identify individual inner membrane protein by SDS-PAGE of crude extractions of whole cell envelope proteins. This is apparently due to the fact that inner membrane proteins are more numerous and

have rather unique forms of distribution in terms of both molecular weights and degree of abundance in each species.

The Aerobic Bacterial Respiratory Chain

The major function of the respiratory chain of aerobically-grown bacteria is the electrogenic translocation of protons out of the cytoplasm. This results in the generation of a proton motive force (PMF) which may then be utilized to drive ATP-synthesis, solute uptake, and other energy-requiring membrane-associated processes such as protein secretion and cell motility (123). The cytoplasmic membrane is the location of the respiratory system as well as the site for these energy transducing and transforming reactions.

In most aerobic and facultatively anaerobic organisms, under aerobic conditions, the bulk of the energy required for anabolic and cellular ion homeostasis is provided by the oxidation-reduction reactions of respiratory substrates through the aerobic electron transport chain. Bacteria have adaptable collections of electron-transporting components that include iron-sulfur clusters, quinones and cytochrome complexes with different heme prosthetic groups, such as aa_3, b, c, d, and o (see Figure 5). These cytochromes make up different terminal oxidases in species specific respiratory chains.

Energy Coupling Hypothesis

In the more than 50 years that electron transport and oxidative phosphorylation have been studied, numerous mechanisms have been proposed to explain how these processes are coupled. These include: (1) The chemical coupling hypothesis formulated in 1953 by Edward Slater (126) that proposed the production of reactive intermediates by electron transport. The subsequent breakdown of these high energy intermediates drives oxidative phosphorylation. (2) The conformational coupling hypothesis formulated by Boyer in 1964 (127) which proposes that electron transport causes protein to adopt 'activated' or 'energized' conformational states. These

FIGURE 5

Several Types of Cytochromes found in Electron-Transport Chains

Legend

Four types of cytochromes are depicted here. They are given the nomenclature a,b,c, and d. A cytochrome is classified by the nature of the side chains on its heme group. The heme group found in cytochrome b (c) in the type present in hemoglobin and myoglobin. The heme group of cytochrome a (a) differs from that of cytochrome b by the replacement of the C-2 vinyl group ($CH=CH_2$) with a polyunsaturated alkene and by the replacement of the C-8 methyl group with a formyl group (CHO). Conversion of both vinyl groups of the heme of cytochrome b to the thiomethyl groups produces the heme group of cytochrome c (d). Lastly, the structure of cytochrome d (b) appears to be derived from cytochrome b by a reductive dihydroxylation in pyrrole ring C involving C- 5 and C-6. Adapted from references 123 and 132.

FIGURE 5
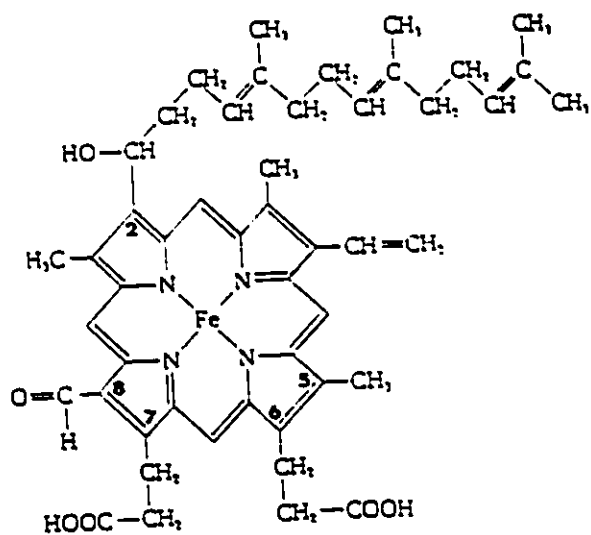
a
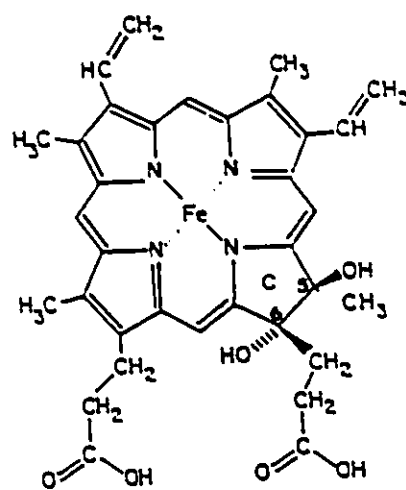
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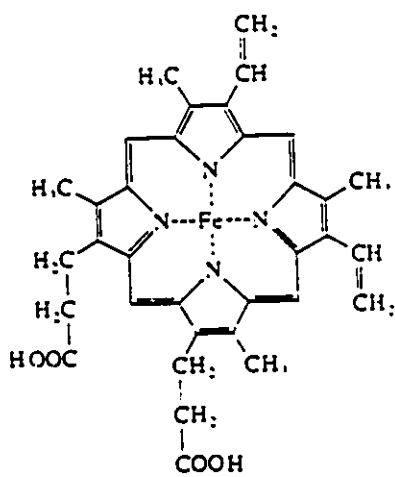
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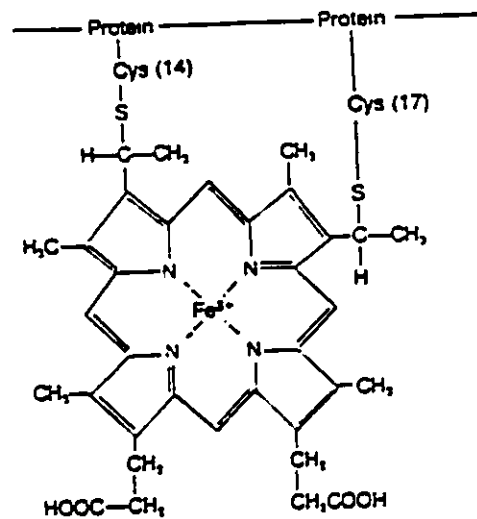
d

proteins are closely associated with ATP synthase such that their relaxation back to a deactivated

conformation drives ATP synthesis. As with the chemical coupling hypothesis the conformational

coupling hypothesis has found little experimental support. (3). The chemiosmotic hypothesis of

oxidative phosphorylation was put forth in 1961 by Peter Mitchell (128). It proposed that an

electrochemical gradient of H^+ ions across the mitochondrial membrane serves as the means of

coupling the energy flow from electron transport to the formation of ATP (Figure 6a). An

essential feature of the chemiosmotic-coupling hypothesis is that it postulates the existence of

vectorial chemical reactions between two osmotic compartments (129). That implies reactions

having a geometric direction in contrast to nondirectional or scalar chemical reactions occurring

in free homogenous solutions. The essence of this proposal, as applied to the oxidative

phosphorylation in mitochondria, is that the electron transport chain is topologically organized

in the inner mitochondrial membrane so that protons are translocated from the inside to the

outside as electrons are passed from substrate to O_2. The electron-transport chain is thus viewed

as a device for converting the energy released by electron transport into the energy of an

electrochemical gradient of H^+ ions. The free energy stored in such a gradient is a function of

the relative concentration of H^+ ions across the membrane; it has been called the proton motive

force (PMF). This energy-rich gradient of H^+ ions is used in the formation of ATP from ADP

and inorganic phosphate (Pi) by a vectorial reaction involving the (F_1 F_o) ATPase complex of the

inner membrane. The original proposal indicated that the transport of $2H^+$ from the external

medium through the ATPase complex into the internal medium driven by this electrochemical

gradient provided the energy for the formation of 1 molecule of ATP from ADP and Pi. More

recent estimations of this H^+/site ratio (number of H^+ ejected per pair of electrons per energy

coupling site) indicate that values closer to 4.0 are common (130). Several key observations are

explained by the chemiosmotic hypothesis. These are: (a) oxidative phosphorylation requires

FIGURE 6

A Generalized Schematic for the Coupling of Solute Transport,

ATP Synthesis and Hydrolysis to a Proton Gradient

Legend

a.   Membrane orientation and direction of proton movement in bacteria. The initial

reduction of a substrate, SH_2, by a specific membrane-bound dehydrogenase results in the transfer

of the equivalent of two hydrogens ($2e^-$ + $2H^+$) through the electron-transport chain to the

terminal electron acceptor O_2 which is subsequently reduced to H_2O. During electron transport,

protons are pumped from the cytoplasmic membrane (CM) inner face externally, creating a

proton gradient (inner $[H^+]$ < < outer $[H^+]$) as well as a membrane potential (negatively charged

inner cytoplasmic face) across the membrane. This proton gradient may now be used for

secondary active transport of essential solutes ($S_o \rightarrow S_i$) by using the influx of protons ($H_o \rightarrow H_i$) to

pull the solute against its concentration gradient. Proton flow in the direction of the

electrochemical gradient through the F_1F_o complexes is also capable of generating ATP from

ADP + Pi (dashed arrows). In contrast, hydrolysis of ATP carried out by the bacterial ATPase

may be used to pump protons from the inner cytoplasmic face to the external environment (solid

arrows) thus generating a proton gradient and membrane potential which may be used by the

bacteria.

b.   The ATP synthase of bacteria, like that of mitochondria and chloroplasts, is a bipartite

enzyme composed of 2 oligomeric complexes F_oF_1. F_o is an integral cytoplasmic membrane

(CM) protein; in bacteria, it consists of three subunits (a, b, and c) which form a proton

conducting channel. F_1 contains three copies each of subunits α and β and is bound to F_o via

subunits γ, δ and ϵ. Synthesis of one ATP molecule from ADP and Pi occurs at the catalytic site

on a β subunits of F_1, due to the tight binding of ATP at this site. Adapted from (124,125).

FIGURE 6
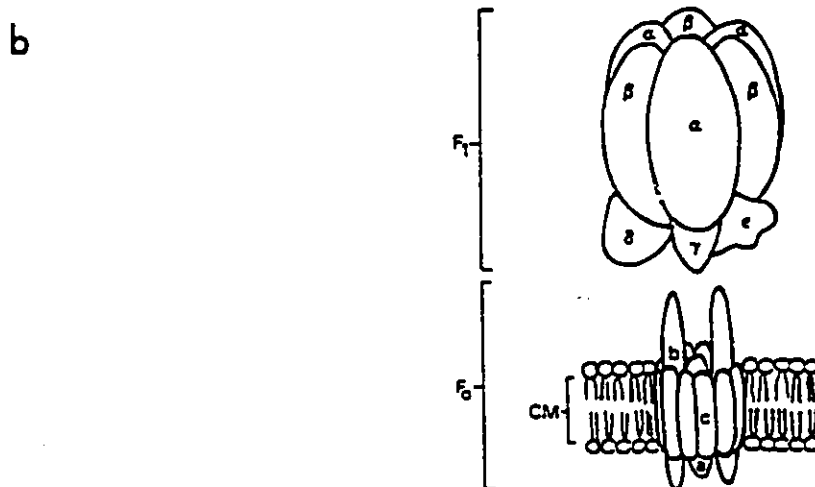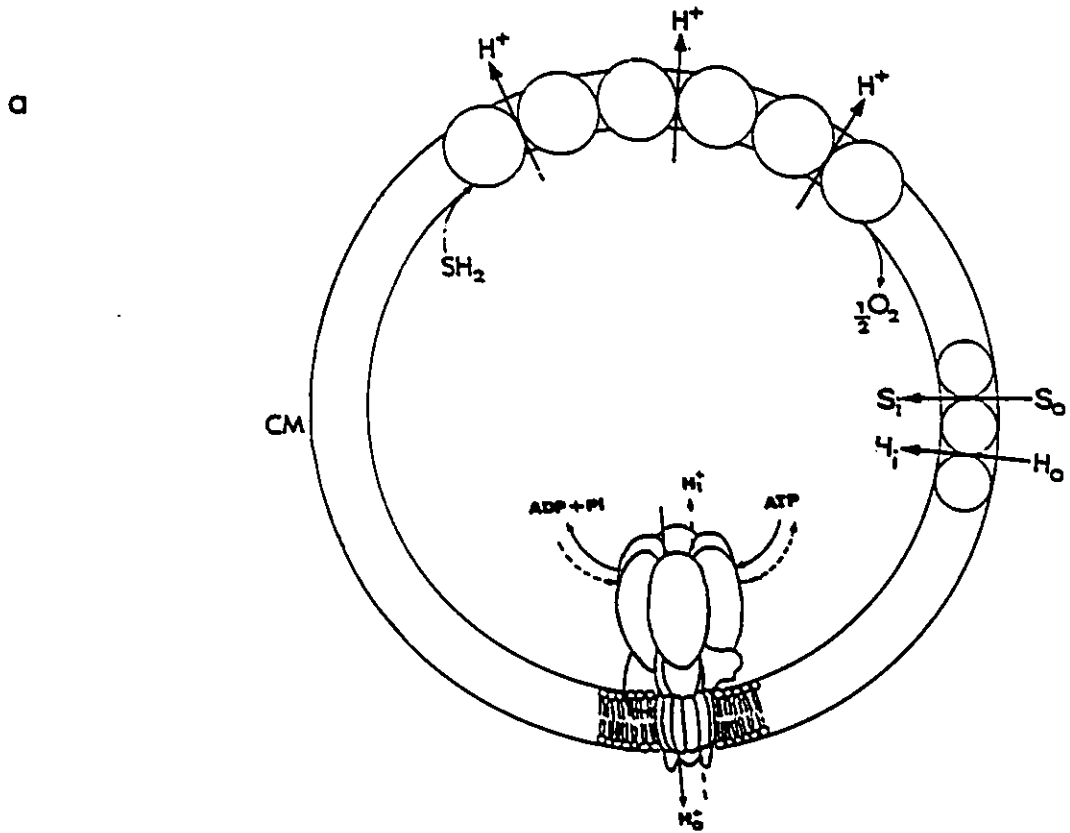
an intact inner mitochondrial membrane; (b) the inner mitochondrial membrane is impermeable to ions such as H^+, OH^-, K^+, and Cl^-. Consequently, the electrogenic pumping of H^+ across the membrane generates an electrochemical potential for H^+ composed of a membrane potential and a chemical concentration gradient of H^+, $\Delta\mu_H^+ = \Delta\Psi - Z \Delta pH$ (where $Z = 2.303RT/F$); (c) electron transport results in the transport out of intact mitochondria producing a measurable gradient across the inner mitochondrial membrane; (d) ionophores such as Nigericin, which exchanges K^+ for H^+ across the membrane, cause the collapse of the electrochemical gradient (ΔpH) without affecting electron transport which would inhibit ATP synthesis; that is, they 'uncouple' electron transport from oxidative phosphorylation. Conversely, increasing the acidity outside the inner mitochondrial membrane stimulates ATP synthesis.

F_1F_o ATPase

Bacteria lack mitochondria; yet aerobic bacteria are capable of carrying out the same processes of oxidative phosphorylation that occur in mitochondria. Enzymes catalyzing the reactions of both the Entner-Doudoroff pathway and the Citric acid cycle are localized in the bacterial cytosol. Enzymes that oxidize NADH to NAD^+ and transfer the electrons to the ultimate acceptor O_2 are localized in the bacterial cytoplasmic membrane. The movement of electrons through those membrane carriers is coupled to the pumping of protons out of the cell (Figure 6). The function of the F_1F_o ATPase (also known as ATP synthase and H^+-ATPase) is to utilize this existing electrochemical gradient and synthesize ATP from ADP and inorganic phosphate (Pi). These enzymes can also hydrolyze ATP and form an electrochemical gradient of protons (131) that can be used for substrate transport and ion co-transport (132).

The membrane proteins of these ATP synthase systems are essentially identical in structure and function as those of the mitochondrial and chloroplast F_1F_o ATPase complexes (133). These systems have been known to be known to be made up of as few as 8 subunits (*E.*

coli) or as many as 13 subunits (bovine heart mitochondria). The enzymes consist of two main components:

(1)   A hydrophilic globular F_1 portion that contains the nucleotide-binding sites. F_1 can be detached from membranes quite easily as a soluble ATPase in aqueous buffers lacking detergent (131).

(2)   A hydrophobic membrane-bound F_o portion functioning as an H^+-conducting 'channel' (133). The F_o of the bacterial synthase is directly linked to F_1 rather than through a connecting stalk as is the situation in the mitochondrial system. This mitochondrial stalk is composed of two subunits:

   a)   The oligomycin-sensitivity conferring protein (OSCP, M_r = 23 kDa), which binds oligomycin thereby prevents phosphoryl group transfer by F_1 when it is membrane-bound (113).

   b)   The F_o subunit (M_r = 8 kDa) (2,113).

(3)   The F_1 inhibitor subunit (M_r = 10 kDa) (2).

These F_1 and F_o portions can be dissociated from each other quite readily, and functionally reconstituted after purification (69). F_1 is comprised of 5 distinct types of subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ (Figure 6b). Of these, the β-subunits have demonstrated the most conserved primary structures with approximately 42% of amino acid residues being identical in the 20 β-subunits sequenced (131). The β-subunits have been observed to function as the three catalytic sites per F_1 (134), and the rapid turnover requires that more than one of these sites be bound to ATP at a given time (135,136). It has become clear that F_1 binds to and blocks the proton channel of F_o and that this binding involves the δ, and, ϵ proteins of F_1 (137-139). When ADP and Pi are added, a gating mechanism in F_1 permits H^+ flux through the channel concomitant with ATP synthesis.

Conversely when ATP is hydrolyzed the gate is opened which fascilitates H^+ flux in the opposite direction (129). Reconstitution studies carried out involving the proton channel-forming protein F_o and purified γ- and ϵ-subunits indicate that the γ-subunit serves as the gate in the ATPase complex (2,130,141).

The F_o portion has the simplest composition, consisting of three subunits [M_r= 24,000, 19,000 and 8,400 for *E. coli* as determined by SDS-gel electrophoretic analysis (131)]. The stoichiometric composition of these three subunits is $a_1b_2c_{10}$ (Figure 6b). All three subunits are essential components of the H^+-conducting channel (69,131). It is believed that F_o is made up of α-helices from the multiple copies of the c-subunit. This subunit is proposed to contain 5-7 transmembrane α-helices while the a and b subunits probably contain one each (131).

The Mechanism of ATP Formation

Boyer *et al.* (141) proposed a binding change mechanism for proton driven ATP synthesis. This mechanism has since been modified to its present form (142-144). It was reasoned that since the three chains interact with just one of the γ, δ, and ϵ chains at any particular moment they then exist in three different and distinct environments. Consequently, this gives rise to three distinct nuclotide-binding sites:

(a)   the O- (open) form that exhibits a low binding affinity for substrates;

(b)   the L- (loose) form that binds substrates but remains catabolically inactive;

(c)   the T- (tight) form which binds substrates tightly and is catabolically active.

In other words, although the three catalytic β-subunits are intrinsically identical they are not functionally equivalent at any given time.

Consider a situation where ATP is bound to the T-site and ADP and Pi are bound to the L-site (Figure 7). An energy input produced by the proton flux causes a conversion of the T-site to the O-form , the L-site adopts the T-conformation while the O-site becomes the new L-site.

FIGURE 7

Schematic of Binding Change Mechanism for ATP Synthesis by F_1F_o ATPase

<u>Legend</u>

The energy-dependent binding change mechanism for ATP synthesis by the proton-translocating F_1F_o ATP synthase. F_1 has three chemically identical but conformationally distinct interacting subunits: β_O, the open conformation; β_L, the loose conformation; and β_T, the tight binding conformation. These three β-subunits alternate between the three conformational states which differ in their binding affinities for ATP, ADP and Pi. In step 1, ADP and Pi bind to the β_L subunits. Step 2 demonstrates the energy-dependent conformational changes in all β-subunits converting binding sites L to T, T to O, and O to L. Step 3 illustrates the synthesis of ATP from ADP and Pi at the new β_T and release of ATP from β_O. Step 4 merely demonstrates that the F_1F_o ATPase complex is now ready to begin the cycle anew. Adapted from references 134 and 144.

FIGURE 7
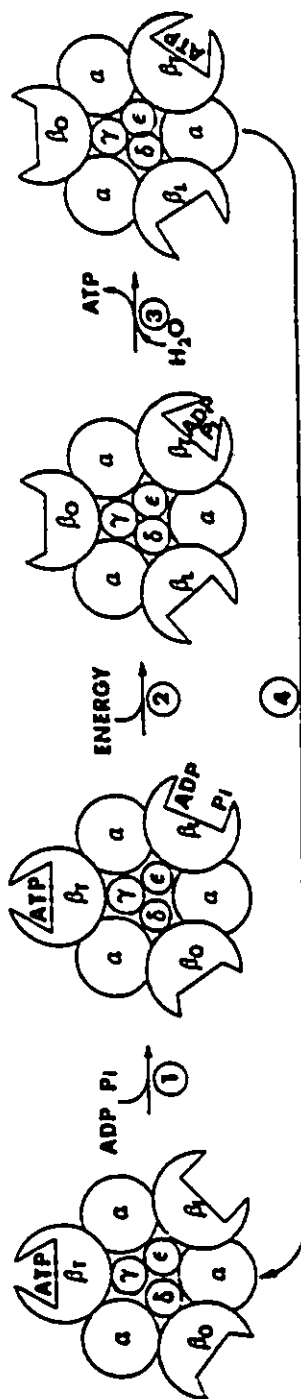
These conversions permit the release of ATP from the new O-site (formerly the T-site), and enable another molecule of ATP to be formed from ADP and Pi in the new T-site (formerly the L-site). Concomitant with the interconversion of the O-, L-, and T-sites is proton flow from the F_o to the F_1 side of the membrane.

Glucose Transport and Utilization in Pseudomonads

General carbohydrate transport and metabolism in bacteria has been extensively reviewed (145,146). Several good reviews have also been written describing the various pathways of carbohydrate catabolism in pseudomonads (147,148). Glucose transport and metabolism in the Group I fluorescent pseudomonads which includes, *P. aeruginosa, P. fluorescens and P. putida,* is distinct from that of enteric bacteria. In these members of the genus Pseudomonas, the Entner-Doudoroff pathway has been shown to play a pivotal role in the metabolism of glucose (149). This pathway has also been demonstrated to function essentially in the degradation of gluconate by enteric bacteria (150).

Pseudomonads are devoid of 6-phosphofructokinase, the enzyme catalyzing the phosphorylation of fructose-1-phosphate at carbon-6 to produce fructose-1,6-diphosphate. As a result they are unable to ferment glucose via the Embden-Meyerhof (glycolytic) pathway (151). The two important enzymes in the Entner-Doudoroff pathway are 6-phosphogluconate dehydratase (**Edd**) and 2-keto-3-deoxy-6-phosphogluconate (**KDPG**) aldolase (**Eda**) which have been characterized extensively (152-154).

In the pathways of glucose dissimilation of pseudomonads (Figure 8) glucose is converted to glyceraldehyde-3-phosphate and pyruvate by the Entner-Doudoroff pathway. The key intermediate in this process is 6-phosphogluconate (6PGA) since it represents a focal point at which the two distinct pathways converge: the direct oxidative pathway which occurs extracellularly and the intracellular phosphorylative route.

FIGURE 8

Pathways Related to Glucose Utilization in Pseudomonads

Legend

Enzymes Related to Glucose Utilization in Pseudomonads. All reactions, except for those indicated by dashed lines have been demonstrated in *P. aeruginosa* . Sugars and intermediates are of the D-configuration. The abbreviation used are: **Gcd** and **Gad**, membrane associated glucose and gluconate dehydrogenases; **Gct, Gat,** and **Kgt** represent transport systems for glucose, gluconate, and 2-ketogluconate respectively; **Gck, Gak,** and **Kgk** represent ATP-dependent kinases for glucose, gluconate, and 2-ketogluconate; **Kgr,** 2-keto-6-phosphogluconate reductase; **Zwf** and **Gnd,** glucose-6-phosphate (G6P) and 6-phosphogluconate (6PGA) dehydrogenases; **Edd** and **Eda** are 6PGA dehydratase and 3-deoxy-2-keto-6-phosphogluconate (KDPG) aldolase; **Tpi,** triose phosphate isomerase; **Fda,** fructose 1,6-diphosphate (FDP) aldolase; F6P, fructose-6-phosphate; and **Pgi,** phosphoglucoisomerase; **Pyc** refers to pyruvate carboxylase. TCA cycle and Pentose phosphate refer to tricarboxylic acid cycle and Pentose Phosphate Pathway. Adapted from (147).

FIGURE 8
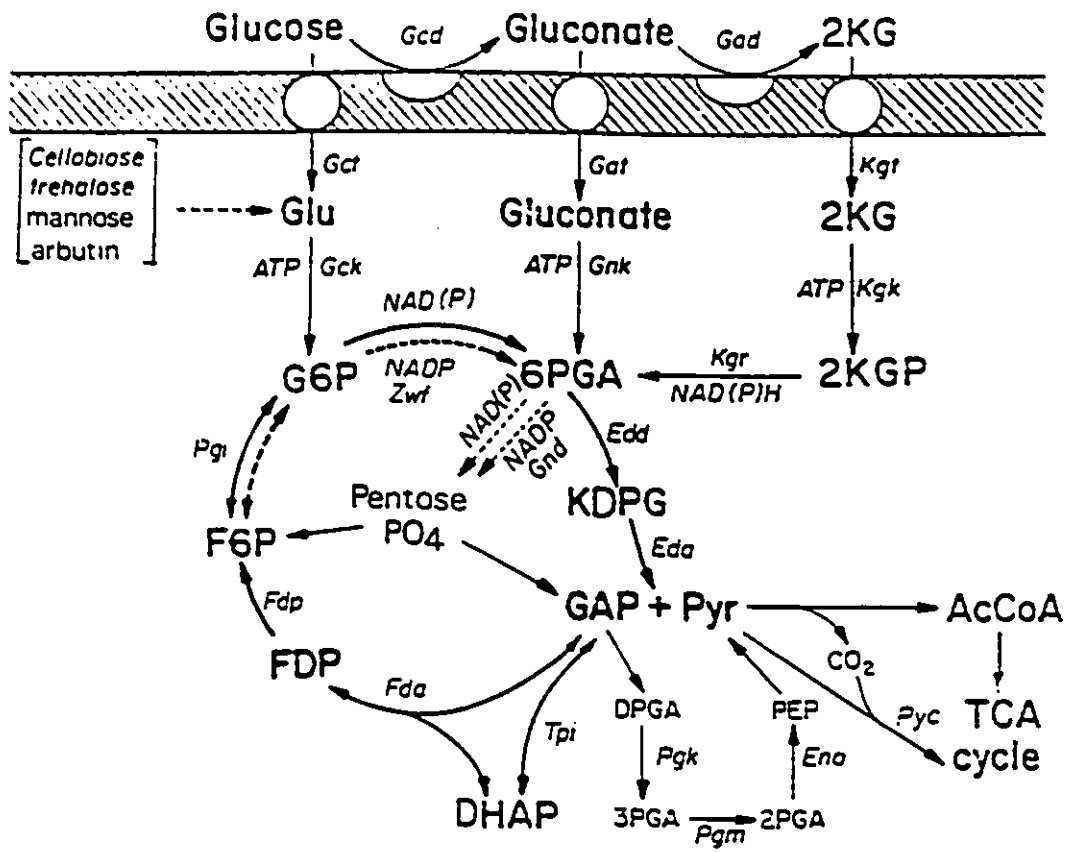
The phosphotransferase system is not utilized for glucose uptake by pseudomonads. Growth under limiting oxygen (155,156), low temperature (157) or limiting glucose concentrations (158) favor glucose uptake with high affinity (K_m= 8 μM) via the phosphorylative pathway. A low affinity transport for gluconate, (K_m= 1 mM) is produced by the action of glucose dehydrogenase (159,160).

Glucose uptake and metabolism is regulated by catabolite and metabolite repression. Growth of pseudomonads on citric acid cycle intermediates such as citrate, succinate or pyruvate, severely repress enzymes of glucose metabolism (161) as well as components of the glucose transport system (159,160,162). Growth of whole cells on gluconate as the sole carbon source supplement or in the presence of gluconate produced by the action of glucose dehydrogenase, repress glucose transport in *P. aeruginosa* and *P. putida* (163,164), the glucose-binding protein in *P. aeruginosa* (111) and outer membrane porin protein OprB (85,106).

The direct oxidative pathway is initiated in the periplasm by the action of membrane-bound, pyridine nucleotide-independent, glucose (Gcd) and gluconate dehydrogenases (Gad) which successively oxidize D-glucose to D-gluconate and 2-keto-D-gluconate respectively (147). Following transport of D-gluconate and 2-keto-D-gluconate across the cytoplasmic membrane via independent active transport carrier systems (162,164), these oxidation products are phosphorylated by their corresponding ATP-dependent kinases (Gnk and Kgk) producing 6-phosphogluconate and 2-keto-6-phosphogluconate respectively. Subsequently, 2-keto-6-phosphogluconate is converted to 6-phosphogluconate (6PGA) by a pyridine nucleotide-dependent reductase (Kgr) (147). The phosphorylative pathway begins when D-glucose is actively transported into the cell where it is phosphorylated in an ATP-dependent reaction to glucose-6-phosphate (G6P). The glucose-6-phosphate then undergoes a pyridine nucleotide-dependent oxidation reaction effected by glucose-6-phosphate dehydrogenase (Zwf) to produce 6-

phosphogluconate (147).

6-phosphogluconate becomes the initial substrate in the Entner-Doudoroff pathway. It is dehydrated to 3-deoxy-2-keto-6-phosphogluconate (KDPG) in a reaction carried out by the enzyme 6-phophogluconate dehydratase (Edd). The dehydratase enzyme Edd from *P. putida* has been observed to promote the irreversible dehydration of 6-phosphogluconate by a mechanism invoving the formation and subsequent rearrangement of the enol to the keto form of 3-deoxy-2-keto-6-phosphogluconate (153). The enzyme is activated by reduced glutathione as well as divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}) and completely inactivated by sodium fluoride, EDTA and *p*-chloromercuribenzoate (152).

Following its formation, 3-deoxy-2-keto-6-phosphogluconate is cleaved to D-glyceraldehyde-3-phosphate and pyruvate by the action of 3-deoxy-2-keto-6-phosphogluconate aldolase (Eda). Eda exibits no requirement for metal ions or cofactors. It is not activated by reduced glutathione and is not affected by *p*-chloromercurybenzoate, EDTA or arsenate (152,165).

The resultant D-glyceraldehyde-3-phosphate may now be further metabolized by undergoing reactions similiar to those it is subject to in the later stages of glycolysis where it yields a second molecule of pyruvate (147). Alternatively, D-glyceraldehyde-3-phosphate may be recycled to 6-phosphogluconate via fructose 1,6 diphosphate (FDP), fructose-6-phosphate (F6P) and glucose-6-phosphate (G6P). Pyruvate is metabolized to acetylCoA by the pyruvate dehydrogenase complex, or to oxaloacetate through the action of an ATP-dependent carboxylation reaction catalyzed by pyruvate carboxylase (147,148). Both these products are then further metabolized through the tricarboxylic acid (TCA) cycle (148). These represent the major pathways for glucose utilization.

The Metabolism of 4-Deoxy-4-fluoro-D-glucose in *Pseudomonas putida*

Interest in the metabolism of 4-deoxy-4-fluoro-D-glucose (4FG) stemmed from observations that glucose-grown whole cell suspensions of *P. putida* incubated with 4FG for 24 hours effected a 95% release of fluoride ion (166). Furthermore, *P. putida* could not utilize 4FG as a sole carbon source and the presence of an equivalent amount of 4FG had a negligible effect on growth yield when the organism was grown on glucose.

Subsequent studies (167) demonstrated that although glucose-grown whole cell suspensions of *P. putida* did not oxidize 4FG to any significant extent, crude cell-free extracts of the same organism were capable of oxidizing 4FG to the extent of two atoms of oxygen per molecule of 4FG in the absence of fluoride release.

Supernatant and intracellular components of the whole-cell incubations with 4FG were analyzed by thin layer chromatography (TLC). The presence of non-fluorinated metabolites could not be detected and it was proposed that a covalent incorporation of the carbon skeleton of 4FG into a cell envelope protein had occured. An alkylation reaction was postulated which was later supported by evidence that defluorination was dependent upon protein synthesis (167).

The K_m and V_{max} values for defluorination reactions in glucose-grown whole cells (3.9 mM and 1nmol F/mg protein/min respectively) were discovered to be identical to the values determined from chloramphenicol-treated glucose-grown whole cells. This suggested that the defluorinating protein was not being directly inhibited but rather that its *de novo* synthesis was prevented (167).

Glucose-, gluconate-, and 2-ketogluconate-grown *P. putida* are capable of C-F bond cleavage. However, the defluorinating activity was repressed when the organism was grown on tricarboxylic acid cycle intermediates such as succinate and citrate (167). Furthermore, the presence of glucose, gluconate or 2-ketogluconate inhibited fluoride release to the extent of 91,

90 and 87% respectively, when added to whole cell incubations with 4FG. Additionally, N-ethylmaleimide (NEM) totally inhibited defluorination, suggesting the importance of protein-SH groups in the defluorination reaction.

From these studies it was concluded that fluoride release from 4FG was dependent upon an inducible-repressible protein associated with the outer membrane. However, while the existence of the inducible-repressible defluorinating protein remains a valid concept, recent studies have ruled out the outer membrane as the site of defluorination (168).

4-Deoxy-4-fluoro-D-[6-^{3}H]-glucose (D-[6-^{3}H]-4FG) was synthesized in order to investigate the mechanism of fluoride release from 4FG. Incubation of whole cells of *P. putida* with 1mM D-[6-^{3}H]-4FG resulted in a 100% release of fluoride. In addition, 70% of the total radioactivity was found in the supernatant fraction, with the remainder in the cell pellet. Extensive analysis revealed approximately 1% of the initial radioactivity to be associated with the cell envelope. Borate anion-exchange column analysis of the supernatant fraction revealed the major contributor of the radioactivity to be tritiated water (^{3}H$_2$O) with a lesser but significant amount (20%) associated with an unidentified tritiated component (195). Problems encountered due to extensive loss of the tritium label when using D-[6-^{3}H]-4FG were resolved by synthesizing 4-deoxy-4-fluoro-D-[U-^{14}C]-glucose (D-[U-^{14}C]-4FG) which was used to trace the metabolic fate of 4FG in *P. putida* (169).

Glucose-grown cell suspensions of the organism incubated with D-[U-^{14}C]-4FG (1mM) for 24 hours at 30°C exhibited extensive release of fluoride (95%) along with a small amount of ^{14}CO$_2$ (4.8 ± 0.2%). The 24 hour supernatant was analyzed by borate anion-exchange chromatography, revealing the presence of two radiolabelled components described as the major and minor peaks. The major peak was subsequently identified as 2,3 dideoxy-D-*glycero*-pentonic acid (2,3-dideoxyribonic acid) using NMR and Fast Atom Bombardment (FAB) mass

spectrometric analysis (169). At this point a pathway was proposed which accounted for the observed loss of tritium from D-[6-^{3}H]-4FG as well as the metabolism of D-[U-^{14}C]-4FG to 2,3-DDRA (13) (Figure 9) (169).

Of moderate interest in these studies was the observation that a small but significant amount of label was found to be incorporated into the envelope fraction (0.42 ± 0.04%). It was considered that if this were the result of a novel carbohydrate analogue (*e.g.* a 4-deoxy-metabolite) derived from 4FG metabolism, then its incorporation into the PG would terminate cell wall biosynthesis and consequently cell growth. Subsequent gel column chromatographic analysis of this envelope fraction gave a high molecular weight (> 400 kDa) radiolabelled fraction. This fraction was defined to be susceptible to lysozyme treatment yielding lower molecular weight (14 kDa) fragments. Results suggested that the radiolabel had been incorporated into a peptidoglycan (PG) protein associated complex. Further treatment of the radiolabelled PG allowing High Pressure Liquid Chromatography (HPLC) analysis revealed that the radiolabel had been incorporated mainly into the amino sugars. The principle PG components were identified as N-acetylglucosamine (NAG), N-acetylmuramic acid (NAM), alanine (ala), glutamic acid (glu), and *m*-diaminopimelic acid (*m*-DAP). In addition, the amino acids glutamic acid (glu), aspartic acid (asp), threonine (thr), and serine (ser) were also identified, and of these, thr was found to contain a majority of the radiolabel. A mechanism for ^{14}CO$_2$ incorporation into these amino acids was proposed making use of established biosynthetic pathways for threonine synthesis in the *Pseudomonas fluorescens* group of the family *Pseudomonadaceae* (170). Of interest in these results was the fact that the amino acids asp, thr, and ser were clearly present after SDS extraction and pronase treatment. This was seen as the result of a strong association or covalent bonding with PG leading to suggestions that asp, thr, and ser could be vestigial remains of a lipoprotein that functions to anchor PG to the outer membrane (171). HPLC analysis of

FIGURE 9

Proposed Pathway for the Formation of 2,3-αideoxy-D-*glycero*-pentonic acid

from 4FG

Legend

Interaction of 4FG with an extra-cellular inducible-repressible protein (168) followed by elimination of HF gives the unsaturated sugar 7. Tautomeric equilibration of the acyclic isomers of 7 (8⇌9⇌10) would allow exchange of tritium at C-6. The transport of 9 into the cell, followed by phosphorylation and action of the appropriate reductase and dehydrogenase, would lead to the formation of 11. The aldonic acid 11 is an attractive analogue substrate for 6-phosphogluconate dehydratase, one of the Entner-Doudoroff enzymes, known to be active in *P. putida* (172). Decarboxylation of the product 12 followed by oxidation and the action of a phosphatase, would account for the observed formation of CO_2 and 2,3-dideoxy-D-*glycero*-pentonic acid (13). Reproduced with permission (169).

FIGURE 9
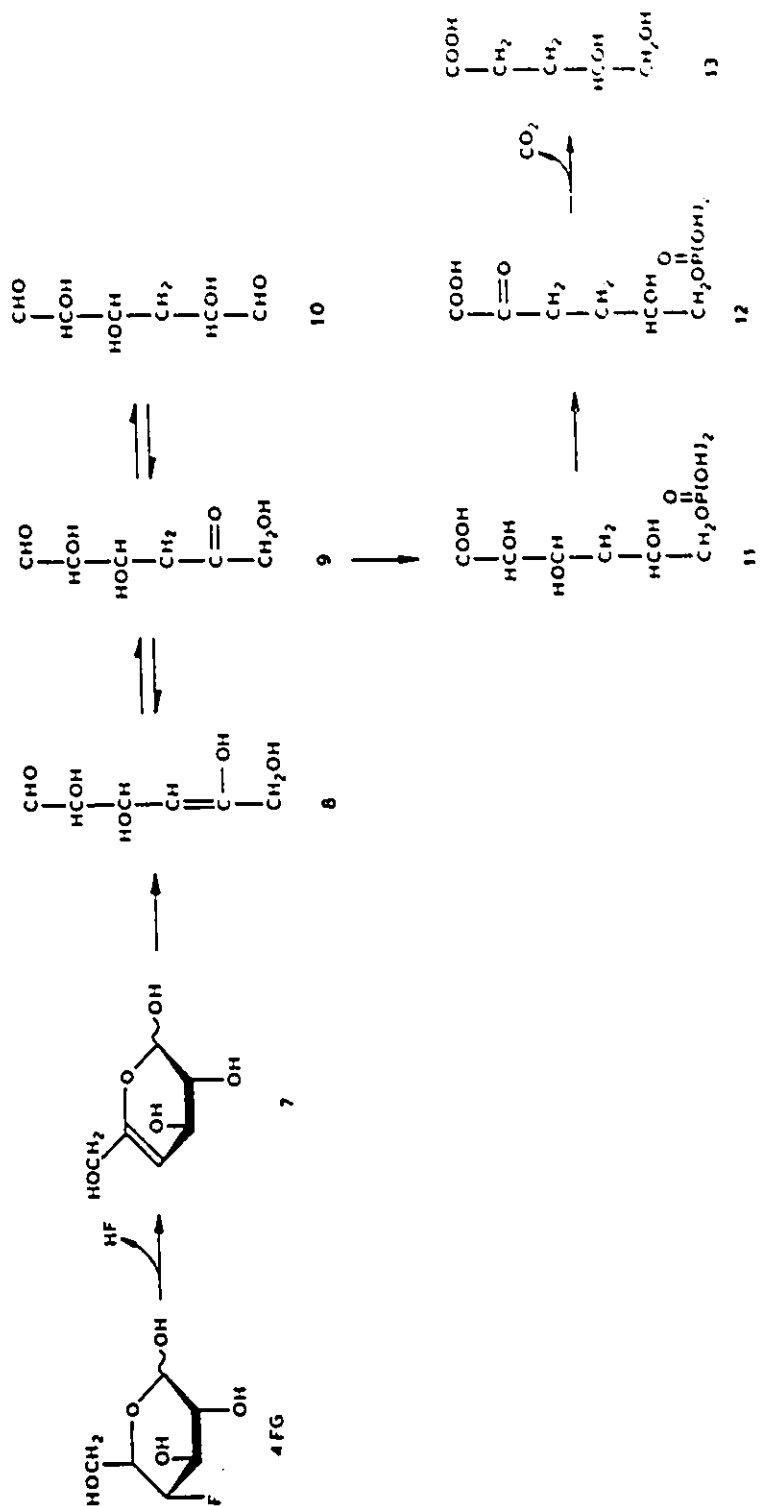
PG isolated from a separate incubation of glucose-grown *P. putida* with $NaH^{14}CO_3$ revealed the presence of NAG, NAM, glucosamine as well as traces of glu and asp. Subsequent physicochemical analysis indicated that the label had been mainly incorporated into the amino sugars NAG and NAM. Pronase treatment during PG isolation was found to cause extensive loss of radiolabel suggesting the incorporation of the radiolabel into protein (170). The results with $NaH^{14}CO_3$ indicate the incorporation of $^{14}CO_2$ into amino acids and carbohydrate of PG.

Further studies of the 'minor peak' components (170) by ^{13}C and ^{1}H-NMR spectroscopy revealed no significant difference in chemical shifts of the minor peak and those of the 'major peak' 2,3-DDRA. However, retention times on borate column analysis were considerably different. Additional observations of the weak affinities for the anion exchanger exhibited by the 'minor peak' as compared to sucrose (internal standard) suggested that the 'minor peak' may be a compound devoid of charge and led to the proposal that this neutral behaviour was indicative of the 1,4-lactone of 2,3-DDRA (170). Further, it was considered plausible that this lactone constituted the predominant species when isolated, but upon borate anion-exchange column chromatography (pH 9.0) alkaline hydrolysis could produce the acyclic structure of 2,3-DDRA. This form would be strongly retained by the borate column (170).

That defluorination might require a proton gradient was suggested by pH effects upon whole cell defluorination. Using 100 mM concentrations of Tris-HCl or potassium phosphate buffers at pH values of 7.1 induced rapid fluoride release activity in the presence of 4FG, however, identical incubations carried out at pH values of 8.2 practically eliminated fluoride release. Subsequent experiments on whole cells using 2,4-dinitrophenol (2,4-DNP) caused virtual arrest of defluorination. A similar inhibition pattern was observed using sodium azide (10 mM). The results suggested involvement of a chemiosmotic energy couple which was essential for defluorination of 4FG. Studies with N,N'-dicyclohexylcarbodiimide (DCCD) or arsenate

have ruled out any involvement of ATP or ATP synthase (170).

Objectives

The principal goals of this study are:

(a) To isolate and identify defluorinated metabolite(s) of 4FG prior to the formation of 2,3-dideoxyribonic acid in *P. putida*.

(b) To determine the cellular location of the defluorinating protein in *P. putida*.

(c) To establish whether an energy couple is required for C-F bond cleavage.

CHAPTER 2
MATERIALS AND METHODS

Materials

The gram-negative organism, *Pseudomonas putida* (ATCC 12633) was obtained as a freeze-dried sample from the American Type Culture Collection (Rockville, Maryland). Bacteriological media, yeast extract and gram-stain reagents were purchased from Difco Laboratories (Detroit, Michigan). D-[U-^{14}C]-4-deoxy-4-fluoro-D-glucopyranose (specific activity, 10, 600 dpm/μmole and 7063 dpm/μmole) and 4-deoxy-4-fluoro-D-glucopyranose were prepared by Dr. N.F. Taylor in this laboratory. D-[6-^{3}H]-glucose (25 Ci/mmole) was obtained from ICN Biomedicals (St. Laurent, Quebec). Polyethylene liquid scintillation vials were obtained from Fisher Scientific Company (Fairlawn, New Jersey). Dupont formula 963 aqueous scintillation fluid was purchased from Dupont, NEM Research Products (Boston, Massachusets 02118). Silica gel thin-layer (plastic backed) chromatography sheets were obtained from the Eastman Kodak Company (Rochester, New York). Fugi RX X-ray Film (Fugi Photo Film Co., Japan). Biogel Wrap System was purchased from Biodesign of New York (Carmel, New York). Deuterium oxide was obtained from MSD isotopes (Division of Merck Frosst Canada Inc., Montréal, Quebec). Whatman 3 MM chromatography paper (Whatman Ltd., Maidstone, England). Copper grids, 200-mesh (J.B. EM Services Inc., Dorval, Quebec). A 2% (w/v) solution of phosphotungstic acid was the generous gift of Mr. Nick Paul (Windsor Public Health Laboratories). Gel blot paper (Mandel Scientific Company Ltd., Guelph, Ontario). Lithium Chloride (J.T. Baker Chemical Co., Phillipsburg, New Jersey), DNase 1 and RNase A were purchased from Boeringer Mannheim (Montréal, Quebec). 5 mm borosilicate NMR tubes were purchased from Wilmad Glass Company, Inc. (Buena, New Jersey).

Dowex 1-×8 (200-400 dry mesh) anion exchange resin in the chloride form, boric acid, nigericin, valinomycin, PMS, FAD, L-malate, ascorbic acid, chloramphenicol, bovine serum

albumin (Fraction V), Folin and Ciocalteau's phenol reagent, deoxycholic acid, Formvar® 15/95

E (registered trademark of Monsanto Company), sodium fluoride, β-D(+) glucose, D-gluconic

acid (sodium salt) (Grade V), glucose-1-phosphate, succinic acid (disodium salt), orcinol,

magnesium chloride hexahydrate, PMSF, ammonium persulfate (electrophoresis grade), Dalton

Mark VI low molecular weight standards, SDS 6H high molecular weight standards, coomassie

brilliant blue G, 2-mercaptoethanol, DL-dithiothreitol, Ponceau S, 5-sulfosalicylic acid, sodium

chloride, egg phosphatidylcholine type XII E (in ethanol), lysozyme, n-octyl β-D-glucopyranoside

and cellulose dialysis bags (6 mm diameter) were all obtained from Sigma Chemical Company

(St. Louis, Missouri).

Borosilicate-glass gravity flow chromatography columns and accessories, Bradford dye

reagent kit, tris (hydroxymethyl) aminomethane-hydrochloride, sodium dodecyl sulfate

(electrophoresis grade), acrylamide, glycine, TEMED (N, N, N', N'-tetramethylethylenediamine)

and the Bio-Rad Trans-Blot® Transfer Medium nitrocellulose membrane were obtained from Bio-

Rad Laboratories (Richmond, California).

Sulfuric acid, diethyl ether (anhydrous), sucrose, dimethyl sulfoxide (DMSO), 2,5-

diphenyloxazole (PPO), and 2,4-dinitrophenol were obtained from BDH Chemical Canada

Limited (Toronto, Ontario).

Ammonium tetraborate tetrahydrate and N, N-methylene-*bis*-acrylamide were obtained

from the Aldrich Chemical Company (Milwaukee, Wisconsin).

Sodium bisulfite, methanol (anhydrous), ammonium sulfate, trichloroacetic acid and

magnesium sulfate were obtained from Fisher Scientific Company (Fairlawn, New Jersey).

The water used, where required for all methods described , was distilled and deionized

using a Barnstead Fi-Streem 2-Liter glass still (Barnstead SYBRON, England) that was fitted with

an ion-exchange resin cartridge filter (Barnstead Thermolyne, Dubuque, Iowa). Unless otherwise

stated, all chemicals and reagents were of the highest possible grade.

Methods

Organism and Culture Conditions

Glucose-, gluconate-, and succinate-grown whole cells of *Pseudomonas putida*, biotype A (ATCC 12633) were used in these studies. This organism was obtained as a freeze-dried culture from the American Type Culture Collection (ATCC) (Rockville Maryland, USA) and usually rehydrated in minimal mineral salts media. Cultures were stored on minimal mineral salts-agar slants by transferring to a fresh slant every 3 months. The cultures were grown for 24 hours at 30°C and then stored at 4°C using the semi-defined minimal mineral salts media of Davis and Mingioli (172) which consisted of:

	g/L (final volume)
K_2HPO_4	7.0
KH_2PO_4	3.0
$(NH_4)_2SO_4$	1.0
$MgSO_4 \cdot 7H_2O$	0.1
Yeast extract	0.2
Trace Solution A	1.0 mL
Trace Solution B	1.0 mL

Solutions of trace elements, made up according to the procedure of Barnett and Ingram contained: (173)

Trace Solution A

	mg/100 mL (stock solution)
$FeSO_4 \cdot H_2O$	80

$MnSO_4 \cdot 4H_2O$	80
NaCl	2000

Trace Solution B

	mg/100 mL (stock solution)
$ZnSO_4 \cdot 7H_2O$	40
$CuSO_4 \cdot 5H_2O$	8
$CoCl_2 \cdot 6H_2O$	8
$CaCl_2$ (anhydrous)	1000
$Na_2MoO_4 \cdot 2H_2O$	10
KI	60

The resulting mineral-salts media which had a pH of about 7.1 was sterilized by autoclaving in batches of 1 or 2 L in 4-L Erlenmeyer flasks for 30 mins. at 126°C and 15-20 pounds per square inch (psi) (103-138 kPa) in a Peltan and Crane Magna-Clave, Model MC (GA Ingram and Co., (Canada) Ltd., Windsor, Ontario). The carbon source (glucose, gluconate or succinate) was prepared as a 2% (w/v) solution autoclaved separately and an approximate amount added asceptically to the mineral salts to give a final concentration of 0.2% (w/v).

Alternatively a 12-L bench top batch fermentation unit (Model SF-116 New Brunswick Scientific Co. Inc., Edison, N.J.) was used to prepare cultures required for larger scale incubations, vesicle preparations and membrane extractions. Mineral salts for 12 L were dissolved in 100 mL of distilled deionized water and sterilized in the fermentor according to the manufacturer's instructions. The carbon source (24 g) was dissolved in 150 mL of distilled deionized water, autoclaved separately and added to the fermentor while the mineral salts were still hot.

For the preparation of glucose-mineral salts agar slants, sterile mineral-salts medium was

prepared and sterilized by autoclaving as described above, except that double-strength solutions of mineral-salts and plain agar [4% (w/v)] were made up and autoclaved separately, mixed with the carbon source and pipetted asceptically into sterile 25 mL borosilicate glass test tubes in 10 mL aliquots. The capped tubes were the allowed to cool in a slanted position at room temperature. These solidified slants were then incubated at 30°C for 24 h. Following this incubation, slants showing any sign of contamination were discarded and uncontaminated slants were stored at 4°C in a sealed plastic bag. Periodically the purity of the cultures of *P. putida* were determined by the following criteria. Initially the cultures were inspected by performing a Gram stain using a commercial Gram-stain kit (Difco Bacto Gram Stain Set, Difco Laboratories, Detroit, Michigan) in accordance with the manufacturer's instructions. The resultant slides were then examined microscopically using an oil immersion lens (magnification 1000 ×) and revealed small gram-negative (red-pink) rods under the microscope. The gram-negative rods should also produce a fluorescent pigment. This was observed by subjecting the culture briefly to an ultraviolet lamp and observing a light blue pigment which is tinted green under visible light. Finally, the fluorescent rods are distinguished from other fluorescent pseudomonads (ie. *P. aeruginosa* and *P. fluorescens*) by observing growth on nutrient gelatin [nutrient broth in 40% (w/v) gelatin]. *P. putida*, unlike the other fluorescent pseudomonads lacks the ability to hydrolyze gelatin. Therefore a 24 hour growth of a sample of the culture in question on nutrient gelatin (30°C), should result in no change to the media. It should remain solid after cooling to 4°C for 15-20 minutes (174,175).

Preparation of Whole Cell Suspensions

Cultures of *P. putida* were routinely maintained in 125 mL flasks containing 50 mL of media with 0.2% glucose, gluconate or succinate. The cells were aseptically transferred daily by adding one inoculating loop full of culture to a fresh flask. For large scale cultures, cell were

grown in either the 4 L flasks containing 1-2 L of mineral salts media or in the 12 L fermentor. The 1-2 L cultures were inoculated with 1-2 mL of 4-8 hour culture respectively, and incubated at 30°C shaking at 150 rpm (Orbit Incubator Shaker Labline Instruments, Melrose Park, Illinois) for 12 hours. The fermentor was inoculated with a 4-8 hour 50 mL culture by aseptic transfer through an inoculating port on the top of the vessel. The large batch culture was aerated at 1.5 L per minute, stirred at 400 rpm and maintained under a slight positive pressure (0.5 psi) at 30°C for 12 hours. At this time, the optical density of the culture as observed at 620 nm was in the range 0.9-1.1 as measured using a Shimadzu UV-240 Spectrophotometer (Shimadzu Corp., Kyoto, Japan) using mineral-salts medium as a blank. At this point the cells are at the mid- to late- logarithmic phase of growth. Whole cells of $P.$ $putida$ were harvested in sterile, one liter bottles by centrifugation at 4000 × g, 20°C for 20 minutes using a Beckman J-6B Centrifuge (Beckman Instruments Inc., Palo Alto, California). The cell pellet was washed in 20-50 mM potassium phosphate buffer (PPB), pH 7.5 and reharvested as described above. Typically, the cell yields were 5-6 g wet weight per liter of media.

Protein Determination

Whole cell suspensions of $P.$ $putida$ were assayed for protein content using a Biorad dye reagent kit which is based upon the method of Bradford (176). For membrane vesicles and crude periplasmic extracts, the method of Lowry et $al.$ (177) was used. In either of these two cases bovine serum albumin (BSA) was used as a standard. In order to determine protein content in crude cytoplasmic membrane extracts and protein incorporation in proteoliposome preparations, the Peterson modification of the Lowry method (178) was employed along with the TCA precipitation step. In these latter two cases standard curves were produced using BSA to which had been added a 15% (w/v) solution of n-octylglucoside (final concentration, 1.25%). An aliquot of non-reconstituted liposome suspension was used as a blank for the proteoliposomal

assay. All absorbance readings were carried out in a Schimadzu UV-240 UV-visible Recording

Spectrophotometer (Schimadzu Corp., Kyoto, Japan). Standard curves generated for the Biorad,

Lowry, and Lowry/Peterson methods are shown in Appendices I, II, and III respectively.

Liquid Scintillation Counting

Radioactivity was measured using a Beckman LS 7500 Liquid Scintillation Counter

(Beckman Instruments Inc., Fullerton, California) according to the manufacturer's instructions.

For all samples, 10 mL of an aqueous scintillation fluid (Formula 963, Dupont Canada) was

added to each 1 mL sample. Smaller sample volumes were made up to that same volume with

distilled deionized water. In all cases a clear homogenous solution was obtained after vigorous

mixing with the scintillation cocktail using a vortex mixer. The level of quenching in each

sample was determined by using the "H-Number" method and counting efficiency axis derived

from a quench correction curve (Appendix V) constructed by plotting counting efficiencies versus

H-number values from a series of carbon-14 standards of varying quench composition (Nuclear

Chicago).

Respirometric Studies

The extent of rate of respiration of glucose and 4FG by whole cells grown on media

supplemented with the different carbon sources was carried out by the Manometric Method (179)

using a Gilson differential respirometer with eight 20 mL calibrated reaction flasks (Gilson

Medical Electronics, Middletown, Wisconsin). Each reaction flask contained in a total volume

of 3 mL; 0.985 mL of 50 mM potassium phosphate buffer, pH 7.5, 2 mL of whole cells (30 mg

protein) and 0.015 mL of a 200 mM sterile aqueous suspension of 4FG in the side-arm. The

center well contained 100 μL of a 20% (w/v) solution of KOH and a folded paper wick

(Whatman 1 filter paper, Whatman limited) to absorb CO_2 evolved during the reaction. After

allowing the contents of the flasks some time to become temperature equilibrated in the water

bath, the reaction was initiated by tipping the contents of the side-arm into the main compartment and the amount of oxygen consumed was monitored as described in the instruction manual (Gilson Medical Electronics). The incubation temperature was 30°C and the gas phase air. The number of moles or gram atoms of oxygen consumed per mole of substrate was determined as shown in Appendix VII.

Trapping of $^{14}CO_2$ During Whole Cell Incubation

The extent of $^{14}CO_2$ release from D-[U-^{14}C]-4FG (specific activity, 10,600 dpm/μmole) by whole cells of *P. putida* grown on glucose or gluconate as carbon sources was conducted in 20-mL Gilson respirometric flasks containing 200 μL of aqueous 20% (w/v) potassium hydroxide in a center well of the main compartment. Each reaction flask contained in a total volume of 3 mL; 0.75 mL of 50 mM potassium phosphate buffer, pH 7.5, 2 mL whole cells (30 mg protein) and 0.25 mL of a 20 mM sterile aqueous suspension of D-[U-^{14}C]-4FG in the side-arm. After allowing the contents of the flask some time to become temperature equilibrated in the water bath, the reaction was initiated by tipping the contents of the side-arm into the main compartment. The 2 and 24-hour incubations were carried out at a temperature of 30°C using air as the gas phase. After the incubations, the 200-μL aliquots of the 20% potassium hydroxide solution from the center well were collected and placed in a scintillation vial. Eight hundred microliters of water was added to bring the final volume to 1 mL and then 10 mL of scintillation cocktail was added to each vial. The resulting mixture was shaken well to obtain a clear homogenous solution and then counted for radioactivity as described in **Liquid Scintillation Counting**. Fluoride release in these 2 and 24-hour incubations was determined directly from the resulting cell suspension in the main compartment as outlined in **Fluoride Ion Measurements**.

Fluoride Ion Measurements

Fluoride ion was measured with a combination fluoride electrode (Orion Research,

Cambridge, Massachusetts) coupled to a Metrohm Herisau E 510 Precision mV/pH Meter (Metrohm Herisau, Switzerland). The electrode was filled with a reference filling solution (saturated AgCl), inverted several times and allowed to equilibrate overnight at room temperature in a solution of sodium fluoride standard (NaF; 10 mM) in potassium phosphate buffer (20 or 50 mM), pH 7.5.

Prior to use, the electrode was rinsed thoroughly and partially immersed in distilled deionized water for an hour at room temperature. Fluoride ion measurements were obtained by inserting the tip of the electrode into the sample for 2 minutes and the electrode potential in the millivolt scale of the meter was recorded to an accuracy of ± 1 mV. The same protocol was followed for constructing a standard curve relating electrode potential to fluoride ion concentration from a series of standard sodium fluoride solutions that had been prepared in 20mM or 50 mM potassium phosphate buffer, pH 7.5. The concentration of fluoride ion in the sample could then be determined from this standard curve with an accuracy of 5% (based upon a possible error of ± 1 mL for the electrode potential reading). A standard curve was produced immediately prior to each intended use of the fluoride electrode (Appendix IV).

Borate Anion-Exchange Column Chromatography

Samples obtained following 80-100 minute incubations of gluconate-grown *P. putida* with D-[U-^{14}C]-4FG were analyzed on a 74 cm × 1.45 cm bed of Dowex 1-×8 200-400 dry-mesh borate anion-exchange column. The resin was prepared from a Dowex 1-×8 200-400 dry-mesh anion-exchange resin in the chloride form by, first, converting it completely to the hydroxide form and then neutralizing with 0.5 M boric acid as described by Floridi (180). Initially, the column was equilibrated with a 29 mM ammonium tetraborate/57 mM boric acid buffer, pH 8.5-8.8 (starting buffer). Prior to the application of radiolabelled samples the columns were standardized in the following manner. Mixtures of various sugar standards including D-

glucose, 4FG, D-glucose-1-phosphate, and D-glucose-6-phosphate (1-2 mL total volume) were applied to the top of the resin bed and permitted to flow into the bed without allowing the top of the bed to run dry. In addition 2 × 1 mL aliquots of starting buffer were used to wash the sample into the resin bed. The column was then topped-up with starting buffer and eluted at a constant flow of 0.5 mL/minute with an increasing linear concentration of 250 mL of starting buffer diluted in a linear manner with 250 mL of 500 mM ammonium tetraborate, pH 8.9-9.2. Fractions of 4 mL were collected with and ISCO Golden Retriever Fraction Collector Model 328 (Instrumentation Specialties Co., Lincoln, Nebraska). Alternate fractions were then analyzed for carbohydrate by Orcinol-Sulfuric acid Colourimetry (180). This consisted of adding approximately 2 mL of orcinol-sulfuric acid reagent [0.1% (w/v) orcinol in 70% (w/v) sulfuric acid] to 1 mL of each alternate fraction, vortexing, heating at 90-95°C for 20-30 minutes, cooling to room temperature and measuring the absorbance of the resulting solution at 420 nm in a Shimadzu UV-240 UV-visible Recording Spectrophotometer (Shimadzu Corp., Kyoto, Japan). Those fractions remaining after orcinol-sulfuric acid colourimetry were used to assay for inorganic phosphate using the method of Clark *et al.* (181) which calls for the addition of 1 mL of acid-molybdate reagent [2.5% (w/v) $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 13.6% (v/v) H_2SO_4] to 1 mL of each fraction followed by 1 mL of reducing agent [3% (w/v) sodium bisulfite and 1% (w/v) *para*-methylaminophenol], vortexing and observing the formation of a dark blue colour after standing for 15-20 minutes at room temperature.

In the case of the radiolabelled metabolites, analysis was carried out by processing 250 μL of every alternate fraction according to the procedure outlined in **Liquid Scintillation Counting.** Those radioactive fractions corresponding to the different metabolite peaks were pooled individually and concentrated to dryness using a BÜCHI Rotavapour EL 130 Rotary Evaporator (Beckman Instruments Ltd., Rexdale, Ontario). Ammonium tetraborate was removed

by repeated addition and evaporation of 5 × 100 mL aliquots of anhydrous methanol. The final residue (metabolite) obtained from this procedure was suspended in 1-2 mL sterile distilled deionized water, concentrated to dryness and resuspended with 500 μL of D_2O to be used for analysis by ^{13}C-NMR and TLC.

Thin Layer Chromatography of Radiolabelled Metabolites

Following isolation, radiolabelled metabolites were analyzed by ascending Thin Layer Chromatography (TLC) on 20 × 20 cm 13181 silica gel (0.2 mm thickness) plastic backed sheets (Eastman Kodak Company, Rochester, New York). The solvent used for development was an ethyl acetate: acetic acid: water (3: 3: 1). This solution was added to the developing tank and the top firmly sealed about 12-16 hrs prior to use, allowing for complete solvent equilibration. After 2-3 hrs, the plates were air dried and subsequently the carbohydrates were detected by spraying with sulphuric acid: ethanol [1: 1 (v/v)] followed by heating at 110°C for 20 minutes.

Periodate-benzidine Spray for Polyols

Following analysis by ascending thin-layer chromatography, the peak h metabolite were subjected to periodate oxidation by the method of Clark et al. (181). The chromatograms were sprayed with 0.5% (w/v) $NaIO_4$ until just damp and allowed to react for 5 minutes at room temperature. The chromatogram was then sprayed with 0.5% benzidine reagent [0.5% benzidine in ethanol:glacial acetic acid (4:1 v/v)].

Reaction with o-phenylenediamine

To 60 μL (~ 100 μg) of the metabolite in water (final volume 2.5 mL), 0.5 mL of o-phenylenediamine reagent (15 mg o-phenylenediamine per mL 0.25 N HCl) was added. The mixture was heated to 100°C for 30 minutes, cooled to room temperature and the absorbance at 338 nm was measured. Additionally, absorbances at 330:360 nm were used to give an extinction ratio of 1.4 consistent with the formation of a hydroxyquinoxaline derivative of an α-ketoaldonic

acid (198).

Fourier Transform (FT) Carbon-13 NMR

^{13}C-NMR spectroscopic analysis of the radiolabelled metabolites resuspended in D_2O was carried out using a Brüker 300 MHz NMR Spectrophotometer (Brüker Instruments, Brüker Canada, Milton, Ontario). Chemical shift values were relative to 1,4-dioxane and expressed in parts per million (ppm) of the main spectrometer frequency (74.469 MHz). DEPT 135 and DEPT 90 analyses of ^{13}C-NMR spectra were run overnight (~ 100 000 scans). See also Appendices XI, XII and XIII.

In Vivo Trace Studies of 4FG Metabolism Using ^{19}F-NMR

Large-scale (12 L) cultures of glucose- and gluconate-grown *P. putida* were prepared as outlined in **Preparation of Whole Cell Suspensions** . The resultant cell pellet was then resuspended in 25 mL total volume of 50 mM potassium phosphate buffer, pH 7.5, to give a final protein concentration of 10 mg/mL. The defluorination reaction was then initiated by the addition of 625 μL of a 200 mM aqueous solution of 4FG (final concentration, 5 mM). Incubations were carried out at 30°C with constant shaking. At 15 minute intervals 500 to 750-μL aliquots of the cell suspension was removed and placed into a 5 mm borosilicate NMR tube and analyzed using a Brüker 200 MHz FT NMR Spectrophotometer in conjunction with a ^{19}F-^{1}H dual probe (Brüker Canada, Milton, Ontario) at 30°C. Digital resolution (Hz/ppm) was 10.17. A line broadening factor of 10-15 Hz was applied routinely for signal enhancement. Chemical shift values were relative to trichlorofluoromethane (CFCl$_3$) (SR= -188.31 ppm).

Osmotic Shock of Whole Cells

The method of osmotic shock was based upon the procedure of Stinson *et al.* (110) for the isolation of a glucose-binding protein from *P. aeruginosa.* Twelve-hour incubations of glucose-, gluconate-, and succinate-grown *P. putida* (mid- to late- exponential growth phase)

were harvested by centrifugation at 2500 × g for 20 minutes at 4°C on a Beckman J-6B centrifuge (Beckman Instruments). The packed cells were rapidly suspended (14 mg of dry cell weight/mL) in a 0.5 M tris (hydroxymethyl) aminomethane-hydrochloride buffer, pH 8.5 containing 0.2 M $MgCl_2$ (extraction buffer). The cell suspensions were incubated at 30°C for 30 minutes with constant stirring and subsequently collected by centrifugation at 3000 × g, 4°C for 10 minutes using a Sorvall Superspeed RC2-B Centrifuge. The supernatant was removed, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.5 mM and it was stored at 4°C. The cells were quickly resuspended in an equal volume of distilled deionized water. After an additional incubation at 30°C for 30 minutes with constant stirring, the cells were centrifuged again at 2500 × g for 20 minutes at 4°C. The resultant supernatant (shock-fluid) was then pooled with the magnesium extract and the final concentration of PMSF adjusted to 0.5 mM. The supernatant was dialyzed against 10 × volume of 1 mM $MgCl_2$-10 mM tris (hydroxymethyl) aminomethane-hydrochloride buffer, pH 7.5 (TM buffer) at 4°C for 20-24 hours. The proteins were precipitated using ammonium sulfate (0 to 95% saturation) (182) followed by centrifugation at 27 000 × g (TY 30 Rotor, Beckman Instruments) for 30 minutes at 4°C. The precipitates were dissolved in a small volume of TM buffer to which PMSF had been added (final concentration, 0.5 mM) and dialyzed against 10 × volume of the same buffer at 4°C for 20-22 hours. After dialysis the crude periplasmic extract was assayed for protein content as described in **Protein Determination**. Subsequently 1 mL aliquots were frozen in liquid nitrogen and stored at -20°C.

Cytoplasmic Membrane Preparations

For studies with isolated membrane vesicles or membrane fractions two different procedures were used. The first involved lysozyme digestion of glucose-grown whole cells of *P. putida* while the second required the French pressure treatment of whole cells to generate a

cytoplasmic membrane fraction. Both procedures included the use of 12-hour incubations of *P. putida* at which time the cells were in the mid-exponential phase of growth. The Lysozyme-generated vesicles were isolated by a procedure originally described for *P. aeruginosa* (183). The cells were harvested as in **Preparation of Whole Cells Suspensions** and the resulting pellet was suspended in 2/3 of the original volume in a solution containing final concentrations of 2.5% (w/v) LiCl, 0.75 M sucrose, 10 mM potassium phosphate, pH 7.0, 10 mM $MgSO_4 \cdot 7H_2O$, and 500 μg/mL lysozyme. The suspension was incubated for 1 hour at 30°C on a rotary shaker, and the resulting osmotically-fragile rods were harvested by centrifugation at 10 000 × g, 30 min, 4°C. The pellet was suspended in the smallest possible volume of 2.5% (w/v) LiCl in 0.75 M sucrose manually, using a loose-fitting teflon rod and glass homogenizer (ACE Glass Inc., Vineland, New Jersey). The suspension was quickly added to 50 volumes of ice-cold 10 mM potassium phosphate buffer, pH 6.6, containing 1 mM $MgSO_4$ in a Waring Blender and blended for 10 seconds. Deoxyribonuclease I (DNase) and ribonuclease A (RNase) were subsequently added to final concentrations of 20 μg/mL each and the mixture incubated at 25°C for 30 minutes with gentle stirring. The mixture was centrifuged at 40 000 × g (TY 30 rotor, Beckman Instruments) for 30 minutes at 4°C. The resultant pellet was resuspended by means of a loose-fitting glass homogenizer in a 1:4 (v/v) ratio in ice-cold 100 mM potassium phosphate buffer, pH 6.6, containing 10 mM $MgSO_4$. Whole cells and large fragments were removed by centrifugation at 800 × g for 30 minutes at 4°C. At this point, an aliquot of the resulting supernatant was removed in order to assay for protein content as outlined in **Protein Determination**. Subsequently, the vesicles were pelleted out by centrifugation at 40 000 × g for 30 minutes at 4°C and the pellets washed twice and suspended in the same buffer to a final concentration of 5-10 mg protein/mL. The vesicles were then transfered into plastic vials, frozen in liquid nitrogen and stored at -80°C. **French-Pressure Generated Cytoplasmic Membrane Fractions**

of glucose-, gluconate- and succinate-grown *P. putida* were processed by a method adapted from Newman and Wilson (184). All steps described were carried out at 4°C. After harvesting of a 12-L cell culture at 2500 × g and 4°C, the pellets were washed in 500 mL of ice-cold 50 mM potassium phosphate buffer, pH 7.5. The cells were resuspended (0.2 g wet weight/mL) in ice-cold 50 mM potassium phosphate pH 7.5, 1 mM dithiothreitol, 5 mM MgSO₄ and then hand homogenized before the addition of 20 µg/mL of DNase and 0.5 mM PMSF. The cells were then disrupted by passage through a prechilled French pressure cell (American Instrument Company, Silver Spring, Maryland) at 19 000 to 20 000 psi total pressure and collected in an ice bath. Unbroken cells and debris were removed by centrifugation at 11 700 × g (TY 30 rotor, Beckman Instruments). The supernatant was carefully removed and the centrifugation was repeated. The resulting supernatants were pooled together and the cytoplasmic membrane fraction was sedimented by ultracentrifugation at 140 000 × g (60 Ti rotor, Beckman Instruments). The pellet was resuspended in 50 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 0.5 mM PMSF. At this point an aliquot was removed to allow determination of protein content as outlined in **Protein Determination** and the centrifugation was repeated. The membrane fraction was resuspended in the same buffer at a protein concentration of 4-5 mg/mL. Fractions were divided into 1 mL aliquots, frozen in liquid nitrogen and stored at -80°C.

Vesicles and membrane extracts were prepared only from organisms kept on slants for less than six months since organisms kept on slants for longer periods were found to have lost their transport activity.

Preparation of Liposomes for Reconstitution

Liposomes were prepared using a modification of a procedure previously published for the reconstitution of LDL receptors (185). One hundred milligrams of egg phosphatidylcholine in ethanol (Sigma Chemical Company, St. Louis Missoury) were placed in a glass flask and the

ethanol removed under a stream of nitrogen. The dry phospholipid was redissolved in diethyl ether, 10 mM 2-mercaptoethanol and then a coat of the lipid covering the inner walls of the flask was formed by evaporating the ether under a stream of nitrogen. Finally, 2 mL of 50 mM potassium phosphate buffer, pH 7.5, 10 mM 2-mercaptoethanol was added and a suspension of liposomes (5 mg/mL) was formed by hand shaking for 5 minutes at room temperature. This suspension is stored at 4°C for up to 1 month according to the reference; however, the liposomes made for these studies were discarded after only two weeks.

Characterization of Liposomes and Proteoliposomes

Electron Microscopy

One drop of a liposome or proteoliposome suspension (final concentration, 1 mg/mL) was placed on to 200-mesh, Formvar®-coated copper grid held by forceps. An equal volume of 2% (w/v) phosphotungstic acid (PTA), pH 6.5, was added to the grid containing the sample and it was left to stand for 30 seconds to 1 minute. The excess solution was removed by blotting with filter paper and the sample allowed to air dry on a piece of filter paper in a petri dish. The grids were examined using a Phillips EM High Resolution Electron Microscope (Findhoven, The Netherlands).

Reconstitution of Defluorinating Activity in Proteoliposomes

C-F bond cleavage activity was reconstituted using a slight modification of the n-octylglucoside dilution procedure of Racker *et al.* (186). The procedure, originally employed to solubilize and reconstitute lactose transport activity in *E. coli* was adapted for extraction and reconstitution of the defluorinating component of the cytoplasmic membrane in *P. putida* . It consisted of three parts:

a) Extraction of Cytoplasmic Membrane Proteins

Two and a half milliliters of 50 mM potassium phosphate buffer, pH 7.5, 40 μL of 100

mM dithiothreitol, 10 mM 2-mercaptoethanol, 1.25 mL of French pressure generated cytoplasmic membrane vesicles (5 mg of protein) and 200 μL of phosphatidylcholine (96 mg/mL stock) were added to a small test tube in an ice bath and the tube was blended on a vortex mixer. Thirty-three microliters of st ck n-octylglucoside [15% (w/v) solution in 50 mM potassium phosphate buffer, pH 7.5] were added and the tube was vortexed gently. The suspension was incubated at 4°C for 20 minutes, blended on a vortex mixer again and then centrifuged at 175 000 × g (65 Ti rotor, Beckman Instruments) at 4°C for 1 hour. The supernatant was carefully removed and protein content was determined according to the modified Lowry procedure of Peterson as described in **Protein Determination.**

b) Incorporation of Membrane Proteins into Liposomes

Two millilitres of solubilized membrane protein (concentration 2-3 mg/mL) were mixed with 1 mL of phosphatidylcholine liposomes (5 mg of lipid) and 270 μL of 15% (w/v) n-octylglucoside (final concentration, ~ 1.25%). The mixture was blended on a vortex mixer and then incubated at 4°C for 20 minutes. The suspension was then pipetted directly into 30 mL of 50 mM potassium phosphate buffer, pH 7.5, 1 mM dithiothreitol, 10 mM 2-mercaptoethanol at room temperature (~ 25°C) and vortexed gently. The resultant proteoliposomes were sedimented by ultracentrifugation at 85 000 × g (60 Ti rotor, Beckman Instruments) , for 1.5 hours. An aliquot of the supernatant was removed in order to measure protein content (protein not incorporated into liposomes) according to the Peterson modification of the Lowry Method as outlined in **Protein Determination.** This determination was carried out in triplicate for three separate extraction-reconstitution assays and it was determined that 32% of the cytoplasmic membrane protein intially added became incorporated into the liposomes provided. The remaining supernatant was decanted and the tube was wiped with a cotton-tipped applicator. At this point, the pellet was resuspended (final protein concentration, 4 mg/mL) in one of three types

of incubation mixtures:

i) 50 mM potassium phosphate, pH 7.5, 12 mM MgSO$_4$

ii) 50 mM potassium phosphate, pH 7.5, 12 mM MgSO$_4$, 10 mM L-malate/50 μM FAD

iii) 50 mM potassium phosphate, pH 7.5, 12 mM MgSO$_4$, 0.1 mM phenazine methosulfate (PMS)/20 mM ascorbate

All of these mixtures contained chloramphenicol (1mg/mL) and were filter-sterilized prior to use.

c) Defluorination Assay

One milliliter of the incubation mixture (protein concentration, 4 mg/mL) was pipetted into a plastic vial and the volume adjusted to 1.99 mL with the same buffer originally used to resuspend the proteoliposome pellet. This suspension was incubated at 30°C for 20 minutes with constant shaking. Assays were initialized with the addition of 10 μL of a 200 mM suspension of 4FG to the incubation mixtures (final concentration ratios, 1 μmole 4FG/2 mg protein/1 mL buffer) and the assay mixtures were incubated at 30°C with constant shaking. Fluoride release was measured as described in **Fluoride Ion Measurements**, at 1 hour intervals.

Ionophore Incubation of Whole Cells

Glucose-grown cells of *P. putida* were prepared and harvested as described in **Preparation of Whole Cell Suspensions**. Concentrated suspensions of the ionophores nigericin and valinomycin were prepared freshly (92 mM and 84 mM respectively) in ethanol. At this point aliquots of a given ionophore suspension (5, 10, 15, 20, and 25 μL) were added to 50 mL (final volume) of filter-sterilized 50 mM potassium phosphate buffer, pH 7.5. The cell pellet was resuspended in this buffer/ionophore mixture at a concentration of 30 mg/mL. Assay mixtures consisted of 1.5 mL of this cell suspension to which had been added a further 1.485 mL of the buffer/ionophore suspension used to suspend the cells initially. These suspensions were then incubated at 30°C, for 30 minutes with constant shaking. Assays were initiated by the addition

of 15 μL of a sterile 200 mM 4FG suspension followed by incubation at 30°C with constant shaking. To determine the reversible nature of treatment with nigericin, nigericin-treated whole cells were washed twice and subsequently resuspended in 50 mM potassium phosphate buffer, pH 7.5 (final protein concentration 30 mg/mL). Defluorination was initiated as previously. Fluoride release was observed as described in **Fluoride Ion Measurements** at intervals of 20 minutes.

SDS-PAGE Analysis

The electrophoretic method used in all studies was a modification (187) of the Laemmli discontinuous buffer system (188). Samples of the crude periplasmic extract were run using a 9 × 8 cm mighty small vertical slab gel unit model SE 200 (Hoefer Scientific Instruments, San Francisco, California). Normally, 10% polyacrylamide gels were run (resolving gel) with a 5% stacking gel. Samples (50 μL) were stacked at 75 V for 35 minutes and then electrophoresed at 100 V for 2.5 hours. Cytoplasmic membrane protein samples were prepared using the same sample buffer system as the periplasmic proteins or in the sample buffer system outlined by Newman *et al.* (189). Non-reduced samples were prepared in the absence of sulfhydryl reducing agents 2-mercaptoethanol or dithiothreitol and incubated at 37°C for 30 minutes before loading on the gel. Reduced samples were prepared by mixing with an equal volume of 2-times concentrated sample buffer (189) which contained 6% SDS and 200 mM dithiothreitol instead of 2-mercaptoethanol. These samples were then heated to 95°C for 5-15 minutes and allowed to cool before being loaded onto the gel. Routine analysis of protein content required a 12% acrylamide gel with a 5% stacking gel. A large BRL-type (Bethesda Research Laboratories, Bethesda, Maryland) 16 × 16 cm gel apparatus fabricated at the University of Windsor, Central Research Shop was used to analyze these samples. These gels were run at 60 V (stacking gel) for 2 hours and then at 90 V (resolving gel) for 5 hours. In general the gels were fixed and

stained overnight in a solution containing 50% (v/v) methanol, 10% (v/v) acetic acid and 2.5% (w/v) Coomassie Brilliant Blue R 250 and then destained in 50% (v/v) methanol, 10% (v/v) acetic acid until the background stain was removed. For storage these gels were dried using the Biogel Wrap System (Biodesign of New York, Carmel, New York). Molecular weight analyses was calculated from a standard curve (Appendix VI) prepared on a Sigma plot version 4.1 (Jandel Scientific, Corte Madera, California).

Non-Denaturing Gel Electrophoresis

Electrophoresis under non-denaturing conditions was carried out using the same procedure oulined under SDS-PAGE Analysis with slight modifications. All of the steps were carried out at 4°C. Water was substituted for SDS and 2-mercaptoethanol where required in the sample buffer as well as the stacking and separating gel preparations. The samples (50 μL) were analyzed using a 10% acrylamide gel and run at a constant 175 V for 5-6 hours at 4°C.

Urea Gel Electrophoretic Analysis

The system used for urea gel analysis was a modification of a procedure published by Barton (187). Samples of the crude periplasmic extract were run using a 9 x 8 cm mighty small vertical slab gel unit model SE 200 (Hoefer Scientific Instruments, San Francisco, California). The samples and the gel were reated as outlined in Appendix X.

Western Blotting

All of the procedures in this section were performed at 4°C. Samples of crude periplasmic protein were prepared as outlined under **Non-Denaturing Gel Electrophoresis** or **Urea Gel Electrophoretic Analysis**. The stacking gel was then cut away and the resolving gel was equilibrated in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol] for 30 minutes. A sheet of Bio-Rad Trans-Blot® Transfer Medium nitrocellulose membrane (Bio-Rad Laboratories, Richmond, California), previously cut to exact gel dimensions and four sheets of

Whatman 3 MM chromatography paper were soaked for 30 minutes in transfer buffer and placed along with the gel in a Bio-Rad Mini Trans-Blot® electrophoretic transfer cell according to the manufacturer's directions (Bio-Rad Laboratories, Richmond, California). The transfer was allowed to run at 30 V, 40 mA overnight. Proteins were visualized by staining in a 1 in 9 dilution mixture of a 10 × stock Ponceau S Solution [2% (w/v) Ponceau S, 30% (w/v) TCA, and 30% (w/v) 5-sulfosalicylic acid] and water. The staining procedure required a 5 minute incubation with constant shaking followed by rinsing the membrane with several changes of water. The Ponceau S was removed by washing the membrane with TBS (20 μM Tris, 0.5 M NaCl, pH 7.5) followed by rinsing with several changes of water. At this point the nitrocellulose membrane from the urea gel transfer was washed with several changes of 20 mM potassium phosphate buffer, pH 7.5 for 10-12 hours. The membrane from either procedure was then incubated for 2 hours at 30°C in a solution mixture composed of D-[6-^{3}H]-glucose and D-glucose (final concentration 1.4 μM, specific activity 154 166 dpm/ng), with constant shaking. The membrane was then washed with several changes of 20 mM potassium phosphate buffer, pH 7.5 for 2 hours. Subsequently the membrane was blotted dry with paper towelling and placed in an autoradiography cassette (Cronex Cassette with Hi Plus intensifying screens, Dupont Canada, Mississauga, Ontario). The membrane was covered with Fugi RX X-ray film (Fugi Photo Film Co., Japan), incubated at -80°C and developed after 30 days exposure.

Radiolabeling and Fractionation of Whole Cells

Glucose-grown whole cells (final protein concentration, 2 mg/mL) of *P. putida* were incubated with a 1 mM final concentration of D-[U-^{14}C]-4FG (specific activity 10,600 dpm/μmole) in sterile 20 mM potassium phosphate buffer, pH 7.5. The incubations were carried out for 24 hours at 30°C in sterile round bottom flasks on a rotary shaker (Lab-line Instruments Inc., Melrose Park, Illinois). The cell suspension was subjected to a fluoride ion determination

in order to determine the extent of fluoride release and subsequently centrifuged at 10 000 × g at 4°C for 15 minutes in a Sorvall RC2-B. The cells were then processed as outlined in the previous section describing the generation of French pressure membrane fractions. The resultant vesicles were then processed as described under **extraction of cytoplasmic membrane proteins.** Aliquots of the cytoplasmic membrane extract were then analyzed for protein content and for incorporation of the ^{14}C radiolabel as outlined in the **Protein Determination** and **Liquid Scintillation Counting** sections.

Fluorography

In order to enhance the intensities of the weak β-emitter, ^{14}C, in the cytoplasmic protein preparation a fluorographic procedure described by Bonner and Laskey (208) was employed. All of the following steps were preformed with gentle shaking at room temperature. The SDS-polyacrylamide gel containing the sample was soaked for 30 minutes to 1 hour in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid. After this initial incubation the gel was soaked in two changes of DMSO for 30 minutes each in order to remove water. Then the gel was placed in a 16% (w/v) solution of PPO in DMSO for 2 hours. Subsequently the gel was washed in distilled deionized water for 30 minutes. The gel was then placed on a sheet of blot paper (Mandel Scientific) that had been presoaked in water and was cut a little bigger than gel size (approximately 1 cm larger per side). The gel was then covered with a sheet of 'Saran Wrap' and dried for 2 hours at 80°C using a Dry gel jr gel dryer (model SE 540, Hoefer Scientific Instruments, San Francisco, California).

Autoradiography

The dried gel was placed in a cassette (Cronex Cassette with Hi Plus intensifying screens, Dupont Canada, Mississauga, Ontario) along with a sheet of Fugi RX X-ray film (Fugi Photo Film Co., Japan), exposed at -80°C and developed after 30 days.

CHAPTER 3
RESULTS AND DISCUSSION

Incubation of glucose-grown whole cells of *P. putida* with 4FG results in the instantaneous release of fluoride without any detectable respiration or transport (167). The specificity of this defluorination is illustrated by the fact that 3FG is oxidized with retention of the C-F bond, to 3FGA and 3F2KGA (45). With 0.5 mM concentrations of 4FG 100% fluoride release occurs in whole cells with no 4FG or defluorinated product being detected in the supernatant by thin-layer chromatography. Fluoride release from 4FG is dependent upon the presence of a protein induced by glucose or gluconate but repressed by succinate, citrate, and other TCA intermediates (167). Cell-free extracts or membrane vesicles prepared from glucose-grown *P. putida* oxidize 4FG to the extent of 2 g atoms oxygen/mol with retention of the C-F bond. Thin-layer chromatography of vesicle or cell-free extracts before and after complete oxidation indicated that 4FG (R_f = 0.62) was converted to a slower moving reducing component (R_f = 0.42) which is consistent with the formation of 4F2KGA (190,191). Fractionation of the cell envelope followed by incubations with 2.5 mM concentrations of 4FG indicated the presence of the inducible-repressible protein associated with the outer membrane of *P. putida* which reacts with 4FG. Subsequent investigations which focused on the isolation and characterization of glucose-grown outer membrane preparations yielded some interesting results (192). It was discovered that any fluoride release activity observed previously was the result of whole cell contamination of the outer membrane preparations which was observed microscopically. Incubation of the outer membrane preparations in the presence of a number of protein inhibitors such as chloramphenicol (shown by D'Amore to inhibit defluorination in whole cells), kanamycin and gentamycin, either eliminated or drastically reduced the cellular contamination (examined microscopically using gram-staining) as well as defluorination after 24-36 hour incubations. The effects brought about by the inhibitors of protein synthesis supported the contention that any

77

defluorination observed in the outer membrane preparations was caused primarily by whole cell contamination. Additionally, the outer membrane protein OprB, originally proposed to be responsible for fluoride release, was shown to be glucose and gluconate inducible, succinate, citrate, maltose, and lactose repressible (106). Growth on gluconate has no effect on fluoride release by whole cells of *P. putida*, thus eliminating OprB as the site of defluorination. Subsequent studies of its substrate specificity indicate that OprB forms small, cation-selective pores which bind glucose ($K_s = 100$ mM) and other carbohydrates (106). In an attempt to elucidate the pathway and mechanism of 4FG metabolism and fluoride release, whole cell suspensions of *P. putida* were incubated in the presence of 1 mM concentrations of D-[U-^{14}C]-4FG (172). The results of 24 hour whole cell incubations in the presence of 1 mM 4FG indicated the release of 95% of the fluoride substituent with $4.8 \pm 0.2\%$ release of the initial radiolabel as $^{14}CO_2$. Borate anion-exchange column chromatography of these 24 hour incubations revealed the presence of 2 radiolabelled metabolites designated a major and minor peak. Subsequent ^{13}C-NMR, ^{1}H-NMR and FAB analysis identified the final metabolite (major peak) as 2,3-dideoxy-D-*glycero*-pentonic acid (2,3-dideoxyribonic acid, 2,3-DDRA). This led to a proposed pathway of 4FG metabolism discussed earlier (Figure 9) (169).

The focus of more recent studies on 4FG metabolism was directed at recovering intermediate metabolites other than 2,3-DDRA in order to provide evidence for the proposed mechanism of 4FG metabolism. Initially in these studies the 'minor peak' originally reported by Sbrissa (169) was studied further. After its isolation by borate anion-exchange column chromatography, the 'minor' peak metabolite was further fractionated by HPLC. The more chromatographically pure sample was analyzed by ^{13}C and ^{1}H-NMR spectroscopy and tentatively identified as the 1,4-lactone of 2,3-DDRA (170). Williams (170) proposed that the lactone form (I) was the predominant species isolated after   initial defluorination and subsequent

decarboxylation of 4FG, however, when subjected to the very alkaline conditions (pH 9.0)

encountered in the borate anion-exchange column, hydrolysis produced the acyclic structure of

2,3-DDRA (II). Further work using cell-free and particle-free extracts of *P. putida* indicated
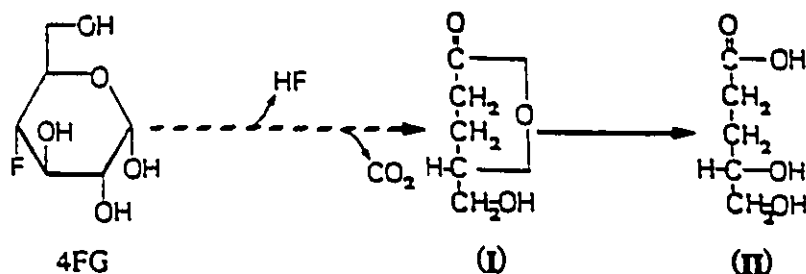
that 4FG is a poor substrate for glucokinase. Under conditions of the glucokinase assay (pH

8.2) 4FG was clearly shown not to be a substrate for phosphorylation. The lack of fluoride

release observed over a 24 hour period in these extracts (1 mM 4FG) was in accordance with the

previous reports (167) and provides further evidence for the contention that fluoride release from

4FG is an extracellular event. Shorter incubation times (40-180 minutes) used to effect the

production of an 'earlier' metabolite other than 2,3DDRA indicated that fluoride release from

4FG is virtually complete within 2-3 hours under the same conditions used by Sbrissa *et al.*

(169). In addition, the release of radiolabel as $^{14}CO_2$ was almost complete after 2 hours which

correlates with the appearance of 2,3-DDRA after 140 minutes (170). Borate anion-exchange

analysis of the supernatants resulting from these incubation time studies indicated the presence

of a putative novel metabolite. Although, follow up ^{13}C and ^{1}H-NMR analysis was insufficient

to provide unequivocal identification of the structure of this 'metabolite X', it allowed for the

tentative structural assignment of 4-deoxy-D-glucose.

The initial focus of these present studies was to generate earlier intermediate metabolites

which would enable the further clarification of the proposed pathway for 4FG metabolism. It was

proposed that a reduction in the rate of whole cell metabolism of 4FG might allow for the

accumulation of one or more intermediate metabolites in the supernatant. It was decided,

therefore, to make use of known patterns of inducibility-repressibility in causing the type of disruptions in metabolism which might result in creating the desired effects. *P. aeruginosa, P. fluorescens* and *P. putida* belong to a group of obligate aerobes that metabolize glucose by the Entner-Doudoroff pathway (163,193). The first is a high affinity system for glucose (K_m = 8 μM) that is inducible by growth on glucose but not on gluconate, succinate or citrate. This system involves the active uptake of glucose into the cell and requires an inducible glucose binding protein component (110,111,194). The second is a low affinity system (K_m = 2-7 mM) that is induced by growth on glucose and gluconate and repressed by succinate, citrate, and other TCA cycle intermediates. This system of glucose transport has a broad specificity and requires glucose dehydrogenase activity to be operative (109,160). The existence of these two distinct pathways for glucose uptake with high and low affinities (K_m = 167 μM and 0.8 mM respectively) has also been demonstrated in glucose-grown cells of *P. putida* (195). In addition it was demonstrated that growth on glucose, gluconate or 2-ketogluconate had no effect on defluorination but that growth on succinate or citrate as carbon sources repressed defluorination (190). Since this pattern of inducibility-repressiblity was reminiscent of that type exhibited by the low affinity transport system it was rationalized that the defluorinating protein may be a component of the said system. Further, it was believed that growth of *P. putida* on gluconate as a carbon source would repress the high affinity system of glucose uptake without eliminating defluorination in a manner that would result in the accumulation of a defluorinated metabolite other than 2,3-DDRA in the supernatant (169). Respirometric comparisons of 4FG oxidation using glucose- and gluconate-grown *P. putida* provided support for this. In the presence of 5 mM concentrations of 4FG the O_2 consumption (indicative of oxidation reactions) of glucose-grown *P. putida* was twice that observed in gluconate-grown cells. After incubation times of 2 and 7 hours, 1.5 and 1.7 μmoles O_2/μmole 4FG were consumed by glucose-grown cells while

0.5 and 0.9 μmoles O_2/μmole 4FG are consumed by gluconate-grown cells. This indicates that

oxidation of 4FG to 4FGA and 4F2KGA occurs to much lower extents in gluconate-grown cells

of *P. putida*. In order to measure quantities of CO_2 evolved as a direct result of 4FG catabolism,

glucose- and gluconate-grown whole cells of *P. putida* were incubated in the presence of D-[U-

^{14}C]-4FG. After incubation periods of 2 hours and 24 hours, in glucose-grown whole cells,

5.18% (2 hour) and 5.3% (24 hour) of the initial radiolabel was released as $^{14}CO_2$. These results

are in direct agreement with those previously published which indicated a release of 4.8% $\pm$

0.2% of the initial radiolabel as $^{14}CO_2$ after a 24 hour incubation (169). The present results also

indicate the almost complete release of $^{14}CO_2$ after 2 hours which correlates with the appearance

of 2,3-DDRA after 140 minutes (170). In the case of the gluconate-grown cells of *P. putida*,

however, approximately 1.95% of the initial radiolabel had evolved as $^{14}CO_2$ in the first 2 hour

period. This value increased to 4.85% after 24 hours. Thus the rate of $^{14}CO_2$ production over

the 2 hour period was significantly reduced in gluconate-grown cells. The O_2 consumption and

CO_2 evolution indicate that in gluconate-grown *P. putida*, rates and extents of oxidation of 4FG

to either 4FGA, 4F2KGA and its metabolism to 2,3-DDRA are significantly reduced.

Large scale incubations of gluconate-grown whole cells of *P. putida* were incubated in

the presence of 1 mM concentrations of D-[U-^{14}C]-4FG and allowed to metabolize the substrate

until ~50% of the fluoride component had been released into the supernatant. At this point, the

supernatant was resolved into four peaks by borate anion-exchange chromatography (Figure 10).

These were referred to as Peaks e, f, g, and h as their elution times were quite different than

those previously reported for 2,3-DDRA (169) or the proposed 1,4-lactone of 2,3-DDRA (170).

These peaks represented putative intermediate metabolites of 4FG. Subsequent thin layer

chromatography and ^{19}F-NMR analysis as well as the elution time from the borate anion-exchange

FIGURE 10

Borate Anion-Exchange Chromatographic Analysis of Radiolabelled Metabolites

in the Supernatant Derived from an Incubation of 1 mM D-[U-^{14}C]-4FG

with Gluconate-grown Whole Cells of *P. putida*

Legend

The supernatant recovered from an incubation of 1 mM D-[U-^{14}C]-4FG with whole cells

of *P. putida* was applied, together with the indicated internal standards to a preparative scale

borate anion-exchange column as outlined in Borate Anion-Exchange Chromatography.

-O-O-   -Radioactivity

-●-●-   -Absorbance at 420 nm

Inorganic phosphate was eluted in fractions 91-164 as observed by Borate Anion-

Exchange Column Chromatography (data not shown). Radioactivity applied was 10 600 ± 100

dpm.

Label	Name	dpm	% Initial Radioactivity
a	4FG	N/A	N/A
b	Glucose	N/A	N/A
c	Glucose-1-phosphate	N/A	N/A
d	Glucose-6-phosphate	N/A	N/A
e	Metabolite 1	1956	18.4
f	Metabolite 2	3751	35.4
g	Metabolite 3	3262	30.8
h	Metabolite 4	1631	15.4

FIGURE 10
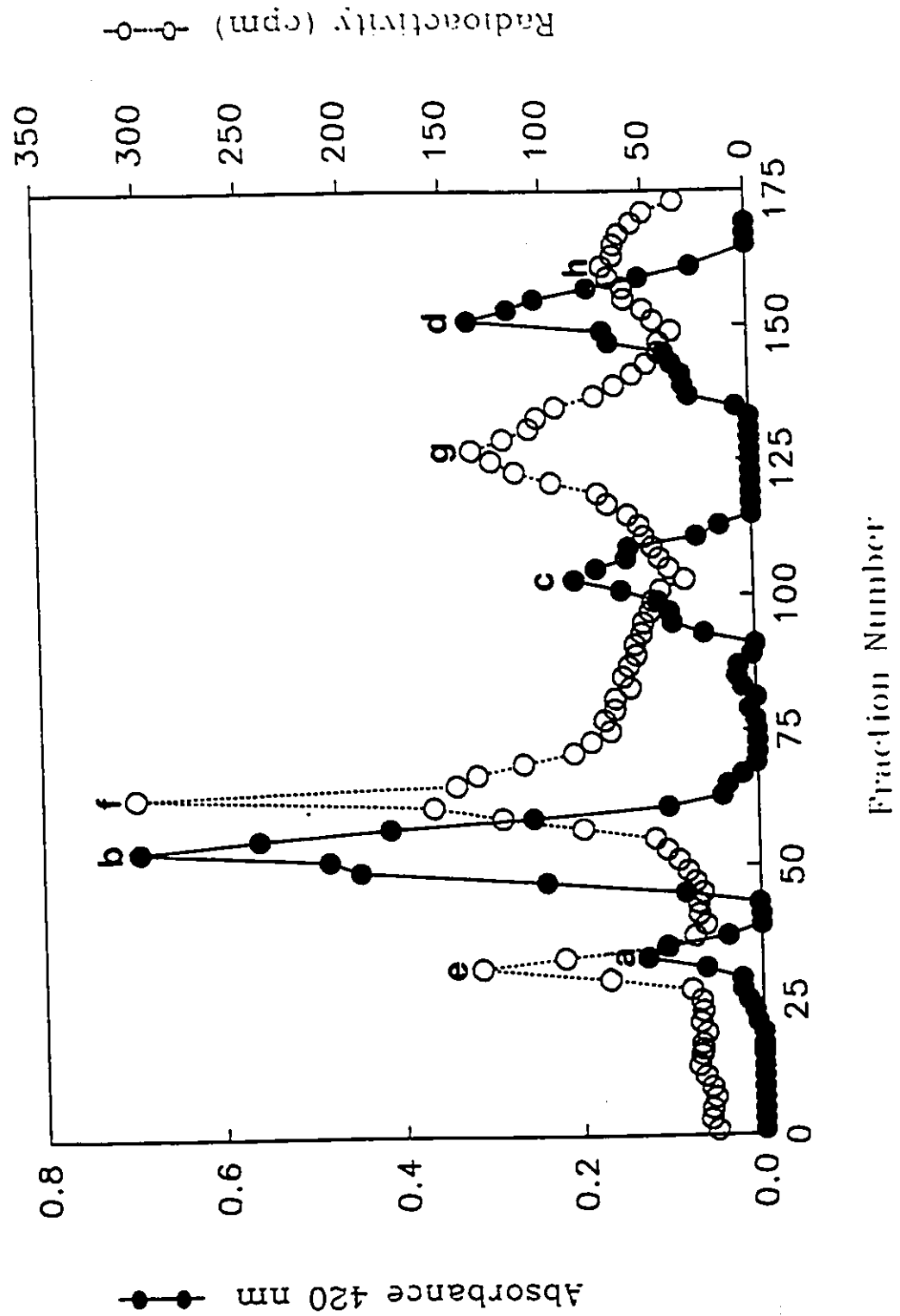
column were used to identify Peak e as 4FG. Peaks f, g, and h gave R_f values of 0.49, 0.58, and 0.74 respectively on thin-layer chromatographic analysis (Figure 11). Under the conditions of a Nelson's test (181), the Peak g metabolite was determined to be a reducing sugar while Peaks f and h were determined to be non-reducing sugars. This indicated that carbon 1 of Peaks f and h may be carboxylic (-COOH) in function while Peak g may be either a hemiacetal or a hemiketal. Furthermore, preliminary analysis of Peaks f and g by proton-decoupled ^{13}C-NMR spectroscopy revealed the presence of 12 to 14 signals for each metabolite. These signals were generally all of the same intensity consistent with the existence of the α and β-anomeric forms of six-carbon intermediate metabolites. In contrast, similar analysis of Peak h produced a set of signals which could be distinguished on the basis of their relative intensities. This allowed the subsequent interpretation of the data permitting the tentative structural designation of Peak h as 3,4-dideoxy-2-keto-D-*glycero*-hexanoic acid (I).

$$
\begin{array}{c}
\overset{O}{\underset{\|}{}} \\
^{1}\text{C-OH} \\
| \\
^{2}\text{C=O} \\
| \\
^{3}\text{CH}_2 \\
| \\
^{4}\text{CH}_2 \\
| \\
\text{H}^{5}\text{C-OH} \\
| \\
^{6}\text{CH}_2\text{OH} \\
\text{(I)}
\end{array}
$$
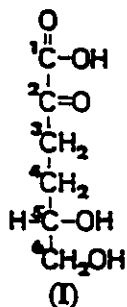
Proton-decoupled ^{13}C-NMR spectra of Peak h produced four intense signals with chemical shifts of 23.9, 30.5, 50.5 and 62.7 ppm (see Appendix XI). Additionally, a relatively weaker signal appeared at 162.8 ppm that could be attributed to the -C=O signal of a carboxyl group. The chemical shifts of these groups appear in the range of 160-180 ppm and are of relatively weak intensities.

Using a Distortionless Enhanced Polarization Transfer (DEPT) 135 program for ^{13}C-NMR provides a spectrum where the methylene (-CH$_2$) groups are shown as negative, *i.e.*, pointing downwards from the baseline of the spectrum (196,197). In contrast, methyne (-CH) and methyl

FIGURE 11

Silica-Gel Thin-layer Chromatographic Analysis of Samples Including

2,3-Dideoxyribonic Acid and the Peak 4 Metabolite Isolated by Preparative

Borate Anion-Exchange Chromatography

Legend

A 40 to 80 minute incubation of glucose-grown whole cells of *P. putida* in the presence

of 1.0 mM D-[U-^{14}C]-4FG (specific activity, 10, 600 dpm/μmole) resulted in approximately 90-

100% release of fluoride anion. Metabolites present in the supernatant were isolated by

preparative borate anion-exchange chromatography as described in Materials and Methods.

Label	Name	Amount Spotted	Rf
a	D-glucose (200 mM)	5 μL	0.62
b	4FG (200 mM)	5 μL	0.68
c	D-gluconate (80 mM)	15 μL	0.61
d	2,3-DDRA (60 mM)	10 μL	0.79
e	Peak h (12 mM)	20 μL	0.74
f	4DOG (78 mM)	15 μL	0.65
g	3FG (80 mM)	15 μL	0.71
h	2-keto-D-gluconate (80 mM)	15 μL	0.31

After development in ethyl acetate-acetic acid-water [3: 3: 1 (v/v)], spots were visualized

by spraying with sulfuric acid-ethanol [1:1 (v/v)] and heating at 140°C for 20 minutes. The lines

expressing the points of origin and the distance travelled by the solvent front were designated as

O and SF respectively.

FIGURE 11
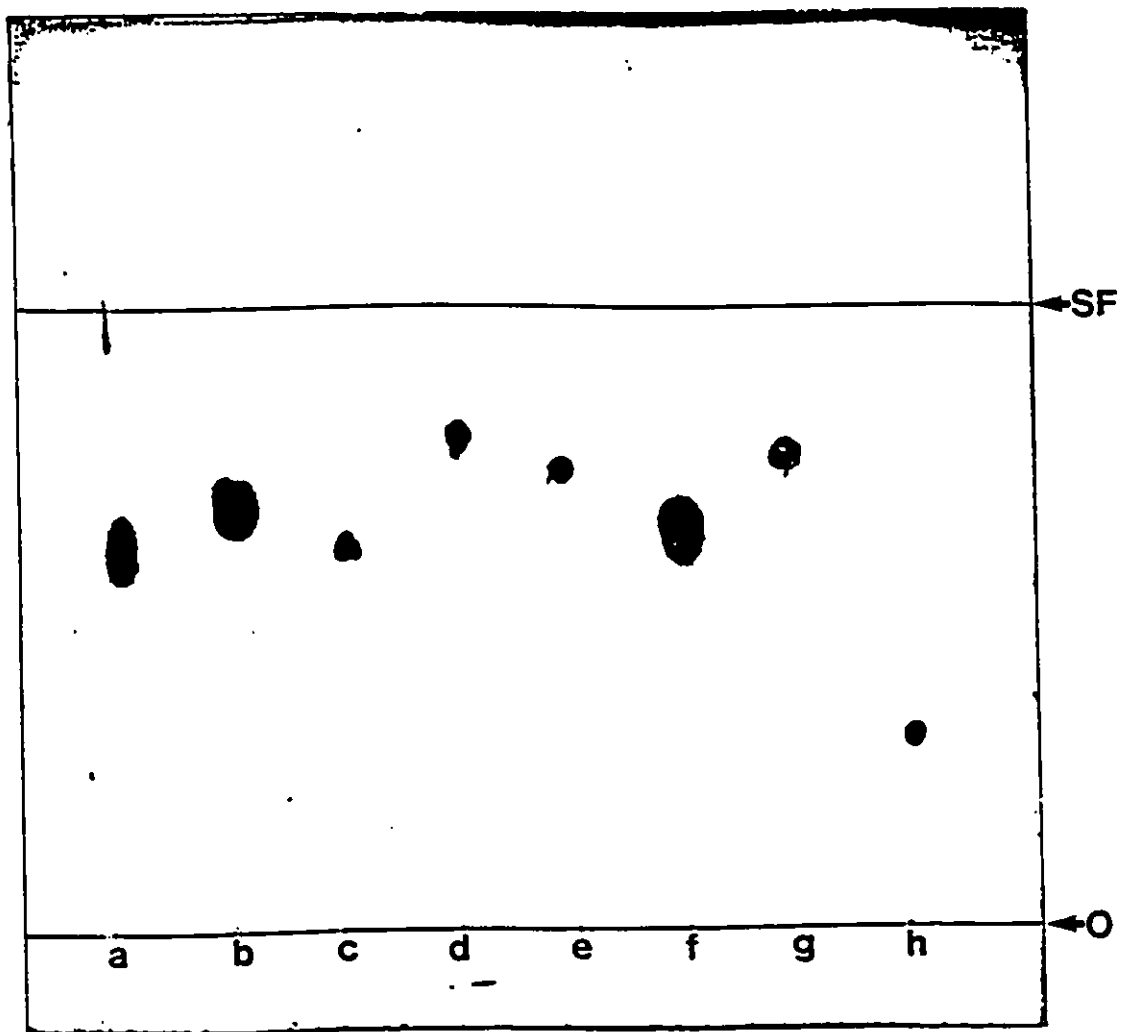
(-CH$_3$) groups appear in the upward direction. Analysis of the data using the DEPT 135 program produced one positive signal at 50.5 ppm (see Appendix XII). The remainder of the signals at 23.9, 30.5 and 62.7 ppm were negative thus allowing carbons 3, 4 and 6 to be designated -CH$_2$ groups. The relatively low chemical shifts indicated that the -CH$_2$ groups were not directly associated with electron-withdrawing substituents although the possibility exhisted that one was associated with a hydroxyl (-OH) group. The data was further analyzed using a DEPT 90 program for ^{13}C-NMR which produces a spectrum where -CH$_3$ and -CH$_2$ signals are nullified and the -CH signals become intensified (196,197). This type of analysis produced a significant increase in the intensity of the signal at 50.5 ppm (see Appendix XIII). This was conclusive evidence for the presence of a -CH group. Although the chemical shift was slightly lower than is normal for carbohydrates in this environment, carbon 5 was designated as -CHOH.

Reaction of the Peak h metabolite with o-phenylenediamine produced a colourimetric reaction with L$_{max}$= 335 nm and a 330/360 nm extinction ratio of 1.36. This is consistent with the formation of a hydroxyquinoxaline derivative of a 2-ketoaldonic acid (45,198). Such a carbonyl group would provide a relatively weak signal in the range of 190-205 ppm not detectable in the ^{13}C-NMR spectra. This would explain why only five signals could be discerned from the original proton-decoupled ^{13}C-NMR spectrum.

Since there was significant uncertainty as to the position of the -CHOH group (50.5 ppm) in the metabolite, a periodate oxidation of Peak h was carried out. Periodate selectively cleaves a molecule between adjacent carbons having a combination of hydroxyls, aldehydes, ketones or primary amine groups. Thus the C-C bonds of cis or $trans$-glycols, hydroxyaldehydes and other combinations common to sugars are oxidatively cleaved by periodate with the stoichiometric reduction of IO$_4^-$ to IO$_3^-$ (181). The reaction of periodate with Peak h produced a positive reaction indicating the presence of a -CHOH group adjacent to the -CH$_2$OH at C-6 of the

metabolite. These results confirmed the structural assignment of 3,4-dideoxy-2-keto-D-*glycero*-hexanoic acid to the Peak h metabolite. The existence of this intermediate metabolite in its phosphorylated form was postulated as a potential precursor to 2,3-DDRA in the previously proposed pathway of 4FG metabolism (Figure 9, metabolite 12). The action of a phosphatase would allow the release of this metabolite into the external environment.

All previous work had revealed that when 4FG is incubated with glucose-grown whole cells of *P. putida*, there was an extensive release of fluoride, but negligible oxidation of 4FG (167). In all these studies no fluorinated metabolite was ever observed using the fluoride electrode at 1 mM concentrations of 4FG. In order to confirm or reject the existence of such intermediate metabolites of 4FG, *in vivo* ^{19}F-NMR spectroscopy was used to track the pattern of metabolism in glucose- and gluconate-grown cells at higher concentrations of 4FG. Moreover, repression of the glucose transporter system occurring in gluconate-grown cells may result in the accumulation of any putative fluorinated metabolites. The results (Figure 12) indicate, that in glucose-grown cells of *P. putida*, at 5 mM concentrations of 4FG, fluoride release is instantaneous concomitant with the appearance of 4-deoxy-4-fluoro-D-gluconic acid (4FGA). Subsequent metabolism of 4FGA to 2-keto-4-deoxy-4-fluoro-D-gluconic acid (4F2KGA) is seen as the metabolite makes an appearance 30 minutes into the incubation reaction. Thin-layer chromatographic analysis of the supernatants corresponding to these incubation times revealed the presence of a single spot (R_f= 0.45) for each trial with identical R_f values. This is in accordance to previously reported R_f values given for 4FGA and 4F2KGA (R_f= 0.42) (190). Further, ^{19}F-NMR observations of the metabolic process indicate that 4FGA and 4F2KGA are also good substrates for the defluorinating protein as their observeable peaks decrease to undetectable levels concomitant with an increase in the fluoride peak. This seems to suggest that the protein effecting fluoride release is a common one for all substrates. The existence of separate

FIGURE 12

In Vivo ¹⁹F-NMR Trace Studies of 4FG Metabolism in

Glucose-Grown Cells of *P. putida*

Legend

An *in vivo* ¹⁹F-NMR trace study of 4FG metabolism in the region of -115.0 ppm and -

210.0 ppm with proton coupling for the first 195 minutes. Experimental conditions were as

described in *In Vivo* Trace Studies of 4FG Metabolism using ¹⁹F-NMR. The whole cells of

P. putida were incubated at 30°C with constant shaking between each NMR recording time.

The number of scans for each spectrum was 500-1000, recycle delay time was 0.5 seconds. The

line broadening factor was 15 Hz for all spectra. In addition data for all spectra was collected

at 30°C. In order of their appearance signals for

    (a)   4FG (2 sets of peaks, doublets each at -198.2 ppm, -198.5 ppm, -200.2 ppm, -

            200.5 ppm)

    (b)   4FGA (-208.1 ppm)

    (c)   fluorine (-118.2 ppm)

    (d)   4F2KGA (single peak split into 2, -201.5 ppm, -201.8 ppm)

FIGURE 12
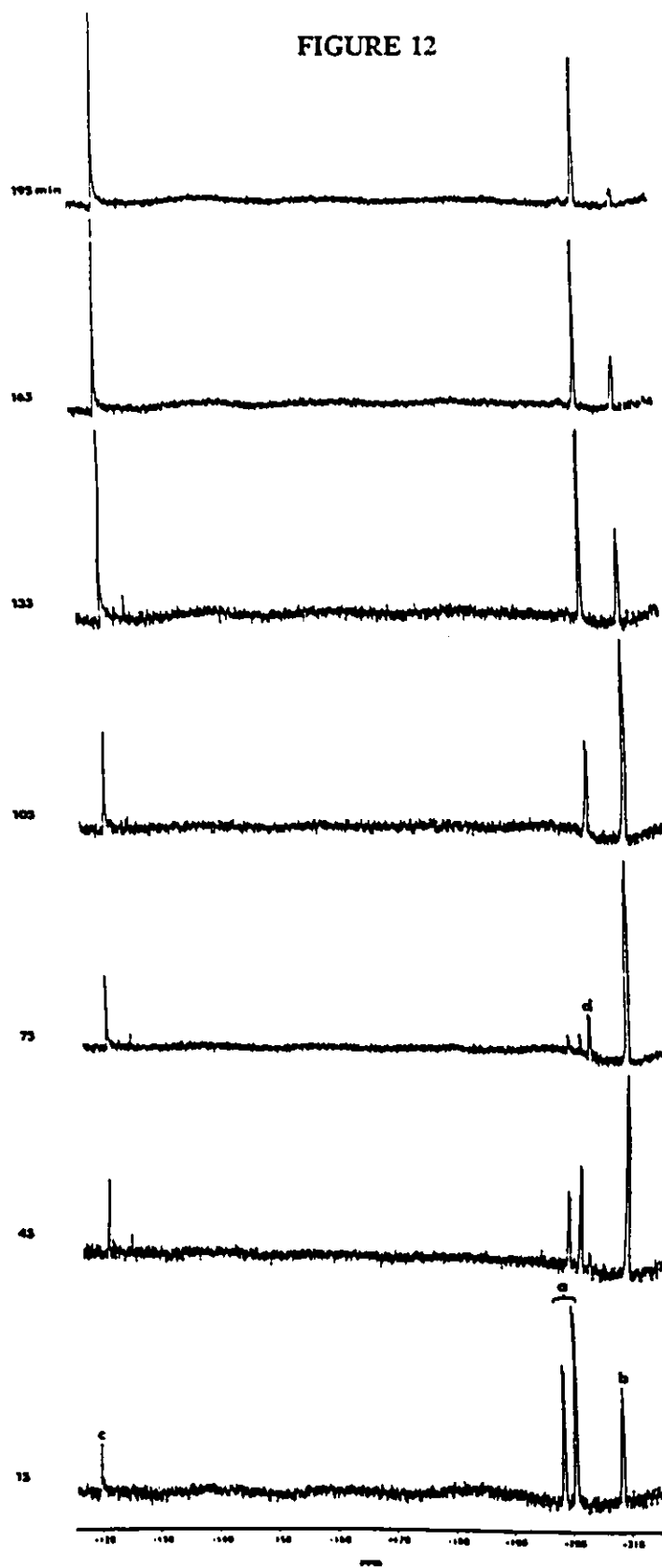
carrier protein components for glucose (K_t= 167 μM, V_{max}= 0.8 nmol/mg protein/min), gluconate (K_t= 0.5μM, V_{max}= 1.0 nmol/mg protein/min) and 2-ketogluconate (K_m= 110.0 $\pm$ 2.9 μM, V_{max}= 0.55 $\pm$ 0.04 nmol/mg protein/min) have been demonstrated using cytoplasmic membrane vesicles prepared from P. putida (162,164) which suggests that the defluorinating protein is distinct and separate from those directly responsible for transport.

In gluconate-grown cells of P. putida the metabolism of 4FG occurs via an identical route but the timing sequence is slightly altered (Figure 13). 4FGA appears after 45 minutes followed by the appearance of 4F2KGA after 120 minutes but fluoride release is not evident until 135 minutes into the reaction. Growth of whole cells of P. putida on gluconate as the carbon source have been shown to have a slight repressive effect on defluorination and present results confirm these previous observations. Other than the presence of 4FGA and 4F2KGA no other fluorinated metabolites were observed in either of the incubations. Defluorination can therefore be seen as a one step process involving the C-F bond cleavage at C-4. This is in contrast to results obtained using the isomeric 3FG, where oxidation was demonstrated, with retention of the C-F bond (45). Both 4FG and 3FG have been found to be poor substrates for oxidation by the membrane-bound glucose dehydrogenase in membrane vesicles with K_m values of 21 mM (190) and 25 mM (195) respectively as compared to 0.8 mM for glucose. In the present studies a 5 mM concentration of 4FG was used which enabled defluorination to occur simultaneously with the oxidation of 4FG by glucose oxidase producing 4FGA which was in turn acted upon by gluconate dehydrogenase producing 4F2KGA.

The effects of osmotic shock on whole cell defluorination were next examined. Crude periplasmic extracts of glucose-, gluconate-, and succinate-grown P. putida were obtained by the shock method of Stinson et al. (110,111) with a slight modification. Previous studies (190,199) using crude periplasmic extracts from glucose-

FIGURE 13

In Vivo ^{19}F-NMR Trace Studies of 4FG Metabolism in

Gluconate-Grown Cells of *P. putida*

Legend

An *in vivo* ^{19}F-NMR trace study of 4FG metabolism in the region of -115.0 ppm and -210.0 ppm with proton coupling for the first 195 minutes. Experimental conditions were as described in *In Vivo* Trace Studies of 4FG Metabolism using ^{19}F-NMR. The whole cells of *P. putida* were incubated at 30°C with constant shaking between each NMR recording time. The number of scans for each spectrum was 500-1000, recycle delay time was 0.5 seconds. The line broadening factor was 15 Hz for all spectra. In addition data for all spectra was collected at 30°C. In order of their appearance signals for

(a)   4FG (2 sets of peaks, douu!cs each at -198.2 ppm, -198.5 ppm, -200.2 ppm, -200.5 ppm)

(b)   4FGA (single peak split into 2, -201.5 ppm, -201.8 ppm)

(c)   fluorine (-118.2 ppm)

(d)   4F2KGA (-208.1 ppm)

FIGURE 13
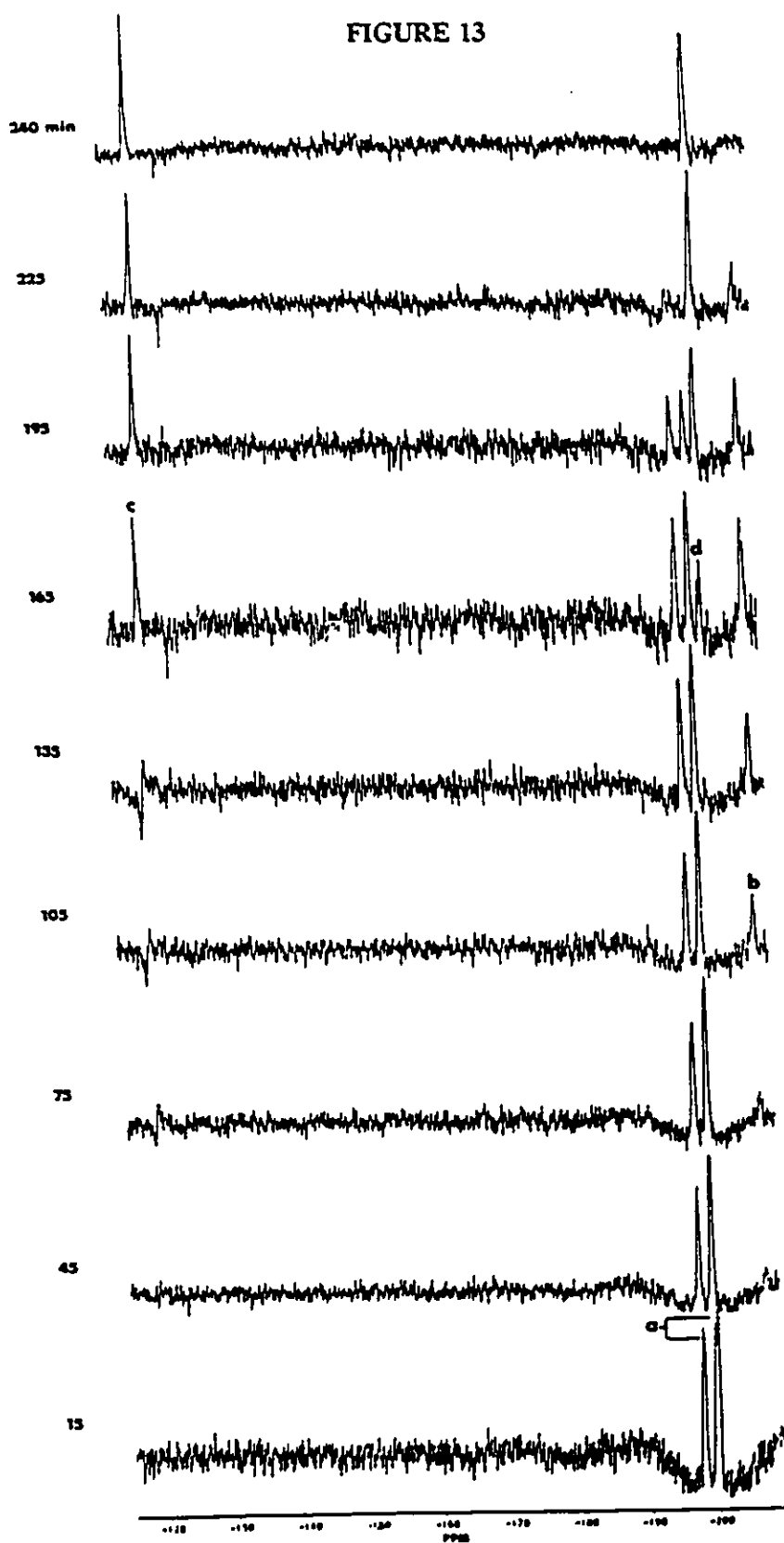
grown *P. putida* have shown the absence of any defluorinating activity in these preparations. Furthermore, it had been shown (190) that any inherent glucose-binding activities in these preparations were negligible (0.04-0.13 nmol glucose/mg protein using a concentration range of 2.5-10 μM D-[6-^{3}H]-glucose) (190,191) when compared to the values reported for the pure glucose-binding protein (GBP) of *P. aeruginosa* (1 mol glucose/mol GBP, K_D=0.35 μM) (111). More recently, work with crude periplasmic extracts from *P. putida* indicate that glucose-binding activity is concentration dependent (199). A range of 0.1 to 0.2 μM D-[6-^{3}H]-glucose resulted in binding activities ranging from 0.143 to 0.613 nmol/mg protein. These values are approximately 3 to 5 times greater than those given previously.

An underlying problem in all previous studies of periplasmic binding protein has been the rapid decrease and subsequent extinction of binding activity as a function of time even in samples stored at -20°C. Gram-negative bacteria are known to contain an abundance of periplasmic proteases (148,182) and so it is conceivable that any protein isolated from the periplasm would be rapidly degraded resulting in the inactivation of glucose binding and/or defluorinating properties. For this reason, the protease inhibitor, PMSF (final concentration 0.5 mM) was included in all steps of the osmotic shock procedure (110,111). Due to the significant glucose-binding activity observed using crude periplasmic extracts *P. putida* (199) it was of interest to see whether the binding could be used in distinguishing the glucose-binding protein from the other components of the crude periplasmic extract. Western blotting assays were attempted using urea gels run under non-reducing conditions (outlined in Urea Gel Electrophoretic Analysis) and polyacrylamide gels run under non-denaturing conditions (outlined under Non-Denaturing Gel Electrophoresis). The results indicated a large amount of non-specific binding and no single band demonstrating glucose-binding was observed. As with the previous reports, crude periplasmic extracts from glucose-, gluconate-, and succinate-grown *P. putida* contained

no defluorinating properties as indicated by fluoride electrode and ^{19}F-NMR spectroscopy. SDS-PAGE analysis of these extracts (Figure 14) revealed the presence of a glucose inducible and succinate repressible protein band with an apparent molecular weight of 44 kDa. These results are very similar to those reported for the glucose-binding protein of *P. aeruginosa* (M_r = 44.5 kDa) which displayed similar induction-repression characteristics (111).

Inhibition studies carried out with crude periplasmic extracts of *P. aeruginosa* and *P. putida* indicate that 4FG competetively inhibits binding of glucose in *P. aeruginosa* but not in *P. putida* (199). Furthermore, 3FG had no effect on binding in either case. This suggests some type of structural differences at least at the glucose binding sites in *P. aeruginosa* and *P. putida*. 3FG and 4FG are both glucose analogues in which the C-3 or C-4 hydroxyl group has been replaced by a fluoride atom. The characteristic feature altered by this substitution is that of hydrogen bonding. Hydrogen bonding is the dominant characteristic in protein carbohydrate interactions. With the exception of the anomeric hydroxyl group which participates solely as a hydrogen bond donor, all the hydroxyl groups of the sugar may serve simultaneously as hydrogen bond donors and acceptors (200). The fluoride substituent may act only as a hydrogen bond acceptor whereas the hydroxyl group is capable of acting as both an acceptor and a donor of hydrogen bonds (16). The results in *P. putida* indicate that 4FG and 3FG are not bound by the glucose binding protein. This suggests that hydrogen bond donors are essential at both carbons 3 and 4. Since C-F bond cleavage in *P. putida* occurs only with 4FG but not 3FG this suggests that in the case of the defluorinating protein a hydrogen bond donor is required at carbon 3 while a hydrogen bond acceptor is essential at C-4. Taken together, the results with *P. putida* indicate that the binding of a periplasmic protein to 4FG is not an essential prerequisite for defluorination. The fact that defluorination of 4FG occurs in 'shocked' cells of *P. putida*, in which the GBP is absent, supports this contention (Table 5). According to Ames (114)

FIGURE 14

10% SDS-PAGE of Periplasmic Extracts from Glucose-,

Gluconate-, and Succinate-Grown *P. putida*

Legend

Aliquots (25 µL) of crude periplasmic protein extracts were obtained as outlined in

Osmotic Shock of Whole Cells. Samples were subjected to 10% SDS-polyacrylamide gel

electrophoresis (100 V, 2.5 hours) and stained with coomassie Brilliant Blue R-250 as outlined

in **SDS-PAGE** analysis.

Lanes 4 and 8:   Low molecular weight (MW) protein standards (Phosphorylase b:

97.4 kDa; bovine serum albumin (BSA): 66.2 kDa; ovalbumin: 45

kDa; bovine carbonic anhydrase: 31 kDa; soybean trypsin inhibitor:

21.5 kDa; hen egg white lysozyme: 14.4 kDa)

Lanes 1,2 and 3:   Crude periplasmic extract obtained from succinate-, gluconate-, and

glucose-grown *P. putida* (12.5 µg protein in each lane) as obtained

by 60-95% ammonium sulfate precipitation

Lanes 5, 6 and 7:   Crude periplasmic extract obtained from succinate-, gluconate-, and

glucose-grown *P. putida* (15 µg protein in each lane) as obtained by

0-60% ammonium sulfate precipitation.

FIGURE 14
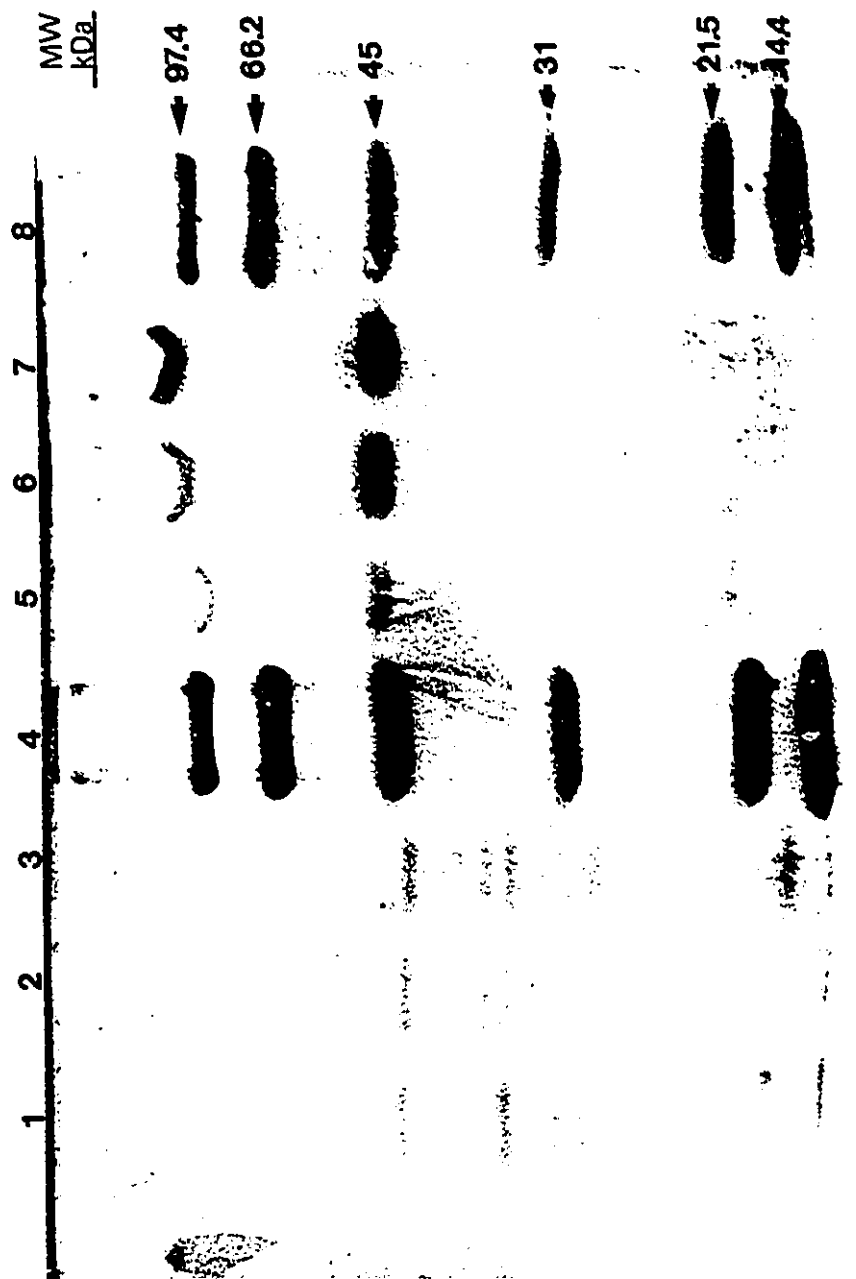
TABLE 5

Defluorination of 4FG by Osmotic Shock Components of Whole Cells of *P. putida*

Time	Glucose-Grown		Gluconate-grown		Succinate-grown		Shock-fluid		
	untreated	shocked	untreated	shocked	untreated	shocked	Glucose	Gluconate	Succinate
				% Defluorination					
0	1.55	1.2	1.6	1.8	1.6	3.0	0.6	0.6	0.6
20	19.4	22.1	15.7	6.5	2.9	3.9	0.8	0.7	0.70
40	36.7	58.3	24.5	9.2	4.2	5.1	0.9	0.90	0.9
60	42.85	88.65	31.5	20.6	5.6	6.9	1.1	1.1	1.1
80	55.8	90.6	36.2	32.5	7.5	8.7	1.0	1.1	1.15
100	68.0	92.6	47.1	44.8	8.3	10.3	1.15	1.20	1.15
120	69.6	92.7	55.5	64.7	10.1	12.5	1.2	1.2	1.2
140	72.5	92.7	57.8	66.3	12.1	13.2	1.2	1.2	1.2

Whole cells of *P. putida* grown on various carbon sources were shocked by the method of Stinson *et al.* (110,111) as outlined under Osmotic Shock of Whole Cells. The components: a) osmotically shocked cells or b) 'shock fluid' were then assayed for defluorinating activity. Concentration ratios of reactants were a) 1 μmole 4FG/10 mg whole cell protein/mL and b) 3 μmole 4FG/1 mg crude periplasmic extract protein/0.5mL.

shock sensitive systems are inactivated during osmotic shock due to the loss of an essential

protein component that binds the transported solute with high affinity. It is reasonable then to

describe defluorination as a shock resistant occurrence. If defluorination is indeed shock resistant

then it may be coupled to one or both components of the proton-motive force (201,202).

Previous experiments indicated that whole cell incubations with N,N'-dicyclohexylcarbodiimide

(DCCD) or arsenate have no effects upon defluorination (170). Consequently, the involvement

of ATP and/or a membrane-bound ATPase was considered unlikely. Subsequent experiments

demonstrated that incubation of glucose-grown whole cells of *P. putida* with 2,4-DNP (2 mM)

or sodium azide (10 mM) prior to the addition of 4FG virtually eliminated defluorination (170).

Both of these substances interfere with a proton gradient (ΔpH) either by mediating proton

conductance back across the inner membrane (2,4-DNP) and eliminating ΔpH, or by binding to

cytochrome a_3 of the cytochrome c oxidase complex and preventing the generation of ΔpH

(sodium azide). Hence it was concluded that a proton gradient (chemiosmotic energy couple) was

the driving force for defluorination. However, the proton-motive force (Δp) is made up of two

components, a pH gradient (ΔpH) and a membrane potential ($\Delta\Psi$), such that $\Delta p = \Delta\Psi - (2.303$

$RT/F) \Delta pH$ (202). It may well be that whole cell incubations with 2,4-DNP and sodium azide

not only abolished the ΔpH component but the membrane potential as well if this component was

dependent on a pH gradient. In order to provide conclusive evidence for the essential nature of

either or both components of Δp, the effects of the ionophores nigericin and valinomycin upon

whole cell defluorination were observed. Valinomycin is a well characterized neutral ionophore

that specifically induces K^+ conductivity in cell membranes (2,113) promoting a collapse of the

membrane potential ($\Delta\Psi$) without affecting the proton gradient. Results (Table 6) indicate that

valinomycin has negligible effects upon defluorination in whole cells. Even at concentrations as

high as 15 and 25 μM the only effects observed can be attributed to ethanol

TABLE 6

Effects of the Neutral Ionophore Valinomycin on the Defluorination of

4FG by Glucose-Grown Whole Cells of *P. putida*

Time (min)	Control 1 1 mM 4FG	Control 2 5 μL EtOH	Control 3 10 μL EtOH	Control 4 15 μL EtOH	Valinomycin		
					5 μM	15 μM	25 μM
			% Defluorination				
20	42.9	13.5	13.2	12.8	13.8	15.6	13.5
40	67.2	29.8	27.5	25	39.5	34.3	29.8
60	92.9	43	41.2	40.5	53.9	47.5	43.8
80	92.9	59.4	57.1	52.5	69.8	64.5	59.5
100	92.9	75.9	72.8	69.5	79.1	77.4	72.8
120	94.8	82.3	80.6	78	90.5	89.2	84
140	94.8	91.1	82.3	80.5	93	91.1	87.4

Whole cell suspensions (3.0 mL, 30 mg protein) of glucose-grown *P. putida* were incubated in 50 mM potassium phosphate buffer, pH 7.5, with 4FG (Control 1); with various volumes of ethanol (controls 2-4) and varying final concentrations of valinomycin in ethanol. All incubations were pre-incubated for 30 min at 30°C in the presence of the ionophore or ethanol prior to the addition of 4FG (final concentration, 1 mM). Fluoride release was measured as described under Fluoride Ion Measurements.

poisoning of the cells which was minimal (controls 2-4, Table 6). However, results of whole

cells defluorination in the presence of nigericin (Table 7) indicate a maximum inhibition of 30.3%

at concentrations of 20 μM nigericin. Nigericin exchanges H^+ ions for K^+ ions resulting in the

collapse of ΔpH while preserving the $\Delta \Psi$ component of Δp. This provides unequivocal proof

for the essential nature of a pH gradient component for the energization of defluorinating

function. In order to distinguish between the direct effects (collapse of ΔpH) versus indirect

effects (inactivation by modification of defluorinating protein) of nigericin treatment, the

reversible nature of the ionophore was examined. Results (post-nigericin, Table 7) indicate that

the observed effects caused by treatment with nigericin are reversible since defluorination of these

whole cells was practically identical to the untreated controls. Due to the very reversible nature

of nigericin treatment it is most likely that the effects observed were due to a collapse of ΔpH

as opposed to some sort of covalent modification of the defluorinating protein which for all

intents and purposes would have been irreversible.

The ^{19}F-nucleus shows spectroscopic behaviour similar to that of the ^{1}H-nucleus (Table

8). It also offers such important benefits as : the isotopic abundance of the nucleus, the high

gyromagnetic ratio and relevant spectroscopic parameters which can be readily extracted from

the spectra. In a recent review Csuk (203) described the ^{19}F-nucleus as a probe that is very

sensitive to changes in the steric and electronic environment. Further he described that extensive

investigations of single groups of fluorinated carbohydrates have not only provided fundamental

information for establishing the structures of the fluorinated derivatives, but have also shown ^{19}F

to be a very sensitive probe and an "amplifier" for showing conformational distortions and

mobility. In light of these attractive characteristics of the ^{19}F-nucleus it was considered possible

that the fluoride substituent at C-4 could be exploited to gauge initially, any binding affinity for

4FG which may exist in the crude periplasmic extracts and subsequently any conformational

TABLE 7

Effects of the Carboxylic Ionophore Nigericin on the Defluorination of 4FG by

Glucose-Grown Whole Cells of *P. putida*

Time (min)	Control 1 1 mM 4FG	Control 2 5 μL EtOH	Control 3 10 μL EtOH	Control 4 15 μL EtOH	Nigericin				Post-Nigericin	
					5 μM	10 μM	15 μM	20 μM		
				% Defluorination						
20	42.9	13.5	13.2	13.8	18.7	15.2	12.5	14.1	25.3	27.5
40	67.2	29.8	27.5	25	33.7	28	27.5	28.5	50.5	41.2
60	92.9	43	41.2	40.5	51.6	43.8	38	37.3	69.9	69.9
80	92.9	59.4	57.1	52.5	59.4	48.7	48.7	42.1	85.7	82.3
100	92.9.	75.9	72.8	69.5	69.9	59.4	58.2	59.5	89.2	85.7
120	94.8	82.3	80.6	78	69.9	63.2	67.2	58.2	92.9	89
140	94.8	91.1	82.3	80.5	71.4	69.9	68.5	64.5	93.5	90.5

Whole cell suspensions (3.0 mL, 30 mg protein) of glucose-grown *P. putida* were incubated in 50 mM potassium phosphate buffer, pH 7.5, with 4FG (Control 1) (Control 1); with various volumes of ethanol (Controls 2-4) and varying final concentrations of nigericin in ethanol. Post-nigericin recovery of defluorination was also assayed as described in Ionophore Incubation of Whole Cells. All incubations were pre-incubated for 30 min at 30°C in the presence of the ionophore or ethanol prior to the addition of 4FG (final concentration, 1 mM). Fluoride release was measured as described under Fluoride Ion Measurements.

TABLE 8

Comparison of the Relevant Parameters of Different Nuclei

Nuclei	Spin	Natural Abundance	Sensitivity		Range[b]	Gyromagnetic Ratio	Frequency[c]
			Relative	Absolute[a]			
^1H	½	99.98	1000	1000	20	2.793	100
^{13}C	½	1.11	15.9	0.18	650	0.702	25.14
^{19}F	½	100	830	830	800	2.627	94.08
^{15}N	½	0.37	1.04	0.004	1000	0.283	10.13

a = Relative sensitivity: at constant field for equal number of nuclei; absolute intensity: product of relative sensitivity and natural abundance

b = In parts per million (ppm)

c = For a field strength of 2.3480 tesla.

Adapted from reference (203).

changes in the 4FG molecule caused by this binding. The results obtained indicate a change in chemical shifting of 2 ppm observed with both α and β-anomeric forms of 4FG in the presence of periplasmic extracts. This was not significant in light of the large range (800 ppm) of the fluorine atom which meant that the shifting observed occurred as 0.25% of that total range. The inability to detect any binding interactions with the periplasmic extracts supports earlier indications that 4FG was a very poor inhibitor of glucose binding (199). This does not minimize the value of ^{19}F-NMR spectroscopy in studies of protein or enzyme binding to fluorinated substrate analogues (19). Since an outer membrane (192) or periplasmic protein had been ruled out as the causitive agent of defluorination (199), attention became focused on the cytoplasmic membrane. The cytoplasmic membrane houses the majority of enzyme components associated with energy coupling (cytochromes, oxidases, dehydrogenases, ubiquinols) as well as the ATP synthase and ATPase systems. Since the C-F bond (485 KJ mol^{-1} or 116 Kcal mol^{-1}) is a high energy bond, it was reasonable to assume that C-F bond cleavage was carried out by a protein component which was very closely associated with the components of energy coupling. Initially, cytoplasmic membrane vesicles from glucose-grown *P. putida* were fabricated according to a procedure described for *P. aeruginosa* (183). The resultant vesicles have previously been characterized for orientation (75% right-side out) and intravesicular volume (3.6 μL/mg protein) (162,164). Incubation of these vesicles (protein concentration 10-20 mg, final volume 3 mL) in the presence of 1 mM concentrations of 4FG produced no observable fluoride release. However, *in vacuo* concentration of the supernatants from these reaction mixtures followed by ^{19}F-NMR analysis indicated peaks at 198 and 200 ppm (4FG), 207.4 ppm (4FGA), and 201 ppm (4F2KGA). The lack of fluoride release agrees with previous results which also indicated that these types of vesicles, generated from *P. putida* grown on glucose as sole carbon source, oxidized 4FG to 4FGA and 4F2KGA with a consumption of 1 μmole O$_2$/μmole 4FG (167).

However these studies were never performed in the presence of electron donors. It was thought that coupling of the defluorination reaction to electron donor oxidation via the respiratory chain would, according to chemiosmotic theory (128,204,205), generate the necessary Δp required to increase any defluorination reactions which would be detectable by fluoride electrode. Subsequent incubations were carried out in the presence of 10 mM L-malate/50 μM FAD under the same conditions used previously. Again there was no detectable fluoride release. *In vacuo* concentration of these supernatants, however, followed by ^{19}F-NMR spectroscopy indicated the presence of the oxidation products 4FGA and 4F2KGA and in addition a single peak at -118.8 ppm which corresponded to a fluoride ion signal. From these studies it was concluded that the defluorinating protein was somehow removed during the fabrication of the cytoplasmic membrane vesicles. This protein is not considered to be very tightly associated with the cytoplasmic membrane since membrane-bound enzymes such as glucose oxidase and gluconate dehydrogenase as well as other membrane associated proteins such as the transport proteins for glucose, gluconate, and 2-ketogluconate were unaffected by vesicle preparation (162,164).

A slightly modified procedure published by Newman and Wilson (184) describing the generation of high pressure vesicles from cells of *Escherichia coli* was used in an attempt to generate a cytoplasmic membrane preparation from *P. putida* which still contained fluoride release activity. The fact that these cytoplasmic membrane preparations were never characterized precluded use of the term "membrane vesicles". Since the membrane studies mainly concerned the reconstitution of defluorinating activity in liposomes, this type of characterization was not considered to be important. After each major step in the procedure described under Cytoplasmic Membrane Preparation, aliquots were tested for fluoride release activity. Results (Figure 15) indicate that as the procedure progresses from the whole cell to the extraction of membrane protein there is a concomitant decrease in fluoride release from 4FG (1 mM). Also evident is

FIGURE 15

Fluoride Release Capabilities of the Various Fractions Produced by

French Pressure Cytoplasmic Membrane Preparation

Legend

Cytoplasmic membrane fractions were produced by the French pressure technique of Newman and Wilson (184) as outlined under Cytoplasmic Membrane Preparations.

○ - Fluoride release in glucose-grown whole cells of *P. putida*. Cell suspensions were prepared as outlined under Preparation of Whole Cell Suspensions. The incubation mixture consisted of 1 μmole 4FG/10 mg whole cell protein/mL 50 mM potassium phosphate buffer, pH 7.5.

▽ - Fluoride release in cytoplasmic membrane fractions incubated with 4FG in the absence of electron-donors. The incubation mixture consisted of 1 μmole 4FG/5 mg membrane protein/mL 50 mM potassium phosphate buffer, pH 7.5.

● - Fluoride release in cytoplasmic membrane fractions incubated in the presence of electron-donors. Incubation mixtures consisted of 1 μmole 4FG/5 mg membrane protein/mL 50 mM potassium phosphate buffer, pH 7.5, 12 mM $MgSO_4$,10 mM-L-malate/50 μM FAD

▼ - Cytoplasmic membrane residue generated by extraction with n-octylglucoside as outlined under Extraction of Cytoplasmic Membrane Proteins. Incubation mixtures consisted of 1 μmole 4FG/5 mg protein/mL 50 mM potassium phosphate buffer, pH 7.5, 12 mM $MgSO_4$, 10 mM-L-malate/50 μM FAD.

Fluoride release was initiated by the addition of 4FG. Subsequent incubation occurred at 30°C with shaking. Fluoride release was measured at 20 minute intervals as oulined in Fluoride Ion Measurements .

FIGURE 15
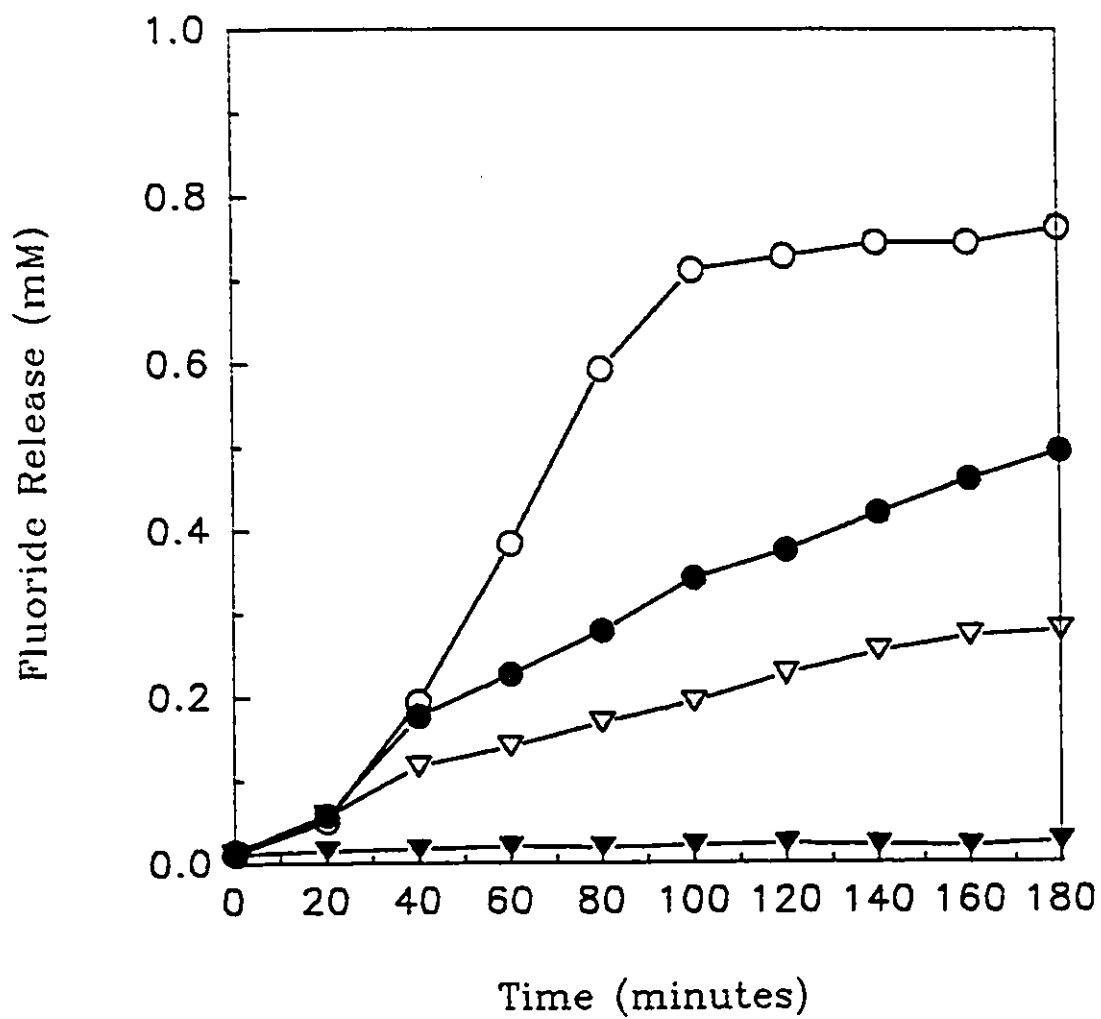
the increase in fluoride release when cytoplasmic membrane fractions are incubated in the presence of electron donors (10 μM L-malate/50 μM FAD) prior to the introduction of 4FG. This observed increase in defluorination which is coupled to the oxidation of natural electron donors provides evidence for the existence of some kind of structural integrity, not unlike that of a vesicle, which is capable of generating and sustaining a proton gradient in order to power C-F bond cleavage. Defluorination is non-existent in residual membrane proteins after n-octylglucoside extraction of the cytoplasmic membrane even in the presence of electron donors. This may be a direct result of the removal of the defluorinating protein or some membrane component essential for chemiosmotic energy coupling. Of relative importance was the rapid degradation of the solubilized protein. Once extracted, the membrane proteins began to break down with concomitant loss of activity. This was observed on SDS-PAGE analysis of a 1 week old preparation of solubilized membrane protein which had been frozen in liquid nitrogen and stored at -20°C. A very noticeable decrease in banding coupled with an increase in fragmentation of protein migrating further in the gel was indicative of this degradation. Incorporation of these membrane preparations into liposomes failed to reconstitute fluoride release activity, even in the presence of electron donors. The membrane extracts, presumed to contain the defluorinating protein, became the focus of follow-up studies.

SDS-PAGE analysis of the crude membrane extracts indicated the existence of a glucose-inducible, gluconate- and succinate-repressible protein band with an apparent molecular weight of 65.4 kDa (Figure 16). As it was the only band that exhibited this type of induction-repression character it will be referred to as the putative defluorinating protein. This band lacked any observable type of heat-modifiable character when heated to 95°C for 5 to 15 minute intervals in the presence of reducing agents (Figure 17) thus distinguishing it from the outer membrane protein (OprB) previously characterized as being heat-modifiable (106). In the absence of

FIGURE 16

12% SDS-PAGE of Cytoplasmic Membrane Extracts from Glucose-,

Gluconate- and Succinate-Grown *P. putida*

Legend

Aliquots (50-70 μL) of membrane protein extracts were obtained as outlined in
Extraction of Cytoplasmic Membrane Proteins. Samples were subjected to 12% SDS-
polyacrylamide gel electrophoresis (90 V, 7 h) and stained with Coomassie Brilliant Blue R-250
as outlined in SDS-PAGE analysis.

|	
Lane 1:	High molecular weight (MW) protein standards (Myosin: 205 kDa; β-galactosidase: 115 kDa; phosphorylase B: 97.4 kDa; bovine serum albumin (BSA): 66 kDa; ovalbumin: 45 kDa; carbonic anhydrase: 29 kDa)
Lane 5:	Mixture of high and low molecular weight (MW) protein standards which consisted of the above as well as Glyceraldehyde-3-phosphate dehydrogenase: 36 kDa; trypsinogen: 24 kDa; trypsin inhibitor: 20.1 kDa; α-lactalbumin: 14.2 kDa
Lanes 2,3,4:	Crude membrane extracts from glucose-, gluconate- and succinate grown *P. putida* (350 μg protein in each lane) as obtained by n-octylglucoside extraction of membrane preparation.

FIGURE 16
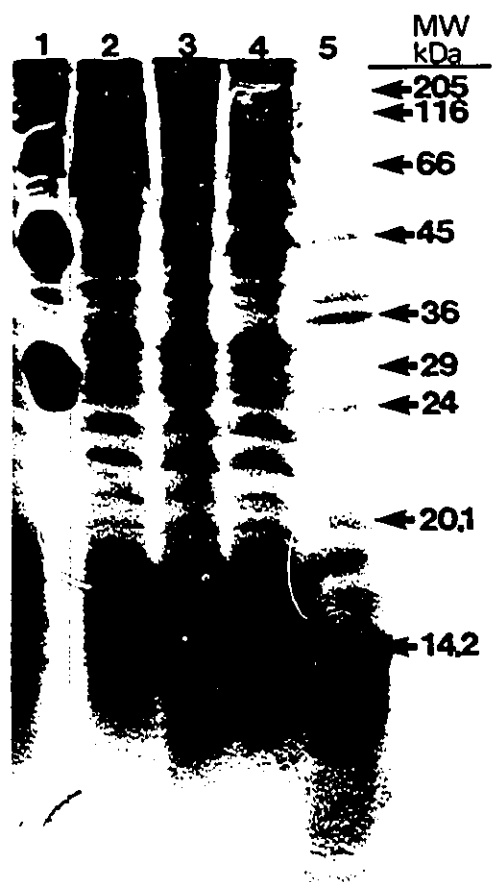
FIGURE 17

Heat-Modifiable Mobilities of Defluorinating Protein from *P. putida*

Cytoplasmic Membrane as Observed by 12% SDS-PAGE Analysis

Legend

Aliquots (50-70 μL) of membrane protein extracts were obtained as outlined in

Extraction of Cytoplasmic membranes Proteins. Samples were combined with equal volumes

of sample buffer [0.063 M Tris-HCl, pH 6.8, 6% (w/v) SDS, 10% (v/v) glycerol, 200 mM

dithiothreitol, 0.002% (w/v) bromophenol blue] and heated at 95°C for 5, 10, and 15 minute

intervals. The samples were then subjected to 12% SDS polyacrylamide gel electrophoresis

(90V, 7h) and stained with Coomassie Brilliant Blue R-250 as outlined in SDS-PAGE Analysis.

Lanes 4, 8, 13: Low molecular weight (MW) protein standards (β-galactosidase: 116 kDa; phosphorylase B: 97.4 kDa; bovine serum albumin (BSA): 66.2 kDa; ovalbumin: 45 kDa; carbonic anhydrase: 29 kDa; lysozyme: 14.3 kDa)

Lanes 1, 2, 3: Crude membrane extracts from glucose-grown *P. putida* (350 μg in each lane) heated at 95°C for 5, 10, and 15 minutes respectively.

Lanes 5, 6, 7: Crude membrane extracts from gluconate-grown *P. putida* (350 μg in each lane) heated at 95°C for 5, 10, and 15 minutes respectively.

Lane 9: Crude membrane extract from glucose-grown *P. putida* (350 μg protein in lane) under non-reducing conditions (2-mercaptoethanol and dithiothreitol are omitted) and heated at 95°C for 5 minutes.

Lanes 10, 11, 12: Crude membrane extracts from succinate-grown *P. putida* (350 μg protein in each lane) heated at 95°C for 5, 10, and 15 minutes respectively.

FIGURE 17

reducing agents even a 5 minute heating of the sample causes a blurring of the protein bands on the gel concomitant with a decrease in the mobility patterns of most of the protein on the top half of the gel. This blurring cannot be due to protein degradation as the resultant protein fragmentation would cause blurring towards the bottom of the gel. The blurring may be the result of cross linking between many different proteins of varying sizes observed in the gel. Conspicuous by its large decrease in mobility, the inducible-repressible band appears to become cross-linked either to itself or other membrane proteins in the absence of reducing agents. This may be the result of auto-oxidation reactions in active sulfhydryl groups of the defluorinating protein in the presence of air. Evidence for the existence of such essential sulfhydryl groups active in the defluorinating protein comes from the inhibition studies of fluoride release which indicated a total protection from defluorination in the presence of N-ethylmaleimide (NEM) (167). Fluoride release activity was reconstituted by the octylglucoside dilution procedure of Racker *et al.* (186). Freshly prepared crude membrane extracts (4-6 mg protein) were mixed with phosphatidylcholine (PC) liposomes (5 mg). These liposomes were prepared by using a modification of a procedure published for the reconstitution of LDL receptors (185). Liposomes made by this pocedure have previously been characterized by electron microscopic techniques as having multilamellar structure with diameters in the range of 3-5 μm (185). Liposomes and proteoliposomes made for these studies were observed under an electron microscope after staining with phosphotungstic acid [2% (w/v)] as outlined under **Electron Microscopy**. The liposome suspension was found to be very homogenous with respect to the size of the liposome stuctures. After an initial solubilization of approximately 52-57% of the total protein in the membrane preparation an average value of 33% of the solubilized protein became incorporated into the liposomes provided. Although these two characteristics were not affected by the presence or absence of PC, the subsequent reconstitution of fluoride release activity was virtually eliminated

by extraction or incorporation in the absence of lipid (Table 9). A requirement for exogenous phospholipid at the time of solubilization has been demonstrated for the reconstitution of bacterial (184) and mammalian (206) transport systems. Although the defluorinating protein is presumed not to be a transport protein it exhibits this same type of requirement for lipid material. The lipid may provide a medium in which the protein resides protecting it against denaturation by stabilizing a particular conformation of the protein during solubilization and extraction (69).

Fluoride release activity was reconstituted in proteoliposomes made from membrane extracts of gluconate-grown *P. putida* in the presence and absence of electron donors (10 mM L-malate/50 μM FAD). The results (Figure 18) indicate an absolute requirement for the provision of electron donors to enable a sufficient amount of fluoride release to be detected. Proteoliposomes were also made from extracts of glucose-, gluconate-, and succinate-grown *P. putida* and incubated with 4FG in the presence of 10 mM L-malate/50 μM FAD. In the proteoliposomes reconstituted using succinate-grown membrane extracts (Figure 19) no fluoride release was detected. In contrast, almost similar rates and extents of fluoride release could be observed in those proteoliposomes made from glucose- and gluconate-grown membrane extracts. In all of these studies chloramphenicol (1 mg/mL) was used to abolish defluorination by whole cell contaminants. In the presence of artificial electron donors (0.1 mM PMS/20 mM ascorbate) fluoride release was also observed in the glucose- and gluconate-grown membrane extracts but not in succinate-grown membrane extracts. However, although there was a slight increase in the rates of fluoride release, the extent of defluorination was generally about half of the value generated with provision of L-malate/FAD. This may be due to the artificial nature of PMS in that the cytochrome component of the electron transport chain may not readily accept electrons from its reduced form. In contrast, the membrane-bound L-malate dehydrogenase acts upon L-malate converting it to oxaloacetate which the concomitant transport of $2H^+$ and $2e^-$ directly into

TABLE 9

Effects of the Presence and Absence of a Lipid Component upon Extraction and

Incorporation Procedures as well as Fluoride Release Activity

Characteristic[a]	Treatment					
	With PC			Without PC		
	Glucose-Grown	Gluconate-Grown	Succinate-Grown	Glucose-Grown	Gluconate-Grown	Succinate-Grown
% Solubilization of Initial Protein Concentration	52.6	49.5	54.2	58.2	60.3	52.2
% Incorporation[b] of solubilized Protein into Liposomes	30.5	32.6	34.2	32.2	30.3	38.2
% Fluoride[c] Release in Proteoliposomes	20	22	2.5	3.2	2.7	1.7

a = values given for each characteristic are the average of duplicate trials

b = % incorporation using a protein to liposome ratio of 0.8 - 1.2 mg protein: 1 mg liposomes

c = Fluoride release measured after incubation times of 17-22 h in the presence of electron donors (L-malate/FAD) at incubation ratios of 1 μmole 4FG/2 mg proteoliposomes/mL 50 mM potassium phosphate buffer, pH 7.5

FIGURE 18

Effects of Electron Donor Upon Reconstitution of Fluoride Release Activity in

Proteoliposomes from Gluconate-Grown Membrane Extracts of *P. putida*

Legend

● - Unreconstituted PC liposomes (5 mg) were incubated in the presence of a 1 mM concentration of 4FG.

▽ - Liposomes were reconstituted for fluoride release activity using membrane extracts from gluconate-grown *P. putida*. Assay mixtures consisted of 1 μmole 4FG/2 mg protein/mL 50 mM potassium phosphate buffer, pH 7.5.

○ - Liposomes were reconstituted for fluoride release activity using membrane extracts from gluconate-grown *P. putida*. Assay mixtures consisted of 1 μmole 4FG/2 mg protein/mL 50 mM potassium phosphate buffer, pH 7.5, 12 mM $MgSO_4$, 10 mM L-malate/50 μM FAD.

All incubation mixtures contained chloramphenicol (1 mg/mL) and were incubated at 30°C with constant shaking. Fluoride release was observed as described under Fluoride Ion Measurements.
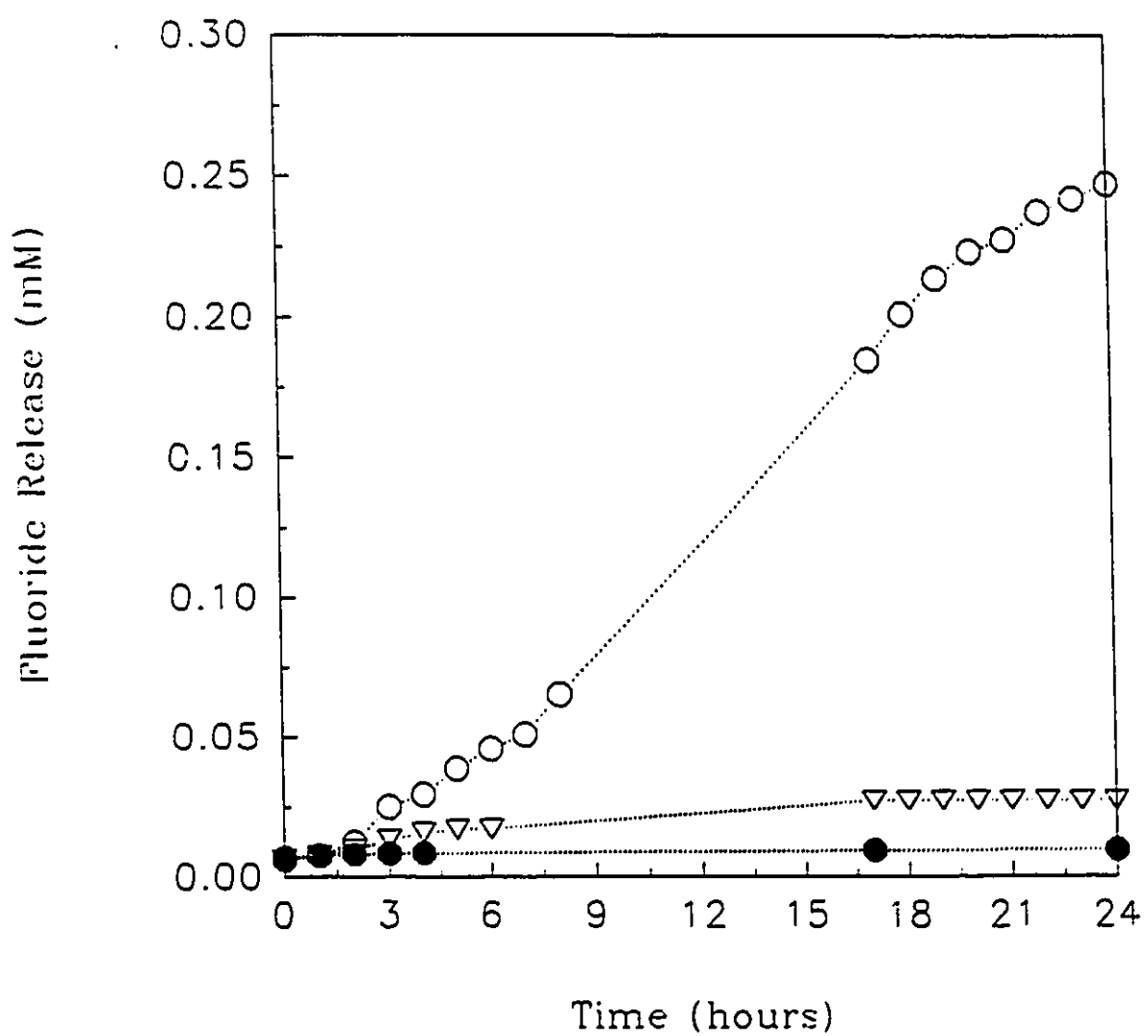
FIGURE 18

FIGURE 19

Fluoride Release Activities in Proteoliposomes Generated from Membrane Extracts of

Glucose-, Gluconate-, and Succinate-Grown *P. putida* in the Presence of Electron-Donors

Legend

Proteoliposomes were reconstituted for fluoride release activities using membrane extracts

from glucose-, gluconate-, and succinate-grown *P. putida*. Incubation mixtures consisted of 1

μmole 4FG/2 mg protein/mL 50 mM potassium phosphate buffer, pH 7.5, 12 mM MgSO₄, 10

mM-L-malate/50 μM FAD.

O - Proteoliposomes reconstituted with membrane extracts of glucose-grown cells.

● - Proteoliposomes reconstituted with membrane extracts of gluconate-grown cells.

▽ - Proteoliposomes reconstituted with membrane extracts of succinate-grown cells.

All incubation mixtures contained chloramphenicol (1 mg/mL). Assays were initialized by the

addition of 4FG (1 mM). Incubations were carried out at 30°C with constant shaking. Fluoride

release was observed at 1 hour intervals as outlined under Fluoride Ion Measurements.

FIGURE 19

Time (hours)
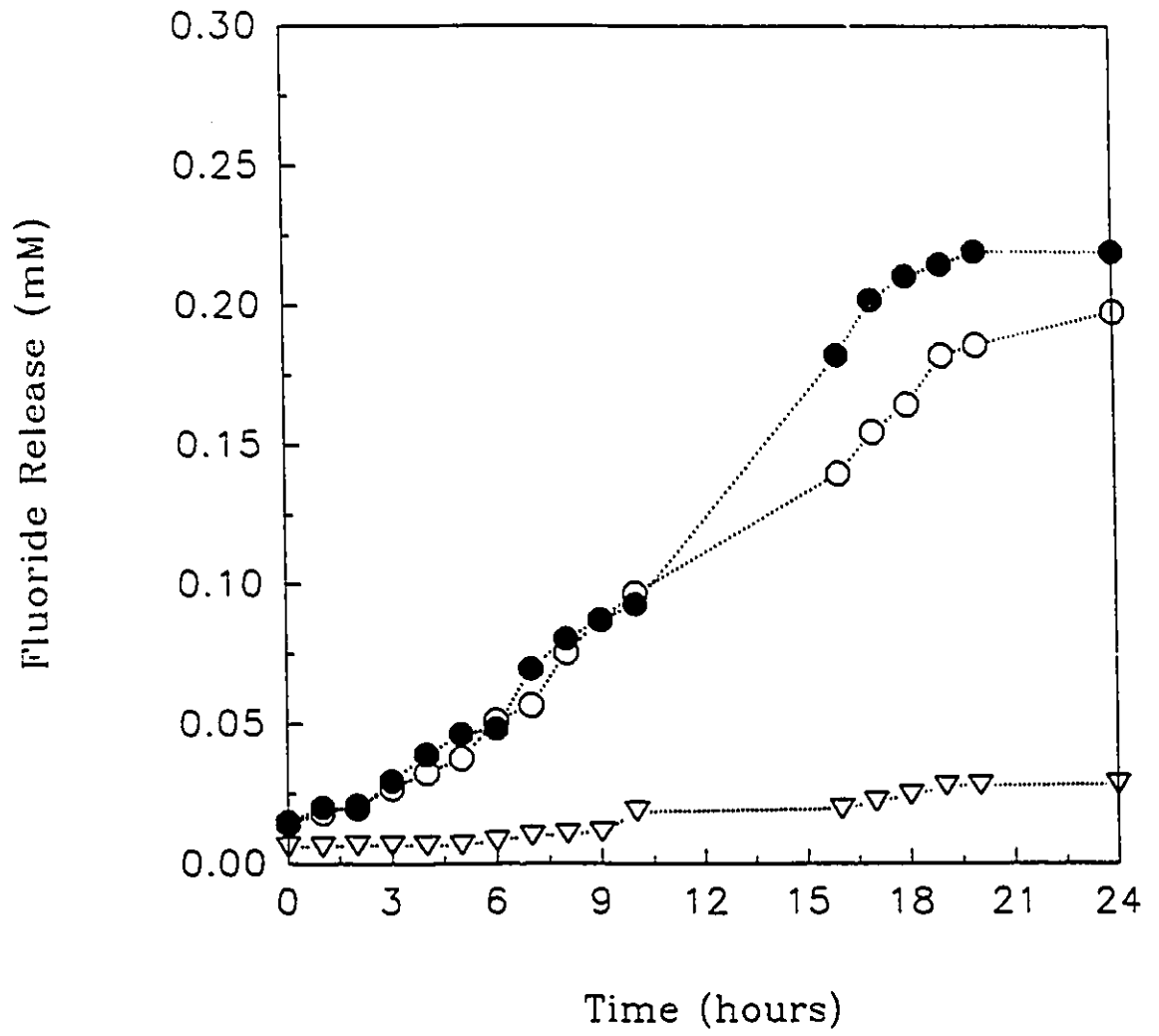
the electron transport chain. Additionally, PMS has been reported to react with -SH compounds including -SH enzymes (207). Since there is evidence for the involvement of reactive -SH groups on the defluorinating protein then inactivation of these by incubation with PMS/ascorbate may result in the lower observed values of fluoride release. The specificity of the defluorinating protein was observed by studies which indicated that the presence of D-glucose inhibited fluoride release (Figure 20). A 0.5 mM concentration of D-glucose was sufficient to effect a greater than 50% inhibition of fluoride release from 4FG (1 mM) while a 1 mM concentration abolished defluorination completely. Glucose inhibition of fluoride release was also observed in whole cells of *P. putida* (190). In these studies the most effective protection of fluoride release was afforded by D-glucose, D-gluconate (GA), and 2-keto-D-gluconate (2KGA) at twice the concentration of 4FG (5 and 2.5 mM respectively). In the present studies a concentration of D-glucose at half the value of 4FG (0.5 and 1 mM respectively) was sufficient to cause an observable decrease in defluorination. This suggests that the defluorinating protein has a higher affinity for glucose as a substrate than for 4FG. The reconstituted systems dispose of the need for corrections resulting from loss of inhibitor concentration due to whole cell metabolism of these inhibitors. Another advantage of these systems is that direct and indirect effects are readily distinguishable. In other words, assuming identical modes of transport into the cell for glucose as well as the fluorinated glucose analogue 4FG, any observed inhibition in a whole-cell system could result from an inhibition in the transport of 4FG into the cell from the external environment. This indirect inhibition of transport would then be erroneously described as an inhibition of defluorination. The reconstituted systems allow the direct observation of inhibitor effects upon the defluorinating protein precluding any transport inhibition. Since 4FGA and 4F2KGA are good substrates for defluorination and GA as well as 2KGA have been observed to inhibit defluorination in whole-cell systems, it is pressumed that they will also inhibit fluoride

121

FIGURE 20

Glucose-Inhibition of Fluoride Release Activity in Proteoliposomes from

Glucose-Grown Membrane Extracts of *P. putida*

<u>Legend</u>

Proteoliposomes reconstituted using membrane extracts from glucose-grown *P. putida*

were incubated in the presence of increasing concentrations of D-glucose. Incubation mixtures

consisted of 1 μmole 4FG/2 mg protein/mL 50 mM potassium phosphate buffer, pH 7.5, 12 mM

MgSO$_4$, 10 mM L-malate/50 μM FAD.

○ - Fluoride release in the absence of D-glucose

● - Fluoride release in the presence of 0.25 mM D-glucose

▽ - Fluoride release in the presence of 0.5 mM D-glucose

▼ - Fluoride release in the presence of 1 mM D-glucose

□ - Fluoride release in the presence of 2 mM D-glucose

All incubation mixtures contained chloramphenicol (1 mg/mL). Assays were initialized by the

addition of corresponding mixtures of 4FG and D-glucose. Incubations were carried out at 30°C

with constant shaking. Fluoride release was observed at 1 hour intervals as oulined under

Fluoride Ion Measurements.

FIGURE 20
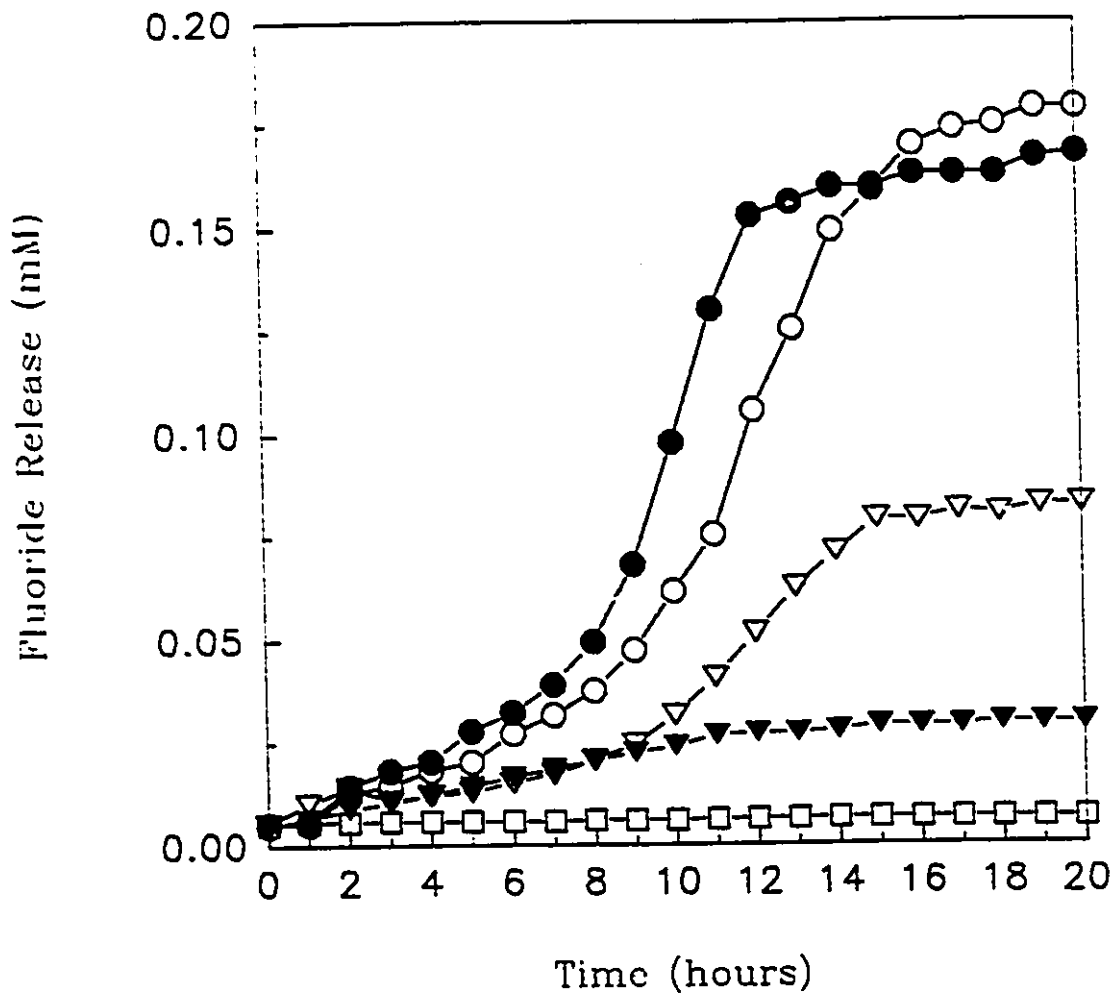
release in the reconstituted systems but at much lower concentrations.

Due to time constraints and the availability of fresh membrane extracts it was only possible to carry out one last set of inhibition studies. Incubations with D-galactose indicate that the of a 1 mM concentration of D- galactose has no effect upon the rate of defluorination initially, but it does cause a 6% decrease in the extent of fluoride release (Figure 21). A 2 mM concentration of D-galactose decreases the initial rate of defluorination as well as decreasing the extent of fluoride release by 10%. The effects observed indicate a lower binding affinity by the defluorinating protein for D-galactose than for 4FG.

The inhibition studies using glucose indicated the substrate specificity of the defluorination reaction. The subsequent studies with D-galactose failed to effect any sort of significant inhibition of fluoride release. This is indicative of the stereospecific requirement for initial binding and subsequent C-F bond cleavage at C-4 by the defluorinating protein. Previous studies of fluoride release inhibition using whole-cell systems (191) emphasized the importance of the carbon 1 and 4 positions as well as the importance of the ring oxygen in the defluorination reactions. Our studies with the reconstituted defluorinating protein directly confirm the stereospecific importance a hydrogen bond acceptor at carbon 4 .

FIGURE 21

Effects of D-Galactose Upon Fluoride Release in Proteoliposomes from

## Glucose-Grown Membrane Extracts of *P. putida*

Legend

Proteoliposomes reconstituted using membrane extracts from glucose-grown *P. putida* were incubated in the presence of increasing concentrations of D-galactose. Incubation mixtures consisted of 1 μmole 4FG/2 mg protein/mL 50 mM potassium phosphate buffer, pH 7.5, 12 mM MgSO$_4$, 10 mM L-malate/50 μM FAD.

O - Fluoride release in the absence of D-galactose

● - Fluoride release in the presence of 1 mM D-galactose

∇ - Fluoride Release in the presence of 2 mM D-galactose

All incubation mixtures contained chloramphenicol (1 mg/mL). Assays were initialized by the addition of corresponding mixtures of 4FG and D-galactose. Incubations were carried out at 30°C with constant shaking. Fluoride release was observed at 1 hour intervals as oulined under Fluoride Ion Measurements.

FIGURE 21
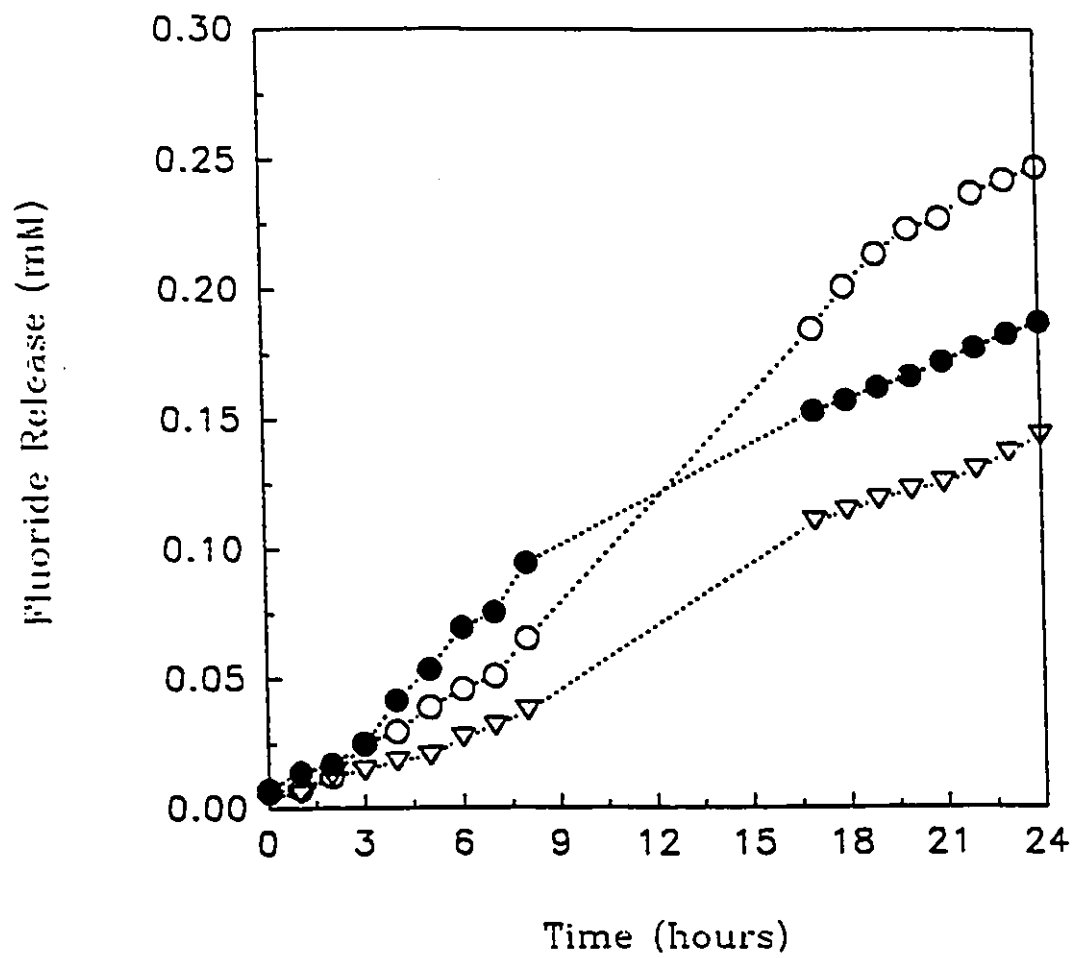
CHAPTER 4
SUMMARY AND FUTURE PERSPECTIVES

When whole cells of *P. putida* are grown using gluconate as the sole carbon source, a certain degree of induction and repression occurs within the cell which results in significant decreases in the rates of O_2 consumption and CO_2 release. Thus, growth on gluconate decreases the rate of 4FG metabolism to 2,3-DDRA by *P. putida*. Borate anion-exchange column chromatographic analysis of the supernatants produced by incubation of 4FG with gluconate-grown cells of *P. putida* indicated the existence of 3 peaks which are distinct from 4FG and 2,3-DDRA. The respective R_f values of these intermediate metabolites are 0.49, 0.58, 0.74. Chemical analysis of these intermediates indicates that while Peak g is a reducing sugar, Peaks f and h are non-reducing. ^{13}C-NMR spectroscopy coupled with spectrophometric analyses and periodate oxidation of the Peak h metabolite has allowed a tentative structural designation corresponding to 3,4-dideoxy-2-keto-D-*glycero*-hexanoic acid (3,4-dideoxy-2-keto-D-gluconic acid, 3,4-DDKGA). Further support for the 3-deoxy-2-keto structural designation may be acquired by reaction of Peak h with 2-thiobarbituric acid (45).

In vivo ^{19}F-NMR time course analyses of 4FG metabolism indicated the absence of any fluorinated intermediate metabolites, other than the oxidation products 4FGA and 4F2KGA, during whole cell metabolism of 4FG. Defluorination is therefore the prelude to 4FG metabolism. Additionally, the rapid dissapearance of the 4FGA and 4F2KGA signals concomitant with the increase in the intensity of the flouride signal indicates that these are also good substrates for defluorination and therefore that the protein involved in C-F bond cleavage must be common for all three substrates. It has been indicated that transport systems for glucose, gluconate and 2-ketogluconate are separate and distinct from each other (164,166) and present results suggest that although the defluorinating protein may be involved with each of these transport systems in

an identical manner, it is a distinctive component. These studies also provided further evidence for the effects proposed to occur in 4FG metabolism as a result of growth on gluconate.

The osmotic shock treatment of whole cells of *P. putida*, grown on a variety of carbon sources, did not affect either the rates or extents of fluoride release by the cells. On the other hand the crude periplasmic extracts exibited no observable fluoride release. No binding of 4FG could be observed by ^{19}F-NMR spectroscopy when these periplasmic extracts were incubated with 4FG. This supported earlier inhibition studies that indicated 4FG was not bound by a periplasmic binding protein in these extracts. The results indicate that binding of a periplasmic protein to 4FG is not essential for defluorination. SDS-PAGE analysis of these crude extracts indicates the existence of a glucose-inducible, gluconate and succinate-repressible periplasmic protein with an apparent molecular weight of 44 kDa. This is consistent with the characteristics published for a glucose-binding protein isolated from *P. aeruginosa* (113).

Metabolic studies using the ionophores valinomycin and nigericin further defined the chemiosmotic energy couple of fluoride release as involving a pH gradient (ΔpH) exclusively. The reversibility of the effects elicited by treatment with nigericin implicate a collapse of the pH gradient and not a covalent modification of the defluorinating protein.

Incubation of cytoplasmic membrane vesicles with 4FG in the presence of the electron donors L-malate/FAD failed to elicit levels of fluoride ion detectable by fluoride electrode. However, *in vacuo* concentration followed by ^{19}F-NMR spectroscopy of the supernatants from these incubations indicated the presence of fluoride ion. It was therefore concluded that the original procedure for generating membrane vesicles (183) resulted in the almost complete loss of defluorinating protein. This may have been due to the absence of any reducing agents during the procedure allowing for the oxidation and inactivation of the defluorinating protein.

The incorporation of cytoplasmic membrane proteins from glucose-grown *P. putida* into

PC liposomes and subsequent incubation in the presence of electron donors succeded in reconstituting active fluoride release upon the introduction of 4FG. Reconstitution of this fluoride release activity was inherent in membrane extracts from glucose-and gluconate-grown *P. putida* but absent from membranes of cells grown on succinate. SDS-PAGE analyses of these extracts indicate the presence of a single glucose-inducible, gluconate and succinate-repressible protein band with an apparent molecular weight of 65.4 kDa. This band is referred to as the putative defluorinating protein. It lacks any type of heat modifiable character, however, it is readily oxidized on heating in the absence of reducing agents resulting in a rapid decrease in activity. The active form of the protein exhibited an absolute requirement for the presence of exogenous phospholipid during the extraction from membrane preparations and subsequent incorporation into liposomes. The lipid may provide a stabilizing medium protecting the protein against denaturation or autooxidation which result in the progressive loss of fluoride release activity.

Glucose inhibition of fluoride release in the reconstituted systems indicate the substrate specificity of the defluorinating protein. The lack of any observed inhibitory effects upon defluorination caused by incubation with D-galactose at concentrations twice that of 4FG indicate the relative stereospecific requirement of the protein for the hydroxyl group at the carbon 4 position.

Perhaps the most important results of this research are: (a) the isolation and structural elucidation of another fluorinated metabolite which is consistent with the proposed metabolism of 4FG and (b) the tentative identification of the membrane-associated protein responsible for C-F bond cleavage. One way to confirm this tentative identification would be to isolate the protein in its pure form, reconstitute this protein into membrane vesicles with a repressed defluorinating function which still maintain an intact respiratory system. For example, incubation of osmotically-shocked succinate-grown *P. putida* with the pure defluorinating protein would allow

the incorporation of this protein into a system with an intact electron transport chain. The provision of electron donors would fascilitate recovery of fluoride release activity. It would also be possible to confirm primary and secondary structures of the protein by amino acid analyses (composition and sequencing) and subsequent circular dichroism spectroscopy. ^{19}F-NMR spectroscopy of the purified protein incubated with 4FG in the presence of electron donors would allow conformational changes due to protein binding of 4FG to be observed. Morever, since the reconstituted systems are devoid of any intracellular metabolic enzymes the activity of the protein would result in the accumulation of the initial defluorinated metabolite thus allowing the structural elucidation of this early intermediate.

APPENDIX I

The Bradford Assay Standard Curve

The standard curve was constructed using a range of concentrations of bovine serum albumin (10 to 125 μg) in 25 or 50 mM potassium phosphate buffer, pH 7.5. The Bio-Rad reagent was diluted 1: 5 before the addition of 100-μL aliquots of protein and allowed to incubate 30 minutes before reading absorbance at 595 nm. Concentrations were determined using the best-fit 1st order regression line of the standard curve data calculated on a Schimadzu UV-240 recording spectrophotometer. Each point represents the average of triplicate readings. Procedure as described in **Protein Determination**.

The equation of the line was given as $y = 0.0072 x - 9.4 \times 10^{-4}$ where y = absorbance at 595 nm, x = μg protein and Rval was 0.999.
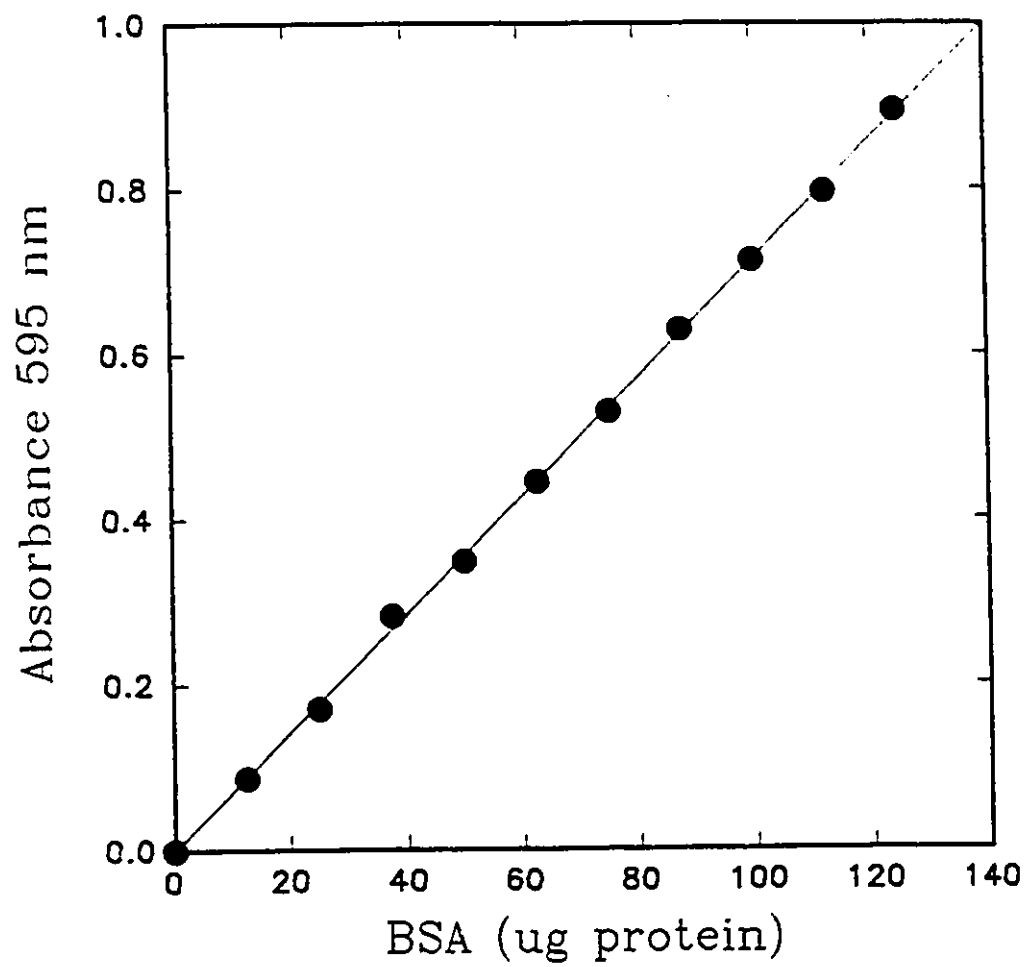
APPENDIX II

The Lowry Protein Assay Standard Curve

The standard curve was constructed using a range of concentrations of bovine serum albumin (0 to 250 μg) in 25 or 50 mM potassium phosphate buffer, pH 7.5. Lowry reagent A [0.5% (w/v) $CuSO_4$ in 1% (w/v) trisodium citrate] was diluted 1: 50 in Lowry reagent B [2% (w/v) Na_2CO_3 in 0.1 M NaOH] to make Lowry reagent C which was prepared daily. Aliquots (250 μL) of protein were mixed with 1.25 mL of reagent C and incubated for 10 minutes prior to the addition of 125 μL of a 1: 2 dilution of Folin Ciocalteau's reagent (Sigma Chemical Company). The mixture was then incubated a further 30 minutes prior to reading absorbance at 750 nm. Concentrations were determined using a best-fit 2nd order regressional analysis of the standard curve data calculated on a Schimadzu UV-240 recording spectrophotometer. Each point represents the average of triplicate readings. Procedure a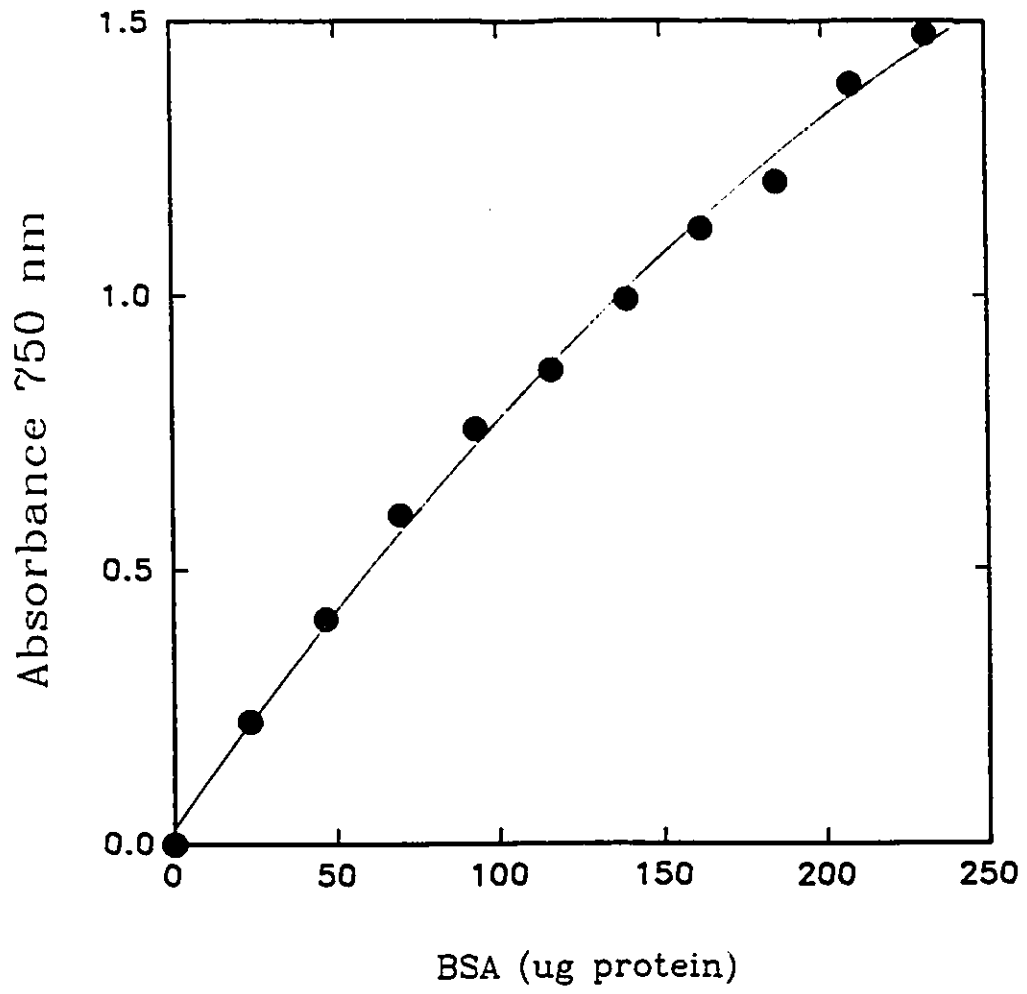s described in **Protein Determination**. The equation of the line was given as $y = -1.0 \times 10^{-5}x^2 + 0.0085 x + 0.028$ where y = absorbance at 750 nm, x = μg protein and Rval was 0.999.

APPENDIX III

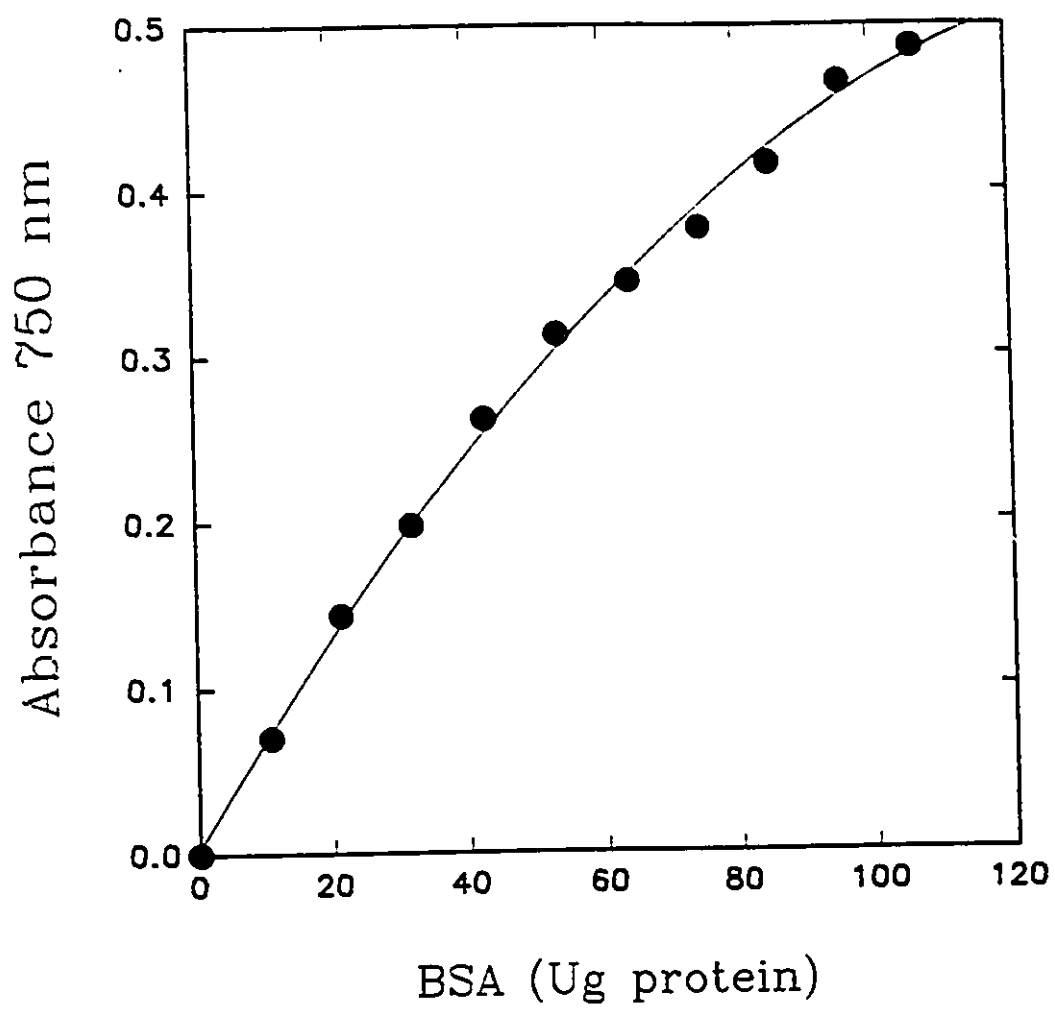### The Peterson Modification of the Lowry Protein Assay Standard Curve

The standard curve was constructed using a range of concentrations of bovine serum albumin (0 to 110 μg) in 25 or 50 mM potassium phosphate buffer, pH 7.5, to which had been added 15% (w/v) n-octylglucoside (final concentration, 1.25%). Aliquots (1 mL) of protein were mixed with 0.1 mL of 0.15% (w/v) deoxycholic acid (DOC), vortexed gently and allowed to stand at room temperature for 10 minutes. To this mixture 0.1 mL of 72% (w/v) trichloroacetic acid (TCA) was added, the samples were mixed and then centrifuged at 3000 $\times$ g for 15 minutes. The supernatant was discarded by immediately decanting after centrifugation and drawing the remaining liquid up using a syringe and 22 gauge needle. The pellet was brought up to 1 mL with distilled deionized water and an equal volume of Reagent A [0.1% (w/v) CuSO$_4$, 0.2% (w/v) potassium tartrate, and 10% (w/v) sodium carbonate mixed with equal volumes of 0.8 N NaOH, 10% SDS and distilled deionized water] was added. After a 10 minute incubation period 0.5 mL of Reagent B (a 1: 5 dilution of Folin Ciocalteau's reagent, Sigma Chemical Company) was added, the mixture vortexed and then allowed to stand for 30 minutes prior to reading the absorbance at 750 nm. Concentrations were determined using a best-fit 2nd order regressional analysis of the standard curve data calculated on a Schimadzu UV-240 recording spectrophotometer. Each point represents the average of triplicate readings. Procedure as described in **Protein Determination**. The equation of the line was given as $y = -2.0 \times 10^{-5} x^2 + 0.0068 x + 0.0035$ where y = absorbance at 750 nm, x = μg protein and the Rval was 0.999.

APPENDIX IV

The Fluoride Ion Standard Curve

The standard curve was constructed using a range of concentrations of sodium fluoride

(NaF) (10^{-1} to 10^{-5} M) in either 20, 25 or 50 mM potassium phosphate buffer, pH 7.5.

Concentrations were determined using a best-fit 1st linear regression analysis of the standard curve

data generated by using a fluoride specific electrode (Metrohm Heriseau, Switzerland). Fluoride

measurements were carried out as described in **Fluoride Ion Measurements**. The equation of

the line was given as $y = -0.0193\,x\ -2.699$ where $y = \log[F^-]$, $x =$ milivolts and the Rval was

0.997.
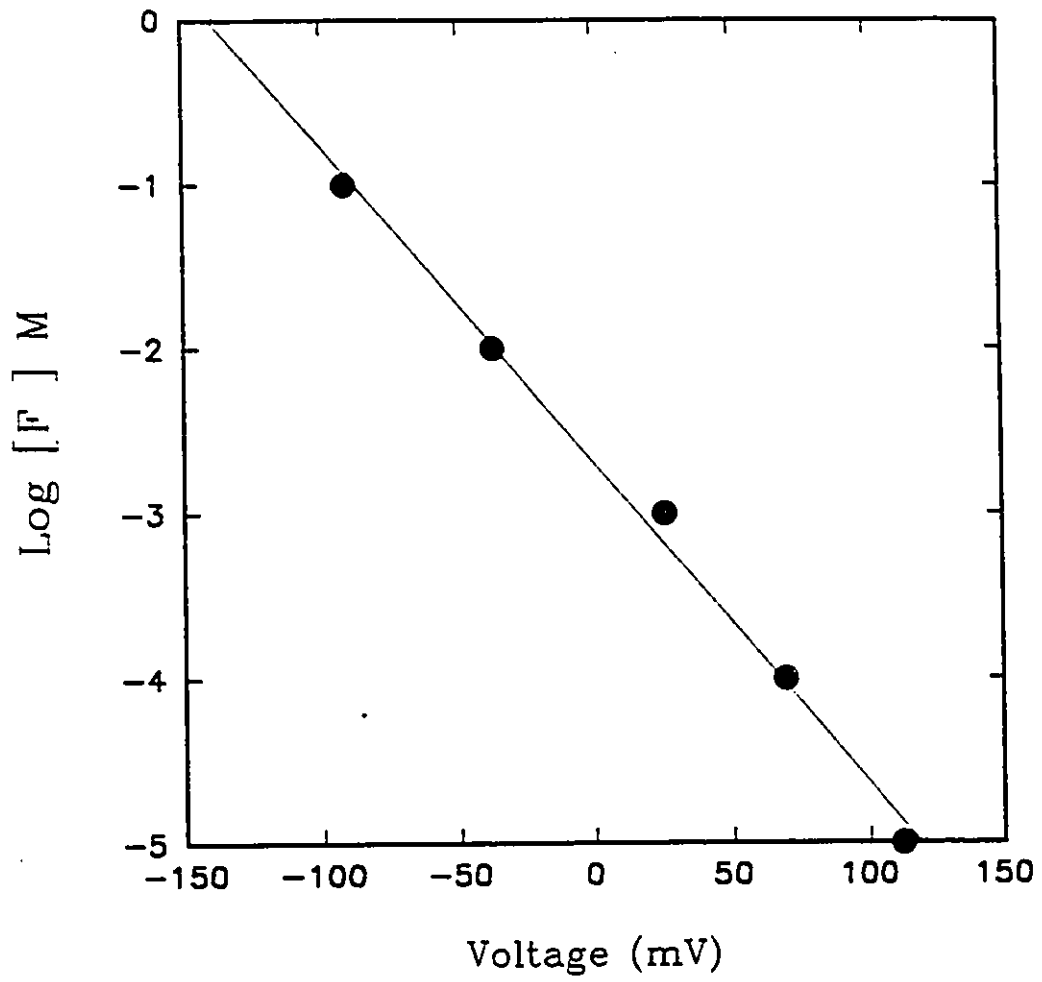
APPENDIX V

The Quench Correction Curve for ^{14}C-labeled Samples

The quench correction curve was produced by counting a series of quenched (190 000 dpm) and unquenched (29 700 dpm) ^{14}C standards (Nuclear Chicago) on a Beckman LS 7500 liquid scintillation counter (see section entitled **Liquid Scintillation Counting**). A full ^{14}C window was used for all three counting channels.

The equation of the line was given as $y = -5.8 \times 10^{-4} x^2 -4.03 \times 10^{-3} x + 95.094$, where $y =$ % counting efficiency, $x =$ H-number and Rval was 0.995.
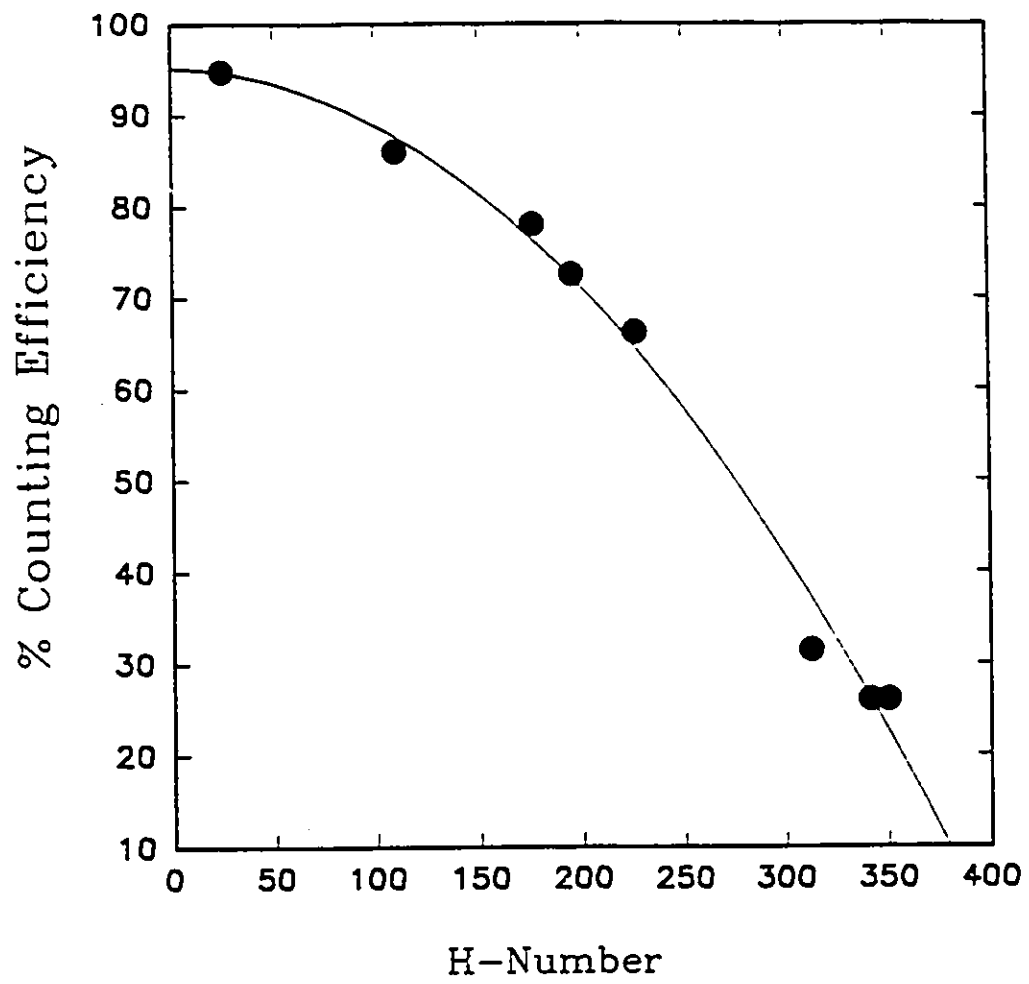
APPENDIX VI

SDS-PAGE Molecular Weight Standard Curve

The molecular weight standards were prepared as described in Materials and Methods. The standards (Sigma Chemical Company) are bovine serum albumin (67 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), soybean trypsin inhibitor (20 100) and lactalbumin (14 200). Molecular weights were determined using a best-fit 1st order linear regression analysis calculated on Sigmaplot 4.1 (Jandel Scientific, Corte Madera, California).

$y = -1.685\ x + 5.104,\ r = 0.993$
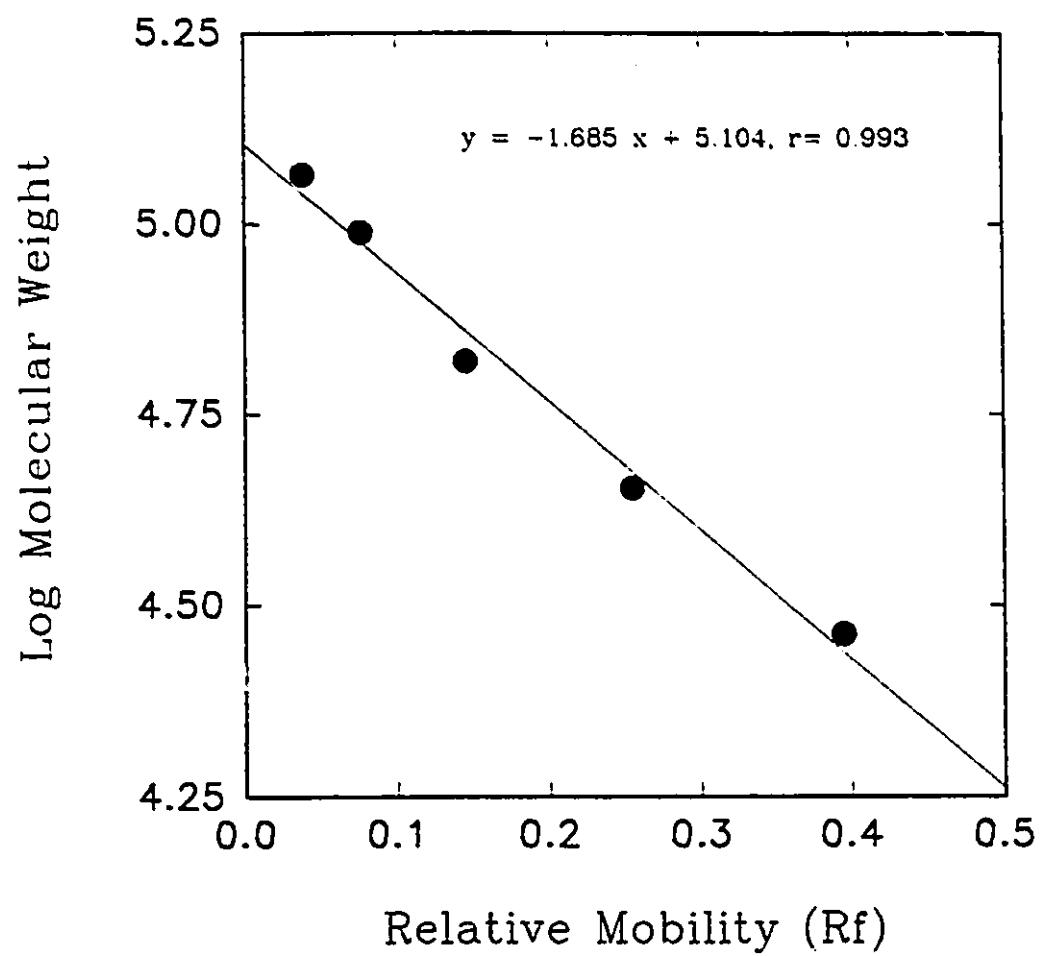
APPENDIX VII

Calculation of Oxygen Consumption

If n moles of substrate consumed x litres of oxygen after a 2 hour incubation, the extent of substrate oxidation as moles of oxygen per mole of substrate was calculated as follows:

x litres of oxygen consumed under the experimental temperature and pressure were converted to x' L of oxygen at STP by the following equation:

$$x' \ L \ (O_2) \ = \ x \ L \ (O_2) \ 273 \ (P_B\text{-}P_w) \ / \ (760 \times T)$$

where P_B and P_w were the experimental and water vapour pressures, respectively, and T was the experimental temperature (K).

Therefore, the number of moles of oxygen consumed at STP $= (P_B \times x') \ / \ (R \times T)$.

From the ideal gas equation; $PV = nRT$, where $P = 101.3$ kPa, $T = 273°C$, $V = x'$ (L of oxygen consumed at STP) and $R = 8.314$ J / mole /K.

Therefore, the extent of substrate oxidation

$$= \frac{(P_B \times x') \ / \ (R \times T)}{n} \qquad = \qquad \frac{\text{moles oxygen consumed}}{\text{moles substrate}}$$

Alternatively, the extent of oxidation was expressed as gram atoms of oxygen per mole of substrate. This was calculated from the following relationships:

a)   One mole of oxygen is equivalent to 32 g or 2 g atoms of oxygen.

b)   The rate of oxygen consumption was similarly calculated by first determining the rate (μL/min).

APPENDIX VIII

Determination of Radiolabel in Various Samples

From the quench correction curve (**Appendix V**), the % counting efficiency of the samples was determined from their H-number. The dpm of the samples was calculated from the following expression:

$$dpm = \frac{cpm}{\%CE} \times 100$$

where CE is the counting efficiency, cpm is counts per minute and dpm is disintegrations per minute.

Using the relationship that 1 nanocurie (nCi) of radioactivity $= 2.22 \times 10^3$ dpm, the amount of radiolabel in the samples

$$= \frac{cpm}{\%CE} \times \frac{100}{2.22 \times 10^3} \ nCi$$

If the specific activity of the radioactive material was A (nCi/mmo), then the amount of radiolabel in the sample per mg of protein

$$= \frac{cpm}{\%CE} \times \frac{1}{22.2 \times A \times B} \quad (nmol\ radiolabel/mg\ protein)$$

where **B** is the mg of protein.

APPENDIX IX

Growth Curve of *Pseudomonas putida*

Cells of *P. putida* were cultured, as described under **Preparation of Whole Cell Suspensions**, in 12 L of sterile minimal mineral salts growth medium supplemented with either gluconate or succinate [0.2% (w/v)] as the sole carbon source. The vessel was aerated with air at a rate of 1.5 L per minute, maintained at a temperature of 30°C, at a stirring rate of 400 rpm and under a slight positive pressure (0.5 psi). Growth was monitored by aseptically withdrawing 1-mL aliquots of medium from the vessel at various times following inoculation and measuring the optical density at 620 nm on a Schimadzu UV-240 Spectrophotometer against a blank composed of sterile mineral-salts medium.

O-gluconate-grown *P. putida*

●-succinate-grown *P. putida*
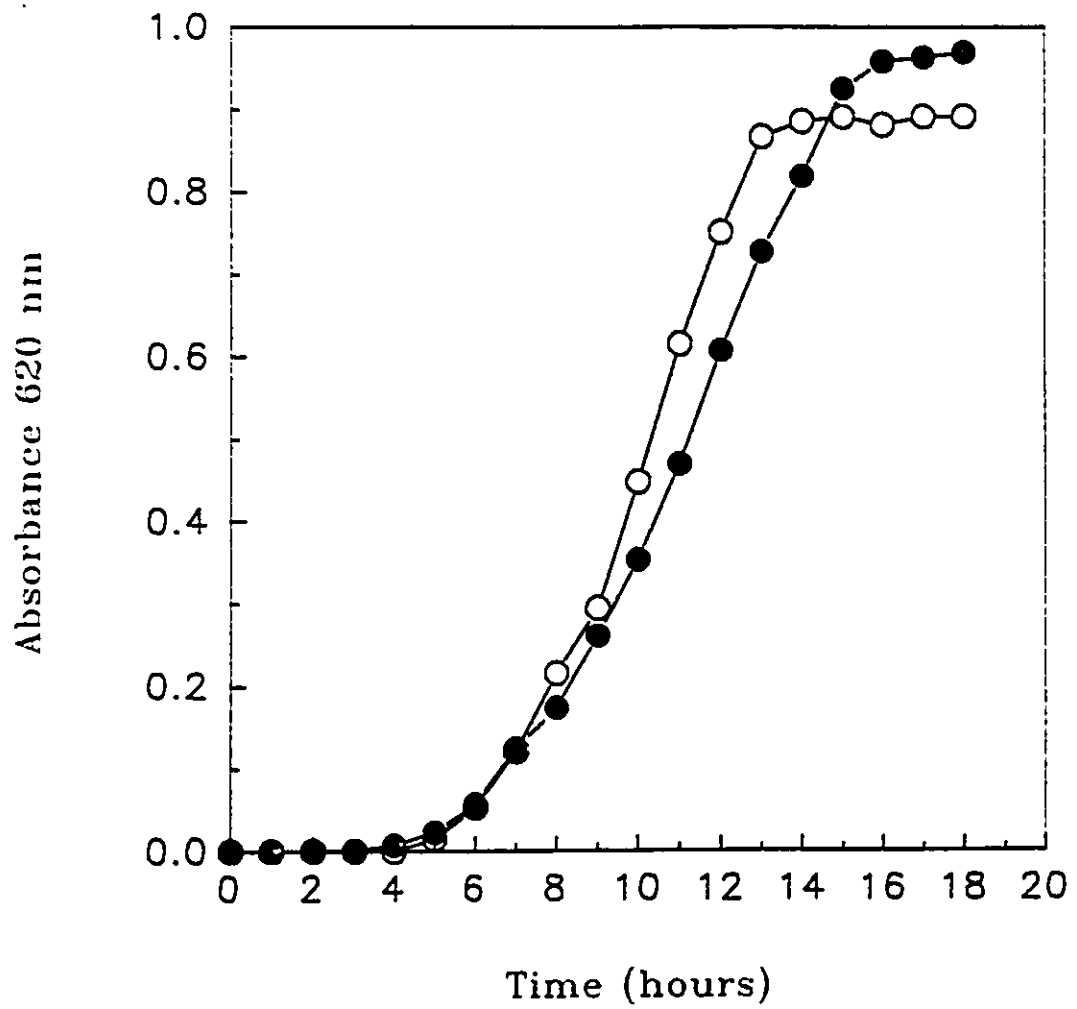
APPENDIX X

Solutions for Urea-Gel Preparations

I. **Stock Solutions**

(filter with Whatman # 1 filter paper to remove urea reagent impurities)

A. Gel Buffer A

Tris base	9.075 g
1N HCl	12 mL
Urea	40.5 g
Water	to 75 mL

B. Acrylamide Solution

Acrylamide	30 g
methylene-bis-acrylamide	0.8 g
Urea	54 g
Water	to 100 mL

C. 9 M Urea

Urea	5.4 g
Water	to 10 mL

D. Riboflavin-5'-phosphate (FMN) Stock Solution

FMN	0.1141 g
Water	to 50 mL

Store in a dark bottle at 4°C. Prepare a 0.13 mM solution of FMN in 9 M urea by adding 150 μL of stock to 6 mL 9 M urea. This is used instead of ammonium persulfate. The gels are photopolymerized with a fluorescent lamp at about 3 inches for 30 minutes

E. _Sample Buffer_

9 M Urea	1 mL
0.005% (w/v) bromophenol blue	50 μL

F. _Tank Buffer_

Tris base	12 g
Glycine	57.6
Water	to 1L

Dilute 1 in 10 prior to use.

II.   Procedure

Only a resolving gel was used. A 10% polyacrylamide gel was prepared as follows:

Solution	mL
Gel buffer (A)	9
Acryiamide solution (B)	8
0.13 mM FMN (D)	3
9 M Urea (C)	4

The gel was poured into a 9 x 8 cm mighty small vertical slab gel unit model SE 200 (Hoefer Scientific Instruments) and allowed to polymerize for 30 minutes. The samples (25 μL) were mixed with equal volumes of sample buffer (E) and allowed to incubate at 37°C for 20-30 minutes. The samples were then applied to the gel and electrophoresed at 150 V , 4°C, for 2.5 hours. Subsequently the gels were stained for 2-3 hours in a solution containing 50% (v/v) methanol, 10% (v/v) acetic acid and 2.5% (w/v) Coomassie Brilliant Blue R 250 and then destained in 50% (v/v) methanol, 10% (v/v) acetic acid until the background stain was removed. Alternatively, the gels were treated as outlined under **Western Blotting** .

APPENDIX XI

PROTON-DECOUPLED ^{13}C-NUCLEAR MAGNETIC RESONANCE

(NMR) SPECTRUM OF PEAK 4 METABOLITE

The proton-decoupled ^{13}C-NMR spectroscopic analyses of the radiolabelled metabolites were carried out using a Brüker 300 MHz NMR Spectrophotometer (Brüker Instruments). The metabolite was resuspended in 500 μL deuterium oxide (D$_2$O) to give a final concentration of 12 mM and scanned overnight ($\sim$90 000 scans). Chemical Shift values were relative to 1,4-dioxane (-275.5 ppm) and expressed in ppm of the main spectrometer frequency (74.469 MHz).
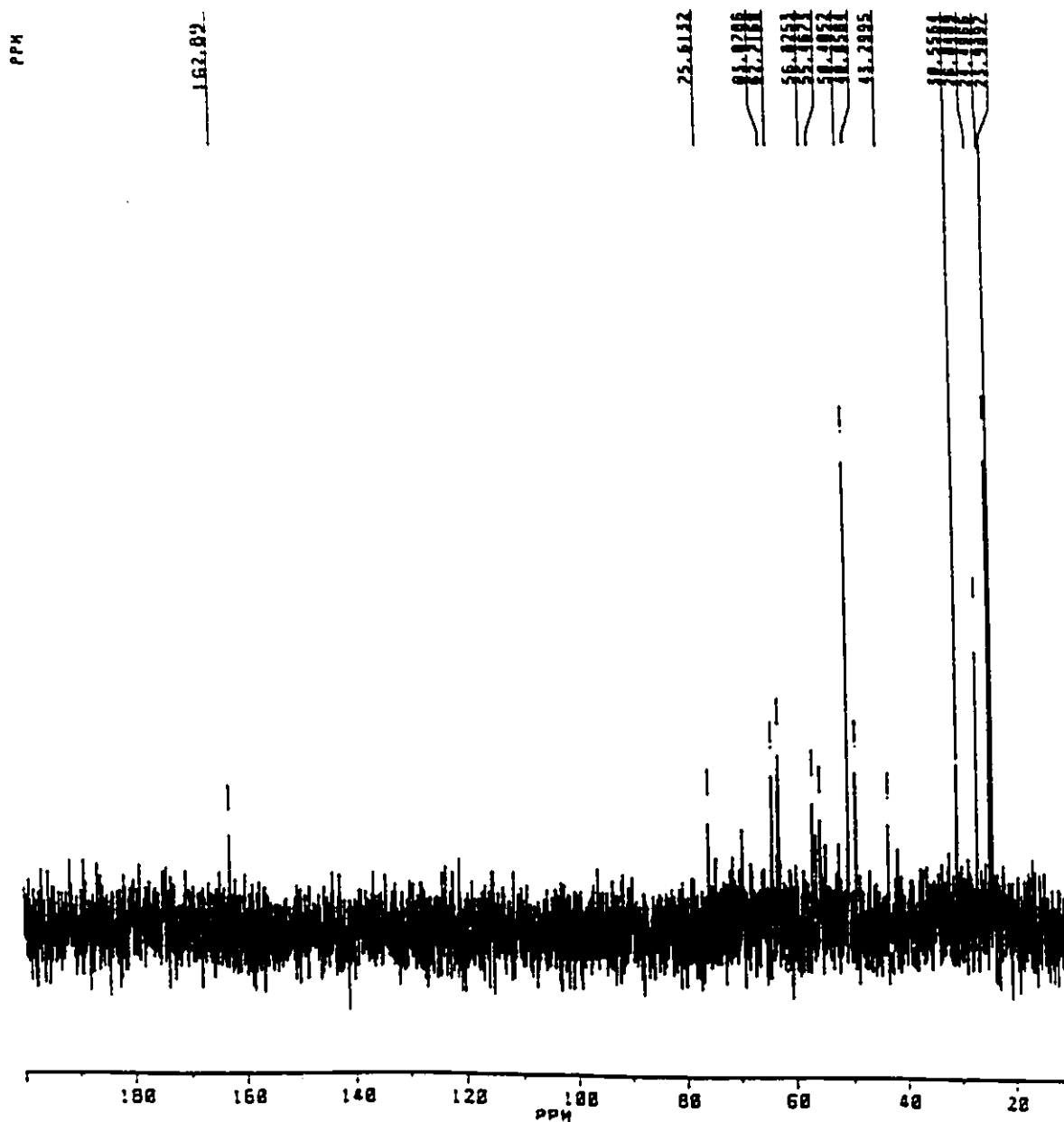
APPENDIX XII

PROTON-DECOUPLED ^{13}C-NUCLEAR MAGNETIC RESONANCE (NMR)

SPECTRUM, USING DEPT 135 ANALYSIS OF PEAK 4 METABOLITE

The Peak h sample used to obtain the ^{13}C-decoupled spectrum shown in Appendix XI was

analyzed using the DEPT 135 program. Procedure as described in Appendix XI.

APPENDIX XIII

PROTON-DECOUPLED ^{13}C-NUCLEAR MAGNETIC RESONANCE (NMR)

SPECTRUM, USING DEPT 90 ANALYSIS OF PEAK 4 METABOLITE

The Peak h sample used to obtain the ^{13}C-decoupled spectrum shown in Appendix XI was analyzed using a DEPT 90 program. Procedure as described in Appendix XI.
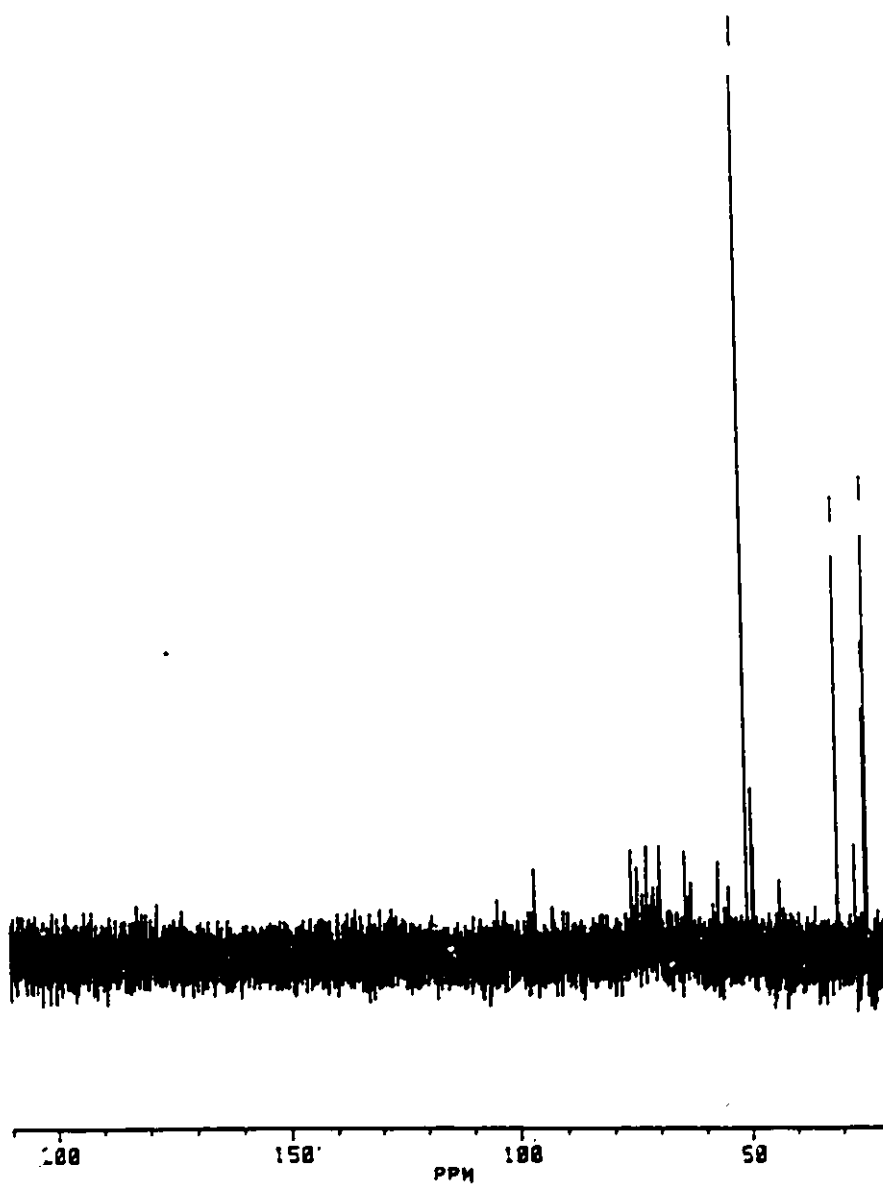
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"The contact poir*s must be dirty . . . just click it up
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