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4-DEOXY-4-FLUORO-D-GLUCOSE: A NOVEL MEMBRANE PROBE IN PSEUDOMONAS PUTIDA.

TONY. D'AMORE

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4-DEOXY-4-FLUORO-D-GLUCOSE: A NOVEL MEMBRANE PROBE IN PSEUDOMONAS PUTIDA

by

Tony D'Amore

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

Windsor, Ontario, Canada

1983
ABSTRACT

4-DEOXY-4-FLUORO-D-GLUCOSE: A NOVEL MEMBRANE PROBE IN PSEUDOMONAS PUTIDA

by

Tony D'Amore

Glucose grown whole cells of Pseudomonas putida do not oxidize 4-deoxy-4-fluoro-D-glucose (4FG). Instead, an extensive release of fluoride ion occurs in which no defluorinated sugar could be detected. Cytoplasmic membrane vesicles and cell-free extracts from this organism oxidize 4FG to the extent of 1 mol oxygen mol substrate$^{-1}$ with retention of the carbon-fluorine bond.

The defluorination reaction displays saturation kinetics with an apparent $K_m$ of 3.9 mM and an apparent $V_{max}$ of 1 nmol fluoride mg protein$^{-1}$ min$^{-1}$. This reaction is inhibited in the presence of glucose, gluconate or 2-ketogluconate. Growth of cells on gluconate or 2-ketogluconate does not affect the rate or extent of fluoride release from 4FG. On the other hand, cells grown on succinate or citrate have drastically reduced rates and extents of fluoride release, demonstrating that defluorination of 4FG is dependent upon the presence of protein.

Pre-incubation of P. putida with chloramphenicol further demonstrates the presence and synthesis of protein responsible for the defluorination of 4FG. Such cells exhibit a large reduction in the extent of fluoride release. The defluor-
minating activity in these cells is shown to be the result of endogenous protein present.

Fractionation of whole cells indicates that the probable site of the defluorinating protein is in the outer membrane. The outer membrane is characterized by electrophoretic and enzyme activity analysis as well as comparison with the outer membrane proteins in *P. aeruginosa*.

The results obtained with D-[6-³H]-4-deoxy-4-fluoro-glucose and a possible mechanism of fluoride release are presented. Such a mechanism accounts for the release of fluoride, the formation of ³H₂O and tritiated-carbohydrates and covalent attachment of the defluorinated sugar residue to the protein. This protein appears to be peptidoglycan-associated and, therefore, may form or be part of the porin channel in the outer membrane. A molecular weight of about 40,000 was estimated for this protein by column chromatography.

The results obtained in this investigation are discussed in relation to the mechanism of glucose transport in whole cells of *P. putida* and other pseudomonads.
DEDICATION

To my wife, Angela
ACKNOWLEDGEMENTS

I would like to express my sincerest thanks to my advisor, Dr. N. F. Taylor, for allowing me the opportunity to expand my knowledge in the area of biochemistry. I would also like to thank him for his guidance and encouragement throughout my graduate studies.

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I would like to thank the Province of Ontario and the Natural Sciences and Engineering Research Council of Canada for financial support during my graduate studies.

I would like to express my undying gratitude to my mother and father for their emotional and financial support.

Finally, I would like to express, with all my love, thanks to my wife for her constant support, encouragement and love throughout this endeavor.
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LIST OF ABBREVIATIONS

ATP - adenosine-5'-triphosphate
CF₃dUMP - 5-trifluoromethyl-2'-deoxyuridylate
CH₂-H₂ folate - 5,10-methylenetetrahydrofolic acid
cpm - counts per minute
CSG - citryl-S-glutathione
DBP - dicarboxylic acid binding protein
DCIP - dichlorophenol indophenol
dpm - disintegrations per minute
dTMP - 2'-deoxythymidylate
DTT - dithiothreitol
dUMP - 2'-deoxyuridylate
EDTA - ethylenediaminetetraacetic acid
FdUMP - 5-fluoro-2'-deoxyuridylate
3FG - 3-deoxy-3-fluoro-D-glucose
3FGA - 3-deoxy-3-fluoro-D-gluconic acid
4FG - 4-deoxy-4-fluoro-D-glucose
D-[6-³H]4FG - D-[6-³H]-4-deoxy-4-fluoroglucose
4FGA - 4-deoxy-4-fluoro-D-gluconic acid
GABA - γ-aminobutyric acid
GBP - glucose binding protein
GS⁰ - glutathione free radical
GSH - glutathione (reduced)
GSSG - glutathione (oxidized)
H₂folate - 7,8-dihydrofolic acid
2K3FGA - 3-deoxy-3-fluoro-2-keto-D-gluconic acid
2K4FGA - 4-deoxy-4-fluoro-2-keto-D-gluconic acid
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<tr>
<td>2KGA</td>
<td>2-keto-D-gluconic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>α-Me4FG</td>
<td>α-methyl-4-deoxy-4-fluoro-D-glucopyranoside</td>
</tr>
<tr>
<td>α-MeG</td>
<td>α-methyl-D-glucopyranoside</td>
</tr>
<tr>
<td>β-MeG</td>
<td>β-methyl-D-glucopyranoside</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethyl maleimide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>PAGB</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAS</td>
<td>periodic acid-Schiff reagent</td>
</tr>
<tr>
<td>PMS</td>
<td>phenazine methosulfate</td>
</tr>
<tr>
<td>POPOP</td>
<td>1,4-bis(5-phenyloxazol-2-yl)-benzene</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
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CHAPTER I
INTRODUCTION

The existence of naturally occurring fluoro-organic compounds was first demonstrated by Marais (1), in 1944, with the isolation of fluoroacetic acid as the toxic component in the leaves of *Dicapetalum cymosum*. This plant has long been responsible for the sporadic poisoning of cattle in South Africa. Subsequently, the same compound has been identified in other toxic plants (2). Other naturally occurring fluoro-compounds include the ω-fluoro-fatty acids, such as ω-fluoromyristic, ω-fluorooleic, ω-fluorocaproic and ω-fluoropalmitic acids (3). Nucleocidin, an antitypanosomal antibiotic, was the first fluoro-sugar derivative to be isolated from any natural source. The fluorine atom replaces the hydrogen atom at the C-4 position of the carbohydrate moiety and through a variety of spectral observations has been assigned the structure of a nucleoside (9-(4-fluoro-5-O-sulfamoylpentofuranosyl)adenine) (4).

Interest in fluorinated compounds arose most likely from the elucidation of the biochemical mode of action of fluoroacetate by Peters et al. (5). Kidney homogenates treated with fluoroacetate were found to accumulate citrate and to produce a new component identified as fluorocitrate. This new compound was found to exert its activity by inhibiting the mitochondrial enzyme aconitase, which catalyzes the conversion of citrate to isocitrate (5). The term 'lethal synthesis' was thus coined by Peters to describe the toxic
effects of fluoroacetate.

Since then a wide range of fluorinated compounds have been synthesized and used as biochemical probes \((6,7)\). Due to the large amount and variety of literature on this subject, only a few examples will be discussed to demonstrate the usefulness of introducing a fluorine atom(s) into compounds to study various biological systems.

**Fluorocitrate as a Biochemical Probe**

Guarriera-Bobyleva and Buffa \((8)\) demonstrated that in vivo administration of toxic doses of fluorocitrate results in almost total inhibition of citrate metabolism in mitochondria. On the other hand, cis-aconitate, which is also a substrate of aconitase, is oxidized with little or no effect. These results would seem to contradict the earlier proposal of Peters \((5)\), that the toxic effect of fluorocitrate is by the inhibition of aconitase. Uncertainties, in both the chemistry of the toxic isomer of fluorocitrate and its subcellular mode of action, led Kun \((9)\) to re-investigate fluorocitrate inhibition of aconitase.

The four possible isomers of fluorocitrate are formed either from fluoroacetyl-CoA and oxaloacetate or from fluorooxaloacetate and acetyl-CoA \((9)\). It was subsequently shown that the toxic isomer is formed only by enzymatic condensation of fluoroacetyl-CoA with oxaloacetate, a reaction catalyzed by citrate synthase \((10)\). All other isomers had no significant inhibitory effects on aconitase. Synthesis and resolution was accomplished in 1969 \((11)\)
where it was shown that the electrophoretically separated
erthro isomers contained the toxic species, which was
further resolved and identified as (-)erythrofluorocitrate;
correctly defined as: 1R:2R 1-fluoro-2-hydroxy-1,2,3-
propane tricarboxylate.

Fluorocitrate was found to behave as a competitive
reversible inhibitor of both cytoplasmic and mitochondrial
aconitase purified from pig liver (12). These isoenzymes
remained fully active without the addition of any artificial
activators. When the bivalent cations, Mg\(^{++}\) and Mn\(^{++}\), are
incubated with either aconitase isoenzyme, inhibition of
enzyme activity results in a time dependent manner (12).
These bivalent cations are necessary constituents of the
frequently used NADP\(^+\)-dependent isocitrate dehydrogenase
assay system. It was this unrecognized effect which accounted
for the earlier in vitro enzymological complications of
aconitase, which gave conflicting results of competitive,
noncompetitive or mixed types of inhibition by fluorocitrate
(9). Furthermore, these earlier preparations of aconitase
required cysteine and Fe\(^{++}\) as artificial activators, resulting
in about 3% fluoride release from fluorocitrate (13). The
preparations without the need of artificial activators
exhibited no defluorination of the substrate (9). These
results emphasize the dangers associated with applying in
vitro biochemical results to the in vivo condition.

If fluorocitrate behaves as an uncomplicated competitive
reversible inhibitor of aconitase, then some other mechanism
must account for the irreversible and toxic effects associated with fluorocitrate administration. When isolated intact liver mitochondria are incubated with citrate, in the presence of (-)-erythrofluoromalate (an activator), rapid citrate influx occurs. Associated with this influx of citrate is a rapid efflux of isocitrate (14). Isocitrate efflux can be monitored spectrophotometrically by the NADP$^+$-dependent isocitrate dehydrogenase coupled assay system, as illustrated in Figure 1. This system provided the basis for establishing the site of fluorocitrate action and toxicity. When intact mitochondria are pre-incubated with less than micromolar concentrations of fluorocitrate, marked irreversible inhibition of isocitrate efflux occurs. Isocitrate efflux is inhibited when either citrate or cis-aconitate are added externally (14). When the mitochondrial structure is disrupted by the nonionic detergent, Triton X-100, full aconitase activity of mitochondria is obtained even in the presence of fluorocitrate, which in the same preparation completely inhibited the flux of citrate into intact mitochondria (15).

The essential prerequisite for inhibition of mitochondrial citrate transfer is pre-incubation with 10-100 mM fluorocitrate in the absence of citrate, which if added simultaneously with fluorocitrate prevents inhibition. Pre-incubation of mitochondria with fluorocitrate also results in selective and irreversible inhibition of citrate-supported ATP synthesis and inhibition of citrate-dependent
Figure 1
Experimental Model System used by Kun (14) in Liver Mitochondria to Establish the Site of Fluorocitrate Toxicity.
Figure 1

citrate

↑ aconitase
cis-aconitate

↓ aconitase
isocitrate

↑ citrate or cis-aconitate

tricarboxylate carrier site

↓ isocitrate

↑ NADP⁺
isoctate dehydrogenase

↑ NADPH

α-ketoglutarate
fatty acid biosynthesis (16). No effect was observed when isocitrate, cis-aconitate or other tricarboxylic acid (TCA) intermediates were used as permeant substrates. These results are consistent with the existence of an inhibitory site of fluorocitrate which is rate-limiting in citrate transport through the inner mitochondrial membrane. Once citrate metabolism is inhibited, no subsequent manipulation can reactivate this specific process. Under these conditions the mitochondria exhibits the classical characteristics associated with fluorocitrate toxicity (17).

Incubation of inner membrane vesicles from mitochondria for one hour with 10 μM [14C]-labeled fluorocitrate results in isolatable protein-fluorocitrate adducts (16). The reaction of fluorocitrate with these membrane proteins was found to involve thioester formation since treatment with neutral hydroxylamine, a thioester bond cleaving agent, results in the release of bound fluorocitrate. Hydrolysis of the protein-fluorocitrate bond with sodium hydroxide is also consistent with the known properties of esters. Furthermore, inhibition of fluorocitrate binding results when vesicles are pre-treated with mersalyl, a specific reagent for thiol groups of proteins (16). The structure of the protein-fluorocitrate thioester adduct formed has not yet been deduced. It is possible that either one or both carboxyl groups of fluorocitrate, which are activated by fluorine substitution, may participate in thioester bond formation (16).
Two distinct proteins containing bound fluorocitrate were isolated from an extract of liver mitoplasts. Mitoplasts are defined as the mitochondrial matrix surrounded by the inner membrane. One has a molecular weight of about 175,000 and the second a molecular weight of about 71,500. These proteins were identified as citryl-S-glutathione (CSG) synthetase and hydrolase, respectively (18). The synthetase catalyzes thioester bond formation between citrate and oxidized glutathione (GSSG), whereas the hydrolase cleaves the thioester bond to give citrate and reduced glutathione (GSH). Pre-incubation of a mitoplast extract with fluorocitrate results in irreversible inhibition of CSG thioester formation. A correlation was found between the degree of inactivation of CSG synthetase and the quantity of protein-fluorocitrate product (18). Furthermore, this enzyme was also found to be inhibited by 1,2,3-propane tricarboxylate, which is generally recognized as a specific inhibitor of citrate transport in intact mitochondria.

The coincidence of inhibition of citrate transport in mitochondria with inhibition of CSG synthetase tends to suggest that this enzyme may be a constituent of the mitochondrial citrate transport system. Such a system could be envisioned to operate as in Figure 2. During the transport across the mitochondrial membrane citrate would be covalently modified by reacting with GSSG to form CSG, a reaction catalyzed by CSG synthetase. Before the thioester can completely traverse the membrane it is cleaved by the
Proposed Transport System for Citrate Across the Mitochondrial Membrane.
Figure 2

citrate → citrlyl-S-glutathione

CSG synthetase

GSSG

GSH

GSO

oxygenase → glutathione sulfinic acid

mitochondrial membrane

CSG hydrolase → citrate
hydrolase, resulting in free citrate and GSH. Inhibition of thioester formation by phenazine methosulfate, a free radical trapping agent, indicates that enzymatic formation of the thioester may involve GS\(^0\) formation via homolytic cleavage of GSSG (18). Associated with this process is the generation of glutathione sulfinic acid on a mole per mole basis with thioester formation, indicating the simultaneous contribution of an oxygenase type of reaction coincidental with the formation of CSG.

Specific enzyme systems were also found to exist in liver mitochondria that catalyzes the glutathione-thioester formation of succinic, malic, α-ketoglutaric, glutamic, pyruvic and isocitric acids and of glutamine (18). This may suggest a general metabolic pathway for the TCA intermediates. In conjunction with this is the identification of hydroxyacyl-S-glutathione, formyl-S-glutathione and succinyl-S-glutathione thioesters and their hydrolases in human liver (19). These enzymes were found to be localized in the cytoplasm and no apparent metabolic function was given.

An alternative function of the thioester synthetase-hydrolase system may be in regulating the concentration of all free carboxylic acid substrates of the TCA cycle. This would operate depending on the GSH-GSSG ratio. GSH is the most abundant thiol present in virtually all cells. It is involved in a wide range of cellular phenomena, such as maintaining protein thiol groups, protection of proteins and cell membranes against peroxides and free radicals,
controlling GSSG levels and in the transport of amino acids across cell membranes (20).

Fluorocitrate has also been used successfully to study citrate transport in whole cells of Salmonella typhimurium (21) and Bacillus subtilis (22). The difficulties in studying transport in whole cells are due to both rapid metabolism and the inherent complications of multiple transport components or systems. Fluorocitrate was found to be an effective probe to kinetically study citrate transport since it is not metabolized by these organisms. Wild-type strains of citrate grown whole cells were found to accumulate fluorocitrate rapidly without metabolism (21, 22).

Enzyme Reaction Mechanism Studies

One enzyme reaction mechanism that has been extensively studied with the use of fluoro-analogues is that of thymidylate synthetase. This enzyme catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP) to 2'-deoxythymidylate (dTMP) with the concomitant conversion of 5,10-methylenetetrahydrofolic acid (CH$_2$-H$_4$folate) to 7,8-di-hydrofolic acid (H$_2$folate) (23). This reaction was proposed by Friedkin (24) to occur via a two step process. The first step was viewed as an electrophilic substitution reaction in which the methylene carbon of CH$_2$-H$_4$folate replaces the hydrogen at the 5-position of dUMP. The second step of this reaction was proposed to be a nucleophilic attack at the incipient methyl group of dTMP by hydride originating from the 6-position of the cofactor to give
dTMP and H₂ folate. As early as 1959, it was recognized that CH₂-H₄ folate serves the dual function of both one carbon carrier and reductant (25). Direct support for major aspects of this proposed mechanism of thymidylate synthetase has been provided by Santi and co-workers (26) from studies of its interaction with 5-fluoro-2'-deoxyuridylate (FdUMP) and 5-trifluoromethyl-2'-deoxyuridylate (CF₃dUMP).

FdUMP has been known for some time to be an extremely potent inhibitor of thymidylate synthetase. Its inhibitory action was suspected to result from reaction with the proposed nucleophilic catalyst of thymidylate synthetase, namely CH₂-H₄ folate (26). In 1974, Santi (27) demonstrated that a tight complex is formed between CH₂-H₄ folate, FdUMP and thymidylate synthetase, which could be isolated by filtration through a nitrocellulose membrane. A scheme for the complex formation was proposed to be as follows:

\[
\begin{align*}
E & \overset{k_+}{\rightleftharpoons} E-\text{CH}_2\text{-H}_4\text{ folate} \\
E & \overset{k_+}{\rightleftharpoons} E-\text{FdUMP} \\
E-\text{CH}_2\text{-H}_4\text{ folate-FdUMP} & \overset{k_-}{\rightarrow} E-\text{CH}_2\text{-H}_4\text{ folate-FdUMP}
\end{align*}
\]

The pathway involves reversible formation of binary complexes which are not sufficiently stable to be isolated. Subsequent formation of a tightly bound ternary complex results as demonstrated by comparing dissociation constants (K_d). K_d for the binary FdUMP complex was calculated by
equilibrium dialysis to be $10^{-5}$ M (27). On the other hand, from studies of the relative rates of association and dissociation of FdUMP with the enzyme-CH$_2$-$\text{H}_4$ folate binary complex, the $K_d$ of the ternary complex was calculated to be $10^{-13}$ M (26). Therefore, once formed, the ternary complex is stable for long periods of time.

Using radioactive FdUMP and CH$_2$-$\text{H}_4$ folate, the enzyme has been titrated and shown to possess two FdUMP and two cofactor binding sites per mole (27). The ternary complex was found to be stable towards protein denaturation and showed a complete loss of absorbance at 269 nm, which corresponds to stoichiometric loss of the pyrimidine chromophore of FdUMP. This strongly suggests that the 5,6-double bond of the pyrimidine is saturated in the bound complex. Based on these findings, together with information gathered from a variety of model chemical studies and $^{19}$F-nuclear magnetic resonance ($^{19}$F-NMR) spectra, the structure of the enzyme-FdUMP-CH$_2$-$\text{H}_4$ folate ternary complex is proposed to be as in Figure 3 (23). Here a nucleophile of the enzyme has added to the 6-position of FdUMP. Furthermore, the 5-position of FdUMP is coupled to the 5-position of CH$_2$-$\text{H}_4$ folate via the methylene group of the cofactor.

Recently, studies of 1-(5-phospho-$\beta$-D-arabinosyl)-5-fluorouracil as a mechanism-based inhibitor of thymidylate synthetase (28) and FdUMP interaction with CH$_2$-$\text{H}_4$ folate and dUMP hydroxymethylase (29), have provided additional support for the proposed structure of the ternary complex. Further-
Figure 3
Proposed Structure of the FdUMP-CH₂-H₄folate-Thymidylate Synthetase Ternary Complex.

Legend:
X - represents a nucleophile of one of the amino acids of the enzyme.
more, FdUMP has also been used in establishing that the
dUMP hydroxymethylase reaction mechanism is analogous to
the mechanism of thymidylate synthetase (29).

As illustrated in Figure 4, the addition of a nucleophile
of thymidylate synthetase to the 6-position of dUMP is the
primary event in the enzyme catalyzed reaction (26). The
resultant carbanion reacts with CH₂-Н₄folate to produce a
steady-state intermediate, as proposed by Friedkin (24),
and analogous to the ternary complex formed with FdUMP,
cofactor and enzyme. Abstraction of the 5-hydrogen, followed
by a series of steps involving reduction of the one carbon
unit and elimination of the nucleophile, results in the
observed products.

The difference between the steady-state intermediate
and the ternary complex is that the steady-state intermediate
possesses a proton at the 5-position of the nucleotide
which is abstracted in a subsequent step. On the other
hand, the ternary complex possesses a stable fluorine atom
at the corresponding position. This fluorine atom prevents
proton abstraction, thus trapping the complex and leading
to inhibition of the enzyme. FdUMP, therefore, behaves as a
'quasi-substrate' (23,26) in the reaction. That is, it
enters into the catalytic reaction, just as the normal
substrate, up to a point where the intermediate is formed
which can proceed no further.

The nucleophile of thymidylate synthetase that attacks
dUMP in the normal enzymatic reaction and FdUMP in the
Figure 4

Proposed Mechanism for the Thymidylate Synthetase Reaction.

Legend:

\[ R = 5\text{-phospho-}2'\text{-deoxyribosyl} \]

\[ X \text{ represents a nucleophile of one of the amino acids of the enzyme.} \]
formation of the ternary complex has been known for some
time to involve a sulfhydryl group. It has recently been
demonstrated by protein degradation studies on the ternary
complex that the most likely candidate for the nucleophile
is cysteine (23).

$\text{CF}_3\text{dUMP}$ has also been shown to be an irreversible
inhibitor of thymidylate synthetase. Reyes and Heidel-
berger (30) proposed that this inhibition was due to the
formation of a covalent bond between the carbon carrying
the $\text{CF}_3$-group and an amino acid near the active site of the
enzyme. As depicted in Figure 5, this nucleophile adds to
the 6-position of $\text{CF}_3\text{dUMP}$, promoting the expulsion of fluoride
ion ($\text{F}^-$) and the formation of a reactive exocyclic difluoro-
methylene. The reactive intermediate would then be trapped
by a nucleophilic group of the enzyme to give the acylated
enzyme, thereby inactivating it.

Santi and co-workers (26) have recently demonstrated
that incubation of thymidylate synthetase with $\text{CF}_3\text{dUMP},$
in the absence of $\text{CH}_2\text{-H}_4\text{folate}$, for twenty minutes results
in 89% inactivation of the enzyme with possible formation
of a 5-acyl derivative of $\text{dUMP}$. From these and model studies
(26), it was proposed that activation of the $\text{C-F}$ bond
requires addition of a nucleophile of the enzyme to the
6-position of the nucleotide. When $\text{CH}_2\text{-H}_4\text{folate}$, $\text{CF}_3\text{dUMP}$
and thymidylate synthetase are incubated together, a
difference spectrum similar to that produced by the $\text{FdUMP-}$
$\text{CH}_2\text{-H}_4\text{folate}$-enzyme ternary complex is observed (26).
Figure 5

Proposed Inactivation Mechanism of Thymidylate Synthetase by CF₃dUMP.

Legend:

\[ R = 5\text{-phospho-2'\text{-deoxyribosyl}} \]

\[ X \] represents a nucleophile of one of the amino acids of the enzyme.
Figure 5

CF₃dUMP → F⁻ → CF₂ → 2F⁻ → H₂O

HN       CF₃
O         N
R

HN       O
O         C
R

HN       O
N         C
R
There is a characteristic increase in absorbance at 330 nm and a decrease at 261 nm. This decrease in absorbance is in accord with the saturation of the 5,6-double bond of the nucleotide. Although preliminary, these results suggest a similar reaction mechanism between thymidylate synthetase and FdUMP or CF3dUMP.

Apart from providing valuable information regarding the general mechanism of folate dependent enzymes, in particular thymidylate synthetase, fluorouracil analogues are currently in use as anti-tumor, anti-viral and antifungal agents (31).

During the past several years, there has been a tremendous increase in the synthesis and biochemical use of a variety of active site directed labeling agents (32-34). The rationale and general criteria for the design of such compounds have been given by Rando (35). Briefly, the design of these inhibitors is based upon the attachment of a chemically reactive group, generally an electrophile, to a molecule which has structural features resembling that of the enzyme's normal substrate. The resultant reaction of the molecule with the active site of the enzyme leads to covalent bond formation and inactivation of the enzyme.

One class of such compounds includes the α-fluoromethylamino acid analogues (36-40). These substrates are non-reactive until fluorine is expelled via enzyme catalyzed decarboxylative elimination. Highly electrophilic species would result which are capable of undergoing addition of
fortuitously positioned nucleophilic groups at or near the enzyme's active site. Stable enzyme-inhibitor covalent adducts would result, thereby inactivating the enzyme. The general scheme for such reactions is as follows:

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_{cat}} EI \xrightarrow{k_{inh.}} E-I \text{ (covalently modified)}$$

These mechanism-based inhibitors have also been referred to as suicide substrates, $k_{cat}$ inhibitors or Trojan-horse inhibitors (33) because of their mode of action.

Fluorine is hard to displace in $S_N2$ reactions, but can undergo $\beta$-elimination from carbanions quite easily (37). It is therefore necessary for such compounds to form reactive intermediates before such reactions can occur. As an example, Figure 6 illustrates the proposed inactivation mechanism of glutamate decarboxylase by $\alpha$-fluoromethylglutamate (36). The first step in this reaction, which is a common step in all pyridoxal phosphate-dependent enzymes, is the formation of a Schiff base between the aldehyde function of the cofactor and the amino group of the substrate. The resulting reactive intermediate generated within the enzyme's active site is unable to dissociate rapidly from the protein (41). This would lead to covalent bond formation between the reactive intermediate and a nucleophilic group of the enzyme, thereby inactivating the enzyme.

The above inactivation reaction occurs for about 80% of the time since nonlethal turnover could also occur.
Proposed Inactivation Mechanism of Glutamate Decarboxylase by \( \alpha \)-Fluoromethylglutamate.

Legend:

X - represents a nucleophile of one of the amino acids of the enzyme.

A - postulated nonlethal turnover leading to the formation of fluoromethyl-GABA.

B - postulated nonlethal turnover leading to the formation of levulinic acid.
It was suggested that direct decarboxylation could occur to form fluoromethyl-GABA or that hydrolysis of the activated intermediate could occur to form levulinic acid (36).

**Carbon- Fluorine Bond Cleavage**

The bond strength of the C-F bond (448 kJ mol⁻¹) is among the highest found in natural products and yet is cleaved in a number of different enzymatic reactions (42). The cleavage of the C-F bond in CF₃UMP and in the α-fluoro-methyl-amino acid analogues was seen to be the result of the formation of reactive intermediates. These compounds were specifically designed with the purpose of forming such reactive intermediates, which would react with and irreversibly inhibit their respective enzymes through covalent bond formation. The release of fluoride, in these and other examples, might therefore be considered purely accidental in terms of the normal role of the enzyme. That is, the release of fluoride is a consequence of the normal enzymatic reaction mechanism.

In contrast to the above, adventitious cleavage of the C-F bond has been shown to occur. By the enrichment culture technique, Goldman (43) has been able to isolate an organism capable of rapid growth with fluoroacetate as the sole source of carbon. A cell-free extract from this pseudomonad was found to contain an enzyme which catalyzes the defluorination of fluoroacetate with concomitant production of glycollate. The enzyme is specific for fluoroacetate although it also catalyzes analogous release of
halide from chloroacetate and iodoacetate. The enzyme was therefore termed haloacetate halidohydrolase (43).

Enzyme reactivity was found to be dependent on the presence of an active thiol group on the enzyme. It was therefore postulated that defluorination proceeds through thioester formation (43) as follows:

\[ \text{ENZ-S}^- + \text{FCH}_2\text{COO}^- \rightarrow \text{ENZ-S-CH}_2\text{COO}^- + \text{F}^- \]

\[ \text{ENZ-S-CH}_2\text{COO}^- + \text{OH}^- \rightarrow \text{ENZ-S}^- + \text{HO-CH}_2\text{COO}^- \]

An intriguing possibility is that this enzyme might be capable of catalyzing reversible formation of fluoroacetate from glycollate and fluoride. If possible, this could give an indication as to how fluoroacetate is formed in nature. Unfortunately though, many attempts have failed to demonstrate the reversibility of this reaction (44).

Organisms capable of metabolizing fluorobenzoates via C-F bond cleavage have also been shown to exist (44,45). A pseudomonad was isolated which could metabolize 2-fluorobenzoic acid with corresponding fluoride release (44). The defluorination reaction was found to take place by the introduction of molecular oxygen in a one-step process as follows:

\[ \text{F} \rightarrow \text{OH} \]
In the above mechanism, 2-fluorobenzoic acid is converted to catechol, which can be further metabolized by the organism. Recently, an enzyme has been isolated from cell-free extracts of *Pseudomonas putida* and shown to catalyze fluoride release from difluoro- and tetrafluoro-derivatives of p-hydroxybenzoic acid, through a similar hydroxylation type reaction (45).

The defluorination reactions discussed thus far represent the few known examples in both microbial and mammalian systems. In contrast to these, the release of fluoride from fluorocarbohydrates has not been observed to any great extent in biological systems, although they have been used extensively as biochemical probes (see next section). One special case though, is the hydrolytic cleavage of the C-F bond in both galacto- and glucopyranosyl fluorides (46,47).

The D-galactopyranosyl fluorides were found to serve as substrates and inducers of the β-galactosidase of *Escherichia coli* (46). The α- and β-forms are hydrolyzed by α- and β-galactosidases, respectively, to D-galactose and F⁻. In either case, the hydroxyl-attack, and thus hydrolytic cleavage, is on the side of the substrate molecule for which the enzyme has specificity.

Similarly, α-D-glucopyranosyl fluoride was found to be hydrolyzed by the action of α-glucosidase to D-glucose and F⁻ (47). The mechanism of α-glucosidase action involves protonation of the glycosidic oxygen followed by nucleophilic attack, either from water leading to inversion of
configuration or by a nucleophilic group on the enzyme which is then displaced by water to give retention of configuration. The enzyme appears to protonate the fluorine atom as well as it does the oxygen atom, leading to the release of fluoride ion (47). α-D-Glucopyranosyl fluoride has also been found to be hydrolyzed by other enzymes such as sucrose phosphorylase and amylomaltase (47). Recently, α- and β-D-glucopyranosyl fluorides have been used as probes to study the enzyme trehalase (48). As in the previous examples, α-D-glucopyranosyl fluoride is hydrolyzed by the action of trehalase to α- or β-D-glucose and F⁻. Furthermore, trehalase also catalyzes the reaction of β-D-glucopyranosyl fluoride with α-D-glucose to give trehalose and F⁻ (48).

**Fluorocarbohydrates as Biochemical Probes**

The rationale, synthesis and use of fluorocarbohydrates as biochemical probes have been reviewed in part (6, 7, 49). Such compounds have been used to probe the specificity of hydrogen-bonding sites between D-glucose and the carrier protein(s) known to be present in the human erythrocyte (50, 51) and the hamster intestine (47). Recently, Grier and Rasmussen (52) have used 3-deoxy-3-fluoro-D-mannose and 4-deoxy-4-fluoro-D-mannose as probes for studying D-mannose transport and metabolism in *Saccharomyces cerevisiae*. 2-Deoxy-2-fluoro-D-glucose and 2-deoxy-2-fluoro-D-mannose have been employed extensively for studies of glycoprotein biosynthesis and function in chick embryo cells (53, 54).
Other studies with fluorocarbohydrates by Taylor have been primarily concerned with enzyme specificity (55), carbohydrate transport in synaptosomes from rat brain cortex (56), carbohydrate metabolism in Locusta migratoria (57) and carbohydrate transport and metabolism in E. coli (58) and P. putida (59).

Previous studies (59) demonstrated that 3-deoxy-3-fluoro-D-glucose (3FG) is metabolized by resting whole cells of glucose grown P. putida, with retention of the C-F bond, to produce 3-deoxy-3-fluoro-D-gluconic acid (3FGA). 3FGA can be further oxidized by whole cells to 3-deoxy-3-fluoro-2-keto-D-gluconic acid (2K3FGA). Cell-free extracts from this organism were found to oxidize 3FG completely to 2K3FGA.

It was also shown that the same cytoplasmic membrane bound enzymes that oxidizes glucose and gluconate, namely glucose oxidase and gluconate dehydrogenase, also oxidizes 3FG and 3FGA, respectively (59). Furthermore, using cytoplasmic membrane vesicles prepared from glucose or succinate grown P. putida, it was shown that 3FG is transported by the same active transport system as glucose (60).

Unlike 3FG, 4-deoxy-4-fluoro-D-glucose (4FG) is not oxidized by whole cells of P. putida. Instead, there is an immediate release of fluoride ion (61). Incubation of a resting whole cell suspension of glucose grown P. putida with 2.5 mM 4FG results in nearly complete fluoride release after 24 hours. Thin layer chromatography of the cell supernatant and intracellular contents failed to reveal the presence of any new carbohydrates. On the other hand, cell-
free extracts were found to oxidize 4FG with the consumption of 2 g atoms of oxygen per mole of substrate, which is consistent with the formation of 4-deoxy-4-fluoro-2-keto-D-gluconic acid (2K4FGA). In this reaction there is virtually complete retention of the C-F bond. Furthermore, \textit{P. putida} is unable to grow on a mineral salts medium with 4FG as the sole carbon source, although fluoride is released into the medium (61). Therefore, the defluorination of 4FG could be considered purely accidental as a consequence of 4FG being initially accepted by the cell as glucose.

These results suggest that cleavage of the C-F bond in 4FG occurs in the cell envelope fraction of \textit{P. putida} and that the defluorination product becomes covalently attached to a protein in the cell envelope (61).

\textbf{The Cell Envelope of Gram-Negative Bacteria}

The cell envelope of gram-negative bacteria consists of two typical membranous structures, outer membrane and cytoplasmic membrane (62). As illustrated in Figure 7, the space between the outer membrane and the cytoplasmic membrane is the periplasmic space, which contains enzymes and binding proteins as well as the peptidoglycan layer.

Kaback and Stadtman (63) were the first to describe a method for obtaining transport active cytoplasmic membrane vesicles from \textit{E. coli}. Since then, vesicles have been used extensively to study and better understand the various bacterial transport systems (64,65). Three distinct classes of active transport systems for sugars, as well as other
33

Figure 7

Proposed Structure of the Cell Envelope of Gram-Negative Bacteria.

Legend:

BP - binding protein
CM - cytoplasmic membrane
OM - outer membrane
PC - porin channel
PG - peptidoglycan
PS - periplasmic space
TP - transport protein
\( \text{\textbullet} \) - lipopolysaccharide
\( \text{\textbullet} \) - phospholipid
substrate molecules, in gram-negative bacteria, have been described (64,65). One class couples the transport of molecules across the cell membrane with chemical modification, a process referred to as group translocation. The second class transports molecules against a concentration gradient with no change in the transported molecule. The third class, which also concentrates substrate molecules without modification, is dependent upon the presence of periplasmic binding proteins. These binding proteins, which can exist in both a free form or bound to the peptidoglycan layer (66), form reversible complexes with specific substrates. Binding proteins have been shown in a number of cases to be involved in the translocation of substrate molecules across the cell envelope of gram-negative bacteria (66-68). In contrast to the above systems, not much attention has been given to the outer membrane as far as transport capabilities are concerned. At best, the outer membrane was generally regarded as a rather inert structure that allows the free diffusion of all low molecular weight substances.

The separation of outer and cytoplasmic membranes was first achieved in *E. coli* by Miura and Mizushima (69). Their technique involves isopycnic sucrose density gradient centrifugation of membranes obtained by lysozyme and EDTA treatment of whole cells, followed by lysis of the resulting osmotically fragile spheroplasts. A similar method modified for *S. typhimurium* was developed by Osborn et al. (70). These techniques have led to a greater understanding of the
structure and function of the outer membrane and outer membrane proteins, especially in the enteric bacteria, as noted by the recent reviews (71-74).

An increasing amount of evidence from the enteric bacteria, E. coli and S. typhimurium, has led to the general conclusion that the outer membrane from these organisms contain two classes of proteins involved with solute uptake (71,75). The first class of proteins, which form hydrophilic aqueous channels, are generally referred to as porins. These proteins vary from strain to strain but generally have molecular weights in the range of 35-40,000 daltons. Furthermore, they allow for the nonspecific free diffusion of small uncharged molecules of less than 600 daltons. These pore-forming proteins have recently been shown by Lugtenberg et al. (76) to be peptidoglycan-associated.

The other class of proteins behave as transmembrane channels with solute specificity. These receptor proteins have been shown to facilitate the diffusion of maltose and maltodextrins, iron-chelated ferrichrome, iron-citrate complexes, nucleosides, vitamin B_{12} as well as others (71). Since most of these nutrients have molecular weights larger than the exclusion limit of the porin channel, specific translocation proteins are a necessity. In examples like maltose, specific proteins are required when the substrates are present in very low concentrations.

Recently, Lo has demonstrated, through the use of various nonpenetrating proteases and inhibitors (77) and a
nonpenetrating substrate analogue (78), the involvement of a cell surface dicarboxylic acid binding protein (DBP) in the translocation of dicarboxylic acids across the outer membrane of E. coli. As illustrated in Figure 8, dicarboxylic acid translocation across the outer membrane is proposed to occur via an outer membrane DBP-porin channel complex (66). It is postulated that DBP associates with the porin channel and confers on it the specificity for dicarboxylic acids. The absence of DBP leaves the porin channel open, resulting in enhanced transmembrane diffusion of molecules other than dicarboxylic acids. A similar mechanism has been postulated to exist between the maltose binding protein and the maltose pore (λ-receptor) for maltose transport in E. coli (79).

In the outer membrane DBP-porin channel complex it is postulated that DBP binds to the porin channel on the outer surface of the outer membrane (66). This, however, may not be true in all cases. It is possible for binding proteins, in general, to confer specificity on the porin channel by binding on the inner surface of the outer membrane, as postulated for maltose translocation (79). Alternatively, binding proteins may be tightly associated within the porin channel (66).

In contrast to the marked accumulation of information on the outer membrane of the enteric bacteria, that of the outer membrane of the aerobic bacteria, such as P. aeruginosa, has only recently been given consideration. The cell envelope of P. aeruginosa has been extensively investigated.
Figure 8

Proposed Model of Lo (66) for the Outer Membrane DBP-Porin Channel Complex.

Legend:
- DBP - dicarboxylic acid binding protein
- OM - outer membrane
- PC - porin channel
- PS - periplasmic space
- - lipopolysaccharide
- - phospholipid
in terms of protein (80), peptidoglycan (81), lipopolysaccharide (82) and lipids (83). However, good separation of outer and cytoplasmic membranes was not always possible due to the hypersensitivity of this and related organisms to EDTA-lysozyme treatment (84). Recently though, several laboratories have reported the isolation of the outer membrane from *P. aeruginosa*, all using different procedures (85-89). As indicated in Table 1, two to nine major proteins were observed in the various outer membrane preparations. Some of these outer membrane proteins, in particular the porin protein F, have been shown to be 'heat-modifiable'. That is, their mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was determined by the solubilization temperature (86,90). Other outer membrane proteins (F, H and I) have been shown to be peptidoglycan-associated (91,92). That is, the protein is tightly, but not covalently, associated with peptidoglycan. The number and molecular weight of many of the outer membrane proteins were found to be dependent on the growth and culture conditions used, the method of outer membrane isolation, the method of electrophoresis employed and the strain used (73,90).

Permeability studies using reconstituted outer membrane vesicles indicated that the outer membrane of *P. aeruginosa* contains porin channels that are permeable to saccharides of up to 6,000 daltons (93). The molecular weight of these porin proteins are also within the range of 35-40,000 daltons. Recently, the *P. aeruginosa* porin
Table 1
Nomenclature and Molecular Weight of *P. aeruginosa*
Outer Membrane Proteins.

<table>
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<tr>
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<th>Molecular Weight (10^3)</th>
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<td>Booth and Curtis (85)</td>
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<td>I (8)</td>
</tr>
<tr>
<td></td>
<td>6 (23)</td>
</tr>
<tr>
<td></td>
<td>7 (19)</td>
</tr>
<tr>
<td></td>
<td>8 (13)</td>
</tr>
<tr>
<td></td>
<td>9 (9.7)</td>
</tr>
</tbody>
</table>
protein has been purified to homogeneity in a functional form (94).

*P. aeruginosa*, *P. fluorescens* and *P. putida* belong to a group of obligate aerobes which metabolize D-glucose by the Entner-Doudoroff pathway (95, 96). The uptake of glucose in *P. aeruginosa* has been shown to involve two distinct inducible pathways with differing affinities for glucose (97, 98). A high affinity (low $K_m$) system with an apparent $K_m$ of 8 µM for glucose is induced by growth on glucose, but not by growth on gluconate, succinate or citrate. This system is only involved with the active transport of glucose into the cell and requires an inducible glucose binding protein (GBP) (67, 68). In addition, a low affinity ($K_m$ in the range of 2-7 mM) glucose transport system exists and is induced by growth of cells on glucose or gluconate, but is repressed by growth on succinate or citrate (97, 98). The low affinity system is an active transport system of rather broad specificity since it not only transports glucose but is also capable of transporting $\alpha$-methyl-D-glucopyranoside ($\alpha$-MeG), 2-deoxyglucose and 6-deoxyglucose as well as others. Furthermore, glucose dehydrogenase activity was required for the low affinity system to be operative (97, 98). Similarly, glucose is transported in glucose grown whole cells of *P. putida* with a low and high $K_m$ of 1.7 µM and 79 µM, respectively (99). In succinate grown cells the low $K_m$ component was found to be present, which may reflect the presence of trace amounts of repressed
glucose carrier protein (99). The high $K_m$ transport system was found to actively transport glucose, 2-deoxyglucose, 3FG and $\alpha$-MeG, whereas the low $K_m$ or high affinity system was found to transport glucose only (99).

When *P. aeruginosa* is grown on minimal medium in the presence of glucose, two major outer membrane proteins, D and E, of very similar molecular weights are induced (86,90). In contrast, the outer membrane of cells grown on succinate are only partially induced for these proteins (90). Protein D is in fact two polypeptides, D1 and D2, of which protein D1 was absent after growth on succinate. Proteins D1 and D2 could only be separated on gels where the acrylamide concentration is 14% or greater (90). Since other proteins associated with glucose transport and metabolism are induced by growth of cells on glucose (67,68,97,98), it is possible that those proteins induced in the outer membrane of *P. aeruginosa* are involved in the translocation of glucose across the outer membrane (90). This would be in the same way that the lambda receptor is associated with maltose uptake (79) and the outer membrane DBP-porin channel complex is associated with dicarboxylic acid transport (66), both in *E. coli*.

Hancock and Carey (100) have proposed that the outer membrane protein D1 and the periplasmic GBP are co-regulated components of the *P. aeruginosa* high affinity transport system. Protein D1 was shown to reconstitute sucrose and glucose permeable pores in LPS-phospholipid vesicles (100).
Furthermore, they also proposed that the low affinity glucose transport system involves uptake across the outer membrane via the porin protein F. No evidence was provided to support these proposals (100).

Therefore, glucose transport in whole cells of P. aeruginosa, and presumably other pseudomonads, may involve the binding of glucose to specific proteins associated with or part of the outer membrane. This interaction allows for the specific translocation of glucose across the outer membrane.

Objectives

This investigation involves the use of 4FG as a biochemical probe to study glucose transport and metabolism in P. putida. The objectives of this investigation are to:
(a) study and gain insight into the nature and mechanism of fluoride release from 4FG (61).
(b) attempt to determine the site in the whole cell responsible for the defluorination reaction.
(c) attempt to demonstrate the covalent bond formation proposed (61) to occur between the defluorination product and a protein.
(d) use these results with a view to obtain an understanding of glucose transport in whole cells of P. putida and, therefore, substrate transport in general.
CHAPTER II
MATERIALS AND METHODS

Materials

Crystalline 4FG, 4-deoxy-D-glucose and α-Me4FG (51) and D-[6-3H]4FG (10.8 mCi/mmol) (101) were made in these laboratories. D-[U-14C]Glucose (283 mCi/mmol) was obtained from Radiochemical Centre (Amersham, United Kingdom). Statzyme Glucose (500 nm) reagent kit was obtained from Worthington Diagnostics, Division of Millipore Corp., (Freehold, New Jersey). SDS-PAGE kit, molecular weight standards, all other electrophoresis reagents and Dowex AG 1x8 (200-400 mesh) resin in the chloride form were obtained from Bio-Rad Laboratories (Richmond, California). Sephadex G-100 resin was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Chloramphenicol, rifampicin, lysozyme, pronase, DTT, NEM, ribonuclease A, deoxyribonuclease I, DCIP and PMS were all obtained from Sigma Chemical Co., (St. Louis, Missouri). Yeast extract, agar and nutrient agar were obtained from Difco Laboratories (Detroit, Michigan). Unless otherwise stated, all other carbohydrates, chemicals and reagents were of the highest grade and purchased from Sigma Chemical Co., or Fisher Scientific Co., (Fairlawn, New Jersey).

The organism used in this study was P. putida (ATCC 12633), formerly classified as P. fluorescens A 3.12. The organism was first isolated by Stanier (102) and has since been used in many different biochemical and physiological studies. The organism was obtained as freeze-dried samples from the American Type Culture Collection (ATCC) (Rockville,
Maryland) and maintained routinely on glucose-mineral salts agar slopes or nutrient agar slopes.

**Methods**

**Growth and Characterization of P. putida**

For growth of *P. putida*, the semi-defined medium of Davis and Mingioli (103) was used throughout these studies:

\[
\begin{align*}
\text{K}_2\text{HPO}_4 & : 7.0 \\
\text{KH}_2\text{PO}_4 & : 3.0 \\
(\text{NH}_4)_2\text{SO}_4 & : 1.0 \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & : 0.1 \\
\text{Yeast extract} & : 0.2 \\
\text{Trace solution A} & : 0.5 \text{ mL} \\
\text{Trace solution B} & : 0.5 \text{ mL} \\
\text{Carbon source} & : 2.0
\end{align*}
\]

Solutions of trace elements, made up according to Barnett and Ingram (104), contained the following:

**Trace solution A**

\[
\begin{align*}
\text{FeSO}_4 \cdot 4\text{H}_2\text{O} & : 80 \\
\text{MnSO}_4 \cdot 4\text{H}_2\text{O} & : 80 \\
\text{NaCl} & : 2000
\end{align*}
\]
Trace solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/100 mL (stock solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>40</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>8</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>8</td>
</tr>
<tr>
<td>CaCl₂ (anhydrous)</td>
<td>1000</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>10</td>
</tr>
<tr>
<td>KI</td>
<td>60</td>
</tr>
</tbody>
</table>

The resulting medium which had a pH of 7.1 was sterilized by autoclaving at 121°C for 15 min in a Pelton and Crane Magna-clave, Model MC (G. A. Ingram Co., Canada) Ltd., Windsor, Ontario), without the carbon source. A 2% (w/v) of carbon source was sterilized by membrane filtration in a Nalgene 0.20 μ filter unit (Nalgene Sybron Corp., Rochester, New York) and the appropriate amount added aseptically to the sterile mineral salts medium to give a final concentration of 0.02%.

The purity of the organism was periodically tested by a variety of biochemical tests as described in Table 2.

Preparation of Whole Cell Suspensions

The necessary cell yields were achieved by growth of *P. putida* in 2 L Erlenmeyer flasks, each containing 500 mL of glucose-, gluconate-, 2-ketogluconate-, citrate-, or succinate-mineral salts medium. Inocula for these cultures were prepared from 24 h slope cultures, by washing with 10 mL of sterile distilled water and aseptically transferring an
Table 2

Biochemical Tests Used in Determining the Purity of *P. putida*.

<table>
<thead>
<tr>
<th>Test</th>
<th><em>P. putida</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of growth in Hugh and Leifsons medium (1% glucose).</td>
<td>Acid production only at top of aerobic tube.</td>
</tr>
<tr>
<td>Methyl red.</td>
<td>-</td>
</tr>
<tr>
<td>Voges Proskauer.</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin liquefaction.</td>
<td>-</td>
</tr>
<tr>
<td>Indole production.</td>
<td>-</td>
</tr>
<tr>
<td>Ammonia from arginine.</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase.</td>
<td>+</td>
</tr>
<tr>
<td>Catalase.</td>
<td>+</td>
</tr>
<tr>
<td>Growth in Koser's citrate medium.</td>
<td>+</td>
</tr>
<tr>
<td>Growth on milk agar* (61).</td>
<td>Growth, no hydrolysis and no pigment.</td>
</tr>
</tbody>
</table>

*Used* to distinguish between *P. fluorescens* and *P. aeruginosa*. 
appropriate aliquot (1 mL) to a 250 mL Erlenmeyer flask containing 50 mL of the appropriate carbon source-mineral salts medium. Cells were grown for 8 h, at which time 1 mL aliquots of the growth solution were aseptically removed and transferred to each of the 2 L flasks. Succinate, citrate, gluconate or 2-ketogluconate grown cells were obtained from cultures trained for at least six sub-cultures through the appropriate carbon source-mineral salts medium. Cultures were incubated at 30°C in an orbital rotary shaker with setting at 4 (Lab-Line Instruments Inc., Melrose Park, Illinois). Using this procedure, cell yields in the range of 2.5 to 3.0 g wet weight per L of culture medium were obtained after 15 h incubation, at which time the cells were in the late exponential phase. Cells from cultures of 1 L or less were harvested in a Sorvall Superspeed RC2-B centrifuge (Ivan Sorvall Inc., Newton, Connecticut) at 10,000 xg for 10 min at 30°C. Cells from cultures of greater than 1 L were harvested in an IEC PR-6000 centrifuge (Damon/IEC Division, Needham Heights, Massachusetts) at 4,200 xg for 20 min at 30°C. In either case, the cells were washed with and suspended in 100 mM potassium phosphate buffer, pH 7.1 (potassium-phosphate buffer), to the desired protein concentration.

Preparation of Cytoplasmic and Outer Membranes

(a) Cytoplasmic Membrane Vesicles

The method for obtaining cytoplasmic membrane vesicles was essentially as that described in (60). Glucose or
succinate grown whole cells were suspended in 2/3 the original culture volume in a solution containing in final concentrations: 2.5% LiCl, 0.75 M sucrose, 10 mM potassium phosphate, pH 7.1, 10 mM MgSO₄ and 0.5 mg lysozyme mL⁻¹. This suspension was incubated for 1 h at 30°C on a rotary shaker and the osmotically fragile spheroplasts were then harvested by centrifugation at 10,000 xg for 15 min at 4°C. The pellet was suspended in the smallest possible volume with 2.5% LiCl in 0.75 M sucrose. The suspension was quickly added to 50 volumes of ice-cold 10 mM potassium phosphate buffer, pH 7.1, containing 1 mM MgSO₄, in a Waring blender and blended for 10 sec. Deoxyribonuclease I (DN'ase) and ribonuclease A (RN'ase) were added to a final concentration each of 20 μg mL⁻¹ and the mixture was incubated at 25°C with gentle stirring for 30 min. This mixture was then centrifuged at 40,000 xg for 30 min at 4°C in a Beckman L8-55 ultracentrifuge (Beckman Instruments Inc., Fullerton, California) and the pellet suspended in ice-cold potassium phosphate buffer containing 10 mM MgSO₄. Large fragments and whole cells were removed by centrifuging the suspension at 800 xg for 30 min at 4°C. The supernatant was then centrifuged at 40,000 xg for 30 min at 4°C and the final pellet, cytoplasmic membrane vesicles, was washed twice with and suspended in the same buffer system and stored at -20°C.

(b) Outer Membranes

The isolation of the outer membrane was based on a modification of the method of Mizunó and Kageyama (86).
Glucose or succinate grown whole cells (4 g wet weight) were suspended in 40 mL of ice-cold 20% sucrose and the following ice-cold reagents added slowly with constant stirring: 20 mL of 2 M sucrose, 10 mL of 100 mM potassium phosphate, pH 7.1, 10 mL of 100 mM MgSO$_4$, 0.8 mg DTT mL$^{-1}$ and 0.5 mg of lysozyme mL$^{-1}$. The suspension was incubated for 1 h at 30°C on a rotary shaker and after 30 min DN'ase was added to a final concentration of 5 μg mL$^{-1}$. The suspension was then centrifuged at 10,000 xg for 15 min at 30°C to remove the osmotically fragile spheroplasts. Cytoplasmic membrane vesicles could be obtained from these spheroplasts by the method described above. Outer membranes were recovered from the supernatant by centrifugation at 100,000 xg for 90 min at 4°C. The outer membrane fraction was suspended in a small amount of potassium phosphate buffer and centrifuged at 800 xg for 30 min at 4°C to remove any large fragments. The supernatant was then centrifuged at 100,000 xg for 90 min at 4°C and the final pellet, which had a whitish opaque appearance and referred to as the crude outer membranes, was washed twice with and suspended in a small volume of the same buffer. For fluoride release studies the outer membranes were used immediately, otherwise they were stored at -20°C.

**Preparation of Cell-Free Extracts and Cell Envelopes**

(a) **Cell-Free Extracts**

Cell-free extracts were produced by ultrasonication (55). A glucose grown whole cell pellet was thoroughly mixed with an equal volume of potassium phosphate buffer to produce a
thick cell suspension. The suspension was cooled to 4°C and ultrasonicated for 8-10 x 1 min periods using a Sonic 300 Dismembrator (ARTEK Systems Corp., Farmingdale, New York) with setting at 60%. The temperature during ultrasonication was maintained at 0-4°C with the use of an ice-salt bath. During ultrasonication the suspension turned reddish-brown, indicating cell breakage and release of cell contents. The ruptured cell suspension was centrifuged at 17,000 xg for 10 min at 4°C. A small dark brown pellet was obtained, consisting of unbroken whole cells and large debris. In some cases no pellet was obtained, suggesting a near complete cell breakage. The reddish supernatant obtained is referred to as the cell-free extract.

(b) Cell Envelopes

Cell envelopes were obtained directly from the cell-free extract. The cell-free extract was centrifuged at 100,000 xg for 90 min at 4°C. The red pellet obtained was washed with and suspended in potassium phosphate buffer. This is referred to as the cell envelope suspension and is made up of the cytoplasmic membrane, outer membrane and the peptido-glycan layer. The supernatant obtained after centrifugation is referred to as the cell envelope-free supernatant and contains many soluble proteins.

SDS-Extraction of Cell Envelopes

This method is based on the procedure of Mizuno and Kageyama (92). Cell envelopes prepared from glucose or succinate grown whole cells of P. putida (5 g wet weight)
were incubated with 15 mL of the SDS-extraction solution (2% SDS–10% glycerol–10 mM Tris–HCl, pH 7.8) for 60 min at 30°C or 70°C. The insoluble fraction was collected by centrifugation at 100,000 xg for 90 min at 25°C. The extraction was repeated once more with 15 mL of the SDS-extraction solution at the appropriate temperatures. Peptidoglycan-associated protein complexes (92) were isolated by centrifugation at 100,000 xg for 90 min at 25°C, washed once with distilled water at 4°C and suspended in a small volume of distilled water. The supernatants from the same extraction temperatures were combined and proteins collected by addition of 2 volumes ice-cold acetone and centrifugation at 17,000 xg for 20 min at 4°C. The pellets were suspended in a small volume of distilled water. Peptidoglycan-associated protein complexes obtained by SDS-extraction at 30°C were further extracted by incubation with 10 mL of the SDS-extraction solution for 60 min at 70°C or by incubation with 10 mL of the SDS-extraction solution supplemented with 0.75 M NaCl for 60 min at 30°C. Insoluble fractions (predominately peptido- glycan) were collected by centrifugation at 100,000 xg for 90 min at 25°C. Proteins from the individual supernatants were collected by addition of 2 volumes ice-cold acetone and centrifugation at 17,000 xg for 20 min at 4°C. Protein pellets were suspended in a small volume of distilled water.

**Osmotic Shock of Whole Cells**

The method of osmotic shock was based on the procedure of Stinson *et al.* (67) for the isolation of GBP from *P.*
aeruginosa. Glucose grown whole cells (1 g wet weight) were suspended in 30 mL of 0.2 M MgCl₂-0.05 M Tris-HCl, pH 8.5, buffer at 25°C. The suspension was incubated for 30 min with constant stirring and then centrifuged at 16,300 xg for 20 min. The cells were rapidly suspended in 30 mL of distilled water and after an additional 30 min stirring at 25°C, the suspension was centrifuged. The pellet was immediately suspended in potassium phosphate buffer and tested for defluorinating activity. The supernatants were combined and dialyzed against 2 L of 1 mM MgCl₂-0.01 M Tris-HCl, pH 7.4, buffer at 4°C overnight. Proteins were collected by 0-95% saturation with (NH₄)₂SO₄ and centrifugation at 17,000 xg for 20 min at 4°C. The sediment was suspended in a small amount of buffer and dialyzed overnight. This crude shock extract was tested for glucose binding and defluorinating activities.

Assay of Glucose Binding Protein

The binding activity of GBP in the crude shock extract was determined by equilibrium dialysis (67). Dialysis tubing (Fisher Scientific Co., 1 cm diameter) were filled with 1.0 mL of extract (1.0 mg protein mL⁻¹) and dialyzed at 4°C for 20 h against 100 mL of buffer containing 2 μM D-[U-¹⁴C]glucose (4.02 mCi/mmol). After dialysis, 0.1 mL samples were removed from each compartment for liquid scintillation counting.

Fluoride Release Studies

Fluoride ion was determined by a fluoride ion specific electrode (Orion Research, Cambridge, Massachusetts) attached
to a Metrohm Herisau E510 mV/pH meter (Brinkman Instruments Ltd., Rexdale, Ontario). A standard curve was constructed with known amounts of NaF in potassium phosphate buffer (APPENDIX I). The protocol adopted for determining fluoride ion in the various reaction mixtures was essentially the same. The reaction was initiated by adding known amounts of 4FG to various whole cell suspensions (5-8 mg protein mL⁻¹), all in potassium phosphate buffer. For inhibition studies, whole cells were incubated simultaneously with 2.5 mM 4FG and 5 mM of the various sugars or 10 mM sulfhydryl alkylating reagents. The total volume of the suspensions was 3 mL, but in some cases 4 mL was used. The suspensions were incubated in 25 mL Erlenmeyer flasks at 30⁰C on a rotary shaker. At various time intervals fluoride release from 4FG was determined by directly submerging the fluoride electrode into the suspensions. After allowing 5 min for equilibration, mV readings were taken. A similar protocol was used in the presence or absence of 1 mM DTT with the various cell envelope fractions. In these cases, however, the total volumes were usually 2 mL (1-3 mg protein mL⁻¹). Incubation of various 4FG concentrations in potassium phosphate buffer at 30⁰C were used as controls. Rates were determined from the slopes of fluoride release curves.

Pre-Incubation of Whole Cells with Chloramphenicol and Pronase

Glucose or succinate grown whole cell suspensions (10 mL, 8-10 mg protein mL⁻¹) were incubated on a rotary shaker for 60 min at 30⁰C in the presence of 1 mg mL⁻¹ chloramphenicol or rifampicin. Appropriate aliquots were then
removed and used directly in fluoride release studies. Similarly, glucose grown whole cells were pre-incubated with Pronase (5 mg mL\(^{-1}\)) for 30 min at 30°C. After incubation, aliquots were removed and used directly in fluoride release studies. The rest of the Pronase treated cells were washed with potassium phosphate buffer and allowed to recover in buffer for 15 min before being used.

**Respirometric Studies**

The extents and rates of respiration of glucose and 4FG by whole cells, cell-free extracts, cell envelopes and cytoplasmic membrane vesicles was carried out by the manometric method (105) using a Gilson differential respirometer with eight 20 mL calibrated reaction flask (Gilson Medical Electronics, Middleton, Wisconsin). Each reaction flask contained in a total volume of 2 mL; 1.0 mL potassium phosphate buffer and 0.5 mL of whole cells, cell-free extract, cell envelopes or vesicles in the main compartment and 0.5 mL of substrate in the side-arm. In the centre well there was placed 0.2 mL of 20% KOH and a folded paper wick to absorb CO\(_2\). 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 g atoms of oxygen consumed per mole of substrate was determined as shown in APPENDIX II.
Incubation of Whole Cells with D-[6-3H]4FG

Glucose grown whole cells (24 mg protein) were incubated in the presence of 1 mM D-[6-3H]4FG (7.2 mCi/mmol) in a total volume of 4 mL at 30°C on a rotary shaker. At appropriate time intervals the suspensions were centrifuged at 10,000 xg for 10 min at 30°C to obtain whole cell pellets and supernatants. The whole cell pellets were either dialyzed against potassium phosphate buffer at 4°C for 24 h or ultrasonicated and centrifuged at 100,000 xg for 90 min at 4°C to obtain a fraction consisting of the intracellular contents. For the determination of the site of the radiolabeled protein(s), glucose grown whole cells (200 mg protein) were incubated in the presence of 1 mM D-[6-3H]4FG (7.2 mCi/mmol) in a total volume of 20 mL for 24 h at 30°C on a rotary shaker, resulting in 100% fluoride release. The whole cell pellet obtained by centrifugation was dialyzed and then fractionated sequentially by the various methods discussed earlier. In some cases, the whole cell pellets obtained from the 4 mL incubation mixtures were used in the fractionation. The specific activities of the various fractions were determined as outlined in APPENDIX III.

Estimation of $^{3}H_2O$ and Tritiated-Carbohydrates

A simple micro-procedure for the estimation of $^{3}H_2O$ and tritiated-carbohydrates ($[^3H]$-carbohydrates) in the various whole cell incubation supernatants and intracellular contents was used as described by Clark (106). Yellow plastic pipet tips (0.5 cm x 5 cm), designed to fit the Pipetman brand.
adjustable pipet, were plugged at the small end using fine glass wool. Each pipet tip was filled to a height of about 4 cm with Dowex AG 1x8 resin in the borate form. This was prepared by washing Dowex AG 1x8 (200-400 mesh) resin in the chloride form with 1 M potassium tetraborate, pH 8.5-9, until the effluent was chloride free (about 30 volumes were required). A subsequent wash with deionized distilled water (10 volumes) was necessary to bring the pH down to 7.5. The columns were loaded with 25 μL of 1 mM D-[U-14C]glucose (4.0 mCi/mmol), 1 mM D-[6-3H]4FG (0.3 mCi/mmol), whole cell incubation supernatants or intracellular contents. 3H2O was estimated by washing the columns with 10 x 0.2 mL aliquots of deionized distilled water. The effluents were collected directly into scintillation vials for counting. These columns were found to retard many carbohydrates and some amino acids, which have been summarized (106). [3H]-Carbohydrates were then estimated by washing the columns with 25 x 0.2 mL aliquots of 0.25 M ammonium tetraborate. The effluents were collected directly into scintillation vials for counting.

Isolation of Tritiated-Carbohydrates

The supernatants obtained from the 24 h incubations were combined (about 60 mL). To this was added 0.5 g decolorizing charcoal and the suspension was stirred for 30 min. The suspension was filtered and reduced to dryness on a rotary evaporator. The residue was suspended in 5 mL of deionized distilled water and then loaded onto a column of the Dowex AG 1x8 borate resin (106) (1 cm x 10 cm bed height). 3H2O
was removed by washing the column with 20 mL of deionized distilled water. [\textsuperscript{3}H]-Carbohydrates were eluted by washing the column with 10 mL of 0.25 M ammonium tetraborate. This fraction was repeatedly mixed with 10 x 100 mL of methanol and evaporated to dryness on a rotary evaporator to remove the volatile methyl borate (107). The pH was checked and adjusted to neutrality with 1 M HCl and the sample was reduced to dryness. The residue was suspended in a final volume of 1 mL with deionized distilled water and was analyzed by chromatography and Fourier transform \textsuperscript{19}F-NMR. To 0.5 mL of this suspension was added 1 mL of 1 M HCl and the solution heated at 100°C for 10 min. After cooling, the solution was neutralized with 1 M NaOH. The solution was reduced to dryness on a rotary evaporator and the residue suspended in 0.5 mL with deionized distilled water. The hydrolyzed product was analyzed by chromatography.

Chromatography

Thin layer chromatography (TLC) was performed using silica gel G 20cm x 20 cm plastic plates (BDH, Toronto, Ontario). The plates were developed in ethyl acetate: acetic acid: water (3:3:1) and the carbohydrates detected by spraying with a 95% solution of concentrated sulfuric acid in ethanol, followed by heating at 110°C for 10 min. Radioactivity on the chromatograms were determined by placing 1 cm x 1 cm scrapings of the sample lane on the dried chromatogram into scintillation vials for counting. Reducing sugars were detected by using aniline hydrogen phthalate (108). Sugar-
phosphates were chromatographed on Whatman #1 chromatography paper in an ascending manner. The paper was pre-washed with 2 M HCl, dried, washed with distilled water and then dried again. The paper was developed in n-propanol: concentrated ammonia: water (6:3:1) and sugar-phosphates detected by the method of Runclies and Krotkov (109). The chromatogram was dipped into a bath of acid-FeCl₃ in acetone (150 mg FeCl₃·6H₂O, 3 mL of 0.3 M HCl and 97 mL of acetone), air dried and then dipped into a bath of 1.25% sulfosalicylic acid in acetone. Sugar-phosphates appeared on the dry chromatogram as white spots in a red-brown field.

**Column Chromatography**

Column chromatography was performed on a Sephadex G-100 column (0.7 cm x 48 cm bed height). The resin was pre-equilibrated with 0.1 M sodium phosphate, pH 7.2, 0.1% SDS, 0.02% sodium azide buffer. 150 µL (500 µg protein, 6,000 dpm) of the radiolabeled cell envelope suspension or 150 µL (150 µg protein, 4,000 dpm) of the radiolabeled peptidoglycan-associated protein complex suspension were applied onto the column and fractions eluted with the same buffer. The samples were heated at 55°C for 30 min prior to application onto the column. The flow rate was set at 1.2 mL h⁻¹ and 0.6 mL fractions were collected on a Gilson model FC-80K microfractionator (Gilson Medical Electronics Inc., Middleton, Wisconsin) at room temperature. Fractions were then determined for radioactivity and protein (absorbance at 280 nm). The molecular weight of the radiolabeled protein was estimated
by using bovine albumin and lysozyme as molecular weight
markers (APPENDIX IV). The void volume \( V_c \) was estimated
with the use of blue dextran. Fractions containing the
highest radioactivities were pooled, lyophilized and the
residue suspended in a small amount of distilled water for
electrophoretic analysis.

**Urea-SDS-Polyacrylamide Gel Electrophoresis**

Urea-SDS-PAGE was performed essentially by the method
of Mizushima and Yamada (110). Gels were prepared by mixing
in final concentrations; 8% acrylamide, 0.2% bis-acrylamide,
0.2% SDS, 8 M urea and 0.1 M sodium phosphate buffer, pH 7.2,
in a total volume of 40 mL. The polymerization of the gel
was initiated by addition of 60 mg of ammonium persulfate
and 24 \( \mu L \) of TEMED. The solution was quickly poured into
0.6 cm x 16 cm glass tubes to a height of about 12 cm and
overlaid with a small amount of water to form a smooth flat
surface. The solution was enough to make about 10 gels
and polymerization occurred within 15 min. Samples were
prepared by dissolving 50-100 \( \mu \)g of protein in 100 \( \mu \)L of 1%
SDS-2% \( \beta \)-mercaptoethanol solution, followed by heating at
100\(^\circ\)C for 5 min and addition of urea (8 M final concentration).
To this was added 10 \( \mu \)L of 0.5% bromophenol blue and 50 or
100 \( \mu \)L of the solution applied onto each gel. Therefore,
about 50 \( \mu \)g of protein were usually applied onto each gel.
This was overlaid with 0.1 M sodium phosphate buffer, pH 7.2.
Electrophoresis was performed in a 15-tube capacity electro-
phoresis cell with the anode in the lower chamber and cathode
in the upper chamber. Electrophoresis was carried out at 4 mA per gel for 30 min at room temperature in 0.1 M sodium phosphate, pH 7.2, 0.1% SDS buffer. Then the current was increased to 6 mA per gel and electrophoresis continued for 5 h until the bromophenol blue had migrated to about 3/4 of the gel. This distance was marked with black drafting ink.

Gels were stained for protein with Coomassie brilliant blue (R-250) or for glycoproteins by the method of Fairbanks et al. (111). In staining for protein the following protocol was used; (1) 25% isopropl alcohol, 10% acetic acid, 0.05% coomassie blue, overnight, (2) 10% isopropl alcohol, 10% acetic acid, 0.005% coomassie blue, 6 h, (3) 10% acetic acid with 0.0025% or less coomassie blue, overnight, and (4) 10% acetic acid for several days until the background is clear.

The protocol for staining for glycoproteins was as follows; (1) 0.5% periodic acid, 2 h, (2) 0.5% sodium arsenite, 5% acetic acid, 1 h, (3) 0.1% sodium arsenite, 5% acetic acid, 3 x 20 min, (4) 5% acetic acid, 10 min, (5) 10 mL of Schiff reagent (111) per gel, overnight, and (6) 0.1% sodium metabisulfite, 3 x 1 h. In staining for glycoproteins about 200 μg of protein were usually applied onto each gel. Gels were scanned on a model 4310 scanning densitometer (Ortex Inc., Oak Ridge, Tennessee) or photographed.

For radioactivity determinations, the gels were sliced into 0.5 cm sections and placed into scintillation vials. 200 μL of H₂O₂ were added to the gel slices which were then incubated at 55°C for 24 h. After digestion of the gel slices,
the solutions were subjected to radioactivity counting. The molecular weight of the protein bands were estimated from a standard curve prepared with known molecular weight proteins (APPENDIX V).

### Preparation of 2K4FGA and 4FGA

2K4FGA and 4FGA were prepared by incubating 10 mM 4FG with cytoplasmic membrane vesicles (10 mg of protein) prepared from glucose and succinate grown whole cells, respectively. The formation of 4FGA was based on the fact that cytoplasmic membrane vesicles from succinate grown whole cells have a defective gluconate dehydrogenase complex (60). The reactions were carried out in volumes of 3 mL and the oxidation of 4FG was monitored on the Gilson differential respirometer. When complete (about 8 h) the vesicles were removed by centrifugation. The products obtained were analyzed by TLC. The concentration of the products was based on the initial concentration of 4FG and assuming that the oxidation of 4FG had gone to completion.

### Liquid Scintillation Counting

Radioactivity was measured on a Beckman LS 7500 liquid scintillation counter (Beckman Instruments Inc., Fullerton, California). The liquid scintillation cocktail was prepared as follows; 5.5 g of PPO, 0.1 g of POPOP, 333 mL of Triton X-100 and 667 mL of scintillation grade toluene. In all cases, 10 mL of the liquid scintillation cocktail was added to the various samples for radioactivity determinations. Quench correction curves (APPENDIX VI and VII) were prepared from
quenched standards (Nuclear Chicago) using the H-number concept of quench correction (see manufacturer's manual for the theoretical discussion on this concept).

**Assay of Enzyme Activities**

The method used for assaying the activities of glucose, gluconate and succinate dehydrogenases was based on the procedure described by Mizuno and Kageyama (86). Reaction mixtures contained 100 mM potassium phosphate, pH 7.1, 10 mM KCN, 10 μg of PMS, 20 μg of DCIP and 50-100 μg protein of outer or cytoplasmic membrane fractions. All reactions were initiated by addition of 100 mM (final concentration) glucose, gluconate or succinate in a total volume of 1.0 mL. The enzyme activities were measured by the decrease in absorbance at 600 nm for DCIP at 25°C on a Beckman model 35 spectrophotometer (Beckman Instruments Inc., Fullerton, California). The activities were calculated using an extinction coefficient for DCIP of 10 mM⁻¹ cm⁻¹.

**Protein Determination**

Protein determinations were by the method of Lowry et al. (112) with bovine serum albumin as a standard. Before the determination of protein in whole cell suspensions, the whole cells were diluted to an absorbance of about 1.0 at 600 nm. This is in the range of about 1.0-1.2 mg of protein mL⁻¹. For column eluates, protein was estimated by measuring the absorbance at 280 nm.

**Estimation of 4FG in Whole Cell Incubation Supernatants**

The removal of 4FG from the supernatant of whole cell
incubation mixtures was monitored with the use of the Statzyme Glucose (500 nm) reagent. 3.0 mL aliquots of this reagent were incubated at 30°C for 10 min. To these were added 200 μL of the various whole cell incubation supernatants and the solutions incubated for an additional 2 h. After incubation 100 μL of 4 M HCl was added to stop the reaction and stabilize the color. The absorbance of the solutions were then determined at 500 nm. 4FG was estimated from a standard curve produced using various concentrations of 4FG (APPENDIX VIII).

Kinetic Treatment of Data

\[ K_m \text{ and } V_{\text{max}} \text{ values were obtained from double reciprocal or Lineweaver-Burk plots (113) and were estimated by linear regression of the kinetic data (APPENDIX IX). The } K_m \text{ and } V_{\text{max}} \text{ values are the average of duplicate trials.} \]

Fourier Transform $^{19}$F-NMR

$^{19}$F-NMR was performed on a Bruker Rulse NMR spectrometer CXP-100 in the Fourier transform mode. Samples were examined for up to 50,000 scans. 0.1 M trifluoroacetic acid and 0.1 M NaF were used as standards.
CHAPTER III
RESULTS AND DISCUSSION

Although fluorocarbohydrates have been used as biochemical probes in a variety of biological studies, few examples of defluorination reactions have been reported. The defluorination of 4FG by glucose grown whole cells of P. putida, therefore, represents a rather unique system which may have biological significance to the organism. Initial investigations, therefore, were concerned with studying the nature and properties of this defluorination reaction.

When thick whole cell suspensions of glucose grown P. putida are incubated with varying concentrations of 4FG, the rates of fluoride release are observed to be virtually proportional to the concentration of 4FG (Table 3). Fluoride release was found to be linear for about the first 4 h with virtually complete defluorination occurring after 24 h incubation. In the case of 10 mM 4FG, further incubation results in the release of more fluoride from 4FG. A double reciprocal plot of the rates of fluoride release against the concentrations of 4FG gives an apparent $K_m$ of 3.9 mM and an apparent $V_{max}$ of 1 nmol fluoride mg protein$^{-1}$ min$^{-1}$ (Figure 9). This suggests that the defluorination reaction follows Michaelis-Menten type kinetics (114). Furthermore, the results give the first indication that the defluorination process is a protein catalyzed reaction, which is dependent on both the concentration of 4FG and the amount of whole cell protein.
Table 3

Fluoride Release from 4FG by Glucose Grown Whole Cells of *P. putida.*

<table>
<thead>
<tr>
<th>[4FG] (mM)</th>
<th>$v$ (nmol fluoride mg protein$^{-1}$ min$^{-1}$)</th>
<th>% fluoride released after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.09</td>
<td>100</td>
</tr>
<tr>
<td>1.0</td>
<td>0.18</td>
<td>100</td>
</tr>
<tr>
<td>2.0</td>
<td>0.30</td>
<td>95</td>
</tr>
<tr>
<td>2.5</td>
<td>0.38</td>
<td>95</td>
</tr>
<tr>
<td>5.0</td>
<td>0.54</td>
<td>88</td>
</tr>
<tr>
<td>10.0</td>
<td>0.60</td>
<td>59</td>
</tr>
</tbody>
</table>

*Whole cell suspensions (24 mg protein) of glucose grown *P. putida* were incubated at 30°C in 100 mM potassium phosphate buffer, pH 7.1, with varying concentrations of 4FG (0.5-10.0 mM) and in a total volume of 4 mL. Rates of fluoride release were determined from the slope of the linear portion of the curves for fluoride release. Fluoride determinations were as described in the MATERIALS AND METHODS section.
Figure 9

Double Reciprocal Plot of Fluoride Release from 4FG by Glucose Grown Whole Cells of *P. putida*.

Legend:

Thick whole cell suspensions (24 mg protein) of glucose grown *P. putida* were incubated with various concentrations of 4FG (0.5-10 mM) in 100 mM potassium phosphate buffer, pH 7.1, at 30°C and in a total volume of 4 mL. Fluoride determinations were as described in the MATERIALS AND METHODS section. The $K_m$ and $V_{max}$ values were estimated by linear regression of the kinetic data and are the average of duplicate trials.

Apparent $K_m = 3.9$ mM

Apparent $V_{max} = 1$ nmol fluoride mg protein$^{-1}$ min$^{-1}$
Figure 9

\[ v^{-1} \text{ (nmol fluoride mg protein}\cdot\text{min}^{-1})^{-1} \]

against

\[ [4\text{FG}]^{-1} \text{ mM}^{-1} \]
No oxygen consumption, above the endogenous level, could be detected in whole cells given 4FG (0.5-2.5 mM) as a substrate, in accord with preliminary studies (61). On the other hand, glucose (0.5-2.5 mM) is oxidized extensively (2.7-3.4 mol oxygen mol glucose\(^{-1}\)). Figure 10 illustrates a typical result obtained with 2.5 mM glucose or 4FG as substrates. Unlike 3FG (61), therefore, 4FG is not oxidized by glucose grown whole cells of \textit{P. putida}, instead an extensive release of fluoride occurs.

In contrast to whole cells, cytoplasmic membrane vesicles or cell-free extracts were found to oxidize 4FG and glucose to the extent of 1 mol oxygen mol substrate\(^{-1}\) (Table 4). In the case of 4FG, oxidation was generally complete within 6 h incubation. Furthermore, negligible fluoride release (<1%) could be detected even after 24 h incubation. Thin layer chromatographic analysis of the vesicle or cell-free extract supernatants before and after complete oxidation indicated that 4FG (\(R_F 0.62\)) was converted to a slower moving component (\(R_F 0.42\)), which is consistent with the formation of 2K4FGA. Likewise, glucose (\(R_F 0.52\)) was converted to a slower moving component (\(R_F 0.36\)), with the formation of 2KGA. These results are similar to those obtained with 3FG (55) and demonstrates that 4FG is a substrate for the cytoplasmic membrane bound enzymes, glucose oxidase and gluconate dehydrogenase. Apparent \(K_m\) values of 21 and 18 mM and apparent \(V_{max}\) values of 138 and 117 nmol oxygen mg protein\(^{-1}\) min\(^{-1}\) were obtained from double reciprocal plots.
Figure 10

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

Legend:

- ▲ - oxidation of 2.5 mM glucose
- ■ - oxidation of 2.5 mM 4FG

Each Gilson flask contained:

- main compartment; 5 mg whole cell protein in 100 mM potassium phosphate buffer, pH 7.1, up to 1.5 mL.
- side-arm; 5 \( \mu \)mol (2.5 mM final concentration) 4FG or glucose in 0.5 mL buffer.
- centre well; 0.2 mL of 20% KOH and paper wick.

The reaction was initiated by tipping the contents of the side-arm into the main compartment. Temperature, 30°C; reaction volume, 2 mL; gas phase, air.
Figure 10

Oxygen Consumption μL(x10^{-2})

Time h
Table 4

The Rates and Extents of D-Glucose and 4FG Oxidation in Cell-Free Extracts and Cytoplasmic Membrane Vesicles.

<table>
<thead>
<tr>
<th>Substrate oxidized</th>
<th>( \nu \text{ (nmol oxygen mg protein}^{-1} \text{ min}^{-1} )</th>
<th>\text{Net oxygen consumption (endogenous subtracted)}</th>
<th>\text{mol oxygen mol substrate oxidized}^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>4FG (1 \mu mol)</td>
<td>3.6</td>
<td>2.7</td>
<td>23</td>
</tr>
<tr>
<td>4FG (2 \mu mol)</td>
<td>6.3</td>
<td>5.7</td>
<td>45</td>
</tr>
<tr>
<td>4FG (5 \mu mol)</td>
<td>11.0</td>
<td>11.7</td>
<td>108</td>
</tr>
<tr>
<td>4FG (10 \mu mol)</td>
<td>20.5</td>
<td>21.2</td>
<td>203</td>
</tr>
<tr>
<td>4FG (20 \mu mol)</td>
<td>nd</td>
<td>45.0</td>
<td>nd</td>
</tr>
<tr>
<td>glucose (5 \mu mol)</td>
<td>50.1</td>
<td>91.5</td>
<td>125</td>
</tr>
<tr>
<td>glucose (10 \mu mol)</td>
<td>95.6</td>
<td>177.0</td>
<td>214</td>
</tr>
</tbody>
</table>

nd - not determined; 1 - cell-free extracts; 2 - vesicles.

The preparation of cell-free extracts and cytoplasmic membrane vesicles and the respirometric studies were as described in the MATERIALS AND METHODS section. The rates and extents of substrate oxidation were determined as outlined in APPENDIX II.
of the rates of 4FG oxidation against the concentration of 4FG in cytoplasmic membrane vesicles and cell-free extracts, respectively (Figures 11 and 12). In comparison, apparent K_m values of 0.8 and 22 mM and apparent V_max values of 160 and 105 nmol oxygen mg protein^{-1} min^{-1} have been previously determined for glucose and 3FG oxidation, respectively, in cytoplasmic membrane vesicles (99). These kinetic values reflect a combination of the various kinetic steps involved in the oxidation reactions.

Cytoplasmic membrane vesicles prepared from succinate grown cells were found to oxidize glucose and 4FG to the extent of 0.5 mol oxygen mol substrate^{-1}, which is consistent with the repression of the gluconate dehydrogenase complex (60). These substrates are only oxidized by the constitutive enzyme glucose oxidase. Thin layer chromatographic analysis of the supernatants indicated that 4FG was converted to a slower moving component (R_F 0.42), indicating the formation of 4FGA. Similarly, glucose was converted to a slower moving component (R_F 0.35), consistent with the formation of gluconic acid. The R_F values for 4FGA and 2K4FGA were observed to be identical in the solvent system used, as were the R_F values for gluconic acid and 2KGA.

When glucose grown whole cell suspensions were incubated simultaneously with 1 or 2.5 mM 4FG and various concentrations of glucose, the rates of fluoride release were found to decrease with increasing concentrations of glucose (Table 5). Although the rates of fluoride release are drastically.
Figure 11

Double Reciprocal Plot of 4FG Oxidation by Cytoplasmic Membrane Vesicles Prepared from Glucose Grown Whole Cells of *P. putida*.

Legend:

Each Gilson flask contained:

- main compartment: 2 mg vesicle protein in 100 mM potassium phosphate buffer, pH 7.1, up to 1.5 mL;
- side-arm: 0.5 mL 4FG solution (0.5-10 mM final concentration);
- centre well: 0.2 mL of 20% KOH and paper wick.

The reaction was initiated by tipping the contents of the side-arm into the main compartment. Temperature, 30°C; reaction volume, 2 mL; gas phase, air. The $K_m$ and $V_{max}$ values were estimated by linear regression of the kinetic data and are the average of duplicate trials.

Apparent $K_m = 21$ mM

Apparent $V_{max} = 138$ nmol oxygen mg protein$^{-1}$ min$^{-1}$
Figure 12

Double Reciprocal Plot of 4FG Oxidation by Cell-Free Extracts Prepared from Glucose Grown Whole Cells of *P. putida*.

Legend:

Each Gilson flask contained:

- **main compartment**: 7 mg cell-free extract protein in 100 mM potassium phosphate buffer, pH 7.1, up to 1.5 mL.
- **side-arm**: 0.5 mL 4FG solution (0.5-5 mM final concentration).
- **centre well**: 0.2 mL of 20% KOH and paper wick.

The reaction was initiated by tipping the contents of the side-arm into the main compartment. Temperature, 30°C; reaction volume, 2 mL; gas phase, air. The $K_m$ and $V_{max}$ values were estimated by linear regression of the kinetic data and are the average of duplicate trials.

Apparent $K_m = 18$ mM

Apparent $V_{max} = 117$ nmol oxygen mg protein$^{-1}$ min$^{-1}$
Figure 12
Table 5

The Effect of D-Glucose on the Rates of Fluoride Release from 4FG in Glucose Grown Whole Cells of P. putida.*

<table>
<thead>
<tr>
<th>[Glucose] (mM)</th>
<th>v (nmol fluoride mg protein⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mM 4FG</td>
</tr>
<tr>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>0.5</td>
<td>0.14</td>
</tr>
<tr>
<td>1.0</td>
<td>0.09</td>
</tr>
<tr>
<td>2.5</td>
<td>0.06</td>
</tr>
<tr>
<td>5.0</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Whole cell suspensions (18 mg protein) of glucose grown P. putida were incubated at 30°C in 100 mM potassium phosphate buffer with 1 or 2.5 mM 4FG + varying concentrations of glucose (0-5.0 mM) and in a total volume of 3 mL. Fluoride determinations were as described in the MATERIALS AND METHODS section.
reduced, there is no effect on the extents of fluoride release. Once glucose has been metabolized by the cells, fluoride release continues as in the control cells (Figure 13). These results suggest that the defluorination reaction involves the interaction or binding of 4FG to the same site as glucose in the whole cell glucose transport system. Furthermore, as would be anticipated, these cells have a preference for glucose over 4FG, presumably in the initial binding step.

The important stereo-specific and hydrogen bonding requirements of 4FG for the defluorination reaction were investigated by using a variety of sugars as inhibitors. The specificity of hydrogen bonding sites between glucose and the carrier protein(s) in the human erythrocyte (50,51) and the hamster intestine (47) have been investigated by this method. Table 6 indicates that the most effective inhibitors of the defluorination reaction are D-glucose, D-gluconate and 2-keto-D-gluconate, suggesting that there is a common binding site for these substrates. As in the case of glucose, once gluconate and 2-ketogluconate are metabolized, fluoride release continues to completion. L-Glucose, α-Me4FG, D-galactose, α- and β-MeG, maltose, fructose, sorbitol, β-D-thiogluco-1se and 5-thioglucose were found to have no inhibitory effect on the defluorination reaction at the concentrations used.

The fact that α-Me4FG and α- and β-MeG do not inhibit fluoride release from 4FG may suggest the importance of the
Figure 13

The Effect of D-Glucose on Fluoride Release from 4FG in Glucose Grown Whole Cells of *P. putida*.

Legend:

- ■ - 2.5 mM 4FG
- ▲ - 2.5 mM 4FG + 5 mM glucose

Whole cell suspensions (20 mg protein) of glucose grown *P. putida* were incubated with 2.5 mM 4FG or 2.5 mM 4FG + 5 mM glucose in 100 mM potassium phosphate buffer, pH 7.1, at 30°C and in a total volume of 3 mL. Fluoride determinations were as described in the MATERIALS AND METHODS section.
Figure 13

nmol fluoride, mg protein^-1 (x10^-2)

Time h
Table 6

The Effect of Various Sugars on Fluoride Release from 4FG in Glucose Grown Whole Cells of *P. putida*.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>% Inhibition of fluoride release after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>L-Glucose</td>
<td>0</td>
</tr>
<tr>
<td>α-Me4FG</td>
<td>0</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>0</td>
</tr>
<tr>
<td>2-Deoxy-D-glucose</td>
<td>57</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>81</td>
</tr>
<tr>
<td>4-Deoxy-D-glucose</td>
<td>30</td>
</tr>
<tr>
<td>D-Gluconate</td>
<td>90</td>
</tr>
<tr>
<td>2-Keto-D-gluconate</td>
<td>90</td>
</tr>
<tr>
<td>α-MeG</td>
<td>0</td>
</tr>
<tr>
<td>β-MeG</td>
<td>0</td>
</tr>
<tr>
<td>Maltose</td>
<td>0</td>
</tr>
<tr>
<td>6-Deoxy-D-glucose</td>
<td>73</td>
</tr>
<tr>
<td>Fructose</td>
<td>0</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0</td>
</tr>
<tr>
<td>β-D-Thioglucose</td>
<td>0</td>
</tr>
<tr>
<td>5-Thioglucose</td>
<td>0</td>
</tr>
</tbody>
</table>

*Whole cell suspensions (15-20 mg protein) of glucose grown *P. putida* were incubated at 30°C in 100 mM potassium phosphate buffer, pH 7.1, with 2.5 mM 4FG + 5 mM of the various sugars, in a total volume of 3 mL. Fluoride determinations were as described in the MATERIALS AND METHODS section.
C-1 position of 4FG in the defluorination reaction. Alternatively, is the realization that these glycosides have a much lower binding affinity for the glucose transport system and, therefore, presumably the defluorinating protein, than glucose and possibly 4FG. It was found that α-MeG had a 1000-fold lower affinity for the glucose transport system than glucose in *P. aeruginosa* (98). Furthermore, glucose transport was only weakly inhibited in the presence of 100-fold excess of α-MeG. An alternative explanation may be that these glycosides are taken up by a transport system different from glucose. This is supported by the evidence for an independent transport system for α-MeG in *P. putida* (99). The lack of inhibition of fluoride release by maltose and β-D-thioglucose may be explained in a similar manner. Furthermore, maltose is not used as a carbon source by *P. aeruginosa* (95) and, therefore, presumably *P. putida*.

2-Deoxy- and 6-deoxy-D-glucose were found to significantly inhibit fluoride release from 4FG, indicating the relative unimportance of the C-2 and C-6 positions of 4FG in the defluorination reaction. As might be anticipated 4-deoxy-D-glucose is a poor inhibitor, confirming the important stereo-specificity and hydrogen bonding requirements at the C-4 position for the binding of sugars with the D-gluco-configuration as well as 4FG to the protein. The importance of the C-4 position is also supported by the lack of inhibition of fluoride release by D-galactose and by the inability of whole cells to defluorinate 3FG (61). Galactose is not
used as a source of carbon for growth and energy by *P. aeruginosa* (95). Furthermore, galactose does not inhibit glucose binding to GBR at a 10-fold excess, although it does at a 100-fold excess (67). The lack of inhibition of fluoride release from 4FG by galactose may indicate a poor affinity, if any, of galactose for the defluorinating protein. The lack of inhibition of fluoride release by sorbitol and fructose supports the requirement for the ring structure of substrates in the D-gluco-configuration for the initial binding. Furthermore, fructose has recently been shown to be transported into *P. aeruginosa* by a phosphoenolpyruvate dependent transport system (115). The lack of inhibition of fluoride release by 5-thioglucose suggests the importance of the ring oxygen in the initial binding.

The above results suggest and provide the first indication that the C-4 position and the ring oxygen of glucose may play critical roles in its transport in *P. putida* and presumably other pseudomonads. In this mechanism, the ring oxygen and the oxygen of the hydroxyl group at the C-4 position of glucose may form hydrogen bonds with appropriate groups of a protein of the glucose transport system. A similar type of hydrogen bonding with the oxygens at the C-1, C-3 positions and the ring oxygen are considered to be important sites for the transport of glucose in the erythrocyte membrane (50,51).

Incubation of glucose grown whole cell suspensions of *P. putida* with 2.5 mM 4FG and 10 mM NEM, iodoacetate or
iodoacetamide results in 100%, 68% or 41% inhibition of fluoride release, respectively, after 30 min. During this time there was only a slight decrease in the cells ability to oxidize glucose, indicating little deleterious effect of these reagents on the cells. After about 2 h incubation though, endogenous respiration ceased, indicating irreversible cell damage. These results suggest the possible involvement of an -SH group on the protein in the defluorination reaction, which may be involved in the formation of a hydrogen bond. Additional support is based on the fact that -SH group participation has been demonstrated in a variety of biological systems as well as those involved with defluorination reactions (6,7,23,42). Direct support for -SH group participation cannot be demonstrated until the defluorinating protein is isolated in a relatively pure form.

If the defluorination reaction is initiated by a protein which is part of the glucose transport system, then growth of cells on various carbon sources should affect the cells ability to defluorinate 4FG. Figure 14 illustrates the results of growing cells on glucose, gluconate, 2-keto-gluconate, succinate or citrate. Cells grown on gluconate or 2-ketogluconate were found to have similar rates and extents of fluoride release as cells grown on glucose. This again suggests the presence of a common binding protein for these substrates. On the other hand, the rates and extents of fluoride release were drastically reduced with cells grown on succinate or citrate, indicating that the protein
The Effect on Fluoride Release from 4FG by Growth of *P. putida* on Various Carbon Sources.

**Legend:**
- ■ glucose grown cells
- ▲ gluconate grown cells
- ● 2-ketogluconate grown cells
- △ succinate grown cells
- □ citrate grown cells

Cells were grown on appropriate carbon sources as described in the MATERIALS AND METHODS section. Whole cell suspensions (15 mg protein) were incubated with 2.5 mM 4FG in 100 mM potassium phosphate buffer, pH 7.1, at 30°C and in a total volume of 3 mL. Fluoride determinations were as described in the MATERIALS AND METHODS section.
Figure 14

nmol fluoride mg protein$^{-1}$(x10$^{-2}$)

Time h
responsible for the defluorination reaction is repressed.
Growth of cells on succinate or citrate results in a greater
than 90\% reduction in the rate of fluoride release.

Exposure of succinate grown cells to 2.5 mM glucose
or 4FG for 4 h results in an increase in the cells' ability
to defluorinate 4FG (Figure 15). Glucose was found to cause
about a 4-fold increase in the rate of fluoride release.
Presumably, this reflects the induction of various proteins
necessary for glucose transport and metabolism. Growth on
succinate has been shown to repress the cytoplasmic glucose
carrier and gluconate dehydrogenase activity (60), an
enzyme having a key regulatory function for glucose transport
and metabolism. It is interesting to note that 4FG also
appears to be acting as an inducer of the protein, although
the effect is much less than glucose.

Glucose grown whole cells of *P. putida* are not only
capable of defluorinating 4FG but also 4FGA and 2K4FGA
(Table 7). The rates and extents of fluoride release were
found to be identical for all three substrates. Furthermore,
glucose was found to inhibit the defluorination of 4FGA and
2K4FGA just as effectively as it does 4FG (about 85\% inhibition
after 60 min). Thin layer chromatographic analysis of the
supernatants failed to detect a defluorination product,
which is consistent with previous results (61). These results
indicate the importance of the C-4 position in the binding
of sugars with the D-gluco-configuration to the protein.
The fact that fluoride is released from 4FG, 4FGA and 2K4FGA
Figure 15

Fluoride Release from 4FG by Succinate Grown Whole Cells of _P. putida_ Pre-Incubated with D-Glucose or 4FG.

Legend:

- **●** - cells pre-incubated with 2.5 mM glucose for 4 h
- **▲** - cells pre-incubated with 2.5 mM 4FG for 4 h
- **■** - cells pre-incubated in buffer for 4 h

Whole cell suspensions (15 mg protein) of succinate grown _P. putida_ were pre-incubated with 2.5 mM glucose or 4FG in 100 mM potassium phosphate buffer, pH 7.1, at 30°C for 4 h and in a total volume of 4 mL. Cells were then washed with and suspended in buffer to the same protein concentration and incubated with 2.5 mM 4FG. Fluoride determinations were as described in the MATERIALS AND METHODS section.
Figure 15
Table 7
Fluoride Release from Various Fluoro-Sugars in Glucose Grown Whole Cells of *P. putida*.

<table>
<thead>
<tr>
<th>Fluoro-sugar (2.5 mM)</th>
<th>v (nmol fluoride mg protein⁻¹ min⁻¹)</th>
<th>% Fluoride released (after 24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4FG</td>
<td>0.65</td>
<td>95</td>
</tr>
<tr>
<td>4FGA</td>
<td>0.65</td>
<td>95</td>
</tr>
<tr>
<td>2K4FGA</td>
<td>0.65</td>
<td>95</td>
</tr>
<tr>
<td>α-Me4FG</td>
<td>&lt;0.01</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3FG (6I)</td>
<td>&lt;0.01</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*Whole cell suspensions (18 mg protein) of glucose grown *P. putida* were incubated at 30°C in 100 mM potassium phosphate buffer, pH 7.1, with various fluoro-sugars (2.5 mM) and in a total volume of 3 mL. 4FGA and 2K4FGA were prepared by incubating 4FG with cytoplasmic membrane vesicles obtained from succinate and glucose grown cells, respectively, as described in the MATERIALS AND METHODS section.
together with the fact that glucose, gluconate and 2-keto-gluconate inhibit the defluorination reaction provides evidence that there is a common binding protein for these substrates. These results provide the first indication that _P. putida_, and presumably other pseudomonads, possess a protein which will bind these sugars with similar affinities.

The uptake of glucose in _P. aeruginosa_ (97,98) and _P. putida_ (99) has been shown to involve two distinct inducible pathways with differing affinities for glucose. A high affinity transport system which is induced by growth of cells on glucose, but not on gluconate, succinate or citrate and a low affinity transport system which is induced by growth of cells on glucose or gluconate, but not on succinate or citrate. The results obtained suggest that the protein, which is responsible for the defluorination of 4PG, is presumably a component of the low affinity glucose transport system. In support of this, it has been demonstrated that gluconate (99) and 2-ketogluconate (116) are actively transported in glucose grown whole cells of _P. putida_. Hancock and Carey (100) have recently proposed that the low affinity glucose transport system involves uptake across the outer membrane of _P. aeruginosa_ via the porin protein F.

Further support for the presence and synthesis of protein responsible for the defluorination reaction is provided when glucose or succinate grown cells are pre-incubated with chloramphenicol (1 mg mL⁻¹) for 1 h at 30°C prior to their
exposure to 4FG (Figure 16). The extents of fluoride release after 24 h were found to be 90% and 18% in the glucose and succinate grown cells, respectively. On the other hand, the extents of fluoride release were found to be 11% and 2% in the glucose and succinate grown chloramphenicol treated cells, respectively. Similar results were obtained with rifampicin, although chloramphenicol was found to be more effective in reducing the extent of fluoride release. The amount of fluoride released in glucose grown chloramphenicol treated cells presumably reflects the amount of endogenous protein present which reacts with 4FG. Fluoride release continues until all the endogenous proteins are saturated and then defluorination stops. Fluoride release above this level, as in the glucose grown untreated cells, is presumably the result of protein synthesis.

To estimate the maximum amount of fluoride release from 4FG, a fixed amount of glucose grown chloramphenicol treated and untreated cells were incubated with various concentrations of 4FG for 24 h. As demonstrated in Figure 17, the extents of fluoride release in the untreated cells is virtually proportional to the concentration of 4FG. There is about 90% or more fluoride release with concentrations of 4FG of 5 mM or less. On the other hand, up to 1.25 mM 4FG results in the production of 133 nmol fluoride mg protein$^{-1}$ in the chloramphenicol treated cells. This value remains constant with increasing concentrations of 4FG. Furthermore, more than 90% fluoride release is achieved with concentrations of
Figure 16

The Effect of Chloramphenicol on Fluoride Release from 4FG by Glucose and Succinate Grown Whole Cells of *P. putida*.

Legend:
- ■ - glucose grown cells
- □ - glucose grown chloramphenicol pre-treated cells
- ▲ - succinate grown cells
- ▼ - succinate grown chloramphenicol pre-treated cells

Cells were pre-treated with chloramphenicol as described in the MATERIALS AND METHODS section. Whole cell suspensions (24 mg protein) of glucose or succinate grown *P. putida* pre-incubated in the presence or absence of chloramphenicol (1 mg mL⁻¹) for 1 h at 30°C were exposed to 2.5 mM 4FG. The suspensions were incubated in 100 mM potassium phosphate buffer, pH 7.1, at 30°C and in a total volume of 4 mL. Fluoride determinations were made as described in the MATERIALS AND METHODS section.
Figure 16

nmol fluoride mg protein$^{-1}$(x10$^{-2}$)

Time h
Figure 17

The Extents of Fluoride Release from 4FG Against the Concentration of 4FG for Glucose Grown and Glucose Grown Chloramphenicol Pre-Treated Whole Cells of *P. putida*.

Legend:

■ - glucose grown cells
□ - glucose grown chloramphenicol pre-treated cells

Whole cell suspensions (24 mg protein) of glucose grown *P. putida* pre-incubated in the absence or presence of chloramphenicol (1 mg mL⁻¹) for 1 h at 30°C were exposed to various concentrations of 4FG (0.1-5 mM). The suspensions were incubated in 100 mM potassium phosphate buffer, pH 7.1, at 30°C and in a total volume of 4 mL. Fluoride determinations were as described in the MATERIALS AND METHODS section. The extent of fluoride release was determined after 24 h incubation.
only 1 mM 4FG or less. The 133 nmol fluoride mg protein\(^{-1}\) value presumably reflects the amount of endogenous protein initially present in the cells which can react with 4FG. It can be calculated (APPENDIX X) from this value that a maximum of 24 μg of 4FG has reacted per mg of whole cell protein. Fluoride release above this value, as in the untreated cells, is the result of protein synthesis.

Fluoride release from 4FG with chloramphenicol treated cells is irreversible since washing the cells after exposure to saturating concentrations of 4FG (2.5 mM 4FG for 6 h) and re-exposure to 2.5 mM 4FG for 24 h results in only 5% fluoride release. Under the same conditions, untreated cells exhibited 78% fluoride release.

Although the extents of fluoride release are reduced by treatment of cells with chloramphenicol, the rates are not significantly affected. A double reciprocal plot of the rates of fluoride release from 4FG against the concentration of 4FG yields an apparent \(K_m\) of 3.6 mM and an apparent \(V_{max}\) of 1 nmol fluoride mg protein\(^{-1}\) min\(^{-1}\) (Figure 18). The values are similar to those obtained in the untreated cells and demonstrates that chloramphenicol only affects the defluorination reaction by inhibiting the synthesis of more defluorinating protein.

Support for a proposed glycosylation reaction is based on the fact that no defluorination sugar could be detected in the supernatants or intracellular contents of cells incubated with 4FG (61). Thin layer chromatographic analysis
Double Reciprocal Plot of Fluoride Release from 4FG by Glucose Grown Chloramphenicol Pre-Treated Whole Cells of *P. putida*.

Legend:
Whole cell suspensions (24 mg protein) of glucose grown *P. putida* pre-incubated in the presence of chloramphenicol (1 mg mL$^{-1}$) for 1 h at 30$^\circ$C were exposed to various concentrations of 4FG (0.1-5 mM). The suspensions were incubated in 100 mM potassium phosphate buffer, pH 7.1, at 30$^\circ$C and in a total volume of 4 mL. Fluoride determinations were as described in the MATERIALS AND METHODS section. The $K_m$ and $V_{max}$ values were estimated by linear regression of the kinetic data and are the average of duplicate trials.

Apparent $K_m = 3.6$ mM
Apparent $V_{max} = 1$ nmol fluoride mg protein$^{-1}$ min$^{-1}$
Figure 18

\[ v^{-1} \text{ (nmol fluoride mg protein}^{-1} \text{ min}^{-1} \times 10^{-1})^{-1} \]

\[ [4FG]^{-1} \text{ mM}^{-1} \]

-2 0 2 4 6 8 10
of supernatants from whole cell suspensions incubated with 5 mM 4FG (88% fluoride release after 24 h) failed to detect any new metabolites. On the other hand, TLC analysis of supernatants from chloramphenicol treated whole cell suspensions incubated with 5 mM 4FG (35% fluoride release after 24 h) demonstrated the presence of one large spot with an Rf value of 0.42. This is consistent with the formation of 4FGA and/or 2K4FGA, which would result by non-specific diffusion of 4FG to the cytoplasmic membrane bound enzymes.

Figure 19 is a comparison of 4FG removal from various supernatants and fluoride release from 4FG against time in glucose and succinate grown cells. This removal of 4FG from the supernatant may be due to the initial binding of 4FG to a protein. After 30 min incubation, about 86 nmol 4FG mg protein⁻¹ are removed from the supernatant of glucose grown cells, while 7.4 nmol fluoride mg protein⁻¹ are released from 4FG. In succinate grown cells, only 9 nmol 4FG mg protein⁻¹ are removed from the supernatant, while only 0.02 nmol fluoride mg protein⁻¹ are released. As previously observed, these residual activities in the succinate grown cells demonstrate the repression of protein. The above results indicate that the defluorination reaction may in fact be a two step process. The first step presumably involves an initial rapid equilibrium binding of 4FG to a protein followed by a slow elimination of fluoride ion. Direct support for this initial binding and events leading to the loss of fluoride
Comparison of Fluoride Release from 4FG and 4FG Removal from the Supernatant by Glucose and Succinate Grown Whole Cells of \textit{P. putida}.

Legend:
- \textbullet{} - amount of 4FG removed from the supernatant of glucose grown cells
- \textbullet{} - amount of fluoride released from 4FG by glucose grown cells
- \textblacksquare{} - amount of 4FG removed from the supernatant of succinate grown cells
- \textsquare{} - amount of fluoride released from 4FG by succinate grown cells

Whole cell suspensions (20 mg protein) of glucose or succinate grown \textit{P. putida} were incubated with 2.5 mM 4FG in 100 mM potassium phosphate buffer, pH 7.1, at 30°C and in a total volume of 3 mL. At 5 min intervals cell suspensions were centrifuged and fluoride and 4FG were determined in the supernatants as described in the MATERIALS AND METHODS section. The results are the average of duplicate trials.
have yet to be deduced.

Figure 20 illustrates the effect of pre-incubating glucose grown cells with Pronase (5 mg mL\(^{-1}\)) for 30 min prior to their exposure to 4FG. There is about a 50\% reduction in the rate of fluoride release with cells treated with Pronase. If these cells are washed and allowed to recover in buffer for 15 min, full defluorinating activity is recovered. Pre-incubation of *E. coli* with Pronase has been shown to inhibit succinate transport (77). It was suggested that this inhibition was due to the action of Pronase on the outer membrane associated DBP. Cells which were washed and allowed to recover in buffer regained the ability to transport succinate. It was also demonstrated that Pronase does not penetrate or cause any significant damage to the outer membrane of *E. coli* (77). Although the permeability and effect of Pronase on the outer membrane of *P. putida* has not been investigated, the above results suggest that the defluorinating protein may be part of or associated with the outer membrane of *P. putida*. It has been previously proposed that the defluorinating protein may be part of the cell envelope (61).

In an attempt to localize the site of the defluorination reaction, glucose grown whole cells were fractionated into various components and each component tested for the ability to defluorinate 4FG. As indicated in Table 8, the most probable site of the defluorinating protein is in the crude outer membrane fraction. Since an -SH group has been
Figure 20

The Effect of Pronase on Fluoride Release from 4FG by Glucose Grown Whole Cells of *P. putida*.

**Legend:**

- ■ - glucose grown cells
- • - glucose grown pronase pre-treated cells
- ▲ - glucose grown pronase pre-treated and washed cells

Whole cell suspensions (20 mg protein) of glucose grown *P. putida* were pre-incubated in the absence or presence of Pronase (5 mg mL⁻¹) for 30 min at 30°C as described in the MATERIALS AND METHODS section. The cells were then exposed to 1 mM 4FG or washed and allowed to recover in buffer for 15 min before exposure to 1 mM 4FG. Cell suspensions were incubated in 100 mM potassium phosphate buffer, pH 7.1, at 30°C in a total volume of 3 mL. Fluoride determinations were as described in the MATERIALS AND METHODS section. The results are the average of duplicate trials.
Table 8
Fluoride Release from 4FG by Various Fractions Obtained from Glucose Grown Whole Cells of *P. putida*.

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>nmol fluoride mg protein$^{-1}$ (after 24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic membrane vesicles</td>
<td>19</td>
</tr>
<tr>
<td>Cell-free extract</td>
<td>6</td>
</tr>
<tr>
<td>Cell envelopes</td>
<td>22</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>60</td>
</tr>
<tr>
<td>Crude shock extract</td>
<td>16</td>
</tr>
<tr>
<td>Crude outer membranes</td>
<td>352</td>
</tr>
</tbody>
</table>

*Fractions (2-6 mg protein) in 100 mM potassium phosphate buffer, pH 7.1, and 1 mM DTT were incubated with 2.5 mM 4FG at 30°C for 24 h, in a total volume of 2 mL. Fluoride determinations and fraction preparations were as described in the MATERIALS AND METHODS section. The results are the average of six trials.*
implicated in the defluorination reaction, DTT was added to the incubates. Although it was later observed that the absence or presence of DTT did not significantly affect the levels of fluoride release in the various fractions, it is still possible that an -SH group is involved.

As in the case of the cytoplasmic membrane vesicles and cell-free extracts, cell envelopes were found to oxidize 4FG (1 mol oxygen mol substrate\(^{-1}\)) with retention of the C-F bond. Although spheroplasts exhibited some defluorinating activity, 4FG was extensively oxidized (0.9 mol oxygen mol substrate\(^{-1}\)). This defluorinating activity is probably due to the presence of whole cells and outer membrane fragments in the spheroplast suspension. The oxidation of 4FG is the result of the exposure of the cytoplasmic membrane bound enzymes.

Proteins obtained by the osmotic shock method of Stinson et al. (67) failed to defluorinate or oxidize 4FG. About 5 mg of protein was obtained from 1 g of wet weight cells. This crude shock extract was found to have negligible amounts of glucose binding activity (0.04 nmol glucose mg protein\(^{-1}\)). The value is about 10-fold lower than that determined for \textit{P. aeruginosa} (67). The small amount of binding activity in the crude shock extract may or may not indicate the presence of GBP. In either case, the shocked cells were not affected in their ability to defluorinate 4FG. The rates and extents of fluoride release were virtually identical to the control cells. It has been previously demonstrated
that P. aeruginosa produces an inducible shock-sensitive GBP (67). Furthermore, this GBP-associated transport system is considered to be energized by phosphate-bond energy (68). Incubation of P. putida with 10 mM sodium cyanide or sodium arsenate had no effect on the rates or extents of fluoride release from 4FG, suggesting that the defluorination reaction is not phosphate-bond energy dependent. The above results indicate that the defluorinating protein is held tightly within the cell, presumably at the outer membrane level, and is not removed by relatively mild procedures.

The outer membrane is preferentially shed during spheroplast formation and recovered by high speed centrifugation. Although this method is convenient, the composition of the released material seems to be significantly different from the average composition of the outer membrane (71). The method of Mizuno and Kageyama (86) involves the use of lysozyme, EDTA and Tris-HCl buffer in a hypertonic solution to isolate the outer membrane form P. aeruginosa. In applying their method to P. putida, cell lysis was observed in which the absorbance of the suspension at 660 nm (117) dropped from 0.9 to 0.6 within 60 min. Furthermore, the suspension became reddish-brown, also indicating the lysis of cells. EDTA has been found to cause severe damage to both the outer and cytoplasmic membranes, resulting in cell lysis (84,87,118,119). EDTA exerts its lytic effect by extracting divalent cations (i.e., Mg²⁺, Ca²⁺, etc.) which anchor a complex of protein and lipopolysaccharide in the
outer membrane. Furthermore, the lysis of cells by EDTA-lysozyme is far more rapid in Tris-HCl buffer than any other buffer, since Tris has a weak metal binding property \( (118) \). Therefore, the isolation of an outer membrane fraction was attempted in the absence of EDTA and Tris.

A whitish-opaque pellet was obtained from the spheroplast formation medium. About 2 mg of protein per 4 g of wet weight cells was obtained. This pellet was defined as crude outer membranes because of the method of isolation and because of the results obtained from specific enzyme studies and electrophoresis. Table 9 demonstrates that this fraction has negligible enzyme activities as compared to the high levels in the cytoplasmic membrane vesicles. In some cases, no enzyme activity could be detected in the crude outer membrane fraction even though fluoride release from 4FG was substantial.

Electrophorhetic analysis of the crude outer membranes gives a rather uncomplicated distribution of 6 major protein bands (a-f), compared to over 30 major protein bands in the cytoplasmic membrane vesicles (Figure 21). The apparent molecular weight of proteins a, b, c, d, e and f were estimated to be 86,000, 55,000, 45,000, 40,000, 22,000 and 16,000, respectively. In some cases, proteins c and d were seen as only one diffuse band. Furthermore, a protein corresponding to an apparent molecular weight of 10,000 was sometimes observed. This protein may correspond to the lipoprotein isolated by Mizuno and Kageyama \( (120) \). The
Table 9
Enzyme Activities in the Crude Outer Membranes and Cytoplasmic Membrane Vesicles.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude Outer Membranes</td>
</tr>
<tr>
<td>Glucose dehydrogenase</td>
<td>7</td>
</tr>
<tr>
<td>Gluconate dehydrogenase</td>
<td>4</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Specific activities are expressed as nmol DCIP reduced min<sup>-1</sup> mg protein<sup>-1</sup>.

Reaction mixtures contained 100 mM potassium phosphate, pH 7.1, 10 mM KCN, 10 μg of PMS, 20 μg of DCIP and 50-100 μg of outer or cytoplasmic membrane protein, all in a total volume of 1.0 mL. All reactions were initiated by addition of 100 mM (final concentration) glucose, gluconate or succinate. The enzyme activities were measured by the decrease in absorbance at 600 nm for DCIP at 25°C. Specific activities were calculated using an extinction coefficient for DCIP of 10 mM<sup>-1</sup> cm<sup>-1</sup>. Outer and cytoplasmic membranes were prepared as described in the MATERIALS AND METHODS section. The results are the average of four trials.
Figure 21

Comparison of the Major Proteins in the Outer and Cyttoplasmic Membranes Obtained from Glucose Grown Whole Cells of *P. putida*.

Legend:

- **A** - cytoplasmic membrane vesicles
- **B** - crude outer membranes
- **O** - represents the origin of the gels

Tracings are reproductions of the densitometer scans of urea-SDS-polyacrylamide gels. Arrows indicate the position of the tracking dye. Samples were prepared and electrophoresis performed as described in the MATERIALS AND METHODS section. The molecular weight of the protein bands were estimated from a standard curve (APPENDIX V).

<table>
<thead>
<tr>
<th>Outer Membrane Protein Band</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>86,000</td>
</tr>
<tr>
<td>b</td>
<td>55,000</td>
</tr>
<tr>
<td>c</td>
<td>45,000</td>
</tr>
<tr>
<td>d</td>
<td>40,000</td>
</tr>
<tr>
<td>e</td>
<td>22,000</td>
</tr>
<tr>
<td>f</td>
<td>16,000</td>
</tr>
</tbody>
</table>
above results are consistent with those obtained for P. aeruginosa (85-89), in which a range of 2-9 proteins were observed in the various outer membrane preparations. The correspondence of the proteins obtained from P. putida to those of P. aeruginosa, if any, have yet to be elucidated. The number, resolution and order of migration of the major outer membrane proteins depends on the SDS-gel system used, the method of outer membrane isolation, strain and culture conditions (73,90). No bands were observed after PAS-staining of the gels, which is specific for glycoprotein. This is in agreement with results obtained for P. aeruginosa (86).

A comparison of the major proteins in crude outer membranes prepared from glucose and succinate grown cells showed a similar distribution (Figure 22). The only noticeable difference is that proteins c and d seem to migrate as one band (c') in the succinate grown cells. This difference may not be significant since proteins c and d were sometimes found to migrate together as one diffuse band in glucose grown cells. On the other hand, protein c' was never observed as two separate bands. Furthermore, the crude outer membranes obtained from succinate grown cells were found to have reduced levels of defluorinating activity (95 nmol fluoride mg protein⁻¹ after 24 h), compared to the crude outer membranes obtained from glucose grown cells (352 nmol fluoride mg protein⁻¹ after 24 h). This indicates the repression of protein responsible for the defluorination of 4FG.

The protein distribution in the outer membranes
Comparison of the Major Proteins in the Crude Outer Membranes Obtained from Glucose and Succinate Grown Whole Cells of P. putida.

Legend:

- **A** - crude outer membranes obtained from succinate grown cells
- **B** - crude outer membranes obtained from glucose grown cells
- **O** - represents the origin of the gels

Tracings are reproductions of the densitometer scans of urea-SDS-polyacrylamide gels. Arrows indicate the position of the tracking dye. Samples were prepared and electrophoresis performed as described in the MATERIALS AND METHODS section. The molecular weight of the protein bands were determined from a standard curve (APPENDIX V).

<table>
<thead>
<tr>
<th>Outer Membrane Protein Band</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>86,000</td>
</tr>
<tr>
<td>b</td>
<td>55,000</td>
</tr>
<tr>
<td>c</td>
<td>45,000</td>
</tr>
<tr>
<td>d</td>
<td>40,000</td>
</tr>
<tr>
<td>e</td>
<td>22,000</td>
</tr>
<tr>
<td>f</td>
<td>16,000</td>
</tr>
<tr>
<td>c'</td>
<td>42,000</td>
</tr>
</tbody>
</table>
obtained from glucose or succinate grown cells of *P. aeruginosa* were observed to be about the same (86,90). Proteins D and E, with molecular weights of 50,000 and 45,000, respectively, were induced by growth on glucose and partially induced by growth on succinate (90). Major differences were only observed when the outer membranes were electrophoresed on gels with concentrations of acrylamide of 14% or greater. Under these conditions protein D was observed as two polypeptides, D1 and D2, of which protein D1 did not appear after growth on succinate (90).

The results discussed thus far suggest the presence of an inducible repressible protein associated with the outer membrane of *P. putida*. This protein, which may be involved with glucose transport, interacts with 4FG to cause fluoride release. It is then possible for the defluorination product to become covalently bound to the defluorinating protein. Therefore, D-[6-\(^3\)H]4FG was synthesized (101) as an aid to confirm and extend the above proposals and to assist with the isolation of the defluorinating protein and elucidation of the mechanism of fluoride release from 4FG.

Incubation of whole cell suspensions of glucose grown *P. putida* with 1 mM D-[6-\(^3\)H]4FG (7.2 mCi/mmol) for 24 h (100% fluoride release) gave an unexpected result. After centrifugation, 70% of the radiolabel used was found in the supernatant with the remainder in the cell pellet. The radiolabeled material in the supernatant was found to be totally dialyzable. After extensive dialysis of the cell
pellet, only about 1% of the total radiolabel used was found to remain cell-associated. If the proposed glycosylation reaction was occurring, it would have been expected that most of the radiolabel would be cell-associated. These results indicate, however, that extensive release of radiolabel may occur as a result of the defluorination reaction.

When the supernatant was reduced to dryness by lyophilization or on a rotary evaporator and the residue suspended to the original volume with distilled water, a 40-50% reduction in the disintegrations per minute (dpm) was observed. Furthermore, a corresponding increase in the dpm was observed in the solvents collected from both processes, indicating the presence of $^3\text{H}_2\text{O}$. To determine more accurately the amount of $^3\text{H}_2\text{O}$ present, a borate column (106) was used. This column has been shown to retain a variety of carbohydrates, which are eluted with ammonium tetraborate. Any $^3\text{H}_2\text{O}$ would be initially eluted with water (106). Figure 23 illustrates the results of applying 25 µL aliquots of 1 mM D-[U-$^{14}$C]-glucose, 1 mM D-[6-$^3$H]4FG and supernatant fraction to the borate column. It can be seen that D-[U-$^{14}$C]glucose and D-[6-$^3$H]4FG were retained by the column and were only eluted with ammonium tetraborate. A large peak corresponding to $^3\text{H}_2\text{O}$ was observed by elution of the supernatant fraction with water. Furthermore, a small peak corresponding to a tritiated carbohydrate(s) ($[^3\text{H}]-\text{carbohydrate}$) was obtained on elution with ammonium tetraborate. A similar distribution of $^3\text{H}_2\text{O}$ and $[^3\text{H}]-\text{carbohydrate}$ was observed in the intra-
Figure 23

Estimation of $^3$H$_2$O and Radiolabeled Carbohydrates on a Micro-Column of Borate Resin.

Legend:

- 25 µL of supernatant (12.3 x 10$^4$ dpm applied onto the column)

- 25 µL of 1 mM D-[U-$^{14}$C]glucose (20.7 x 10$^4$ dpm applied onto column)

- 25 µL of 1 mM D-[6-$^3$H]4FG (24.4 x 10$^4$ dpm applied onto the column)

The preparation of the borate column was as described in the MATERIALS AND METHODS section. 25 µL of 1 mM D-[U-$^{14}$C]glucose (4.0 mCi/mmol), 1 mM D-[6-$^3$H]4FG (0.3 mCi/mmol) or supernatant were applied onto the borate column. $^3$H$_2$O was estimated by elution with 10 x 0.2 mL aliquots of deionized distilled water. Radiolabeled carbohydrates were then estimated by elution with 25 x 0.2 mL aliquots of 0.25 M ammonium tetraborate. Effluents were collected directly into scintillation vials for radioactivity determinations. The results are the average of duplicate trials.
cellular contents prepared from the undialyzed whole cell pellets. Table 10 summarizes the amount and distribution of $^{3}\text{H}_{2}\text{O}$ and $[^{3}\text{H}]$-carbohydrate after incubation of whole cells with 1 mM D-[6-$^{3}\text{H}$]4FG for 24 h. The results indicate that about 67% of the total radiolabel used is released in the form of $^{3}\text{H}_{2}\text{O}$. Furthermore, about 20% of the total radiolabel used corresponds to a $[^{3}\text{H}]$-carbohydrate. This distribution of radiolabel may result from the mechanism of fluoride release. The remainder of the radiolabel, except for the 1% that remains cell-associated, may be lost as a result of quenching and/or the assay procedure.

Although the results from the borate column indicated the presence of a $[^{3}\text{H}]$-carbohydrate in the supernatant, no carbohydrate could be detected by TLC analysis. Concentration of the supernatants, followed by isolation and purification of the radiolabeled material with the use of the borate column, resulted in the appearance of a single radiolabeled spot by TLC analysis (Figure 24). The compound was observed to migrate slower ($R_f$ 0.3) than most of the standard sugars used, indicating a more polar compound. Furthermore, this material gave a positive reaction with aniline hydrogen phthalate (108), indicating that it is a reducing sugar. No fluorine could be detected in the sample by Fourier transform $^{19}\text{F}$-NMR, consistent with the fact that there was 100% defluorination. Chromatographic detection by the acid-$\text{FeCl}_3$-sulfosalicylic acid reagents (109) indicated that the isolated carbohydrate is a sugar-phosphate (Figure 25a).
Table 10

Distribution of Radiolabel from D-[6-\textsuperscript{3}H]4FG in the Whole Cell Incubation Mixture.*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Label present (%)</th>
<th>( \textsuperscript{3}\text{H}_{2}\text{O} ) (%)</th>
<th>([\textsuperscript{3}H])-Carbohydrate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>70</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>Whole cell pellet\textsuperscript{a}</td>
<td>25</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
<td>67</td>
<td>20</td>
</tr>
</tbody>
</table>

*Whole cell suspensions (24 mg protein) of glucose grown \emph{P. putida} in 100 mM potassium phosphate buffer, pH 7.1, at 30\(^\circ\)C were incubated with 1 mM D-[6-\textsuperscript{3}H]4FG (7.2 mCi/mmol), in a total volume of 4 mL. After 24 h incubation the suspensions were centrifuged to obtain the whole cell pellets and supernatants. Results are expressed as percentages of the total dpm used.

\textsuperscript{a}Results are from undialyzed intracellular contents of whole cell pellets. 1% of radiolabel was found to remain cell-bound after extensive dialysis of the whole cell pellets. Fraction preparations and \( \textsuperscript{3}\text{H}_{2}\text{O} \) and \([\textsuperscript{3}H]\)-carbohydrate determinations were as described in the MATERIALS AND METHODS section.
Figure 24

Thin Layer Chromatographic Analysis of the Isolated Radiolabeled Sample and Various Standard Sugars.

Legend:

S - radiolabeled sample
4FG - 4-deoxy-4-fluoro-D-glucose
GLU - D-glucose
ALL - D-allose
G6P - glucose-6-phosphate
G1P - glucose-1-phosphate
IDO - D-idose
ALT - D-altrose
GAL - D-galactose
2KGA - 2-ketogluconate

TLC analysis was as described in the MATERIALS AND METHODS section. Solvent system; ethyl acetate: acetic acid: water (3:3:1). Carbohydrates were detected by sulfuric acid spray. The numbers on the left hand column represent the counts per minute (cpm) detected from the 1 cm x 1 cm squares scraped from the sample lane.
Figure 24

Solvent Front

S  4FG  GLU  ALL  G6P  G1P  IDO  ALT  GAL  2KGA

897  361  1630  1844  11078  5396  1476  672  1160  185  63  36  25
Figure 25a

Chromatographic Analysis of the Isolated Radiolabeled Carbohydrate by the Sugar-Phosphate Detection Method.

Legend:

G6P - glucose-6-phosphate  
G1P - glucose-1-phosphate  
S - radiolabeled carbohydrate  
PPB - potassium phosphate buffer

Sugar-phosphates were detected on Whatman #1 chromatography paper as described in the MATERIALS AND METHODS section. Solvent system; n-propanol: concentrated ammonia: water (6:3:1).

Figure 25b


Legend:

S - radiolabeled carbohydrate  
HS - hydrolysis product  
4FG - 4-deoxy-4-fluoro-D-glucose  
GLU - D-glucose  
ALT - D-altrose  
ALL - D-allose  
IDO - D-idose

TLC analysis and hydrolysis of the sugar-phosphate was as described in the MATERIALS AND METHODS section. Solvent system; ethyl acetate: acetic acid: water (3:3:1). Carbohydrates were detected by sulfuric acid spray.
Furthermore, hydrolysis of this sugar-phosphate with 1 M HCl resulted in a new component ($R_f$ 0.5) being produced (Figure 25b). This sugar-phosphate may be formed by diffusion of the defluorinating sugar into the cell and subsequent interaction with a phosphorylating enzyme. Although the identity of the sugar has not yet been deduced, identification may provide evidence for the mechanism of fluoride release.

The amount of $^3$H$_2$O and total $[^3$H]-carbohydrates in the supernatant and the amount of radiolabel incorporated into the cells were compared to fluoride release from 4FG with respect to time (Figure 26). The formation of $^3$H$_2$O was observed to be virtually linear up to 24 h, although a slight lag is noticeable. On the other hand, the release of fluoride is much more extensive and begins to level off after 4 h.

The total $[^3$H]-carbohydrates, which would be a measurement of both D-[6-$^3$H]4FG and the defluorination sugar, reaches a minimum value after 4 h and then remains fairly constant. The incorporation of radiolabel into whole cells (presumably by a glycosylation reaction) was found to follow the same profile as the defluorination reaction, reaching a maximum value after 24 h.

Based on the above results, a mechanism for fluoride release, $^3$H$_2$O and $[^3$H]-carbohydrate formation and protein glycosylation can be proposed (Figure 27). Thus, D-[6-$^3$H]4FG first binds to a protein (I) (presumably by fluorine-hydrogen bonding) which eventually results in the loss of fluoride. The 4,5-glucopyranoseen (II) may now either
Figure 26

Determination of $^3$H$_2$O, Total Tritiated-Carbohydrates and Fluoride in the Supernatants and Tritium-Incorporated into Whole Cells with Respect to Time.

Legend:
- ● - nmol total [3H]-carbohydrates mg protein$^{-1}$
- ▲ - nmol $^3$H$_2$O mg protein$^{-1}$
- ▲ - nmol fluoride mg protein$^{-1}$
- ■ - nmol [3H]-incorporated into whole cells mg protein$^{-1}$

Whole cell suspensions (24 mg protein) of glucose grown P. putida were incubated with 1 mM D-[6-3H]4FG (7.2 mCi/mmol) for various time intervals in 100 mM potassium phosphate buffer, pH 7.1, at 30°C and in a total volume of 3 mL. $^3$H$_2$O, total [3H]-carbohydrates and fluoride in the supernatants and [3H]-incorporated into whole cells were determined as described in the MATERIALS AND METHODS section.
- nmol total $[^3H]$-carbohydrates mg protein$^{-1}$

$\triangle$ nmol $^3$H$_2$O mg protein$^{-1}$

$\blacktriangle$ nmol fluoride mg protein$^{-1}$

$\blacksquare$ nmol $[^3H]$-incorporated into whole cells mg protein$^{-1}$
Figure 27

Possible Mechanism of Fluoride Release from D-[6-³H]4FG and Covalent Attachment of the Defluorination Sugar to the Protein.

Legend:

- protein

A - direct reaction with the -SH group of the protein resulting in covalent attachment

B - ring opening leading to prototropy and tritium exchange with water
directly react with the -SH group of the protein or in chain form (III) undergo prototropy (IV and V) thereby allowing tritium exchange with water to occur. Such a rearrangement may possibly provide alternative substrates (III or V) for nucleophilic attack by the -SH group. The rates and sequence of these reactions will be determined by kinetic factors. In any event the mechanism accounts for fluoride release, the formation of $^{3}$H$_{2}$O and $[^{3}$H]-carbohydrate and suggests that the covalent attachment to the protein may be predominately at the C-4 position of the sugar residue. Recovery of the sugar residue by hydrogenation of the sugar-protein adduct will be required to determine the attachment site(s). To obtain enough sugar-protein adduct for this, $^{14}$C-4FG would be required.

In an attempt to isolate the radiolabeled protein(s), dialyzed whole cell pellets containing bound radiolabel were fractionated. As indicated in Table 11, the highest specific activity of radiolabel was obtained in the cell envelope fraction, supporting previous proposals (61). Electrophoretic analysis of the cell envelopes revealed a distribution of about 30 major protein bands. Digestion of 0.5 cm gel slices with $H_{2}O_{2}$ for 24 h at 55°C and subsequent radioactivity determination demonstrated that at least 65% of the radiolabel applied onto the gels was lost. The remainder of the radiolabel was found to be confined within the top 0.5 cm of the gel (Figure 28). Slices of 0.1 cm indicated that the radiolabeled material is at the origin or surface
Table 11

Distribution of Cell-Bound Radiolabel from D-[6-\(^3\)H]4FG in Whole Cells of Glucose Grown \textit{P. putida}.*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>nmol radiolabel mg protein(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzed whole cells</td>
<td>0.6</td>
</tr>
<tr>
<td>Cell-free extracts</td>
<td>0.7</td>
</tr>
<tr>
<td>Cell envelopes</td>
<td>2.3</td>
</tr>
<tr>
<td>Supernatant of cell envelopes</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*Whole cell suspensions (200 mg protein) of glucose grown \textit{P. putida} in 100 mM potassium phosphate buffer, pH 7.1, were incubated with 1 mM D-[6-\(^3\)H]4FG (7.2 mCi/mmol) at 30°C, in a total volume of 20 mL. After 24 h, the suspensions were centrifuged and cells fractionated as described in the MATERIALS AND METHODS section. The amount of radiolabel bound per mg of protein were determined as outlined in APPENDIX III. The results are the average of three trials.
Radioactivity Determination in Urea-SDS-Polyacrylamide Gels of Radiolabeled Cell Envelopes.

Legend:

A - radiolabeled cell envelopes

Gels were sliced into 0.5 cm sections and each section placed into scintillation vials. To each vial was added 200 μL of H₂O₂ and the suspensions were then incubated at 55°C for 24 h. 10 mL of liquid scintillation cocktail was then added to the digested material and radioactivity determined. Tracing is a reproduction of the densitometer scans of the urea-SDS-polyacrylamide gels. Gels were sliced beginning from the origin. The arrows indicate the position of the tracking dye on the gels. Samples were prepared and electrophoresis performed as described in the MATERIALS AND METHODS section. The results are the average of six trials.
of the gel. This would tend to suggest that the radiolabeled material is a relatively large or uncharged protein. A protein of about 150,000 daltons or more would be expected to remain at the origin in the gel system used (Appendix V).

No significant protein band could be observed at the origin. Furthermore, reducing the acrylamide concentration to as low as 4%, which would allow larger proteins to penetrate into the gel, was found to have no effect on the migration of the radiolabeled material. Using different electrophoretic methods, such as changing the pH of the electrode buffer, omission of SDS and urea in the gels and samples, had no effect on the position of the radiolabeled material on the gels. This may indicate that the radiolabel remaining at the origin is probably an artifact rather than due to the presence of a radiolabeled protein.

Since strong evidence for the presence of a radiolabeled protein could not be demonstrated by electrophoresis, the radiolabeled cell envelopes were subjected to column chromatography. As illustrated in Figure 29, virtually no radioactivity could be detected in the void volume, indicating that the radiolabeled material is not as large as implicated by electrophoresis. Furthermore, about 90% of the radiolabel applied onto the column was recovered. A molecular weight about 38,000 could be estimated for the radioactive peak using bovine albumin and lysozyme as molecular weight markers. The peak of radioactivity was found to correspond to the protein peak, indicating the presence of a radiolabeled
Figure 29

Elution Profile of Radiolabeled Cell Envelopes on a Sephadex G-100 Column.

Legend:

BA - bovine albumin (molecular weight of 66,000)
L - lysozyme (molecular weight of 14,300)
V₀ - void volume

150 μL of a radiolabeled cell envelope suspension (500 μg protein, 6,000 dpm) was applied onto a Sephadex G-100 column (0.7 cm x 48 cm bed height). The flow rate was set at 1.2 mL h⁻¹ and 0.6 mL fractions were collected. 0.1 mL was removed from each fraction for the determination of radioactivity. The remainder of the solutions were diluted to 1 mL with distilled water and protein qualitatively measured at an absorbance of 280 nm. The V₀ (6 mL) was determined with blue dextran. Bovine albumin and lysozyme were used as molecular weight markers (APPENDIX IV). The radioactive peak corresponds to a molecular weight of about 38,000. Samples treated with and gel permeation column chromatography performed in the presence of SDS as described in the MATERIALS AND METHODS section.
protein. In support of this is the fact that the radio-
labeled material can be precipitated with trichloroacetic
acid. Lyophilization of the fraction containing the highest
radioactivity and subsequent electrophoretic analysis gave
similar results as before. Whereas most of the radiolabel
applied onto the gels was lost, the remainder was found at
the origin of the gels. Although the use of the Sephadex
G-100 column gave an indication as to the presence and
molecular weight of the radiolabeled protein, isolation of
this protein could not be achieved by the column. For the
isolation of this protein further fractionation of the fraction
containing the highest radioactivity would be required.

Alternatively, cell envelopes could be further fraction-
ated by the SDS-extraction method of Mizuno and Kageyama (92).
According to their method, a peptidoglycan-associated protein
complex is obtained by incubation of cell envelopes from
*P. aeruginosa* with 2% SDS-10% glycerol-10 mM Tris-HCl, pH 7.8,
extraction solution for 60 min at 30°C, followed by centri-
fugation. Similar methods have been used by Heilmann (81)
and Hancock et al. (91) for *P. aeruginosa* and Lugtenberg
et al. (79) for *E. coli*. The proteins that remain peptido-
glycan-associated in *P. aeruginosa* have been shown to
correspond to the outer membrane proteins F, H and I, with
molecular weights of 35,000, 21,000 and 8,000, respectively.
Furthermore, proteins F and H were found to form hydrophilic
aqueous channels in the outer membrane (91,92,100). Indeed,
proteins attached to the peptidoglycan are outer membrane
proteins which may form or be part of the pores or hydrophilic channels through which numerous molecules permeate (121). These proteins remain attached to the peptidoglycan by treatment with SDS at or below 60°C, but are removed by heating above 60°C (92,121). Furthermore, these proteins are released from the peptidoglycan by treatment with NaCl (92) demonstrating that they are not covalently attached to the peptidoglycan, but held together by strong ionic interactions (121). In P. aeruginosa, proteins F, H and I have been shown to be held together by strong ionic interactions (91,92).

The results of applying the above SDS-extraction procedure to the radiolabeled cell envelopes of P. putida are demonstrated in Table 12. It can be seen that the highest specific activity of radiolabel is in the peptidoglycan-associated protein complex obtained by SDS-extraction of cell envelopes at 30°C. SDS-extraction of cell envelopes at 70°C resulted in a substantial reduction in the amount of radiolabel in the peptidoglycan fraction. Associated with this is a greater than 95% release of protein from the peptidoglycan fraction. Although the above comparisons were from SDS-extractions carried out on different radio-labeled cell envelopes, the results suggest that the radio-labeled protein is associated with peptidoglycan.

Analysis of the radiolabeled peptidoglycan-associated protein complex by electrophoresis and column chromatography yielded results similar to the cell envelopes. Although
Table 12
SDS-Extraction of Cell Envelopes and the Distribution of Radiolabel from D-[6-\(^3\)H]4FG.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total dpm((\times 10^5))</th>
<th>Total mg protein</th>
<th>nmol radiolabel/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidoglycan-associated proteins (SDS-extraction of cell envelopes A at 30°C).</td>
<td>0.5</td>
<td>0.7</td>
<td>5.0</td>
</tr>
<tr>
<td>Proteins from supernatant of SDS-extraction of cell envelopes A at 30°C.</td>
<td>0.14</td>
<td>3.0</td>
<td>0.29</td>
</tr>
<tr>
<td>Peptidoglycan-associated proteins (SDS-extraction of cell envelopes B at 70°C).</td>
<td>0.005</td>
<td>0.03</td>
<td>0.90</td>
</tr>
<tr>
<td>Proteins from supernatant of SDS-extraction of cell envelopes B at 70°C.</td>
<td>0.4</td>
<td>4.5</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Whole cell suspensions (24 mg protein) of glucose grown *P. putida* were incubated at 30°C in 100 mM potassium phosphate buffer, pH 7.1, with 1 mM D-[6-\(^3\)H]4FG (7.2 mCi/mmol), in a total volume of 4 mL. After 24 h the suspensions were centrifuged and cell envelopes prepared by ultrasonication. SDS-extraction of cell envelopes were as described in the MATERIALS AND METHODS section. Cell envelopes A and B refer to cell envelopes prepared from two different whole cell incubation mixtures under the same conditions. The amount of radiolabel in cell envelopes A and B were not determined, but subjected to SDS-extraction at 30°C and 70°C, respectively.
most of the radiolabel applied onto the gels was lost, the remainder was found at the origin. This may be due to the high content of peptidoglycan in the sample, although there is no evidence to support this effect of peptidoglycan on electrophoresis. An alternative procedure may be to treat the cell envelopes and peptidoglycan-associated protein complex with lysozyme before analysis. An apparent molecular weight of 43,000 could be estimated for the peak of radioactivity of the peptidoglycan-associated protein complex on the Sephadex G-100 column. Furthermore, this peak corresponds to the protein peak.

A whitish-opaque pellet, similar to the outer membrane pellet, is obtained by SDS-extraction of cell envelopes of \textit{P. putida} at 30°C. Figure 30 is a comparison of the protein distribution in the outer membrane and peptidoglycan-associated protein complex isolated from \textit{P. putida}. The peptidoglycan-associated protein complex was observed to have 4 major protein bands with apparent molecular weights of 47,000, 42,000, 22,000 and 9,000. The 47,000, 42,000 and 22,000 molecular weight proteins were observed to correspond to the outer membrane proteins c, d and e, respectively. These proteins may in fact form pores or hydrophilic channels in the outer membrane. The 9,000 molecular weight protein most likely corresponds to the lipoprotein (120), which functions to anchor the outer membrane to the peptido- glycan (92,121). Virtually no difference was observed in the protein distribution in the peptidoglycan-associated
Figure 30

Comparison of the Major Proteins in the Crude Outer Membranes and Peptidoglycan-Associated Protein Complex Obtained from Glucose Grown Whole Cells of P. putida.

Legend:

- B - crude outer membranes
- A - peptidoglycan-associated protein complex obtained by SDS-extraction of cell envelopes at 30°C

O - represents the origin of the gels

Tracings are reproductions of the densitometer scans of urea-SDS-polyacrylamide gels. Arrows indicate the position of the tracking dye. Samples were prepared and electrophoresis performed as described in the MATERIALS AND METHODS section.

<table>
<thead>
<tr>
<th>Outer Membrane Protein Band</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>86,000</td>
</tr>
<tr>
<td>b</td>
<td>55,000</td>
</tr>
<tr>
<td>c</td>
<td>45,000</td>
</tr>
<tr>
<td>d</td>
<td>40,000</td>
</tr>
<tr>
<td>e</td>
<td>22,000</td>
</tr>
<tr>
<td>f</td>
<td>16,000</td>
</tr>
</tbody>
</table>
protein complex obtained from succinate grown cells.

When the SDS-extraction procedure is carried out at 70°C, a transparent pellet of similar size to the pellet obtained by SDS-extraction at 30°C is obtained. This pellet should be made up of predominately peptidoglycan (92,121). As previously mentioned, greater than 95% of the protein was found to be released from the peptidoglycan by SDS-extraction at 70°C. As can be seen in Figure 31, no proteins could be observed in the pellet by electrophoresis, but could be recovered from the supernatant. Similarly, about 80% of the proteins were released from the peptidoglycan by treatment with the SDS-extraction solution supplemented with 0.75 M NaCl at 30°C. Although some residual proteins could be detected in the peptidoglycan pellet, the majority of the proteins could be recovered from the supernatant. These results demonstrate that the peptidoglycan-associated proteins are not held together by covalent attachment, but by strong ionic interactions. Furthermore, these results indicate that a peptidoglycan-associated protein(s) may be involved in the defluorination reaction with 4FG.

The above SDS-extraction method may provide a simple and rapid procedure for the isolation and identification of the radiolabeled protein. It may be possible that more than one of the peptidoglycan-associated proteins are radiolabeled. Extraction of the radiolabeled cell envelopes with SDS at 30°C will yield the peptidoglycan-associated protein complex. This complex may then be treated with the SDS-extraction
Urea-SDS-Polyacrylamide Gel Electrophoretic Comparison of Fractions Obtained by SDS-Extraction of Cell Envelopes with the Crude Outer Membranes.

Legend:

- lane 1 - cell envelopes
- lane 2 - proteins from supernatant of SDS-extraction of cell envelopes at 30°C
- lane 3 - crude outer membranes
- lane 4 - pellet obtained from SDS-extraction of cell envelopes at 30°C
- lane 5 - pellet obtained from SDS-extraction of cell envelopes at 70°C
- lane 6 - proteins from supernatant of SDS-treatment of peptidoglycan-associated protein complex at 70°C
- lane 7 - pellet obtained from 6
- lane 8 - proteins from supernatant of SDS-0.75 M NaCl treatment of peptidoglycan-associated protein complex at 30°C
- lane 9 - pellet obtained from 8

<table>
<thead>
<tr>
<th>Protein Band</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>86,000</td>
</tr>
<tr>
<td>b</td>
<td>55,000</td>
</tr>
<tr>
<td>c</td>
<td>45,000</td>
</tr>
<tr>
<td>d</td>
<td>40,000</td>
</tr>
<tr>
<td>e</td>
<td>22,000</td>
</tr>
<tr>
<td>f</td>
<td>16,000</td>
</tr>
<tr>
<td>g</td>
<td>9,000</td>
</tr>
</tbody>
</table>

M<sub>r</sub> - molecular weight markers used:
- bovine albumin - 66,000
- egg albumin - 45,000
- pepsin - 37,500
- trypsinogen - 24,000
- β-lactoglobulin - 18,400
- lysozyme - 14,300

See APPENDIX V for the standard curve of the above proteins.

Samples were prepared and electrophoresis performed as described in the MATERIALS AND METHODS section.
solution for 60 min at 70°C or with the SDS-extraction solution supplemented with 0.75 M NaCl for 60 min at 30°C. This will result in the release of proteins from the peptidoglycan, which can be recovered by precipitation with 2 volumes ice-cold acetone. This fraction may then be further fractionated by ammonium sulfate precipitation, ion-exchange chromatography, column chromatography or by a combination of these methods. Isolation of this protein may provide a better understanding of the mechanism and precise location of fluoride release.
CHAPTER IV
SUMMARY AND CONCLUSIONS

The defluorination of 4FG in glucose grown whole cells of *P. putida* displays saturation kinetics. This defluorination reaction was found to be effectively inhibited by sugars with the D-gluco-configuration. Glucose, gluconate and 2-ketogluconate exhibited the highest levels of inhibition. Growth of cells on gluconate or 2-ketogluconate had no effect on the defluorination of 4FG. On the other hand, cells grown on succinate or citrate exhibited drastically reduced levels of defluorinating activity. Treatment of cells with chloramphenicol or pronase indicated the presence and synthesis of protein required for the defluorination reaction.

Fractionation of whole cells indicated that the defluorinating protein may be part of or associated with the outer membrane. The use of D-[6-3H]4FG provided evidence for a possible mechanism of fluoride release in which there is formation of \({{^3}}\text{H}_2\text{O}\) and [\(^3\text{H}\)]-carbohydrate and covalent attachment of the protein by the defluorinated sugar residue. Extraction of cell envelopes with SDS indicated that this protein may be associated with the peptidoglycan layer and, therefore, possibly linked to the outer membrane. A molecular weight of about 40,000 was estimated for this protein by column chromatography. Therefore, the most likely candidates for this protein are the outer membrane proteins c and/or d.

This outer membrane protein may form or be part of the porin channel. Furthermore, the protein may be involved in the specific translocation of glucose, gluconate or 2-keto-
gluconate across the outer membrane. Such a specific translocation system may be envisioned to operate analogous to the maltose (79) or dicarboxylic acid (66) transport systems in E. coli.

Future studies may require the use of cells defective in the ability to defluorinate 4FG to support the above proposals. Such mutants may be defective in or exhibit reduced levels of glucose transport. The use of 4FG as an inhibitor of glucose transport may provide further evidence for the presence of a common protein for defluorination and glucose transport. The use of $^{125}\text{I}$-lactoperoxidase as a labeling agent may provide support for the involvement of an outer membrane protein in the defluorination reaction. This reagent has been shown to label the outer membrane or cell surface proteins in P. aeruginosa (122) and E. coli (77).

The isolation of the defluorinating protein will be required to determine the mechanism and precise location of fluoride release. This protein may be isolated in large amounts by the use of an affinity column or by the use of antibodies directed against the protein. The protein may also be isolated by fractionation of the outer membranes, but this will require a large number of cells.
APPENDIX I

Calibration Curve of Voltage Potential Versus Fluoride Ion Concentration.

Legend:
The calibration curve was constructed with various concentrations of NaF \((10^{-6} - 10^{-1} \text{ M})\) in 100 mM potassium phosphate buffer, pH 7.1. Fluoride was measured with a fluoride ion specific electrode (Orion Research) as described in the MATERIALS AND METHODS section.
APPENDIX II

Calculation of Oxygen Consumption

If A moles of substrate consumed B L of oxygen after 8 h incubation, the extent of substrate oxidation as moles of oxygen per mole of substrate was calculated as follows:

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

\[ B' \ L(O_2) = B \ L(O_2) \times \frac{273(P-P_w)}{760xT} \]

where \( P \) 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 = \( (P \times B')/(R \times T) \).

From the ideal gas equation: \( PV = nRT \), where \( P = 101.3 \) kPa, \( T = 273^\circ C \), \( V = B' \) (L of oxygen consumed at STP) and \( R = 8.314 \) J mol\(^{-1}\) K\(^{-1}\).

Therefore, the extent of substrate oxidation

\[ \frac{(P \times B')}{(R \times T)} = \frac{\text{moles oxygen consumed}}{\text{moles substrate}} \]

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

1 mole of oxygen is equivalent to 32 g or 2 g atoms of oxygen.

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

Determination of Radioactivity in Various Samples

From quench correction curves (APPENDIX VI and VII), the % Efficiency of the samples were determined from the H-number. The dpm of the samples were calculated from the following expression:

\[ \text{dpm} = \frac{\text{cpm}}{\text{E}} \times 100 \]

where E is the 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

\[ = \frac{\text{cpm}}{\text{E}} \times \frac{100}{2.22 \times 10^3} \text{ nCi} \]

If the specific activity of the radioactive material was A nCi/mmol, then the amount of radiolabel in the samples per mg of protein

\[ = \frac{\text{cpm}}{\text{E}} \times \frac{1}{22.2 \times A \times B} \text{ (nmol radiolabel/mg protein)} \]

where B is the mg of protein.
Molecular Weight Calibration Curve for Sephadex G-100 Column.

Legend:

\[ V_e \] - elution volume
\[ V_o \] - void volume

The void volume (6 mL) was determined with blue dextran. The elution volumes were determined as the protein peak from the elution profile of the molecular weight markers or from the radioactivity peak of the radiolabeled samples. The preparation and operation of the column was as described in the MATERIALS AND METHODS section.

Molecular weight markers used:

- bovine albumin - 66,000
- lysozyme - 14,300

This resin has an exclusion limit of 150,000 daltons.
APPENDIX V

Molecular Weight Calibration Curve for Urea-SDS-Poly-acrylamide Gel Electrophoresis.

Legend:
The relative mobilities were calculated as follows:
relative mobility = \frac{\text{distance of protein band from origin (cm)}}{\text{distance of tracking dye from origin (cm)}}

Electrophoresis was performed as described in the MATERIALS AND METHODS section.
Molecular weight markers used:
- bovine albumin - 66,000
- egg albumin - 45,000
- pepsin - 37,500
- trypsinogen - 24,000
- β-lactoglobulin - 18,400
- lysozyme - 14,300
APPENDIX VI

Quench Correction Curve for $^{14}\text{C}$-Samples.

Legend:

$^{14}\text{C}$-toluene quenched standards containing 190,000 dpm (Nuclear Chicago) were used to construct the quench correction curve (%Efficiency versus H-number). The instrument used was the Beckman LS 7500 liquid scintillation counter. The H-number concept of quench correction was used (see manufacturer's instruction manual for theoretical discussion of the H-number concept).
APPENDIX VI

![Graph]

- % Efficiency
- H-number

0 50 100 150 200 250 300 350 400

100 200 300 400
APPENDIX VIII

Calibration Curve for 4FG Concentration.

Legend:

4FG concentration was determined with the Statzyme Glucose (500 nm) reagent. 3.0 mL aliquots of this reagent were incubated at 30°C for 10 min. To these were added 200 µL of the 4FG standard solutions (0.25-5 mM) and the solutions incubated for an additional 2 h. After incubation 100 µL of 4 M HCl was added to stop the reaction and stabilize the color. The absorbance of the solutions were measured at 500 nm.
APPENDIX IX

Linear Regression Analysis of the Kinetic Data

All linear plots were analyzed by linear regression analysis. It was assumed that the set of data points fit the equation \( y = mx + b \), where \( x \) and \( y \) are the independent and dependent variables, respectively, \( b \) is the \( y \)-intercept and \( m \) the slope of the resulting line.

The slope and the \( y \)-intercept of the regression line are determined as follows:

\[
m = \frac{\sum xy - \left( \frac{\sum x \sum y}{N} \right)}{\sum x^2 - \left( \frac{(\sum x)^2}{N} \right)}
\]

\[
b = \frac{\sum y - m \sum x}{N}
\]

Additional data points can be predicted simply by choosing some new \( x \) or \( y \) value and the calculator* computes a corresponding \( y \) or \( x \) value on the regression line.

*The linear regression routine was performed on a Texas Instrument TI-58 programmable calculator.
APPENDIX X

Maximum Defluorination in Chloramphenicol Treated Cells

The maximum amount of fluoride release in glucose grown chloramphenicol treated cells was found to be 133 nmol fluoride mg protein\(^{-1}\). It can be assumed, therefore, that 133 nmol 4FG has reacted per mg of protein.

Therefore, \[
\frac{133 \text{ nmol}}{\text{mg protein}} \times \frac{182 \text{ ng 4FG}}{\text{nmol}}
\]

\[
= 24,200 \text{ ng 4FG mg protein}^{-1}
\]

\[
= 24 \text{ µg 4FG mg protein}^{-1}
\]

Therefore, 24 µg of 4FG has reacted per mg of whole cell protein.
REFERENCES


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Publications:


Presentations:


Societies: Canadian Biochemical Society

Chemical Institute of Canada

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