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**THE METABOLISM OF 4-DEOXY-4-FLUORO-D-GLUCOSE
IN *PSEUDOMONAS PUTIDA***

by

© Diego Sbrissa

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

Windsor, Ontario, Canada

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ISBN 0-315-50530-3

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ABSTRACT
THE METABOLISM OF 4-DEOXY-4-FLUORO-D-GLUCOSE
IN *PSEUDOMONAS PUTIDA*

by

Diego Sbrissa

Uniformly carbon-14-labelled 4-deoxy-4-fluoro-D-glucose (D-[U-¹⁴C]4FG) is synthesized for the first time and used to follow the fate of 4-deoxy-4-fluoro-D-glucose (4FG) in *Pseudomonas putida*.

Following a 24-hour incubation of a suspension of glucose-grown whole cells of *P. putida* with 1 millimolar (mM) D-[U-¹⁴C]4FG under conditions giving a 95% release of F⁻, less than 1% of the radiolabel is found to be covalently associated with the peptidoglycan present in the cell-envelope fraction, more than 50% of the radiolabel accumulates in the suspending medium, and as much as 5% of the radiolabel is recovered as a volatile product which appears to be ¹⁴CO₂. Analysis of the radiolabelled cell-envelope fraction by gel filtration gives no evidence that the peptidoglycan-associated radiolabel is incorporated into protein. When the suspending medium is analyzed by borate anion-exchange column chromatography, the radiolabel is resolved into two components: 1) a poorly retained minor-peak metabolite; and 2) a more strongly retained major-peak metabolite which contains 90% of the radiolabel recovered in these two metabolites.

Isolation of the major-peak metabolite by preparative borate anion-exchange chromatography yields a single radiolabelled product which appears to be pure by thin-layer chromatographic analysis. This radiolabelled product is identified as 2,3-dideoxyribonic acid by proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy and mass spectrometric analyses. This identification was later confirmed by synthesizing the sodium salt of 2,3-dideoxyribonic acid and showing that the proton and carbon-13 NMR spectra were identical to those obtained for the

isolated major-peak metabolite. A similar isolation and thin-layer chromatographic analysis of the minor-peak metabolite yields an impure product which is tentatively identified as a deoxycarbohydrate derivative.

Following a 24-hour incubation of a crude outer-membrane preparation derived from glucose-grown *P. putida* with 1 mM D-[U-¹⁴C]4FG under conditions giving a 10% release of F⁻, all of the radiolabel is recovered in the suspending medium. Analysis of the suspending medium by borate anion-exchange chromatography reveals that all of the radiolabelled 4FG is converted to two highly anionic, possibly phosphorylated, components: 1) a major component which is probably a fluorinated metabolite; and 2) a minor component which is probably a non-fluorinated metabolite. Both metabolites differ from the metabolites derived from whole-cell incubations and have retentions that are probably characteristic of phosphorylated aldonic acids.

The results of this study, especially the formation of 2,3-dideoxyribonic acid, implicate an intracellular pathway in the metabolism of 4FG. The mechanism by which this occurs is presented and discussed.

DEDICATION

To Livia and Delfino

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. N. F. Taylor for extending to me his inordinate patience, guidance, financial support, and the opportunity to have developed an interest in and to have contributed to the study of fluorinated carbohydrate metabolism.

I gratefully acknowledge the help and friendship extended to me by the graduate students, members of faculty, the staff, and technicians of the Department of Chemistry and Biochemistry, extend special thanks to my doctoral committee for having spent their valuable time to examine this dissertation, and graciously extend my gratitude to the Ontario Ministry of Colleges and Universities for an Ontario Graduate Scholarship.

I wish to thank: Dr. John M. McIntosh for his provision and interpretation of NMR spectra; Dr. Ole Mols, Wayne State University, Detroit, Michigan, and Mr. Michael Puerth, University of Windsor, for their expertise in providing the fine NMR spectral data; Edward Saravolac for his help with outer membrane preparations.

Special thanks go to: Dr. Ajit Sharma, Dr. Nadarajah Karuppiah, Gerardo M. Castillo, and "professor" John W. Williams for their friendship and intelligent discussions over the years; Ovidio Sbrissa Architects, Ottawa, Ont., for preparation of the final printed form of this dissertation.

Lastly, I express my gratitude to my family for their help throughout all my years of study, and I especially extend my unsurpassed thanks to my parents for their lifelong encouragement and support.

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
°C	degrees Celsius
CH ₂ FAH ₂	5,10-methylenetetrahydrofolic acid
cm	centimetre
CoA	coenzyme A
cpm	counts per minute
D ₂ O	deuterated water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpm	decompositions per minute
dTMP	2'-deoxythymidine-5'-monophosphate
dUMP	2'-deoxyuridine-5'-monophosphate
ED	Entner-Doudoroff
EDA	3-deoxy-2-keto-6-phosphogluconate aldolase
EDD	6-phosphogluconate dehydrase
EDTA	ethylenediaminetetraacetic acid
FAB	fast atom bombardment
FAH ₂	7,8-dihydrofolic acid
FdUMP	5-fluoro-2'-deoxyuridine-5'-monophosphate
3FG	3-deoxy-3-fluoro-D-glucose
4FG	4-deoxy-4-fluoro-D-glucose
4FGA	4-deoxy-4-fluoro-D-gluconic acid

g	gram
KDO	3-deoxy-2-keto-octulosonic acid
4F2KGA	4-deoxy-4-fluoro-2-keto-D-gluconic acid
K_i	inhibitor dissociation constant
K_m	Michaelis-Menten constant
kV	kilovolt
L	litre
mA	milliampere
mg	milligram
mL	millilitre
mm	millimetre
mM	millimolar
M	molar
MHz	megahertz
mV	millivolt
nm	nanometre
NMR	nuclear magnetic resonance
PPB	potassium phosphate buffer
re	two-dimensional equivalent of R stereochemical configuration
rpm	revolutions per minute
S.D.	standard deviation
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
si	two-dimensional equivalent of S stereochemical configuration

2

SPB	sodium phosphate buffer
TLC	thin-layer chromatography
$V_{\max}$	maximal rate
v/v	millilitre per millilitre
w/v	grams per millilitre
~	approximately
≈	approximately equals

CHAPTER I

INTRODUCTION

Fluorinated Analogues as Biochemical Probes

The study of the interaction of diverse natural and synthetic fluorinated organic compounds with a variety of biochemical systems has provided useful information in such areas as metabolism, toxicology, chemotherapy, enzyme specificity and mechanism, transport, and antibody-antigen specificity (1,2,3). Fluorine is of unique value in the design of synthetic analogues which can closely mimic the activity of natural biological compounds because the bond-length and size of fluorine bonded to carbon is intermediate between that of hydrogen and that of oxygen; thus, fluorine can be used to replace either hydrogen or a hydroxyl group with a minimal perturbation of molecular structure and with retention of biochemical reactivity (4). This biochemical reactivity can sometimes give rise to either a stimulatory or an inhibitory biological effect which can differ dramatically from that of the natural compound. Therefore, fluorinated analogues of this kind can be useful in medicine as specific therapeutic agents. However, since biochemical reactivity normally involves a specific binding interaction between a molecule and a protein (such as an enzyme, membrane transport or receptor protein, or antibody), fluorinated analogues can be extremely valuable as sensors, or probes, to elucidate specific structural and stereochemical features of the molecule which are required for that interaction and, by inference, may provide some notion of the stereochemistry of the protein at the binding site. In the case of enzymes, this may give some insight into reaction mechanisms involved in certain kinds of enzymatic catalysis (5). Additionally, the fluorine nucleus is a sensitive nuclear magnetic resonance probe which permits a study of substrate-protein interactions by fluorine-19 nuclear magnetic resonance spectroscopy (6).

Interest in the biochemistry of fluorinated organic compounds was undoubtedly catalyzed by the discovery of Marais (7), in 1944, that fluoroacetate, the first carbon-fluorine compound isolated in Nature, was the toxic principle in

the leaves of the South African plant, *Dichapetalum cymosum*, and later, by the extensive studies of Sir Rudolph Peters and his collaborators which made it clear that the characteristic accumulation of citrate induced by fluoroacetate was related to an inhibition of citrate metabolism by the action of fluorocitrate, a potent inhibitor of the mitochondrial enzyme, aconitase (8,9). Apart from providing evidence for the operation of the citric acid stage of the tricarboxylic acid cycle *in vivo*, an important outcome of these studies was the demonstration of the concept of a "lethal synthesis" whereby the non-toxic fluorinated compound, fluoroacetate, was enzymatically transformed into a toxic product, fluorocitrate, by deceiving enzymes responsible for synthesizing citrate from acetate into an analogous synthesis of fluorocitrate from fluoroacetate (9,10).

The extensive studies conducted by Kun and his co-workers suggested that of the four possible isomers of fluorocitric acid only (-)-*L-erythro*-fluorocitric acid, correctly designated as (1R,2R)-1-fluoro-2-hydroxy-1,2,3-propanetricarboxylic acid, which is formed from fluoroacetyl-CoA and oxaloacetic acid by citrate (*si*)-synthase, was a strong inhibitor of aconitase (11,12,13).

An early X-ray crystallographic study of a rubidium ammonium hydrogen fluorocitrate dihydrate salt showed the crystals were actually those of a racemate and, therefore, only allowed the relative configuration of the inhibitory isomer to be determined (14,15). The absolute configuration of the isomer of fluorocitrate that inhibits aconitase was recently shown to be 1R,2R by an X-ray crystallographic study which revealed that the absolute configuration of the (-)-methylbenzylamine complex of the diethylester of the non-inhibitory (+)-*erythro* isomer of fluorocitrate was 1S,2S (16). A stereocontrolled chemical synthesis of the 1S,2S isomer by a synthetic sequence that mimics the reaction of citrate (*re*)-synthase, an enzyme isolated from several anaerobic bacteria which catalyzes the addition of acetyl-CoA to the *re* diastereotopic face of the ketonic carbonyl group of oxaloacetate, has also lent further support for the 1R,2R configuration of the inhibitory isomer of fluorocitric acid (17). A recent reinvestigation of the stereoselective synthesis of fluorocitrate from fluoroacetyl-CoA and oxaloacetate with pig heart citrate (*si*)-synthase has

revealed the presence of the 1S,2R isomer as a minor product which amounted to 2-3% of the 1R,2R isomeric major product (18). This suggests that the selective abstraction of the *pro*-S hydrogen of the fluoroacetyl group (19) predominates (97-98%), but it is not absolutely specific. Taken together, these studies have established that the enzyme catalyzes an inversion of configuration in the fluoroacetyl group during the synthesis of the 1R,2R-*erythro*-fluorocitrate isomer (Figure 1) and concurs with previous findings that an inversion also occurs in the normal reaction with acetyl-CoA (18,20).

Citrate synthase is a very interesting enzyme which is coded by nuclear DNA, translated in the cytoplasm as a precursor, and transported into the mitochondria where it becomes bound to the inner mitochondrial membrane (21). The functional enzyme is a 100,000-dalton dimer composed of identical subunits, exists in various conformational states having different enzymatic activities, and is known to have a biphasic rate dependence on enzymatic concentration and to exhibit hysteretic behaviour (21).

As was pointed out by Kun and his colleagues (22), the interpretation of early studies determined to elucidate the molecular toxicological mechanism of action of fluorocitrate in terms of aconitase inhibition was complicated by several factors (22). Firstly, the majority of the early work concerning the inhibition of aconitase by fluorocitrate had been performed with fluorocitrate preparations of varying degrees of purity and with aconitase-containing tissue extracts which were relatively poorly defined, and this resulted in considerable variation in the reported K_i values for fluorocitrate and the types of inhibitions observed (22). Secondly, notorious problems of enzyme instability during advanced stages of purification necessitated a reactivation of the enzyme by the addition of ferrous ions and a reducing agent such as ascorbate or cysteine; in the presence of these artificial activators, a purified aconitase preparation from pig heart cytoplasm caused a nearly stoichiometric release of fluoride ion from (-)-*erythro*-fluorocitrate (0.8 moles of F^- released per mole of aconitase) and a rapid and stoichiometric loss of enzymatic activity (90% loss of activity after 1 minute) (23), whereas enzyme preparations

Figure 1. Possible Mode of Formation of the Toxic Isomer of Fluorocitric Acid by Citrate (si)-Synthase from Fluoroacetyl-CoA and Oxaloacetate

— : represents a bond within the plane of the page

||||| : represents a bond behind the plane of the page

▴ : represents a bond before the plane of the page

His 274 and His 320 are histidine residues found by X-ray crystallographic analysis of pig heart citrate synthase to be located at positions in the active site of the enzyme and would be ideal for promoting the condensation reaction. Phe 397 is a phenylalanine residue at the active site which forms an unusual edge-on interaction with the citrate molecule and may help to confer the extreme substrate specificity and stereoselectivity observed with citrate synthase. The proposed scheme is based on an interpretation given in a recent review by Wiegand and Remington (21). A Fischer projection of the absolute configuration of the toxic isomer is shown.

requiring no activators did not defluorinate fluorocitrate (11).

The inactivation accompanying the defluorination of fluorocitrate in the presence of these artificial activators is apparently reversible, since aconitase inactivated by carbon-14-labelled fluorocitrate did not retain radioactivity after gel filtration or precipitation with ammonium sulfate, and full enzymatic activity could be regained after either of these treatments by activation with cysteine and ferrous salts (23). The authors suggested that the inactivated enzyme may be a very labile covalent complex formed by a metal (ferrous)-ion-assisted removal of fluoride ion and substitution by a basic proton-abstracting group of the enzyme. This would be consistent with a previously proposed model (Figure 2) for both the competitive inhibition and the time-dependent inactivation of the enzyme based on a comparison of the crystallographically determined conformations of the tridentate metal chelates of citrate, isocitrate, and fluorocitrate. Only the inhibitory (1R,2R) isomer of fluorocitrate could fit in the active site in a manner similar to that proposed for citrate or isocitrate in an enzyme-ferrous ion-substrate ternary complex whereby the fluorine atom, the oxygen atom of the hydroxyl group, and the oxygen atom of the terminal carboxyl group adjacent to the —CHF— would be coordinated to the enzyme-bound ferrous ion (14,24). Alternatively, it was suggested that inactivation could occur by formation of a very tight complex between the enzyme and fluorocis-aconitate or a tight complex between a defluorinated hydroxycitrate derivative of fluorocitrate which could arise either from the labile covalent complex mentioned previously or from the hydrolysis of the putative fluoro-cis-aconitate—a fluorinated compound which would be expected to readily lose allylic fluorine by undergoing a nucleophilic (Michael-type) attack at the β -carbon and elimination of fluoride ion. Interestingly, one of the possible isomers of the hydroxycitrates that could arise from these processes is known to be a potent competitive inhibitor (inhibition constant, $K_i = 52 \pm 6$ nanomolar) of the cytosolic enzyme, ATP citrate lyase from rat liver (25). This inhibitor would also correspond to the hydroxyl analogue of the 1S,2R isomer of fluorocitrate. That fluorocitrate may be a substrate for aconitase is an intriguing possibility which does not seem to have been examined.

Figure 2. *Proposed Mode of Binding of (-)-erythro-Fluorocitrate Leading to Inhibition or Inactivation of Aconitase*

(a) Fluorocitrate acting as a competitive inhibitor by binding the "wrong way" with fluorine coordinated to the ferrous ion at the active site.

(b) Fluorocitrate acting as an inactivator by alkylating the active site near or at a hydrogen-abstracting group (normally required for the dehydration and hydration reactions catalyzed by aconitase), designated B, as a result of a ferrous-ion-assisted cleavage of the C-F bond. Adapted from Glusker⁽²⁴⁾.

Finally, an additional complication was the finding that the apparent time-dependent inhibitory effect of fluorocitrate on aconitase, observed in early studies, only occurred when the enzyme was assayed in the presence of Mg^{2+} or Mn^{2+} by the isocitrate dehydrogenase assay procedure (22). A further complication was the occurrence of both cytoplasmic and mitochondrial aconitase isoenzymes having greatly differing isoelectric points and stabilities (22). Therefore, in an attempt to clarify the mode of action of (-)-*erythro*-fluorocitrate on aconitase isoenzymes, Eanes and Kun demonstrated in 1974 that in the absence of any artificial activator (e.g., ferrous ion and cysteine) both a purified cytosolic aconitase and a partially purified mitochondrial aconitase, both isolated from pig liver, were inhibited in a strictly competitive and reversible manner by (-)-*erythro*-fluorocitrate (average $K_i = 22$ -45 micromolar) with respect to either citrate or isocitrate as substrates in the absence of any bivalent cations when the reaction was monitored by the rate of *cis*-aconitate formation at 240 nanometres (22). When either aconitase isoenzyme was incubated with the bivalent cations, Mg^{2+} and especially Mn^{2+} , enzymatic activity was inhibited in a time-dependent manner, and this inhibition could be reversed by addition of a high concentration of citrate (22). Presumably this inhibition reflects a competition between bivalent cations and ferrous ions for the same metal-binding site on the enzyme, since manganous ions have been reported to compete for the same metal-binding site of aconitase as ferrous ions and to decrease the rate and extent of activation of aconitase by ferrous ions (24).

It may be of interest to mention that recent studies with mitochondrial beef heart aconitase have revealed that the enzyme contains an iron-sulfur cluster that can be readily interconverted between an inactive 3Fe-4S cluster and a catalytically active 4Fe-4S cluster. This is due to the presence of a single labile iron site which undergoes changes in coordination number upon addition of substrate to the enzyme (26). The labile iron atom is readily lost upon oxidation of the 4Fe-4S cluster to yield an inactive aconitase. This 3Fe-4S cluster can be reactivated anaerobically by various reducing agents with re-incorporation of the labile iron atom (27). The presence of thiol agents facilitates the 3Fe-4Fe cluster interconversion (27).

Important progress in understanding the *in vivo* toxicology of fluorocitrate was made by Kun and his co-workers in 1978 (28). They demonstrated that the toxicity of fluoroacetate poisoning is probably due to the irreversible inhibition by fluorocitrate of the citrate-porter system of the inner mitochondrial membrane. Thus, when mitoplasts (mitochondria devoid of their outer membrane) were pre-incubated for 5-15 minutes with 100 nanomolar (-)-*erythro*-fluorocitrate, a 77-90% inhibition of citrate-dependent ATP synthesis and a 70-90% inhibition of citrate-supported fatty acid biosynthesis was observed (28). Evidence was also presented which demonstrated the stable, covalent incorporation of less than 1% of the total radiolabel into inner-membrane proteins following an incubation of inner-mitochondrial-membrane vesicles with [3,4,5,6-¹⁴C](-)-*erythro*-fluorocitrate (28). Inhibition of incorporation of the radiolabel into inner-membrane-vesicle protein by mersalyl and the release of 70% of the radiolabel bound to inner-membrane-vesicle protein by neutral hydroxylamine suggested the formation of a radiolabel-protein thioester adduct; however, evidence for the precise structure of this fluorocitrate-derived thioester adduct was not presented (28). The authors concluded (28):

"We observed that a limited degree of defluorination of fluorocitrate to hydroxycitrate did occur when mitochondria were incubated with fluorocitrate. Electrophoretic reisolation of fluorocitrate from this incubation system revealed the appearance of traces of hydroxycitrate, which at pH 1.85 separated from unreacted fluorocitrate. However, the formation of hydroxycitrate from fluorocitrate is a separate reaction and bears no apparent relationship to the formation of fluorocitryl thioesters of two specific proteins of mitoplasts."

These studies concluded that (-)-*erythro*-fluorocitrate specifically and irreversibly inhibits mitochondrial citrate transport by its covalent binding to two protein fractions (molecular weights, 175,000 and 71,500) associated with mitoplasts (28). Evidence of a direct correlation between inhibition of citrate transport and the formation of radiolabelled protein adducts was not given.

In a related study, Kun *et al.* (29) demonstrated the presence in rat liver mitoplast extracts of a citryl-S-glutathione thioester synthetase (molecular weight, 171,000) and citryl-S-glutathione thioester hydrolase (molecular weight, 71,000) system which catalyzes a rapid biphasic synthesis and hydrolysis of the 1:1 mole ratio thioester adduct of citric acid and glutathione. By using [3,4,5,6-¹⁴C](-)-erythro-fluorocitrate, a correlation was found between the degree of inactivation of the citryl-S-glutathione thioester synthetase activity (50% inactivation with 225 nanomolar fluorocitrate after a 5 minute pre-incubation) and the formation of a stable trichloroacetic-acid-precipitated radiolabelled protein adduct. The thioester-forming system, which apparently involves formation of the glutathione free radical by the homolytic cleavage of oxidized glutathione, was inhibited about 95% by pre-incubation with 0.5 millimolar mersalyl and more than 80% by 1 millimolar phenazine methosulfate, a free-radical-trapping agent, and 1 millimolar 1,2,3-propanetricarboxylic acid, which is recognized as a specific inhibitor of mitochondrial citrate transport (29). It was suggested that the coincident inhibition of the soluble citryl-S-glutathione thioester synthetase and mitochondrial citrate transport by fluorocitrate may indicate that the thioester synthetase may be a constituent of the mitochondrial citrate-transport system (29).

An alternative possible role that the glutathione thioester enzyme system might have is the regulation of the concentrations of all free carboxylic acid substrates of the Krebs cycle, since specific enzyme systems exist in mitochondria that also catalyze the formation of glutathione thioesters of succinic, malic, α -ketoglutaric, glutamic, pyruvic, and isocitric acids (29).

Thus, the use of fluorocitrate as a biochemical probe has not only stimulated an interest in and contributed to a better understanding of the enzymology of citrate synthase and aconitase but it has also led to the discovery of the irreversible inhibition of the mitochondrial citrate transporter as a new site of toxic action. Furthermore, a citryl-S-glutathione synthase/hydrolase enzyme system which may be involved in the translocation of citrate across the inner mitochondrial membrane has been identified. The recognition of the irreversible inhibition of mitochondrial

citrate transport as a site of toxic action now may offer an explanation for the apparently irreversible lethal neurotoxic effects associated with fluoroacetate or fluorocitrate poisoning. Since acetylcholine synthesis is a cytoplasmic process which depends on the mitochondrial translocation of citrate into the cytoplasm as a source for acetyl-CoA, the neurotoxicity of fluorocitrate may be caused by an inhibition of acetylcholine synthesis in nerve cells (28). Experimental proof of this possibility has not been reported.

It may be of interest to note that the citrate carrier of the inner mitochondrial membrane has been recently recognized as playing a conspicuous role in supplying neoplastic cells with cytosolic acetyl-CoA (30). This is required for fatty acid and cholesterol biosynthesis and, therefore, is an important component in fuelling the proliferation of tumour cells. A direct relationship has also been found between the operation of the citrate carrier in tumour mitochondria and the observation that tumour mitochondrial membranes are rich in cholesterol (30). A possible future medical application for fluorocitrate would be to selectively eradicate, by means of a specific drug-delivery system (e.g., monoclonal antibodies), proliferating tumour cells by "jamming" their seemingly overactive mitochondrial citrate carrier. At last, Sir Rudolph would have found his clinical application (10)!

Palmieri *et al.* (31) have recently reported the functional reconstitution of a partially purified tricarboxylate (citrate) carrier which was isolated from rat liver mitochondria and which closely resembles in its properties the transport system of intact mitochondria. Benzene-1,2,3-tricarboxylate (2.7 millimolar), a specific competitive inhibitor of the carrier with respect to citrate transport ($K_i = 0.13$ millimolar), and *para*-hydroxymercuribenzoate (0.2 millimolar and 2-minute pre-incubation) caused more than 90% inhibition of citrate (0.3 millimolar) transport by the reconstituted carrier ($K_m = 0.28$ millimolar; $V_{max} = 338$ nanomoles citrate/minute/mg protein). An SDS-PAGE analysis of the partially purified carrier showed at least 6 protein bands in the 28-35 kilodalton region, with three of those six components having been identified as the adenine nucleotide carrier, the phosphate carrier and the mitochondrial porin (31).

Another example of a fluorinated analogue which also demonstrates the principle of lethal synthesis and which has given considerable insight into the mechanism of catalysis of the enzyme, thymidylate synthetase, is exemplified by the *in vivo* conversion of 5-fluorouracil to 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), a potent inhibitor ($K_i \approx 0.1$ picomolar) of the enzyme (32).

Thymidylate synthetase normally catalyzes the conversion of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP) by the concomitant transfer and reduction of the one-carbon unit of the cofactor, 5,10-methylenetetrahydrofolic acid (CH_2FAH_2), and formation of 7,8-dihydrofolic acid (FAH_2) (32). The net result of this process is the introduction of a methyl group into the pyrimidine ring at the carbon-5 position of dUMP to yield dTMP, the methylated analogue which serves as a source of the thymidine-nucleotide component required for DNA synthesis. In the case of FdUMP, an analogous reaction occurs; however, the fluorinated analogue is enzymatically modified only during the early stages of the catalytic cycle up to a point where the enzyme is irreversibly inactivated by the formation of a stable, covalently linked enzyme-FdUMP-cofactor ternary complex (Figure 3), with retention of the carbon-fluorine bond (33). The stable ternary complex formed by FdUMP is believed to be analogous to the steady-state ternary-complex intermediate formed with dUMP in the natural enzymatic reaction. This provided direct evidence in support of a previously proposed mechanism of catalysis for thymidylate synthetase which had been inferred from investigations of chemical models and had suggested that the reaction is initiated by attack of an enzyme nucleophile at the carbon-6 position of the pyrimidine moiety of dUMP (32). The nucleophile of the enzyme initiating this attack appears to be a sulfhydryl group of a cysteine residue (34). 5-fluorouracil is a clinically useful anticancer drug which is particularly effective in the cure of skin cancer (35).

The type of inhibition discussed above for FdUMP is characteristic of a class of irreversible inhibitors known variously as suicide inhibitors, k_{cat} inhibitors, mechanism-based inhibitors, trojan-horse inhibitors and enzyme-activated substrate inhibitors (36). Suicide inhibitors of this type are in themselves unreactive

Figure 3. *Structure of the Thymidylate Synthetase-FdUMP-Methylenetetrahydrofolate Ternary Complex.*

S represents a cysteinyl residue at the active site of the enzyme. Adapted from Stryer (34).

compounds which behave as close analogues of a natural substrate and contain an appropriately placed group which promotes the formation of a reactive intermediate only after interacting with the enzyme's active site and participating in a catalytic transformation that is a normal part of the enzyme's catalytic process (37).

Most suicide inhibitors are based on the generation of an intermediate which is inherently reactive due to the presence of conjugated double bonds that are susceptible to a Michael addition reaction (36). A nucleophilic group at the enzyme's active site may then attack the intermediate to form a stable, inactive, alkylated enzyme (36):

Enzymes containing pyridoxal phosphate as a cofactor are excellent targets for suicide inhibition because of the extended system of conjugated double bonds formed by the pyridoxal ring.

Although fluorine is difficult to displace in bimolecular nucleophilic substitution (S_N2) reactions, it combines a good leaving-group ability in elimination reactions with a small size—properties which have accounted for its constant use in the design of suicide inhibitors (37). The usefulness of fluorinated analogues as suicide inhibitors is probably best exemplified by the α -fluoromethyl amino acid substrate analogues which have been found to be very potent irreversible inhibitors of pyridoxal-phosphate-linked amino acid decarboxylases (38). Many amines with important roles in mammalian physiology are formed by the enzymatic decarboxylation of various amino acids by specific amino acid decarboxylases. The development

of various α -fluoromethyl amino acid substrate analogues, which act as selective suicide inhibitors for these key enzymes, will undoubtedly provide useful tools for studying the physiology of biologically active amines and may also be of therapeutic value in medicine by ameliorating pathological conditions that may be related to an abnormality in their metabolism (38).

The development of the α -fluoromethyl amino acid inhibitors discussed above was initially prompted by earlier observations that β -D-fluoroalanine was an effective bactericidal agent as a consequence of its action as a suicide inhibitor of alanine racemase (38,39). β -D-fluoroalanine is an effective bactericidal agent exhibiting a wide spectrum of antibacterial activity. By inactivating alanine racemase, it can block the biosynthesis of D-alanine, which is an important component of bacterial cell walls (peptidoglycan) and appears to be formed directly from L-alanine by the action of an alanine racemase in many species of bacteria (40,41). Thus, β -D-fluoroalanine has been an important conceptual "steppingstone" in the design of suicide inhibitors for pyridoxal-phosphate-dependent enzymes.

Recent investigations into the mechanism of action of suicide inactivators of pyridoxal-phosphate-dependent enzymes by Metzler and colleagues (42) and by Walsh and co-workers (43) have uncovered a new mechanism of inactivation. This involves the generation of a reactive α -aminoacrylate intermediate which partitions between hydrolysis to form pyruvate, ammonia, and active enzyme and a nucleophilic attack by the β carbon of the α -aminoacrylate on the "internal" Schiff base of the active enzyme-pyridoxal-phosphate complex. This yields an inactive, stable ternary adduct which contains a non-hydrolyzable secondary amine linkage to an active-site lysine residue of the enzyme (Figure 4). A similar mechanism also seems to operate in the inactivation of γ -aminobutyrate aminotransferase (an enzyme catalyzing the degradation of the inhibitory neurotransmitter, γ -aminobutyric acid, to succinic semialdehyde) by 4-amino-5-fluoropentanoic acid (44,45).

Figure 4. Newly Proposed Mechanism of Action of Suicide Inhibitors of Pyridoxal-Phosphate-Dependent Enzymes

The mechanism shown is specifically that which was proposed by Metzler and his co-workers (42) for the inactivation of glutamate decarboxylase of *Escherichia coli* by L-serine-O-sulfate; however, the formation of a reactive free aminoacrylate or enamine intermediate which then nucleophilically attacks the carbon-nitrogen double bond of the "internal" Schiff base (imine) formed between an active-site lysyl residue of the enzyme and the pyridoxal phosphate coenzyme may be a more general mechanism pertaining to the mechanism of action of many other suicide substrate inhibitors directed at other pyridoxal-phosphate-dependent enzymes (e.g., α -fluoromethyl amino acid inhibitors). The inactivated enzyme, 1, upon treatment with base liberates compound 2. Prior treatment of 1 with borohydride prevents the base-promoted release of 2. Previously, it had been assumed that inactivation of the enzyme was a result of addition of a nucleophilic group, designated Y, from the enzyme to the carbon-carbon double bond as indicated by the arrow crossed out by an X. Adapted from Likos *et al.* (42).

The above findings suggest that previously proposed mechanisms of action of other suicide inhibitors of pyridoxal-phosphate-linked enzymes may need to be re-investigated. In the past, it had been generally assumed that inactivation of the enzyme was due to a Michael-type addition of a nucleophilic group of the enzyme to the terminal carbon-carbon double bond of the reactive intermediate (Figure 4).

Recently, 3-fluoropyruvate has been reported to be a substrate for the pyruvate dehydrogenase complex of *Escherichia coli*, giving rise to acetyl-CoA, as well as being a suicide substrate; however, the suicide-inactivation mechanism was not reported (46).

In many instances where fluorinated analogues have been shown to have useful and dramatically altered biological properties with respect to their natural counterparts, it is a hydrogen atom that has been replaced by fluorine. For example, replacement of hydrogen by fluorine as in fluoroacetate or 5-fluorouracil results in a "lethal synthesis". However, this is not to underestimate the usefulness of replacing hydroxyl groups in biological compounds of interest by fluorine. The utility of fluorine as a substituent for oxygen has been exemplified particularly by the use of fluorinated carbohydrates as biochemical probes in a variety of biochemical studies which have been recently reviewed (3).

The basic strategy behind the use of deoxyfluoro carbohydrates as biochemical probes resides in the fact that whereas the hydroxyl group in a carbohydrate may act either as a donor or acceptor in hydrogen bonding, the fluorine of a deoxyfluoro-carbohydrate analogue can only hydrogen-bond unidirectionally as an acceptor. As a consequence, the selective introduction of fluorine in place of a hydroxyl group allows the use of the deoxyfluoro analogue as a probe to infer the direction of hydrogen bonding that occurs between the natural carbohydrate and a protein to which it is bound (47).

Thus, by a comparison of kinetic parameters associated with the transport of various deoxyfluoro and deoxyglucose analogues, the specificity of hydrogen bonding

and the structural requirements for the binding and transport of glucose has been determined for the glucose carrier of the human erythrocyte (48,49), the hamster intestine (50) and rat brain synaptosomes (51). Other transport-related studies employing deoxyfluorosugars include: the use of 3-deoxy-3-fluoro-D-glucose and the study of glucose transport mutants in *Escherichia coli* (52); the transport and metabolism of 3-deoxy-3-fluoro-D-mannose and 4-deoxy-4-fluoro-D-mannose by *Saccharomyces cerevisiae* (53); the use of 3-deoxy-3-fluoro-D-glucose and 4-deoxy-4-fluoro-D-glucose in the characterization of D-glucose, D-gluconate and 2-keto-D-gluconate transport systems in membrane vesicles of *Pseudomonas putida* (54,55); 2-deoxy-2-fluoro-D-glucose and the study of hexose transport mutants in L6 rat myoblasts (56,57); the use of deoxyfluoro-sucroses to determine the specificity of the sucrose carrier protein in plants (58).

Deoxyfluoro carbohydrates have also been useful as probes to obtain information about binding interactions between a substrate and the catalytic site of an enzyme. For example, a number of enzymes including glycerol kinase from *Candida mycoderma* (59), yeast hexokinase (60), glucose phosphate isomerase (59), galactokinase (61), UDPG-pyrophosphorylase (62), rabbit muscle glycogen phosphorylase (63), the glucansucrases of *Leuconostoc mesenteroides* (64), β -galactosidase of *Escherichia coli* (65), trehalase (66), glucose and gluconate dehydrogenases and gluconokinase of *Pseudomonas putida* (67), and pyranose-2-oxidase of *Polyporus obtusus* (47), have been investigated by this approach.

The use of glycosyl fluorides has also provided information about the mechanisms of hydrolysis of glycosidases (68,69,70) and glycosyltransferases including the enzymes, sucrose phosphorylase (71) and trehalase (72).

A number of metabolic studies with deoxyfluoro carbohydrates have been undertaken to examine their antibacterial, antiviral and insecticidal potential. Thus, 3-deoxy-3-fluoro-D-glucose has been found to inhibit glucose and galactose metabolism and polysaccharide synthesis in *Saccharomyces cerevisiae* (73,74); 3-deoxy-3-fluoro-D-glucose and 4-deoxy-4-fluoro-D-glucose were found to inhibit the growth

of *Escherichia coli* by uncompetitive inhibition of β -galactosidase activity and repression of β -galactosidase synthesis (75,76); 3-deoxy-3-fluoro-D-glucose and 4-deoxy-4-fluoro-D-glucose inhibit the growth of *Pseudomonas putida* with release of fluoride ion from the latter by an inducible-repressible outer-membrane protein (77,78,79). Both 3-deoxy-3-fluoro-D-glucose and 4-deoxy-4-fluoro-D-glucose are toxic to *Locusta migratoria*, with an LD₅₀ of 4.8 milligram/gram and 0.6 milligram/gram, respectively (80,81,82,83); 2-deoxy-2-fluoro-D-glucose and 2-deoxy-2-fluoro-D-mannose are inhibitors of enveloped-virus multiplication and glycoprotein biosynthesis (84); and 4-deoxy-4-fluoro-D-mannose had been found to interfere with glycosylation of viral protein (85).

Fluorine has been used extensively in a variety of therapeutic agents as a means of improving their efficacy (41); included in this category are a number of fluorinated carbohydrate derivatives. For example, the use of a 2'-fluoro substituent instead of a 2'-hydroxyl group in 5-substituted pyrimidine (uracil or cytosine) nucleosides containing the 1-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl) moiety confers more potent antiviral activity and makes them very effective agents against herpes simplex viruses (HSV-1 and HSV-2), presumably due to the increased stability of the N-glycosidic linkage induced by the highly electronegative 2'-fluorine substituent (41,86). A number of fluorinated derivatives of the antibacterial aminoglycoside antibiotics have been prepared (87). However, the first instance of a hydroxyl group being replaced by fluorine in order to avert the inactivation of the antibiotic by resistant bacteria has only recently been reported for 3'-deoxy-3'-fluorokanamycin A and B (87).

Last, but not least, the first and only naturally occurring fluorinated carbohydrate known, nucleocidin, is a protein-synthesis inhibitor which forms a complex with ribosomes to hinder peptide-bond formation and which has a broad antibacterial spectrum and high antiprotozoal action (88).

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

Interest in studying the metabolism of 4FG, in contrast to most fluorinated analogues, was not so much for its toxicity, since an earlier study conducted by Hill (78) indicated that *Pseudomonas putida* was still able to grow on glucose even in the presence of an equivalent amount of 4FG, with only a small reduction in the net growth yield. What was of interest, however, was the observation that *P. putida* caused a release of fluoride ion in cultures containing 4FG and was unable to utilize 4FG as a carbon source for growth (78). It was also demonstrated that unlike D-glucose, 4FG was only negligibly oxidized by resting glucose-grown whole-cell suspensions of *P. putida*; instead, there was a progressive release of fluoride ion, with 94% of the covalently bound fluorine being released after 24 hours (78). Based on these results, a non-fluorinated metabolite of 4FG was expected to accumulate, but a thin-layer chromatographic analysis of the suspending buffer or of the intracellular contents of cells obtained from glucose-grown whole-cell incubations with 4FG, failed to detect any new metabolites (78). Furthermore, in contrast to whole-cell incubations, crude cell-free extracts prepared from glucose-grown *P. putida* were able to oxidize 4FG to an extent of two atoms of oxygen per molecule of 4FG, without the release of fluoride ion (78).

From these early studies (78), it was proposed that the inability to detect a non-fluorinated metabolite arising from 4FG may be explained by a covalent incorporation of the carbon skeleton of 4FG into a cell-envelope protein, perhaps a glucose-transport protein, by an alkylation reaction whereby 4FG was envisioned to lose hydrogen fluoride to form a 3,4-epoxide intermediate which could then alkylate the protein through an opening of the epoxide ring by a nucleophilic group of an amino acid residue of the protein (78).

In support of this proposed protein-alkylation theory, later studies performed by D'Amore (79,89) revealed that the defluorination of 4FG depended on the synthesis of protein, since the presence of chloramphenicol, a well-known inhibitor of protein synthesis, severely inhibited the defluorination of 4FG by glucose-grown

whole cells of *P. putida*. The finding that the kinetic parameters, K_m and V_{max} for the defluorination of 4FG by glucose-grown whole cells (apparent $K_m = 3.9$ mM; apparent $V_{max} = 1$ nanomole fluoride/mg protein/minute) were nearly identical to those obtained with glucose-grown chloramphenicol-treated whole cells (apparent $K_m = 3.6$ mM; apparent $V_{max} = 1$ nanomole fluoride/mg protein/minute) demonstrated that chloramphenicol only affects the defluorination reaction by inhibiting the synthesis of protein and not by a direct inhibition of the defluorinating protein *per se* (89). Rifampicin was also reported to inhibit the defluorination reaction (89). The defluorinating protein is induced when *P. putida* is grown on glucose, gluconate or 2-ketogluconate but is repressed when the organism is grown on succinate or citrate (89). In addition, it was shown that glucose, gluconate and 2-ketogluconate extensively inhibited the release of fluoride from 4FG and that N-ethylmaleimide completely inhibited fluoride release (79,89).

Also consistent with the proposed alkylation theory was the finding that the defluorinating activity in various sub-cellular fractions isolated from glucose-grown whole cells of *P. putida* was highest in a crude outer-membrane fraction, suggesting that the defluorinating protein is localized in the outer membrane (79,89). Furthermore, incubation of glucose-grown whole cells of *P. putida* with 1 mM D-[6-³H]4FG, under conditions giving 100% fluoride release after 24 hours, revealed that a small amount, less than 1%, of the radiolabel was incorporated into the cell-envelope fraction (89). From a gel-filtration analysis of this radiolabelled cell envelope, it was suggested that the label had been incorporated into a cell-envelope protein (89). However, a straightforward interpretation of this finding may have been complicated by the fact that a substantial amount, approximately 70%, of the radiolabel was released in the form of tritiated water during the incubation (89). In addition, evidence for the presence of an unidentified radiolabelled metabolite in the suspending medium was also presented (89).

Since earlier studies in these laboratories had suggested that the site of the extensive defluorination of 4-deoxy-4-fluoro-D-glucose was due to a repressible/inducible protein associated with the outer membrane of the cell envelope of glucose-

grown *Pseudomonas putida* (79,89), a brief review of the structure and function of the Gram-negative bacterial cell envelope will now be given.

The Cell Envelope of Gram-Negative Bacteria

Bacteria are generally classified as being either Gram-positive or Gram-negative according to a differential staining procedure named the Gram stain, in honour of its inventor, the Danish bacteriologist, Christian Gram (90). This differential staining really reflects a fundamental difference in the molecular architecture of the entire enveloping structure, or cell envelope, which forms a receptacle for the internal metabolic milieu, or cytoplasm, in these two types of bacterial cells.

In addition to an innermost cytoplasmic membrane, both Gram-positive and Gram-negative bacteria are surrounded by a rigid polymeric molecule known as the peptidoglycan, murein, or cell-wall layer, which is typically much thicker in Gram-positive bacterial cells (91). The importance of this layer to the survival of the bacterium is demonstrated by the fact that the enzyme, lysozyme, which is found in many of our body fluids (e.g., tears, saliva, serum) protects us particularly against Gram-positive bacterial infections because it specifically can hydrolyze the long mucopolysaccharide chains that form a major part of the peptidoglycan, and in doing so it induces a susceptibility to osmotic lysis. Furthermore, the peptidoglycan is an important bacterial "Achilles heel" upon which a number of antibiotics, including the well-known β -lactam antibiotics (e.g., penicillins, cephalosporins), depend for exerting their lethal action (91).

The most salient feature of the Gram-negative cell envelope that distinguishes it from the Gram-positive cell envelope is the presence of an additional external membrane, called the outer membrane, which largely serves as a protective barrier by physically excluding potentially harmful high-molecular-weight hydrophilic compounds (like lysozyme, for example) as well as by being impermeable to

hydrophobic compounds, including many antibiotics (like the β -lactams), hydrophobic dyes like crystal violet, and detergents (92,93). At the same time, this outer membrane can allow low-molecular-weight hydrophilic solutes, including various nutrients like sugars, amino acids, nucleotides and ions, to diffuse freely through small, water-filled, hydrophilic channels, or pores, which are formed by a special group of outer-membrane proteins referred to as "porins" (93,94). Although these permeability properties have largely been derived from the study of the enterobacteria, particularly *Escherichia coli* and *Salmonella typhimurium*, it appears that they may be common to other Gram-negative bacterial species as well (95).

Another important role of the outer membrane is to provide the bacterial cell with resistance against host immunological defence mechanisms that are elicited during infection (e.g., phagocytosis by white blood cells and the bactericidal action of serum complement). This property is conferred by the presence of a glycolipid known as lipopolysaccharide (92). Lipopolysaccharide is unique to the cell envelope of Gram-negative bacteria and appears to be located exclusively in the outer leaflet of the outer membrane, whereas phospholipid, primarily phosphatidyl ethanolamine, is found only in the inner leaflet (96,97). By formation of tightly linked divalent-metal-ion cross-bridges, lipopolysaccharide is responsible for maintaining the permeability-barrier function of the outer membrane (95,97).

The general structure of the Gram-negative bacterial cell envelope that emerges is shown in Figure 5. Basically, it consists of two membranes, the innermost cytoplasmic membrane and the outer membrane, separated by the peptidoglycan layer, which is located in the inter-membrane region known as the periplasm or periplasmic space.

The cytoplasmic membrane contains a variety of proteins which function in energy transduction (i.e., the respiratory chain), in active and facilitated transport of nutrients and export of toxic metabolic by-products, in enzymatic synthesis and translocation of cell-envelope components, in DNA replication, and in transduction of chemotactic signals (95,97,98). The cytoplasmic membrane also provides the

Figure 5. General Structure of the Cell Envelope of Gram-Negative Bacteria

The cytoplasmic membrane (CM) is a phospholipid bilayer containing a variety of proteins (CP) which function in energy production, active and facilitated transport of nutrients and export of toxic metabolites, and in enzymatic synthesis and translocation of cell-envelope components. The outer membrane (OM), consisting of an asymmetric lipid bilayer composed of lipopolysaccharide (LPS) at the outer leaflet and phospholipid (PL) and lipoprotein (LP) at the inner leaflet, is anchored to the underlying peptidoglycan (PG) by covalent attachment of a portion of the lipoprotein to the peptidoglycan and by strong ionic bonds between peptidoglycan and transmembrane trimeric pore-forming proteins, or porins (PP), which allow the passage of small hydrophilic molecules across the outer membrane. In the enteric bacteria, the transmembrane OmpA protein (A) also plays a role in stabilizing the structure of the outer membrane by association with lipoprotein and interaction with peptidoglycan via ionic bonds; it also functions in stabilizing cell-to-cell contacts during F-pilus-mediated conjugation and serves as a receptor for bacterial viruses (phages). The periplasm, or periplasmic space (PPS), between the OM and CM contains proteins which may function in catabolism (CAT) of various nutrients, detoxification (Dtox) of antimicrobial agents, or in the binding (BP) of nutrients to prevent their leakage back-out through the outer membrane. Adapted from de Maagd and Lugtenberg (93).

phospholipid bilayer which serves as the major permeability barrier for hydrophilic or charged molecules, while hydrophobic molecules are generally regarded as being able to cross the lipid bilayer (95).

Moving outwards from the cytoplasmic membrane there is the periplasm (periplasmic space). This region, in addition to containing the peptidoglycan layer, harbours a variety of proteins which may function as catabolic enzymes that are required for the conversion of nutrients into a form that is suitable for transport, as enzymes involved in the detoxification of antibacterial agents (e.g., hydrolysis of β -lactam antibiotics by β -lactamases), or as specific binding proteins for a wide variety of nutrients (95,97). A number of the latter proteins, referred to as periplasmic binding proteins, have been identified and are thought to play an important role in chemotaxis as well as being essential components of many cytoplasmic-membrane transport systems, e.g., the maltose/maltodextrin active-transport system of the cytoplasmic membrane of enteric bacteria does not transport free substrate but, rather, it only transports substrate that is bound by the periplasmic maltose-binding protein (97). In addition, the periplasmic space contains low-molecular-weight oligosaccharides (membrane-derived oligosaccharides) which are synthesized and secreted into the periplasm only in response to growth in a medium of low osmolarity and are believed to play an important protective role in regulating the internal osmotic pressure of Gram-negative cells (97).

The peptidoglycan is a unique feature found only in bacteria and is an important structure which protects bacterial cells against osmotic lysis. In addition to mechanical strength, it also confers shape to the bacterial cell (91). A consistent feature of peptidoglycan found in both Gram-positive and Gram-negative bacteria is the finding that it is composed of a network of linear polysaccharide chains, consisting of alternating N-acetylglucosamine and N-acetylmuramic acid joined by a β -1,4 glycosidic linkage, cross-linked by short peptide chains (91).

In Gram-negative bacteria, the ether-linked D-lactyl group at the carbon-3 position of each N-acetylmuramic acid residue in the polysaccharide chains is linked through an amide bond to a short tetrapeptide substituent which consists of an alternating sequence of L and D amino acids in the order, beginning from the innermost D-lactyl-linked residue, L-alanine, D-glutamic acid, D,L-*meso*-diaminopimelic acid and D-alanine (91). In most Gram-negative bacteria, these tetrapeptide substituents serve to cross-link adjacent mucopolysaccharide chains by formation of a direct amide bond between the free carboxyl group of the terminal D-alanine and the free amino group of *meso*-diaminopimelic acid (91). Chemical analysis of the isolated peptidoglycan sacculi of several species of *Pseudomonas* revealed a composition which is compatible with this generalized structure (99). The extent of cross-linking of the tetrapeptide chains in *Pseudomonas aeruginosa* has been estimated to be between 24% and 30% depending on conditions used for growth, and it is similar to values reported for other Gram-negative bacteria (99).

Finally, closely associated with and beyond the peptidoglycan lies the outer membrane, with its asymmetrically distributed lipopolysaccharide at the outer leaflet, phospholipid at the inner leaflet, and a variety of outer-membrane-associated proteins having either a structural role or being involved in solute permeation across the lipopolysaccharide-phospholipid bilayer. As alluded to earlier, the effectiveness of the outer membrane as a protective barrier against the action of degradative enzymes, hydrophobic antibiotics and immunological defence mechanisms is made possible by lipopolysaccharide.

The bacterial lipopolysaccharide is composed of three well-defined regions:

- 1) **lipid A**, a glycolipid which is common to a wide range of Gram-negative bacteria;
- 2) **the core**, a short oligosaccharide which characteristically contains the sugar, L-*glycero*-D-*manno*-heptose, and the sugar acid, 3-deoxy-2-keto-octulosonic acid (KDO);
- 3) **the O-side chain**, a highly variable polysaccharide chain often containing rare sugars such as deoxyhexoses, dideoxyhexoses, and unusual amino sugars (92).

Lipid A is an unusual glycolipid having a glucosamine disaccharide backbone to which a number of short (10-16 carbon atoms) saturated hydroxy fatty acids (usually β -hydroxy fatty acids) are linked either by amide linkages (at the C-2 position) or by ester linkages (at the C-3 position) at both glucosamine residues; in addition, the free hydroxyl group of the hydroxy fatty acid may itself bear an ester-linked saturated non-hydroxy fatty acid or hydroxy fatty acid, and the glucosamine disaccharide is also substituted at the C-1 and C-4 position with phosphate groups (92). Lipid A serves to anchor the lipopolysaccharide molecule in the outer leaflet of the lipid bilayer; in addition, the predominance of saturated fatty acid chains encourages a close packing of hydrocarbon chains in the outer leaflet and, therefore, causes hydrophobic compounds to have greater difficulty in penetrating through this relatively rigid region of hydrocarbon chains (97).

The glucosamine disaccharide portion of lipid A is covalently linked to the core oligosaccharide via a ketosidic (glycosidic) linkage to KDO. Together, the lipid A portion and the adjacent KDO residues carry a number of negatively charged residues which are responsible for binding of divalent cations (e.g., magnesium, calcium) and the formation of a tightly cross-linked lattice of lipopolysaccharide molecules through divalent cation cross-bridges (97). The importance of divalent cation cross-bridges between lipopolysaccharide molecules is readily proven by the loss of lipopolysaccharide and the concomitant decrease in the ability of the outer membrane to function as a permeability barrier when cells are treated by a divalent-metal-ion chelating agent like ethylenediaminetetraacetic acid (EDTA) (95,97).

When outer membranes from Gram-negative bacteria are analyzed for their protein composition by SDS PAGE, their protein profile is less complex than that of the cytoplasmic membrane and consists of only a few (typically four to six) major proteins which are expressed at high copy numbers, and many more minor proteins which are present in low copy number (92,97).

The most abundant outer-membrane protein of *Escherichia coli* is a low-molecular-weight (7,200 daltons) lipoprotein bearing an N-terminal cysteine linked

to a diacylglycerol by a thioether linkage and to a fatty acid by an amide linkage. This N-terminal end is then incorporated into the inner leaflet of the outer-membrane lipid bilayer via hydrophobic interactions of its three fatty acid chains (97). About one-third of the ϵ -amino groups of the C-terminal lysine residue of lipoprotein are covalently linked to the free L carboxyl group of a *meso*-diaminopimelic acid residue of the tetrapeptide chains of peptidoglycan (92).

Lipoprotein is believed to function mainly to give structure and stability to the outer membrane by anchoring it to the peptidoglycan, since lipoprotein-deficient mutants show a significant loss of outer membrane in the form of vesicles. These mutants display surface vesicles or "blebs" on electron-microscopic examination, are hypersensitive to EDTA and detergents, and tend to leak periplasmic proteins into their environment (92,93,95,96,97). Very similar lipoproteins have been found in most of the *Enterobacteriaceae* (97). Mizuno and Kageyama (100) have reported the presence in *Pseudomonas aeruginosa* of a lipoprotein (8,000 daltons) in the outer membrane that was analogous to the lipoprotein of *Escherichia coli* and existed in both a free form and a form which is covalently bound to the peptidoglycan. Presumably, the lipoprotein of *Pseudomonas aeruginosa* may offer a stabilizing function in the outer membrane similar to that of the lipoprotein of the enteric bacteria.

Another major outer-membrane protein found in *Escherichia coli* which appears to have a stabilizing influence on the outer membrane is a protein designated OmpA. Again, mutants lacking this protein produce unstable outer membranes (97). The OmpA protein is a transmembrane, 35-kilodalton protein which may exert its stabilizing effects by associating with lipoprotein and by interacting with the peptidoglycan through ionic bonds (97). Additionally, the protein plays a role in stabilizing cell-to-cell contacts during F-pilus-mediated conjugation, and it serves as a receptor for phages (97).

Most other outer-membrane proteins with known functions are involved in the uptake of nutrients. The entry of nutrients through the outer membrane is

accommodated in two ways: 1) passive diffusion of low-molecular-weight solutes present at relatively high concentrations (about micromolar) through water-filled pore-forming proteins, the so-called porins; 2) specific binding of the solute or a solute-chelator complex to an outer-membrane receptor (transport) protein.

The outer-membrane proteins referred to as porins derive their name from their ability to form relatively nonspecific pores or channels that allow the passage of small hydrophilic molecules across the outer membrane (96). The porins as a group are transmembrane proteins having molecular weights between approximately 35,000 and 45,000 daltons and appear to function *in vivo* and *in vitro* as trimers (97). The porins have a strong tendency to maintain their trimeric association even in the presence of sodium dodecyl sulfate (1%) at moderately elevated temperatures (below 60°C) and have a strong association with the underlying peptidoglycan due to either a direct interaction via ionic bonds or indirectly via lipoprotein or OmpA (97).

In *Escherichia coli* several different porins have been reported: OmpC, OmpF, Tsx, PhoE, and LamB (93,96,97). The OmpF and OmpC porins are constitutive proteins obtained under normal growth conditions, forming general pores that allow the ready passage of a variety of small hydrophilic solutes with a molecular weight up to about 700 daltons (97). Both of these porins are also major outer-membrane proteins (92).

The Tsx porin is a constitutive minor outer-membrane protein which enhances the diffusion of nucleosides across the outer membrane; however, it may act as a more general porin as well, in view of its ability to allow the diffusion of amino acids (96,97).

The PhoE porin is an inducible/repressible minor protein which is specifically induced by growth at low phosphate concentrations and appears to be specialized for allowing the passage of anionic solutes, but it can function as a general pore for nonspecific diffusion of other hydrophilic solutes as well (97).

Finally, the LamB porin is also an inducible/repressible minor outer-membrane protein which is induced by growth on maltose, has a selectivity for the permeation of maltose and maltodextrins up to maltoheptaose, and can act as a nonspecific general pore for the diffusion of various monosaccharides and other unrelated small molecules (96,97).

Constitutive and inducible/repressible pore-forming outer-membrane proteins have been reported also in the non-enteric bacterium, *Pseudomonas aeruginosa* (92,96). A constitutive major outer-membrane protein of this bacterium, protein F, with an apparent molecular weight of 35,000 to 40,000 daltons (101) was responsible for the formation of transmembrane pores that were freely permeable to saccharides having molecular weights below 9,000 daltons in reconstituted lipopolysaccharide-phospholipid vesicles (102). The high exclusion limit of this general hydrophilic pore may seem difficult to reconcile with the fact that *Pseudomonas aeruginosa* shows a higher resistance to hydrophilic antibiotics due to a lowered outer-membrane permeability as compared to *Escherichia coli* (95). However, it has been estimated that only 0.2-1% of the available protein F porin molecules form open functional channels, and this would account for the lowered permeability to hydrophilic antibiotics (103).

The inducible/repressible major outer-membrane protein, protein D1, having an apparent molecular weight of 46,000 daltons (101) and being specifically induced by growth of *Pseudomonas aeruginosa* on glucose, was also reported to reconstitute sucrose-and-glucose-permeable pores in lipopolysaccharide-phospholipid vesicles (104). When the organism is grown on glucose, the pore-forming protein D1 is apparently induced simultaneously along with a co-inducible, 44,500-dalton periplasmic glucose-binding glycoprotein (105) and with the induction of the high-affinity glucose-transport system that has been reported to be present in *Pseudomonas aeruginosa* (104,106). It was suggested, therefore, that the outer-membrane porin, protein D1, and the periplasmic glucose-binding glycoprotein are co-regulated components of the high-affinity glucose-transport system in *Pseudomonas aeruginosa*, and that the low-affinity glucose-transport system may involve uptake of glucose

across the outer membrane via the protein F porin (104). These features of glucose uptake in *Pseudomonas aeruginosa* are analogous to those of maltose transport in *Escherichia coli*. This consists of a co-inducible periplasmic maltose-binding protein, a high-affinity maltose-uptake system when the inducible/repressible LamB outer-membrane porin protein is present, and a low-affinity maltose-uptake system when the LamB porin is absent (104).

Since previous studies (79,89) have suggested that glucose-grown whole cells of *Pseudomonas putida* are metabolizing 4-deoxy-4-fluoro-D-glucose as a glucose analogue, some of the salient pathways for glucose utilization in pseudomonads will now be reviewed briefly.

Pathways of Glucose Utilization in Pseudomonads

The multifarious pathways of carbohydrate catabolism in the nutritionally versatile pseudomonads has been extensively reviewed (107,108,109). In members of the genus *Pseudomonas*, the Entner-Doudoroff pathway plays a central role in the pathways related to glucose utilization (Figure 6). Since they are devoid of the enzyme, 6-phosphofructokinase, pseudomonads are unable to metabolize glucose by the well-known Embden-Meyerhof (glycolytic) pathway. However, the Entner-Doudoroff pathway, first discovered by Entner and Doudoroff in *Pseudomonas saccharophila* (110), enables pseudomonads to utilize glucose by a novel route which has an analogous role to that of the glycolytic pathway (107).

In the pathways related to glucose utilization in pseudomonads (Figure 6), 6-phosphogluconate is a key intermediate because it represents a focal point at which two distinct pathways of glucose metabolism converge: the direct oxidative pathway and the phosphorylative pathway. In addition, 6-phosphogluconate is also the starting point for the Entner-Doudoroff pathway (107).

Figure 6. Pathways Related to Glucose Utilization in Pseudomonads.

The scheme presented here was adapted from a recent review of *Pseudomonas* carbohydrate catabolism by Lessie and Phibbs (107). All reactions, except those indicated by broken lines, have been demonstrated in *P. aeruginosa*. Sugars and intermediates are of the D configuration. The abbreviations used are, respectively, as follows: Gcd and Gad are membrane-associated glucose and gluconate dehydrogenases; Gct, Gat and Kgt represent transport systems for glucose, gluconate and 2-ketogluconate; Gck, Gnk and Kgk represent ATP-dependent kinases for glucose (Glu), gluconate and 2-ketogluconate (2KG); Kgr, 2-keto-6-phosphogluconate (2KGP) reductase; Zwf and Gnd are glucose-6-phosphate (G6P) and 6-phosphogluconate (6PGA) dehydrogenases; Edd and Eda are 6PGA dehydrase and 3-deoxy-2-keto-6-phosphogluconate (KDPGA) aldolase; F6P, fructose-6-phosphate; Pgi, Fdp and Fda represent phosphoglucosomerase, fructose 1,6-diphosphatase and fructose 1,6-diphosphate (FDP) aldolase; DHAP, dihydroxyacetone phosphate; Tpi, Gap and Pyk refer to triose phosphate isomerase, glyceraldehyde-3-phosphate (GAP) dehydrogenase and pyruvate (Pyr) kinase; DPGA, 1,3-diphosphoglycerate; Pgc, Pgm and Eno are 3-phosphoglycerate (3PGA) kinase, phosphoglyceromutase and enolase; 2PGA and PEP are 2-phosphoglycerate and phosphoenolpyruvate; Pyc, pyruvate carboxylase. NAD, NADP and AcCoA are nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate and acetyl-coenzyme A; TCA cycle and Pentose PO₄ refer to tricarboxylic acid cycle and pentose-phosphate pathway.

Through the action of membrane-bound glucose and gluconate dehydrogenases, the direct oxidative pathway is initiated by an extracellular oxidation of D-glucose to D-gluconate and 2-keto-D-gluconate, respectively (Figure 6). Following transport of D-gluconate and 2-keto-D-gluconate across the cytoplasmic membrane, by independent carrier systems, these oxidation products are then phosphorylated by their corresponding ATP-dependent kinases to yield, respectively, 6-phosphogluconate and 2-keto-6-phosphogluconate. The latter is then reduced to 6-phosphogluconate through the action of a pyridine-nucleotide-dependent reductase (107).

The non-oxidative, phosphorylative pathway begins when D-glucose is transported across the cytoplasmic membrane, by a glucose-carrier system (Figure 6). Subsequently, an intracellular phosphorylation by an ATP-dependent kinase yields D-glucose-6-phosphate. Finally, a pyridine-nucleotide-dependent oxidation of D-glucose-6-phosphate to 6-phospho-D-gluconate is effected by glucose-6-phosphate dehydrogenase (107).

The 6-phosphogluconate formed by either the phosphorylative pathway or the direct oxidative pathway can now follow one of two fates. In some pseudomonads, 6-phosphogluconate may undergo an oxidative decarboxylation which is mediated by a pyridine-nucleotide-dependent dehydrogenase and gives rise to D-ribulose-5-phosphate; thus, significant amounts of 6-phosphogluconate may be diverted to the pentose-phosphate pathway (107) (Figure 6). While there are some conflicting reports in the literature regarding the existence of 6-phosphogluconate dehydrogenase in various species, it appears that several pseudomonads, including *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Pseudomonas saccharophila*, are devoid of 6-phosphogluconate dehydrogenase and, therefore, are unable to metabolize 6-phosphogluconate via this oxidative portion of the pentose-phosphate pathway (107,111). Alternatively, 6-phosphogluconate can be metabolized by the Entner-Doudoroff pathway.

The first step in the Entner-Doudoroff (ED) pathway is the conversion of 6-phosphogluconate to 3-deoxy-2-keto-6-phosphogluconate (Figure 6). This dehydra-

tion reaction is catalyzed by the enzyme, 6-phosphogluconate dehydrase (EDD). Radioisotopic experiments conducted by Meloche and Wood (112) have shown that the EDD enzyme from *P. putida* promotes an irreversible dehydration of 6-phosphogluconate by a mechanism involving a formation and subsequent ketonization of the enol of 3-deoxy-2-keto-6-phosphogluconate. The enzyme is activated by reduced glutathione and ferrous, manganous or magnesium ions; and it is inactivated completely by sodium fluoride (50 mM), *p*-chloromercuribenzoate (1 mM) or EDTA (1 mM) (113,114).

The second step comprising the ED pathway involves a cleavage of 3-deoxy-2-keto-6-phosphogluconate to pyruvate and D-glyceraldehyde-3-phosphate (Figure 6). This reaction is catalyzed by the enzyme, 3-deoxy-2-keto-6-phosphogluconate aldolase (EDA). The resulting D-glyceraldehyde-3-phosphate can then be further metabolized to a second molecule of pyruvate either by entering reactions that are common to the later stages of glycolysis or by being recycled through the ED pathway via fructose 1,6-diphosphate (107). Pyruvate can be further metabolized to acetyl-CoA, through the action of the pyruvate dehydrogenase complex, or to oxaloacetate, via an ATP-dependent carboxylation catalyzed by pyruvate carboxylase (115). Both of these products are then utilized through the tricarboxylic acid cycle, which serves a vital role in catabolism and biosynthesis (115).

Largely due to the efforts of Wood and his colleagues, the EDA enzyme from *P. putida* has been extensively investigated with regard to substrate specificity, structure and the mechanism and stereochemistry of catalysis (116,117). The enzyme was shown to catalyze a reversible aldol condensation of pyruvate and D-glyceraldehyde-3-phosphate by a mechanism involving formation of an azomethine (Schiff base) between the ketonic carbonyl group of the substrate and an ϵ -amino group of an active-site lysine residue of the aldolase (118,119). By the use of bromopyruvate as an active-site-specific alkylating agent, a glutamate and a cysteine residue at the active site of the enzyme have been implicated in catalysis (116,117). The enzyme is not inactivated by EDTA, N-ethylmaleimide, *p*-chloromercuribenzoate, iodoacetate or arsenite; furthermore, neither a requirement for cofactors nor an activation by

metal ions and reduced glutathione has been observed (117,120). Studies with substrate analogues have established that the enzyme is highly specific and that a 6-phosphate group, a 4-hydroxyl group in the *gluco* configuration and a 3-deoxy group are all essential features required for promoting substrate cleavage (117).

Objectives

The investigation presented herein is concerned with certain aspects of the metabolism of the fluorinated glucose analogue, 4-deoxy-4-fluoro-D-glucose (4FG), by the bacterium, *Pseudomonas putida* (ATCC 12633). Specifically, the objectives of this investigation are:

- (a) To prepare uniformly carbon-14-labelled 4FG and use it to follow the metabolism of 4FG in glucose-grown whole cells of *P. putida* (ATCC 12633) in order to overcome the complication of the extensive loss of tritium as tritiated water, encountered in previous studies with specifically carbon-6-tritiated 4FG (89).
- (b) To attempt to determine whether 4FG is alkylating a cell-envelope protein of glucose-grown whole cells of *P. putida* as was previously proposed to occur (78,89).
- (c) To isolate and identify a defluorinated metabolite(s) of 4FG which had previously been shown to accumulate in the suspending buffer following a 24-hour incubation of glucose-grown whole cells of *P. putida* with D-[6-³H]4FG under conditions giving 100% fluoride release (89).
- (d) To compare the products obtained from incubation of glucose-grown whole cells of *P. putida* and 4FG with those obtained from incubation of 4FG and outer membranes derived from glucose-grown whole cells of *P. putida*. Previous studies had implicated the outer membrane of glucose-grown *P. putida* in the defluorination of 4FG (79,89).

CHAPTER II

MATERIALS AND METHODS

Materials

Crystalline 4FG and D-[U-¹⁴C]4FG (specific activity, $10,600 \pm 100$ dpm/micromole) were synthesized by a slight modification of published methods (49,121,122) as outlined in Appendix I.

D-[U-¹⁴C] galactose (specific activity, 57 millicuries/millimole) was purchased from Amersham Corporation (Amersham, U.K.).

5-hydroxy-4-valerolactone (δ -hydroxy- γ -valerolactone) was synthesized by Dr. N.F. Taylor as previously reported (123) and was kindly provided for the preparation of the sodium salt of 4,5-dihydroxypentanoic acid as described in Appendix II.

Diethylaminosulfur trifluoride (DAST) and **4-pentenol acid** were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin).

Benzoyl chloride was obtained from Fisher Scientific Company (Fair Lawn, New Jersey).

Kieselguhr was from J.T. Baker Chemical Company (Phillipsburg, New Jersey) or from BDH Chemicals Canada Limited (Toronto, Ontario).

Pyridine, calcium gluconate and silica gel-60 thin-layer plastic-backed chromatography sheets were obtained from BDH Chemicals Canada Limited.

Sodium dodecyl sulfate (SDS), Bio-Gel A-1.5m (200-400 wet mesh) and low-dead-volume, borosilicate-glass, gravity-flow chromatography columns and accessories were obtained from Bio-Rad Laboratories (Richmond, California).

Dowex 1-X8 (200-400 dry mesh) anion-exchange resin in the chloride form, lysozyme, bovine serum albumin, ovalbumin, calcium 2-keto-D-gluconate, monosodium D-glucose-6-phosphate, dipotassium D-glucose-1-phosphate, sucrose, deoxyribonuclease (DNase) I, ammonium tetraborate, Blue Dextran (average molecular weight, 2,000,000), and Triton X-100 were obtained from Sigma Chemical Company (St. Louis, Missouri).

Bacteriological media, yeast extract and Gram-stain reagents were obtained from Difco Laboratories (Detroit, Michigan).

Scintillation-grade toluene, 2,5-diphenyloxazole (PPO) and 1,4-bis(5-phenyloxazol-2-yl)-benzene (POPOP) were obtained from Fisher Scientific Company or BDH Chemicals Canada Limited.

Polyethylene liquid scintillation vials were purchased from Packard Instrument Canada Ltd., Mississauga, Ontario.

Other chemicals and reagents were normally obtained from Fisher Scientific Company, BDH Chemicals Canada Limited, Canlab (Mississauga, Ontario) or Sigma Chemical Company.

All chemicals and reagents were of A.C.S. grade or the best grade available. Distilled-deionized or distilled water was used at all times.

Methods

Organism and Culture Conditions

Glucose-grown whole cells of *Pseudomonas putida*, biotype A (ATCC 12633) were used throughout this study. This organism which was previously designated as *Pseudomonas fluorescens*, strain A.3.12 and first isolated by Stanier (124) was obtained as a freeze-dried culture from the American Type Culture Collection (ATCC), Rockville, Maryland, USA. The organism was routinely maintained on glucose-mineral-salts agar slants by transferring to a fresh slant every 2 to 6 months, allowing growth to occur by incubating at room temperature or 30°C for 24 hours, and then storing the resulting 24-hour culture at 4°C.

The mineral-salts growth medium used for obtaining glucose-grown whole cells of *P. putida* was the semi-defined medium of Davis and Mingioli (125). This

consisted of:

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

Solutions of trace elements, made up according to Barnett and Ingram (126), contained:

TRACE SOLUTION A

	mg/100 mL (stock solution)
$FeSO_4 \cdot 4H_2O$	40
$MnSO_4 \cdot 4H_2O$	40
NaCl	1,000

TRACE SOLUTION B

	mg/100 mL (stock solution)
$ZnSO_4 \cdot 7H_2O$	20
$CuSO_4 \cdot 5H_2O$	4
$CoCl_2 \cdot 6H_2O$	4
$CaCl_2$ (anhydrous)	500
$Na_2MoO_4 \cdot 4H_2O$	5
KI	30

The resulting mineral-salts medium which had a pH of about 7.1 was then sterilized by autoclaving in batches of 2 L in 4-L Erlenmeyer flasks for 15-20 minutes at 121°C or by sterilizing 12 L of medium in a large batch-fermentation unit (New

Brunswick Scientific Bench-top Fermentor, Model SF-116, New Brunswick Scientific Company, Inc., Edison, New Jersey) at 121°C for 30 minutes, always without glucose. An appropriate amount of an aqueous solution of D-glucose which had been sterilized either by autoclaving for 15-20 minutes at 121°C or by membrane filtration in a Nalgene 0.20-micrometre filter unit (Nalgene Sybron Corporation, Rochester, New York) was then added aseptically to the hot sterile mineral-salts medium to give a final glucose concentration of 0.2% (w/v).

For the preparation of glucose-mineral-salts agar slants, sterile mineral-salts medium was prepared and sterilized by autoclaving as described above, except that double-strength mineral-salts solution was used. A 2% (w/v) solution of plain agar in water was prepared by sterilizing at 121°C for 15-20 minutes. While all solutions were still hot, equal volumes of sterile 2% agar solution and sterile mineral-salts solution were combined, an appropriate amount of a sterile aqueous glucose solution was then added to give a final glucose concentration of 0.2% (w/v), and the resulting mixture was mixed well. The mixture, while still hot, was pipetted aseptically into sterile 25-mL borosilicate-glass tubes in 10-mL aliquots, and the capped tubes were then allowed to cool in a slanted position at room temperature. The solidified slants were then incubated at 30°C for 24 hours. Following this incubation, slants showing any sign of contamination were discarded, and the uncontaminated slants were then stored at 4°C in a sealed plastic bag.

Preparation of Whole-Cell Suspensions

Glucose-grown whole-cell suspensions of *P. putida* were obtained by culturing at 30°C in sterile glucose-mineral-salts growth medium in either 4-L Erlenmeyer flasks containing 2 L of growth medium or in a bench-top batch-fermentation unit containing 12 L of growth medium. The inoculum for these cultures was prepared by transferring 2 "loops" of cells from a fresh 24-hour culture of *P. putida* grown on a glucose-mineral-salts agar slant, to 10 mL of sterile mineral-salts solution and allowing the resulting suspension to stand at room temperature for 1 hour. A 1-mL

aliquot of this suspension was aseptically transferred to each 4-L Erlenmeyer flask containing 2 L of sterile glucose-mineral-salts growth medium to initiate growth. The flasks were then incubated at 30°C and shaken on a rotary shaker at a rate of about 100-200 revolutions per minute. When cells were cultured in the large bench-top batch-fermentation unit, containing 12 L of sterile glucose-mineral-salts growth medium, the entire 10-mL inoculum was transferred aseptically into the fermentation unit through one of the inoculating ports at the top of the vessel to initiate growth. The vessel was maintained at a temperature of 30°C, aerated with air at a rate of 1-2 L per minute, stirred at a rate of 400 revolutions per minute, and maintained at a slight positive pressure of 0.5-1 pound per square inch. Cultures were grown overnight for 13-14 hours in the bench-top batch-fermentation unit or for 18 hours in the 4-L Erlenmeyer flasks; after this time, the optical density of the culture at 620 nanometres was approximately 1.0 (measured with a Bausch & Lomb Spectronic 20 spectrophotometer using either mineral-salts medium or distilled water as a blank), indicating that the culture was in the pre-stationary phase of growth (Appendix III). At this time the cells were then harvested in sterile 1-L centrifuge bottles by centrifugation at 4,700 x g at 25°C for 35-40 minutes in an IEC PR-6000 centrifuge (Damon/IEC Division, Needham Heights, Massachusetts). The cell pellet obtained from each litre of culture was washed twice with 250 mL of sterile 0.1 M potassium phosphate buffer (PPB), pH 7.1, and reharvested as described above. The washed cells from 12 L of culture were then resuspended with about 120 mL of sterile 0.1 M PPB, pH 7.1. Protein was determined by the method of Lowry *et al.* (127) using bovine serum albumin as a standard. The resulting whole-cell suspension was then used immediately for whole-cell incubations.

The purity of the cultures and of the stock cultures was determined by performing a Gram stain using a commercial Gram-stain kit (Difco Bacto Gram Stain Set, Difco Laboratories, Detroit, Michigan) as directed by the manufacturer's instructions; the only exception was that the decolorization step was performed for 10 seconds. A freshly grown 24-hour culture of yogurt occasionally served as a Gram-positive internal standard (control). Of all the cultures of *P. putida* examined by this procedure, no contamination by other bacterial species was observed.

Microscopic examination, under an oil-immersion lens (magnification, 1,000), showed uniformly short, Gram-negative rods.

Labelling and Fractionation of Whole Cells

Glucose-grown whole cells (600 mg whole-cell protein) were incubated with 1.0 mM D-[U-¹⁴C]4FG (specific activity, 10,600 dpm/micromole) in sterile 0.1 M potassium phosphate buffer (PPB), pH 7.1, to give a total incubation volume of 100 mL. The incubation was conducted for 24 hours at 30°C in a sterile 500-mL Erlenmeyer flask on a rotary shaker (Lab-Line Instruments Inc., Melrose Park, Illinois). An identical whole-cell control incubation was performed in the absence of D-[U-¹⁴C]4FG and was processed in a manner that was identical to that which will now be described for the whole-cell incubations that contained D-[U-¹⁴C]4FG. After the 24-hour incubation, the cells were collected by centrifugation at 4,000 x g for 25 minutes at 25°C, and the resulting supernatant was frozen at -20°C until it could later be analyzed for radioactivity, fluoride ion and radiolabelled metabolites. The remaining whole-cell pellet was then suspended with 10 mL of 0.1 M PPB, pH 7.1, transferred to and sealed in a boiled dialysis bag (molecular weight cut-off, 12,000 daltons; Fisher Cat. No. 8-667D, Fisher Scientific Company), and then dialyzed against 1.25 L of 0.01 M or 0.1 M PPB, pH 7.1, containing 0.1% (w/v) sodium azide, for three 12-hour periods at 4°C. During dialysis, the dialyzing buffer was constantly stirred with a magnetic stirrer and at the end of the first and second dialysis period it was replaced with a fresh 1.25 L of dialyzing buffer. Samples of the dialysates were stored at -20°C for future radioactivity measurements.

Dialyzed whole cells were then collected by centrifugation at 4,000 x g for 25 minutes at 4°C. The resulting supernatant, referred to as the "supernatant from dialysis bag", was retained and stored at -20°C for radioactivity measurements. The resulting cell pellet was then resuspended in 5 mL of sterile 0.1 M, pH 7.1, sodium phosphate buffer (SPB) and ultrasonicated using an Artek Sonic 300 Dismembrator (Artek Systems Corporation, Farmingdale, New York) to obtain a cell-free extract

(67). Sonication was performed by cooling the suspension on an ice bath to about 4°C with the sonicating probe immersed into the suspension and then sonicating for ten 1-minute intervals, with 2 minutes allowed for cooling between sonication periods. The sonicating probe was rinsed with 10 mL of sterile 0.1 M SPB, pH 7.1, and the rinsings were added to the sonicated suspension.

After removal of unbroken cells and large cell fragments, referred to collectively as "sonication debris", by centrifugation at 17,000 x g for 10 minutes at 4°C, subsequent centrifugation at 100,000 x g for 90 minutes at 4°C yielded a supernatant, referred to as the cell-free extract, which was frozen at -20°C until it was later analyzed for radioactivity. The resulting pellet, referred to as the cell envelope, was resuspended with 15 mL of sterile 0.1 M SPB, pH 7.1, and reisolated by centrifugation at 100,000 x g for 90 minutes at 4°C in a Beckman L8-55 Ultracentrifuge (Beckman Instruments Inc., Palo Alto, California). The resulting cell-envelope fraction was suspended with 10 mL of sterile 0.1 M, pH 7.1, SPB and the suspension was stored at -20°C for radioactivity and gel-filtration analysis. Non-fractionated material, *i.e.*, residual radiolabelled material remaining behind in the incubation flask, centrifuge tube, or dialysis bag, was collected with 10-mL portions of water and also stored at -20°C for future radioactivity measurements; this material is collectively referred to as "residual material".

As an additional control, 1.0 millimolar D-[U-¹⁴C]4FG (specific activity, 10,600 dpm/micromole) was incubated in 0.1 M PPB, pH 7.1, in a total volume of 20 mL for 24 hours at 30°C. This incubation mixture was then stored at -20°C and later submitted to fluoride, radioactivity and chromatographic analysis.

Liquid Scintillation Counting

Radioactivity was measured with a Beckman LS 7500 liquid scintillation counter (Beckman Instruments Inc., Fullerton, California) according to the manufacturer's instructions. For all samples, 10 mL of a liquid scintillation cocktail

consisting of 2 volumes of scintillation-grade toluene, 1 volume of Triton X-100, 10 g/L of PPO and 0.2 g/L of POPOP, was added. In all cases, enough water was added to the samples to ensure that a clear, homogeneous solution was obtained after vigorous mixing with the scintillation cocktail. The level of quenching in each sample was determined by the "H-number" method and counting efficiency was derived from a quench correction curve (Appendix IV). This was constructed by plotting the counting efficiency versus H-number values obtained for a series of sealed, quenched carbon-14 standards (Nuclear Chicago, 190,000 dpm/Mar. 15, 1968) and a sealed, unquenched carbon-14 standard (Beckman, 29,700 dpm/Jan. 1, 1979). A full carbon-14 window was used in all three counting channels for all radioactivity determinations.

Fluoride Ion Measurements

Fluoride ion was measured with a combination fluoride electrode (Orion Research, Cambridge, Massachusetts) coupled to a Metrohm Herisau E510 Precision mV/pH Meter (Metrohm Herisau, Switzerland). After allowing samples to fully equilibrate to room temperature, the electrode was inserted into the sample for 5 minutes and the electrode potential on the millivolt scale of the meter was recorded to an accuracy of ± 1 mV. The same protocol was followed for constructing a standard curve relating electrode potential to fluoride ion concentration from a series of standard sodium fluoride solutions that had been prepared in 0.1 M potassium phosphate buffer, pH 7.1. The concentration of fluoride ion in the samples could then be determined from this standard curve with an accuracy of about 5% (based on a possible error of ± 1 mV for the electrode potential reading). In a study using either D-[U- ^{14}C]4FG (specific activity, 10,600 dpm/micromole) or non-radiolabelled 4FG in whole-cell incubations (performed as described above), at a concentration of 1.0 millimolar, the fluoride-electrode potential readings obtained for each supernatant after 24 hours were nearly identical (within 0.5 mV of each other); apparently, the presence of radiolabel, at least up to a level of 10,600 dpm/mL, did not have any effect on the fluoride-electrode potential.

Gel-Filtration Chromatography

Gel-filtration analysis of radiolabelled cell envelope was performed on a 180 ± 4 -mL bed of Bio-Gel A-1.5 m (exclusion limit, 1.5 million daltons) by gravity-flow through an Econo-Column (Bio-Rad Laboratories, Richmond, California) borosilicate-glass column having a nominal inner diameter of 1.5 cm. The total dead-volume of this column and its associated stopcock and tubing was found to be less than 2 mL and, therefore, was neglected in calculating elution volumes. The column was equilibrated and eluted at room temperature using 0.1 M SPB, pH 7.2, containing 0.1% (w/v) sodium dodecyl sulfate (SDS) and 0.05% (w/v) sodium azide. The void volume of the bed and the exclusion limit of the gel under these conditions was determined by running a sample containing Blue Dextran (average molecular weight, 2 million daltons) and several molecular-weight protein standards (bovine serum albumin, chicken egg ovalbumin and chicken egg white lysozyme) as described in Appendix V and Appendix VI.

The general procedure adopted for analysis of radiolabelled cell envelope obtained by ultrasonication (as described above under "Labelling and Fractionation of Whole Cells") was as follows. Two equal portions of a suspension of radiolabelled cell envelope were incubated separately for 1 to 2 hours at 30°C in 0.1 M SPB, pH 7.1, in the absence or presence of lysozyme (1 mg/mL). Subsequently, SDS was added to both the untreated and lysozyme-treated radiolabelled cell envelope to a final concentration of 1% (w/v), and the resulting clear solution was heated at 65°C or 70°C for 30 minutes. The resulting solubilized samples of untreated and lysozyme-treated radiolabelled cell envelope were then both stored at -20°C prior to gel filtration. Either the untreated or lysozyme-treated SDS-solubilized radiolabelled cell envelope was then thawed and mixed with 0.05 volumes of a 0.05% (w/v) aqueous solution of bromophenol blue (Fisher Scientific Co.). An aliquot of 2 to 4 mL of the resulting sample was then carefully layered onto the top of the gel bed with the aid of a tuberculin-type syringe fitted with a 12-cm-long piece of Teflon capillary tubing. The sample was eluted at a flow rate of 3.6 to 4.2 mL per hour while 1.2 to 1.4-mL fractions were collected with an ISCO Model 328 Golden

Retriever fraction collector (Instrumentation Specialties Company, Lincoln, Nebraska). The fraction volumes quoted throughout these studies represent an average of volumes measured from at least 5 different fractions for any particular chromatographic analysis, while the corresponding uncertainty represents the maximum observed deviation from that average volume. Fractions were assayed spectrophotometrically for protein at a wavelength of 280 nanometres by transferring a fraction to a quartz cuvette and measuring the absorbance in a Shimadzu UV-240 UV-visible recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Following each absorbance measurement, the fraction was placed into a scintillation vial; afterwards, to each vial was added 0.5 mL of water, followed by 10 mL of scintillation cocktail. After shaking vigorously, each vial was submitted to liquid scintillation counting (as described above under "Liquid Scintillation Counting") to determine the radioactivity in the fraction.

Borate Anion-Exchange Column Chromatography

Samples obtained following 24-hour incubations with D-[U- ^{14}C] 4FG (as described above under "Labelling and Fractionation of Whole Cells") were analyzed on a 108-cm x 1-cm bed of Dowex 1-X8 borate anion-exchange resin by gravity-flow through an Econo-Column (Bio-Rad Laboratories) borosilicate-glass column. The resin was prepared from a Dowex 1-X8 200-400 dry-mesh anion-exchange resin in the chloride form by, first, converting it completely to the hydroxide form and then neutralizing with 0.5 M boric acid as described by Floridi (128). All analyses were performed at room temperature as follows. Prior to every analysis, the column was equilibrated with a 0.029 M ammonium tetraborate/0.057 M boric acid buffer, pH 8.5-8.8; this will be referred to as "starting buffer". Prior to application of radiolabelled samples onto the column, various sugars including sucrose, 4FG, D-fructose, D-glucose, D-glucose-1-phosphate and D-glucose-6-phosphate were added to the sample as internal standards. The elution order of these sugar standards was previously determined by running each one individually under the same conditions as described here. Approximately 2 mL of the resulting radiolabelled sample was then applied

to the top of the resin bed and was allowed to flow into the bed without allowing the top of the bed to run dry. This was followed by a similar application of 1 to 2 mL of starting buffer which had been used to rinse out the container from which the radiolabelled sample had been taken. This was then followed by an additional application of 1 to 2 mL of starting buffer to rinse down any radiolabelled material from the inner wall of the column into the bed. The column was then topped-up with starting buffer and eluted with an increasing linear concentration gradient of ammonium tetraborate; this consisted of 600 mL of starting buffer diluted linearly with 600 mL of 0.5 M ammonium tetraborate, pH 8.9-9.2. Approximately 4-mL fractions were collected with an LKB Bromma 2112 Redirac Fraction Collector (LKB-Produkter AB, Bromma, Sweden) at a flow rate of about 7 mL/hour. Actual fraction volumes quoted represent the average of at least 5 different fractions within a given chromatographic analysis, with the associated uncertainty representing the maximum observed deviation from that average volume. Fractions were then analyzed for radioactivity by taking 1.0 mL from each fraction, placing it in a scintillation vial, adding to it 0.5 to 2 mL of water, adding 10 mL of scintillation cocktail, shaking vigorously to obtain a clear, homogeneous solution and then counting for radioactivity as previously described (*vide* "Liquid Scintillation Counting"). Subsequently, alternate fractions were analyzed for carbohydrate by orcinol-sulfuric acid colorimetry (128). This consisted of adding approximately 2 volumes of orcinol-sulfuric acid reagent (0.1% w/v orcinol in 70% v/v sulfuric acid), vortexing, heating at 90-95°C for 15-20 minutes, cooling to room temperature and then measuring the absorbance of the resulting solution at 420 nm in a Shimadzu UV-240 UV-visible recording spectrophotometer. The alternate fractions remaining after orcinol-sulfuric acid colorimetry were used to assay for inorganic phosphate as described below.

For the preparative isolation of radiolabelled metabolites from supernatants derived from 24-hour whole-cell incubations with D-[U-¹⁴C]4FG (as described above under "Labelling and Fractionation of Whole Cells"), a 112-cm x 1.5-cm bed of Dowex 1-X8 borate anion-exchange resin in an Econo-Column borosilicate-glass column was used. The method of sample application and the gradient-elution

conditions used for this column were identical to that given above for the analysis of radiolabelled samples on the 108-cm x 1-cm borate anion-exchange column. All isolations were performed under gravity-flow, at room temperature, using an increasing linear ammonium-tetraborate gradient consisting of 600 mL of starting buffer diluted linearly with 600 mL of 0.5 M, pH 8.9-9.2, ammonium tetraborate. Prior to its application on the column, 30 mL of the radiolabelled supernatant was concentrated, 10-fold, to 3 mL by rotary evaporation under vacuum at 40°C. This concentrated radiolabelled supernatant was then applied directly to the column; no internal standards were added to this sample. The column was eluted at a flow rate of about 16 mL/hour and 4.2 ± 0.2 -mL fractions were collected. Radioactivity in fractions corresponding to either the radiolabelled "minor-peak metabolite" or the radiolabelled "major-peak metabolite" was monitored by withdrawing a 0.1-mL aliquot from these fractions and measuring its radioactivity by liquid scintillation counting. Radioactive fractions corresponding to either the minor-peak or major-peak metabolite were pooled and stored at -20°C. Two more identical preparative chromatographic runs, again using 3 mL of 10-fold concentrated radiolabelled supernatant, were conducted as above. Between consecutive preparative runs, the following protocol was followed in preparing the column for a subsequent preparative run. After completion of the elution of inorganic phosphate from the column, the column was eluted with 1 column volume of 0.5 M, pH 8.9-9.2, ammonium tetraborate. The column was then re-equilibrated by elution with 3 column volumes of starting buffer. Inorganic phosphate in the late-eluting fractions from preparative runs was monitored, as described by Clark *et al.* (129), by adding 1 mL of acid-molybdate reagent (2.5% w/v $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in 13.6% v/v sulfuric acid) to each fraction, vortexing, adding 1 mL of reducing agent (3% w/v sodium bisulfite and 1% w/v *para*-methylaminophenol), vortexing and noting the formation of a strong dark blue color after standing at room temperature for 20 minutes. Inorganic phosphate was found to elute in fractions 206-240.

Fluoride ion in early-eluting fractions from one preparative run was monitored with a combination fluoride electrode as described under "Fluoride Ion Measurements". Fluoride ion was detected in fractions 118-128.

The radioactive fractions corresponding to either the minor-peak or major-peak radiolabelled metabolite, obtained by pooling fractions from 3 preparative runs, were then concentrated to dryness under vacuum at $40^{\circ} \pm 3^{\circ}\text{C}$ on a Buchi Rotavapor EL130 Rotary Evaporator (Brinkmann Instruments Limited, Rexdale, Ontario). Ammonium tetraborate was then removed by repeated addition and evaporation to dryness of 100-mL portions of anhydrous methanol (J.T. Baker Chemical Company) for a total of 5 times. Completeness of borate removal was checked by using a green-flame test on the distillate. Finally, the residue obtained from the major-peak-metabolite fractions was solubilized with 1 mL of deuterated water (D_2O) and stored at -20°C for future analysis. Similarly, the residue obtained from the minor-peak-metabolite fractions was also solubilized with 1 mL of D_2O , but this was then concentrated to dryness in a vacuum desiccator and re-solubilized with 200 microlitres of D_2O . Control samples referred to as the minor-peak control and the major-peak control were isolated from the *non*-radiolabelled supernatant of a 24-hour whole-cell incubation conducted in the absence of 4FG (as described under "Labelling and Fractionation of Whole Cells"), by the same procedures given above for the isolation of the radiolabelled minor-peak and major-peak metabolites. The minor-peak control consisted of 4.2 ± 0.1 -mL fractions that had been pooled from the same region where the minor-peak radiolabelled metabolite had eluted: specifically, fractions 30 to 38 (Appendix VII). Similarly, the major-peak control consisted of 4.2 ± 0.1 -mL fractions that had been pooled from the same region where the major-peak radiolabelled metabolite had eluted: specifically, fractions 163 to 188 (Appendix VII). These controls were derived from pooling of appropriate fractions from 3 separate chromatographic runs of 3 mL of 10-fold concentrated *non*-radiolabelled supernatant. After removal of ammonium tetraborate and solubilizing with D_2O as described above, these controls were also stored at -20°C for further analysis. The above D_2O solutions of the radiolabelled major-peak and minor-peak metabolite and their respective controls were used directly in all subsequent analyses without further treatment.

Analysis of the isolated major-peak metabolite and the major-peak control for inorganic phosphate before and after 7-minute hydrolysis in 1 N HCl and after total

hydrolysis was performed by the modified Fiske-Subbarow method according to the procedure of Clark *et al.* (129).

Thin-Layer Chromatography of Radiolabelled Metabolites

After isolation, the major-peak and minor-peak metabolites and their respective controls were analyzed by thin-layer chromatography (TLC) on 20-cm x 20-cm silica gel-60 (0.2 mm layer thickness) plastic-backed TLC sheets (Merck, Darmstadt, Germany). The developing solvent was ethyl acetate-acetic acid-water (3:3:1 v/v). After air drying, the spots on the chromatogram were visualized by spraying with sulfuric acid-ethanol (1:1 v/v) and then placing the chromatogram in an oven at 140°C for 20 minutes. For detection of 4-pentenoic acid, the chromatogram was placed in a chamber containing iodine vapour for 10-15 minutes; it was then heated at 130°C for 1 minute to remove iodine vapour prior to visualizing the other components as described above.

Radioactivity in a developed silica-gel TLC sheet was determined by taking the appropriate vertical section from the air-dried chromatogram and taping it to a glass plate with Scotch Brand Magic Transparent Tape (3M Canada Inc., London, Ontario) so that the entire surface of the silica-gel layer was covered with the tape. The resulting vertical section of the chromatogram was then sectioned horizontally, starting from the bottom, into 1.0-cm sections. Each of the resulting sections was placed separately into a scintillation vial, 1.2 mL of water was added to the vial, 10 mL of scintillation cocktail was added and the mixture was then vigorously shaken to obtain a clear homogeneous liquid phase. The radioactivity of each of these was then determined by liquid scintillation counting as previously described under "Liquid Scintillation Counting".

Fourier Transform Carbon-13 and Proton NMR

Carbon-13 and proton Fourier transform (FT) nuclear magnetic resonance (NMR) spectroscopic analysis of the isolated radiolabelled major-peak metabolite was performed on a 300 MHz General Electric QE-300 FT NMR spectrometer. Carbon-13 and proton FT NMR spectroscopic analysis of the synthetic sodium salt of 4,5-dihydroxypentanoic acid was performed on a Bruker 300 MHz FT NMR spectrometer. D₂O was the solvent for both samples. All chemical-shift values are relative to tetramethylsilane and are expressed in parts per million (PPM) of the main spectrometer frequency.

Fast-Atom-Bombardment (FAB) Mass Spectrometry

Negative-ion FAB mass spectrometric analysis of a sample of the isolated radiolabelled major-peak metabolite in D₂O was performed on a Finnigan-mat CH5-DF magnetic sector mass spectrometer. Xenon atoms from a FAB gun set at a power of 4 watts (4 kV x 1 mA) were used as the projectiles, while glycerol was used as a sample matrix. The major-peak control sample was also analyzed under the same conditions.

Labelling and Fluorine-19 NMR Analysis of Cell Envelope

Glucose-grown whole cells (120 mg whole-cell protein) were incubated with 10.0 mM D-[U-¹⁴C]4FG (specific radioactivity, 10,600 dpm/micromole) in sterile 0.1 M, pH 7.1, potassium phosphate buffer (PPB) to give a final volume of 20 mL. The incubation was conducted in a sterile 125-mL Erlenmeyer flask for 24 hours at 30°C on a rotary shaker. An identical whole-cell incubation in the absence of 4FG was also performed and was subsequently processed, as described below, in the same manner. After the 24-hour incubation, the cells were collected by centrifugation at

10,000 x g for 10 minutes at 25°C. Fluoride ion measurements of the resulting supernatants indicated there was a $42 \pm 5\%$ release of fluoride ion. The resulting cell pellets, after storing at -20°C, were then separately suspended with 10 mL of sterile 0.1 M sodium phosphate buffer (SPB), pH 7.1, and ultrasonicated to obtain a cell-envelope pellet as previously described (see above under "Labelling and Fractionation of Whole Cells"). The resulting cell-envelope pellets, after storing at -20°C, were washed twice by resuspending the pellet with 20 mL of sterile 0.1 M SPB, pH 7.1, and collecting the cell envelope by centrifugation at 100,000 x g for 90 minutes at 4°C. The resulting washed cell-envelope pellets were stored at -20°C before being solubilized as described below.

Each cell-envelope pellet was solubilized separately by adding to each pellet 0.6 mL of 1% (w/v) sodium dodecyl sulfate (SDS) in 0.1 M SPB, pH 7.1, and 40 mg of SDS, followed by heating at 80-90°C. An additional 20 mg of SDS along with about 2 mL of water was later added to each pellet to aid in solubilization, and heating was continued at 90-100°C until the pellet was completely dissolved. Heating at about 90°C was then continued in order to allow the resulting solutions to concentrate to a syrupy consistency. Radioactivity measurements on the resulting solubilized sample of radiolabelled cell envelope (0.77 ± 0.01 mL final volume) indicated the radiolabel was present at a level of $1,500 \pm 100$ dpm/mL. Therefore, as a control for a subsequent attempt to detect fluorine in this solubilized sample of radiolabelled cell envelope, the solubilized sample of non-radiolabelled cell envelope (0.97 ± 0.01 mL final volume) was mixed with 10 ± 0.5 microlitres of a 20.0 ± 0.1 mM aqueous solution of D-[U-¹⁴C]4FG (specific activity, $10,600 \pm 100$ dpm/micromole) to give approximately 0.2 micromoles of fluorine (or 2,000 dpm of radiolabel) per mL. The resulting solutions were stored at 4°C until they could be analyzed for fluorine by fluorine-19 NMR.

Analysis of the above solubilized samples of radiolabelled cell envelope or non-radiolabelled cell envelope (containing added radiolabel) for fluorine was performed by Fourier transform (FT) fluorine-19 nuclear magnetic resonance (NMR) spectroscopy on a Bruker 200 MHz FT NMR spectrometer. 5-mm NMR

tubes were used for both samples. The scanning range was from +440 to -440 parts per million (PPM) relative to an external trichlorofluoromethane (neat) reference which was assigned a chemical shift of 0.000 PPM. After at least 50,000 scans, no fluorine signal could be found in either of the above samples.

Crude Outer-Membrane Preparation and Incubation with D-[U-¹⁴C]4FG

The isolation of crude outer membrane, based on a modification of the method of Mizuno and Kageyama (130), was performed according to the procedure of D'Amore (89). After harvesting glucose-grown whole cells from 12 L of culture, the cells were washed once with about 500 mL of 20% (w/v) sucrose for every 2 L of culture harvested, and the cells were collected by centrifugation at 4,700 x g for 40 minutes at 25°C. Every 4 g of wet cells was suspended with 40 mL of ice-cold 20% (w/v) sucrose, and the following ice-cold sterile solutions were slowly added to the suspension with constant stirring: 20 mL of 2 M sucrose, 10 mL of 100 mM potassium phosphate buffer (PPB), pH 7.1, and 10 mL of 100 mM MgSO₄. Lysozyme was then added to a final concentration of 0.5 mg/mL. The suspension was quickly warmed to room temperature and then incubated at 30°C on a rotary shaker (180 rpm) for 30 minutes. After this time, DNase I was added to a final concentration of 5 micrograms/mL; the incubation was continued for another 30 minutes as before. Spheroplasts were then removed by centrifugation at 10,000 x g for 15 minutes at 30°C or at 4,700 x g for 2 hours at 20°C. Crude outer membranes were then recovered from the resulting decanted supernatant by centrifugation at 100,000 x g for 60 minutes at 4°C in a Beckman L8-55 Ultracentrifuge. The resulting pellets were washed once by resuspending with 120 mL of ice-cold sterile 0.1 M PPB, pH 7.1, and crude outer membranes were again collected as above. The washed crude-outer-membrane pellets were then resuspended with 5-6 mL of ice-cold sterile 0.1 M PPB, pH 7.1, by vigorous vortexing at 4°C. Following a determination of crude-outer-membrane protein by the method of Lowry *et al.* (127), the resulting suspension of crude outer membrane was used immediately for incubations with D-[U-¹⁴C]4FG. Typically, about 10 mg of crude-outer-membrane

protein was obtained from about 40 grams of wet cells by this procedure.

Crude outer membranes (1.6-1.7 mg crude-outer-membrane protein per mL) were incubated with 1.0 mM or 2.5 mM D-[U- 14 C]4FG in sterile 0.1 M PPB, pH 7.1, in sterile, capped 25-mL polycarbonate centrifuge tubes for 24 hours at 30°C on a rotary shaker. Crude outer membranes were then removed by centrifugation at 100,000 x g for 60 minutes at 25°C. The resulting supernatants were analyzed for fluoride ion and then stored at -20°C for subsequent radioactivity measurements and for analysis of radiolabelled metabolites by borate anion-exchange column chromatography.

Trapping of $^{14}\text{CO}_2$ During Whole-Cell Incubations

Glucose-grown whole cells (24-26 mg whole-cell protein) were incubated with 1.0 mM D-[U- 14 C]4FG (specific activity, 10,600 dpm/micromole) in 0.1 M potassium phosphate buffer, pH 7.1, for 24 hours at 30°C in a final volume of 4.0 mL. The incubations were conducted in 20-mL Gilson respirometer flasks containing 400 microlitres of aqueous 20% (w/v) potassium hydroxide in a centre well and 4.0 mL of the above incubation mixture in the main compartment. Incubations were initiated by adding the D-[U- 14 C]4FG last, to the main compartment, and then quickly placing the flask on its holder on a Gilson Differential Respirometer (Gilson Medical Electronics, Middleton, Wisconsin) with gentle shaking. After the 24-hour incubation, two 200-microlitre aliquots of the 20% potassium hydroxide solution from the centre well were collected and dispensed into separate scintillation vials. 3 mL of water and 10 mL of scintillation cocktail was added to each vial, and the resulting mixture was shaken well to obtain a clear homogeneous solution. These solutions were stored overnight for at least 18 hours to allow chemiluminescence to subside and then counted for radioactivity by liquid scintillation counting. In some cases, the 200-microlitre samples of 20% potassium hydroxide were acidified by the addition of 630 microlitres of 1 N HCl and placed under vacuum in a vacuum desiccator containing NaOH pellets, for 24 hours at room temperature; these samples were

then counted for radioactivity as described for the non-acidified samples. The amount of fluoride ion released in these incubations after 24 hours was determined directly with the resulting cell suspension from the main compartment.

CHAPTER III

RESULTS AND DISCUSSION

Distribution of Radiolabel in Whole-Cell Incubations after 24 Hours

In an effort to overcome the complication of the extensive loss of tritium encountered with D-[6-³H]4FG, and to further elucidate the possibility that 4FG may be alkylating a cell-envelope protein, D-[U-¹⁴C]4FG was synthesized in these laboratories (Appendix I) and used in incubations with glucose-grown whole cells of *P. putida* (Table 1).

Table 1 shows the distribution of radioactivity in various fractions obtained from two identical 24-hour incubations of glucose-grown whole cells of *P. putida* (600 mg protein) with 1.0 mM D-[U-¹⁴C]4FG (specific activity, 10,600 dpm/micromole) in 0.1 M potassium phosphate buffer (PPB), pH 7.1, at 30°C. Fluoride ion measurements on the supernatant fractions from both incubations revealed a fluoride ion concentration of 0.95 ± 0.05 mM, indicating that there was a $95 \pm 5\%$ release of F⁻ (assuming the cell pellet, which accounted for about 4% of the total volume of the incubation, were to have the same concentration of F⁻). A control consisting of a 24-hour incubation of 1.0 mM D-[U-¹⁴C]4FG in 0.1 M PPB, pH 7.1, at 30°C in the absence of cells gave less than 1% release of F⁻. Whereas more than 50% of the radiolabel was found in the suspending buffer, or supernatant fraction, less than 1% of the total radioactivity was incorporated into the cell-envelope fraction. Additionally, the results of two separate 24-hour incubations of glucose-grown whole cells of *P. putida* (26 mg protein) with 1.0 mM D-[U-¹⁴C]4FG (specific activity, 10,600 dpm/micromole) in 0.1 M PPB, pH 7.1, at 30°C, revealed that $3.8 \pm 0.2\%$ and $4.8 \pm 0.2\%$ of the total radioactivity could be recovered as a volatile product by using an aqueous solution of 20% (w/v) potassium hydroxide as a trapping agent; under these conditions, there was a $98 \pm 5\%$ release of F⁻ in both incubations after 24 hours. When the trapping agent obtained from these 24-hour incubations was acidified and placed under reduced pressure for 24 hours, there was a complete loss of radioactivity. These latter results would be consistent with the conversion of

Table 1. Distribution of Radioactivity Found in Various Fractions Obtained from 24-Hour Incubations of Glucose-Grown Whole Cells of *Pseudomonas putida* with 4-Deoxy-4-fluoro-D-[U-¹⁴C]glucose.

Fraction	Radioactivity (dpm)		% of Initial Radioactivity	
	Incubate No. 1	Incubate No. 2	Incubate No. 1	Incubate No. 2
Supernatant from 24-Hour Incubation	556,000 ± 12,000	566,000 ± 12,000	52 ± 1	53 ± 1
Whole-Cell Dialysate	369,000 ± 48,000	380,000 ± 49,000	35 ± 5	36 ± 5
Supernatant from Dialysis Bag	8,200 ± 300	2,800 ± 200	0.77 ± 0.03	0.27 ± 0.02
Sonication Debris	1,100 ± 100	850 ± 80	0.11 ± 0.01	0.08 ± 0.01
Cell-Free Extract	54,800 ± 1,400	29,500 ± 900	5.2 ± 0.2	2.8 ± 0.1
Cell Envelope	4,500 ± 400	4,500 ± 500	0.42 ± 0.04	0.43 ± 0.05
Cell-Envelope Washings	1,600 ± 300	1,100 ± 300	0.16 ± 0.03	0.10 ± 0.03
Residual Material (non-fractionated)	15,800 ± 600	10,800 ± 500	1.5 ± 0.06	1.0 ± 0.05
Total Recovery	1,010,000 ± 60,000	995,000 ± 63,000	95 ± 6	94 ± 6

Glucose-grown whole cells (600 mg protein) of *P. putida* were prepared as described under "Preparation of Whole-Cell Suspensions" in Materials and Methods and were incubated with 1.0 mM D-[U-¹⁴C]4FG (specific activity, 10,600 dpm/micromole) in sterile 0.1 M potassium phosphate buffer, pH 7.1, in a final volume of 100 mL, at 30°C, on a rotary shaker, for 24 hours. After collecting the supernatant fraction by centrifugation at 4,000 × g for 25 minutes at 25°C, the resulting cell pellet was dialyzed for 3 consecutive 12-hour periods at 4°C against a fresh 1.25-L portion of 0.1 M (Incubate No. 1) or 0.01 M (Incubate No. 2) potassium phosphate buffer, pH 7.1, containing 0.1% (w/v) sodium azide. The dialyzed whole cells, following ultrasonic disruption, were then further fractionated, as described under "Labelling and Fractionation of Whole Cells" in Materials and Methods, to yield a cell-free extract and a cell-envelope fraction. Radioactivity in each fraction was measured by liquid scintillation counting (as described in Materials and Methods) and was calculated as shown in Appendix VIII.

radiolabel to volatile $^{14}\text{CO}_2$.

Gel-Filtration Analysis of Radiolabelled Cell Envelope

Analysis of an SDS-solubilized sample of the radiolabelled cell-envelope fraction (Table 1) by SDS-gel-filtration chromatography on Bio-Gel A-1.5m (Figure 7) revealed that most of the radiolabel in this fraction was covalently associated with a high-molecular-weight component which eluted in the void volume (Appendix V) and, therefore, must have had a molecular weight close to or in excess of the exclusion limit of the gel, which was about 400,000 daltons under the conditions of analysis used (Appendix VI). On treatment of the same radiolabelled cell-envelope fraction with lysozyme prior to solubilizing with SDS, an identical gel-filtration analysis now revealed that most of the recovered radiolabel, about 70%, eluted as low-molecular-weight components (fractions 110-140) having molecular weights below 14,000 daltons (Figure 8). Except for a barely detectable amount of radioactivity which eluted in the void volume and accounted for only about 1% of the recovered radiolabel, the remaining radiolabel eluted in the molecular-weight range of 14,000-66,000 daltons (Figure 8). These results clearly demonstrated that there was a covalent association of radiolabel with the peptidoglycan which is present in the cell-envelope fraction.

In an attempt to determine whether the low-molecular-weight radiolabelled components obtained from lysozyme-treated radiolabelled cell envelope might contain protein, a gel-filtration analysis identical to the one described above was repeated, and the 280-nanometre absorbance profiles of untreated (Figure 9) and lysozyme-treated (Figure 10) radiolabelled cell envelope were compared. The SDS-gel-filtration analysis of the lysozyme-treated radiolabelled cell envelope showed a small 280-nanometre absorbance peak (fractions 115-130) which eluted in the region corresponding to the low-molecular-weight radiolabelled components (fractions 110-135). However, an identical SDS-gel-filtration analysis of the same radiolabelled cell envelope which was not treated with lysozyme also contained an identical 280-

Figure 7. SDS-Gel-Filtration Chromatographic Analysis of Untreated Radiolabelled Cell Envelope Isolated from Glucose-Grown Whole Cells of *Pseudomonas putida* after 24-Hour Incubation with 4-Deoxy-4-fluoro-D-[U- 14 C]glucose.

Glucose-grown whole cells of *P. putida* were incubated with 1.0 mM D-[U- 14 C]4FG for 24 hours, dialyzed and then fractionated as described in the legend under Table 1. A suspension of radiolabelled cell envelope (Incubate No. 2, Table 1) was divided into two equal portions which were incubated for 1 hour at 30°C in 0.1 M sodium phosphate buffer (SPB), pH 7.1, either with no lysozyme added, as shown here for the untreated radiolabelled cell envelope, or with lysozyme added to a final concentration of 1 mg/mL in the case of the lysozyme-treated radiolabelled cell envelope (*vide infra*, Figure 8). Subsequently, a denaturing SDS gel filtration of untreated or lysozyme-treated radiolabelled cell envelope, after each had been solubilized with 1% (w/v) SDS in 0.1 M SPB, pH 7.1, at 65°C for 30 minutes, was performed on a 180 ± 4 -mL bed of Bio-Gel A-1.5m as described in Materials and Methods, using 0.1 M SPB, pH 7.2, containing 0.1% (w/v) SDS and 0.05% (w/v) sodium azide, for equilibration and elution. 1.22 ± 0.05 -mL fractions were collected, assayed spectrophotometrically at 280 nanometres for protein and then counted for radioactivity by liquid scintillation counting as described in Materials and Methods. Virtually all of the radiolabel in the untreated radiolabelled cell envelope eluted as a single peak in the void volume. The void volume (61 ± 3 mL) and the approximate exclusion limit (400,000 daltons) of the gel were determined as described in Appendix V and Appendix VI. Bromophenol blue eluted in fractions 160-167. Radioactivity applied: 870 dpm. Radioactivity recovered in fractions 48-56: 710 dpm (~82%).

Figure 8. SDS-Gel-Filtration Chromatographic Analysis of Lysozyme-Treated Radiolabelled Cell Envelope Isolated from Glucose-Grown Whole Cells of *Pseudomonas putida* after 24-Hour Incubation with 4-Deoxy-4-fluoro-D-[U- ^{14}C]glucose.

The isolation and lysozyme treatment of radiolabelled cell envelope and its subsequent gel filtration on a 180 ± 4 -mL bed of Bio-Gel A-1.5m was performed as described in the legend under Figure 7 (*vide supra*). 1.22 ± 0.05 -mL fractions were collected and assayed for protein by direct spectrophotometric measurement of their absorbance at 280 nanometres and for radioactivity by liquid scintillation counting as described in Materials and Methods. The elution volumes of several protein standards were determined from separate chromatographic runs on the same column as described in Appendix V and are indicated by vertical bars which are labelled with the molecular weight of the corresponding protein standard. The molecular weights of 14,600 and 66,000 represent chicken egg white lysozyme and bovine serum albumin, respectively. The approximate exclusion limit of the gel and the void volume (61 ± 3 mL) of the gel bed, both of which are represented by the vertical bar at 400,000 daltons, were determined as described in Appendix V and Appendix VI. Bromophenol blue eluted in fractions 160-167. Radioactivity applied: 890 dpm. Radioactivity recovered: fractions 48-56, 10 dpm ($\sim 1\%$); fractions 75-100, 250 dpm ($\sim 28\%$); fractions 110-140, 610 dpm ($\sim 69\%$).

Figure 9. SDS-Gel-Filtration Chromatographic Analysis of Untreated Radiolabelled Cell Envelope Isolated from Glucose-Grown Whole Cells of *Pseudomonas putida* after 24-Hour Incubation with 4-Deoxy-4-fluoro-D-[U- 14 C]glucose.

Glucose-grown whole cells (600 mg protein) of *P. putida* were incubated with 1.0 mM D-[U- 14 C]4FG (specific activity, 10,600 dpm/micromole) in sterile 0.1 M potassium phosphate buffer, pH 7.1, in a final volume of 100 mL, at 30°C, on a rotary shaker, for 24 hours. After collecting the supernatant fraction by centrifugation at 10,000 $\times$ g for 10 minutes at 30°C, the resulting cell pellet was ultrasonicated and fractionated to obtain a radiolabelled cell-envelope fraction as described in Materials and Methods. Two equal portions of a suspension of radiolabelled cell envelope were then incubated for 2 hours at 30°C in 0.1 M sodium phosphate buffer (SPB), pH 7.1, either with no lysozyme or with lysozyme added to a final concentration of 1 mg/mL. After solubilizing with 1% (w/v) SDS in 0.1 M SPB, pH 7.1, at 70°C for 30 minutes, the untreated and lysozyme-treated (*vide infra*, Figure 10) radiolabelled cell envelope were analyzed by denaturing SDS gel filtration on a 182 $\pm$ 4-mL bed of Bio-Gel A-1.5m as described in Materials and Methods. 1.38 $\pm$ 0.02-mL fractions were collected, assayed for protein at 280 nanometres and then counted for radioactivity by liquid scintillation counting as described in Materials and Methods. Bromophenol blue eluted in fractions 147-164. Radioactivity applied: 560 dpm.

Figure 10. SDS-Gel-Filtration Chromatographic Analysis of Lysozyme-Treated Radiolabelled Cell Envelope Isolated from Glucose-Grown Whole Cells of *Pseudomonas putida* after 24-Hour Incubation with 4-Deoxy-4-fluoro-D-[U- ^{14}C]glucose.

The isolation and lysozyme treatment of radiolabelled cell envelope and its subsequent gel filtration on a 181 ± 4 -mL bed of Bio-Gel A-1.5m was performed as described in the legend under Figure 9 (*vide supra*). 1.37 ± 0.02 -mL fractions were collected, assayed for protein at 280 nanometres and then counted for radioactivity by liquid scintillation counting as described in Materials and Methods. Bromophenol blue eluted in fractions 147-163. Radioactivity applied: 630 dpm.

nanometre absorbance peak (fractions 115-130) in that same region. Based on this comparative analysis, therefore, no evidence for the presence of a radiolabelled protein could be demonstrated. Again, the results of this analysis is consistent with the incorporation of radiolabel into a low-molecular-weight (less than 14,000 daltons) component which appears to be covalently associated with the peptidoglycan.

Does 4FG Alkylate a Cell-Envelope Protein?

The results presented above do not exclude the possibility that the radiolabel may have been incorporated into a low-molecular-weight protein or peptide which could be covalently linked to the peptidoglycan, since such a protein or peptide may not have been present in sufficient quantity or may have been lacking in the amino acids (tyrosine, tryptophan) required for detection at 280 nanometres. Therefore, the possibility that 4FG may be alkylating a cell-envelope protein as proposed in earlier studies (78,89) cannot be excluded. A candidate protein which would fulfil the requirements of having a low molecular weight and being covalently linked to the peptidoglycan would be an outer-membrane lipoprotein analogous to the 7,200-dalton lipoprotein of *Escherichia coli* (131) or the 8,000-dalton lipoprotein of *Pseudomonas aeruginosa* (100). Whether an analogous protein exists in *P. putida* is not known.

As was previously mentioned, the alkylation of a cell-envelope protein by 4FG was suggested to occur with a loss of fluorine as free fluoride ion. According to this view, the putative alkylation of a cell-envelope protein would proceed without incorporation of covalently bonded fluorine into the cell envelope. An analysis of an SDS-solubilized preparation of radiolabelled cell envelope by Fourier transform fluorine-19 nuclear magnetic resonance (NMR) spectroscopy failed to ascertain the presence of fluorine (for details refer to "Labelling and Fluorine-19 NMR Analysis of Cell Envelope" in Materials and Methods). Due to the low level of incorporation of radiolabel into the cell envelope and limitations in the quantity of sample which could be analyzed, it was concluded that there was not a sufficient quantity of

radiolabel present that would enable fluorine to be detected.

Although the chemical nature of the peptidoglycan-associated radiolabel and the biochemical events leading to its incorporation into the peptidoglycan remain to be established, it may be worth speculating on another plausible hypothesis that could account for the incorporation of radiolabel into peptidoglycan as an alternative to the previously proposed alkylation theory. One plausible explanation which would be consistent with the results presented thus far would be an incorporation of carbon-14-labelled amino acids into the peptidoglycan *per se* or into a low-molecular-weight peptide or protein which is covalently attached to the peptidoglycan. As mentioned earlier, as much as about 5% of the total radiolabel from D-[U- ^{14}C]4FG was converted to $^{14}\text{CO}_2$ under conditions similar to those used in preparing radiolabelled cell envelope. It is possible, therefore, that some of the liberated $^{14}\text{CO}_2$ was incorporated into the amino acid biosynthetic pathways via carboxylation of pyruvate to yield radiolabelled oxaloacetate (132). Oxaloacetate could then give rise to aspartate and glutamate, both of which serve as precursors for the synthesis of numerous other amino acids (glutamate—glutamine, proline and arginine; aspartate—asparagine, diaminopimelate, lysine, methionine, threonine and isoleucine) (132).

In support of this hypothesis, recent studies in these laboratories have indicated that the peptidoglycan-associated radiolabel is due to the presence of radiolabelled threonine and radiolabelled glutamate and/or aspartate (133). These studies were based on a high-pressure-liquid-chromatographic analysis of acid-hydrolyzed, purified radiolabelled peptidoglycan isolated from radiolabelled cell envelope which was prepared from a 24-hour incubation of glucose-grown whole cells of *P. putida* with 1 mM D-[U- ^{14}C]4FG under the same conditions reported here. Whether these radiolabelled amino acids are normal constituents of the peptidoglycan or whether they are remnants of covalently attached peptidoglycan-associated protein, which may have survived the proteolytic digestion with pronase used for purification of the peptidoglycan, remains to be established.

By analogy with the proposed incorporation of $^{14}\text{CO}_2$ into amino acids, a similar process may have accounted for the incorporation of radiolabel from D-[6- ^3H]4FG into the cell envelope in previous studies (89). The extensive liberation of tritiated water from D-[6- ^3H]4FG that was encountered in those earlier studies raises the possibility of an incorporation of tritium into amino acids, a possibility which had not been considered previously (89). For example, the alanine racemase of *E. coli* catalyzes an incorporation of tritium into alanine when the racemization is conducted in the presence of $^3\text{H}_2\text{O}$ (40). The radiolabelled alanine might then be incorporated into peptidoglycan or proteins present in the cell envelope. In fact, electrophoretic analyses of radiolabelled cell envelope by urea-SDS PAGE in these previous studies revealed that all of the recovered radiolabel remained at the origin and had been excluded from penetrating into a gel system which had an exclusion limit of about 150,000 daltons (89). Although the radiolabel remaining at the origin was interpreted as being "probably an artifact", this finding would be consistent with an incorporation of tritium into the peptidoglycan and would also be consistent with the results of the gel-filtration analyses of radiolabelled cell envelopes obtained by labelling with D-[U- ^{14}C]4FG (Figures 7-10).

Borate Anion-Exchange Chromatography of Radiolabelled Supernatants

Since more than 50% of the total radioactivity was recovered in the supernatant fraction following 24-hour incubations of glucose-grown whole cells of *P. putida* with D-[U- ^{14}C]4FG, the supernatant fractions (Table 1) were analyzed for radiolabelled metabolites by borate anion-exchange column chromatography. A previous gel-filtration analysis (not shown) of this supernatant fraction had revealed that it contained only low-molecular-weight radiolabelled material under 14,000 daltons. When supernatant fractions were eluted on a borate anion-exchange column with an increasing linear ammonium-tetraborate gradient, the radiolabel was resolved into a poorly retained component, referred to as the minor-peak metabolite, and a more strongly retained component, referred to as the major-peak metabolite (Figure 11). About 90% of the radiolabel recovered in these two components was

present in the major-peak metabolite. An identical chromatographic analysis of a sample obtained from a 24-hour incubation of 1.0 mM D-[U- 14 C]4FG with 0.1 M PPB, pH 7.1, at 30°C in the absence of cells, which resulted in less than 1% release of F⁻, yielded a single peak of radioactivity corresponding to unchanged 4FG (Figure 12). In contrast to the results from previous studies using D-[6- 3 H]4FG, where a single unidentified radiolabelled metabolite had been detected in the supernatant fraction (89), the results presented here clearly indicate the presence of two radiolabelled metabolites of D-[U- 14 C]4FG in the supernatant fraction.

It may be appropriate to note that, based on the similarity in retention between the major-peak metabolite and glucose-1-phosphate in the borate anion-exchange chromatographic system (Figure 11), the chromatographic behaviour of the major-peak metabolite is consistent with a compound which elutes as a divalent anionic species. In contrast to this, the minor-peak metabolite, eluting just slightly before the poorly retained non-anionic sucrose, has a chromatographic demeanour consistent with a non-anionic compound which is incapable of forming an anionic borate complex. It may be of interest to note that, after reviewing some of the literature on borate anion-exchange chromatography of carbohydrates, two compounds which have been reported to have a similar retention in borate anion-exchange chromatographic systems to that of the minor-peak metabolite are hydroxymethyl furfural (134) and deoxyribose (presumably 2-deoxyribose) (135).

Identification of the Major Metabolite Present in Supernatants

Following an isolation of the radioactive fractions corresponding to the major-peak metabolite from a preparative borate anion-exchange column (Appendix VII), a single radiolabelled product which appeared to be pure by silica-gel thin-layer chromatography ($R_f = 0.77$ using an ethyl acetate-acetic acid-water, 3:3:1 v/v, solvent system) was obtained (Figure 13). In that same thin-layer chromatographic analysis, a control sample, referred to as the major-peak control, isolated from the supernatant of a glucose-grown whole-cell suspension of *P. putida* which had been

Figure 11. Borate Anion-Exchange Chromatographic Analysis of Radiolabelled Metabolites in the Supernatant Fraction Obtained after a 24-Hour Incubation of Glucose-Grown Whole Cells of *Pseudomonas putida* with 4-Deoxy-4-fluoro-D-[U- 14 C]glucose.

Supernatant fractions obtained from the 24-hour incubations of glucose-grown whole cells of *P. putida* with D-[U- 14 C]4FG, presented in Table I (*vide supra*), were analyzed on a Dowex 1-X8 borate anion-exchange column (108-cm x 1-cm bed) as described in Materials and Methods. An increasing, linear gradient of ammonium tetraborate (600 mL of 0.029 M ammonium tetraborate/0.057 M boric acid, pH 8.5-8.8, diluted linearly with 600 mL of 0.5 M ammonium tetraborate, pH 8.9-9.2) was used for elution. Sucrose, 4FG (4-fluoroglucose), fructose, glucose, glucose-1-phosphate and glucose-6-phosphate were added to a sample of the radiolabelled supernatant prior to its application onto the column and served as internal standards. 4.2 ± 0.2 -mL fractions were collected and analyzed for inorganic phosphate, for carbohydrate by orcinol-sulfuric acid colorimetry and for radioactivity by liquid scintillation counting as described in Materials and Methods. The radioactivity, monitored in each fraction from 1-250 and expressed in counts per minute (cpm), represents the count rate obtained from a 1.00 ± 0.01 -mL aliquot of each collected fraction. Inorganic phosphate (not shown) eluted in fractions 142-152. Glucose-6-phosphate (also not shown) eluted in fractions 181-201. Radioactivity applied: 9,630 dpm. Radioactivity recovered: fractions 13-19, 390 dpm (~4%); fractions 98-115, 3,740 dpm (~39%).

Figure 12. Borate Anion-Exchange Chromatographic Analysis of 4-Deoxy-4-fluoro-D-[U- 14 C]glucose after a 24-Hour Incubation in the Absence of *Pseudomonas putida*.

1.0 mM D-[U- 14 C]4FG (specific activity, 10,600 dpm/micromole) was incubated with sterile 0.1 M potassium phosphate buffer, pH 7.1, in a final volume of 20 mL for 24 hours at 30°C on a rotary shaker. This incubate was subsequently analyzed on a Dowex 1-X8 borate anion-exchange column (108-cm x 1-cm bed), using the same internal standards and methods of elution and detection described for the chromatographic analysis presented in Figure 11 (*vide supra*). 3.5 ± 0.1 -mL fractions were collected in fractions 1-85, and 4.0 ± 0.1 -mL fractions were collected in fractions 86-220. A single radioactive peak which coincides precisely with the elution profile of the 4FG (4-fluoroglucose) standard was observed. No other radioactive peaks were detected. The radioactivity of each collected fraction represents the count rate, expressed in counts per minute (cpm), obtained from a 1.00 ± 0.01 -mL aliquot. Radioactivity applied: 10,900 dpm. Radioactivity recovered in fractions 35-52: 9,350 dpm (~86%).

Figure 13. Silica-Gel Thin-Layer Chromatographic Analysis of Samples of the Major-Peak Metabolite (MJP) and the Major-Peak Control (MJC) Isolated by Preparative Borate Anion-Exchange Chromatography.

After a 24-hour incubation of glucose-grown whole cells of *P. putida* in the presence or absence, respectively, of 1.0 mM D-[U- ^{14}C]4FG (specific activity, 10,600 dpm/micromole) as described under "Labelling and Fractionation of Whole Cells" in Materials and Methods, samples of the MJP and the MJC were isolated by preparative borate anion-exchange chromatography (Appendix VII) as described in Materials and Methods. 2 microlitres of each of the resulting D_2O solutions of the MJP and MJC were spotted in duplicate and air-dried onto a silica gel-60 plastic-backed TLC sheet. 1 microlitre of an aqueous solution of 20.0 mM 4FG and 1 microlitre of a 1% (w/v) aqueous solution of each of the following standards was also applied in the same manner: calcium gluconate monohydrate (GA), dipotassium D-glucose-1-phosphate trihydrate (G1P) and D-glucose (GLU). After development in ethyl acetate-acetic acid-water (3:3:1 v/v), spots were visualized by spraying with sulfuric acid-ethanol (1:1 v/v) and heating at 140°C for 20 minutes. GA and the MJP were the last spots to appear. The two lanes which correspond to the MJP and the MJC and are delineated by vertical broken lines were removed prior to visualization and were later cut into 1-cm sections, delineated by horizontal broken lines, which were counted for radioactivity as described in Materials and Methods. The number in each section represents the radioactivity, expressed in counts per minute, of the section in which the number appears. Radioactivity applied: 390 dpm. Radioactivity recovered in sections A and B: 340 dpm (~87%).

incubated for 24 hours in the absence of 4FG and processed by the same purification procedure used for isolating the radiolabelled major-peak metabolite, did not show any component corresponding to the major-peak metabolite (Figure 13).

An analysis of a sample of the isolated radiolabelled major-peak metabolite by carbon-13 NMR spectroscopy revealed the presence of a 5-carbon compound whose proton-decoupled spectrum (Figure 14) was consistent with the structure of 4,5-dihydropentanoic acid (2,3-dideoxyribonic acid). The proton-coupled carbon-13 NMR spectrum of that same sample (Figure 15) displayed a splitting pattern which was also consistent with a structural assignment of 2,3-dideoxyribonic acid. The proton NMR spectrum of the isolated radiolabelled major-peak metabolite (Figure 16) yielded an integration for 7 carbon-bound protons whose structural assignments were entirely consistent with those of the carbon-13 NMR spectra. A two-dimensional (COSY) proton NMR spectroscopic analysis (not shown) also supported the conclusion that the major-peak metabolite was 2,3-dideoxyribonic acid.

In support of these findings, a subsequent negative-ion fast-atom-bombardment (FAB) mass spectrum of the isolated radiolabelled major-peak metabolite was obtained (Figure 17) and revealed an intense peak with a mass/charge (M/E) ratio of 133. This peak would correspond to the expected molecular weight of the carboxylate anion of 2,3-dideoxyribonic acid. A negative-ion FAB mass spectrum of the major-peak control did not show a peak at a M/E ratio of 133 (Figure 18). Instead, the only major new peaks observed appeared to correspond to borate-glycerol complexes, indicating the presence of residual borate salts. Similarly, the negative-ion FAB mass spectrum of the glycerol which was used as an energy transfer matrix in obtaining these spectra also did not display a peak at a M/E ratio of 133 (Figure 19).

To further confirm the identity of the major-peak metabolite, the sodium salt of 2,3-dideoxyribonic acid was synthesized (Appendix II) from a synthetic preparation of 5-hydroxy-4-valerolactone which had been obtained by a performic acid oxidation of 4-pentenoic acid (123). The proton-decoupled carbon-13 NMR

spectrum of this synthetic sodium salt of 2,3-dideoxyribonic acid (Figure 20) was found to be identical to the proton-decoupled carbon-13 NMR spectrum which had been obtained for the major-peak metabolite (Figure 14). Similarly, the proton-coupled carbon-13 NMR spectrum of this synthetic sodium salt (Figure 21) was identical to the proton-coupled carbon-13 NMR spectrum which had been acquired for the major-peak metabolite (Figure 15). In addition, the proton NMR spectrum of this synthetic sodium 2,3-dideoxyribonate (Figure 22) was also found to be identical to the proton NMR spectrum of the major-peak metabolite (Figure 16). Also, in a silica-gel thin-layer chromatographic analysis (Figure 23), the major-peak metabolite and the synthetic sodium 2,3-dideoxyribonate had identical mobilities ($R_f = 0.76$ in an ethyl acetate-acetic acid-water, 3:3:1 v/v, solvent system).

In previous studies with D-[6-³H]4FG, it was suggested that the radiolabelled metabolite isolated from the supernatant fraction was phosphorylated and reducing, based on the use of iron-sulfosalicylic acid and aniline-hydrogen phthalate chromatographic detection reagents, respectively (89). However, this is inconsistent with the chromatographic and spectroscopic data obtained for the major-peak metabolite. By the use of paper chromatography and iron-sulfosalicylic acid and aniline-acid oxalate detection reagents, there was no indication that the major-peak metabolite was phosphorylated and no indication that it was reducing. An *in vitro* assay of the isolated major-peak metabolite for inorganic phosphate present both before and after a 7-minute hydrolysis in 1 N HCl and after a total hydrolysis, was also consistent with the major-peak metabolite not being phosphorylated. Furthermore, the isolated major-peak metabolite did not give a positive color reaction by the orcinol-sulfuric acid colorimetric assay which had been used to monitor "reducing" sugar standards used for borate anion-exchange chromatography.

The identification of the major-peak metabolite as 2,3-dideoxyribonic acid would be consistent with the chromatographic behaviour of this metabolite in the borate anion-exchange chromatographic system described here. As mentioned earlier, the major-peak metabolite appears to elute as a divalent anion in this chromatographic system. Under the basic conditions of this chromatographic system,

Figure 14. Proton-Decoupled Carbon-13 Nuclear Magnetic Resonance (NMR) Spectrum of the Radiolabelled Major-Peak Metabolite Isolated from Supernatant Fractions Obtained after a 24-Hour Incubation of Glucose-Grown Whole Cells of *Pseudomonas putida* with 4-Deoxy-4-fluoro-D-[U- ^{14}C]glucose.

Following a 24-hour incubation of glucose-grown whole cells of *P. putida* with 1.0 mM D-[U- ^{14}C]4FG, the radiolabelled major-peak metabolite present in the supernatant fraction (obtained from Incubate No. 1 and No. 2 in Table 1) was purified by preparative borate anion-exchange chromatography (Appendix VII) as described under "Borate Anion-Exchange Column Chromatography" in Materials and Methods. A proton-decoupled carbon-13 NMR spectrum of the resulting D_2O solution of the isolated radiolabelled major-peak metabolite was obtained on a 300 MHz General Electric Fourier transform NMR spectrometer after 34,604 scans. Chemical-shift values are relative to tetramethylsilane and are expressed in parts per million (PPM) of the main spectrometer frequency (75.480824 MHz). The structural assignment for a given peak is indicated in the structural formula and corresponds to the carbon atom which is directly to the right of the chemical-shift value of the peak.

Figure 15. Proton-Coupled Carbon-13 Nuclear Magnetic Resonance (NMR) Spectrum of the Radiolabelled Major-Peak Metabolite Isolated from Supernatant Fractions Obtained after a 24-Hour Incubation of Glucose-Grown Whole Cells of *Pseudomonas putida* with 4-Deoxy-4-fluoro-D-[U-¹⁴C]glucose.

The same sample of the D₂O solution of the radiolabelled major-peak metabolite which was used to obtain the proton-decoupled carbon-13 NMR spectrum shown in Figure 14 was used to generate this proton-coupled carbon-13 NMR spectrum on a 300 MHz General Electric Fourier transform NMR spectrometer after 36,000 scans. Chemical-shift values are relative to tetramethylsilane and are expressed in parts per million (PPM) of the main spectrometer frequency (75.480824 MHz). The structural assignment for a given peak is indicated in the structural formula and corresponds to the carbon atom which is directly to the right of the observed splitting pattern and chemical-shift value of the peak.

Figure 16. Proton Nuclear Magnetic Resonance (NMR) Spectrum of the Radiolabelled Major-Peak Metabolite Isolated from Supernatant Fractions Obtained after a 24-Hour Incubation of Glucose-Grown Whole Cells of *Pseudomonas putida* with 4-Deoxy-4-fluoro-D-[U- ^{14}C]glucose.

From the same sample of the D_2O solution of the radiolabelled major-peak metabolite which was used to obtain the proton-decoupled and proton-coupled carbon-13 NMR spectra shown in Figure 14 and Figure 15, respectively, this proton NMR spectrum was acquired on a 300 MHz General Electric Fourier transform NMR spectrometer after 8 scans. Chemical-shift values are relative to tetramethylsilane and are expressed in parts per million (PPM) of the main spectrometer frequency (300.3 MHz). The structural assignment for a given peak is indicated in the structural formula and corresponds to the carbon-bound proton (shown in bold) which is directly to the left of the chemical-shift value at which the peak was centered. The peaks occurring at 1.90 and 3.34 PPM represent minor impurities which were ignored in the interpretation of the spectrum.

Figure 17. Negative-Ion Fast-Atom-Bombardment (FAB) Mass Spectrum of the Radiolabelled Major-Peak Metabolite (M/E 133) Isolated from Supernatant Fractions Obtained after a 24-Hour Incubation of Glucose-Grown Whole Cells of *Pseudomonas putida* with 4-Deoxy-4-fluoro-D-[U- ^{14}C]glucose.

The same sample of the D_2O solution of the radiolabelled major-peak metabolite which had been isolated by preparative borate anion-exchange chromatography and analyzed by carbon-13 and proton NMR as described in Figures 14, 15 and 16, was submitted to negative-ion FAB mass spectrometry on a Finnigan-mat CH5-DF magnetic sector mass spectrometer. Xenon atoms from a FAB gun set at a power of 4 watts (4 kV x 1 mA) were used as the projectiles, while glycerol was used as a sample matrix. The ordinate scale represents the relative percent abundance of negative ions, and the abscissa represents the mass to charge (M/E) ratio of a given negative ion. The intense peak (at the arrow) with a M/E ratio of 133, which was absent in both the negative-ion FAB mass spectrum of the major-peak control (*vide infra*, Figure 18) and of glycerol (*vide infra*, Figure 19), corresponds to the expected molecular weight of the carboxylate anion of 2,3-dideoxyribonic acid (molecular weight, 134). The intense peaks with M/E ratios of 91 and 183 represent monomeric and dimeric anions of glycerol, respectively (cf. Figure 19). The peak with a M/E ratio of 191 is probably a 2:1 glycerol-borate complex which was also observed in the major-peak control (cf. Figure 18).

Figure 18. Negative-Ion Fast-Atom-Bombardment (FAB) Mass Spectrum of the Major-Peak Control Isolated from the Supernatant Fraction Obtained after a 24-Hour Incubation of Glucose-Grown Whole Cells of *Pseudomonas putida* in the Absence of 4-Deoxy-4-fluoro-D-[U- 14 C]glucose.

A supernatant fraction was obtained from a 24-hour incubation of glucose-grown whole cells of *P. putida* in the absence of D-[U- 14 C]4FG as described under "Labelling and Fractionation of Whole Cells" in Materials and Methods. The major-peak control was isolated from this supernatant by the same procedures of preparative borate anion-exchange chromatography and purification which were used for isolating the radiolabelled major-peak metabolite and are described under "Borate Anion-Exchange Column Chromatography" in Materials and Methods. The resulting D₂O solution of the major-peak control was then analyzed by negative-ion FAB mass spectrometry with the same instrumentation and under the same conditions which were used for the negative-ion mass spectrometric analysis of the radiolabelled major-peak metabolite and are described in the legend under Figure 17. The peaks (at the arrows) with a M/E ratio of 117 and 191 were interpreted as corresponding to the glycerol-borate complexes whose structural formulas are depicted.

Figure 19. Negative-Ion Fast-Atom-Bombardment (FAB) Mass Spectrum of Glycerol.

The instrumentation and conditions used in this negative-ion FAB mass spectrometric analysis of the glycerol which was used as an energy transfer matrix in the negative-ion FAB mass spectrometric analysis of the radiolabelled major-peak metabolite (*vide supra*, Figure 17) and the major-peak control (*vide supra*, Figure 18) were as described in the legend under Figure 17. The intense peaks with a M/E ratio of 91 and 183 correspond to monovalent anions of monomeric and dimeric glycerol, i.e., glycerol minus a proton and a glycerol dimer minus a proton, respectively.

Figure 20. Proton-Decoupled Carbon-13 Nuclear Magnetic Resonance (NMR) Spectrum of the Synthetic Sodium 2,3-Dideoxyribonate.

The sodium salt of 2,3-dideoxyribonic acid was prepared from synthetic 5-hydroxy-4-valerolactone (123) as described in Appendix II. A proton-decoupled carbon-13 NMR spectrum of a solution of the resulting purified syrup of sodium 2,3-dideoxyribonate in D₂O was acquired after 907 scans on a Bruker 300 MHz Fourier transform NMR spectrometer. 1,4-Dioxane in D₂O was used as an external chemical-shift standard. Chemical-shift values are relative to tetramethylsilane and are expressed in parts per million (PPM) of the main spectrometer frequency (75.469 MHz).

Figure 21. Proton-Coupled Carbon-13 Nuclear Magnetic Resonance (NMR) Spectrum of the Synthetic Sodium 2,3-Dideoxyribonate.

The same sample of the synthetic sodium 2,3-dideoxyribonate in D_2O which was used to obtain the proton-decoupled carbon-13 NMR spectrum presented in Figure 20 was used to acquire this proton-coupled carbon-13 NMR spectrum after 1,255 scans on a Bruker 300 MHz Fourier transform NMR spectrometer. 1,4-Dioxane in D_2O was used as an external chemical-shift standard. Chemical-shift values are relative to tetramethylsilane and are expressed in parts per million (PPM) of the main spectrometer frequency (75.469 MHz).

Figure 22. Proton Nuclear Magnetic Resonance (NMR) Spectrum of the Synthetic Sodium 2,3-Dideoxyribonate.

The same sample of the solution of the sodium 2,3-dideoxyribonate in D_2O which was used to generate the proton-decoupled carbon-13 NMR spectrum presented in Figure 20 (*vide supra*) was used to acquire this proton NMR spectrum after 16 scans on a Bruker 300 MHz Fourier transform NMR spectrometer. Methanol in D_2O was used as an external chemical-shift standard. Chemical-shift values are relative to tetramethylsilane and are expressed in parts per million (PPM) of the main spectrometer frequency (300.134 MHz).

Figure 23. Silica-Gel Thin-Layer Chromatography (TLC) of the Major-Peak (MJP) and Minor-Peak (MNP) Metabolites, the Minor-Peak Control (MNP-CTRL), the Synthetic Sodium 2,3-Dideoxyribonate (4,5-DHPA SS) and Synthetic 5-Hydroxy-4-valerolactone (L).

The radiolabelled major-peak metabolite (MJP), the radiolabelled minor-peak metabolite (MNP) and the minor-peak control (MNP-CTRL) were obtained as described under "Labelling and Fractionation of Whole Cells" and "Borate Anion-Exchange Column Chromatography" in Materials and Methods. The synthetic sodium salt of 2,3-dideoxyribonic acid (4,5-DHPA SS) was prepared from synthetic 5-hydroxy-4-valerolactone (L) as described in Appendix II. The following samples were spotted and air-dried onto a silica gel-60 plastic-backed TLC sheet: 2 microlitres each of a 1% (w/v) aqueous solution (aq. sol.) of 4-pentenoic acid (4PA), a 1% (w/v) aq. sol. of L, an aq. sol. of purified L (PL) obtained after lyophilization (*vide* Appendix II), and an aq. sol. of 20 mM 4FG; 4 microlitres each of a 1% (w/v) aq. sol. of the purified syrup of 4,5-DHPA SS and a D₂O solution of MJP; 6 microlitres each of a D₂O solution of MNP and a D₂O solution of MNP-CTRL. After development in ethyl acetate-acetic acid-water (3:3:1 v/v), 4PA was visualized with iodine vapour prior to detection of the other spots by spraying with sulfuric acid-ethanol (1:1 v/v) and charring at 140°C for 20 minutes as described in Materials and Methods. The first spots to appear after about 5 minutes of charring at 140°C were the one from 4FG, the two spots from MNP and the spot from MNP-CTRL. All other spots, except 4PA, were detected, later, after 20 minutes of charring at 140°C.

2,3-dideoxyribonic acid, with its two vicinal *cis* hydroxyl groups, would be expected to combine with an equivalent of borate ion (136), thus forming a divalent anionic complex.

Characterization of the Minor Metabolite Present in Supernatants

A silica-gel thin-layer chromatographic analysis (Figure 23) of the radiolabelled minor-peak metabolite which was isolated by preparative borate anion-exchange chromatography (Appendix VII) revealed that it had a mobility ($R_f = 0.75$ in an ethyl acetate-acetic acid-water, 3:3:1 v/v, solvent system) nearly identical to that of the major-peak metabolite. The supernatant fraction from a 24-hour incubation of glucose-grown whole cells of *P. putida* in the absence of 4FG yielded, by the same methods used for isolating the minor-peak metabolite, a minor-peak control which did not contain a component corresponding to the minor-peak metabolite (Figure 23). A notable difference between the minor-peak metabolite and either the major-peak metabolite, the synthetic sodium 2,3-dideoxyribonate, or the synthetic 5-hydroxy-4-valerolactone was observed in this thin-layer chromatographic analysis: namely, the greater susceptibility of the minor-peak metabolite to charring at 140°C after the chromatogram had been sprayed with sulfuric acid-ethanol (Figure 23).

As mentioned previously, the behaviour of the minor-peak metabolite in the borate-anion-exchange chromatographic system used in these studies would be consistent with a non-anionic compound which does not complex with borate ion. Based on the identification of the major-peak metabolite as 2,3-dideoxyribonic acid, one might have anticipated, therefore, that the minor-peak metabolite could be a lactonized form of the major-peak metabolite. However, the differential susceptibility to charring which was noted above strongly suggests that the minor-peak metabolite is neither 2,3-dideoxyribonic acid nor a lactone of 2,3-dideoxyribonic acid (*i.e.*, 5-hydroxy-4-valerolactone).

The relatively high mobility and facile detection by charring observed for the minor-peak metabolite suggests that it may be a deoxycarbohydrate (137). In conjunction with its poor retention on the borate anion-exchange column, this implies that the minor-peak metabolite may be a neutral deoxycarbohydrate which does not form a complex with borate ion. Work is now in progress in these laboratories to purify the minor-peak metabolite and ascertain its identity.

Incubation of D-[U-¹⁴C]4FG with Crude Outer Membranes

Since previous studies had implicated the outer membrane of glucose-grown *P. putida* in the defluorination of 4FG (79,89), a comparison of the products obtained from incubations of outer membranes and 4FG to those which had been obtained from incubations of whole cells and 4FG was considered to be of interest. Crude outer membranes from glucose-grown whole cells of *P. putida* were isolated, therefore, and incubated with D-[U-¹⁴C]4FG by the same method and under the same conditions used previously (79,89). The preparation of crude outer membranes was based on the release of outer membranes during the lysozyme-induced formation of spheroplasts and recovering the outer membranes from the supernatant of the spheroplast formation medium by ultracentrifugation.

Following a 24-hour incubation of a crude-outer-membrane preparation (5.1 mg protein) with 1.0 mM D-[U-¹⁴C]4FG (specific activity, 10,600 dpm/micromole) in 0.1 M PPB, pH 7.1, at 30°C, and in a final incubation volume of 3.0 mL, a fluoride ion determination on the supernatant, obtained after removal of crude outer membranes, indicated there had been a $9.7 \pm 0.5\%$ release of F⁻. In terms of specific defluorinating activity, this amounted to 57 nanomoles of F⁻ released per milligram of outer-membrane protein—a value that is about fivefold to sixfold lower than previously reported (79,89).

The supernatant fraction, which accounted for all of the radioactivity used for this incubation, was then analyzed for radiolabelled metabolites by borate anion-

exchange chromatography (Figure 24). Surprisingly, this analysis revealed that all of the D-[U-¹⁴C]4FG had disappeared and had been converted to a highly anionic minor component which accounted for about 12% of the applied radioactivity and, therefore, is labelled "NFM" to indicate that it is probably a non-fluorinated metabolite, and to a highly anionic major component which is labelled "FM" to indicate that it is probably a fluorinated metabolite (Figure 24). Clearly, these metabolites differ from those obtained from whole-cell incubations.

The strong retention of the metabolites obtained from this incubation with outer membranes was unexpected. One possibility that may account for the apparently strong anionic character of these metabolites could be that they may be aldonic acids arising from the oxidation of 4FG. Previous studies have demonstrated that cell-free extracts or membrane vesicles prepared from glucose-grown *P. putida* are able to oxidize 4FG to 4-deoxy-4-fluoro-2-keto-D-gluconic acid (4F2KGA) (78,79,89). This oxidation is mediated by cytoplasmic-membrane-bound glucose and gluconate dehydrogenases which constitute an extracellular oxidative pathway for glucose utilization in pseudomonads (138,139). It is possible that some contaminating cytoplasmic-membrane-bound dehydrogenases, present in the crude-outer-membrane preparation, may have caused an oxidation of 4FG to 4-deoxy-4-fluoro-D-gluconic acid (4FGA) or 4F2KGA. Since 4FGA and 4F2KGA would be expected to form a trivalent and a divalent anionic borate complex, respectively, they would be expected to have a retention equal to or less than, respectively, that of glucose-6-phosphate (which presumably forms a trivalent borate complex) under the conditions used for borate anion-exchange chromatographic analysis (Figure 24). For example, in a similar borate anion-exchange chromatographic analysis, it was found that gluconic acid and 2-ketogluconic acid eluted, respectively, slightly after and slightly before the glucose-6-phosphate peak which was incompletely separated from both. However, the radiolabelled metabolites obtained from this incubation with crude outer membranes were well separated from and eluted well after glucose-6-phosphate, suggesting that they may be more negatively charged, possibly tetravalent, species which have a greater retention than would be anticipated for 4FGA and 4F2KGA or even defluorinated derivatives of 4FGA and 4F2KGA. This

Figure 24. Borate Anion-Exchange Chromatographic Analysis of a Supernatant Fraction Obtained after a 24-Hour Incubation of 4-Deoxy-4-fluoro-D-[U- 14 C]glucose with a Crude-Outer-Membrane Preparation Derived from Glucose-Grown Whole Cells of *Pseudomonas putida*.

Crude outer membrane was prepared from glucose-grown whole cells of *P. putida* as described in Materials and Methods. Following a 24-hour incubation of crude outer membranes (5.1 mg protein) with 1.0 mM D-[U- 14 C]4FG (specific activity, 10,600 dpm/micromole) in 0.1 M PPB, pH 7.1, at 30°C, and in a final incubation volume of 3.0 mL, the supernatant fraction was collected by centrifugation at 100,000 $\times$ g for 60 minutes at 25°C. Fluoride ion measurements (see Materials and Methods) on this supernatant fraction revealed a $9.7 \pm 0.5\%$ release of F^- had occurred. A determination of the radioactivity in this supernatant by liquid scintillation counting (see Materials and Methods) indicated 100% of the initial radioactivity was recovered in the supernatant fraction. The supernatant was then analyzed for radiolabelled metabolites on a Dowex 1-X8 borate anion-exchange column (108-cm $\times$ 1-cm bed) as described in Materials and Methods. 5.1 $\pm$ 0.1-mL fractions were collected and analyzed for carbohydrate by orcinol-sulfuric acid colorimetry and for radioactivity by liquid scintillation counting as described in Materials and Methods. The radioactivity in each collected fraction is expressed in counts per minute (cpm) and represents the count rate obtained from a 1.00 $\pm$ 0.01-mL aliquot of fraction. Fractions 170-220 gave no color reaction by the orcinol-sulfuric acid colorimetric assay. Radioactivity applied: 27,300 dpm. Radioactivity recovered: fractions 170-191, 3,250 dpm ($\sim 12\%$); fractions 192-220, 18,200 dpm ($\sim 67\%$). NFM is presumably a non-fluorinated metabolite, whereas FM is presumably a fluorinated metabolite.

greater retention, therefore, would seem to argue against these radiolabelled metabolites being 4FGA and 4F2KGA or their defluorinated derivatives.

An alternative possibility that would appear to be more consistent with the retention of these radiolabelled metabolites is that they may be phosphorylated aldonic acid derivatives. For example, C-6 phosphorylated 4FGA and 4F2KGA or their defluorinated derivatives would be expected to form tetravalent anionic borate complexes which may have retentions similar to those observed for the radiolabelled metabolites. Hopefully, this possibility will be examined in future studies after an appropriate purification of these radiolabelled metabolites. The borate anion-exchange chromatographic system would appear to be a useful means for their isolation.

The possibility of phosphorylated metabolites arising from incubation of crude outer membranes with 4FG raises an important question. Since phosphorylated metabolites are normally products of intracellular metabolism, is it possible that phosphorylated metabolites may have arisen from a cellular contaminant which was present in the crude-outer-membrane preparation? In fact, the possibility that the seemingly low defluorinating activity attributed to crude-outer-membrane preparations may have been due to a spheroplast or whole-cell contaminant is a matter which had not been considered in the past (89). Since the defluorination of 4FG by glucose-grown whole cells of *P. putida* is known to be inhibited substantially by chloramphenicol, this possibility could readily be tested by conducting incubations of crude-outer-membrane preparations and 4FG in the presence and absence of chloramphenicol. If spheroplast or whole-cell contamination is responsible for the defluorinating activity observed in crude-outer-membrane preparations, then the presence of chloramphenicol would be expected to considerably inhibit the defluorination of 4FG. Recent studies, in these laboratories, which tend to substantiate this view have revealed, in fact, that the presence of chloramphenicol completely inhibits the defluorination of 4FG by crude-outer-membrane preparations and substantially reduces the amount of cellular contamination present in these preparations after a 24-hour incubation (140).

Clues to a Metabolic Pathway for 4FG in *Pseudomonas putida*

Many unanswered questions, particularly regarding transport and formation of intracellular metabolites, must be answered before a definitive pathway for the metabolism of 4FG in *P. putida* can be proposed. The results presented thus far, however, may provide some important clues for future studies.

Firstly, it appears that the 6-carbon 4FG is being mainly metabolized to the 5-carbon 2,3-dideoxyribonic acid by a process which involves the loss of one of the terminal carbon atoms. Since approximately as much as 5% of the total radiolabel recovered from incubations of glucose-grown whole cells of *P. putida* with D-[U-¹⁴C]4FG was found in a volatile product which was presumably ¹⁴CO₂, it would seem reasonable to suggest that the loss of a terminal carbon atom probably occurs through a decarboxylation reaction. The formation of ¹⁴CO₂ might be confirmed in future studies by gas or anion-exchange chromatographic analysis. Furthermore, future studies could establish whether the loss of terminal carbon occurs at the C-1 terminus or whether it occurs at the C-6 terminus by the use of D-[1-¹⁴C]4FG.

Secondly, the 5-carbon metabolite, 2,3-dideoxyribonic acid, may in itself contain an important structural clue which could help to elucidate the pathway of 4FG metabolism; namely, the two adjacent methylene groups occurring at the C-2 and C-3 positions. These adjacent methylene groups could be derived from the C-3 and C-4 positions of 4FG. In previous studies with D-[6-³H]4FG, the introduction of a methylene group at the C-4 position of 4FG had already been postulated to occur in order to account for the extensive loss of tritium (47,89). Thus, in the scheme proposed for the release of fluoride and the formation of tritiated water from D-[6-³H]4FG, the defluorination of 4FG was viewed as an elimination of HF between C-4 and C-5 to form a 4,5-unsaturated hexose which, by tautomeric rearrangements, could then allow an exchange of tritium at C-6 with water (47,89). As a result of these tautomeric rearrangements, a methylene group could be introduced at the C-4 position; this methylene group could then account for one of the methylene groups present in 2,3-dideoxyribonic acid. However, it is now

clear that a second, adjacent methylene group derived from the C-3 position of 4FG must be accounted for in the formation of 2,3-dideoxyribonic acid.

Since the results from earlier studies suggest that 4FG is being metabolized as a glucose analogue (e.g., defluorination is induced in cells grown on glucose and is protected by glucose), it would seem appropriate to interpret these metabolic clues in the light of some of the known pathways for glucose utilization in pseudomonads. In members of the genus *Pseudomonas*, glucose utilization occurs through a unique pathway which was first discovered by Entner and Doudoroff (110) and is known as the Entner-Doudoroff (ED) pathway (107). In this pathway, 6-phosphogluconate is dehydrated to 3-deoxy-2-keto-6-phosphogluconate by 6-phosphogluconate dehydrase (EDD), the first enzyme of the ED pathway. The 3-deoxy-2-keto-6-phosphogluconate is then cleaved to glyceraldehyde-3-phosphate and pyruvate by the action of 3-deoxy-2-keto-6-phosphogluconate aldolase (EDA), the other enzyme comprising the ED pathway. Apparently, the reaction catalyzed by EDD is the only step in the known pathways for glucose catabolism in pseudomonads where a methylene group can be introduced into the C-3 position of glucose. Assuming 4FG is being metabolized as a glucose analogue, this step is an obvious candidate for the incorporation of a methylene group into the C-3 position of 4FG and could, therefore, account for the second methylene group of 2,3-dideoxyribonic acid. The possible involvement of the EDD enzyme would also imply that 4FG may be metabolized to its 6-phosphogluconate analogue via an intracellular pathway (107).

A Plausible Route for the Defluorination of 4FG in *Pseudomonas putida*

Although there is at this time no direct evidence for the participation of the Entner-Doudoroff pathway or of any intracellular pathway in the metabolism of 4FG, the implied involvement of the Entner-Doudoroff pathway would seem to offer an attractive metabolic route for the defluorination of 4FG and the formation of 2,3-dideoxyribonic acid (Figure 25). Assuming 4FG is metabolized as far as its 6-phosphogluconate analogue (i), it may then act as a substrate for EDD. The EDD

enzyme from *P. putida*, which requires both ferrous ions and reduced glutathione for activity and is inhibited by EDTA, fluoride, cyanide, iodoacetate, and *p*-chloromercuribenzoate (113), has been shown to catalyze the dehydration of 6-phosphogluconate to the enol form of 3-deoxy-2-keto-6-phosphogluconate, which spontaneously and irreversibly rearranges to the keto form and stably incorporates a proton from water at the C-3 position in a random fashion (112). By an analogous mechanism, the 6-phosphogluconate analogue of 4FG could yield an enol which, bearing an *allylic* fluorine, could be expected to readily lose fluorine as fluoride ion (32) (this is not shown in Figure 25) or to rearrange to the corresponding 3-deoxy-2-keto-6-phosphogluconate analogue (ii). This may then act as a substrate for the EDA enzyme, which would form a Schiff-base intermediate (iii) through an ϵ -amino group of a lysine residue in the active site (119) (Figure 25).

The extensive studies of Wood and his colleagues have shown that the EDA from *P. putida* can form a Schiff base with a large number of 3-deoxy-2-keto carbonyl analogues (141), but a 6-phosphate group, a 4-hydroxyl group in the *gluco* configuration and a 3-deoxy group are all required for cleavage (117). In addition, it has been shown that the aldolase catalyzes an exchange of protons from water with all three hydrogen atoms at C-3 of pyruvate and stereospecifically with a single hydrogen atom at the corresponding carbon atom of α -ketobutyrate, bromopyruvate and β -bromo- α -ketobutyrate (142). Both proton exchange and cleavage have been proposed to be mediated by a single active-site glutamate residue (143) which, by a rotation about a carbon-carbon bond of the side chain, can bring its γ -carboxyl group into a position that would allow proton exchange to occur at the C-3 hydrogen or would promote a cleavage by deprotonation of the hydroxyl group at C-4 of 3-deoxy-2-keto-6-phosphogluconate (116,144).

The 3-deoxy-2-keto-6-phosphogluconate analogue (ii) of 4FG, because it lacks a hydroxyl group at C-4, would not be expected to undergo a cleavage by EDA. Instead, it may be deprotonated at C-3 to yield an enamine intermediate (iv) which, bearing allylic fluorine, would be expected to readily lose fluorine as F^- and form a

Figure 25. A Possible Metabolic Pathway for the Defluorination of 4-Deoxy-4-fluoro-D-glucose and the Formation of 2,3-Dideoxyribonic Acid in *Pseudomonas putida*.

In this proposed pathway, 4FG is assumed to be metabolized to its 6-phosphogluconate analogue (i). This fluorinated analogue may then act as a substrate for the first enzyme of the Entner-Doudoroff (ED) pathway: 6-phosphogluconate dehydratase (EDD). At this stage, the fluoro analogue is dehydrated to an intermediate (not shown) which either may lose allylic fluorine as F⁻ (not shown) or may be converted to the corresponding fluorinated 3-deoxy-2-keto-6-phosphogluconate analogue (ii). This latter fluoro analogue may then form a Schiff base (iii) with the other enzyme of the ED pathway: 3-deoxy-2-keto-6-phosphogluconate aldolase (EDA). The enamine form (iv) of the resulting Schiff base may then lose allylic fluorine as F⁻ to yield a β,γ-unsaturated, Schiff-base intermediate (v). This intermediate then may undergo either a series of tautomeric rearrangements via pathway B or a decarboxylation via pathway A to yield, ultimately, 2,3-dideoxyribonic acid (viii) (via pathway A1) and a 2-deoxypentose (x) (via pathway A2). Further details of the pathway are described in the text.

β,γ -unsaturated, protonated Schiff-base derivative of 3-deoxy-2-keto-6-phosphogluconate (v) (Figure 25). This β,γ -unsaturated intermediate (v) may then undergo either a series of tautomeric rearrangements (pathway B, Figure 25) which could account for the previously observed loss of tritium at C-6 of D-[6- ^3H]4FG (47,89), or a decarboxylation (pathway A, Figure 25) at C-1 to liberate carbon dioxide and to yield a carbanion-like intermediate (vi) which would presumably facilitate decarboxylation because of its stabilization through an inductive effect of the positively charged nitrogen (145,146). The resulting carbanion-like intermediate (vi) may then undergo either a rearrangement to a keteneimine intermediate (pathway A1, Figure 25) which upon hydration would yield a phosphorylated derivative of 2,3-dideoxyribonic acid that is linked to the enzyme via an amide bond (vii), a subsequent hydrolysis, and a dephosphorylation to yield 2,3-dideoxyribonic acid (viii); or a direct protonation to a Schiff-base intermediate (pathway A2, Figure 25), a hydrolysis and dephosphorylation to an α,β -unsaturated ribose derivative (ix), and a subsequent hydration by a Michael-type addition reaction, which perhaps may occur under alkaline conditions such as those used for borate anion-exchange chromatography, to yield 2-deoxyribose and/or 2-deoxyxylose (x).

The pathway for 4FG metabolism proposed above would be consistent with the formation of the two radiolabelled metabolites which were obtained from glucose-grown whole-cell incubations of *P. putida* with D-[U- ^{14}C]4FG. Firstly, the pathway offers a plausible route to 2,3-dideoxyribonic acid, the major whole-cell metabolite identified in these studies. Secondly, the pathway suggests the formation of a second metabolic product (Figure 11), a 2-deoxypentose, which would seem consistent with the observed chromatographic properties of the minor whole-cell metabolite. Additionally, the pathway suggests a decarboxylative loss of C-1 to yield CO_2 , which is consistent with the volatile radiolabelled product described in these studies. Although an oxidative decarboxylation of the 6-phosphogluconate analogue of 4FG by 6-phosphogluconate dehydrogenase would offer an alternative route to the formation of CO_2 and a pentose derivative, it has been reported that *P. putida* lacks a 6-phosphogluconate dehydrogenase (107,111).

The proposed pathway would also be consistent with a number of observations from previous studies of 4FG metabolism in *P. putida*. Thus, the induction of a defluorinating protein in cells grown on glucose, gluconate or 2-ketogluconate and its repression in cells grown on succinate or citrate (89) is consistent with the known pattern of induction and repression of the two enzymes of the Entner-Doudoroff pathway (107). Glucose, gluconate or 2-ketogluconate—all shown to extensively protect 4FG from defluorination (79,89)—would be expected to effectively inhibit defluorination because they are all converted to 6-phosphogluconate (107), which could then compete with the 6-phosphogluconate analogue of 4FG for the dehydrase. The complete protection afforded by N-ethylmaleimide against the defluorination of 4FG (79,89) is also consistent with the known inhibition of the dehydrase by iodoacetate and *p*-chloromercuribenzoate and the requirement of reduced glutathione for activation of the dehydrase (113).

The time-dependent loss of residual defluorinating activity observed in chloramphenicol-treated, glucose-grown whole cells of *P. putida* (79,89) would be consistent with another feature suggested by this pathway: the possibility of forming either an intermediate which is inherently reactive (*e.g.*, v, Figure 25) due to the presence of conjugated double bonds that are susceptible to a Michael addition reaction, or an intermediate which may be relatively stably linked to the enzyme (*e.g.*, vii). Since these intermediates might be expected to cause an irreversible loss of enzymatic activity, it is possible that the observed loss of residual defluorinating activity in chloramphenicol-treated cells may be explained by the action of the 3-deoxy-2-keto-6-phosphogluconate analogue of 4FG as a suicide inhibitor for the aldolase. To test this possibility, future studies should be performed to determine whether this time-dependent loss of defluorinating activity is caused by the metabolism of 4FG and subsequent inactivation of the aldolase or whether it is due to an unrelated, endogenous process (*e.g.*, proteolysis) which may have occurred even in the absence of 4FG.

The negligible oxidation and extensive defluorination of 4FG by glucose-grown whole cells of *P. putida* (78) could be explained by its active transport via the

glucose carrier (54) and subsequent metabolism to its 6-phosphogluconate analogue via the non-oxidative, phosphorylative pathway of glucose utilization which is present in pseudomonads (107). In order to confirm this possibility, it is hoped that future studies will be directed at determining whether 4FG is transported and identifying the early intracellular phosphorylated, fluorinated metabolites which would be formed if such a pathway were operative.

It is possible that a transport of 4FG may be preferred over its oxidation because of a low affinity (high K_m) of the membrane-bound glucose dehydrogenase for 4FG relative to the affinity of the glucose carrier for 4FG. For example, previous studies have shown that 4FG and 3-deoxy-3-fluoro-D-glucose (3FG) are, relative to glucose, comparatively poorer substrates which have a low affinity for the membrane-bound glucose dehydrogenase. The respective kinetic parameters for the oxidation of 4FG, 3FG or glucose by membrane vesicles are: apparent $K_m = 21$ (89), 25 and 0.833 μM (147); apparent $V_{max} = 138$ (89), 105 and 160 nanomoles oxygen/minute/mg protein (147). A comparison of these values with the kinetic parameters for the transport of 3FG or glucose by membrane vesicles reveals that, although the glucose carrier transports 3FG about as well as glucose, the apparent affinity of the glucose carrier for 3FG is much greater than the affinity of the membrane-bound glucose dehydrogenase for 3FG. The respective kinetic parameters for the transport of 3FG or glucose by membrane vesicles are: apparent $K_m = 400$ and 167 micromolar (54); apparent $V_{max} = 2.7$ and 0.8 nanomoles/minute/mg protein (54). It may also be worth noting that the transport of glucose in whole cells of *P. putida* appears to occur more rapidly than in membrane vesicles, possibly indicating a loss of transport activity during membrane-vesicle preparation. The kinetic parameters for glucose uptake in whole cells, which contain both a "low- K_m " and a "high- K_m " glucose-uptake system, are: apparent $K_m = 1.7$ (low- K_m) and 76.9 (high- K_m) micromolar (147); apparent $V_{max} = 5.15$ (low- K_m) and 11 (high- K_m) nanomoles/minute/mg protein (147).

If 4FG were transported with kinetic parameters comparable to those cited above for 3FG or glucose, then it is conceivable that 4FG could be preferentially

transported by the glucose carrier rather than being oxidized by the low-affinity, membrane-bound glucose dehydrogenase. Furthermore, since membrane vesicles from *P. putida* have been shown to accumulate glucose by an active-transport mechanism which can be coupled to the respiration of electron donors (e.g., L-malate) via the electron transport chain (54), it is possible that an active transport and accumulation of 4FG analogous to glucose would tend to minimize the availability of 4FG for the membrane-bound glucose dehydrogenase which is considered to be located at the outer surface of the cytoplasmic membrane (138,107). Finally, the preferential transport of 4FG prior to its defluorination would also be consistent with the demonstrated repression of both the glucose carrier in membrane vesicles prepared from succinate-grown cells of *P. putida* (54) and the defluorination of 4FG in succinate-grown whole cells of *P. putida* (89).

Previously, it was reported that glucose-grown whole cells of *P. putida* were also capable of defluorinating both 4FGA and 4F2KGA to the same extent (95%) as 4FG (89). In terms of the pathway proposed above, the defluorination of 4FGA and 4F2KGA could be explained by their transport by the gluconate and 2-ketogluconate carrier systems (54,55), respectively, and subsequent conversion to their 6-phosphogluconate analogue via the later, non-oxidative segments of the direct oxidative pathway of glucose metabolism which is present in pseudomonads (107). In this context, 4F2KGA has been shown to competitively inhibit (apparent $K_i = 50$ micromolar) the active transport of 2-ketogluconate (apparent $K_m = 50$ micromolar; apparent $V_{max} = 0.55$ nanomoles/minute/mg protein) by membrane vesicles prepared from glucose-grown *P. putida* (55).

In the later segments of the direct oxidative pathway and in the phosphorylative pathway of glucose metabolism in pseudomonads (Figure 6), the formation of 6-phosphogluconate from glucose, gluconate or 2-ketogluconate would require a phosphorylation which is mediated by their corresponding ATP-dependent kinases (107). However, the utilization of these pathways for the metabolism of 4FG, 4FGA or 4F2KGA would seem to be inconsistent with previous studies which revealed that cell-free extracts from glucose-grown whole cells of *P. putida* were incapable of

defluorinating 4FG; instead, 4FG was oxidized to an extent of 2 atoms of oxygen per molecule, with retention of the C—F bond, to yield 4F2KGA (79,89). The following considerations may offer an explanation for this apparent inconsistency.

Earlier studies conducted by Wood and Schwerdt (148) have demonstrated that cell-free extracts from glucose-grown *P. putida* (*P. fluorescens*, strain A.3.12, ATCC 12633) were devoid of hexokinase (glucokinase) and gluconokinase activity, as measured at pH 7.4 in 0.025 M glycylglycine buffer. In addition, these extracts were incapable of oxidizing 2-ketogluconate (Veronal buffer, pH 7), and it was shown that the ability of these extracts to oxidize glucose-6-phosphate and 6-phosphogluconate (Veronal buffer, pH 7) was completely lost when incubations were conducted in 0.1 M phosphate buffer, pH 7, whereas glucose and gluconate oxidation was unaffected and proceeded to an extent of 2 and 1 atoms of oxygen per molecule, respectively (148). Later, Narrod and Wood (149) reported the presence of ATP-dependent gluconokinase and 2-ketogluconokinase activities which required Mg^{+2} (10 mM, in 0.025 M glycylglycine buffer, pH 7.4) for maximal activity in cell-free extracts of glucose-grown *P. putida* (ATCC 12633, formerly *P. fluorescens*, strain A.3.12). These extracts, suitably supplemented with ATP and Mg^{+2} , could convert both gluconate (149) and 2-ketogluconate (150) to 6-phosphogluconate, which was then further degraded to pyruvate and glyceraldehyde-3-phosphate via the ED pathway. Eisenberg *et al.* (151) later reported that an earlier inability to detect glucose kinase activity in extracts of *P. fluorescens* (ATCC 13525) could be overcome by simply using a higher pH in the enzyme-assay reaction mixture (pH 8.2, Tris-hydrochloride buffer). Also, in a personal communication to these authors, it was noted that P. V. Phibbs had found cytoplasmic glucose kinase activity in *P. putida* (ATCC 12633), although the details of the assay conditions were not given.

In view of these findings, it is possible that the inability of cell-free extracts of *P. putida* to defluorinate 4FG, 4FGA or 4F2KGA may have been due to unfavourable conditions of pH and buffer (0.1 M potassium phosphate, pH 7.1) which could have interfered with their phosphorylation and subsequent metabolism to their 6-phosphogluconate analogue. In addition, since these phosphorylations

would be mediated by ATP-dependent kinases, it is possible that a lack of ATP in cell-free-extract preparations may have been another factor which could have prevented the phosphorylation and, hence, subsequent defluorination of these compounds. Perhaps the use of a higher pH (e.g., 8.2), the avoidance of phosphate buffer, and the supplementation of cell-free extracts with ATP and Mg^{+2} could provide conditions which may be more favourable for the defluorination of 4FG in cell-free-extract preparations. In this regard, it may be of interest to note that in his paper, "Buffers for Enzymes", Blanchard (152) remarks:

"Unfortunately, many buffers have intrinsic handicaps associated with their use in biochemical systems, e.g., interactions with substrates or enzymes, resulting in reduced activity. Because buffers are generally present in much higher concentration than any other component in reaction mixtures, interactions of any sort can seriously influence the interpretation of enzymological data. ...The problem of specific buffer interaction with other reaction components is most prevalent when inorganic buffers are used. Phosphate, for example, inhibits many kinases and dehydrogenases as well as enzymes with phosphate esters as substrates."

It is hoped that these matters will be given due consideration in future studies of the metabolism of 4FG in *P. putida*.

CHAPTER IV

SUMMARY AND CONCLUSIONS

It has been shown that incubating glucose-grown whole cells of *Pseudomonas putida* with D-[U- ^{14}C]4FG under conditions giving rise to nearly complete (95%) release of fluoride ion results in a covalent association of a small amount, less than 1%, of radiolabel with the peptidoglycan, the accumulation of more than 50% of the radiolabel in the suspending medium, and a liberation of as much as 5% of the radiolabel as a volatile product which appears to be $^{14}\text{CO}_2$. A gel-filtration analysis of the peptidoglycan-associated radiolabel gave no indication that the radiolabel was incorporated into a peptidoglycan-associated protein. Therefore, evidence for the previous proposal (78,89) that 4FG may be alkylating a cell-envelope protein was not obtained. Chromatographic analysis of the suspending medium revealed that the radiolabel was primarily associated with two low-molecular-weight metabolites: a minor metabolite which has properties that appear to be consistent with a neutral deoxycarbohydrate, and a major metabolite which was identified as 2,3-dideoxyribonic acid by NMR spectroscopic and mass spectrometric analyses.

It has been shown that incubating a crude-outer-membrane preparation derived from glucose-grown whole cells of *P. putida* with D-[U- ^{14}C]4FG results in limited (10%) release of fluoride ion, a complete consumption of 4FG, and the formation of highly anionic fluorinated and non-fluorinated metabolites which appear to behave chromatographically as phosphorylated aldonic acids and clearly differ from the two metabolites obtained from incubations of whole cells with 4FG. Although previous studies had implicated the outer membrane in the defluorination of 4FG (79,89), the possible formation of phosphorylated metabolites raises the possibility that the defluorinating activity attributed to crude-outer-membrane preparations may have been caused by spheroplast and/or whole-cell contaminants.

The formation of 2,3-dideoxyribonic acid may provide an important clue towards elucidating the metabolic pathway responsible for its formation and for the

defluorination of 4FG. In particular, the Entner-Doudoroff pathway would appear to offer an attractive route for the formation of 2,3-dideoxyribonic acid and the defluorination of 4FG. The possible involvement of the Entner-Doudoroff pathway in the defluorination of 4FG would necessitate the formation of 4-deoxy-4-fluoro-6-phosphogluconate as an intracellular fluorinated, phosphorylated intermediate. The availability of carbon-14-labelled 4FG should readily facilitate the identification of such an intermediate especially in the early phase of incubation. In addition, the identification of this intermediate and support for the involvement of the Entner-Doudoroff pathway could also be aided by the use of specific metabolic poisons. For example, sodium fluoride, which is a known inhibitor of the 6-phosphogluconate dehydrase of *P. putida* (113), would be expected to inhibit the further metabolism and, hence, the defluorination of this fluorinated intermediate via the Entner-Doudoroff pathway. That sodium fluoride may inhibit the defluorination of 4FG is an intriguing and heretofore unexplored possibility.

APPENDIX I

Synthesis of Uniformly Carbon-14-Labelled 4-Deoxy-4-fluoro-D-glucose

Methyl α -D-[U-¹⁴C]galactopyranoside monohydrate (1)

The procedure used for the preparation of 1 was based on the reported synthesis of methyl α -D-galactopyranoside monohydrate (153). To 200 microcuries of D-[U-¹⁴C]galactose (specific activity, 57 microcuries/micromole) was added 7 g of cold D-galactose and 56 mL of a 2% (w/v) solution of hydrogen chloride in pure methanol. The resulting mixture was refluxed for 8 hours while stirring magnetically. The warm solution was then neutralized with 8.0 g of lead carbonate for 3 hours with stirring. The resulting solution, which was neutral to blue litmus paper, was then filtered over a bed of Kieselguhr to remove lead salts. The resulting filtrate and methanolic washings (200 mL) were combined and evaporated to a syrup. The warm syrup was mixed with 2.0 mL of water, and crystallization was allowed to proceed at room temperature for 24 hours and then at 4°C for 48 hours. Crystals of 1 were removed from the syrupy mother liquor by suction filtration and were washed with about 30 mL of methanol chilled to 4°C. The crystals were air-dried by suction on a filtration funnel to yield 2.1 g of crystalline product. The remaining mother liquor and methanolic washings were combined and dried by repeated distillation with methanol (4 x 100 mL) under reduced pressure at 40-45°C to yield a syrup. The resulting syrup was refluxed again with 56 mL of 2% (w/v) of hydrogen chloride in methanol, and following the same procedures given above, another 1.3 g of crystalline 1 was collected. Again, the remaining mother liquor and methanolic washings were combined and treated as described above to yield an additional 1.1 g of crystalline product. The remaining mother liquor and methanolic washings were combined and treated again in the same manner to yield a final crop of 0.85 g of crystalline product which had a melting range of 80-100°C on a Fisher-Johns Melting Point Apparatus (Fisher Scientific Company). This melting range agreed well with a melting range of 76-98°C obtained for synthesized, non-

radiolabelled methyl α -D-galactopyranoside monohydrate (obtained as the first crop, from 7 g of cold D-galactose, by the same procedures given above); literature melting range, 75-99°C (153). The overall yield of 1 was 5.3 g or 64%.

Methyl 2,3,6-tri-O-benzoyl- α -D-[U- 14 C]galactopyranoside (2)

Synthesis of 2 was based on the reported synthesis of methyl α -D-galactopyranoside 2,3,6-tri-O-benzoate (154). A magnetically stirred solution of 1 (3.6 g) in pyridine (110 mL) was cooled to -30°C by means of a solid CO₂ (dry ice)-acetone bath. Benzoyl chloride (9.6 mL) was added drop-wise (10-30 minutes), with exclusion of moisture and magnetic stirring at -30°C. Stirring was continued at -30°C for 2 hours, at 4°C for 24 hours, and then at room temperature for 2 days. Pyridine was then removed under reduced pressure at 40°C, and the residue was dissolved in chloroform (250 mL). The chloroform solution was washed successively with 2 N hydrochloric acid (3 x 100 mL), a saturated aqueous solution of sodium bicarbonate (2 x 150 mL), and finally with water (2 x 150 mL). The chloroform layer was collected and solvent was removed under reduced pressure to yield a thick syrup. The syrup was dissolved in hot ethanol (70 mL), and crystallization was allowed to proceed at room temperature for 18 hours. The crystalline product was collected, washed with ethanol (2 x 25 mL), and air-dried by suction filtration. The mother liquor and ethanolic washings were combined, evaporated to a thin syrup, and allowed to stand at room temperature to yield additional crops of crystalline product. The overall yield of 2 was 6.0 g or 69%. The crystals of 2 melted at 138-140°C (as determined by a Fisher-Johns Melting Point Apparatus). Non-radiolabelled methyl α -D-galactopyranoside 2,3,6-tri-O-benzoate synthesized by an identical procedure had a melting point of 136-138°C; literature, 139-140°C (154).

Methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-fluoro- α -D-[U- 14 C]glucopyranoside (3)

Preparation of 3 was based on the reported synthesis of methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-fluoro- α -D-glucopyranoside (121,83). A magnetically stirred solution of 2 (4.8 g) in methylene chloride (22 mL) was cooled to -40°C in a dry ice-acetone bath. DAST (3.5 mL) was added and the solution was allowed to warm gradually to room temperature while constantly stirring. After stirring at room temperature for 28 hours, the reaction was cooled to 0°C and quenched by adding 15 mL of methanol (30 minutes), with stirring. After allowing to warm to room temperature, the solution was poured into 100 mL of a saturated aqueous solution of sodium bicarbonate. The resulting mixture was then extracted with methylene chloride (400 mL). The organic layer was collected and washed with a second portion of saturated sodium bicarbonate solution (100 mL) and then with water (2 x 100 mL). Concentration of the organic phase under reduced pressure yielded an amber-colored syrup. After allowing the syrup to stand overnight at room temperature a few crystals were deposited. The entire lot was then dissolved in 80 mL of hot absolute ethanol, and crystallization was allowed to proceed at room temperature for 4 hours and then at 4°C for 20 hours. After decanting the mother liquor, the crude crystals were recrystallized in hot ethanol (50 mL). The resulting slightly yellowish crystals were collected by filtration and were immediately recrystallized again in hot ethanol (50 mL). The resulting crystals were collected, washed with several portions (2 x 25 mL) of cold absolute ethanol, and air-dried by suction on a filtration funnel. The resulting crystals of 3 were obtained in an overall yield of 3.2 g or 66%. Non-radiolabelled methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-fluoro- α -D-glucopyranoside synthesized by an identical procedure had a melting point of $141\text{--}142^{\circ}\text{C}$ (determined by a Fisher-Johns Melting Point Apparatus); literature, $139\text{--}141^{\circ}\text{C}$ (121).

Methyl 4-deoxy-4-fluoro- α -D-[U- 14 C]glucopyranoside (4)

The synthesis of 4 was based on the method reported for the preparation of methyl 4-deoxy-4-fluoro- α -D-glucopyranoside (83,122). A magnetically stirred suspension of 3 (3.2 g) in anhydrous methanol (24 mL) was cooled to 0-4°C in an ice bath. To the stirring suspension a freshly prepared solution of sodium methoxide (0.24 g of sodium metal in 4.1 mL of anhydrous methanol) was added drop-wise. The reaction was stirred continuously at 4°C for 16 hours. The reaction was subsequently neutralized with Amberlite 1R-120 (H^+) cation-exchange resin while stirring at room temperature; completeness of neutralization was tested with red litmus paper. The resin was removed by filtration and washed with several portions of methanol (2 x 50 mL). The neutralized solution and methanolic washings were combined and concentrated under reduced pressure to give a syrup. The syrup was extracted with a mixture of chloroform (100 mL) and water (100 mL), and the chloroform layer was then washed with water (2 x 100 mL). The aqueous layers were collected and evaporated under reduced pressure. The resulting solid residue was then crystallized from a minimal volume of a hot ethyl acetate-acetone mixture (acetone being added to the hot ethyl acetate solution until dissolution was complete). The crystallized product was collected, washed with a minimal volume of cold ethyl acetate, and air-dried by suction filtration. After recrystallization in ethyl acetate-acetone and reisolation as above, 4 was obtained in an overall yield of 1.0 g or 81%. Non-radiolabelled methyl 4-deoxy-4-fluoro- α -D-glucopyranoside synthesized by an identical procedure had a melting point of 123-124°C; literature, 129-130°C (49,122).

4-Deoxy-4-fluoro-D-[U- 14 C]glucose (5)

The synthesis of 5 was performed according to the reported synthesis of 4-deoxy-4-fluoro-D-glucose (49). A solution of 4 (1.0 g) in 2 M sulfuric acid (63 mL) was refluxed gently for 2 hours. The reaction was then neutralized with a hot suspension of barium carbonate (31 g) in water (800 mL), and the resulting mixture

was stirred overnight. Barium salts were then removed by filtration over a bed of Kieselguhr, and the filtrate was evaporated to dryness under reduced pressure. The resulting residue was dissolved in hot absolute ethanol (200 mL), and the solution was decolorized with finely powdered carbon and filtered over a bed of Kieselguhr. The solution was then evaporated to a syrup from which 5 crystallized. Crystals were collected, washed with cold ethanol (3 x 15 mL), and air-dried by suction filtration on a sintered-glass funnel. Recrystallization from a minimal volume of hot, boiling ethanol and reisolation as above yielded 0.55 g of 5. The air-dried product melted at 186-192°C, with a slight browning. Further recrystallization of this product in hot ethanol and reisolation as above yielded 0.39 g (42% yield) of fine crystalline material which melted at 188-191°C without browning. Non-radiolabelled 4-deoxy-4-fluoro-D-glucose prepared by the same method had a melting point of 190-192°C; literature, 189-190°C (49). A chromatographic analysis of 5 by silica-gel thin-layer chromatography in ethyl acetate-acetic acid-water (3:3:1 v/v) yielded a single peak of radioactivity following a radiochromatographic scan on a Packard Model 7220/21 Radiochromatogram Scanner (Packard Instrument Company, Inc., Downers Grove, Illinois). Subsequently, after the very same chromatogram was visualized by spraying with sulfuric acid-ethanol (1:1 v/v) and charring over a hotplate, a single spot ($R_f = 0.70$) which coincided exactly with the single peak revealed by radiochromatographic scanning was detected. Non-radiolabelled 4FG which was run on the same chromatogram also gave a single spot ($R_f = 0.70$) which coincided with the radioactive peak and with the visually detected spot of 5.

Determination of Specific Activity of D-[U- 14 C]4FG

A 200 ± 2 microlitre aliquot of a 20.0 ± 0.1 millimolar aqueous stock solution of 5 and a 200 ± 2 microlitre aliquot of a 20.0 ± 0.1 millimolar aqueous stock solution of non-radiolabelled ("COLD") 4FG were counted for radioactivity by liquid scintillation counting as described under "Liquid Scintillation Counting" in Materials and Methods. The results of this counting are given below. The average count rate and average H number both represent the average of at least

four independent countings, while the corresponding uncertainties represents the standard deviation (S.D.) calculated from the equation defining the S.D. for small sets of data:

$$\text{standard deviation, S.D.} = \sqrt{\frac{\sum_{i=1}^N (x_i - \bar{x})^2}{N - 1}}$$

where: x_i is the measured value of an unknown quantity, x

$\bar{x}$ is the average value computed from a set of values of x_i

N is the total number of measured values of x

The percentage counting efficiency (%E) value and its corresponding uncertainty were determined from the quench correction curve shown in Appendix IV.

SAMPLE COUNTED	AVERAGE COUNT RATE (cpm)	H NUMBER	%E
200 ± 2 microlitres of 20.0 ± 0.1 mM aqueous solution of D-[U- ¹⁴ C]4FG	39,663.23 ± 56.8 (S.D.)	64.9 ± 0.7 (S.D.)	93.45 ± 0.65
200 ± 2 microlitres of 20.0 ± 0.1 mM aqueous solution of "COLD" 4FG	29.31 ± 1.1 (S.D.)	63.2 ± 1.7 (S.D.)	

The specific activity of 5 can, therefore, be calculated as follows:

$$\begin{aligned}
 \text{specific activity} &= \frac{\left(\begin{array}{c} 39,663.23 \\ \pm 56.8 \end{array} - \begin{array}{c} 29.31 \\ \pm 1.1 \end{array} \right) \left(\begin{array}{c} 100 \\ \pm 0.65 \end{array} \right)}{\left(\begin{array}{c} 200 \times 10^{-6} \\ \pm 2 \times 10^{-6} \end{array} \text{ L} \right) \left(\begin{array}{c} 20.0 \times 10^{-3} \\ \pm 0.1 \times 10^{-3} \end{array} \text{ moles/L} \right)} \\
 &= 10,603 \pm 140 \text{ dpm/micromole} \\
 &\quad \text{or } 10,600 \pm 100 \text{ dpm/micromole}
 \end{aligned}$$

Due to the relatively low count rates obtained and the small coincidence resolving time for the instrument used (15 nanoseconds for the Beckman LS 7500), corrections for coincidence losses were neglected. Similarly, corrections for radioactive decay were neglected due to the relatively long half-life of carbon-14 (5,730 years) (155).

Summary of the Synthesis of 4-Deoxy-4-fluoro-D-glucose (4FG)

APPENDIX II

Synthesis of the Sodium Salt of 4,5-Dihydroxypentanoic Acid

Impure 5-hydroxy-4-valerolactone (1.0 g) (123) was dissolved in water (100 mL), and 5.0-mL aliquots of the solution were heated to 70-80°C and titrated with 0.2 N aqueous sodium hydroxide to a light-pink phenolphthalein end-point which was stable for more than 1 hour at 70-80°C. To the remaining non-titrated solution was added 0.2 N aqueous sodium hydroxide in the same proportion that was required for titration to a phenolphthalein end-point as described above. This solution was then heated for 1 hour at 80°C and allowed to cool overnight to room temperature. The resulting solution, which had a pH of 8.0, was then concentrated under reduced pressure at 40°C to yield a clear colorless syrup. The syrup was then applied to a 70-mL bed (42 cm x 1.5 cm) of Amberlite 1R-120 (H⁺) cation-exchange resin which was previously equilibrated with 2 volumes of water. The sample was eluted from the column with water, and the effluent was collected until it was no longer acidic to blue litmus paper (125 mL). The collected effluent was then frozen in a liquid nitrogen bath and lyophilized on a Labconco lyophilizing unit (Labconco Corporation, Kansas City, Missouri) to remove contaminating formic acid. The resulting clear, colorless syrup obtained after lyophilization was then mixed with 100 mL of water. 5.0-mL aliquots of the solution were heated to 70-80°C and titrated with 0.2 N sodium hydroxide to a light-pink phenolphthalein end-point which persisted for more than 1 hour at 70-80°C. To the remaining non-titrated solution was added 0.2 N sodium hydroxide in the same proportion required for its titration to the phenolphthalein end-point in the preceding step. The solution was then heated at 80°C for 1 hour and allowed to cool to room temperature overnight. The resulting solution, which had a pH of 8.1, was extracted with chloroform (2 x 100 mL), and the top aqueous layer was collected and concentrated under reduced pressure at 40-45°C to yield a thick syrup. After desiccating overnight under vacuum over phosphorus pentoxide, a thick, clear, colorless syrup was obtained. 65 mg of

this syrup was mixed with 0.5 mL of D₂O in an NMR tube and analyzed by proton and carbon-13 NMR as described under "Fourier Transform Carbon-13 and Proton NMR" in Materials and Methods. The remaining syrup was stored at -20°C for future thin-layer chromatographic analysis as described under "Thin-Layer Chromatography of Radiolabelled Metabolites" in Materials and Methods.

APPENDIX III

Growth Curve for *Pseudomonas putida*

Cells of glucose-grown *P. putida* were cultured in 12 L of sterile glucose-mineral-salts growth medium in a large bench-top fermentation unit (New Brunswick Scientific Company, Inc.) as described in Materials and Methods. The vessel, aerated with air at a rate of 1-2 L per minute, was maintained at a temperature of 30°C, at a stirring rate of 400 revolutions per minute, and at a slight positive pressure of 0.5-1 pounds per square inch. Growth was monitored by withdrawing 5-mL aliquots of medium from the vessel at various times following inoculation and measuring their optical density at 620 nanometres on a Bausch & Lomb Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, New York) against a blank of distilled water or mineral-salts medium.

APPENDIX IV

Quench Correction Curve for Carbon-14-Labelled Samples

A series of sealed quenched carbon-14 standards (Nuclear Chicago, 190,000 dpm/Mar. 15, 1968) and a sealed unquenched carbon-14 standard (Beckman, 29,700 dpm/Jan. 1, 1979) were counted for radioactivity on a Beckman LS 7500 liquid scintillation counter (Beckman Instruments Inc.), against a sealed background reference (Beckman), using a full carbon-14 window in all three counting channels. The counting efficiency, expressed as a percentage, and H number for each standard is an average of two independent countings; the uncertainty associated with these values is displayed in the curve as vertical and horizontal bars, respectively, and is based on the standard deviation (as defined in Appendix I). Broken lines represent uncertainty limits used to estimate the error associated with values of counting efficiency interpolated from the curve.

APPENDIX V

Gel-Filtration Chromatography of Blue Dextran and Molecular-Weight Protein Standards on Bio-Gel A-1.5m Column

Bovine serum albumin (3 mg), chicken egg ovalbumin (4 mg) and chicken egg white lysozyme (1 mg) were heated in 1 mL of 0.1 M sodium phosphate buffer, pH 7.2, containing 1% (w/v) SDS and 0.05% (w/v) sodium azide, for 30 minutes at 65°C. Blue Dextran (1 mg) and sucrose (10 mg) were then added, and the resulting solution was applied to a 180 $\pm$ 4-mL bed of Bio-Gel A-1.5m (Bio-Rad Laboratories) equilibrated with 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% (w/v) SDS and 0.05% (w/v) sodium azide. The sample was eluted from the column, with the same buffer used for equilibration, at a flow rate of 3.6 mL per hour, and fractions of 1.26 $\pm$ 0.05 mL were collected and assayed manually for protein by measuring the absorbance at 280 nanometres as described in Materials and Methods under "Gel-Filtration Chromatography". The void volume, determined from the elution volume corresponding to the top of the peak of Blue Dextran, was 61 $\pm$ 3 mL. Numbers in parentheses refer to molecular weights.

APPENDIX VI

Molecular-Weight Calibration Curve for Bio-Gel A-1.5m Gel-Filtration Column

The data for this curve was derived from the gel-filtration analysis presented in Appendix V. V_0 represents the void volume determined as described in Appendix V. V_e represents the elution volume corresponding to the top of the elution peak of a given protein standard. The V_e/V_0 ratio for each protein was then plotted against the log_{10} of its molecular weight. The line plotted represents the line-of-best-fit obtained from linear regression of the data by the method of least-squares analysis (156). Extrapolation of the regression equation, $V_e/V_0 = -0.665 \text{log}_{10}(\text{molecular weight}) + 4.75$, to $V_e/V_0 = 1$ yields an exclusion limit of about 400,000 daltons. Numbers in parentheses indicate molecular weights.

APPENDIX VII

Chromatography of Radiolabelled Supernatant on Dowex 1-X8 Borate Anion-Exchange Column Used for Preparative Isolation of Minor-Peak and Major-Peak Metabolites

Radiolabelled supernatant was obtained from a 24-hour incubation of glucose-grown whole cells of *P. putida* with D-[U- ^{14}C]4FG as described under "Labelling and Fractionation of Whole Cells" in Materials and Methods. An aliquot of 3.0 mL of the supernatant was mixed with the indicated carbohydrate standards, and the mixture was applied to a column containing a 112-cm x 1.5-cm bed of Dowex 1-X8 borate anion-exchange resin and eluted with an increasing linear concentration gradient of ammonium tetraborate (600 mL of 0.029 M ammonium tetraborate/0.057 M boric acid, pH 8.5-8.8, diluted linearly with 600 mL of 0.5 M ammonium tetraborate, pH 8.9-9.2). Fractions of 4.4 ± 0.2 mL were collected at a flow rate of 15 mL per hour. The radioactivity in fractions 1 to 240 was determined by liquid scintillation counting as described in Materials and Methods and represents the count rate, expressed in counts per minute (cpm), in a 1.00 ± 0.01 -mL aliquot of fraction. Carbohydrate in even-numbered fractions was assayed by sulfuric acid-ornicinal colorimetry at 420 nanometres as described in Materials and Methods. Inorganic phosphate in odd-numbered fractions, determined as described in Materials and Methods, eluted in fractions 203 to 217. The preparative isolation of the minor-peak and major-peak metabolites was performed on this column as described in Materials and Methods.

APPENDIX VIII

Calculation of Radioactivity in Radiolabelled Fractions and Propagation of Errors

After determining the sample count rate (S) and the counting efficiency (E) as described in Materials and Methods (under "Liquid Scintillation Counting"), the radioactivity (dpm) in the total volume (V_t) of a radiolabelled fraction isolated following incubations with D-[U- ^{14}C]4FG was calculated using the equation,

$$\text{dpm} = \frac{(S - \text{background})/E}{V_s} \times V_t$$

where V_s is the volume of sample used for counting, and background is the count rate (in cpm) obtained for a non-radiolabelled fraction isolated from an identical incubation performed without D-[U- ^{14}C]4FG. Uncertainties in the sample and background count rates were based on the "2 sigma" (2 x standard deviation) relative percentage error which was given for each count rate value in the counting data provided by the Beckman LS 7500 liquid scintillation counter and was calculated according to the equation,

$$\text{2 sigma relative \% error} = \frac{2 (R/t)^{1/2}}{R} \times 100$$

where t is the total counting time in minutes and R is the count rate in cpm. The count rate value and its associated error was calculated as an average of the values obtained from each of three separate counting channels. In calculating the total radioactivity in a radiolabelled fraction, the uncertainty propagated through various mathematical operations was calculated according to the equations (157) given below. Considering two numbers and their uncertainties (standard deviations), $A \pm a$ and $B \pm b$, the uncertainty in the result of some mathematical operation is:

Operation	Answer	Uncertainty
Addition	$A + B$	$(a^2 + b^2)^{1/2}$
Subtraction	$A - B$	$(a^2 + b^2)^{1/2}$
Multiplication	$A \times B$	$A \times B [(a/A)^2 + (b/B)^2]^{1/2}$
Division	A/B	$A/B [(a/A)^2 + (b/B)^2]^{1/2}$

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