Further studies on the metabolism of 4-deoxy-4-fluoro-D-glucose in Pseudomonas putida.

John Watcyn. Williams
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FURTHER STUDIES ON THE METABOLISM OF
4-DEOXY-4-FLUORO-D-GLUCOSE IN
PSEUDOMONAS PUTIDA

by

JOHN WATCYN WILLIAMS

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

Windsor, Ontario, Canada
1990
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ABSTRACT

FURTHER STUDIES ON THE METABOLISM OF
4-DEOXY-4-FLUORO-D-GLUCOSE IN

PSEUDOMONAS PUTIDA

by

John Watcyn Williams

Following a 24 h incubation of glucose-grown whole cells of Pseudomonas putida with D-[U-14C] 4-deoxy-4-fluoro-D-glucose (4FG), there is an extensive release of fluoride ion, (95 ± 5%). The isolated peptidoglycan (PG) fraction contains less than 1% of the radiolabel. This small, but significant amount of radioactivity is not due to the presence of a novel carbohydrate derived from 4FG. HPLC analysis with an Aminex HPX-87C (Bio-Rad) carbohydrate analytical column reveals the presence of the radioactive amino acids threonine, serine, glutamate and aspartate. These are considered to have arisen from the observed 14CO2 from 4FG. When the incubation is performed with NaH14CO3 an identical isolation and analysis of the PG shows the presence of radioactive N-acetylglucosamine, muramic acid, glucosamine, glutamate and aspartate. Pathways for 14CO2 incorporation into PG are presented.

Preparative scale isolation of a previously unknown minor metabolite resulting from a 24 h incubation of P. putida and 4FG reveals the γ-lactone of 2,3-dideoxy-D-glycero-pentonic acid.

Metabolic studies suggest that a chemiosmotic energy couple (ΔpH) is required for the defluorination of 4FG, which is not dependent upon ATP synthesis. These studies suggest the possibility that a periplasmic
shock-resistant binding protein could be implicated in the mechanism of fluoride release.

Time course studies reveal that within the first 80 min of incubation of D-[U-14C]4FG with P. putida, the removal of 4FG from the suspending medium is concomitant with the appearance of a non-fluorinated intermediate metabolite in the supernatant. NMR and mass spectrometry data suggest the structure of 4-deoxy-5-keto-D-glucose and/or 4-deoxy-D-glucose. An examination of the intracellular contents of P. putida over the same time profile reveals the presence of 4FG and an unknown metabolite.

The site, possible mechanism of defluorination of 4FG and its subsequent metabolism to 2,3-dideoxy-D-glycero-pentonic acid are discussed in relation to glucose transport and metabolism in pseudomonads.
ACKNOWLEDGEMENTS

I wish to express sincere gratitude to my supervisor Dr. Norman F. Taylor for his support, wise counsel and sense of humour.

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To Michael Fuerth, Jim Olsen and the ubiquitous David Hill, I append my appreciation for the knowledgeable and prompt attention they afforded me at all times.

I thank Dr. Roger Thibert, Dr. Hugh Fackrell, Dr. Keith Taylor, Dr. Ken Rutherford and Dr. Robert Rumfeldt for the edifying conversations over the years.

To Miss Maeve Doyle for her ability to transpose hieroglyphics into legible print, a special word of thanks.

To my parents, my wife Loes and daughter Angela, you have indeed endured.
DEDICATION

To my wife Loes
and daughter Angela
Forsan et haec olim meminisse juvabit.

Virgil


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<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>CM</td>
<td>Cytoplasmic Membrane</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>2,3DDRA</td>
<td>2,3-dideoxyribonucleic acid</td>
</tr>
<tr>
<td>DAP</td>
<td>Diaminopimelic acid</td>
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<tr>
<td>DNP</td>
<td>Dinitrophenol</td>
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<tr>
<td>4DOG</td>
<td>4-deoxy-D-glucose</td>
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<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
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<td>ED</td>
<td>Entner Doudoroff</td>
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<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
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<td>3FG</td>
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<td>Leu</td>
<td>Leucine</td>
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<tr>
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<tr>
<td>NAG</td>
<td>N-acetylglicosamine</td>
</tr>
<tr>
<td>NAM</td>
<td>N-acetyl muramic</td>
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<tr>
<td>PG</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PPB</td>
<td>Potassium Phosphate Buffer</td>
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<td>SDS</td>
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CHAPTER 1
INTRODUCTION

1.1 Some Biochemical Aspects of the C-F Bond

The ingenuity of a Belgian organic chemist in preparing fluoroacetic acid (F\textsubscript{2}CH\textsubscript{2}COOH) in 1896 (1) probably heralded in the era of investigation into carbon-fluorine (C-F) chemistry. Inevitably, Teutonic curiosity was stimulated when it was realized that fluorinated organic compounds had potential applications as insecticides.

The onset of World War II led to some exigent research into the toxicity of these compounds by Saunders and his group at Cambridge (2,3,4). It was feared that such compounds could be used as weapons against the Allies. Their work on C-F compounds was parallel with, but distinct from their researches on phosphorus-carbon compounds - the nerve gases (2,4,5).

The interest in fluorinated compounds burgeoned following the discovery in 1943 by Marais (6) that potassium fluoroacetate was the toxic principle of the plant Dichapetalum cymosum (gifblaar) found in the Transvaal region of South Africa. Another variety, D. toxicarium, although containing traces of fluoroacetate, owed its toxicity to fluoroooleic acid found in its seeds. Further, it had been earlier reported (7) that the seeds had been used for criminal intent.

The mechanism of the toxicity of fluoroacetate was eventually elucidated by Sir Rudolf Peters, et al. (8,9,10) who showed it was metabolically transformed into the more toxic fluorocitric acid, a potent inhibitor of the tricarboxylic acid cycle (Krebs). Hence, the term
"Lethal Synthesis" was coined by Peters to describe a biochemical phenomenon in which a potential toxic agent could be transformed by existing pathways into a toxic metabolite. Subsequently, it was shown that fluoro-citrate also exerted its toxic effect by irreversibly alkylating the carrier protein for citrate in the mitochondrial membrane (11).

It was reported that aerobic micro-organisms were capable of utilizing fluoroacetate as a sole carbon source following cleavage of the carbon-fluorine bond to release fluoride ion (12) to produce glycolate. Using a culture enrichment technique, a pseudomonad was shown to possess a defluorinating enzyme. The same enzyme was also capable of releasing the halide from chloroacetate and iodoacetate; kinetic data revealed that the preferred substrate was fluoroacetate ($K_m$ 2.4 mM) whereas for chloroacetate it was 20 mM. Similarly, another pseudomonad was isolated that grew on fluorocitrate after fluoride release (13).

The prospect of fluorinated sugars being metabolized to fluoroacetate initiated research into their chemical syntheses (14). The efficiency of these synthetic operations was increased by the advent of powerful fluorinating agents such as diethylaminosulphur trifluoride (15).

It was suggested that the replacement of a hydroxyl (-OH) group by fluorine would cause minimum perturbation of molecular structure and conformation, whilst still conferring subtle differences in chemical and biochemical reactivities (16). This idea emanated from an earlier observation that there was a close similarity in size and electronegativity between fluorine (F) and an -OH group attached to carbon (17). This was subsequently confirmed by physico-chemical analysis (18).
In addition, it was pointed out that the -OH group in a carbohydrate may act as a donor or acceptor of hydrogen bonding, whilst a deoxyfluoro substitute may act only as a hydrogen bond acceptor. This could in turn provide information regarding the direction and stereospecificity of binding to a variety of proteins (19). Furthermore, the presence and sensitivity of F permits its location in any substrate to be detected by \(^{19}\)F NMR spectroscopy (20). The rationale for the introduction of F into carbohydrates has been exhaustively reviewed (21).

The biological effects of fluorinated monosaccharides are diverse. They are rarely if ever metabolized to fluoroacetate, but enter a number of biochemical pathways (14). 2-Deoxy-2-fluoro hexoses caused oligosaccharide chain-shortening in glycolipids of influenza A infected chick cells and in yeast (22). The consequent alteration to the molecular architecture of the membrane suggested a possible application for reducing viral infectivity. 2-Deoxy-2-fluoro-glucose and other fluorinated monosaccharides promoted a reduction in cell growth for a number of tumour cell lines in culture, but had little or no effect on tumours in vivo (23).

3FG was shown to be a specific competitive inhibitor of mammalian glucose transport (18). In *Locusta migratoria* (24) 3FG irreversibly inhibited glycolysis, which resulted in the death of the insect. In addition, the release of fluoride and \(^{3}\)H\(_2\)O from D-[3-\(^3\)H]-3FG together with an accumulation of tritium labelled fructose suggested an unusual catabolism. It was concluded that 3FG was metabolized to monofluorohydroxyacetone phosphate and glyceraldehyde-3-phosphate. Further, the inhibition of enolase by fluoride ion (25) and
the action of aldolase in the glucogenic mode would explain the accumulation of fructose. It was also demonstrated that D-[3-\textsuperscript{3}H]-3FG was incorporated into tritium labelled glycogen and trehalose with retention of the C-F bond. These studies were cited as an example of a novel "Lethal Synthesis"; 3FG utilized various anabolic and catabolic enzymes, involving both retention and cleavage of the C-F bond (26).

In glucose-grown whole cells or cell-free extracts of Pseudomonas putida (29,30) 3FG was oxidized to 3FGA and 3F2KGA by the membrane-bound glucose and gluconate dehydrogenases respectively (29,30). In addition, there was retention of the C-F bond. There was no evidence of phosphorylation, but both 3FGA and 3F2KGA were competitive inhibitors of the cytosolic gluconokinase (28). Evidence for further metabolism of these fluorinated sugars via the Entner-Doudoroff pathway was not found (31).

In Escherichia coli, 3FG (32) and 4FG were both transported and converted to their 6-phosphates by the phosphoenolpyruvate phosphotransferase (PTS) system, without further metabolism ensuing. There was retention of the C-F bond and both sugars inhibited growth of cells grown on glucose or lactose. The latter phenomenon was explained by the uncompetitive inhibition of β-D-galactosidase (inducible) by 3FG and 4FG (33). Further, the fluorinated sugar phosphates 3FG-6-P and 4FG-6-P were shown to repress β-D-galactosidase synthesis. These results supported the Peterkofsky proposal (34) that catabolite repression could be produced by compounds that are not metabolized further than the hexose 6-phosphates.

Since the pioneer work of the 1940s the research into the application
of compounds containing the C-F bond has flourished. In addition to the drugs based on the β-lactam ring - the penicillins and cephalosporins (35), the use of fluorinated quinolones (36) has expanded the antimicrobial drug arsenal. Some examples are shown in Fig. 1. Norfloxacin (37) and Ciprofloxacin possess 6-fluorine substituents. Lomefloxacin has fluorine substituents at positions 6 and 8 and exhibits greater metabolic stability in addition to microbiological activity that rivals the mono-fluorinated compounds. These anti-microbial agents have demonstrated enhanced activity against both Gram-negative and Gram-positive organisms, including P. aeruginosa. The fluorinated quinolones exert their action on bacterial cells by interfering with a wide variety of DNA-related processes. They complex specifically with DNA-gyrase, thus preventing DNA polymerase from proceeding at the replication fork and therefore, stopping DNA synthesis (38). The future of synthetic antibiotics is, of necessity, continually expanding; to date only one naturally occurring antibiotic, nucleocidin (Fig. 2), a fluorinated carbohydrate has been reported (39,40).

It would appear that the potential of fluoro-containing biological analogues will continue to be of interest. Deoxyfluorinated sugars, with retention of the C-F bond can serve as probes for membrane and metabolic studies. In addition to their use as antibiotic compounds, recent work (41) has seen fluorinated sugar derivatives being considered as in vivo anti-viral agents. The distinctive chemical shifts arising from in vivo $^{19}$F NMR studies, suggest methods for locating F-containing molecules of pharmacological interest (14). More recently, $^{19}$F fluorinated carbohydrates have been used in Positron Emission Transaxial Tomography
FIGURE 1

Fluorinated Quinolones

Norfloxacin

Ciprofloxacin

Lomefloxacin
FIGURE 2

Structure of Nucleocidin

\[
\begin{align*}
\text{NH}_2 \\
\text{H}_2\text{N}-\text{SO}_2-\text{O} \\
\text{F} & \quad \text{H} \\
\text{OH} & \quad \text{OH}
\end{align*}
\]

Nucleocidin
(PETT), a technique that permits the in vivo use of fluorinated sugars as non-invasive probes of metabolic flux in animals and humans (19,42).

In order to study the transport and metabolism of fluorinated carbohydrates, a brief review of the structure of the cell envelope and carbohydrate metabolism in pseudomonads is appropriate.

1.2 The Cell Envelope of Gram-Negative Bacteria

Bacteria are generally classified as either Gram-positive or Gram-negative. These are distinguished by a differential staining technique, the Gram stain, introduced in 1884 by the Danish bacteriologist Christian Gram. Although special stains are used for certain organisms (e.g., mycobacteria) the Gram stain remains the preferred method (43). In addition to its use in classification, it is regularly employed as a means of detecting unwanted bacterial contaminants.

High resolution electron micrographs reveal gross morphological differences between Gram-negative and Gram-positive bacteria (Fig. 3). The cytoplasmic membrane of Gram-positive bacteria contains teichoic acids, which are linear polymers of glycerol or ribitol phosphate. These are covalently linked to and encased within a thick peptidoglycan layer. In some species there is an extra-cellular capsule, composed of polysaccharide (44). The envelope of Gram-negative bacteria is more complex. The peptidoglycan is less substantial and not as closely associated with the cytoplasmic membrane as in Gram-positive bacteria (Fig. 4). The cell envelope of Gram-negative bacteria consists of three distinct structures: the outer membrane (OM); the peptidoglycan (PG) and the inner, or cytoplasmic membrane (CM). The OM consists of three components, lipopolysaccharide (LPS), protein and phospholipid. LPS,
FIGURE 3

Diagrammatic Representation of the Cell Surface of
Gram-Positive and Gram-Negative Bacteria (75)

GRAM POSITIVE  GRAM NEGATIVE
FIGURE 4

The Gram-Negative Cell Envelope (46)
which is characteristic for Gram-negative bacteria is an amphipathic molecule possessing three covalently linked regions (45). The hydrophobic component Lipid A, is embedded in the phospholipid bilayer of the OM. The hydrophilic part is composed of the core, which is a short carbohydrate chain that contains 3-deoxy-2-keto-octulosonic acid (KDO). Extending from the core is the third component of the LPS unit, the O-side chain, which is the serologically dominant part of the LPS molecule. It is composed of repeating oligosaccharide units, containing rare sugars (e.g., deoxyhexoses) within which the serological determinants reside (46). This membrane constitutes an important barrier against a variety of assaults such as enzymatic attack and antibiotic entry (46). Lysozyme, a vital component of host defence and present in biological fluids (tears, serum) rapidly degrades peptidoglycan. However, it is prevented from entry into a Gram-negative bacterium by the outer membrane. In contrast, lysozyme has unrestricted access to the peptidoglycan of Gram-positive bacteria. Lysozyme can exert its action only if the permeability of the outer membrane has been increased by treatment with a chelating agent such as EDTA. The outer membrane makes the Gram-negative envelope impermeable to hydrophobic compounds and higher molecular weight hydrophilic compounds. *Pseudomonas aeruginosa* and other pseudomonads differ from the enteric bacteria in that they are super-sensitive to EDTA; extremely low concentrations causing lysis of the bacterium. This suggests therefore that the outer membrane of pseudomonads may differ significantly from that of other Gram-negative bacteria (46). Limited permeability to small solutes is available through the outer membrane by way of proteinaceous channels, composed of a special class of proteins called
porins. These can be either substrate-specific or substrate non-specific (47).

Lipoprotein accounts for about 6% of total bacterial cell protein. Usually one third of it is covalently bound to peptidoglycan (PG), whilst the remainder exists freely in the OM. The C-terminal lysine of the PG-bound form is covalently linked through the ε-amino group to the carboxyl group of diaminopimelic acid, linked to a muramic residue of PG (46). Braun and Rehn (48) showed that lipoprotein was covalently bound to the PG in Escherichia coli and this preceded the finding of other lipoproteins by a decade. After characterization (49) it became known as Braun's lipoprotein or the major lipoprotein - largely because it was shown to be the most abundant protein in E. coli. The occurrence of the free form was subsequently demonstrated (50) to be part of the outer membrane (OM). The discovery of pro-lipoprotein (51), the precursor form of PG lipoprotein, led to major advances in the knowledge of the biogenesis of membrane lipoprotein. The structure of pro-lipoprotein differed from the mature lipoprotein in a number of ways, (e.g., the proteolytic removal of signal peptide), which indicated that a series of post-translational and processing reactions must occur prior to assembly of the lipoprotein in the OM.

The OM of Pseudomonas aeruginosa contains six major proteins designated D, E, F, G, H, and I. F (mol. wt. 33,000) and H (21,000) were shown to be retained by the PG layer following SDS (2%) extraction of the cell envelope at 35°C (52). Both F and H exhibited a similar amino acid composition. A protein of the same molecular weight as protein H was found in the PG-associated fraction of E. coli and was designated, protein
21K (53). Furthermore, when *P. fluorescens* (ATCC 17397) and *P. putida* (CIS) were examined using the same extraction conditions, each contained a protein with an approximate molecular weight of 21,000 (SDS-PAGE) in their respective PG-protein complexes. The 21K protein of *E. coli* and protein H of *P. aeruginosa* were designated as novel lipoproteins (52) and were shown to differ from the so-called Braun lipoprotein (54) in molecular weight (7000) and amino acid composition. Both 21K and protein H revealed substantial quantities of Asp, Thr, Ser, Glu, Gly, Ala, Leu, Tyr and Arg (52).

Both the bound and free form of the major lipoprotein were shown to be in the OM after lysozyme digestion of the PG (55). The bound form (covalently linked) had been presumed to be important in the interaction between OM and PG (56). However, recent evidence (57) has suggested that the free form played a role in OM/PG interaction.

Although anchorage of the OM to the PG through lipoprotein is important for maintenance of structure and functional integrity, the lack of major lipoprotein is not lethal to the cell. In a lipoprotein-negative mutant, "blebbing" of OM vesicles from the cell surface was taken as evidence of a weakened PG/OM association (58). Since the physical association of the PG was not grossly disrupted in the lipoprotein-negative mutant, it was suggested that OM components other than the major lipoprotein are involved in the interaction with PG (59).

When the *E. coli* cell envelope was treated with SDS, an ordered lattice sheet was observed to cover the entire surface of the PG layer (60). The lattice was composed of outer membrane proteins F and C and lipopolysaccharide. When the lipoprotein-negative mutant was used, SDS
treatment released the lattice sheet from the PG (61), indicating that the lipoprotein held the OM lattice on the PG.

The cytoplasmic membrane is composed of protein (60-70%), lipid (20-30%, mainly phosphatides) and carbohydrates. It provides an osmotic barrier which retains metabolites and excludes external compounds. Ions and non-ionized molecules larger than glycerol penetrate very slowly. The CM provides an insulatory barrier across which energy can be built up in the form of a membrane potential. The CM contains the enzyme systems of the electron transport chain (cytochromes) and oxidative phosphorylation. In addition, there are systems for the active transport of solutes and excretion of waste products plus the synthetic apparatus necessary for the production of exterior layers (62).

The region between the cytoplasmic and outer membrane is known as the periplasmic space, and it constitutes 20-40% of the total cell volume. The periplasm possesses a unique series of proteins, which are prevented from leaking into the environment by the restrictive permeability of the outer membrane. Essential solutes which permeate the outer membrane are sequestered by soluble periplasmic proteins, which are specific for a wide variety of organic acids, amino acids and sugars. These binding proteins are closely associated with the periplasmic face of the cytoplasmic membrane. There are scavenging hydrolytic periplasmic enzymes (e.g., alkaline phosphatase) which are involved in the degradation of metabolisable compounds, which are too large or too highly charged to pass through the cytoplasmic membrane. Another group of periplasmic enzymes catalyse the destruction of some antibacterial agents able to penetrate the outer membrane. This detoxification system is exemplified by the β-
lactamase enzyme which degrades penicillins and cephalosporins and is common in both gram-negative and gram-positive bacteria. Certain pseudomonads possess enzymes capable of degrading toxic detergents. The containment of detoxifying enzymes within the periplasm ensures that the inactivating enzyme concentration is greatest where it is needed most, to prevent entry of toxic solutes into the cell. Only drug molecules that are directly threatening the life of the organism are degraded. This confers an advantage to gram-negative bacteria since, unlike gram-positive bacteria, they do not need to produce large quantities of a drug-destroying enzyme which is constantly lost to the environment (63).

The periplasmic contents are released by a mild osmotic shock procedure. The bacteria are plasmolysed by hypertonic sucrose/EDTA and re-suspended in cold distilled water. The bacteria remain viable, though unable to perform any function dependent upon the lost periplasmic binding protein (47).

Transport systems can be categorized according to their response to osmotic shock (64) into shock-sensitive and shock-resistant permeases (proteins). In a recent review Ames (65) cautions that the use of the term 'periplasmic proteins' should be used as an operational definition rather than an indication of physical location. A number of phosphatases, which are typical periplasmic enzymes are poorly released by osmotic shock (66).

Shock-sensitive permeases (periplasmic permeases) are inactivated during osmotic shock due to the loss of an essential protein component, referred to as the periplasmic component, which binds the transported solute with high affinity. The first of these permeases to be shown to
be released by osmotic shock was the sulphate-binding protein, part of a sulphate permease in *Salmonella typhimurium* (67). Shock-resistant permeases retain all of their activity (and therefore, their components) upon osmotic shock. The classic representatives of this class is the β-galactoside permease which has been well studied (68,69).

The nature of the mechanism of energy coupling has been used as an additional characteristic to distinguish between the two classes of permeases. Shock-resistant permeases are powered by the proton-motive force (PMF), (68,69), while energy coupling in shock-sensitive permeases has been postulated to depend on substrate-level phosphorylation (70,71). Berger and Heppel (71) presented evidence that the energy donors for the two classes of transport systems in *E. coli* were fundamentally different. The activity of shock-sensitive permeases was abolished by arsenate, which had earlier been shown to cause a drastic depletion of intracellular ATP levels (72), by deprivation of glycolytic sources of high-energy phosphate. The shock-resistant class remained immune from the effects of arsenate, but were strongly inhibited by the uncoupler 2,4-dinitrophenol (DNP). Shock-sensitive permeases were far less affected by DNP (71).

Peptidoglycan is a highly cross-linked aminosugar polymer which overlays the cytoplasmic membrane. Together they constitute the bacterial cell wall, which is responsible for the maintenance of the shape and integrity of the cell. Peptidoglycan (murein, mucoproteptide and glycopeptide are synonyms) accounts for 50% of the weight of the gram-positive cell wall, but only 10-20% of that of gram-negative species. It is, however, of paramount importance to both classes.

The basic structure of peptidoglycan is essentially the same for most
bacterial species, despite much variation in detailed composition in different organisms. It is made up of a network of linear polysaccharide chains (glycan strands), up to 200 disaccharide units in length, cross-linked by short peptide chains. The glycan strands are composed of alternating units of N-acetylg glucosamine (NAG) and N-acetylmuramic acid (NAM) joined by a 1,4-β glycosidic linkage (Fig. 5). NAM is unique to peptidoglycan (PG) and is a derivative of NAG, bearing an ether-linked D-lactyl residue on carbon 3. A chain of four amino acids, L-alanine, D-glutamic, meso-diaminopimelic and D-alanine, is joined by a peptide bond to the carboxyl group of each NAM residue. Both NAM and meso-diaminopimelic acid are unique to peptidoglycan. In most strains of *Pseudomonas* and other Gram-negative bacteria, the tetrapeptide chains on adjacent glycan strands are cross-linked by a direct peptide linkage between the carboxyl group of D-alanine (fourth amino acid) on one glycan strand and the free amino group of the third amino acid on an adjacent chain, i.e., meso-diaminopimelic acid (Fig. 6). Similar linkages at other points along the glycan strands build up the cross-linked structure of PG. The degree of cross-linking exhibits great variability. In *E. coli* it can be as low as 20%, whilst in *Staphylococcus aureus* it can exceed 90%. The latter organism possesses a bridge of five glycine residues between the D-alanine (-COOH group) of one chain and the free amino group of the third amino acid on an adjacent chain, which is L-lysine (substituted for meso-diaminopimelic acid).

The enzyme lysozyme attacks the β-1-4 linkages between NAG and NAM units. In addition, N-acetylglucosaminidase will also cleave the glycan chains. Nearly all bacteria produce autolysins, which are enzymes
FIGURE 5

Linkage of N-acetylmuramic Acid and N-Acetylglucosamine in

the Glycan Strands and Attachment of Amino Acids to

Muramic Acid (46)
FIGURE 6
Formation of the Peptide Cross-Links Between
Adjacent Glycan Strands

The linkage is formed by a peptide bond between the \( \epsilon \)-NH\(_2\) group of diaminopimelic acid on chain 1 and the carboxyl group of the terminal D-alanines on chain 2 (46).
capable of PG degradation. Their function appears to be to modify existing PG thus permitting cell wall extension during growth (73).

The biosynthesis of peptidoglycan occurs in three stages: synthesis of precursors in the cytoplasm; transfer of precursors to a lipid carrier for transportation across the membrane; insertion into the wall and coupling to existing PG (74).

The precursors synthesized in the cytoplasm are uridinediphospho-N-acetylglucosamine (UDP-NAG) and UDP-N-acetylmuramylpentapeptide. The latter pentapeptide includes an extra D-alanine residue which is removed during incorporation into PG, leaving the familiar tetrapeptide. The lipid carrier is a C\textsubscript{35}-isoprenoid alcohol phosphate (undecaprenol phosphate). The final stage of PG biosynthesis involves the formation of new peptide bonds between nascent glycan strands and existing PG. The process is called transpeptidation and it occurs outside the cytoplasmic membrane and therefore outside direct metabolic control of the cell (73).

A wide range of antibiotics are known to inhibit PG synthesis (Table 1). The unusual chemical composition of PG makes it a good selective target for antibiotic action. The most recent explanation for the mode of action of the β-lactam antibiotics is that they are structural analogues of D-alanyl-D-alanine. The antibiotic molecules are mistaken for genuine substrate by the various enzymes. β-lactams bind covalently to their target enzymes and research interests are centred on enzyme-antibiotic complexes or penicillin binding proteins (PBP) which are produced following antibiotic treatment of bacterial cells. These include \textit{E. coli} and other Gram-negative species (75).
**TABLE 1**

Some Inhibitors of Peptidoglycan Synthesis

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Site of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>phosphomycin</td>
<td>inhibits synthesis of UDP-N-acetylmuramic acid from UDP-N-acetylglucosamine and phosphoenolpyruvate</td>
</tr>
<tr>
<td>D-cycloserine</td>
<td>inhibits conversion of L-alanine into D-alanine by alanine racemase and coupling of two D-alanines by D-alanine: D-alanine ligase</td>
</tr>
<tr>
<td>bacitracin</td>
<td>prevents de-phosphorylation of undecaprenyl pyrophosphate thereby inhibiting the lipid carrier cycle</td>
</tr>
<tr>
<td>vancomycin</td>
<td>binds to acyl-D-alanyl-D-alanine region of the lipid-linked precursors preventing incorporation into the wall</td>
</tr>
<tr>
<td>β-lactam antibiotics e.g., penicillins and cephalosporins</td>
<td>prevent cross linking by inhibition of transpeptidase/carboxypeptidase</td>
</tr>
</tbody>
</table>

Adapted from (73).
1.3 Glucose Utilization in Pseudomonads

General carbohydrate metabolism in bacteria has been exhaustively reviewed (76,77). The Entner-Doudoroff (ED) pathway (78) was shown to occupy a pivotal role in glucose utilization in pseudomonads. This pathway was also demonstrated to play an important function in the degradation of gluconate in enteric bacteria (79). As shown in Fig. 7, the two enzymes that comprise the pathway are 6-phosphogluconate dehydratase (Edd) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (Eda). Both enzymes were extensively characterized by Wood et al. (81,82,83). Pseudomonads are unable to metabolize glucose by the Embden-Meyerhof pathway due to a lack of phosphofructokinase (84).

In pseudomonads, glucose is converted to glyceraldehyde-3-phosphate and pyruvate via the ED pathway. The key intermediate in this process is 6-phosphogluconate (6PGA). There are two possible routes for the conversion of glucose into 6PGA: the direct oxidative pathway which is extracellular and the intracellular phosphorylation pathway. The formation of 6PGA represents the point of convergence of the two pathways, and physiological conditions dictate which one (direct oxidative or phosphorylative) predominates. Both pathways are shown in Fig. 7.

In the oxidative pathway, glucose is successively oxidized to gluconate and 2-ketogluconate in the periplasm by the action of membrane-associated, pyridine nucleotide-independent glucose (Gcd) and gluconate dehydrogenases (Gad) respectively (85,86,87). 2-Ketogluconate is actively transported into the cell (88,89,90) and phosphorylated by a kinase (Kkg) to 2-keto-6-phosphogluconate (91,92) which is converted to 6-phosphogluconate (6PGA) by a reductase (Kgr). Glucose can also be
FIGURE 7

Pathways Related to Glucose Utilization in Pseudomonads

The above scheme was adapted from a review of *Pseudomonas* carbohydrate catabolism by Lessie and Phibbs (80). 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-ketogluco-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 phosphoglucoisomerase, 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; Pgk. 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.
FIGURE 7
Pathways Related to Glucose Utilization in Pseudomonads

[Diagram showing metabolic pathways involving glucose, gluconate, and related metabolites.]
actively transported into the cell of most pseudomonads (93), where it is phosphorylated in an ATP-dependent reaction to glucose-6-phosphate (G6P). The G6P is converted into 6PGA by pyridine nucleotide-dependent oxidation (94,95). 6PGA can also be produced by the transport and phosphorylation (Gmk) of gluconate (94).

The first substrate in the Entner-Doudoroff (ED) pathway is 6PGA (Fig. 5). It is dehydrated by 6-phosphogluconate dehydratase (Edd) to 3-deoxy-2-keto-6-phosphogluconate (KDPG) (81). The enol-KDPG formed spontaneously rearranged to the keto form (82). The enzyme (Edd) has a requirement for divalent cations; is further activated by reduced glutathione and is totally and irreversibly inactivated by p-chloromercuribenzoate (81). The KDPG thus formed is cleaved to pyruvate and D-glyceraldehyde-3-phosphate by KDPG aldolase (Eda). It exhibits no requirement for metal ions, is not activated by reduced glutathione and was not affected by p-chloromercuribenzoate (81).

Mutants of P. putida and P. aeruginosa blocked in the ED pathway (deficient in Edd and Eda) were unable to utilize glucose or gluconate (96,84). Allenza and Lessie (97) cautioned against interpreting the failure of such mutants to utilize various carbohydrates as evidence for exclusive degradation via the ED pathway. It was postulated that accumulation of 6PGA and/or KDPG could lead to hexose phosphate toxicity. In P. putida A.3.12 (ATCC 12633), the physiological inducers of Edd and Eda were gluconate or 2-ketogluconate and KDPG (96). The hexosemonophosphate (HMP) pathway has no major role in the dissimilation of 6PGA in most pseudomonads, including P. putida (98,99). There is a natural deficiency of 6-phosphogluconate dehydrogenase (6PGAD) in P.
aeruginosa (81). However, in the nutritionally versatile P. cepacia (98) and the closely related P. marginata, 6PGAD is a prominent enzyme of glucose and gluconate degradation. There is both a constitutive and an inducible 6PGAD species in both pseudomonads (100,101).

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

Interest in the metabolism of 4-deoxy-4-fluoro-D-glucose (4FG) emanated from the demonstration that when glucose-grown whole cell suspensions of P. putida were incubated with 4FG for 24 h, there was a 95% release of fluoride ion (102). Further, although P. putida could not utilize 4FG as a sole carbon source, the presence of an equivalent amount of 4FG had a negligible effect on growth yield, when the organism was grown on glucose.

Glucose-grown whole cell suspensions of P. putida did not oxidize 4FG to any significant extent. However, crude cell-free extracts of the same organism were shown to oxidize 4FG to the extent of two atoms of oxygen per molecule of 4FG, but without fluoride release (103).

Following incubations of 4FG with whole cell preparations of P. putida, the supernatants and intracellular contents were analyzed by thin-layer chromatography for the presence of non-fluorinated metabolites. The absence of such compounds led to the proposal that there had been a covalent incorporation of the carbon skeleton of 4FG into a cell envelope protein. An alkylation reaction was postulated and later supported by evidence that defluorination was dependent upon protein synthesis (103).

The $K_m$ and $V_{max}$ for the defluorination reaction by glucose-grown whole cells (3.9 mM and 1 nmol F'/mg Prot/min respectively) were identical to chloramphenicol-treated glucose-grown whole cells. This suggested that
the putative defluorinating protein was not directly inhibited by chloramphenicol but that de novo synthesis of protein was prevented (103).

When P. putida was grown on glucose, gluconate or 2-ketogluconate the organism was capable of defluorinating 4FG. However, this defluorinating activity was repressed when the organism was grown on succinate or citrate (103). Glucose, gluconate and 2-ketogluconate inhibited fluoride release to the extent of 91, 90 and 87% respectively, when added to whole cell/4FG incubations. In addition, N-ethyl maleimide produced a 100% inhibition. It was concluded that fluoride release from 4FG was dependent upon an inducible/repressible protein associated with the outer membrane of the organism. Whilst the inducible/repressible protein remains a viable entity, recent evidence has eliminated the outer membrane as the site of defluorination. It appears that the outer membrane preparation (104) was contaminated with whole cells.

To investigate the mechanism of fluoride release from 4FG, 4-deoxy-4-fluoro-D-[6-3H]-glucose (D-[6-3H]-4FG) was synthesized (105). When whole cells of P. putida were incubated with 1 mM D-[6-3H]-4FG, there was a 100% release of fluoride. In addition, 70% of the total radioactivity was found in the supernatant fraction, with the remainder in the cell pellet. After extensive dialysis of the pellet, approximately 1% of the radioactivity remained associated with the cell envelope. Borate anion-exchange column analysis of the supernatant fraction revealed that the radioactivity was essentially due to tritiated water ($^3$H$_2$O), with a lesser but significant amount (20%) associated with an unidentified tritiated component (19).

The problem of extensive loss of tritium with D-[6-3H]-4FG was
circumvented by synthesizing $^{14}$C-labelled 4-deoxy-4-fluoro-D-glucose (D-
[U-$^{14}$C]-4FG), which was used to trace the fate of 4FG metabolism in *P.
putida*. When glucose-grown whole cell suspensions of the organism were
incubated with $^{14}$C-4FG (1 mM) for 24 h at 30°C, there was a 95% ± 5%
release of fluoride ion and the loss of CO$_2$. The 24-h supernatant was
analyzed by borate anion-exchange chromatography, revealing the presence
of two radiolabelled components. These were described as the "major and
minor peaks". The major peak was subsequently identified as 2,3-dideoxy-
D-glycero-pentonic acid (2,3-dideoxyribononic acid) using NMR and mass
spectrometric analyses (106). In addition, a small but significant amount
of radioactivity was incorporated into a peptidoglycan (< 1%) associated
complex (106).

1.5 Objectives

As part of the continuing investigation into the metabolism of 4FG
in *P. putida* (ATCC 12633) the principal goals of this study are:

1. To confirm the presence or absence of a novel radiolabelled
fluorinated or non-fluorinated component in the peptidoglycan of *P.
putida*.

2. To isolate and identify the "minor peak" metabolite previously
reported (107).

3. The effect of different inhibitors, buffers and conditions of pH on
defluorination of 4FG in whole cell suspensions.

4. To establish whether 4FG is transported into the cell and metabolized
prior to defluorination.

5. Isolate and identify 4FG metabolites prior to the formation of 2,3-
dideoxyribononic acid.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Bacteriological media, yeast extract and Gram-stain reagents were purchased from Difco Laboratories, Detroit, MI USA. HPX-87C (Ca\(^{2+}\) form) cation-exchange analytical column (300 x 7.8 mm), Carbo-C Micro-Guard cartridge, obtained from Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON. L-Amino acid standards, N-acetylglucosamine (NAG), N-acetylmuramic acid (NAM), Muramic acid, Bovine serum albumin fraction V, Ammonium Tetraborate, Dowex 1-X8 (200-400 dry mesh) Anion-exchange resin, Triton X-100 and Pronase (S. griseus) Type XIV were obtained from Sigma Chemical Company, St. Louis, MO USA. Deoxyribonuclease (DNase) I, Grade II and Ribonuclease (RNase) A, Type IAS (both from bovine pancreas) were purchased from Boehringer Mannheim Canada, Ltd., Dorval, Quebec.

Calcium sulphate, calcium nitrate and 2,4-Dinitrophenol were purchased from BDH Chemicals Canada, Ltd. 1,3-Dicyclohexylcarbodiimide (DCCD) was purchased from Aldrich Chemical Company, Milwaukee, WI USA. Crystalline 4FG and D-\([U^{14}C]\)4FG (specific activity 10,600 dpm/\(\mu\)mole) was previously synthesized in this laboratory (106). Scintillation-grade toluene, 2,5-diphenyloxazole (PPO) and 1,4-bis(5-phenyloxazol-2-yl)-benzene (POPOP) and scintillation vials were obtained from Fisher Scientific Co. Ltd. Dupont Formula 963 aqueous scintillation fluid was purchased from DuPont, NEM Research Products, Boston, MA 02118 USA.

The Gram-negative organism, *Pseudomonas putida* (ATCC 12633) (formerly classified as *P. fluorescens* A3.12) was obtained as a freeze-dried sample
from American Type Culture Collection (ATCC), Rockville, Maryland.

All other chemicals and reagents were of "Analar" or HPLC grade and were obtained from BDH Chemicals Canada, J.T. Baker Chemical Co., NJ USA or Sigma Chemical Co. Deionized water used in these studies was obtained from a Zenopure Laboratory Water System (Zenon Environmental Inc., Burlington, ON).

2.2 Methods

2.2.1 Culture Conditions

Glucose-grown whole cells and cell-free extracts of *Pseudomonas putida* biotype A (ATCC 12633) were used in this investigation. The organism was routinely maintained on glucose-mineral salts-agar slants by transferring to a fresh slant every 2-3 months or more frequently when required. The cultures were grown for 24 h at 30°C and then stored at 4°C in sealed plastic bags.

*Escherichia coli* (ATCC 23227) was also used. The organism was maintained on glucose and gluconate mineral salts agar slants by daily transfer for 10 days prior to use. Each culture was grown for 24 h at 30°C.

2.2.2 Growth Media

The semi-defined medium of Davis and Mingioli (107) was used throughout these studies.

<table>
<thead>
<tr>
<th></th>
<th>g/L (final volume)</th>
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<tbody>
<tr>
<td>$\text{K}_2\text{HPO}_4$</td>
<td>7.0</td>
</tr>
<tr>
<td>$\text{KH}_2\text{PO}_4$</td>
<td>3.0</td>
</tr>
<tr>
<td>$(\text{NH}_4)_2\text{SO}_4$</td>
<td>1.0</td>
</tr>
<tr>
<td>$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Yeast extract 0.2
Trace solution A 0.5 mL
Trace solution B 0.5 mL
Carbon source 2.0

Solutions of trace elements, made up according to Barnett and Ingram (108), contained the following:

**Trace solution A**

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/100 mL (stock solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO$_4$.4H$_2$O</td>
<td>80</td>
</tr>
<tr>
<td>MnSO$_4$.4H$_2$O</td>
<td>80</td>
</tr>
<tr>
<td>NaCl</td>
<td>2000</td>
</tr>
</tbody>
</table>

**Trace solution B**

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/100 mL (stock solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>40</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>8</td>
</tr>
<tr>
<td>CoCl$_2$.6H$_2$O</td>
<td>8</td>
</tr>
<tr>
<td>CaCl$_2$(anhydrous)</td>
<td>1000</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>10</td>
</tr>
<tr>
<td>KI</td>
<td>60</td>
</tr>
</tbody>
</table>

The resulting medium (pH 7.1) was sterilized by autoclaving in batches of 1 L in 4 L Erlenmeyer flasks for 20 min at 121°C in a Peltan and Crane Magna-Clave, Model MC (GA Ingram and Co., (Canada) Ltd., Windsor, ON). Alternatively, 12 L of media were sterilized in a bench top batch fermentation unit (Model SF-116, New Brunswick Scientific Co. Inc., Edison, NJ, USA) for 30 min at 121°C. A 2% (w/v) of carbon source, D-glucose was autoclaved separately and an appropriate amount was
aseptically transferred to the mineral salts medium to give a final concentration of 0.2% (w/v).

2.2.3 Mineral Salts Agar Slants

The following solutions were prepared:

Mineral Salts growth media 200 mL
Carbon Source (D-glucose or D-gluconate) 2% (w/v) 1 g/50 mL
Plain Agar 2% (w/v) 10 g/250 mL

The solutions were autoclaved separately for 20 min at 121°C. Whilst still hot, the sterile solution of mineral salts and agar were combined. The carbon source (D-glucose) was then added and the solution thoroughly mixed. The hot mixture, in 10 mL aliquots, was pipetted into 25 mL glass tubes. The tubes were capped and allowed to cool (20-24 h) in a slanted position at RT. All of the above transfers were performed under aseptic conditions. The slants were then stored in a sealed plastic bag at 4°C.

2.2.4 Gram-staining Procedures

Cultures of *P. putida* were routinely tested for contamination by performing a Gram stain (Difco Bacto Gram Stain set, Difco Labs. Detroit, MI) in accordance with the manufacturers recommendations. Microscopich examination using an oil immersion lens (mg x 1000) revealed short Gram-negative rods.

2.2.5 Preparation of Whole Cell Suspensions

The whole cells were grown in sterile glucose mineral salts media either in batches of 1 L in 4-L Erlenmeyer flasks or in a 12 L bench top fermentation unit. The source of the inoculum for these cultures was obtained from 24 hour grown agar slants (previously described). This was
accomplished by aseptically transferring 1 loop of cells from the agar slants to 10 mL of sterile mineral salts solution and incubating for 1 h at 30°C on a rotary shaker (300-400 rpm). Growth was subsequently initiated by aseptically transferring the inoculum (10 mL) to each flask. The 4 L flasks containing the inoculated media were placed in a laboratory Orbital shaker (Lab-Line Instruments Inc., Melrose Park, IL) 150 rpm and allowed to grow for 14 h at 30°C.

An identical inoculating protocol was employed when 12 L of cells were grown in a bench top fermentation unit. The inoculum (10 mL) was administered to the unit by aseptic transfer through an inoculating port on top of the vessel. The large batch culture was aerated at 1.5 L/min, stirred at 400 rpm and maintained with a slight positive pressure (0.5 lbs/sq. inch) at 30°C for 14 h. At this time, with either method, an absorbance of about 1.0 (620 nm) indicated that the culture had attained the pre-stationary phase of growth. This measurement was performed in a Bausch and Lomb Spectronic 20 Spectrophotometer using mineral salts medium as a blank.

Whole cells of *Pseudomonas putida* (*P. putida*) were harvested in sterile 1L centrifuge bottles by centrifugation at 4500 x g for 25 min at 4°C in a Beckman JX8 (Beckman Instruments., Palo Alto, CA 94304). The cell pellet was washed in phosphate or Tris-HCl buffer (100 mM, or 50 mM, pH 7.1) and re-suspended with 20-25 mL of the chosen buffer. The average wet weight yield of cells was 3-3.6 g/L of media.

Certain visual criteria were useful for assessing the healthy growth of *P. putida*. At 14 h, the growth media had a distinctive lime-green colour, whilst the harvested cell pellet possessed a pink-red appearance.
In addition, the cells exhibited fluorescence when subjected to a very short pulse of irradiation from a UV source.

Whole cells of *Escherichia coli* (*E. coli*) were grown in sterile glucose and gluconate mineral salts media. All procedures were as described for *P. putida*.

### 2.2.6 Protein Determination

Whole cell suspension of *P. putida* were assayed for protein content by the Coomassie Brilliant Blue G-250 dye-binding method of Bradford (133), as formulated by Bio-Rad Laboratories. Bovine Serum Albumin was used for the production of a standard curve (Appendix 1). All absorbance measurements were carried out in a Hewlett Packard (8451A Diode Array Spectrophotometer). When re-suspended with 20-25 mL of the chosen buffer (Tris or Phosphate) the whole cell protein content was 5-6 g/L media.

### 2.2.7 Cell Free Extracts

Glucose grown whole-cell pellets were re-suspended with an equal volume of PPB pH 7.1 and homogenized. The cell suspension was maintained at 4°C in an ice-bath and sonicated (Sonic 300 Dismembranator, Artek Systems Corp. Farmingdale, NY) for 10 min, using 20 sec bursts (100%) followed by 1 min cooling. During the procedure, the cream colored suspension progressively changed to a reddish-brown appearance, providing an indicator of cell disruption. After centrifugation at 15000 x g at 4°C for 15 min, there was usually no pellet. The amber colored supernatant was defined as the ‘crude cell-free extract’, and was assayed for both defluorinating and soluble enzyme activity.
2.2.8 Particle Free Extract

The crude cell-free extract was centrifuged at 200,000 × g at 4°C for 2.5 h. The resultant supernatant was designated as the particle free extract (109) and retained for soluble enzyme analysis. The 200,000 × g pellet was defined as the particulate fraction.

2.2.9 Glucokinase Enzyme Assay

The crude cell-free and particle-free preparations were assayed for glucokinase activity using the method of Eisenberg et al. (93). Enzyme activity was estimated by coupling with glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: nicotinamide adenine dinucleotide phosphate (NADP) oxidoreductase, EC 1.1.1.49) and measuring the production of reduced NADP (NADPH) at 340 nm in a Shimadzu UV 240 Spectrophotometer (Shimadzu Corp., Kyoto, Japan) at room temperature (~25°C).

Glucokinase reaction mixtures of 1 mL contained (final concentration): Tris-HCl, 200 mM pH 8.2, β-mercaptoethanol 2.5 mM, magnesium chloride 10 mM, NADP 5 mM, D-glucose or 4-deoxy-4-fluoro-D-glucose (4FG), 1 mM and 25 mM, ATP 4 mM and G6PDH 2 units. The latter was in excess so that glucokinase was rate limiting.

2.2.10 Fluoride Ion Measurement

Fluoride ion was measured with a combination fluoride electrode (Orion Research, Cambridge, Mass) coupled to a E510 Precision mV/pH Meter (Metrohm Herisau, Switzerland). The electrode was replenished with reference filling solution (saturated AgCl), inverted several times and
allowed to equilibrate overnight at room temperature in a solution of sodium fluoride standard (NaF, 10 mM) in the chosen buffer. Potassium phosphate buffer (PPB), 100 or 50 mM, pH 7.1; Tris-HCl, 100 or 50 mM, pH 7.1.

Prior to use, the electrode was thoroughly rinsed and partially immersed in deionized water for about an hour at room temperature. Fluoride ion measurements were facilitated by inserting the tip of the electrode into the suspensions or solutions. Standard curves relating electrode potential to fluoride ion concentration, \( [F^-] \) were obtained from a series of NaF standards prepared in the chosen buffer. A standard curve was produced immediately prior to each intended use of the fluoride electrode (Appendix 2).

2.2.11 Scintillation Counting

A Beckman LS 7500 liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA) was used to measure radioactivity. Aqueous scintillation fluid (10 mL, Formula 963 DuPont Canada) was added to each 1 mL sample. Smaller sample volumes were made up to that same volume with deionized water. A homogeneous solution was obtained by vortexing each vial for about 20 sec. Quenching was determined by the "H-number" method. Counting efficiency was derived from a quench correction curve (Appendix 3) constructed by plotting counting efficiency against H-number values obtained from a series of standards (\(^{14}C\)) of varying quench composition (Nuclear Chicago). Column fractionated samples were usually counted for 50 min, with a "2 sigma % error" which provided a confidence level of 95%. 
2.2.12 Labelling and Fractionation of Whole Cells

Glucose-grown whole cells (100-950 mg whole cell protein) were incubated with 1 mM D-[U-\(^{14}\)C]4FG (SA. 10600 dpm/\(\mu\)mol) or Na\(^{14}\)CO\(_3\) in sterile potassium phosphate buffer (PPB) (50 mM or 100 mM, pH 7.1) or sterile Tris-HCl (100 mM, pH 7.1) in total incubation volumes of 10-200 mL. The incubation times varied from 40 min to 24 hours and were carried out at 30°C in sterile Erlenmeyer flasks on a rotary shaker (Lab-line Instr. Inc. Melrose Park, Ill). Upon completion of the incubation, the cell suspension was quickly subjected to a fluoride ion (F\(^-\)) determination and then centrifuged at 10,000 x g at 4°C for 15 min in a Sorvall-Superspeed RC2-B (Ivan Sorvall Inc., Newton, CT 06470). The decanted supernatant was again checked for [F\(^-\)] and a sample removed for radioactivity measurement. The supernatant and cell pellets were either, immediately frozen using liquid nitrogen and stored at -20°C or prepared (without freezing) for metabolite isolation by anion exchange chromatography.

2.2.13 Isolation of Peptidoglycan

The whole-cell pellet was processed by a method adapted from Kolenbrander (110) for the extraction and isolation of peptidoglycan. The procedure, shown below, was modified with regard to centrifugation conditions. Following SDS-solubilization, it was found that 200,000 x g (as opposed to 13,000 x g) was necessary in order to clarify the suspensions.
PROCEDURE FOR THE ISOLATION OF PEPTIDOGLYCAN FROM P. putida

Whole Cell Pellet

Washing PPB (100 mM, pH 7.1)

Re-suspended in PPB; Sonication on ice

Crude Extract incubated with DNase (20 mg), RNase (5 mg) and MgSO₄ (25 mg) for 2 h, 30°C (shaken) in PPB. SDS added to 2% boiling H₂O bath centrifugation 200,000 x g, 4°C, 2 h.

Supernatant (Spnt.) Pellet resuspended with SDS 1%
Boiling H₂O bath 1 h cooled-stirred
12 h at RT
Centrifugation 200,000 x g, 4°C, 2 h.

Spnt. Pellet washed 8 times with deionized H₂O. Centrifugation 200,000 x g, 4°C, 2 h.

Spnt. Washed pellet re-suspended in Tris-HCl (25 μM, pH 7.1), incubated with Pronase (100 mg) and CaCl₂ (10 mM) 4 h, 30°C (vol 5 mL). Boiling H₂O bath 30 min.
Centrifugation 200,000 x g, 4°C, 2 h.

Spnt. Pellet washed successively with 150 μM EDTA (4 times) and deionized H₂O (4 times). Centrifugation 200,000 x g, 4°C, 2 h.
Pellet hydrolyzed with 6 N HCl, 106°C, 12 h.

2.2.14 Acid Hydrolysis of Peptidoglycan

The washed pellet of peptidoglycan (PG) was suspended in 1 mL of deionized water and 200 μL aliquots were hydrolysed with 6 N HCl in sealed tubes at 100°C for 10 h. The resultant hydrolysates were evaporated to dryness under N₂, re-solubilized in deionized water (200 μL), pooled and taken to dryness by rotary evaporation. The residue was re-solubilized
in 500 µL deionized water and used for HPLC analysis. To serve as a control, a mixture containing Mur, Glu, Asp, Thr, Ser, Ala, Gly, Glucosamine, meso-diaminopimelic acid (DAP), Leu was subjected to identical hydrolytic conditions as the PG.

2.2.15 HPLC Analysis of Peptidoglycan

The analyses were performed with a Waters Millipore System, using a Bio-Rad HPX-87C column fitted with a Carbo-C guard column. Using RI detection, the peaks were recorded with a Spectra Physics SP 4290 computing integrator. The column was operated at 85°C and at a flow rate of 1 mL/min. The column was standardized with a mixture of the appropriate amino acids and amino sugars (Fig. 10), which had been subjected to the same hydrolytic conditions as the peptidoglycan.

700 µL (129 ± 4 dpm) of hydrolysed PG from incubation 1 was applied to the column in 7 x 100 µL injections. Fractions were collected at 1 mL/min. The 7 mL fractions were individually concentrated 10-fold by rotary evaporation (35°C) and counted for radioactivity. The radiolabelled fractions were re-injected in the presence/absence of appropriate amino acid standards to confirm chromatographic identity (Table 5). The efficiency of this semi-preparative mode was shown in the label recovery of 121 ± 4 dpm (94%). An identical protocol was used for the analysis of incubates 2 and 3.

2.2.16 Orcinol-Sulphuric Acid Colorimetry

Borate column eluted fractions were analyzed for carbohydrate content by orcinol-sulphuric acid (H₂SO₄) colorimetry (111). This consisted of adding 2 volumes of orcinol-H₂SO₄ reagent (0.1 % w/v orcinol in 70% v/v H₂SO₄) to each fraction, vortexing, heating at 95°C for 20-30 min, cooling
to room temperature and measuring the absorbance at 420 nm in a Shimadzu UV-240 UV-Visible recording spectrophotometer.

2.2.17 Inorganic Phosphate Determination

Inorganic phosphate in borate column eluted fractions was monitored by the method of Clark et al (112). Acid-molybdate reagent (1 mL of 2.5% w/v (NH₄)₆Mo₇O₂₄·4H₂O in 13.6% w/v H₂SO₄) was added to each fraction followed by 1 mL of reducing agent (3% w/v sodium bisulphite and 1% w/v para-methylaminophenol), vortexing and observing the formation of a dark blue colour after standing for 15-20 min at room temperature.

2.2.18 Time Course Study of Defluorination

A whole cell suspension of *P. putida* (24 mL, 230 mg whole cell protein) was incubated in 50 mM Tris-HCl pH 7.1 with 1 mM D-[U-¹⁴C]4FG at 30°C (shaken). At certain time intervals 3 mL aliquots were removed and immediately centrifuged in an Eppendorf 5914, (Brinkman Instr. Div., Rexdale, ON) at 15600 x g for 5 min at 30°C. The resulting supernatants and pellets were frozen in liquid nitrogen prior to storage at -20°C. Subsequently, the supernatants were analyzed for fluoride ion concentration (Method 2.2.10) and radiolabel distribution by borate anion-exchange chromatography.

2.2.19 Isolation of Extra-Cellular Metabolites

The supernatant from an 80 min whole-cell incubation was concentrated to about 1 mL under vacuum at 35°C on a Buchi Rotavapor EL 130 Rotary Evaporator (Brinkman Instruments Ltd., Rexdale, ON). After removing a small sample for radioactivity measurement, non-radiolabelled D-glucose and 4FG (internal standards) were added to the supernatant and the mixture applied to a Dowex 1-X8 200-400 dry mesh borate anion-exchange column (25
x 1 cm). In addition, 2 x 1 mL aliquots of starting buffer were used to rinse the flask containing the supernatant and this was used to wash the sample into the resin bed. The column was topped up with starting buffer and percolated, by gravity flow at 24 mL/h with an increasing linear concentration gradient of ammonium tetraborate; 200 mL of 29 mM Ammonium Tetraborate/57 mM Boric Acid (starting buffer) linearly diluted with 200 mL of 500 mM Ammonium Tetraborate. (Total Linear Gradient volume, 400 mL.) Fractions (4 mL) were collected with an LKB Bromma 2112 Redirac Fraction Collector (LKB Produkter AB, Bromma, Sweden). Each fraction was analyzed for radioactivity (250 μL → 1 mL). Even numbered fractions were analyzed for carbohydrates by Orcinol-H₂SO₄ colorimetry (Methods 2.1.16). Odd numbered fractions were assayed for inorganic phosphate (Methods 2.1.17).

The radioactive fractions corresponding to the metabolite peak were pooled and concentrated to dryness under vacuum in a rotary evaporator. Ammonium tetraborate was removed by repeated addition and evaporation of 5 x 100 mL aliquots of anhydrous methanol. The final residue (metabolite) obtained from this procedure was solubilized with 1-2 mL of D₂O, concentrated to dryness and re-solubilized with 500 μL of D₂O and used for analysis by NMR, Mass Spectrometry and TLC.

2.2.20 Isolation and HPLC Analysis of the Minor Peak

The "Minor Peak" metabolite was isolated from the pooled supernatants derived from several 24 h incubations by an identical procedure to that described above. From a 500 mL pool which had been stored at -20°C for several months, 50 mL portions were removed, concentrated to about 2 mL and analyzed on a preparative scale borate anion-exchange column (112 x
1.5 cm; Bed volume 198 cm³. Total Linear Gradient volume of Ammonium Tetraborate 1200 mL) at a flow rate of 20 mL/h. It required 10 cycles of this procedure to complete the isolation. The radioactive fractions (4 mL corresponding to the minor peak were pooled, concentrated and re-solubilized in deionized water (800 μL). This pool was fractionated (20 injections) by HPLC (HPX 87C) using the same operating conditions as described earlier (Methods 2.2.15) except, deionized water was employed as the mobile phase. From each of the resulting 20 mL fractions, 200 μL aliquots were monitored for radioactivity by liquid scintillation counting (Methods 2.2.11). Those fractions containing radiolabel were pooled, concentrated to dryness and re-solubilized in 450 μL of D₂O. A 10 μL sample was re-injected onto the HPLC column and the remainder analyzed by TLC and both ¹³C and ¹H NMR.

The "Major Peak" metabolite previously identified as 2,3-dideoxy-D-glycero-pentonic acid (106) was also isolated during these chromatographic separations and stored at -20°C for future use as a standard.

2.2.21 Thin Layer Chromatography of Radiolabelled Metabolites

Following isolation, radiolabelled metabolites were analyzed by ascending Thin layer chromatography (TLC) on 20 x 20 cm silica gel-60 (0.2 mm thickness) plastic backed sheets (Merck, Darmstadt, Germany). The solvent used for development was ethyl acetate-acetic acid-H₂O (3:3:1 v/v). This solution was added to the developing tank and the top firmly sealed about 12-16 h prior to use, thus allowing complete solvent equilibration. After 2-2.5 h, the plates were air dried (suspended in a fume cupboard), sprayed with sulphuric acid:ethanol (1:1 v/v) and incubated at 140°C for 20 min to visualize the spots.
To ascertain the distribution of radioactivity in a developed silica gel TLC, the entire chromatogram was taped (cellophane) to a glass plate. Each vertical section was cut into 1 cm squares and each square was placed in a scintillation vial together with 1 mL of de-ionized water and 10 mL of scintillation cocktail (Formula 963) and vortexed. An adjacent vertical section, devoid of radioactivity was treated identically and served as a background control. Radioactivity was determined as described under 'scintillation counting' (Methods 2.1.11).

2.2.22 Procedure for the Isolation of Intra-Cellular Metabolites

The pellet derived from whole-cell incubations was processed according to the protocol shown in the flow diagram.

Whole Cell Pellet

3 washes with PPB (50 mM, pH 7.1)  
Centrifugation 15000 x g, 4°C 10 min

Washed whole cell pellet
Re-suspended in PPB (50 mM, pH 7.1)  
Sonication on ice, 20 sec bursts (100%) with 1 min cooling for 10 min  
Centrifugation 15000 x g, 4°C, 10 min

Small dark  
amber pellet  
with red halo  

Spnt. (Post Sonicated)  
Protein precipitation with 3-4 drops of cold 10% TCA  
Centrifugation 15000 x g, 4°C, 10 min

Cream-white  
pellet (protein)  

Spnt. (Post TCA)  
- extract TCA into H2O-saturated ether  
- lower aqueous layer concentrated by rotary evaporation and applied to borate anion-exchange column
2.2.23 Fast Atom Bombardment (FAB) Mass Spectrometry

The isolated radiolabelled metabolite in deuterium oxide was analyzed by positive and negative ion FAB mass spectrometry. The instrument used was a VG-ZAB magnetic sector, reverse geometry machine with an 8 kV accelerating voltage, at the UCR Mass Spectrometry Facility, Department of Chemistry, University of California, Riverside CA 92521. The sample matrix was glycerol and the beam of Xenon atoms was run at 8 kV x 1 mA (8 watts).

2.2.24 Fourier Transform (FT) Carbon-13 and Proton NMR

$^{13}$C and $^1$H NMR spectroscopic analysis of the radiolabelled metabolites in D$_2$O was carried out on a Bruker 300 MHz FT NMR Spectrometer. Chemical shift values were relative to dioxane and expressed in ppm.
CHAPTER 3
RESULTS AND DISCUSSION

3.1 Isolation and Analysis of Radiolabelled Peptidoglycan

In previously reported work (105) it was shown that following a 24 h incubation of 1 mM D-[U-\(^{14}\)C]4FG with glucose grown whole cells of \(P. \textit{putida}\), there was a 95% release of fluoride ion from the sugar. In addition, almost 90% of the radiolabel was accounted for in the supernatant and whole cell dialysates, whilst 5% was recovered as \(^{14}\)CO\(_2\). It was also established that 0.42% of the radiolabel was covalently associated with the cell envelope, defined as the pellet resulting from centrifugation at 100,000 x g of a cell-free extract. Gel column chromatography of this SDS-solubilized cell envelope gave a high molecular weight (> 400 kD) radiolabelled fraction. This fraction was susceptible to lysozyme treatment to yield lower molecular weight (14 kD) fragments. It was concluded, therefore, that the radiolabel had been incorporated into a peptidoglycan (PG)-protein associated complex.

The presence of the small amount of radioactivity in the PG was potentially significant. It was important to establish whether or not it was due to the presence of a novel carbohydrate analogue, derived from 4FG metabolism. If a 4-deoxy metabolite derived from 4FG (e.g., N-acetyl-4-deoxy-D-glucosamine) were to be incorporated into the PG, it would terminate cell wall biosynthesis and hence, cell growth.

Table 2 shows the amount of radioactivity recovered in acid-hydrolyzed PG following isolation from a 200 mL, 24 h incubation of \(P. \textit{putida}\) with D-[U-\(^{14}\)C]4FG (Incubation 1) and a \(^{14}\)C-labelled crude cell-free extract (Incubate 2) prepared earlier and stored at -20\(^\circ\)C. Since this had been
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incubation 1</th>
<th>Incubation 2</th>
<th>Incubation 3</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4FG/Whole Cells</td>
<td>4FG/Whole Cells</td>
<td>NaH¹⁴CO₃/Whole Cells</td>
<td>Inc. 2 Inc. 3</td>
</tr>
<tr>
<td>Pre-Pronase Treatment of PG</td>
<td>nd</td>
<td>1795±42</td>
<td>1489±62</td>
<td>-</td>
</tr>
<tr>
<td>Post-Pronase Treatment of PG</td>
<td>nd</td>
<td>303±10</td>
<td>190±8</td>
<td>16.9</td>
</tr>
<tr>
<td>PG Acid Hydrolysate</td>
<td>129±4</td>
<td>254±9</td>
<td>nd</td>
<td>14.2</td>
</tr>
<tr>
<td>HPLC of Acid Hydrolysate PG</td>
<td>121±4</td>
<td>162±6</td>
<td>149±9</td>
<td>6.6</td>
</tr>
</tbody>
</table>

nd - not determined

Radiolabelled peptidoglycan (PG) was isolated as described in Method 2.2.13. The PG acid hydrolysates (2.2.14) were analyzed by HPLC (2.2.15). Radioactivity is expressed in dpm above background.
previously sonicated, its subsequent processing began with the DNase/RNase/SDS treatment as shown in the flow diagram for PG isolation (Methods 2.2.13). Table 2 also shows the amount of radioactivity recovered in acid-hydrolyzed PG following a similar 200 mL incubation of whole cells with NaH\(^{14}\)CO\(_3\). The acid hydrolyzed PG was analyzed and subsequently fractionated using a Bio-Rad HPX-87C cation exchange column.

In accordance with the manufacturers recommendations, CaSO\(_4\) (10 mM, pH 5.5) was initially used as the mobile phase, but resolution of muramic acid and glutamic acid was only achieved after progressively decreasing the pH to 3.6. However, due to its greater solubility, Ca(NO\(_3\))\(_2\); (50 mM, pH 3.6) was subsequently chosen as the mobile phase.

HPLC analysis of the isolated PG fraction (Fig. 8) showed all the commonly accepted amino-acid and amino-sugar constituents of gram-negative PG; glucosamine, muramic acid, glutamic acid, alanine, glycine and diaminopimelic acid (DAP). In addition, aspartate threonine and serine were also identified. Aspartic and glutamic acids co-eluted (peak e, Fig. 8). They were, however, resolved by using Ca(NO\(_3\))\(_2\) 200 mM, pH 3.6 (Fig. 9). The presence of muramic acid and DAP which are unique to PG were regarded as appropriate diagnostic features. The PG was therefore, deemed to be chromatographically pure.

The identity of the amino-acid and amino-sugar components of the PG was achieved by comparing their retention time to those of appropriate standards (Table 3). The standards were subjected to the same
FIGURE 8
HPLC Analysis of Acid Hydrolysed Peptidoglycan from

P. putida following D-[U-14C]-4FG Incubation

The chromatogram was obtained from the HPLC analysis of acid hydrolysed peptidoglycan as described in Methods 2.2.15. The letter code for each peak is shown in Table 3. Horizontal axis - 1 cm rep 2.7 min.
FIGURE 9

Resolution of Peak e (Fig. 8) into Glutamate and Aspartate

Peak e (Fig. 8) was resolved into glutamate and aspartate using the same HPLC conditions as previously described except that 200 mM Ca(NO₃)₂, pH 3.6 was used as the mobile phase.

y - Glutamate

z - Aspartate
TABLE 3
RetentionPolicy from HPLC Analyses of Hydrolysed PG and Standards

<table>
<thead>
<tr>
<th>Amino Acid/Sugar Inorganic Ions</th>
<th>Letter</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Acid Hydrolysed PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAG</td>
<td>c</td>
<td></td>
<td>6.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAM</td>
<td></td>
<td></td>
<td>10.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl⁻</td>
<td>a</td>
<td>4.97</td>
<td>4.95</td>
<td>4.95</td>
<td></td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>b</td>
<td></td>
<td></td>
<td></td>
<td>5.01</td>
</tr>
<tr>
<td>Muramic</td>
<td>d</td>
<td>9.27±0.02</td>
<td>9.28±0.03</td>
<td>9.24</td>
<td>9.26±0.03</td>
</tr>
<tr>
<td>Glutamic</td>
<td>e</td>
<td>12.60±0.03</td>
<td>12.53±0.07</td>
<td>12.20</td>
<td>12.38±0.06</td>
</tr>
<tr>
<td>Aspartic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>f</td>
<td>15.10±0.04</td>
<td>15.13±0.06</td>
<td>15.16</td>
<td>15.12±0.05</td>
</tr>
<tr>
<td>Serine</td>
<td>g</td>
<td>15.99±0.03</td>
<td>16.00±0.03</td>
<td>16.04</td>
<td>15.97±0.03</td>
</tr>
<tr>
<td>Alanine</td>
<td>h</td>
<td>17.30±0.08</td>
<td>17.20±0.12</td>
<td>17.16</td>
<td>17.28±0.07</td>
</tr>
<tr>
<td>Glycine</td>
<td>j</td>
<td>20.1±0.4</td>
<td>19.56±0.4</td>
<td>19.64</td>
<td>19.77±0.3</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>k</td>
<td>22.63±0.2</td>
<td>22.71±0.3</td>
<td>22.57</td>
<td>22.37±0.6</td>
</tr>
<tr>
<td>DAP</td>
<td>m</td>
<td>30.2±0.2</td>
<td>30.1±0.2</td>
<td>29.98</td>
<td>29.95±0.04</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamic</td>
<td></td>
<td></td>
<td></td>
<td>11.22±0.06</td>
<td></td>
</tr>
<tr>
<td>Aspartic</td>
<td></td>
<td></td>
<td></td>
<td>12.17±0.05</td>
<td></td>
</tr>
<tr>
<td>DAP</td>
<td></td>
<td></td>
<td></td>
<td>25.40</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td></td>
<td></td>
<td></td>
<td>28.38</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td></td>
<td></td>
<td></td>
<td>31.16</td>
<td></td>
</tr>
</tbody>
</table>

1. Mixture of standards subjected to HCl hydrolysis.

2. Same mixture of standards as 1 but not subjected to HCl hydrolysis.

3. Retention times of individually run standards.

HPLC analysis as described in Methods 2.2.15. (a) and (b) represent retention times obtained with respectively, 50 and 200 mM Ca(NO₃)₂ as the mobile phase.
hydrolytic conditions (6N HCl, 106°C, 12 h) as the isolated PG, and thus served as the closest approximation to an absolute control for hydrolysed PG (Fig. 10).

HPLC fractionation of the radiolabelled PG from incubates 1, 2 and 3, revealed the specific location of the radiolabel. The fractions containing radioactivity (Table 4) were injected onto the HPLC in the absence/presence of standards (Table 5). A comparison of retention times showed that the radiolabel from incubates 1 and 2 (4PG incubation) resided in the amino-acids glutamate, aspartate, threonine and serine. The major radioactive amino-acid was threonine.

HPLC analysis of the PG isolated from the NaH\(^{14}\)CO\(_3\) incubation (incubation 3) (Fig. 11) reveals the presence of NAG, muramic acid, glucosamine and barely detectable amounts of glutamate/aspartate. Peaks f and k remained unidentified. However, fractionation of the PG acid hydrolysate and the subsequent identification of the radiolabelled fractions (Tables 4 and 5) showed that the label had been mainly incorporated into the amino-sugars. Table 6 shows a summary of the recovered radiolabel distribution in the PG components.

The results show no evidence for the incorporation into the PG of a novel metabolite derived from the metabolism of 4PG. The principle PG components were identified as NAG, NAM, Ala, Glu, and DAP. There was evidence however, that the \(^{14}\)CO\(_2\), derived from 4PG metabolism, had been incorporated into amino acid biosynthetic pathways. The radiolabelled amino-acids were Glu, Asp, Thr and Ser (Table 6). In addition, the extensive loss of radiolabel following the pronase treatment during the PG isolation also indicated an incorporation of radiolabel into protein.
FIGURE 10

HPLC Analysis of Amino Acid/Sugar Standards

The chromatogram was obtained from the HPLC analysis (Method 2.2.15) of a mixture of standards which had been subjected to the same hydrolytic conditions as the peptidoglycan. The letter code for each peak is shown in Table 3.

Horizontal axis, 1 cm rep 2.3 min.
### TABLE 4

**Distribution of Radioactivity of Acid Hydrolysed PG by HPLC**

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Total DPM</th>
<th>Total DPM Applied</th>
<th>Rec'd. Rec'</th>
<th>Incubation 1</th>
<th>Incubation 2</th>
<th>Incubation 3</th>
<th>Amino Acid/Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>129±5</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>Glu/Asp</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td>43</td>
<td></td>
<td></td>
<td>Thr</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td>53</td>
<td></td>
<td></td>
<td>Ser</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>-</strong></td>
<td><strong>121±4</strong></td>
<td><strong>93.8±3.5%</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td>43</td>
<td></td>
<td></td>
<td>Glu/Asp</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td>91</td>
<td></td>
<td></td>
<td>Thr</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td>21</td>
<td></td>
<td></td>
<td>Ser</td>
</tr>
<tr>
<td><strong>-</strong></td>
<td><strong>162±6</strong></td>
<td><strong>71±5%</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td></td>
<td></td>
<td>NAG</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td>22</td>
<td></td>
<td></td>
<td>NAG</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
<td></td>
<td>Mur/Glu</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td></td>
<td></td>
<td>Glu/Asp</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
<td></td>
<td>Glu</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
<td></td>
<td>U</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td></td>
<td></td>
<td>U</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td>GlcNH₂</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td></td>
<td></td>
<td>GlcNH₂</td>
</tr>
<tr>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td>GlcNH₂</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
<td></td>
<td>U</td>
</tr>
<tr>
<td><strong>-</strong></td>
<td><strong>149±5</strong></td>
<td><strong>78±5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Incubations 1, 2 and 3 are as shown in Table 2.

All radioactivity is expressed in dpm, above background.

The isolated radiolabelled PG from each incubation was fractionated by HPLC as described in Method 2.2.15.

± SD, standard deviations
### TABLE 5

**HPLC Analysis of Acid Hydrolysed PG Plus or Minus Standards**

<table>
<thead>
<tr>
<th>Incub. No.</th>
<th>Active</th>
<th>50 mM Ca(NO$_3$)$_2$</th>
<th>200 mM Ca(NO$_3$)$_2$</th>
<th>Amino Acid/Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Radio- Frac. No.</td>
<td>Glu</td>
<td>Asp</td>
<td>Thr</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>15.10</td>
<td>15.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>15.10</td>
<td>15.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>16.00</td>
<td>15.96</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>15.14</td>
<td>15.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>15.21</td>
<td>15.11</td>
<td></td>
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<tr>
<td></td>
<td>16</td>
<td>16.01</td>
<td>16.09</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Glu</td>
<td>Asp</td>
<td>Thr</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>6.60</td>
<td>6.64</td>
<td>6.67</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>9.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>12.70</td>
<td>12.50</td>
<td>12.74</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>12.57</td>
<td>12.54</td>
<td>12.74</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>22.52</td>
<td>22.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>22.46</td>
<td></td>
<td></td>
</tr>
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<td></td>
<td>27</td>
<td>22.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>27.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The radioactive fractions obtained from HPLC analysis were re-injected onto HPLC in the absence/presence of standards.
FIGURE 11

HPLC Analysis of Acid Hydrolysed PG from P. putida

Following NaH\textsuperscript{14}CO\textsubscript{3} incubation

Peak a - Cl\textsuperscript{-}
Peak b - nitrate
Peak c - NAG
Peak d - Muramic Acid
Peak e - Aspartate/Glutamic
Peak k - Glucosamine

Peaks f and r were unidentified

Peak e was resolved (not shown) using 200 mM Ca(NO\textsubscript{3})\textsubscript{2} pH 3.6 as previously described in the legend to Figure 9.

HPLC analysis as previously described (Method 2.2.15).
FIGURE 11

HPLC Analysis of Acid Hydrolysed PG from P. putida

Following NaH\textsuperscript{14}CO\textsubscript{3} Incubation
TABLE 6
Summary of Amino Acid and Amino Sugar Composition of Peptidoglycan of *P. putida*

<table>
<thead>
<tr>
<th>Component</th>
<th>Incubation 1</th>
<th>Incubation 2</th>
<th>Incubation 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic Acid</td>
<td>23%</td>
<td>21%</td>
<td>11.3%</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>11.6%</td>
<td>6.5%</td>
<td>6%</td>
</tr>
<tr>
<td>Threonine</td>
<td>53.7%</td>
<td>58.7%</td>
<td>--</td>
</tr>
<tr>
<td>Serine</td>
<td>9%</td>
<td>13.5%</td>
<td>--</td>
</tr>
<tr>
<td>Muramic Acid</td>
<td>--</td>
<td>--</td>
<td>7.4%</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>--</td>
<td>--</td>
<td>22%</td>
</tr>
<tr>
<td>NAG</td>
<td>--</td>
<td>--</td>
<td>30.8%</td>
</tr>
</tbody>
</table>

* Based on integrated HPLC peak areas.

Incubations 1, 2 and 3 are as shown in Table 2.
It is feasible for $^{14}$CO$_2$ to be incorporated into a biosynthetic pathway, leading eventually to labelled threonine. The first step of $^{14}$CO$_2$ incorporation would be a direct carboxylation of pyruvate by an ATP-dependent pyruvate carboxylase which is present in most species of *Pseudomonas* (113).

\[
\text{CH}_3\text{CO.COOH} + ^{14}\text{CO}_2 + \text{ATP} \rightarrow \text{HOOC}^{14}\text{CH}_2\text{CO.COOH} + \text{ADP} + \text{Pi}
\]

The labelled oxaloacetate (OA) thus produced is the starting point for subsequent biosynthetic events. As shown in Appendix IV, glutamate is formed from the reductive amination of $\alpha$-ketoglutarate. Aspartate arises from OA by transamination from glutamate. Threonine, together with methionine and lysine form the aspartate family of amino acids. The formation of aspartyl phosphate is a key point in the regulation of the biosynthesis of threonine (114). Cohen *et al.* (115) found that all *Pseudomonas* species had a single aspartate kinase regulated by concerted feedback inhibition by lysine and threonine.

The incubation with NaH$^{14}$CO$_3$ produced an unexpected result in that a major proportion of the radiolabel was associated with the amino sugar components (Table 6). A further point of contrast was the absence of labelled threonine. Although the bacteria were subjected to conditions of nutritional deprivation, there would be some turnover, such as cell wall components, which would utilize endogenous sources, such as threonine. A pathway for threonine degradation is shown in Appendix V. Threonine can give rise to amino-acetone in an NAD-dependent oxidation, followed by a decarboxylation. The amino-acetone is deaminated to methylglyoxal, which is converted to pyruvate either directly or via the action of glyoxylase and LDH. This is the major route of threonine
catabolism in most organisms (116). The pyruvate orthophosphate dikinase (EC 2.7.9.1; ATP: pyruvate phosphate diphosphotransferase) which catalyzes the reaction; Pyruvate + ATP + Pi = PEP + AMP + PPi. This enzyme is present in _E. coli_ (117) and propionibacteria (118) and has been reported to function in gluconeogenesis (119) due to its favourable equilibrium in the direction of PEP synthesis (120). The PEP formed, (labelled in the C-3 position) would be converted to F6P (branch point for amino sugar synthesis) by the known gluconeogenic pathway. Ultimately, NAG + NAM would be labelled in their C1 and C6 positions (Appendix V).

The composition of the isolated PG from _P. putida_ poses some points for consideration. In addition to the accepted components of PG, NAG, NAM acid, Ala, and DAP, the amino acids Asp, Thr and Ser are clearly present. The latter have survived SDS extraction and pronase treatment, so that suggests a strong association or a covalent bond with PG. It is therefore feasible that Asp, Thr and Ser are the vestigial remains of a lipoprotein, which functions to anchor the PG to the outer membrane. There is evidence of a novel PG-associated lipoprotein in the cell envelope of _P. aeruginosa_ (53). This was isolated using a temperature dependent SDS extraction; at temperatures greater than 55°C no proteins were retained by the PG, but below this value, protein remained associated with PG. An amino acid analysis revealed the presence, amongst others, of Asp, Thr and Ser. In another study, the PG of _P. aeruginosa_ (OSU 64) was isolated and characterized (121). The method used to extract the PG was similar to the one used in this study, except that lysozyme was used to break down the cell wall. Further, it involved an overnight incubation with trypsin/chymotrypsin to remove covalently bound protein. Amino acid
analysis of the purified PG revealed the presence of Asp, Thr, Ser, Gly, Leu and Lys, in addition to the usual cell wall constituents. In the isolation of PG from *Spirillum serpens* (110) it was shown that following SDS extraction (twice) and pronase digestion (4 h), Asp and Gly were present in addition to Ala, GluNH₂, DAP and muramic acid.

The original impetus of this investigation, however, was the possibility of a novel carbohydrate metabolite becoming incorporated into the cell wall. Although this was not found, the study revealed that ¹⁴CO₂ released during 4FG metabolism was exclusively incorporated into amino acids. The incubation of *P. putida* with NaH¹⁴CO₃ also confirmed that ¹⁴CO₂ was incorporated into PG. In this case, however, a major proportion of the radiolabel was found in NAG and Muramic acid. Both these studies illustrate the highly adaptive behaviour of *P. putida*.

3.2 Isolation of the Minor Peak

The supernatant obtained from a 24 h incubation of D-[U-¹⁴C]4FG with glucose-grown whole cell of *P. putida* is resolved into two peaks of radioactivity, when analyzed by borate anion exchange chromatography (Peaks G and A, Fig. 12). These were previously designated "Major" and "Minor" respectively, with the former identified as 2,3-dideoxy-D-glycero-pentonic acid (2,3-dideoxyribo nic acid) (106).

The Minor peak of radioactivity was isolated by borate column chromatography. When fractionated using HPLC, the concentrated pool of radiolabelled fractions corresponding to the Minor peak was resolved into several components (Fig. 13). However, only the fraction corresponding to the peak d (Fig. 13) was found to contain radioactivity.
FIGURE 12
Borate Anion-Exchange Chromatographic Analysis of Radiolabelled Metabolites in the Supernatant Derived from a 24 h Incubation of D-[U-14C]-4FG with Glucose-Grown whole cells of P. putida

The supernatant recovered from a 24 h incubation of D-[U-14C]4FG with whole cells of P. putida was applied, together with the indicated internal standards to a preparative-scale borate anion-exchange column (Methods 2.2.20).

- Radioactivity (cpm)
- Absorbance at 420 nm

Inorganic phosphate (Method 2.2.17) was eluted in fractions 207-230 (not shown).

Radioactivity applied - 10258 ± 205 dpm.

Minor peak (fractions 29-36) - 999 ± 37 dpm 9.6% of recovered label.

A - Minor Peak
B - Sucrose
C - 4FG
D - Fructose
E - Glucose
F - Glucose-1-PO₄
G - Major Peak
FIGURE 12

Borate Anion-Exchange Chromatographic Analysis of Radiolabelled Metabolites in the Supernatant Derived from a 24 h Incubation of D-[U-14C]-4FG with Glucose-Grown whole cells of P. putida

[Diagram showing chromatographic analysis with labeled peaks A through E and a graph labeled 'Total cpm/fraction' and 'Absorbance 420 nm'.]
FIGURE 13

HPLC Analysis of Minor Peak Metabolite Fraction

The isolated, pooled and concentrated Minor peak metabolite was fractionated by HPLC (Methods 2.2.15) and resolved into the following components. The retention times (min) of standards are also shown.

<table>
<thead>
<tr>
<th>Letter Code</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>4.36</td>
</tr>
<tr>
<td>b</td>
<td>4.73</td>
</tr>
<tr>
<td>c</td>
<td>5.96</td>
</tr>
<tr>
<td>d</td>
<td>7.28 - radioactive</td>
</tr>
<tr>
<td>e</td>
<td>8.65</td>
</tr>
<tr>
<td>f</td>
<td>10.41</td>
</tr>
<tr>
<td>h</td>
<td>13.72</td>
</tr>
<tr>
<td>glucose</td>
<td>6.22</td>
</tr>
<tr>
<td>4FG</td>
<td>6.61 standards (not shown)</td>
</tr>
<tr>
<td>2-deoxyribose</td>
<td>8.58</td>
</tr>
</tbody>
</table>

Applied radioactivity (680 µL) - 69496 ± 2356 dpm

Recovered radioactivity (680 µL) - 63220 ± 1990 dpm

- 91 ± 3.2%

Horizontal axis - 1 cm rep 2 min
FIGURE 13

HPLC Analysis of Minor Peak Metabolite Fraction
This fraction was pooled, concentrated and a sample re-analyzed by HPLC to give one component (Fig. 14). Subsequent analysis by TLC revealed one spot of radioactivity (Fig. 15). The remainder of the Minor peak was analyzed by $^{13}$C and $^1$H NMR spectroscopy (Appendix VI and Appendix VII). There was no significant difference between the chemical shifts of the Minor peak and those of the previously identified Major peak 2,3-dideoxyribononic acid (Table 7). However, the borate column retention times were quite different. In addition, the Minor peak and sucrose (internal standard) exhibit a similarly weak affinity for the anion exchanger (Fig. 12). This poorly retained nature of the Minor peak suggested the presence of a compound devoid of charge. Hence, this neutral behaviour was considered to be indicative of the 1,4-lactone of 2,3-dideoxyribononic acid. Both compounds would exist in the equilibrium shown below.

\[
\begin{align*}
&\text{COO}^- \\
&\text{CH}_2 \\
&\text{CH}_2 \\
&\text{CHOH} \\
&\text{CH}_2\text{OH} \\
&\leftrightarrow \\
&\text{CO} \\
&\text{CH}_2 \\
&\text{O} \\
&\text{CH}_2 \\
&\text{CH} \\
&\text{CH}_2\text{OH}
\end{align*}
\]
FIGURE 14

HPLC Analysis of the Purified Minor Peak

Following HPLC fractionation of the Minor peak metabolite (Fig. 13), peak d was isolated and analyzed by HPLC, as previously described in Methods 2.2.15.
FIGURE 15
Silica-Gel TLC Analysis of the Minor Peak

The isolated radiolabelled Minor peak (Fig. 14) and various standards were applied to a Silica-Gel 60 TLC plate (Methods 2.2.21). The columns A (Minor peak) and D (Control) show the radioactive distribution of these vertical sections of the TLC plate, which were monitored for radioactivity as previously described.

<table>
<thead>
<tr>
<th>Letter Code</th>
<th>Compound</th>
<th>Applied</th>
<th>A</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a</strong></td>
<td>Minor peak</td>
<td>4 ( \mu L )</td>
<td>28.8</td>
<td>27.2</td>
</tr>
<tr>
<td>b</td>
<td>2-deoxyribose</td>
<td>1 ( \mu L ) (20 mM)</td>
<td>28.2</td>
<td>31.8</td>
</tr>
<tr>
<td>c</td>
<td>Minor peak</td>
<td>4 ( \mu L )</td>
<td>29.1</td>
<td>31.9</td>
</tr>
<tr>
<td>d</td>
<td>Control</td>
<td>-</td>
<td>31.2</td>
<td>29.7</td>
</tr>
<tr>
<td>e</td>
<td>4FG</td>
<td>2 ( \mu L ) (20 mM)</td>
<td>33.6</td>
<td>34.6</td>
</tr>
<tr>
<td>f</td>
<td>Glucose</td>
<td>1 ( \mu L ) (20 mM)</td>
<td>33.9</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*124.4</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*87.2</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>36.2</td>
<td>30.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>39.3</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>32.4</td>
<td>35.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>34.7</td>
<td>32.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>35.2</td>
<td>34.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>33</td>
<td>34.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28.8</td>
<td>33.6</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>32.6</td>
<td>31.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28.2</td>
<td>32.9</td>
</tr>
</tbody>
</table>

**Radioactivity applied - 170 dpm
Radioactivity recovered - 151 dpm - 88.2%
*Radioactivity (cpm) in 1 cm² section of TLC plate.
FIGURE 15

Silica-Gel TLC Analysis of the Minor Peak
TABLE 7

Chemical Shifts

<table>
<thead>
<tr>
<th>Minor Peak</th>
<th>$^{13}$C</th>
<th>$^1$H</th>
<th>2,3-Dideoxyribonic acid</th>
<th>$^{13}$C</th>
<th>$^1$H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>183.91</td>
<td>3.63</td>
<td>183.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72.54</td>
<td></td>
<td></td>
<td>72.03</td>
<td>3.67</td>
<td></td>
</tr>
<tr>
<td>66.16</td>
<td></td>
<td>3.54</td>
<td>65.65</td>
<td>3.53</td>
<td></td>
</tr>
<tr>
<td>34.57</td>
<td></td>
<td>2.28</td>
<td>34.03</td>
<td>2.27</td>
<td></td>
</tr>
<tr>
<td>30.09</td>
<td></td>
<td>1.72</td>
<td>29.57</td>
<td></td>
<td>1.69</td>
</tr>
</tbody>
</table>

Comparison of chemical shift values obtained from $^{13}$C and $^1$H NMR spectra (Methods 2.2.24) of Minor and Major peak (2,3-dideoxyribonic acid) metabolites.
It is possible that the lactone form was the predominant species when isolated. However, when applied to the borate anion-exchange column (pH 9.0), alkaline hydrolysis would produce the acyclic structure of 2,3-dideoxyribonucleic acid. This would be strongly retained by the borate column.

3.3 Metabolism of 4FG in *P. putida*

Previous work revealed that when 4FG was incubated with glucose-grown whole cells of *P. putida*, there was an extensive release of F⁻, but negligible oxidation of 4FG (103). This was in contrast to the isomeric 3FG, where oxidation was demonstrated, but with retention of the C-F bond (28). Both 4FG and 3FG are comparatively poor substrates for oxidation by the membrane bound glucose dehydrogenase in membrane vesicles. They exhibit *Kₘ* values of 21 (103) and 25 mM (122) respectively, when compared to 0.8 mM for glucose (122). It is possible that 4FG could be preferentially transported into the cell especially at 1 mM concentration used for the incubation experiments. It has been shown that the ED pathway features two routes for the assimilation of glucose; the extracellular direct oxidative and the intracellular phosphorylation route. The loss of one route is compensated by the operation of the alternative and, depending upon prevailing physiological conditions, one or the other predominates (80). Interestingly, Lynch and Franklin (89) showed that when *P. fluorescens* E.20 was grown on glucose at 30°C, the phosphorylation pathway for glucose (and gluconate) catabolism was predominant. With a decrease in growth temperature to 5°C, the direct oxidative route assumed precedence. It was also demonstrated that glucose dehydrogenase was not induced, when the same organism was grown on glucose
at 30°C (123). Whiting et al. reported that in glucose-grown *P. aeruginosa* PAO, low concentrations of glucose were catabolized preferentially by the phosphorylative route. During the current study, *P. putida* was glucose-grown at 30°C and all incubations with 4FG were carried out at a concentration of 1 mM. Previous studies have shown that glucose and 3FG are accumulated by an active transport system (124). It is not inconceivable that 4FG (a glucose analogue) should also be actively transported by, the glucose carrier protein. Therefore, if 4FG is transported, it becomes a potential substrate for ATP-dependent phosphorylation to its 6-phosphate.

Eisenberg et al. (93) showed that in *P. fluorescens* glucose was rapidly transported, metabolized to glucose-6-phosphate (G6P) by the action of a glucokinase, and subsequently oxidized to 6-phosphogluconate (6PGA). They also asserted that particulate glucose oxidase was not an obligatory first step for glucose uptake. The latter conclusion was drawn from the observation that a glucose oxidase deficient mutant accumulated glucose and metabolized it to G6P and 6-PGA. This result contradicted their earlier report that glucokinase activity was absent (109). Other groups had also reported a failure to detect glucokinase (125,126). Amongst them, Wood (127) failed to detect the enzyme in cell-free extracts of *P. putida* using a glycyglycine buffer at pH 7.4. However, Eisenberg et al. (93) showed that glucokinase activity could be detected by increasing the enzyme assay reaction pH from 7.4 to 8.2. The detection of the kinase activity vindicated the work of Eagon and Phibbs (127) who had demonstrated that glucose was actively transported as a free sugar and trapped by intracellular phosphorylation in *P. aeruginosa*.
It was important, therefore, to establish the presence of glucokinase activity in *P. putida*. If 4FG was preferentially transported prior to defluorination in a similar manner to that of glucose, then it may be subject to phosphorylation. Cell-free and particle-free extracts of *P. putida* were prepared and the same assay procedure for glucokinase was used as reported by Eisenberg *et al.* (93). The distribution of kinase activity in both the crude extract and particle-free supernatent is consistent with the presence of a soluble cytoplasmic enzyme in *P. putida*. Table 8 shows that with glucose as a substrate, kinase activity is apparent. However, 4FG proved to be a poor substrate for glucokinase. Under the conditions of the glucokinase assay (pH 8.2) 4FG is clearly not a substrate for phosphorylation. The same cell-free extracts were monitored for defluorination over a period of 24 h (data not shown) and no fluoride release was detected.

A series of whole cell suspensions of *P. putida* were prepared and washed with the appropriate buffer prior to incubation with 1 mM 4FG. As shown in Table 9, the pH of the incubation had a profound effect on defluorination. At pH 7.1, the whole cells defluorinated 4FG with alacrity, but at pH 8.2, the defluorination reaction was virtually stopped. The inference drawn from these results is that defluorination may require a proton gradient. The metabolic poison 2,4-dinitrophenol (DNP), functions as a membrane transporter of protons (H⁺) and uncouples the oxidation of NADH from ATP synthesis. It therefore dissipates the proton gradient generated by electron transport.
**TABLE 8**

Glucose Kinase Activity in Crude Extracts of *P. putida*

<table>
<thead>
<tr>
<th>Cell Extract</th>
<th>Fraction Volume (mL)</th>
<th>Protein Concentration (mg/mL)</th>
<th>Total Units of Kinase Activity</th>
<th>SA U/mg Protein(^a)</th>
<th>Glucose 1 mM</th>
<th>Glucose 25 mM</th>
<th>4PG 1 mM</th>
<th>4PG 25 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>0.675</td>
<td>3.84</td>
<td>26.5</td>
<td></td>
<td>0.012</td>
<td>0.011</td>
<td>5x10^-4</td>
<td>8.3x10^-4</td>
</tr>
<tr>
<td>Supernatant from Crude Extract</td>
<td>0.675</td>
<td>1.24</td>
<td>21.4</td>
<td></td>
<td>n.d.</td>
<td>0.026</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

\(^a\) 1 unit of activity represents 1 \(\mu\)mol NADPH/min.

\(^b\) Specific activity (SA) = 1 \(\mu\)mol NADPH/min/mg protein.

n.d. not determined

-- no activity detected

Glucokinase activity was estimated by coupling with glucose-6-phosphate dehydrogenase (G6PDH) and measuring NADPH production at 340 nm (\(-30^\circ\)C) (Methods 2.2.9).
TABLE 9

The effect of pH on the defluorination of 4FG by glucose grown whole cells of *P. putida*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Tris-HCl Buffer pH 7.1</th>
<th>Tris-HCl Buffer pH 8.2</th>
<th>Potassium Phosphate Buffer pH 7.1</th>
<th>Potassium Phosphate Buffer pH 8.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47</td>
<td>1.3</td>
<td>36</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>7</td>
<td>65</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>83</td>
<td>7</td>
<td>86</td>
<td>--</td>
</tr>
<tr>
<td>4</td>
<td>85</td>
<td>8</td>
<td>86</td>
<td>16</td>
</tr>
</tbody>
</table>

Whole cell suspensions, (4 mL, 34 mg protein) of glucose grown *P. putida* were incubated with 4FG (1 mM) at 30°C (shaken) in the buffer and at the pH as indicated. Fluoride in determination and standard curves were carried out as described in Materials and Methods.
Table 10 shows that of the three different concentrations of DNP used, 2 mM was sufficient to virtually abolish defluorination. A similar inhibition was obtained with sodium azide (10 mM), which binds to cytochrome a₃, a component of the cytochrome c oxidase complex. This is the last complex in the electron transport chain; the energy release from the transfer of a pair of electrons to oxygen, is utilized for the pumping of protons across the membrane. These results suggest that a chemiosmotic energy couple (ΔpH) is required for the defluorination of 4FG. When whole cells were treated with N,N'-dicyclohexylcarbodiimide (DCCD) or arsenate, no inhibition of fluoride release from 4FG was observed (Tables 11 and 12). The involvement of ATP and/or a membrane bound ATPase is considered therefore, to be unlikely.

The interpretation of these data show that 4FG is defluorinated when incubated with glucose-grown whole cells of P. putida, but not with cell-free extracts of the same organism. There is evidence for the requirement of a proton motive force (chemiosmotic energy couple) for when the proton gradient was dissipated by the use of 2,4-DNF, defluorination was abolished. This also indicated that the defluorinating activity was not dependent upon ATP synthesis, an inference that was consistent with the lack of inhibition of F⁻ release from 4FG in the presence of DCCD (ATP synthetase inhibitor). Incubations in the presence of arsenate showed that phosphate bond energy, derived from substrate-level phosphorylation, was also not required for defluorination.
**TABLE 10**

The Effect of The Inhibitors DNP and Sodium Azide on The Defluorination of 4FG by Glucose Grown Whole Cells of *P. putida*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>+ DNP 1 mM</th>
<th>+ DNP 2 mM</th>
<th>+ DNP 5 mM</th>
<th>+ Azide 10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73</td>
<td>6</td>
<td>1.1</td>
<td>1.4</td>
<td>4.7</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>10</td>
<td>1.8</td>
<td>1.4</td>
<td>--</td>
</tr>
<tr>
<td>8</td>
<td>82</td>
<td>-</td>
<td>-</td>
<td>1.7</td>
<td>6.5</td>
</tr>
<tr>
<td>18</td>
<td>84</td>
<td>26</td>
<td>2.6</td>
<td>-</td>
<td>6.5</td>
</tr>
<tr>
<td>24</td>
<td>86</td>
<td>30</td>
<td>5</td>
<td>2</td>
<td>7.8</td>
</tr>
</tbody>
</table>

% Defluorination

Whole cell suspension (4.2 mL, 38 mg protein) of glucose grown *P. putida* were incubated in 100 mM Tris-HCl, pH 7.1 with 4FG and the inhibitor shown (in column) at 30°C on a rotary shaker. All incubations were pre-incubated for 1 h at 30°C in the presence of the inhibitor prior to the addition of 4FG.

Fluoride ion determinations were carried out as described in Methods 2.2.10.
TABLE 11

The Effect of DCCD, on The Defluorination of 4FG by Glucose Grown Whole Cells of *P. putida*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control 1</th>
<th>Control 2 + EtOH</th>
<th>+ EtOH/DCCD 1 mM</th>
<th>+ EtOH/DCCD 2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>21</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>61</td>
<td>58</td>
<td>42</td>
<td>42</td>
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<td>18</td>
<td>82</td>
<td>91</td>
<td>87</td>
<td>85</td>
</tr>
<tr>
<td>24</td>
<td>91</td>
<td>91</td>
<td>87</td>
<td>87</td>
</tr>
</tbody>
</table>

% Defluorination

Conditions and procedures as described in legend to Table 10.
TABLE 12

The effect of sodium arsenate on the defluorination of 4FG by glucose grown whole cells of *P. putida*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>Arsenate</th>
<th>Tris-HCl, 100 mM, pH 7.1</th>
<th>Glycyl-Glycine, 20 mM, pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
<td>-</td>
<td>Arsenate</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>85</td>
<td>57</td>
<td>85</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>89</td>
<td>62</td>
<td>85</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arsenate</td>
<td>Arsenate</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>100</td>
<td>88</td>
<td>82</td>
</tr>
<tr>
<td>24</td>
<td>100</td>
<td>100</td>
<td>90</td>
<td>86</td>
</tr>
</tbody>
</table>

% Fluoride Release

All incubations with 4FG (1 mM) were carried out with whole cell suspensions (4.2 mL, 36 mg protein) of *P. putida* in the indicated buffers and in the presence/absence (+/-) of 10 mM sodium arsenate. Incubations 2, 3, 4 were pre-incubated with arsenate for 1 h at 30°C (shaken) prior to the addition of 4FG. Incubation 2 was washed free of arsenate prior to addition of 4FG. Incubation 5 served as a control for incubation A.
Both *P. aeruginosa* (29,128) and *P. putida* (124) possess two inducible transport systems with different affinities for glucose. A high affinity system of broad specificity (*K_m* ~ 8 µM) which is induced by growth on glucose, but repressed on gluconate, succinate and citrate. A low affinity transport system (*K_m* ~ 1 mM) which is induced by growth on glucose or gluconate, but not on succinate or citrate. It was subsequently postulated (129) that the low affinity glucose transport system involved uptake across the outer membrane (OM) via the porin protein F. Since defluorination is observed when 4FG is incubated with either glucose or gluconate grown whole cells, then the defluorinating protein could be a component of the low affinity system.

Previous work (103) demonstrated that growth on gluconate or 2-ketogluconate had no effect on the defluorination of 4FG. However, when succinate or citrate was utilized as the carbon source, defluorination was repressed. The most effective inhibitors of defluorination were, D-glucose, D-gluconate and 2-ketogluconate, which suggested a common binding site for these substrates. In addition, 6-deoxy-D-glucose, 2-deoxy-D-glucose and 4-deoxy-D-glucose (4-DOG) inhibited defluorination to the extent of 73, 57 and 30% respectively. The poor inhibition exhibited by 4-DOG reflects the importance of the C-4 position and the hydrogen bonding requirements for binding to the putative defluorinating protein. The inference is therefore, that there is retention of the carbon-fluorine bond when 4FG is prevented from binding to an extracellular inducible/repressible protein. Further, if the low affinity system is involved, it is conceivable that there is coordinate regulation of an outer membrane protein and a periplasmic binding protein.
The sensitivity of these binding proteins to osmotic shock can be used to
differentiate them into two broad classes, shock-sensitive and shock-
resistant (65). Shock-sensitive permeases require ATP or some form of
phosphate bond energy, usually derived from substrate-level
phosphorylation. This renders them susceptible to inhibition by arsenate.
They are also relatively insensitive to uncouplers of oxidative
phosphorylation. In contrast, shock-resistant permeases are strongly
inhibited by uncouplers such as 2,4-DNP and are dependent upon the energy
derived from a PMF. They are insensitive to arsenate but highly sensitive
to sulphhydryl reagents such as N-ethylmaleimide (NEM) and p-
mercuribenzoate (PMB).

Recently, 4FG was demonstrated to inhibit the glucose-binding protein
(GBP) of P. aeruginosa, extracted by osmotic shock (shock sensitive
permease). Significantly, there was no detectable release of fluoride ion
(D. Francescutti - personal communication). A similar study using P.
putida is currently under investigation. During our study, it was
demonstrated that defluorination of 4FG in P. putida required a Δ pH and
in addition, the reaction was not dependent upon phosphate bond energy.
Earlier results (103) showed that defluorination in P. putida was
abolished in the presence of NEM. These results are therefore consistent
with the properties of a shock-resistant binding protein.

In all previous studies, the phenomenon of the defluorination of 4FG
has only been observed in the three species, P. aeruginosa, P. fluorescens
and P. putida. Furthermore, the release of fluoride from 4FG was induced
when the bacteria were grown on glucose, gluconate or 2-ketogluconate.
The ED pathway is the principal route for glucose metabolism in pseudomonads and it appears to play a role in the subsequent metabolism of the defluorinated product of 4FG. The enzymes of the pathway, 6-phosphogluconate dehydrase (Edd) and 3-deoxy-2-keto-6-phosphogluconate aldolase (Eda), although non-coordinately regulated, are induced by, gluconate or 2-ketogluconate and KDPG respectively. Since it has been shown that gluconate induces the ED pathway in *E. coli* (79), the intriguing possibility that defluorination could be induced in *E. coli* by gluconate was examined. Glucose and gluconate-grown whole cells of *E. coli* were incubated with 1 mM 4FG. Since both glucose and gluconate-grown cells only exhibit 12% defluorination (Table 13), it would appear that gluconate does not specifically induce defluorination in *E. coli*.

Previous studies revealed the presence of 2,3-dideoxyribonucleic acid (DDRA) in the supernatant following a 24 h incubation of 4FG with whole cells of *P. putida*. To detect the presence of other metabolites of 4FG formed prior to DDRA, it was necessary to establish its time of appearance in the supernatant. The current studies demonstrated that the defluorination of 4FG is virtually completed with in about 2-3 h, therefore a time course study using D-[U-14C]4FG was undertaken. As shown, the maximum defluorination of 95% was constant from 260 min to 24 h (Table 14). The control (2) incubation showed that non-specific defluorination was ~1% throughout the 24 h period. Each supernatant obtained from the time course study was applied, together with 4FG and glucose.
<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control Phosphate Buffer 1 mM 4FG</th>
<th>Glucose-Grown E. coli 1 mM 4FG</th>
<th>Gluconate Grown E. coli 1 mM 4FG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Defluorination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.3</td>
<td>1.1</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>1.85</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>2.73</td>
<td>2.97</td>
</tr>
<tr>
<td>5</td>
<td>0.3</td>
<td>4</td>
<td>4.2</td>
</tr>
<tr>
<td>6</td>
<td>0.3</td>
<td>5.2</td>
<td>4.98</td>
</tr>
<tr>
<td>22</td>
<td>0.38</td>
<td>12.3</td>
<td>10.82</td>
</tr>
<tr>
<td>27</td>
<td>0.3</td>
<td>12.3</td>
<td>11.3</td>
</tr>
</tbody>
</table>

Glucose and gluconate grown whole cell suspensions (4 mL, 36 mg protein) of E. coli (ATCC 23227) were incubated in 100 mM Tris-HCl, pH 7.1 with 1 mM 4FG and monitored for defluorination (Method 2.2.10) at the times shown.
TABLE 14

Time Course Study of the Defluorination of 4FG by

Glucose-Grown *P. putida*

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control 1 Whole Cells Buffer mV</th>
<th>Control 2 Buffer + 4FG mV</th>
<th>Incubation 14C 4FG + Whole Cells [F(^-)] mV</th>
<th>[F(^-)] mM</th>
<th>% F(^-) Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 min</td>
<td>+130</td>
<td>+126</td>
<td>+39.5</td>
<td>0.45 mM</td>
<td>45</td>
</tr>
<tr>
<td>80 min</td>
<td>+131</td>
<td>+126</td>
<td>+24</td>
<td>0.86 mM</td>
<td>86</td>
</tr>
<tr>
<td>140 min</td>
<td>+135</td>
<td>+125</td>
<td>+22</td>
<td>0.93 mM</td>
<td>93</td>
</tr>
<tr>
<td>200 min</td>
<td>+135</td>
<td>+126</td>
<td>+22</td>
<td>0.93 mM</td>
<td>93</td>
</tr>
<tr>
<td>260 min</td>
<td>+135</td>
<td>+125</td>
<td>+21.5</td>
<td>0.95 mM</td>
<td>95</td>
</tr>
<tr>
<td>320 min</td>
<td>+134</td>
<td>+127</td>
<td>+21.5</td>
<td>0.95 mM</td>
<td>95</td>
</tr>
<tr>
<td>380 min</td>
<td>+135</td>
<td>+126</td>
<td>+21.5</td>
<td>0.95 mM</td>
<td>95</td>
</tr>
<tr>
<td>24 h</td>
<td>+135</td>
<td>+126</td>
<td>+21.5</td>
<td>0.95 mM</td>
<td>95</td>
</tr>
</tbody>
</table>

Procedure as described in Method 2.2.18.
(internal standards) to an analytical borate anion exchange column. Each fraction was analyzed for carbohydrate and radioactivity. The borate column analyses (Figs. 16 and 17) reveal the presence of three peaks of radioactivity. The first, being poorly retained by the borate column was considered to be a lactone, possibly the lactone of 2,3-dIDEOxyribonucleic acid, since similar chromatographic behaviour was observed with the minor peak (Fig. 12). The second peak of radioactivity coincided with the carbohydrate profile of the internal standard (cold 4FG). The third radiolabelled peak was designated Metabolite X. The 140 min supernatant is virtually devoid of radiolabelled 4FG. In addition, the 140 min supernatant also reveals the first trace of 2,3-DDRA which is present in the 260 min and 380 min supernatants (Fig. 17). This was confirmed by using 2,3-DEPDA previously prepared) as an internal standard. Table 15 summarizes the distribution of the radiolabelled components found in each supernatant. The whole cell uptake of 4FG is reflected in the decrease of the radioactive peak between 40 and 80 min from 20% to 3% in the supernatant. Concomitantly, there is an increase in the putative lactone component (2.4 to 8%) and Metabolite X, which increased from 33.9 to 71%. During the same time period (40 to 80 min), 4FG was defluorinated by 41%. The formation of 2,3-dIDEOxyribonucleic acid which first appeared in the 140 min supernatant increased progressively to become the sole product of 4FG metabolism after 24 h.

Since 80 min appeared to be the optimum time for the formation of metabolite X a larger scale incubation was used to isolate this species.
FIGURE 16
Borate Anion-Exchange Chromatography of
Time Course Supernatants

The $^{14}$C-radiolabelled supernatents obtained from the time course study were applied, together with the indicated internal standards, to a borate anion-exchange column as described in Method 2.2.19. Each fraction was assayed for radioactivity (Method 2.2.11) and carbohydrate (Method 2.2.16).

- open circles - Radioactivity (dpm) at 40 min
- closed circles - Radioactivity (dpm) at 80 min
- open squares - Absorbance at 420 nm
- dashed lines - Internal standards (absorbance at 420 nm)

Inorganic phosphate (Method 2.2.17) eluted in fractions 45-52 (not shown).

A - Lactone
B - 4FG
C - Glucose
X - Metabolite X
Absorbance at 420 nm

Figure 16

A

B

C

Total dpm/Fraction

Fraction No.

0.00 0.25 0.50 0.75 1.00 1.25 1.50

40 min 80 min

1500 1250 1000 750 500 250
FIGURE 17
Borate Anion-Exchange Chromatography of
Time Course Supernatants

Procedure as described in legend to Figure 18.
open circles - Radioactivity (dpm) at 140 min
closed circles - Radioactivity (dpm) at 260 min
triangles - Radioactivity (dpm) at 380 min
dashed lines - Internal standards (absorbance 420 nm)

Inorganic phosphate eluted in fractions 46-54 (not shown).

A - Lactone
B - 4FG
C - Glucose
D - 2,3-dideoxyribonic acid
X - Metabolite X
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Lactone</th>
<th>4FG</th>
<th>Metabolite</th>
<th>% Total Recovery</th>
<th>% F Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>2.4</td>
<td>20.4</td>
<td>33.9</td>
<td>56</td>
<td>45</td>
</tr>
<tr>
<td>80</td>
<td>8</td>
<td>3</td>
<td>71</td>
<td>82</td>
<td>86</td>
</tr>
<tr>
<td>140</td>
<td>9</td>
<td>0.5</td>
<td>73.8</td>
<td>Trace</td>
<td>83</td>
</tr>
<tr>
<td>260</td>
<td>22.7</td>
<td>-</td>
<td>52</td>
<td>26</td>
<td>100</td>
</tr>
<tr>
<td>380</td>
<td>13</td>
<td>-</td>
<td>29</td>
<td>32</td>
<td>74</td>
</tr>
<tr>
<td>24 h</td>
<td>*8.7</td>
<td>-</td>
<td>-</td>
<td>*41.27</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 15 shows each radiolabelled component (Fig. 16 and Fig. 17) expressed as a % of the total radioactivity recovered in each supernatant obtained from the time course study (Methods 2.2.18).

* Figures from Borate column analysis (Fig. 12).

2,3DDRA - 2,3-dideoxyribonic acid
Table 16 summarizes the isolation procedure. It shows that the net yield of metabolite X from the supernatant of a whole cell incubation was 31%. The concentration of Metabolite X was estimated at \( \geq 13 \text{ mM} \) (1 \( \mu \text{mol} \) D-[U-\(^{14}\text{C}]4FG = 10,600 \text{ dpm} \)). To ascertain if the metabolite consisted of one or more radioactive components, it was applied to a silica-gel TLC plate (Fig. 18). Metabolite X migrated as one spot.

Table 17 (columns 4 and 5) shows the chemical shifts arising from a proton decoupled \(^{13}\text{C} \) NMR analyses of 20 \( \text{ mM} \) solutions (in \( \text{D}_2\text{O} \)) of \( \beta\)-D-glucose and 4FG respectively. The assignments were made by comparing the chemical shifts to those of published data (130) shown in columns 1, 2 and 3 (Table 17). The chemical shifts obtained from a similar \(^{13}\text{C} \) NMR analysis of metabolite X appear in column 6, and the actual spectrum is shown in Appendix VIII. The same data was also analyzed using a 'Distortionless Enhanced Polarization Transfer' (DEPT) programme for \(^{13}\text{C} \) NMR, Appendix IX. The salient feature of the DEPT (131) technique is that it produces a spectrum where methylene (\(-\text{CH}_2\)) groups are shown as negative, i.e., pointing downwards from the baseline of the spectrum. In contrast, \(-\text{CH}\) and \(-\text{CH}_3\) groups appear in the upward direction. A proton NMR spectrum of metabolite X was also obtained (Appendix X). A sample of metabolite X (in \( \text{D}_2\text{O} \)) was submitted to negative ion fast atom bombardment (FAB) mass spectrometry, using glycerol as the sample matrix. A peak with a mass/charge \((m/e)\) ratio of 163 was shown (Appendix XI) which would correspond to a molecular weight of 164. An appropriate control using borate buffer, was prepared in an identical manner to the metabolite.

Although the NMR data was insufficient to provide an unequivocal identification of the structure of metabolite X, a partial elucidation was
TABLE 16

Isolation of Radiolabelled Metabolite X

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (mL)</th>
<th>Total DPM ($^{14}$C)</th>
<th>% Recovery From Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>45</td>
<td>288,774</td>
<td>-</td>
</tr>
<tr>
<td>Concentrated Supernatant</td>
<td>1.7</td>
<td>270,904</td>
<td>94</td>
</tr>
<tr>
<td>Pooled Fractions from Borate Column</td>
<td>52</td>
<td>125,276</td>
<td>43</td>
</tr>
<tr>
<td>Metabolite X in D$_2$O</td>
<td>600 µL</td>
<td>88,130</td>
<td>31</td>
</tr>
</tbody>
</table>

A whole-cell suspension of P. putida (48 mL, 100 mg protein) was incubated in 50 mM Tris-HCl pH 7.1, with 1 mM D-[U-$^{14}$C]4FG for 85 min (86% defluorination). Isolation procedure as described in Methods 2.2.19.
FIGURE 18  
Silica-Gel TLC Analysis of Metabolite X

Following isolation by borate anion-exchange chromatography, $^{14}$C-Metabolite X and non-radiolabelled standards were applied to a Silica-gel 60 TLC plate (Method 2.2.21). The distribution of radioactivity in the entire verticle section containing Metabolite X is shown.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_f$</th>
<th>Applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolite X</td>
<td>0.47</td>
<td>8 $\mu$L</td>
</tr>
<tr>
<td>4PG</td>
<td>0.43</td>
<td>4 $\mu$L (20 mM)</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.62</td>
<td>4 $\mu$L (20 mM)</td>
</tr>
</tbody>
</table>

Radioactivity applied - 552 dpm

Radioactivity recovered - 422 dpm (76%)
FIGURE 18
Silica-Gel TLC Analysis of Metabolite X
### TABLE 17
Proton Decoupled $^{13}$C NMR Analyses

<table>
<thead>
<tr>
<th>Chemical Shifts (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

D-Glucose* (Published Data)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th>C1</th>
<th>96</th>
<th>96.74</th>
<th>189.3-CHO (C1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>B</td>
<td>Assign</td>
<td>α-β-D-Glucose</td>
<td>4FG</td>
<td>Metabolite X</td>
<td></td>
</tr>
<tr>
<td>93.6</td>
<td>97.4</td>
<td>C1</td>
<td>96</td>
<td>96.74</td>
<td>189.3-CHO (C1)</td>
<td></td>
</tr>
<tr>
<td>93.93</td>
<td>92.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>73.2</td>
<td>75.9</td>
<td>C2</td>
<td>73.8</td>
<td>74.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>74.5</td>
<td>77.5</td>
<td>C3</td>
<td>72.2</td>
<td>86.63-CH$_2$ (C5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>71.5</td>
<td>81.05-CH$_2$ (C3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>71.4</td>
<td>71.3</td>
<td>C4</td>
<td>70.5</td>
<td>69.86</td>
<td>72-CH$_2$ (C4)</td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>77.4</td>
<td>C5</td>
<td>75</td>
<td>88.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>62.3</td>
<td>62.5</td>
<td>C6</td>
<td>61.35</td>
<td>60.92</td>
<td>66.06</td>
<td></td>
</tr>
<tr>
<td>60.77</td>
<td>65.35 (C6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>64.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Obtained from published data (130).

Col 4 + 5 - 20 mM solutions in D20

Col 6 - estimated concentration 13 mM in D$_2$O

Procedure as described in Method 2.2.24.
possible. The integrated proton NMR spectrum revealed the presence of eleven protons. The cluster of signals in the 3.4 to 4 ppm range are indicative of alcohols (-CHOH) and the chemical shift at 1.8 ppm (and possibly at 2.2 ppm) suggests a -CH₂ group. The ¹³C decoupled analysis (DEPT programme) clearly indicated the presence of two -CH₂ groups and the chemical shifts in the 64-66 ppm range suggest a primary alcohol group. The downfield signal at 185 ppm is characteristic of an aldehyde (-CHO) group. Its presence suggests that the species is an acyclic structure. The chemical shifts arising from cyclic sugars are shown in Table 17. The mass spectrometric data (Appendix XI) revealed a molecular weight of 164. Analyses with ¹⁹F and ³²P NMR (not shown) showed that the compound was neither fluorinated nor phosphorylated. An acyclic structure consistent with these data would be 4-deoxy-D-glucose. A likely pathway for the formation of 2,3-dideoxy-D-glycero-pentonic acid, which also allows for the previously reported (103) exchange of tritium at C6 of D-[6-³H]4FG subsequent to defluorination is shown (Fig. 19). The elimination of HF from 4FG would give the unsaturated sugar 7. Tautomeric equilibrium of the acyclic isomers of 7 (8 = 9 = 10) would allow the tritium exchange. After transport of 4-deoxy-5-keto-D-glucose, 9, into the cell, it would be reduced to 4-deoxy-D-glucose, 11, which would be transported out of the cell until equilibrium was attained. Intracellular phosphorylation and a dehydrogenase would produce, 4-deoxy-6-phospho-D-gluconic acid, 12. This metabolite would be a potential substrate for the ED pathway enzyme, 6-phosphogluconate dehydrase (Fig. 7). Following decarboxylation and oxidation of 3,4-dideoxy-2-keto-6-phosphogluconic acid, 13, the action of a phosphatase would produce 2,3-dideoxy-D-glycero-pentonic acid, the 24
FIGURE 19

A Feasible Pathway for the Defluorination of 4-Deoxy-4-fluoro-D-glucose and the formation of 2,3-Dideoxy-D-glycero-pentonic acid

9 - 4-deoxy-5-keto-D-glucose
11 - 4-deoxy-D-glucose
12 - 4-deoxy-6-phosphogluconic acid
13 - 3,4-dideoxy-2-keto-6-phosphogluconic acid
14 - 2,3-dideoxy-D-glycero-pentonic acid

CM - cytoplasmic membrane
h product of 4FG metabolism.

The possibility that 4FG is preferentially transported into the cell prior to defluorination prompted an investigation of the intracellular contents of P. putida. Using the same time profile established by the time course study (40 and 80 min), a whole cell suspension of P. putida was incubated with 1 mM D-[U-14C]4FG. The distribution of the radioactivity during the isolation of the intracellular contents is shown in Table 18. The rationale behind the extensive pellet washing was based on observations made during preliminary experiments. When the cell pellet was isolated and frozen with liquid nitrogen (to cease metabolism) the subsequent thawing revealed that the cells were ruptured. It was therefore difficult to differentiate between intra- and extra-cellular components. Each wash of the cell pellet revealed decreasing amounts of radioactive components which were washed from, presumably the periplasm. This reflected the permeability of the outer membrane (OM) and it has been shown that the OM of P. aeruginosa allows compounds with molecular weights of 150-194 to penetrate the outer barrier (132). Upon sonication, the cell pellet released radioactive components which were contained within the confines of the highly selective cytoplasmic membrane and therefore, intracellular. These components obtained from sonication of the cell pellet after 40 and 80 min incubation, were resolved into two peaks of radioactivity (Fig. 20). The first peak was identified as 4FG, using non-radiolabelled 4FG as an internal standard. The more strongly retained second peak was demonstrated to be a carbohydrate by the orcinol-sulphuric acid procedure. There is, therefore, evidence for an intracellular presence of 4FG between 40 and 80 min (Table 19). At these times, the
### TABLE 18

Isolation of Radio-labeled Intracellular Metabolites

<table>
<thead>
<tr>
<th></th>
<th>40 min</th>
<th></th>
<th>80 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% F^-</td>
<td>Total Radio-</td>
<td>% of</td>
</tr>
<tr>
<td>Release Activity</td>
<td>dpm</td>
<td>Activity Radio-</td>
<td>dpm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Release Activity</td>
<td></td>
</tr>
</tbody>
</table>

|                  |        |                  |        |        |                  |        |
| Supernatant      | 50     | 39338            | 39.8   | 80     | 28136            | 28.5   |
| Pellet Washes (7)| -      | 35274            | 35.7   | -      | 42522            | 43     |
| Sonicated Pellet (Intracellular) | - | 14666            | 14.85  | -      | 23750            | 24     |
| Recovery         | -      | 89278            | 90.1   | -      | 94408            | 95     |

A 10 mL whole cell suspension of *P. putida* (100 mg, whole cell protein) was incubated with 1 mM [U-14C]-4FG. At 40 and 80 min, the suspensions were processed as described in Method 2.2.22.

* Initial Radioactivity 98750 dpm.
FIGURE 20

Borate Anion-Exchange Chromatography of Intracellular Metabolites

Whole cell suspension of *P. putida* (2 x 10 mL, 100 mg/suspension) were incubated in 50 mM PPB, pH 7.1 at 30°C with 1 mM D-[U-¹⁴C]FG for 40 and 80 min. The intracellular contents were isolated (Method 2.2.22) and analyzed on a borate anion-exchange column (Method 2.2.19). Each fraction was monitored for radioactivity (Method 2.2.11) and carbohydrate (Method 2.2.16).

open circles - Radioactivity (dpm) at 40 min

closed circles - Radioactivity (dpm) at 80 min

Inorganic phosphate (Method 2.2.17) eluted in fractions 45-52 (not shown).

Recoveries of radioactivity are shown in Table 19.
FIGURE 20

Borate Anion-Exchange Chromatography of Intracellular Metabolites

[Graph showing chromatography results with markers for 40 min and 80 min, with axes labeled Total dpm/fraction and Fraction No.]
### TABLE 19

Intracellular Radioactivity Distribution following Borate Anion Exchange Chromatography

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Initial Radioactivity (dpm)</th>
<th>% of Initial Radioactivity</th>
<th>Unknown Metabolite (dpm)</th>
<th>% of Initial Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>14666</td>
<td>1420</td>
<td>8626</td>
<td>8.74</td>
</tr>
<tr>
<td>80</td>
<td>23750</td>
<td>6352</td>
<td>10714</td>
<td>10.85</td>
</tr>
</tbody>
</table>

The intracellular contents obtained from the sonicated cell pellets (Table 20) were analyzed by borate anion exchange chromatography (Methods 2.2.19).
fluoride release from 1 mM 4FG was 40 and 80%. Therefore, the concentrations of the remaining 4FG (500 μM and 200 μM) would still be at half-saturating levels if the kinetic parameters of its transport were comparable to those of 3FG or glucose in vesicles; apparent $K_m = 400$ μM and 167 μM respectively (124); or in whole cells where glucose uptake was via a low $K_m$ (1.7 μM) and a high $K_m$ (76.6 μM) system (122). Since 4FG does not appear to be defluorinated intracellularly, an equilibrium between intracellular and extracellular 4FG may be established. This equilibrium would be ultimately governed by the rate of fluoride release in the periplasm, which is markedly slower after 80 min. This could be explained by the potential competition between 4FG and the defluorinated product for the same transport system. Therefore, depending upon its specificity, the glucose carrier system could transport the putative defluorinated product, 4-deoxy-5-keto-D-glucose, 9 and/or 4-deoxy-D-glucose 11 into the cell. Metabolites 12 and 13 (Fig. 19) are both likely to be retained by the borate anion-exchange column and therefore consistent with the second peak of radioactivity (Fig. 20). Recent evidence (Max L. Tejada, personal communication) supports the view that defluorination occurs in the periplasm. When 4FG is incubated with gluconate-grown whole cells of P. putida, fluoride release ensues, but without the evolution of carbon dioxide. Since gluconate is known to repress the glucose carrier system, it was concluded that 4FG was not being transported efficiently into the cell. Its subsequent metabolism to 2,3-dideoxyribononic acid, which would involve a decarboxylation step, was thus significantly retarded. A
possible mechanism of fluoride release and metabolism to 4-deoxy-D-glucose is shown (Fig. 21.).
FIGURE 21

Possible Mechanism of Fluoride Release and Metabolism of
4FG to 4-Deoxy-D-glucose

A diagrammatic mechanism for the transport of 4FG into the cell of P. putida is shown.

G - Glucose carrier system
CM - Cytoplasmic membrane
P - Periplasm
CHAPTER 4
SUMMARY AND FUTURE PERSPECTIVES

When whole cells of P. putida are incubated with 1 mM D-[U-\(^{14}\)C]4FG for 24 h, there is an extensive release of fluoride ion (95 ± 5%) and about 50% of the radiolabel accumulated in the suspending medium. In addition, about 5% of the radiolabel is liberated as \(^{14}\)CO\(_2\) and less than 1% is associated with the PG (106). This small, but significant amount of radioactivity was possibly attributable to the presence of a novel radiolabelled fluorinated or non-fluorinated component in the PG. No evidence was found to support this postulate. However, analysis of the isolated PG from P. putida has revealed that the radiolabel is incorporated into the amino acids threonine, serine, glutamate and aspartate. The main radiolabelled amino acid was threonine. This is considered to have arisen from \(^{14}\)CO\(_2\) incorporation into amino acid biosynthetic pathways. When P. putida was incubated with NaH\(^{14}\)CO\(_3\), the pattern of radiolabel exhibited some deviation. The major proportion of the radiolabel was found in the amino sugars NAG and GlcNH\(_2\), with a lesser amount of glutamate and aspartate and no threonine. It was concluded that under conditions of nutritional deprivation, the bacteria utilized threonine as an energy source for metabolic turnover. The use of the Aminex HPX-87C (Bio-Rad) carbohydrate analytical column with Ca(NO\(_3\))\(_2\) as the mobile phase was demonstrated to be particularly useful for the assessment of the composition of peptidoglycan. The main principles of separation were ligand exchange and size exclusion.
The previously unknown minor metabolite (106) resulting from a 24 h incubation of *P. putida* and D-\([U-^{14}C]\)4FG was isolated on a preparative scale and identified as the lactone of 2,3-dideoxyribonic acid.

Metabolic studies suggest that a chemiosmotic energy couple (ΔpH) is required for the defluorination of 4FG. The inhibition studies indicated that the defluorinating activity was not dependent upon ATP synthesis. These studies also suggested the possibility that a periplasmic shock-resistant binding protein could be implicated in the mechanism of fluoride release.

A time-course study with radiolabelled 4FG showed that between 40 and 80 min, there was removal of 4FG from the suspending medium with the concomitant appearance of a non-fluorinated intermediate metabolite X in the supernatant. Structural data obtained from NMR and mass spectrometry indicates that this metabolite may be 4-deoxy-D-glucose and/or 4-deoxy-5-keto-D-glucose. To unequivocally demonstrate the structure of this metabolite it will be necessary to isolate it on a large scale. It may be advantageous to use gluconate grown whole cells of *P. putida*, because it appears that under these conditions, defluorination is observed, but without the subsequent evolution of CO₂. The gluconate repressed glucose carrier system would significantly retard the transport and metabolism of 4-deoxy-D-glucose or 4-deoxy-5-keto-D-glucose.

A preliminary examination of the intracellular contents of *P. putida*, after incubation with 4FG over the same time profile revealed the presence of 4FG and an unknown metabolite (Fig. 20). It is likely therefore that 4FG is transported by the known glucose carrier and that defluorination is a prelude to metabolism. The elucidation of the structure of the
unknown intracellular component (Fig. 20) would again require large-scale isolation procedures.

In an apt piece of understatement, Vicente and Canovas (99) reflected that the picture of hexose catabolism in the pseudomonads is far from simple. This could unquestionably be extended to include 4FG, for much remains to be unravelled before a complete understanding of its metabolic fate in *P. putida* is achieved. In particular, the mechanism of carbon-fluorine bond cleavage still remains to be determined.
APPENDIX I

PROTEIN STANDARD CURVE

![Graph showing protein standard curve with absorbance at 595 nm on the x-axis and concentration in mg/mL on the y-axis.](image-url)
The standard curve was constructed with a range of concentrations of bovine serum albumin (0.1-1.0 mg/mL) in either potassium phosphate or Tris-HCl buffer, 100 or 50 mM, pH 7.1. Each point represents the average of duplicate readings. Procedure as described in Method 2.2.6.
APPENDIX II

FLUORIDE ION STANDARD CURVE

[Graph showing a log-log plot with voltage on the y-axis and log [F⁻] on the x-axis. The graph is linear with data points graphed.]
The standard curve was constructed with a range of concentrations of NaF (10^{-1} - 10^{-5} M) in either potassium phosphate or Tris-HCl buffer, 100 or 50 mM, pH 7.1. Fluoride was measured with a fluoride specific electrode (Orion Research) as described in Method 2.2.10.
APPENDIX III

QUENCH CORRECTION CURVE FOR $^{14}$C-LABELLED SAMPLES

![Graph showing quench correction curve](image-url)
The quench correction curve was produced by counting a series of quenched (190,000 dpm) and unquenched (29,700 dpm) $^{14}$C standards (Nuclear Chicago) on a Beckman LS 7500 liquid scintillation counter (Method 2.2.11). A full $^{14}$C window was used for all three counting channels. The counting efficiency (%) and H-number is an average of ten independent countings. Error bars represent the uncertainties in the values and is based on the "2 sigma" (2x standard deviation) relative percentage error give in the data provided by the scintillation counter.
APPENDIX IV

PATHWAY FOR $^{14}$CO$_2$ INCORPORATION INTO
GLUTAMATE, ASPARTATE AND THREONINE

\begin{align*}
\text{Oxaloacetate} & \xrightarrow{1} \text{Glutamate} \\
& \quad \text{Via TCA Cycle and Glutamate Dehydrogenase} \\
\text{Aspartate} & \xrightarrow{2} \text{Aspartyl Phosphate}
\end{align*}
Aspartic Semialdehyde

Homoserine

Homoserine Phosphate

Theonine
The numbers shown in the pathway refer to:

1. TCA cycle and glutamate dehydrogenase
2. Aspartate aminotransferase
3. Aspartic kinase
4. Aspartate semialdehyde dehydrogenase
5. homoserine dehydrogenase
6. homoserine kinase
7. threonine synthase
APPENDIX V

\[ ^{14}C\text{-RADIOLABELLED PATHWAY FROM THREONINE TO SERINE, NAG AND NAM} \]

Threonine

\[ \text{CHO} \quad \text{COOH} \quad \text{CH}_2\text{NH}_2 \]
\[ \text{CO}_2 \quad \text{NAD}^+ \quad \text{NADH} \]

Amino Acetone

\[ \text{CHO} \quad \text{COOH} \quad \text{CHOH} \]
\[ \text{CH}_3 \quad \text{CH}_3 \]

Methylglyoxal

\[ \text{CHO} \quad \text{COOH} \quad \text{OP} \]
\[ \text{CH}_2 \quad \text{CH}_3 \]

PEP

\[ \text{COOH} \quad \text{CHO} \quad \text{CH}_3 \]

Pyruvate
F6P $\rightarrow$ Glucosamine 6-phosphate $\rightarrow$ NAG-6-P $\rightarrow$ NAG-1-P
The numbers shown in the pathway refer to:

1. Threonine dehydrogenase
2. Glyoxylase
3. Lactate dehydrogenase
4. Aldehyde dehydrogenase
5. Pyruvate orthophosphate dikinase
6. Enolase
7. Phosphoglyceromutase
8. Glycerate kinase and dehydrogenase; serine aminotransferase and dehydrogenase
9. Phosphoglycerate kinase
10. Triosephosphate isomerase
11. Enolase
12. Fructose diphosphatase
13. Glucosaminephosphate isomerase
14. Glucosaminephosphate acetyltransferase
15. Acetylg glucosamine phosphomutase
16. UDP acetylg glucosamine pyrophosphorylase
17. Epimerase
18. NADPH reductase
APPENDIX VI

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

THE ISOLATED RADIOLABELLED MINOR PEAK METABOLITE

183.91 - Singlet - $\text{C-O}^-$
34.57 - Triplet - $\text{CH}_2$
29.37 - Triplet - $\text{CH}_2$
72.54 - Doublet - $\text{CN-OH}$
66.16 - Triplet - $\text{CH}_2\text{OH}$
Following isolation and HPLC analysis as previously described (Method 2.2.20), the Minor peak, in D$_2$O, was used to obtain this proton-decoupled $^{13}$C spectrum. The analysis was performed on a Bruker 300 MHz FT NMR Spectrometer and required 48,000 scans. The assigned structure, together with the data obtained from a $^{13}$C-proton coupled spectrum is shown in the figure. Chemical shifts are relative to 1,4-dioxane and are expressed in ppm of the main spectrometer frequency (74.469 MHz).
APPENDIX VII

PROTON NUCLEAR MAGNETIC RESONANCE (NMR) SPECTRUM OF
THE ISOLATED RADIONLABELLED MINOR PEAK METABOLITE
The isolated Minor peak, in D$_2$O, that was used to obtain the $^{13}$C decoupled spectrum shown in Appendix VI was also used to acquire this proton NMR spectrum. It was carried out on the same Bruker FT NMR spectrometer and required 64 scans. Chemical shifts are relative to 1,4-dioxane and expressed in ppm of the main frequency (130-134 MHz).
APPENDIX VIII

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

SPECTRUM OF METABOLITE X
Following isolation (Method 2.2.19) the Metabolite X in D$_2$O was used to obtain this proton-decoupled $^{13}$C spectrum. The analysis (42,000 scans) was performed on a Bruker 300 MH FT NMR Spectrometer. Chemical shifts are relative to 1,4-dioxane and are expressed in ppm of the main spectrometer frequency (74.469 MHz).
APPENDIX IX

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

SPECTRUM, USING DEPT* ANALYSIS OF METABOLITE X
The same sample of Metabolite X in D$_2$O used to obtain the $^{13}$C-decoupled spectrum shown in Appendix VIII was analyzed using the *Distortionless Enhanced Polarization Transfer* (DEPT) programme. Procedure as described in Appendix VIII.
APPENDIX X

PROTON NUCLEAR MAGNETIC RESONANCE (NMR) SPECTRUM OF METABOLITE X

130
The sample used for the previous $^{13}C$ spectra was also used to obtain this proton NMR spectrum. The procedure and instrumentation was as reported in the legends to Appendices VI and VII. It required 16 scans and the chemical shifts are expressed in ppm of the main frequency (120-134 MHz).
APPENDIX XI
NEGATIVE-ION FAST ATOM BOMBARDMENT (FAB) MASS SPECTRUM OF METABOLITE X
The Metabolite X was isolated by borate anion-exchange chromatography and prepared as described in Method 2.2.19. Metabolite X in D$_2$O was analyzed by negative-ion FAB mass spectrometry on a VG (Vacuum Generator) Leeds, UK.

ZAB magnetic sector reverse geometry machine. The sample matrix was glycerol and the beam of Xenon atoms was run at 8 kV x 1 mA (8 watts). The ordinate scale represents the relative % abundance of negative ions and the abscissa shows the mass to charge (M/e) ratio of a given negative ion.

The peak with an M/e ratio of 163 would correspond to a molecular weight of 164. Another peak within the cluster showed an M/e ratio of 161 which would correspond to the molecular weight of 162.
APPENDIX XII

NEGATIVE-ION FAST ATOM BOMBARDMENT (FAB) MASS SPECTRUM OF METHANOL-

EXTRACTED BORATE BUFFER IN THE ABSENCE OF METABOLITE X
Ammonium tetraborate buffer (pH 8.9), which was used to elute the borate anion-exchange column, was extracted with methanol and solubilized in D$_2$O (Method 2.2.19). This was designated the Metabolite X control and was analyzed under the same procedural conditions described in Appendix IX.
Felix qui potuit rerum cognoscere causas.

(Virgil)
REFERENCES

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CURRICULUM VITAE

JOHN WATCYN WILLIAMS

PLACE OF BIRTH: Neath, West Glamorgan, South Wales, United Kingdom

DATE OF BIRTH: September 14, 1942

MARRITAL STATUS: Married with one daughter

EDUCATION:

University College Swansea
University of Wales, UK
1977-1980, B.Sc. (Hons.) Biochemistry

University of Western Ontario
London, Ontario, Canada

University of Windsor
London, Ontario, Canada
1985-1990, Ph.D. Biochemistry

AWARDS:

William A. Redmond Memorial Bursary in Chemistry 1989-90

William A. Redmond Memorial Bursary in Chemistry 1988-89

University of Windsor Summer Research Scholarship 1989

PROFESSIONAL QUALIFICATIONS:

Chartered Chemist, Member of Royal Society of Chemistry (UK), C.Chem., MRSC., (UK)

PROFESSIONAL ORGANIZATIONS:

Royal Society of Chemistry (UK)
The Biochemical Society (UK)
The Canadian Biochemical Society

INDUSTRIAL EXPERIENCE:

Prior to entry to the University of Wales, 1977, I held the post of Shift Chemist with the British Steel Corporation at the Port Talbot plant in S. Wales. My work centred on the chemistry of water treatment and its relationship to power plant operation. The main responsibilities were:
1. Monitoring of water treatment/power plant conditions by chemical analysis (largely inorganic) and method development.

2. Supervisory duties e.g., junior laboratory staff, labour force of water-treatment plants.

3. Responsibility for safety aspects of certain work areas of plant e.g., toxic gas level quantitation.

TEACHING EXPERIENCE:

Teaching Assistant, University of Windsor, 1985-1990

PUBLICATION: