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4-DEOXY-4-FLUORO-D-GLUCOSE: A NOVEL MEMBRANE

PROBE IN PSEUDOMONAS PUTIDA

by

Tony D'Amore

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

1983

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ABSTRACT

4-DEOXY-4-FLUORO-D-GLUCOSE: A NOVEL MEMBRANE

PROBE IN PSEUDOMONAS PUTIDA

by

Tony D'Amore

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

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

Pre-incubation of P. putida with chloramphenicol further demonstrates the presence and synthesis of protein responsible for the defluorination of 4FG. Such cells exhibit a large reduction in the extent of fluoride release. The defluor-

inating activity in these cells is shown to be the result of endogenous protein present.

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

The results obtained with D-[6-^{3}H]-4-deoxy-4-fluoro-glucose and a possible mechanism of fluoride release are presented. Such a mechanism accounts for the release of fluoride, the formation of ^{3}H$_2$O and tritiated-carbohydrates and covalent attachment of the defluorinated sugar residue to the protein. This protein appears to be peptidoglycan-associated and, therefore, may form or be part of the porin channel in the outer membrane. A molecular weight of about 40,000 was estimated for this protein by column chromatography.

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

DEDICATION

To my wife, Angela

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ATP	-	adenosine-5'-triphosphate
CF_3dUMP	-	5-trifluoromethyl-2'-deoxyuridylate
$CH_2-H_4folate$	-	5,10-methylenetetrahydrofolic acid
cpm	-	counts per minute
CSG	-	citryl-S-glutathione
DBP	-	dicarboxylic acid binding protein
DCIP	-	dichlorophenol indophenol
dpm	-	disintegrations per minute
dTMP	-	2'-deoxythymidylate
DTT	-	dithiothreitol
dUMP	-	2'-deoxyuridylate
EDTA	-	ethylenediaminetetraacetic acid
FdUMP	-	5-fluoro-2'-deoxyuridylate
3FG	-	3-deoxy-3-fluoro-D-glucose
3FGA	-	3-deoxy-3-fluoro-D-gluconic acid
4FG	-	4-deoxy-4-fluoro-D-glucose
$D-[6-^3H]4FG$	-	$D-[6-^3H]$-4-deoxy-4-fluoroglucose
4FGA	-	4-deoxy-4-fluoro-D-gluconic acid
GABA	-	γ-aminobutyric acid
GBP	-	glucose binding protein
GS^O	-	glutathione free radical
GSH	-	glutathione (reduced)
GSSG	-	glutathione (oxidized)
$H_2folate$	-	7,8-dihydrofolic acid
2K3FGA	-	3-deoxy-3-fluoro-2-keto-D-gluconic acid
2K4FGA	-	4-deoxy-4-fluoro-2-keto-D-gluconic acid

2KGA	– 2-keto-D-gluconic acid
LPS	– lipopolysaccharide
α-Me4FG	– α-methyl-4-deoxy-4-fluoro-D-glucopyranoside
α-MeG	– α-methyl-D-glucopyranoside
β-MeG	– β-methyl-D-glucopyranoside
$NADP^+$	– nicotinamide adenine dinucleotide phosphate
NADPH	– nicotinamide adenine dinucleotide phosphate (reduced form)
NEM	– N-ethyl maleimide
NMR	– nuclear magnetic resonance
PAGE	– polyacrylamide gel electrophoresis
PAS	– periodic acid-Schiff reagent
PMS	– phenazine methosulfate
POPOP	– 1,4-bis(5-phenyloxazol-2-yl)-benzene
PPO	– 2,5-diphenyloxazole
SDS	– sodium dodecyl sulfate
TCA	– tricarboxylic acid
TEMED	– N,N,N',N'-tetramethylethylenediamine

CHAPTER I

INTRODUCTION

General

The existence of naturally occurring fluoro-organic compounds was first demonstrated by Marais (1), in 1944, with the isolation of fluoroacetic acid as the toxic component in the leaves of Dicapetalum cymosum. This plant has long been responsible for the sporadic poisoning of cattle in South Africa. Subsequently, the same compound has been identified in other toxic plants (2). Other naturally occurring fluoro-compounds include the ω-fluoro-fatty acids, such as ω-fluoro-myristic, ω-fluorooleic, ω-fluorocaproic and ω-fluoro-palmitic acids (3). Nucleocidin, an antitrypanosomal antibiotic, was the first fluoro-sugar derivative to be isolated from any natural source. The fluorine atom replaces the hydrogen atom at the C-4 position of the carbohydrate moiety and through a variety of spectral observations has been assigned the structure of a nucleoside (9-(4'-fluoro-5-0-sulfamoylpentofuranosyl)adenine) (4).

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

1

effects of fluoroacetate.

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

Fluorocitrate as a Biochemical Probe

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

The four possible isomers of fluorocitrate are formed
either from fluoroacetyl-CoA and oxaloacetate or from
fluorooxaloacetate and acetyl-CoA (9). It was subsequently
shown that the toxic isomer is formed only by enzymatic
condensation of fluoroacetyl-CoA with oxaloacetate, a
reaction catalyzed by citrate synthase (10). All other
isomers had no significant inhibitory effects on aconitase.
Synthesis and resolution was accomplished in 1969 (11)

where it was shown that the electophoretically separated erythro isomers contained the toxic species, which was further resolved and identified as (-)erythrofluorocitrate; correctly defined as: 1R:2R 1-fluoro-2-hydroxy-1,2,3-propane tricarboxylate.

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

If fluorocitrate behaves as an uncomplicated competitive reversible inhibitor of aconitase, then some other mechanism

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

The essential prerequisite for inhibition of mitochon-
drial citrate transfer is pre-incubation with 10-100 nM
fluorocitrate in the absence of citrate, which if added
simultaneously with fluorocitrate prevents inhibition.
Pre-incubation of mitochondria with fluorocitrate also
results in selective and irreversible inhibition of citrate-
supported ATP synthesis and inhibition of citrate-dependent

Figure 1

Experimental Model System used by Kun (14) in Liver Mito-chondria to Establish the Site of Fluorocitrate Toxicity.

Figure 1

citrate ← ——————— citrate or cis-aconitate

tricarboxylate
carrier site

⇅ aconitase

cis-aconitate

→ isocitrate

⇅ aconitase

isocitrate

NADP⁺

isocitrate
dehydrogenase

NADPH

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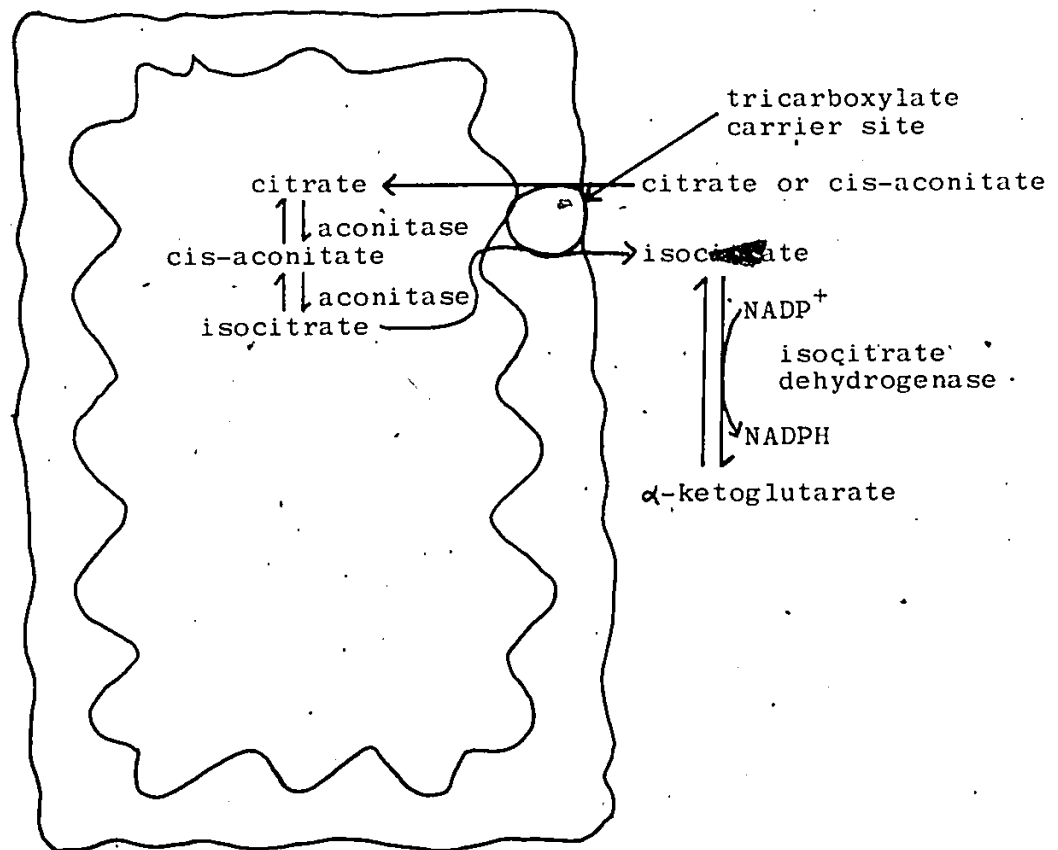
fatty acid biosynthesis (16). No effect was observed when isocitrate, cis-aconitate or other tricarboxylic acid (TCA) intermediates were used as permeant substrates. These results are consistent with the existence of an inhibitory site of fluorocitrate which is rate-limiting in citrate transport through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

Incubation of inner membrane vesicles from mitochondria for one hour with 10 μM [^{14}C]-labeled fluorocitrate results in isolatable protein-fluorocitrate adducts (16). The reaction of fluorocitrate with these membrane proteins was found to involve thioester formation since treatment with neutral hydroxylamine, a thioester bond cleaving agent, results in the release of bound fluorocitrate. Hydrolysis of the protein-fluorocitrate bond with sodium hydroxide is also consistent with the known properties of esters. Furthermore, inhibition of fluorocitrate binding results when vesicles are pre-treated with mersalyl, a specific reagent for thiol groups of proteins (16). The structure of the protein-fluorocitrate thioester adduct formed has not yet been deduced. It is possible that either one or both carboxyl groups of fluorocitrate, which are activated by fluorine substitution, may participate in thioester bond formation (16).

Two distinct proteins containing bound fluorocitrate
were isolated from an extract of liver mitoplasts. Mitoplasts
are defined as the mitochondrial matrix surrounded by the
inner membrane. One has a molecular weight of about
175,000 and the second a molecular weight of about 71,500.
These proteins were identified as citryl-S-glutathione (CSG)
synthetase and hydrolase, respectively (18). The synthetase
catalyzes thioester bond formation between citrate and
oxidized glutathione (GSSG), whereas the hydrolase cleaves
the thioester bond to give citrate and reduced glutathione
(GSH). Pre-incubation of a mitoplast extract with fluoro-
citrate results in irreversible inhibition of CSG thioester
formation. A correlation was found between the degree of
inactivation of CSG synthetase and the quantity of protein-
fluorocitrate product (18). Furthermore, this enzyme was
also found to be inhibited by 1,2,3-propane tricarboxylate,
which is generally recognized as a specific inhibitor of
citrate transport in intact mitochondria.

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

9

Figure 2

Proposed Transport System for Citrate Across the Mito-
chondrial Membrane.

Figure 2
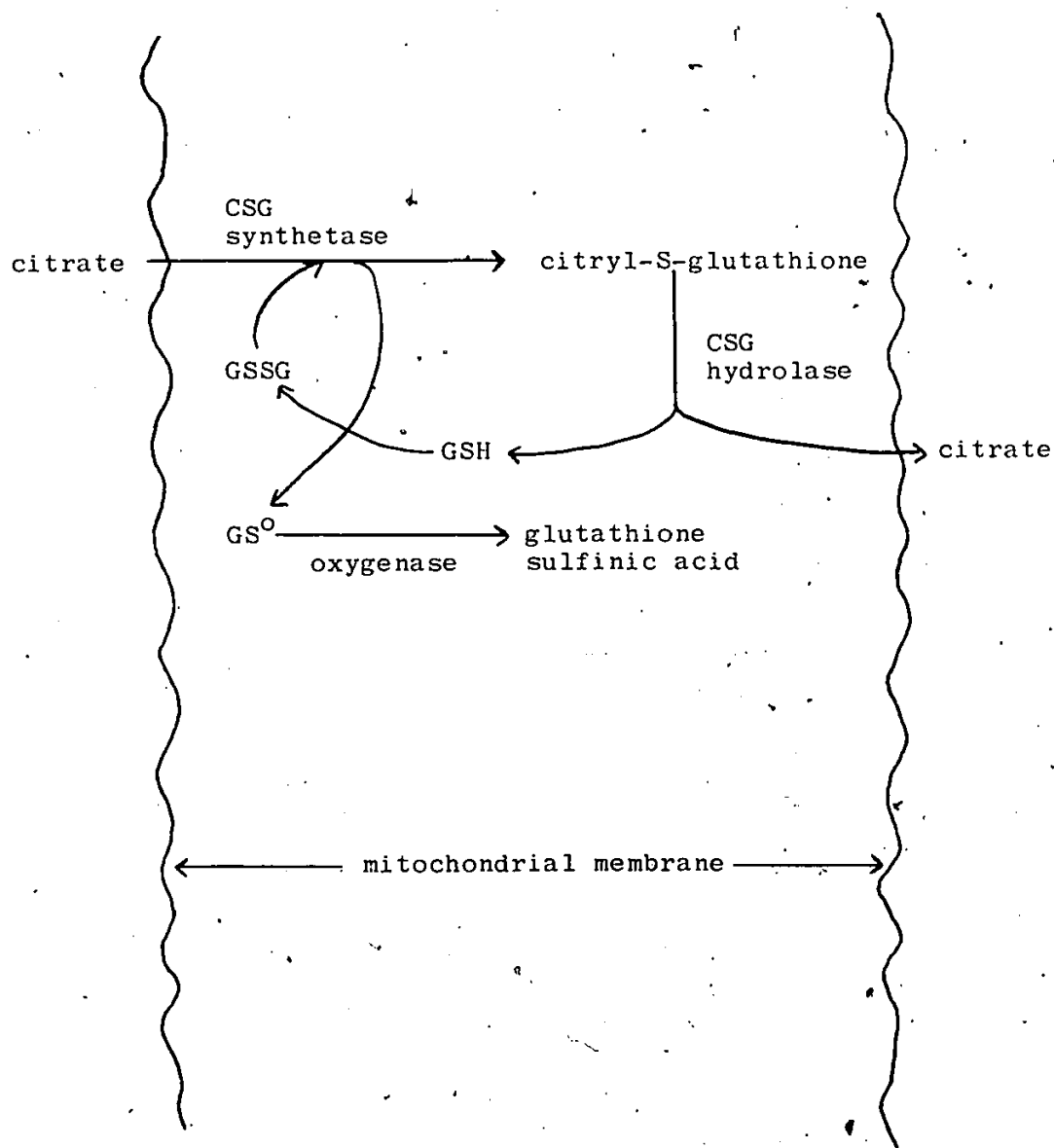
hydrolase, resulting in free citrate and GSH. Inhibition

of thioester formation by phenazine methosulfate, a free

radical trapping agent, indicates that enzymatic formation

of the thioester may involve GS^O formation via homolytic

cleavage of GSSG (18). Associated with this process is the

generation of glutathione sulfinic acid on a mole per mole

basis with thioester formation, indicating the simultaneous

contribution of an oxygenase type of reaction coincidental

with the formation of CSG.

Specific enzyme systems were also found to exist in

liver mitochondria that catalyzes the glutathione-thioester

formation of succinic, malic, α-ketoglutaric, glutamic,

pyruvic and isocitric acids and of glutamine (18). This

may suggest a general metabolic pathway for the TCA inter-

mediates. In conjunction with this is the identification of

hydroxyacyl-S-glutathione, formyl-S-glutathione and succinyl-

S-glutathione thioesters and their hydrolases in human

liver (19). These enzymes were found to be localized in

the cytoplasm and no apparent metabolic function was given.

An alternative function of the thioester synthetase-

hydrolase system may be in regulating the concentration of

all free carboxylic acid substrates of the TCA cycle. This

would operate depending on the GSH-GSSG ratio. GSH is the

most abundant thiol present in virtually all cells. It

is involved in a wide range of cellular phenomena, such as

maintaining protein thiol groups, protection of proteins

and cell membranes against peroxides and free radicals,

controlling GSSG levels and in the transport of amino acids across cell membranes (20).

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

Enzyme Reaction Mechanism Studies

One enzyme reaction mechanism that has been extensively studied with the use of fluoro-analogues is that of thymidylate synthetase. This enzyme catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP) to 2'-deoxythymidylate (dTMP) with the concomitant conversion of 5,10-methylenetetrahydrofolic acid (CH_2-H_4folate) to 7,8-dihydrofolic acid (H_2folate) (23). This reaction was proposed by Friedkin (24) to occur via a two step process. The first step was viewed as an electrophilic substitution reaction in which the methylene carbon of CH_2-H_4folate replaces the hydrogen at the 5-position of dUMP. The second step of this reaction was proposed to be a nucleophilic attack at the incipient methyl group of dTMP by hydride originating from the 6-position of the cofactor to give

dTMP and. H_2 folate. As early as 1959, it was recognized that CH_2-H_4 folate serves the dual function of both one carbon carrier and reductant (25). Direct support for major aspects of this proposed mechanism of thymidylate synthetase has been provided by Santi and co-workers (26) from studies of its interaction with 5-fluoro-2'-deoxyuridylate (FdUMP) and 5-trifluoromethyl-2'-deoxyuridylate (CF_3dUMP).

FdUMP has been known for some time to be an extremely potent inhibitor of thymidylate synthetase. Its inhibitory action was suspected to result from reaction with the proposed nucleophilic catalyst of thymidylate synthetase, namely CH_2-H_4 folate (26). In 1974, Santi (27) demonstrated that a tight complex is formed between CH_2-H_4 folate, FdUMP and thymidylate synthetase, which could be isolated by filtration through a nitrocellulose membrane. A scheme for the complex formation was proposed to be as follows:
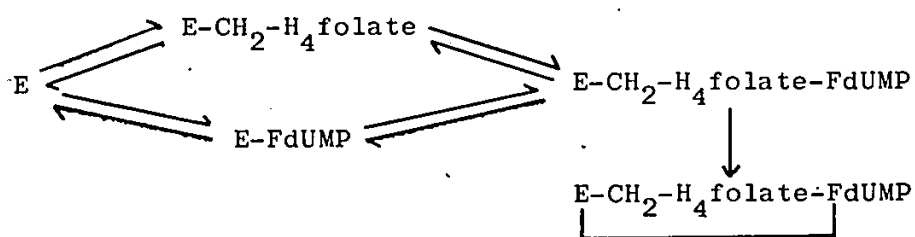
The pathway involves reversible formation of binary complexes which are not sufficiently stable to be isolated. Subsequent formation of a tightly bound ternary complex results as demonstrated by comparing dissociation constants (K_d). K_d for the binary FdUMP complex was calculated by

equilibrium dialysis to be 10^{-5} M (27). On the other hand, from studies of the relative rates of association and dissociation of FdUMP with the enzyme-CH_2-H_4folate binary complex, the K_d of the ternary complex was calculated to be 10^{-13} M (26). Therefore, once formed, the ternary complex is stable for long periods of time.

Using radioactive FdUMP and CH_2-H_4folate, the enzyme has been titrated and shown to possess two FdUMP and two cofactor binding sites per mole (27). The ternary complex was found to be stable towards protein denaturation and showed a complete loss of absorbance at 269 nm, which corresponds to stoichiometric loss of the pyrimidine chromophore of FdUMP. This strongly suggests that the 5,6-double bond of the pyrimidine is saturated in the bound complex. Based on these findings, together with information gathered from a variety of model chemical studies and ^{19}F-nuclear magnetic resonance (^{19}F-NMR) spectra, the structure of the enzyme-FdUMP-CH_2-H_4folate ternary complex is proposed to be as in Figure 3 (23). Here a nucleophile of the enzyme has added to the 6-position of FdUMP. Furthermore, the 5-position of FdUMP is coupled to the 5-position of CH_2-H_4folate via the methylene group of the cofactor.

Recently, studies of 1-(5-phospho-β-D-arabinosyl)-5-fluorouracil as a mechanism-based inhibitor of thymidylate synthetase (28) and FdUMP interaction with CH_2-H_4folate and dUMP hydroxymethylase (29), have provided additional support for the proposed structure of the ternary complex. Further-

Figure 3

Proposed Structure of the FdUMP-CH_2-H_4folate-Thymidylate
Synthetase Ternary Complex.

Legend:

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

Figure 3
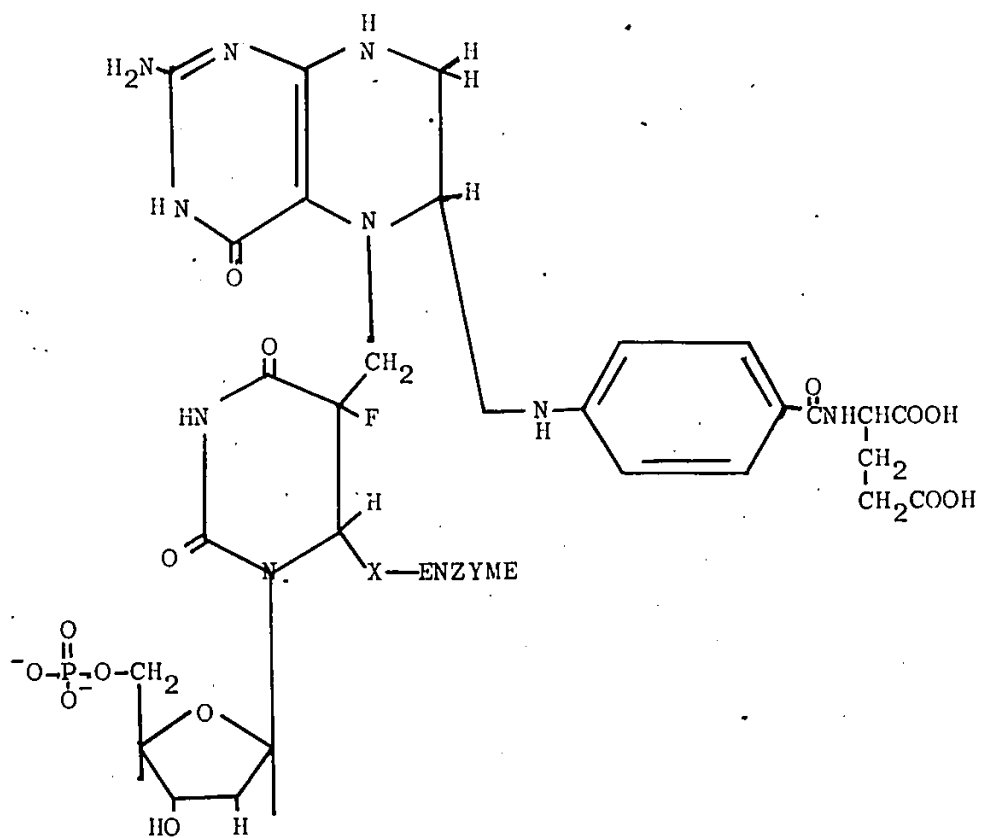
more, FdUMP has also been used in establishing that the
dUMP hydroxymethylase reaction mechanism is analogous to
the mechanism of thymidylate synthetase (29).

As illustrated in Figure 4, the addition of a nucleophile
of thymidylate synthetase to the 6-position of dUMP is the
primary event in the enzyme catalyzed reaction (26). The
resultant carbanion reacts with CH_2-H_4folate to produce a
steady-state intermediate, as proposed by Friedkin (24),
and analogous to the ternary complex formed with FdUMP,
cofactor and enzyme. Abstraction of the 5-hydrogen, followed
·by a series of steps involving reduction of the one carbon
unit and elimination of the nucleophile, results in the
observed products.

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

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

Figure 4

Proposed Mechanism for the Thymidylate Synthetase Reaction.

Legend:

    R = 5-phospho-2'-deoxyribosyl

    X - represents a nucleophile of one of the amino

       acids of the enzyme.

Figure 4

dUMP

ENZYME-X:

CH_2-H_4folate

CH_2-H_4folate

X-ENZYME

CH_2-H_4folate

X-ENZYME

dTMP

H_2folate

+

ENZYME-X:
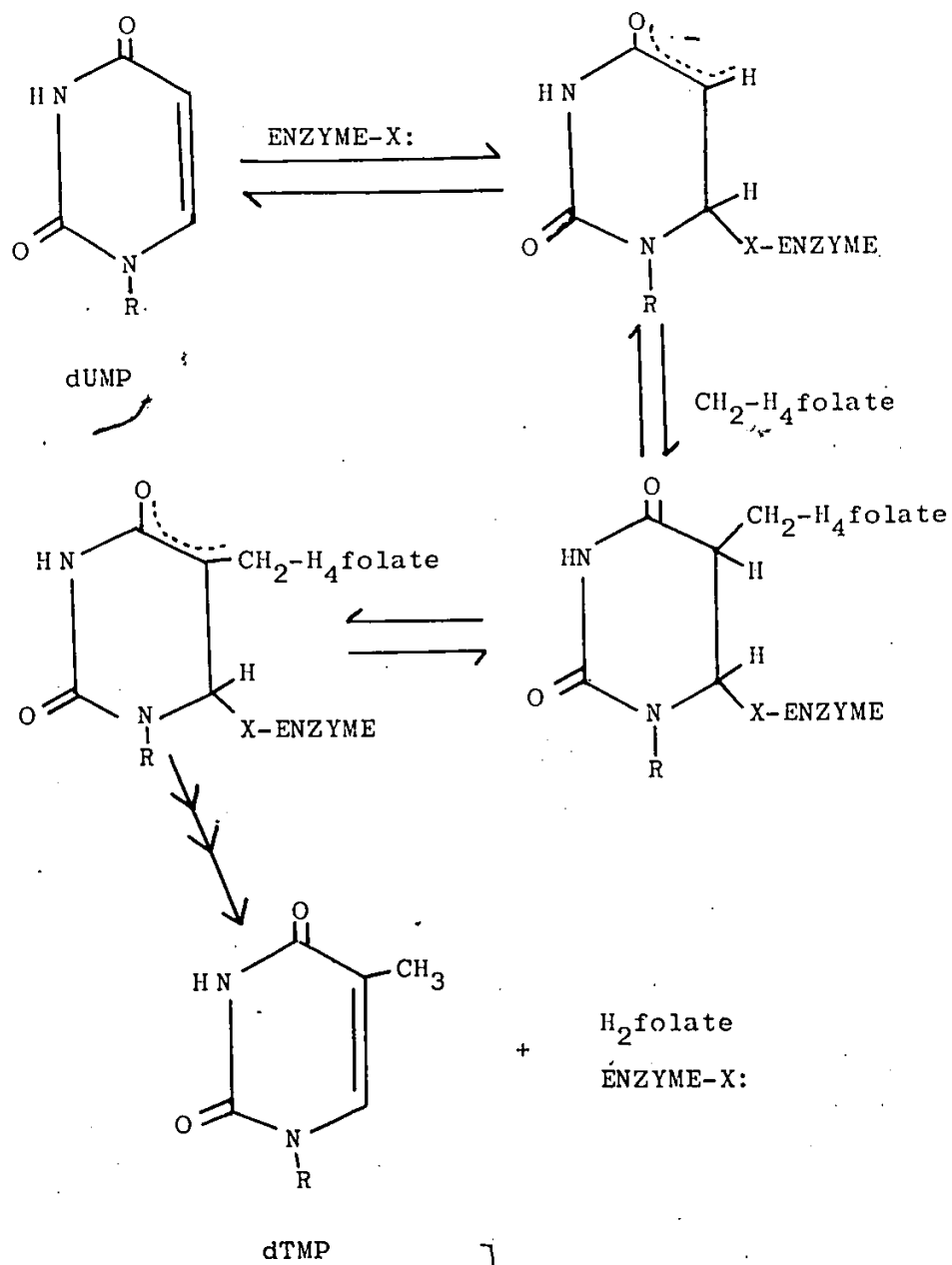
formation of the ternary complex has been known for some time to involve a sulfhydryl group. It has recently been demonstrated by protein degradation studies on the ternary complex that the most likely candidate for the nucleophile is cysteine (23).

CF_3dUMP has also been shown to be an irreversible inhibitor of thymidylate synthetase. Reyes and Heidelberger (30) proposed that this inhibition was due to the formation of a covalent bond between the carbon carrying the CF_3-group and an amino acid near the active site of the enzyme. As depicted in Figure 5, this nucleophile adds to the 6-position of CF_3dUMP, promoting the expulsion of fluoride ion (F^-) and the formation of a reactive exocyclic difluoro-methylene. The reactive intermediate would then be trapped by a nucleophilic group of the enzyme to give the acylated enzyme, thereby inactivating it.

Santi and co-workers (26) have recently demonstrated that incubation of thymidylate synthetase with CF_3dUMP, in the absence of $CH_2-H_4folate$, for twenty minutes results in 89% inactivation of the enzyme with possible formation of a 5-acyl derivative of dUMP. From these and model studies (26), it was proposed that activation of the C-F bond requires addition of a nucleophile of the enzyme to the 6-position of the nucleotide. When $CH_2-H_4folate$, CF_3dUMP and thymidylate synthetase are incubated together, a difference spectrum similar to that produced by the FdUMP-$CH_2-H_4folate$-enzyme ternary complex is observed (26).

Figure 5

Proposed Inactivation Mechanism of Thymidylate Synthetase
by CF_3dUMP.

Legend:

    R = 5-phospho-2'-deoxyribosyl

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

Figure 5
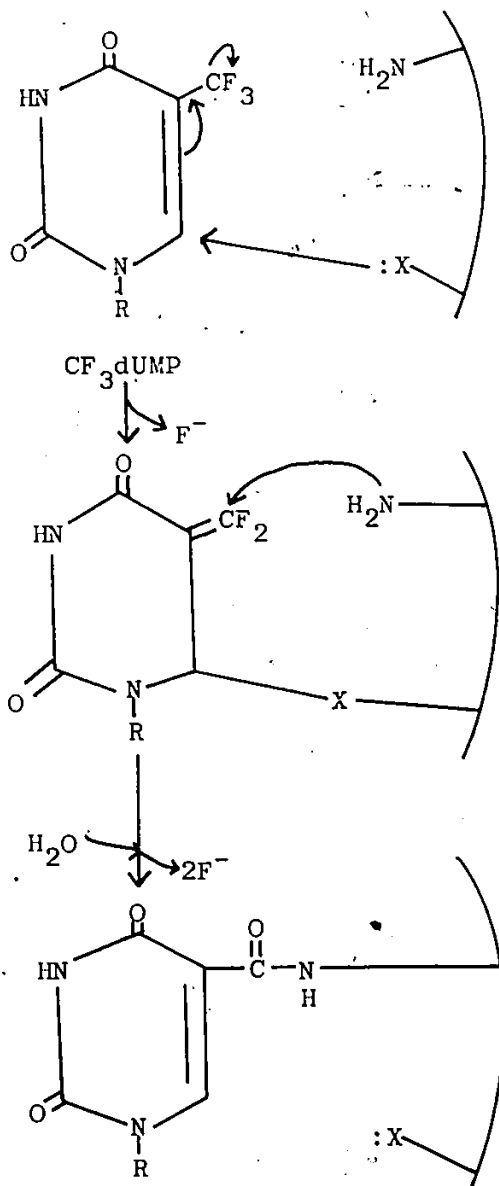
There is a characteristic increase in absorbance at 330 nm

and a decrease at 261 nm. This decrease in absorbance is

in accord with the saturation of the 5,6-double bond of

the nucleotide. Although preliminary, these results

suggest a similar rection mechanism between thymidylate

synthetase and FdUMP or CF$_3$dUMP.

Apart from providing valuable information regarding

the general mechanism of folate dependent enzymes, in

particular thymidylate synthetase, fluorouracil analogues

are currently in use as anti-tumor, anti-viral and anti-

fungal agents (31).

During the past several years, there has been a

tremendous increase in the synthesis and biochemical use

of a variety of active site directed labeling agents (32-

34). The rationale and general criteria for the design of

such compounds have been given by Rando (35). Briefly,

the design of these inhibitors is based upon the attachment

of a chemically reactive group, generally an electrophile,

to a molecule which has structural features resembling that

of the enzyme's normal substrate. The resultant reaction

of the molecule with the active site of the enzyme leads

to covalent bond formation and inactivation of the enzyme.

One class of such compounds includes the α-fluoromethyl-

amino acid analogues (36-40). These substrates are non-

reactive until fluorine is expelled via enzyme catalyzed

decarboxylative elimination. Highly electrophilic species

would result which are capable of undergoing addition of

fortuitously positioned nucleophilic groups at or near the
enzyme's active site. Stable enzyme-inhibitor covalent
adducts would result, thereby inactivating the enzyme.
The general scheme for such reactions is as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} EI \xrightarrow{k_{inh.}} E\text{-}I \text{ (covalently modified)}$$
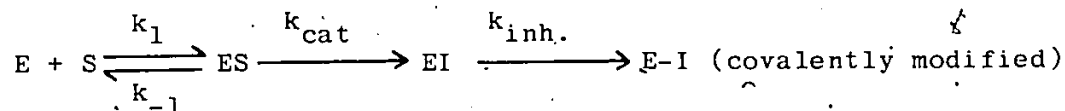
These mechanism-based inhibitors have also been referred
to as suicide substrates, k_{cat} inhibitors or Trojan-horse
inhibitors (33) because of their mode of action.

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

The above inactivation reaction occurs for about 80%
of the time since nonlethal turnover could also occur.

Figure 6

Proposed Inactivation Mechanism of Glutamate Decarboxylase
by α-Fluoromethylglutamate.

Legend:

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

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

    B - postulated nonlethal turnover leading to the
        formation of levulinic acid.

Figure 6
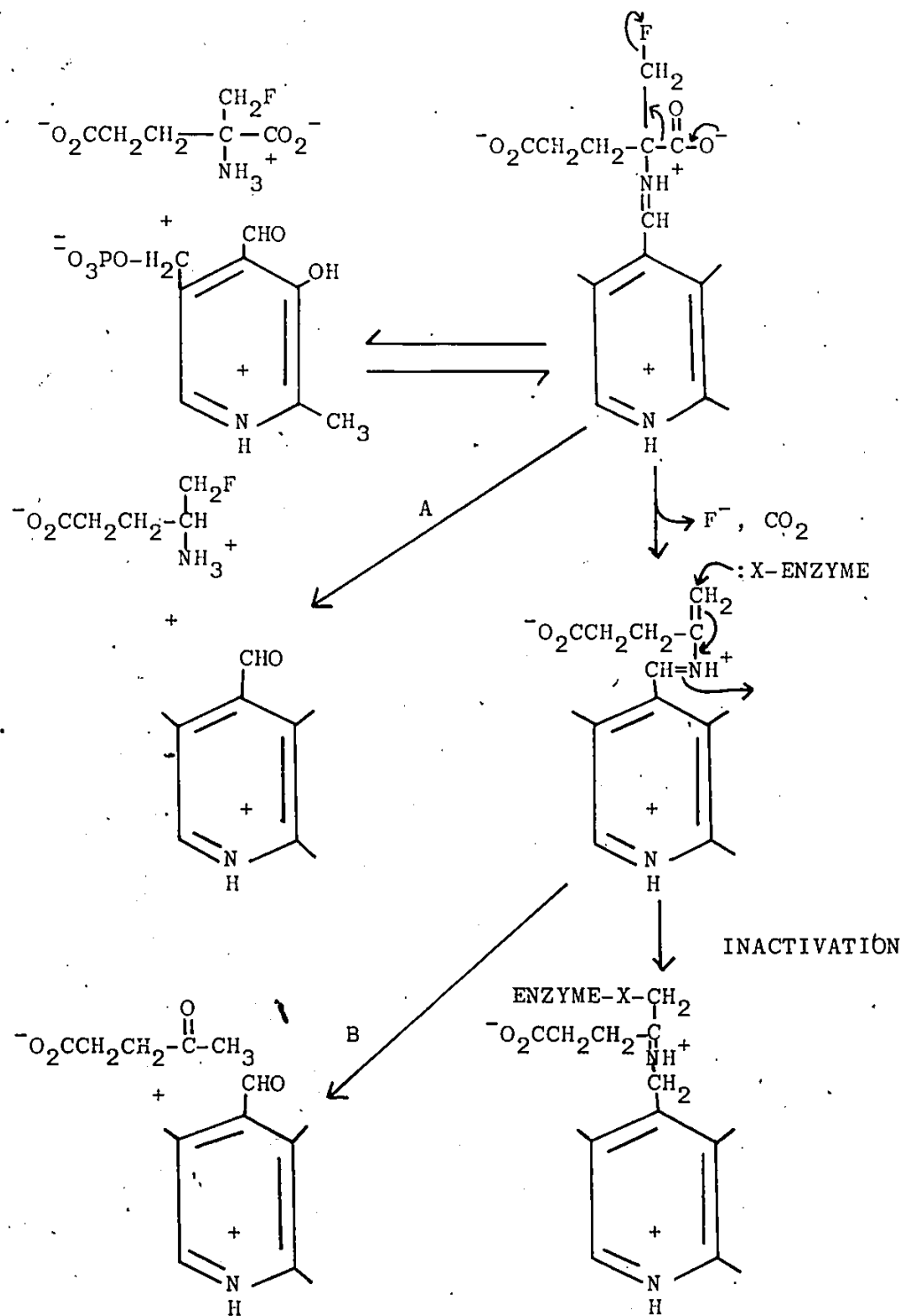
It was suggested that direct decarboxylation could occur
to form fluoromethyl-GABA or that hydrolysis of the activated
intermediate could occur to form levulinic acid (36).

Carbon-Fluorine Bond Cleavage

The bond strength of the C-F bond (448 kJ mol^{-1}) is
among the highest found in natural products and yet is
cleaved in a number of different enzymatic reactions (42).
The cleavage of the C-F bond in CF_3dUMP and in the α-fluoro-
methyl-amino acid analogues was seen to be the result of the
formation of reactive intermediates. These compounds were
specifically designed with the purpose of forming such
reactive intermediates, which would react with and irreversibly
inhibit their respective enzymes through covalent bond
formation. The release of fluoride, in these and other
examples, might therefore be considered purely accidental
in terms of the normal role of the enzyme. That is, the
release of fluoride is a consequence of the normal enzymatic
reaction mechanism.

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

halide from chloroacetate and iodoacetate. The enzyme

was therefore termed haloacetate halidohydrolase (43).

Enzyme reactivity was found to be dependent on the

presence of an active thiol group on the enzyme. It was

therefore postulated that defluorination proceeds through

thioester formation (43) as follows:

$$ENZ-S^- + FCH_2COO^- \longrightarrow ENZ-S-CH_2COO^- + F^-$$

$$ENZ-S-CH_2COO^- + OH^- \longrightarrow ENZ-S^- + HO-CH_2COO^-$$
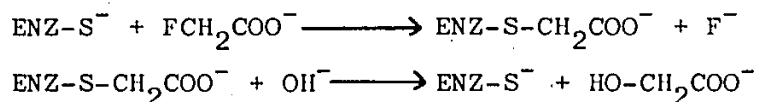
An intriguing possibility is that this enzyme might be

capable of catalyzing reversible formation of fluoroacetate

from glycollate and fluoride. If possible, this could

give an indication as to how fluoroacetate is formed in

nature. Unfortunately though, many attempts have failed

to demonstrate the revesibility of this reaction (44).

Organisms capable of metabolizing fluorobenzoates via

C-F bond cleavage have also been shown to exist (44,45).

A pseudomonad was isolated which could metabolize 2-fluoro-

benzoic acid with corresponding fluoride release (44).

The defluorination reaction was found to take place by

the introduction of molecular oxygen in a one-step process

as follows:
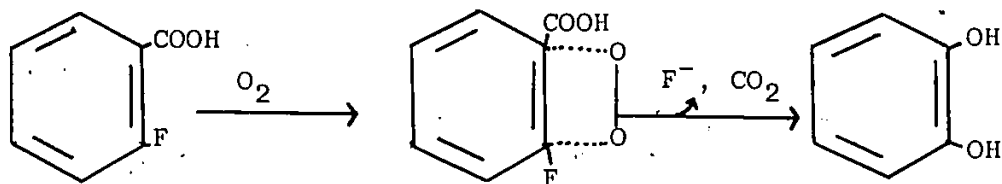
In the above mechanism, 2-fluorobenzoic acid is converted to catechol, which can be further metabolized by the organism. Recently, an enzyme has been isolated from cell-free extracts of _Pseudomonas putida_ and shown to catalyze fluoride release from difluoro- and tetrafluoro-derivatives of p-hydroxybenzoic acid, through a similar hydroxylation type reaction (45).

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

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

Similarly, α-D-glucopyranosyl fluoride was found to be hydrolyzed by the action of α-glucosidase to D-glucose and F^- (47). The mechanism of α-glucosidase action involves protonation of the glycosidic oxygen followed by nucleophilic attack, either from water leading to inversion of

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

Fluorocarbohydrates as Biochemical Probes

The rationale, synthesis and use of fluorocarbohydrates as biochemical probes have been reviewed in part (6,7,49). Such compounds have been used to probe the specificity of hydrogen-bonding sites between D-glucose and the carrier protein(s) known to be present in the human erythrocyte (50, 51) and the hamster intestine (47). Recently, Grier and Rasmussen (52) have used 3-deoxy-3-fluoro-D-mannose and 4-deoxy-4-fluoro-D-mannose as probes for studying D-mannose transport and metabolism in Saccharomyces cerevisiae. 2-Deoxy-2-fluoro-D-glucose and 2-deoxy-2-fluoro-D-mannose have been employed extensively for studies of glycoprotein biosynthesis and function in chick embryo cells (53,54).

Other studies with fluorocarbohydrates by Taylor have been
primarily concerned with enzyme specificity (55), carbohydrate
transport in synaptosomes from rat brain cortex (56), carbo-
hydrate metabolism in Locusta migratoria (57) and carbohydrate
transport and metabolism in E. coli (58) and P. putida (59).

Previous studies (59) demonstrated that 3-deoxy-3-fluoro-
D-glucose (3FG) is metabolized by resting whole cells of
glucose grown P. putida, with retention of the C-F bond, to
produce 3-deoxy-3-fluoro-D-gluconic acid (3FGA). 3FGA can
be further oxidized by whole cells to 3-deoxy-3-fluoro-2-
keto-D-gluconic acid (2K3FGA). Cell-free extracts from this
organism were found to oxidize 3FG completely to 2K3FGA.
It was also shown that the same cytoplasmic membrane bound
enzymes that oxidizes glucose and gluconate, namely glucose
oxidase and gluconate dehydrogenase, also oxidizes 3FG and
3FGA, respectively (59). Furthermore, using cytoplasmic
membrane vesicles prepared from glucose or succinate grown
P. putida, it was shown that 3FG is transported by the same
active transport system as glucose (60).

Unlike 3FG, 4-deoxy-4-fluoro-D-glucose (4FG) is not
oxidized by whole cells of P. putida. Instead, there is an
immediate release of fluoride ion (61). Incubation of a
resting whole cell suspension of glucose grown P. putida
with 2.5 mM 4FG results in nearly complete fluoride release
after 24 hours. Thin layer chromatography of the cell
supernatant and intracellular contents failed to reveal the
presence of any new carbohydrates. On the other hand, cell-

free extracts were found to oxidize 4FG with the consumption
of 2 g atoms of oxygen per mole of substrate, which is con-
sistent with the formation of 4-deoxy-4-fluoro-2-keto-D-
gluconic acid (2K4FGA). In this reaction there is virtually
complete retention of the C-F bond. Furthermore, P. putida
is unable to grow on a mineral salts medium with 4FG as the
sole carbon source, although fluoride is released into the
medium (61). Therefore, the defluorination of 4FG could be
considered purely accidental as a consequence of 4FG being
initially accepted by the cell as glucose.

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

The Cell Envelope of Gram-Negative Bacteria

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

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

Figure 7

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

Legend:

      BP - binding protein

      CM - cytoplasmic membrane

      OM - outer membrane

      PC - porin channel

      PG - peptidoglycan

      PS - periplasmic space

      TP - transport protein

         - lipopolysaccharide

         - phospholipid

Figure 7
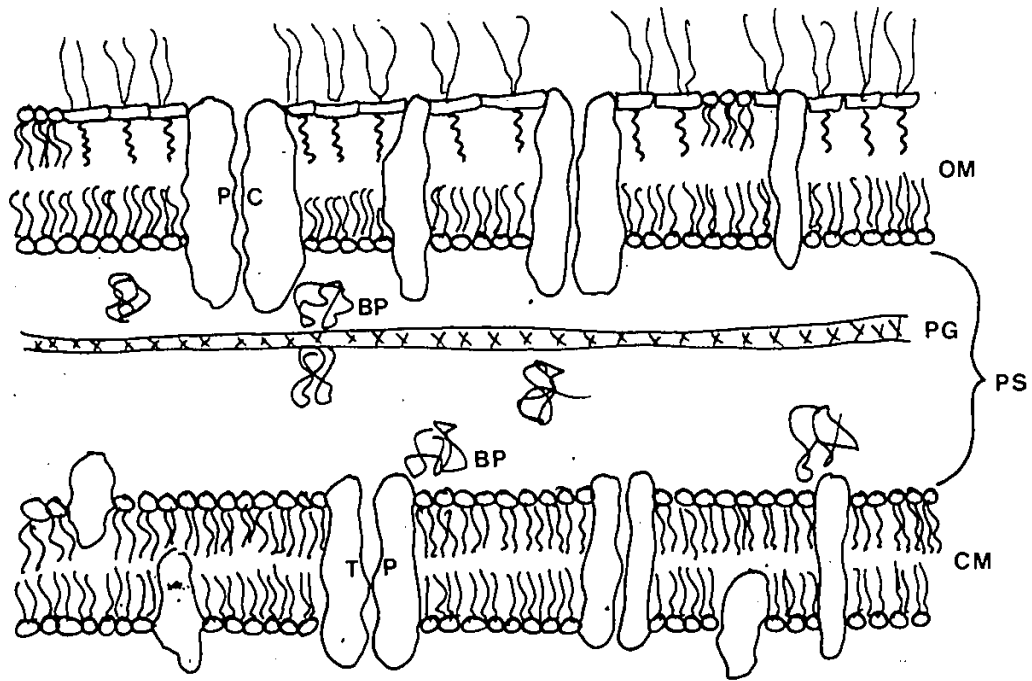
substrate molecules, in gram-negative bacteria, have been
described (64,65). One class couples the transport of mole-
cules across the cell membrane with chemical modification, a
process referred to as group translocation. The second class
transports molecules against a concentration gradient with no
change in the transported molecule. The third class, which
also concentrates substrate molecules without modification,
is dependent upon the presence of periplasmic binding proteins.
These binding proteins, which can exist in both a free form
or bound to the peptidoglycan layer (66), form reversible
complexes with specific substrates. Binding proteins have
been shown in a number of cases to be involved in the trans-
location of substrate molecules across the cell envelope
of gram-negative bacteria (66-68). In contrast to the above
systems, not much attention has been given to the outer
membrane as far as transport capabilities are concerned.
At best, the outer membrane was generally regarded as a
rather inert structure that allows the free diffusion of all
low molecular weight substances.

The separation of outer and cytoplasmic membranes was
first achieved in E. coli by Miura and Mizushima (69). Their
technique involves isopycnic sucrose density gradient centri-
fugation of membranes obtained by lysozyme and EDTA treat-
ment of whole cells, followed by lysis of the resulting
osmotically fragile spheroplasts. A similar method modified
for S. typhimurium was developed by Osborn et al. (70).
These techniques have led to a greater understanding of the

structure and function of the outer membrane and outer

membrane proteins, especially in the enteric bacteria, as

noted by the recent reviews (71-74). .

An increasing amount of evidence from the enteric

bacteria, E. coli and S. typhimurium, has led to the general

conclusion that the outer membrane from these organisms

contain two classes of proteins involved with solute

uptake (71,75). The first class of proteins, which form

hydrophilic aqueous channels, are generally referred to as

porins. These proteins vary from strain to strain but

generally have molecular weights in the range of 35-40,000

daltons. Furthermore, they allow for the nonspecific free

diffusion of small uncharged molecules of less than 600

daltons. These pore-forming proteins have recently been

shown by Lugtenberg et al. (76) to be peptidoglycan-associated.

The other class of proteins behave as transmembrane

channels with solute specificity. These receptor proteins

have been shown to facilitate the diffusion of maltose and

maltodextrins, iron-chelated ferrichrome, iron-citrate

complexes, nucleosides, vitamin B_{12} as well as others (71).

Since most of these nutrients have molecular weights larger

than the exclusion limit of the porin channel, specific

translocation proteins are a necessity. In examples like

maltose, specific proteins are required when the substrates

are present in very low concentrations.

Recently, Lo has demonstrated, through the use of

various nonpenetrating proteases and inhibitors (77) and a

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

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

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

Figure 8

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

Legend:

    DBP – dicarboxylic acid binding protein

    OM – outer membrane

    PC – porin channel

    PS – periplasmic space

    – lipopolysaccharide

    – phospholipid

Figure 8
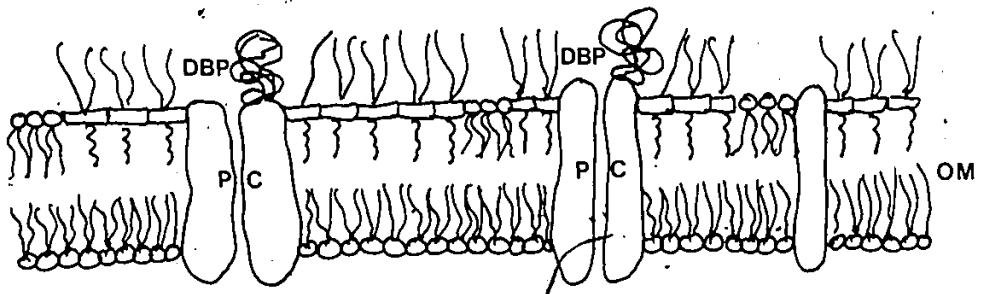
in terms of protein (80), peptidoglycan (81), lipopolysaccharide
(82) and lipids (83). However, good separation of outer and
cytoplasmic membranes was not always possible due to the
hypersensitivity of this and related organisms to EDTA-lysozyme
treatment (84). Recently though, several laboratories have
reported the isolation of the outer membrane from P. aeruginosa,
all using different procedures (85-89). As indicated in
Table 1, two to nine major proteins were observed in the
various outer membrane preparations. Some of these, outer
membrane proteins, in particular the porin protein F, have
been shown to be 'heat-modifiable'. That is, their mobility
on sodium dodecyl sulfate-polyacrylamide gel electrophoresis
(SDS-PAGE) was determined by the solubilization temperature
(86,90). Other outer membrane proteins (F, H and I) have
been shown to be peptidoglycan-associated (91,92). That is,
the protein is tightly, but not covalently, associated with
peptidoglycan. The number and molecular weight of many of
the outer membrane proteins were found to be dependent on
the growth and culture conditions used, the method of outer
membrane isolation, the method of electrophoresis employed
and the strain used (73,90).

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

Table 1

Nomenclature and Molecular Weight of P. aeruginosa

Outer Membrane Proteins.

Molecular Weight (10^3)				
Booth and Curtis (85)	Mizuno and Kageyama (86)	Matsushita et al. (87)	Hancock and Nikaido (88)	Stinnet and Eagon (89)
I (56)	D (50)	1 (95)	E (44)	A (43)
II (53)	E (45)	3 (74)	F (35)	B (16.5)
III (38)	F (33)	4 (53)	G (21)	
1V (21)	G (21)	4 (49)	H (17)	
V (16)	H (21)	5 (34)		
	I (8)	6 (23)		
		7 (19)		
		8 (13)		
		9 (9.7)		

protein has been purified to homogeneity in a functional form (94).

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

glucose carrier protein (99). The high K_m transport system was found to actively transport glucose, 2-deoxyglucose, 3FG and α-MeG, whereas the low K_m or high affinity system was found to transport glucose only (99).

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

Hancock and Carey (100) have proposed that the outer membrane protein D1 and the periplasmic GBP are co-regulated components of the P. aeruginosa high affinity transport system. Protein D1 was shown to reconstitute sucrose and glucose permeable pores in LPS-phospholipid vesicles (100).

Furthermore, they also proposed that the low affinity glucose transport system involves uptake across the outer membrane via the porin protein F. No evidence was provided to support these proposals (100).

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

Objectives

This investigation involves the use of 4FG as a biochemical probe to study glucose transport and metabolism in P. putida. The objectives of this investigation are to:

(a) study and gain insight into the nature and mechanism of fluoride release from 4FG (61).

(b) attempt to determine the site in the whole cell responsible for the defluorination reaction.

(c) attempt to demonstrate the covalent bond formation proposed (61) to occur between the defluorination product and a protein.

(d) use these results with a view to obtain an understanding of glucose transport in whole cells of P. putida and, therefore, substrate transport in general.

CHAPTER II

MATERIALS AND METHODS

Materials

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

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

Maryland) and maintained routinely on glucose-mineral salts agar slopes or nutrient agar slopes.

Methods

Growth and Characterization of P. putida

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

	g/L (final volume)
K_2HPO_4	7.0
KH_2PO_4	3.0
$(NH_4)_2SO_4$	1.0
$MgSO_4 \cdot 7H_2O$	0.1
Yeast extract	0.2
Trace solution A	0.5 mL
Trace solution B	0.5 mL
Carbon source	2.0

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

Trace solution A

	mg/100 mL (stock solution)
$FeSO_4 \cdot 4H_2O$	80
$MnSO_4 \cdot 4H_2O$	80
NaCl	2000

Trace solution B

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

The resulting medium which had a pH of 7.1 was sterilized by autoclaving at $121^{\circ}C$ for 15 min in a Pelton and Crane Magna-clave, Model MC (G. A. Ingram Co., (Canada) Ltd., Windsor, Ontario), without the carbon source. A 2% (w/v) of carbon source was sterilized by membrane filtration in a Nalgene 0.20 µ filter unit (Nalgene Sybron Corp., Rochester, New York) and the appropriate amount added aseptically to the sterile mineral salts medium to give a final concentration of 0.2%.

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

Preparation of Whole Cell Suspensions

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

Table 2

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

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

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

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

Preparation of Cytoplasmic and Outer Membranes

(a) Cytoplasmic Membrane Vesicles

The method for obtaining cytoplasmic membrane vesicles
was essentially as that described in (60). Glucose or

succinate grown whole cells were suspended in 2/3 the

original culture volume in a solution containing in final

concentrations; 2.5% LiCl, 0.75 M sucrose, 10 mM potassium

phosphate, pH 7.1, 10 mM $MgSO_4$ and 0.5 mg lysozyme mL^{-1}.

This suspension was incubated for 1 h at $30^O C$ on a rotary

shaker and the osmotically fragile spheroplasts were then

harvested by centrifugation at 10,000 xg for 15 min at $4^O C$.

The pellet was suspended in the smallest possible volume

with 2.5% LiCl in 0.75 M sucrose. The suspension was quickly

added to 50 volumes of ice-cold 10 mM potassium phosphate

buffer, pH 7.1, containing 1 mM $MgSO_4$, in a Waring blender

and blended for 10 sec. Deoxyribonuclease I (DN'ase) and

ribonuclease A (RN'ase) were added to a final concentration

each of 20 μg mL^{-1} and the mixture was incubated at $25^O C$

with gentle stirring for 30 min. This mixture was then

centrifuged at 40,000 xg for 30 min at $4^O C$ in a Beckman L8-55

ultracentrifuge (Beckman Instruments Inc., Fullerton,

California) and the pellet suspended in ice-cold potassium

phosphate buffer containing 10 mM $MgSO_4$. Large fragments

and whole cells were removed by centrifuging the suspension

at 800 xg for 30 min at $4^O C$. The supernatant was then

centrifuged at 40,000 xg for 30 min at $4^O C$ and the final

pellet, cytoplasmic membrane vesicles, was washed twice with

and suspended in the same buffer system and stored at $-20^O C$.

(b) Outer Membranes

The isolation of the outer membrane was based on a

modification of the method of Mizuno and Kageyama (86).

Glucose or succinate grown whole cells (4 g wet weight) were suspended in 40 mL of ice-cold 20% sucrose and the following ice-cold reagents added slowly with constant stirring; 20 mL of 2 M sucrose, 10 mL of 100 mM potassium phosphate, pH 7.1, 10 mL of 100 mM $MgSO_4$, 0.8 mg DTT mL^{-1} and 0.5 mg of lysozyme mL^{-1}. The suspension was incubated for 1 h at 30°C on a rotary shaker and after 30 min DN'ase was added to a final concentration of 5 μg mL^{-1}. The suspension was then centrifuged at 10,000 xg for 15 min at 30°C to remove the osmotically fragile spheroplasts. Cytoplasmic membrane vesicles could be obtained from these spheroplasts by the method described above. Outer membranes were recovered from the supernatant by centrifugation at 100,000 xg for 90 min at 4°C. The outer membrane fraction was suspended in a small amount of potassium phosphate buffer and centrifuged at 800 xg for 30 min at 4°C to remove any large fragments. The supernatant was then centrifuged at 100,000 xg for 90 min at 4°C and the final pellet, which had a whitish opaque appearance and referred to as the crude outer membranes, was washed twice with and suspended in a small volume of the same buffer. For fluoride release studies the outer membranes were used immediately, otherwise they were stored at -20°C.

Preparation of Cell-Free Extracts and Cell Envelopes

(a) Cell-Free Extracts

Cell-free extracts were produced by ultrasonication (55). A glucose grown whole cell pellet was thoroughly mixed with an equal volume ~~~~ tassium phosphate buffer to produce a

thick cell suspension. The suspension was cooled to $4^{\circ}C$ and ultrasonicated for 8-10 x 1 min periods using a Sonic 300 Dismembrator (ARTEK Systems Corp., Farmingdale, New York) with setting at 60%. The temperature during ultrasonication was maintained at $0-4^{\circ}C$ with the use of an ice-salt bath. During ultrasonication the suspension turned reddish-brown, indicating cell breakage and release of cell contents. The ruptured cell suspension was centrifuged at 17,000 xg for 10 min at $4^{\circ}C$. A small dark brown pellet was obtained, consisting of unbroken whole cells and large debris. In some cases no pellet was obtained, suggesting a near complete cell breakage. The reddish supernatant obtained is referred to as the cell-free extract.

(b) Cell Envelopes

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

SDS-Extraction of Cell Envelopes

This method is based on the procedure of Mizuno and Kageyama (93). Cell envelopes prepared from glucose or succinate grown whole cells of P. putida (5 g wet weight)

were incubated with 15 mL of the SDS-extraction solution
(2% SDS-10% glycerol-10 mM Tris-HCl, pH 7.8) for 60 min at
30°C or 70°C. The insoluble fraction was collected by centri-
fugation at 100,000 xg for 90 min at 25°C. The extraction
was repeated once more with 15 mL of the SDS-extraction
solution at the appropriate temperatures. Peptidoglycan-
associated protein complexes (92) were isolated by centri-
fugation at 100,000 xg for 90 min at 25°C, washed once with
distilled water at 4°C and suspended in a small volume of
distilled water. The supernatants from the same extraction
temperatures were combined and proteins collected by addition
of 2 volumes ice-cold acetone and centrifugation at 17,000 xg
for 20 min at 4°C. The pellets were suspended in a small
volume of distilled water. Peptidoglycan-associated protein
complexes obtained by SDS-extraction at 30°C were further
extracted by incubation with 10 mL of the SDS-extraction
solution for 60 min at 70°C or by incubation with 10 mL of
the SDS-extraction solution supplemented with 0.75 M NaCl for
60 min at 30°C. Insoluble fractions (predominately peptido-
glycan) were collected by centrifugation at 100,000 xg for
90 min at 25°C. Proteins from the individual supernatants
were collected by addition of 2 volumes ice-cold acetone and
centrifugation at 17,000 xg for 20 min at 4°C. Protein
pellets were suspended in a small volume of distilled water.

Osmotic Shock of Whole Cells

The method of osmotic shock was based on the procedure
of Stinson et al. (67) for the isolation of GBP from P.

aeruginosa. Glucose grown whole cells (1 g wet weight) were suspended in 30 mL of 0.2 M $MgCl_2$-0.05 M Tris-HCl, pH 8.5, buffer at $25^{\circ}C$. The suspension was incubated for 30 min with constant stirring and then centrifuged at 16,300 xg for 20 min. The cells were rapidly suspended in 30 mL of distilled water and after-an additional 30 min stirring at $25^{\circ}C$, the suspension was centrifuged. The pellet was immediately suspended in potassium phosphate buffer and tested for defluorinating activity. The supernatants were combined and dialyzed against 2 L of 1 mM $MgCl_2$-0.01 M Tris-HCl, pH 7.4, buffer at $4^{\circ}C$ overnight. Proteins were collected by 0-95% saturation with $(NH_4)_2SO_4$ and centrifugation at 17,000 xg for 20 min at $4^{\circ}C$. The sediment was suspended in a small amount of buffer and dialyzed overnight. This crude shock extract was tested for glucose binding and defluorinating activities.

Assay of Glucose Binding Protein

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

Fluoride Release Studies

Fluoride ion was determined by a fluoride ion specific electrode (Orion Research, Cambridge, Massachusetts) attached

to a Metrohm Herisau E510 mV/pH meter (Brinkman Instruments
Ltd., Rexdale, Ontario). A standard curve was constructed
with known amounts of NaF in potassium phosphate buffer
(APPENDIX I). The protocol adopted for determining fluoride
ion in the various reaction mixtures was essentially the same.
The reaction was initiated by adding known amounts of 4FG to
various whole cell suspensions (5-8 mg protein mL^{-1}), all in
potassium phosphate buffer. For inhibition studies, whole
cells were incubated simultaneously with 2.5 mM 4FG and 5 mM
of the various sugars or 10 mM sulfhydryl alkylating reagents.
The total volume of the suspensions was 3 mL, but in some cases
4 mL was used. The suspensions were incubated in 25 mL
Erlenmeyer flasks at 30°C on a rotary shaker. At various
time intervals fluoride release from 4FG was determined by
directly submerging the fluoride electrode into the suspen-
sions. After allowing 5 min for equilibration, mV readings
were taken. A similar protocol was used in the presence or
absence of 1 mM DTT with the various cell envelope fractions.
In these cases, however, the total volumes were usually 2 mL
(1-3 mg protein mL^{-1}). Incubation of various 4FG concentrations
in potassium phosphate buffer at 30°C were used as controls.
Rates were determined from the slopes of fluoride release curves.

Pre-Incubation of Whole Cells with Chloramphenicol and Pronase

Glucose or succinate grown whole cell suspensions
(10 mL, 8-10 mg protein mL^{-1}) were incubated on a rotary
shaker for 60 min at 30°C in the presence of 1 mg mL^{-1} chlor-
amphenicol or rifampicin. Appropriate aliquots were then

removed and used directly in fluoride release studies.
Similarly, glucose grown whole cells were pre-incubated
with Pronase (5 mg mL^{-1}) for 30 min at 30°C. After incubation,
aliquots were removed and used directly in fluoride release
studies. The rest of the Pronase treated cells were washed
with potassium phosphate buffer and allowed to recover in
buffer for 15 min before being used.

Respirometric Studies

The extents and rates of respiration of glucose and 4FG
by whole cells, cell-free extracts, cell envelopes and
cytoplasmic membrane vesicles was carried out by the manometric
method (105) using a Gilson differential respirometer with
eight 20 mL calibrated reaction flask (Gilson Medical
Electronics, Middleton, Wisconsin). Each reaction flask
contained in a total volume of 2 mL; 1.0 mL potassium phosphate
buffer and 0.5 mL of whole cells, cell-free extract, cell
envelopes or vesicles in the main compartment and 0.5 mL of
substrate in the side-arm. In the centre well there was
placed 0.2 mL of 20% KOH and a folded paper wick to absorb CO_2.
The reaction was initiated by tipping the contents of the
side-arm into the main compartment and the amount of oxygen
consumed was monitored as described in the instruction manual
(Gilson Medical Electronics). The incubation temperature was
30°C and the gas phase air. The number of moles or g atoms
of oxygen consumed per mole of substrate was determined as
shown in APPENDIX II.

Incubation of Whole Cells with D-[6-^{3}H]4FG

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

Estimation of ^{3}H$_2$O and Tritiated-Carbohydrates

A simple micro-procedure for the estimation of ^{3}H$_2$O and tritiated-carbohydrates ([^{3}H]-carbohydrates) in the various whole cell incubation supernatants and intracellular contents was used as described by Clark (106). .Yellow plastic pipet tips (0.5 cm x 5 cm), designed to fit the Pipetman brand·

adjustable pipet, were plugged at the small end using fine
glass wool. Each pipet tip was filled to a height of about
4 cm with Dowex AG 1x8 resin in the borate form. This was
prepared by washing Dowex AG 1x8 (200-400 mesh) resin in the
chloride form with 1 M potassium tetraborate, pH 8.5-9, until
the effluent was chloride free (about 30 volumes were required).
A subsequent wash with deionized distilled water (10 volumes)
was necessary to bring the pH down to 7.5. The columns were
loaded with 25 μL of 1.mM D-[U-^{14}C]glucose (4.0 mCi/mmol),
1 mM D-[6-^{3}H]4FG (0.3 mCi/mmol), whole cell incubation super
natants or intracellular contents. ^{3}H$_2$O was estimated by
washing the columns with 10 x 0.2 mL aliquots of deionized
distilled water. The effluents were collected directly into
scintillation vials for counting. These columns were found
to retard many carbohydrates and some amino acids, which have
been summarized (106). [^{3}H]-Carbohydrates were then estimated
by washing the columns with 25 x 0.2 mL aliquots of 0.25 M
ammonium tetraborate. The effluents were collected directly
into scintillation vials for counting.

Isolation of Tritiated-Carbohydrates

The supernatants obtained from the 24 h incubations were
combined (about 60 mL). To this was added 0.5 g decolorizing
charcoal and the suspension was stirred for 30 min. The
suspension was filtered and reduced to dryness on a rotary
evaporator. The residue was suspended in 5 mL of deionized
distilled water and then loaded onto a column of the Dowex
AG 1x8 borate resin (106) (1 cm x 10 cm bed height). ^{3}H$_2$O

was removed by washing the column with 20 mL of deionized
distilled water. [^{3}H]-Carbohydrates were eluted by washing
the column with 10 mL of 0.25 M ammonium tetraborate. This
fraction was repeatedly mixed with 10 x 100 mL of methanol
and evaporated to dryness on a rotary evaporator to remove
the volatile methyl borate (107). The pH was checked and
adjusted to neutrality with 1 M HCl and the sample was reduced
to dryness. The residue was suspended in a final volume of
1 mL with deionized distilled water and was analyzed by
chromatography and Fourier transform ^{19}F-NMR. To 0.5 mL
of this suspension was added 1 mL of 1 M HCl and the solution
heated at 100°C for 10 min. After cooling, the solution
was neutralized with 1 M NaOH. The solution was reduced to
dryness on a rotary evaporator and the residue suspended in
0.5 mL with deionized distilled water. The hydrolyzed
product was analyzed by chromatography.

Chromatography

Thin layer chromatography (TLC) was performed using
silica gel G 20cm x 20 cm plastic plates (BDH, Toronto,
Ontario). The plates were developed in ethyl acetate: acetic
acid: water (3:3:1) and the carbohydrates detected by spraying
with a 95% solution of concentrated sulfuric acid in ethanol,
followed by heating at 110°C for 10 min. Radioactivity on
the chromatograms were determined by placing 1 cm x 1 cm
scrapings of the sample lane on the dried chromatogram
into scintillation vials for counting. Reducing sugars were
detected by using aniline hydrogen phthalate (108). Sugar-

phosphates were chromatographed on Whatman ≠ 1 chromatography paper in an ascending manner. The paper was pre-washed with 2 M HCl, dried, washed with distilled water and then dried again. The paper was developed in n-propanol: concentrated ammonia: water (6:3:1) and sugar-phosphates detected by the method of Runeckles and Krotkov (109). The chromatogram was dipped into a bath of acid-$FeCl_3$ in acetone (150 mg $FeCl_3 \cdot 6H_2O$, 3 mL of 0.3 M HCl and 97 mL of acetone), air dried and then dipped into a bath of 1.25% sulfosalicylic acid in acetone. Sugar-phosphates appeared on the dry chromatogram as white spots in a red-brown field.

Column Chromatography

Column chromatography was performed on a Sephadex G-100 column (0.7 cm x 48 cm bed height). The resin was pre-equilibrated with 0.1 M sodium phosphate, pH 7.2,-0.1% SDS-0.02% sodium azide buffer. 150 µL (500 µg protein, 6,000 dpm) of the radiolabeled cell envelope suspension or 150 µL (150 µg protein, 4,000 dpm) of the radiolabeled peptidoglycan-associated protein complex suspension were applied onto the column and fractions eluted with the same buffer. The samples were heated at 55°C for 30 min prior to application onto the column. The flow rate was set at 1.2 mL h^{-1} and 0.6 mL fractions were collected on a Gilson model FC-80K micro-fractionator (Gilson Medical Electronics Inc., Middleton, Wisconsin) at room temperature. Fractions were then determined for radioactivity and protein (absorbance at 280 nm). The molecular weight of the radiolabeled protein was estimated

by using bovine albumin and lysozyme as molecular weight

markers (APPENDIX IV). The void volume (V_o) was estimated

with the use of blue dextran. Fractions containing the

highest radioactivities were pooled, lyophilized and the

residue suspended in a small amount of distilled water for

electrophoretic analysis.

Urea-SDS-Polyacrylamide Gel Electrophoresis

Urea-SDS-PAGE was performed essentially by the method

of Mizushima and Yamada (110). Gels were prepared by mixing

in final concentrations; 8% acrylamide, 0.2% bis-acrylamide,

0.2% SDS, 8 M urea and 0.1 M sodium phosphate buffer, pH 7.2,

in a total volume of 40 mL. The polymerization of the gel

was initiated by addition of 60 mg of ammonium persulfate

and 24 μL of TEMED. The solution was quickly poured into

0.6 cm x 16 cm glass tubes to a height of about 12 cm and

overlaid with a small amount of water to form a smooth flat

surface. The solution was enough to make about 10 gels

and polymerization occurred within 15 min. Samples were

prepared by dissolving 50-100 μg of protein in 100 μL of 1%

SDS-2% β-mercaptoethanol solution, followed by heating at

100°C for 5 min and addition of urea (8 M final concentration).

To this was added 10 μL of 0.5% bromophenol blue and 50 or

100 μL of the solution applied onto each gel. Therefore,

about 50 μg of protein were usually applied onto each gel.

This was overlaid with 0.1 M sodium phosphate buffer, pH 7.2.

Electrophoresis was performed in a 15-tube capacity electro-

phoresis cell with the anode in the lower chamber and cathode

in the upper chamber. Electrophoresis was carried out at
4 mA per gel for 30 min at room temperature in 0.1 M sodium
phosphate, pH 7.2, 0.1% SDS buffer. Then the current was
increased to 6 mA per gel and electrophoresis continued for
5 h until the bromophenol blue had migrated to about 3/4
of the gel. This distance was marked with black drafting ink.

Gels were stained for protein with Coomassie brillant
blue (R-250) or for glycoproteins by the method of Fairbanks
et al. (111). In staining for protein the following protocol
was used; (1) 25% isopropyl alcohol, 10% acetic acid, 0.05%
coomassie blue, overnight; (2) 10% isopropyl alcohol, 10%
acetic acid, 0.005% coomassie blue, 6 h, (3) 10% acetic acid
with 0.0025% or less coomassie blue, overnight, and (4) 10%
acetic acid for several days until the background is clear.
The protocol for staining for glycoproteins was as follows; (1)
0.5% periodic acid, 2 h, (2) 0.5% sodium arsenite, 5% acetic
acid, 1 h, (3) 0.1% sodium arsenite, 5% acetic acid, 3 x 20
min, (4) 5% acetic acid, 10 min, (5) 10 mL of Schiff reagent
(111) per gel, overnight, and (6) 0.1% sodium metabisulfite,
3 x 1 h. In staining for glycoproteins about 200 μg of
protein were usually applied onto each gel. Gels were
scanned on a model 4310 scanning densitometer (Ortex Inc.;
Oak Ridge, Tennessee) or photographed.

For radioactivity determinations, the gels were sliced
into 0.5 cm sections and placed into scintillation vials.
200 μL of H_2O_2 were added to the gel slices which were then
incubated at 55°C for 24 h. After digestion of the gel slices,

the solutions were subjected to radioactivity counting. The
molecular weight of the protein bands were estimated from a
standard curve prepared with known molecular weight proteins
(APPENDIX V).

Preparation of 2K4FGA and 4FGA

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

Liquid Scintillation Counting

Radioactivity was measured on a Beckman LS 7500 liquid
scintillation counter (Beckman Instruments Inc., Fullerton,
California). The liquid scintillation cocktail was prepared
as follows; 5.5 g of PPO, 0.1 g of POPOP, 333 mL of Triton
X-100 and 667 mL of scintillation grade toluene. In all cases,
10 mL of the liquid scintillation cocktail was added to the
various samples for radioactivity determinations. Quench
correction curves (APPENDIX VI and VII) were prepared from

quenched standards (Nuclear Chicago) using the H-number

concept of quench correction (see manufacturer's manual for

the theoretical discussion on this concept).

Assay of Enzyme Activities

The method used for assaying the activities of glucose,

gluconate and succinate dehydrogenases was based on the

procedure described by Mizuno and Kageyama (86). Reaction

mixtures contained 100 mM potassium phosphate, pH 7.1, 10 mM

KCN, 10 μg of PMS, 20 μg of DCIP and 50-100 μg protein of

outer or cytoplasmic membrane fractions. All reactions were

initiated by addition of 100 mM (final concentration) glucose,

gluconate or succinate in a total volume of 1.0 mL. The

enzyme activities were measured by the decrease in absorbance

at 600 nm for DCIP at $25^{\circ}C$ on a Beckman model 35 spectro-

photometer (Beckman Instruments Inc., Fullerton, California).

The activities were calculated using an extinction coefficient

for DCIP of 10 mM^{-1} cm^{-1}.

Protein Determination

Protein determinations were by the method of Lowry, et al.

(112) with bovine serum albumin as a standard. Before the

determination of protein in whole cell suspensions, the

whole cells were diluted to an absorbance of about 1.0 at

600 nm. This is in the range of about 1.0-1.2 mg of protein

mL^{-1}. For column eluates, protein was estimated by measuring

the absorbance at 280 nm.

Estimation of 4FG in Whole Cell Incubation Supernatants

The removal of 4FG from the supernatant of whole cell

incubation mixtures was monitored with the use of the Statzyme

Glucose (500.nm) reagent. 3.0 mL aliquots of this reagent

were incubated at 30°C for 10 min. To these were added

200 μL of the various whole cell incubation supernatants and

the solutions incubated for an additional 2 h. After incubation

100 μL of 4 M HCl was added to stop the reaction and stabilize

the color. The absorbance of the solutions were then determined

at 500 nm. 4FG was estimated from a standard curve produced

using various concentrations of 4FG (APPENDIX VIII).

Kinetic Treatment of Data

K_m and V_{max} values were obtained from double reciprocal

or Lineweaver-Burk plots (113) and were estimated by linear

regression of the kinetic data (APPENDIX IX). The K_m and

V_{max} values are the average of duplicate trials.

Fourier Transform ^{19}F-NMR

^{19}F-NMR was performed on a Bruker pulse NMR spectro-

meter CXP-100 in the Fourier transform mode. Samples were

examined for up to 50,000 scans. 0.1 M trifluoroacetic acid

and 0.1 M NaF were used as standards.

CHAPTER III

RESULTS AND DISCUSSION

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

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

66

Table 3

Fluoride Release from 4FG by Glucose Grown Whole

Cells of P. putida.*

[4FG] (mM)	v(nmol fluoride mg protein^{-1} min^{-1})	% fluoride released after 24 h
0.5	0.09	100
1.0	0.18	100
2.0	0.30	95
2.5	0.38	95
5.0	0.54	88
10.0	0.60	59

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

Figure 9

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

Legend:

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

Apparent K_m = 3.9 mM

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

Figure 9
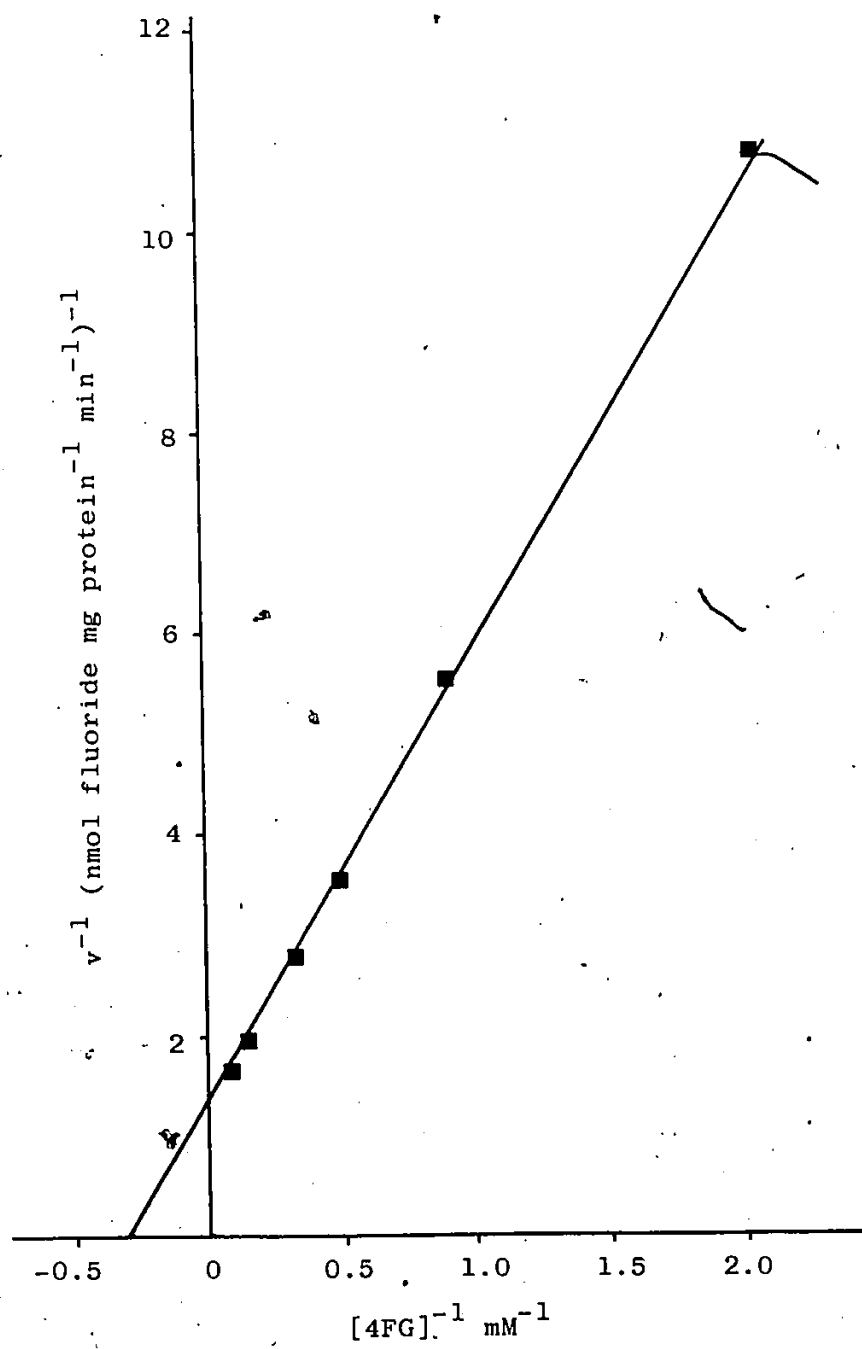
No oxygen consumption, above the endogenous level, could be detected in whole cells given 4FG (0.5-2.5 mM) as a substrate, in accord with preliminary studies (61). On the other hand, glucose (0.5-2.5 mM) is oxidized extensively (2.7-3.4 mol oxygen mol glucose^{-1}). Figure 10 illustrates a typical result obtained with 2.5 mM glucose or 4FG as substrates. Unlike 3FG (61), therfore, 4FG is not oxidized by glucose grown whole cells of P. putida, instead an extensive release of fluoride occurs.

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

Figure 10

Oxidation of D-Glucose and 4FG by Glucose Grown Whole Cells

of P. putida.

Legend:

    ▲ - oxidation of 2.5 mM glucose

    ■ - oxidation of 2.5 mM 4FG

Each Gilson flask contained:

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

The reaction was initiated by tipping the contents of the

side-arm into the main compartment. Temperature, 30°C;

reaction volume, 2 mL; gas phase, air.

Figure 10
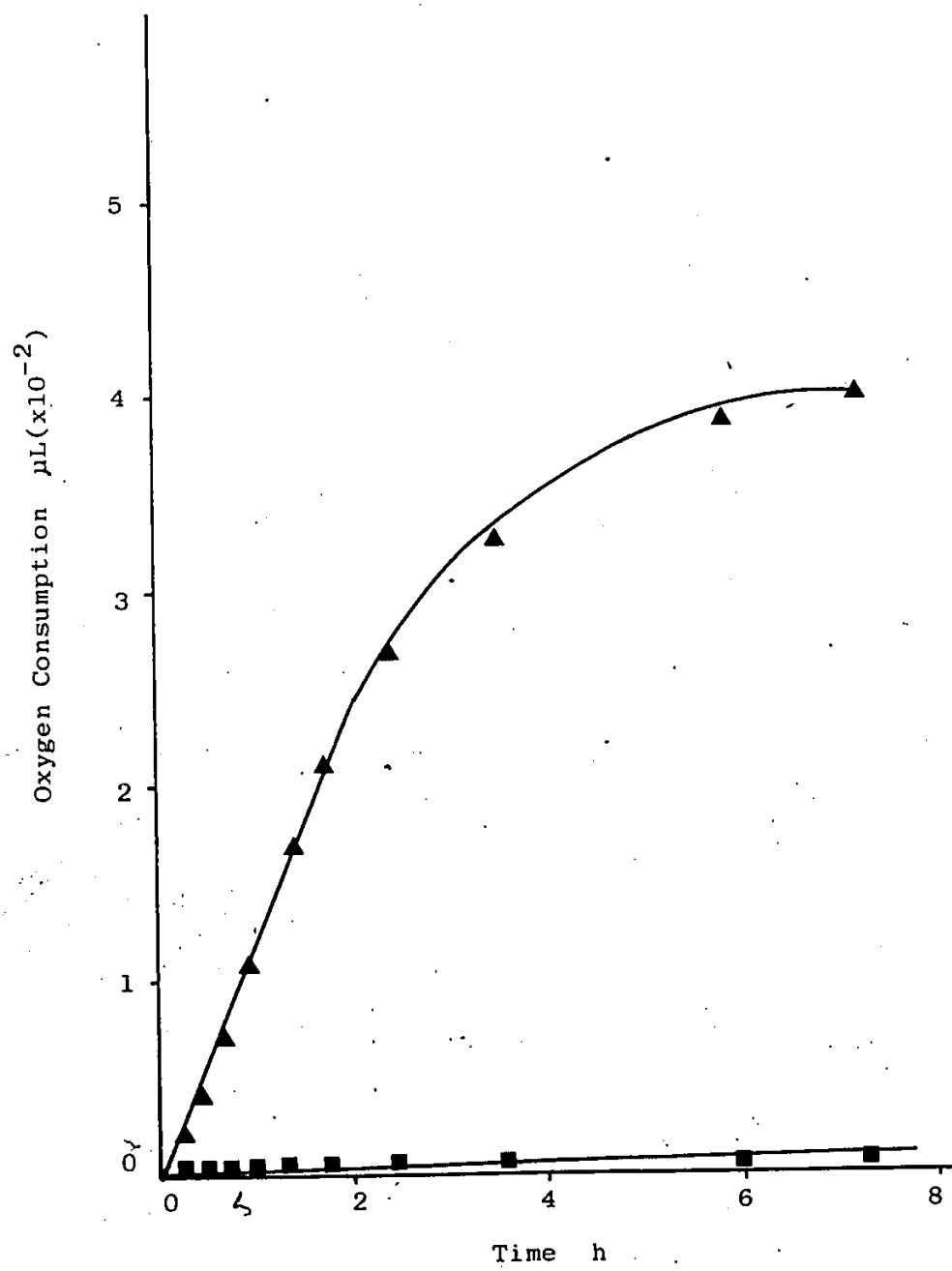
Table 4

The Rates and Extents of D-Glucose and 4FG Oxidation in

Cell-Free Extracts and Cytoplasmic Membrane Vesicles.

Substrate oxidized	v(nmol oxygen mg protein^{-1} min^{-1})		Net oxygen consumption (endogenous subtracted)		mol oxygen· mol substrate oxidized^{-1}	
	1	2	1	2	1	2
4FG (1 μmol)	3.6	2.7	23	21	1.05	0.95
4FG (2 μmol)	6.3	5.7	45	48	1.0	1.07
4FG (5 μmol)	11.0	11.7	108	118	0.90	1.05
4FG (10 μmol)	20.5	21.2	203	212	0.90	0.95
4FG (20 μmol)	nd	45.0	nd	424	nd	0.95
glucose (5 μmol)	50.1	91.5	125	118	1.1	1.05
glucose (10 μmol)	95.6	177.0	214	223	0.96	0.99

nd - not determined; 1 - cell-free extracts; 2 - vesicles.
The preparation of cell-free extracts and cytoplasmic
membrane vesicles and the respirometric studies were as
described in the MATERIALS AND METHODS section. The rates
and extents of substrate oxidation were determined as
outlined in APPENDIX II.

of the rates of 4FG oxidation against the concentration of
4FG in cytoplasmic membrane vesicles and cell-free extracts,
respectively (Figures 11 and 12). In comparison, apparent
K_m values of 0.8 and 22 mM and apparent V_{max} values of
160 and 105 nmol oxygen mg protein^{-1} min^{-1} have been pre-
viously determined for glucose and 3FG oxidation, respec-
tively, in cytoplasmic membrane vesicles (99). These kinetic
values reflect a combination of the various kinetic steps
involved in the oxidation reactions.

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

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

Figure 11

Double Reciprocal Plot of 4FG Oxidation by Cytoplasmic

Membrane Vesicles Prepared from Glucose Grown Whole Cells

of P. putida.

Legend:

Each Gilson flask contained:

    main compartment;   2 mg vesicle protein in 100 mM
                            potassium phosphate buffer, pH 7.1,
                            up to 1.5 mL.

    side-arm;   0.5 mL 4FG solution (0.5-10 mM final
                            concentration).

    centre well;   0.2 mL of 20% KOH and paper wick.

The reaction was initiated by tipping the contents of the

side-arm into the main compartment. Temperature, $30^{\circ}C$;

reaction volume, 2 mL; gas phase, air. The K_m and V_{max} values

were estimated by linear regression of the kinetic data

and are the average of duplicate trials.

Apparent K_m = 21 mM

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

Figure 11

Y-axis: v^{-1} (nmol oxygen mg protein^{-1} min^{-1}(x10^2))$^{-1}$

X-axis: [4FG]$^{-1}$ mM^{-1}
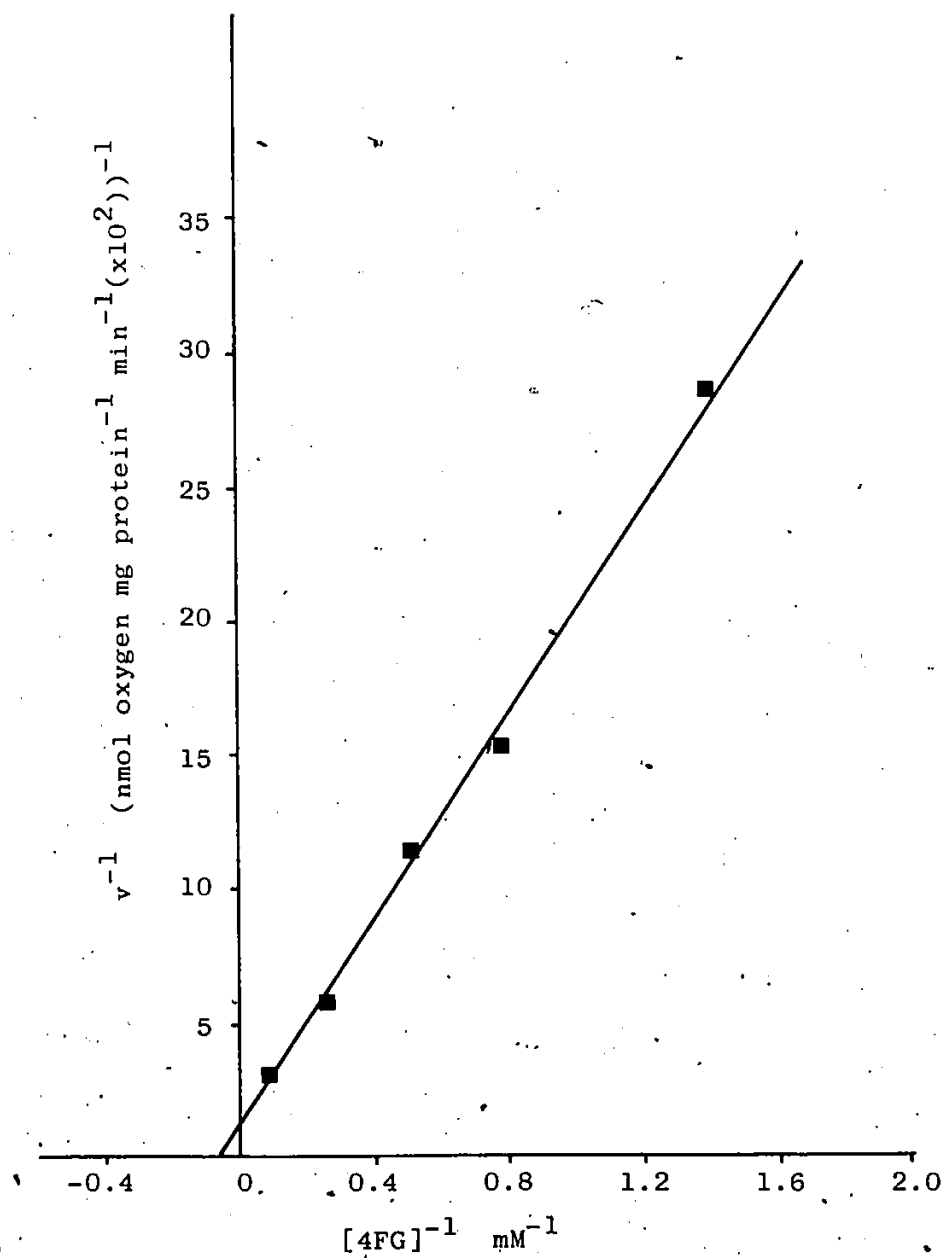
Figure 12

Double Reciprocal Plot of 4FG Oxidation by Cell-Free Extracts

Prepared from Glucose Grown Whole Cells of P. putida.

Legend:

Each Gilson flask contained:

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

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

Apparent K_m = 18 mM

Apparent V_{max} = 117 nmol oxygen mg protein^{-1} min^{-1}

Figure 12
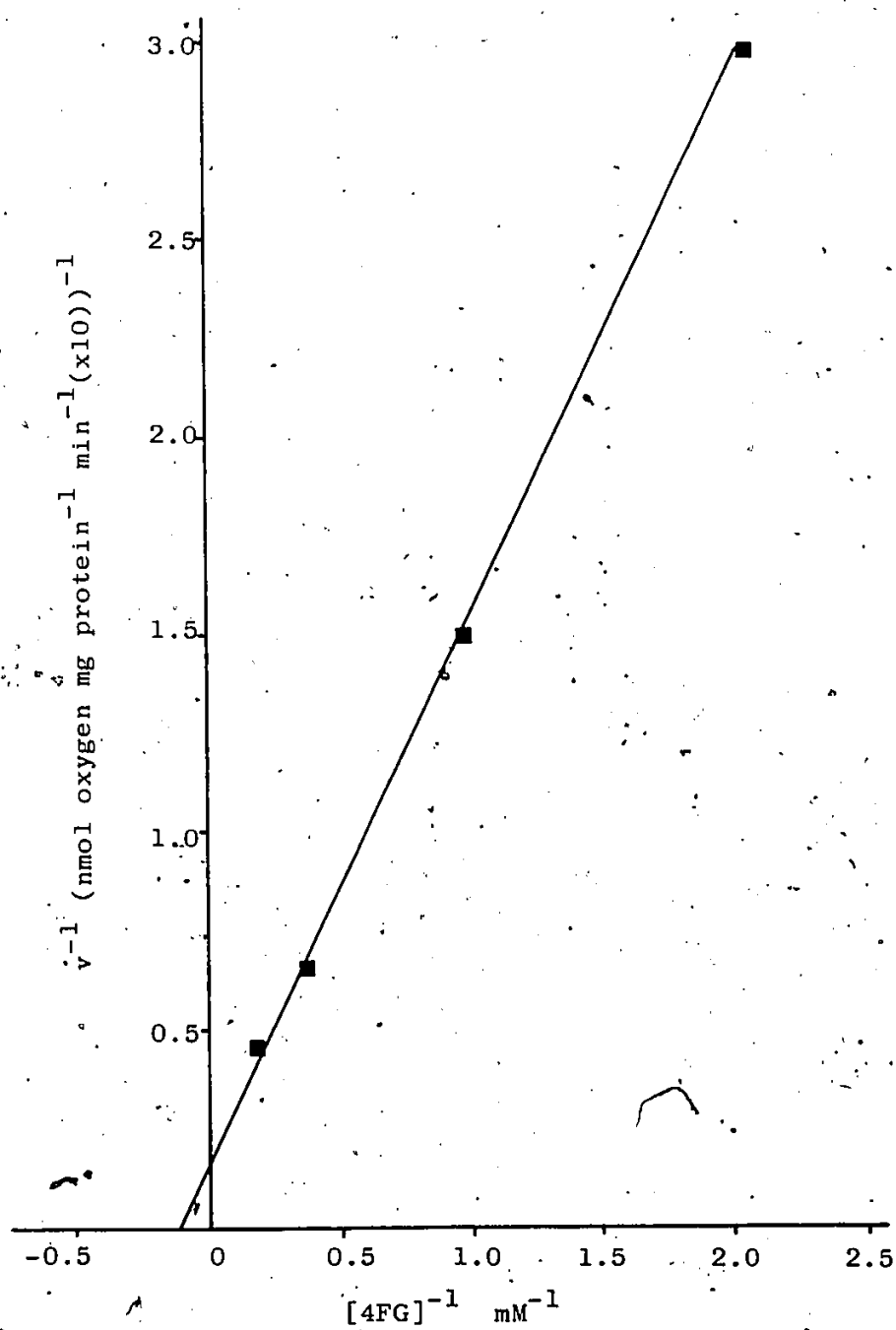
Table 5

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

[Glucose] (mM)	v(nmol fluoride mg protein^{-1} min^{-1})	
	1 mM 4FG	2.5 mM 4FG
0	0.25	0.43
0.5	0.14	0.29
1.0	0.09	0.14
2.5	0.06	0.08
5.0	0.05	0.06

*Whole cell suspensions (18 mg protein) of glucose grown
P. putida were incubated at 30°C in 100 mM potassium
phosphate buffer with 1 or 2.5 mM 4FG + varying
concentrations of glucose (0-5.0 mM) and in a total
volume of 3 mL. Fluoride determinations were as described
in the MATERIALS AND METHODS section.

reduced, there is no effect on the extents of fluoride release. Once glucose has been metabolized by the cells, fluoride release continues as in the control cells (Figure 13). These results suggest that the defluorination reaction involves the interaction or binding of 4FG to the same site as glucose in the whole cell glucose transport system. Furthermore, as would be anticipated, these cells have a preference for glucose over 4FG, presumably in the initial binding step.

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

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

Figure 13

The Effect of D-Glucose on Fluoride Release from 4FG in

Glucose Grown Whole Cells of P. putida.

Legend:

    ■ - 2.5 mM 4FG

    ▲ - 2.5 mM 4FG + 5 mM glucose

Whole cell suspensions (20 mg protein) of glucose grown

P. putida were incubated with 2.5 mM 4FG or 2.5 mM 4FG +

5 mM glucose in 100 mM potassium phosphate buffer, pH 7.1,

at 30°C and in a total volume of 3 mL. Fluoride determin-

ations were as described in the MATERIALS AND METHODS

section.

Figure 13

Time h
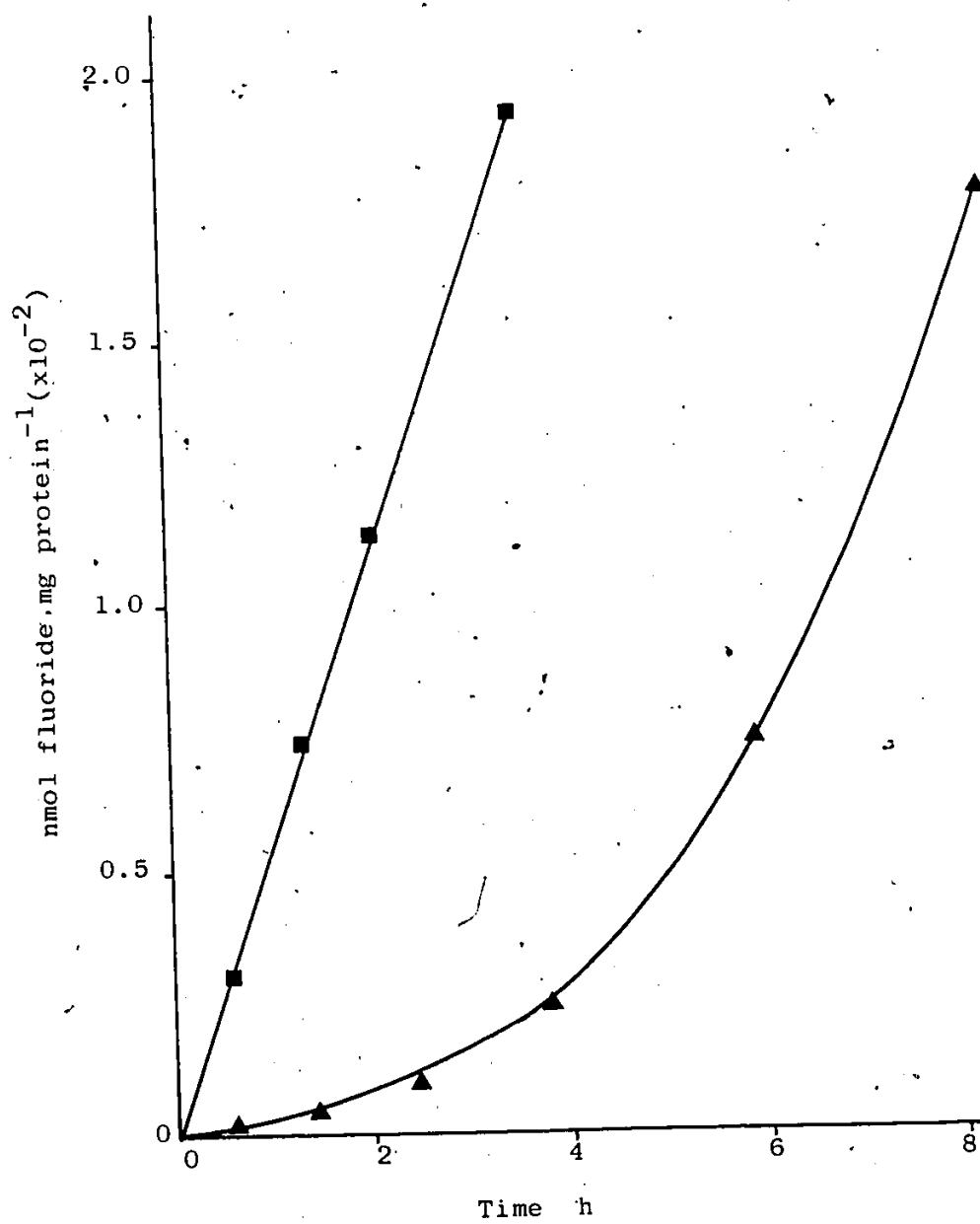
Table 6

The Effect of Various Sugars on Fluoride Release from 4FG

in Glucose Grown Whole Cells of P. putida.*

2.5 mM 4FG + 5 mM sugar added to incubate	% Inhibition of fluoride release after	
	30 min	60 min
L-Glucose	0	0
α-Me4FG	0	0
D-Galactose	0	0
2-Deoxy-D-glucose	57	55
D-Glucose	81	91
4-Deoxy-D-glucose	30	28
D-Gluconate	90	90
2-Keto-D-gluconate	90	87
α-MeG	0	0
β-MeG	0	0
Maltose	0	0
6-Deoxy-D-glucose	73	74
Fructose	0	0
Sorbitol	0	0
β-D-Thioglucose	0	0
5-Thioglucose	0	0

*Whole cell suspensions (15-20 mg protein) of glucose grown
P. putida were incubated at 30°C in 100 mM potassium
phosphate buffer, pH 7.1, with 2.5 mM 4FG + 5 mM of the
various sugars, in a total volume of 3 mL. Fluoride deter-
minations were as described in the MATERIALS AND METHODS
section.

C-1 position of 4FG in the defluorination reaction. Alter-
natively, is the realization that these glycosides have a
much lower binding affinity for the glucose transport system
and, therefore, presumably the defluorinating protein, than
glucose and possibly 4FG. It was found that α-MeG had a
1000-fold lower affinity for the glucose transport system
than glucose in P. aeruginosa (98). Furthermore, glucose
transport was only weakly inhibited in the presence of 100-
fold excess of α-MeG. An alternative explanation may be
that these glycosides are taken up by a transport system
different from glucose. This is supported by the evidence
for an independent transport system for α-MeG in P. putida (99).
The lack of inhibition of fluoride release by maltose and
β-D-thioglucose may be explained in a similar manner. Further-
more, maltose is not used as a carbon source by P. aeruginosa
(95) and, therefore, presumably P. putida.

2-Deoxy- and 6-deoxy-D-glucose were found to significantly
inhibit fluoride release from 4FG, indicating the relative
unimportance of the C-2 and C-6 positions of 4FG in the
defluorination reaction. As might be anticipated 4-deoxy-
D-glucose is a poor inhibitor, confirming the important
stereo-specificity and hydrogen bonding requirements at the
C-4 position for the binding of sugars with the D-gluco-
configuration as well as 4FG to the protein. The importance
of the C-4 position is also supported by the lack of inhi-
bition of fluoride release by D-galactose and by the inability
of whole cells to defluorinate 3FG (61). Galactose is not

used as a source of carbon for growth and energy by P. aeruginosa (95). Furthermore, galactose does not inhibit glucose binding to GBP at a 10-fold excess, although it does at a 100-fold excess (67). The lack of inhibition of fluoride release from 4FG by galactose may indicate a poor affinity, if any, of galactose for the defluorinating protein. The lack of inhibition of fluoride release by sorbitol and fructose supports the requirement for the ring structure of substrates in the D-gluco-configuration for the initial binding. Furthermore, fructose has recently been shown to be transported into P. aeruginosa by a phosphoenolpyruvate dependent transport system (115). The lack of inhibition of fluoride release by 5-thioglucose suggests the importance of the ring oxygen in the initial binding.

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

Incubation of glucose grown whole cell suspensions of P. putida with 2.5 mM 4FG and 10 mM NEM, iodoacetate or

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

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

Figure 14

The Effect on Fluoride Release from 4FG by Growth of

P. putida on Various Carbon Sources.

Legend:

      ■ - glucose grown cells

      ▲ - gluconate grown cells

      ● - 2-ketogluconate grown cells

      △ - succinate grown cells

      □ - citrate grown cells

Cells were grown on appropriate carbon sources as described

in the MATERIALS AND METHODS section. Whole cell suspensions

(15 mg protein) were incubated with 2.5 mM 4FG in 100 mM

potassium phosphate buffer, pH 7.1, at 30°C and in a

total volume of 3 mL. Fluoride determinations were as

described in the MATERIALS AND METHODS section.

Figure 14
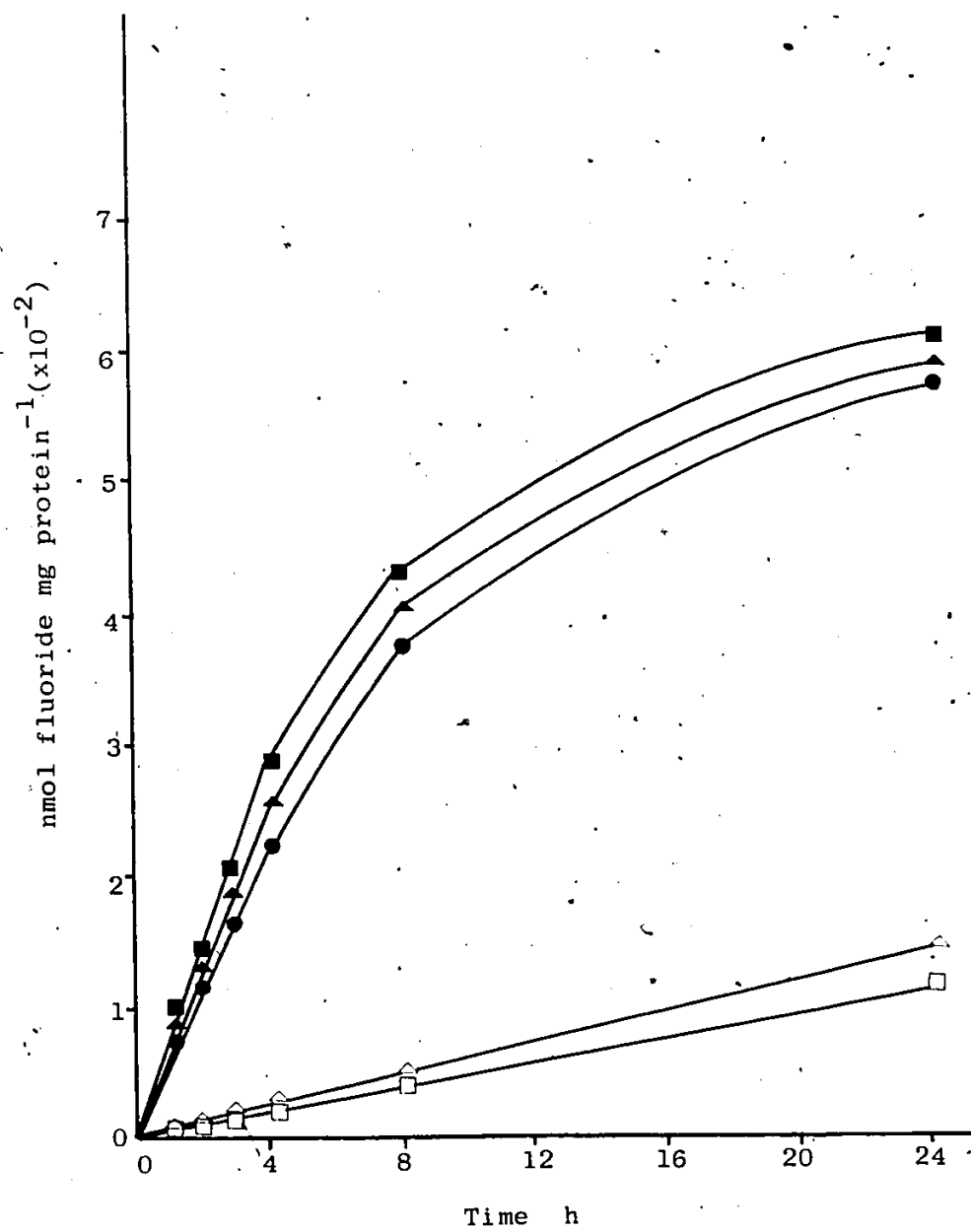
responsible for the defluorination reaction is repressed. Growth of cells on succinate or citrate results in a greater than 90% reduction in the rate of fluoride release.

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

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

Figure 15

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

Legend:

●.- cells pre-incubated with 2.5 mM glucose for 4 h

▲ - cells pre-incubated with 2.5 mM 4FG for 4 h

■ - cells pre-incubared in buffer for 4 h

.Whole cell suspensions (15 mg protein) of succinate grown
P. putida were pre-incubated with 2.5 mM glucose or 4FG
in 100 mM potassium phosphate buffer, pH 7.1, at 30°C for
4 h and in a total volume of 4 mL. Cells were then washed
with and suspended in buffer to the same protein concen-
tration and incubated with 2.5 mM 4FG. Fluoride determin-
ations were as described in the MATERIALS AND METHODS
section.

Figure 15
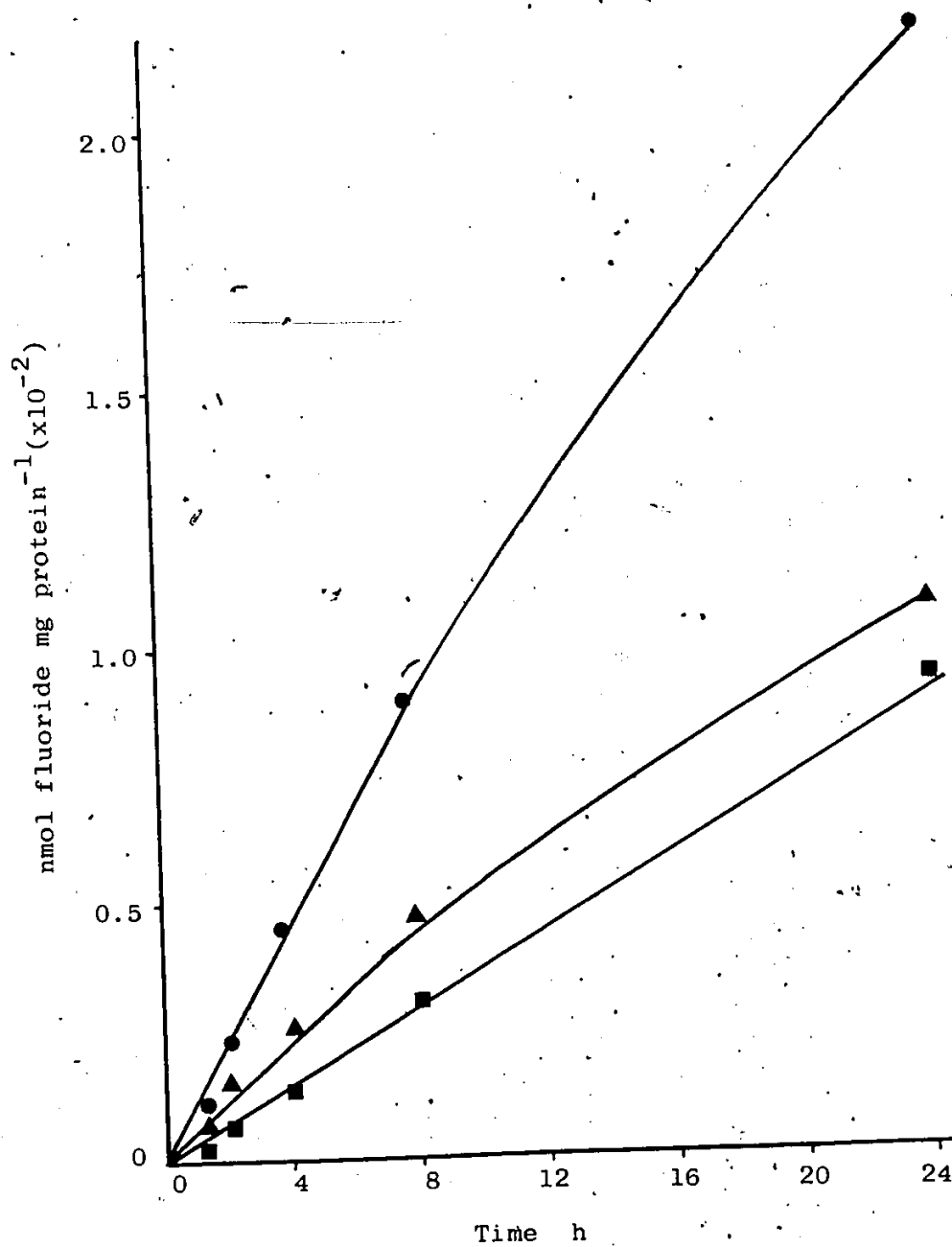
Table 7

Fluoride Release from Various Fluoro-Sugars in Glucose

Grown Whole Cells of P. putida.*

Fluoro-sugar (2.5 mM)	v(nmol fluoride mg protein^{-1} min^{-1})	% Fluoride released (after 24 h)
4FG	0.65	95
4FGA	0.65	95
2K4FGA	0.65	95
α-Me4FG	<0.01	<1
3FG (61)	<0.01	<1

*Whole cell suspensions (18 mg protein) of glucose grown

P. putida were incubated at 30°C in 100 mM potassium

phosphate buffer, pH 7.1, with various fluoro-sugars

(2.5 mM) and in a total volume of 3 mL. 4FGA and 2K4FGA

were prepared by incubating 4FG with cytoplasmic membrane

vesicles obtained from succinate and glucose grown cells,

respectively, as described in the MATERIALS AND METHODS

section.

together with the fact that glucose, gluconate and 2-keto-
gluconate inhibit the defluorination reaction provides
evidence that there is a common binding protein for these
substrates. These results provide the first indication that
P. putida, and presumably other pseudomonads, possess a
protein which will bind these sugars with similar affinities.

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

Further support for the presence and synthesis of protein
responsible for the defluorination reaction is provided when
glucose or succinate grown cells are pre-incubated with
chloramphenicol (1 mg mL^{-1}) for 1 h at 30°C prior to their

exposure to 4FG (Figure 16). The extents of fluoride

release after 24 h were found to be 90% and 18% in the

glucose and succinate grown cells, respectively. On the

other hand, the extents of fluoride release were found to be

11% and 2% in the glucose and succinate grown chloram-

phenicol treated cells, respectively. Similar results

were obtained with rifampicin, although chloramphenicol

was found to be more effective in reducing the extent of

fluoride release. The amount of fluoride released in glucose

grown chloramphenicol treated cells presumably reflects the

amount of endogenous protein present which reacts with 4FG.

Fluoride release continues until all the endogenous proteins

are saturated and then defluorination stops. Fluoride release

above this level, as in the glucose grown untreated cells,

is presumably the result of protein synthesis.

To estimate the maximum amount of fluoride release from

4FG, a fixed amount of glucose grown chloramphenicol treated

and untreated cells were incubated with various concentrations

of 4FG for 24 h. As demonstrated in Figure 17, the extents

of fluoride release in the untreated cells is virtually

proportional to the concentration of 4FG. There is about

90% or more fluoride release with concentrations of 4FG of

5 mM or less. On the other hand, up to 1.25 mM 4FG results

in the production of 133 nmol fluoride mg protein^{-1} in the

chloramphenicol treated cells. This value remains constant

with increasing concentrations of 4FG. Furthermore, more

than 90% fluoride release is achieved with concentrations of

Figure 16

The Effect of Chloramphenicol on Fluoride Release from 4FG

by Glucose and Succinate Grown Whole Cells of P. putida.

Legend:

■ - glucose grown cells

❏ - glucose grown chloramphenicol pre-treated cells

▲ - succinate grown cells

△ - succinate grown chloramphenicol pre-treated cells

Cells were pre-treated with chloramphenicol as described

in the MATERIALS AND METHODS section. Whole cell suspen-

sions (24 mg protein) of glucose or succinate grown P.

putida pre-incubated in the presence or absence of chlor-

amphenicol (1 mg mL^{-1}) for 1 h at 30°C were exposed to

2.5 mM 4FG. The suspensions were incubated in 100 mM

potassium phosphate buffer, pH 7.1, at 30°C and in a total

volume of 4 mL. Fluoride determinations were made as

described in the MATERIALS AND METHODS section.

Figure 16
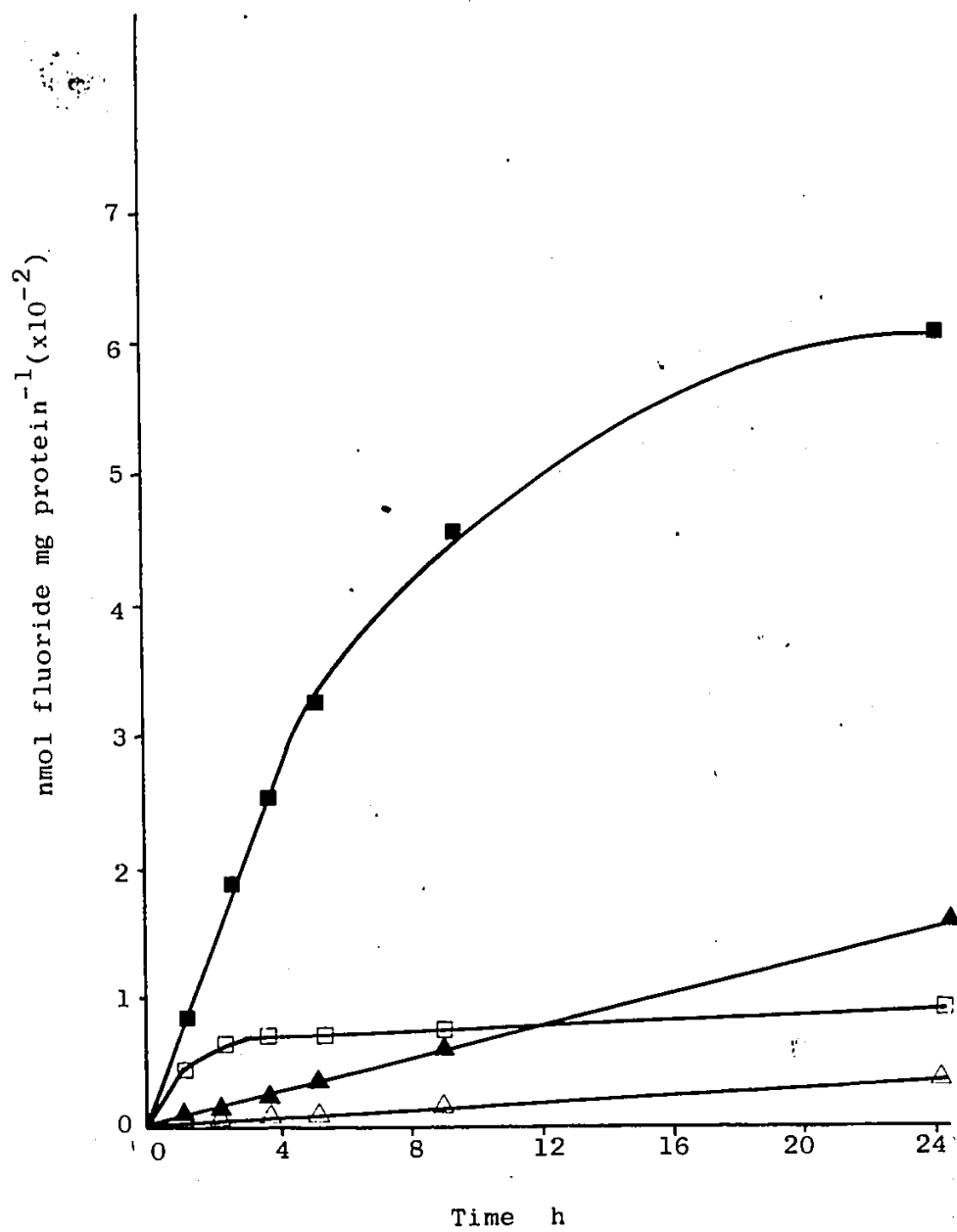
Figure 17

The Extents of Fluoride Release from 4FG Against the

Concentration of 4FG for Glucose Grown and Glucose Grown

Chloramphenicol Pre-Treated Whole Cells of P. putida.

Legend:

■ – glucose grown cells

□ – glucose grown chloramphenicol pre-treated cells

Whole cell suspensions (24 mg protein) of glucose grown

P. putida pre-incubated in the absence or presence of

chloramphenicol (1 mg mL^{-1}) for 1 h at $30^{\circ}C$ were exposed

to various concentrations of 4FG (0.1-5 mM). The suspen-

sions were incubated in 100 mM potassium phosphate buffer,

pH 7.1, at $30^{\circ}C$ and in a total volume of 4 mL. Fluoride

determinations were as described in the MATERIALS AND

METHODS section. The extent of fluoride release was

determined after 24 h incubation.

Figure 17

[4FG]   mM
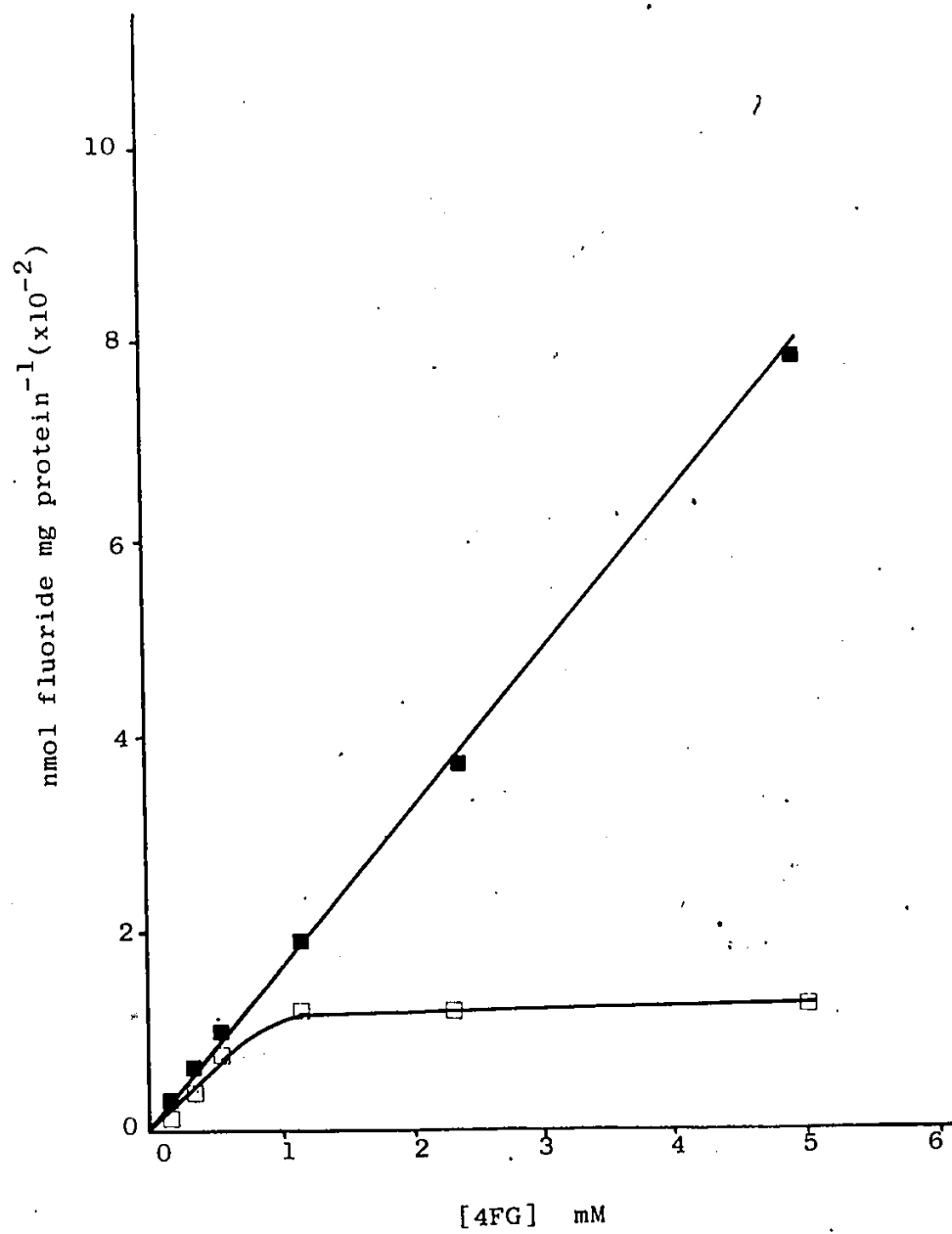
only 1 mM 4FG or less. The 133 nmol fluoride mg protein^{-1}
value presumably reflects the amount of endogenous protein
initially present in the cells which can react with 4FG.
It can be calculated (APPENDIX X) from this value that a
maximum of 24 μg of 4FG has reacted per mg of whole cell
protein. Fluoride release above this value, as in the
untreated cells, is the result of protein synthesis.

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

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

Support for a proposed glycosylation reaction is based
on the fact that no defluorination sugar could be detected
in the supernatants or intracellular contents of cells
incubated with 4FG (61). Thin layer chromatographic analysis

Figure 18

Double Reciprocal Plot of Fluoride Release from 4FG by

Glucose Grown Choramphenicol Pre-Treated Whole Cells of

P. putida.

Legend:

Whole cell suspensions (24 mg protein) of glucose

grown P. putida pre-incubated in the presence of chloram-

phenicol (1 mg mL^{-1}) for 1 h at 30°C were exposed to various

concentrations of 4FG (0.1-5 mM). The suspensions were

incubated in 100 mM potassium phosphate buffer, pH 7.1,

at 30°C and in a total volume of 4 mL. Fluoride determin-

ations were as described in the MATERIALS AND METHODS

section. The K_m and V_{max} values were estimated by linear

regression of the kinetic data and are the average of

duplicate trials.

Apparent K_m = 3.6 mM

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

Figure 18
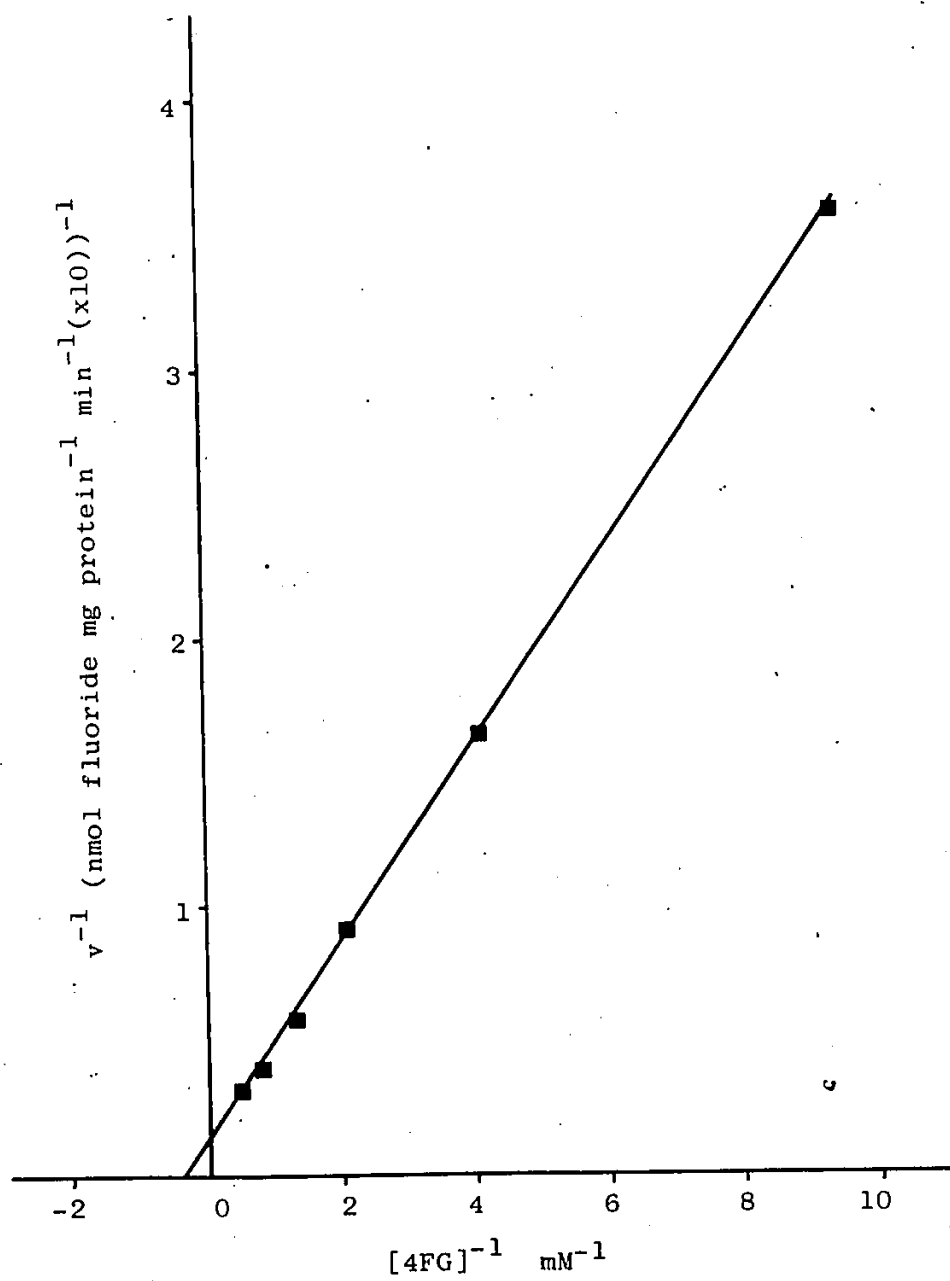
of supernatants from whole cell suspensions incubated with

5 mM 4FG (88% fluoride release after 24 h) failed to detect

any new metabolites. On the other hand, TLC analysis of

supernatants from chloramphenicol treated whole cell

suspensions incubated with 5 mM 4FG (35% fluoride release

after 24 h) demonstrated the presence of one large spot

with an R_F value of 0.42. This is consistent with the

formation of 4FGA and/or 2K4FGA, which would result by non-

specific diffusion of 4FG to the cytoplasmic membrane bound

enzymes.

Figure 19 is a comparison of 4FG removal from various

supernatants and fluoride release from 4FG against time in

glucose and succinate grown cells. This removal of 4FG

from the supernatant may be due to the initial binding of

4FG to a protein. After 30 min incubation, about 86 nmol 4FG

mg protein^{-1} are removed from the supernatant of glucose grown

cells, while 7.4 nmol fluoride mg protein^{-1} are released from

4FG. In succinate grown cells, only 9 nmol 4FG mg protein^{-1}

are removed from the supernatant, while only 0.02 nmol fluoride

mg protein^{-1} are released. As previously observed, these

residual activities in the succinate grown cells demonstrate

the repression of protein. The above results indicate that

the defluorination reaction may in fact be a two step

process. The first step presumably involves an initial

rapid equilibrium binding of 4FG to a protein followed by a

slow elimination of fluoride ion. Direct support for this

initial binding and events leading to the loss of fluoride

Figure 19

Comparison of Fluoride Release from 4FG and 4FG Removal

from the Supernatant by Glucose and Succinate Grown Whole

Cells of P. putida.

Legend:

● - amount of 4FG removed from the supernatant of
    glucose grown cells

○ - amount of fluoride released from 4FG by glucose
    grown cells

■ - amount of 4FG removed from the supernatant of
    succinate grown cells

□ - amount of fluoride released from 4FG by succinate
    grown cells

Whole cell suspensions (20 mg protein) of glucose or succinate

grown P. putida were incubated with 2.5 mM 4FG in 100 mM

potassium phosphate buffer, pH 7.1, at $30^{\circ}C$ and in a total

volume of 3 mL. At 5 min intervals cell suspensions were

centrifuged and fluoride and 4FG were determined in the

supernatants as described in the MATERIALS AND METHODS

section. The results are the average of duplicate trials.

Figure 19
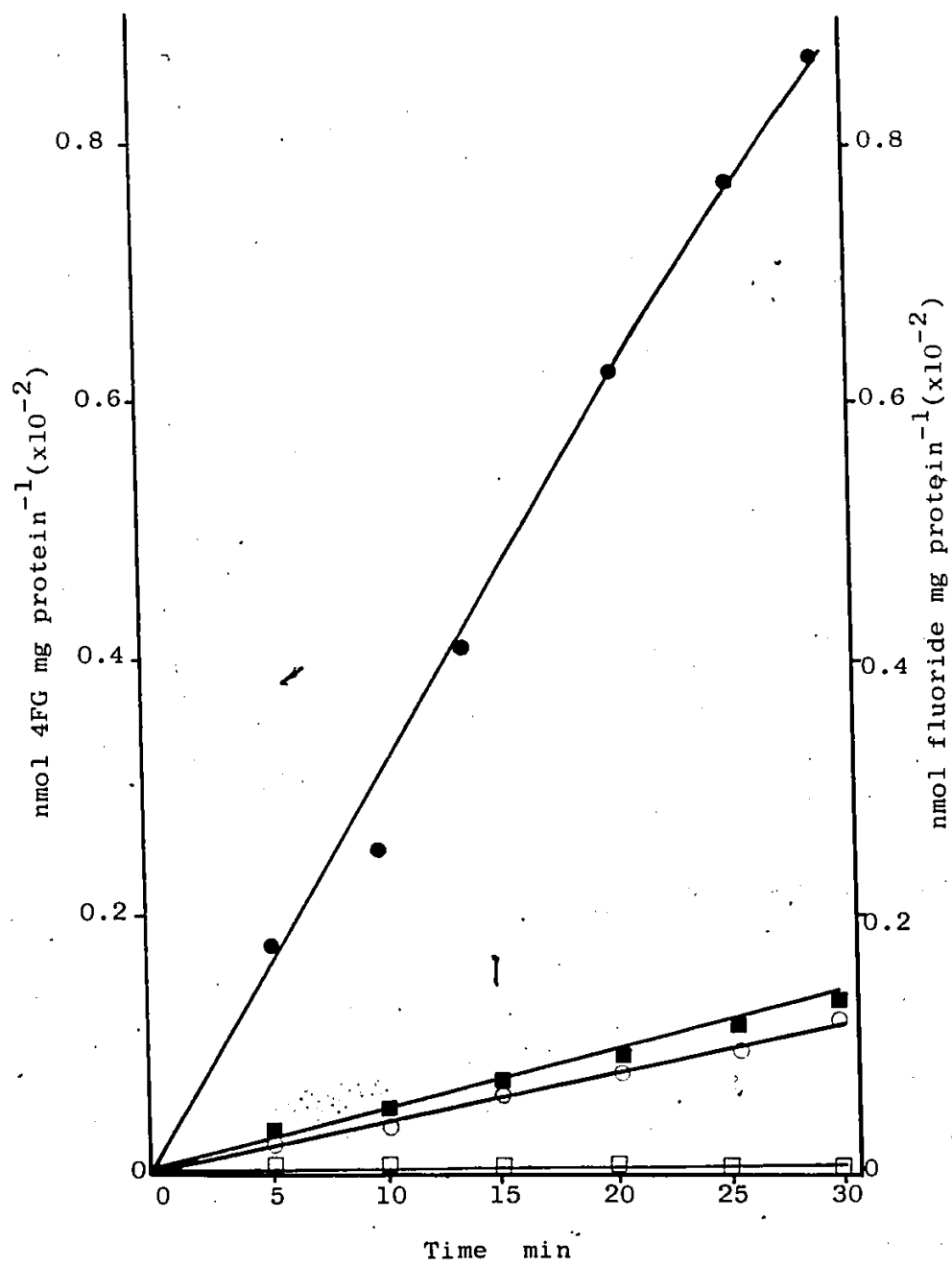
have yet to be deduced.

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

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

Figure 20

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

Legend:

■ – glucose grown cells

● – glucose grown pronase pre-treated cells

▲ – glucose grown pronase pre-treated and washed cells

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

Figure 20
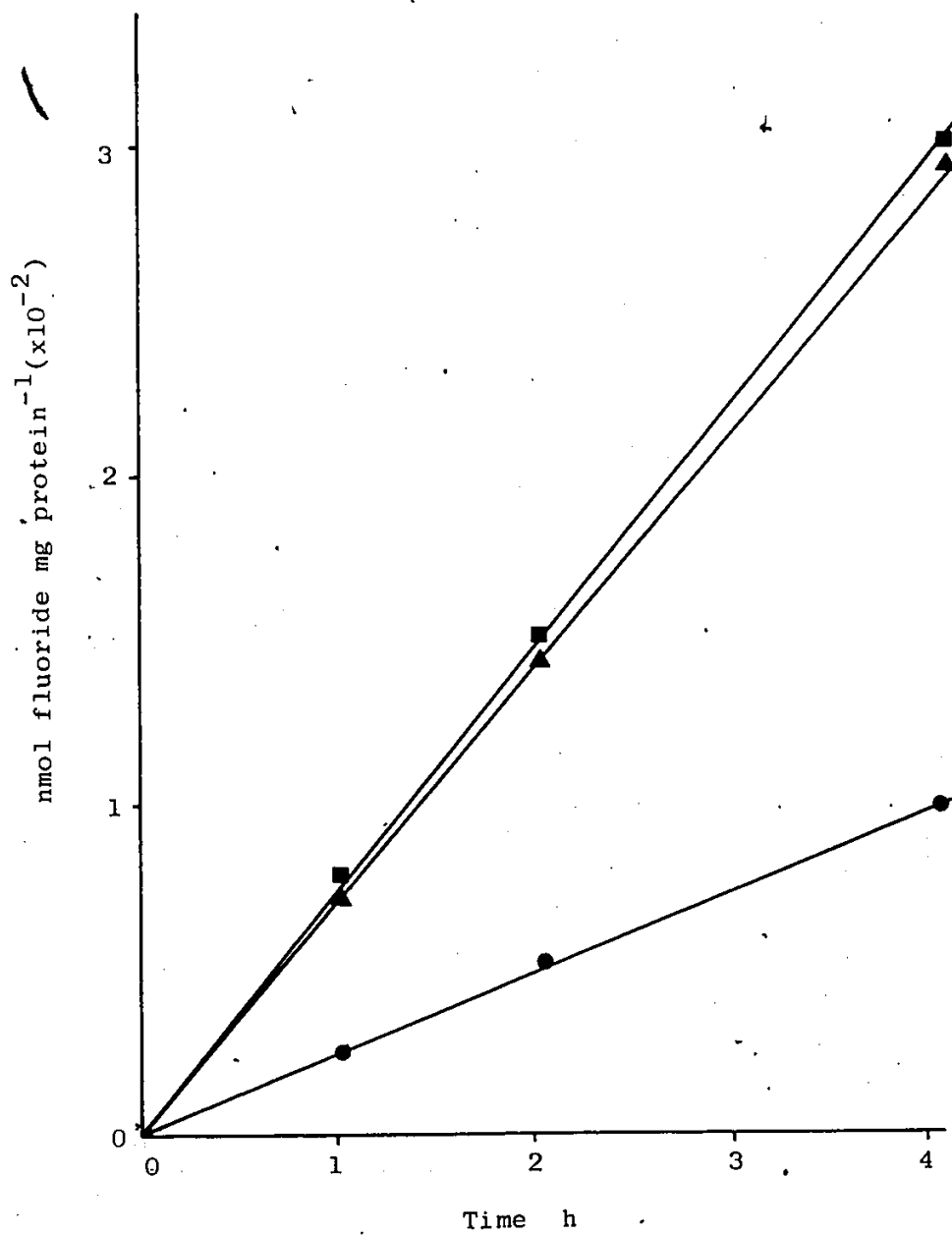
Table 8

Fluoride Release from 4FG by Various Fractions Obtained

from Glucose Grown Whole Cells of P. putida.

Fraction*	nmol fluoride mg protein^{-1} (after 24 h)
Cytoplasmic membrane vesicles	19
Cell-free extract	6
Cell envelopes	22
Spheroplasts	60
Crude shock extract	16
Crude outer membranes	352

*Fractions (2-6 mg protein) in 100 mM potassium phosphate

buffer, pH 7.1, and 1 mM DTT were incubated with 2.5 mM

4FG at 30°C for 24 h, in a total volume of 2 mL. Fluoride

determinations and fraction preparations were as described

in the MATERIALS AND METHODS section. The results are

the average of six trials.

implicated in the defluorination reaction, DTT was added to
the incubates. Although it was later observed that the
absence or presence of DTT did not significantly affect
the levels of fluoride release in the various fractions,
it is still possible that an -SH group is involved.

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

Proteins obtained by the osmotic shock method of Stinson
et al. (67) failed to defluorinate or oxidize 4FG. About
5 mg of protein was obtained from 1 g of wet weight cells.
This crude shock extract was found to have negligible amounts
of glucose binding activity (0.04 nmol glucose mg protein^{-1}).
The value is about 10-fold lower than that determined for
P. aeruginosa (67). The small amount of binding activity
in the crude shock extract may or may not indicate the
presence of GBP. In either case, the shocked cells were not
affected in their ability to defluorinate 4FG. The rates
and extents of fluoride release were virtually identical
to the control cells. It has been previously demonstrated

that P. aeruginosa produces an inducible shock-sensitive

GBP (67). Furthermore, this GBP-associated transport system

is considered to be energized by phosphate-bond energy (68).

Incubation of P. putida with 10 mM sodium cyanide or sodium

arsenate had no effect on the rates or extents of fluoride

release from 4FG, suggesting that the defluorination reaction

is not phosphate-bond energy dependent. The above results

indicate that the defluorinating protein is held tightly

within the cell, presumably at the outer membrane level,

and is not removed by relatively mild procedures.

The outer membrane is preferentially shed during sphero-

plast formation and recovered by high speed centrifugation.

Although this method is convenient, the composition of the

released material seems to be significantly different from

the average composition of the outer membrane (71). The

method of Mizuno and Kageyama (86) involves the use of

lysozyme, EDTA and Tris-HCl buffer in a hypertonic solution

to isolate the outer membrane form P. aeruginosa. In

applying their method to P. putida, cell lysis was observed

in which the absorbance of the suspension at 660 nm (117)

dropped from 0.9 to 0.6 within 60 min. Furthermore, the

suspension became reddish-brown, also indicating the lysis

of cells. EDTA has been found to cause severe damage to

both the outer and cytoplasmic membranes, resulting in cell

lysis (84,87,118,119). EDTA exerts its lytic effect by

extracting divalent cations (i.e., Mg^{++}, Ca^{++}, etc.) which

anchor a complex of protein and lipopolysaccharide in the

outer membrane. Furthermore, the lysis of cells by EDTA-lysozyme is far more rapid in Tris-HCl buffer than any other buffer, since Tris has a weak metal binding property (118). Therefore, the isolation of an outer membrane fraction was attempted in the absence of EDTA and Tris.

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

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

Table 9

Enzyme Activities in the Crude Outer Membranes and

Cytoplasmic Membrane Vesicles.

Enzyme	Specific Activity[a]	
	Crude Outer Membranes	Cytoplasmic Membrane Vesicles
Glucose dehydrogenase	7	440
Gluconate dehydrogenase	4	285
Succinate dehydrogenase	<1	7

[a]Specific activities are expressed as nmol DCIP reduced min^{-1} mg $protein^{-1}$.

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

Figure 21

Comparison of the Major Proteins in the Outer and Cyto-

plasmic Membranes Obtained from Glucose Grown Whole Cells

of P. putida.

Legend:

    A - cytoplasmic membrane vesicles

    B - crude outer membranes

    0 - represents the origin of the gels

Tracings are reproductions of the densitometer scans of

urea-SDS-polyacrylamide gels. Arrows indicate the position

of the tracking dye. Samples were prepared and electo-

phoresis performed as described in the MATERIALS AND

METHODS section. The molecular weight of the protein

bands were estimated from a standard curve (APPENDIX V).

Outer Membrane Protein Band	Molecular Weight
a	86,000
b	55,000
c	45,000
d	40,000
e	22,000
f	16,000

Figure 21

above results are consistent with those obtained for *P.*
aeruginosa (85-89), in which a range of 2-9 proteins were
observed in the various outer membrane preparations. The
correspondence of the proteins obtained from P. putida to
those of P. aeruginosa, if any, have yet to be elucidated.
The number, resolution and order of migration of the major
outer membrane proteins depends on the SDS-gel system used,
the method of outer membrane isolation, strain and culture
conditions (73,90). No bands were observed after PAS-
staining of the gels, which is specific for glycoprotein.
This is in agreement with results obtained for P. aeruginosa (86).

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

The protein distribution in the outer membranes

Figure 22

Comparison of the Major Proteins in the Crude Outer

Membranes Obtained from Glucose and Succinate Grown

Whole Cells of P. putida.

Legend:

A - crude outer membranes obtained from succinate
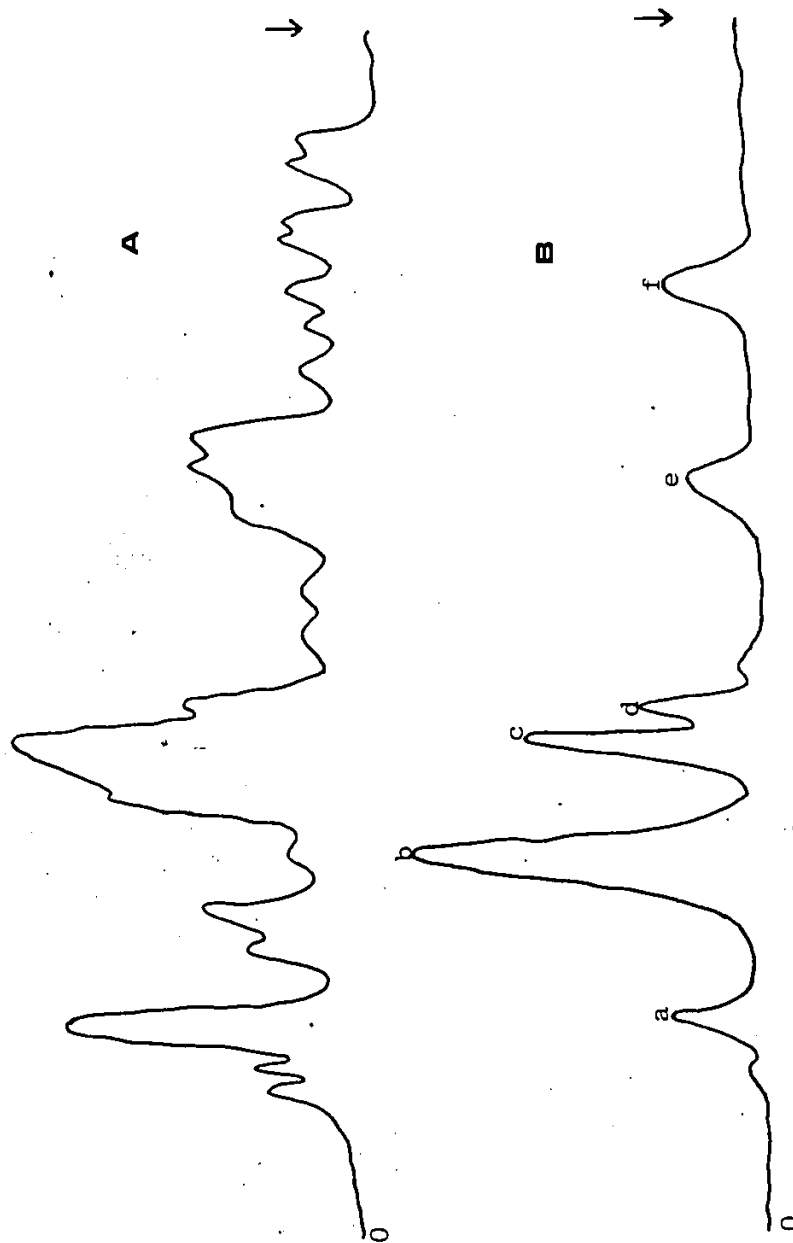   grown cells

B - crude outer membranes obtained from glucose
    grown cells

O - represents the origin of the gels

Tracings are reproductions of the densitometer scans of

urea-SDS-polyacrylamide gels. Arrows indicate the position

of the tracking dye. Samples were prepared and electro-

phoresis performed as described in the MATERIALS AND

METHODS section. The molecular weight of the protein

bands were determined from a standard curve (APPENDIX V).

Outer Membrane Protein Band	Molecular Weight
a	86,000
b	55,000
c	45,000
d	40,000
e	22,000
f	16,000
c'	42,000

Figure 22
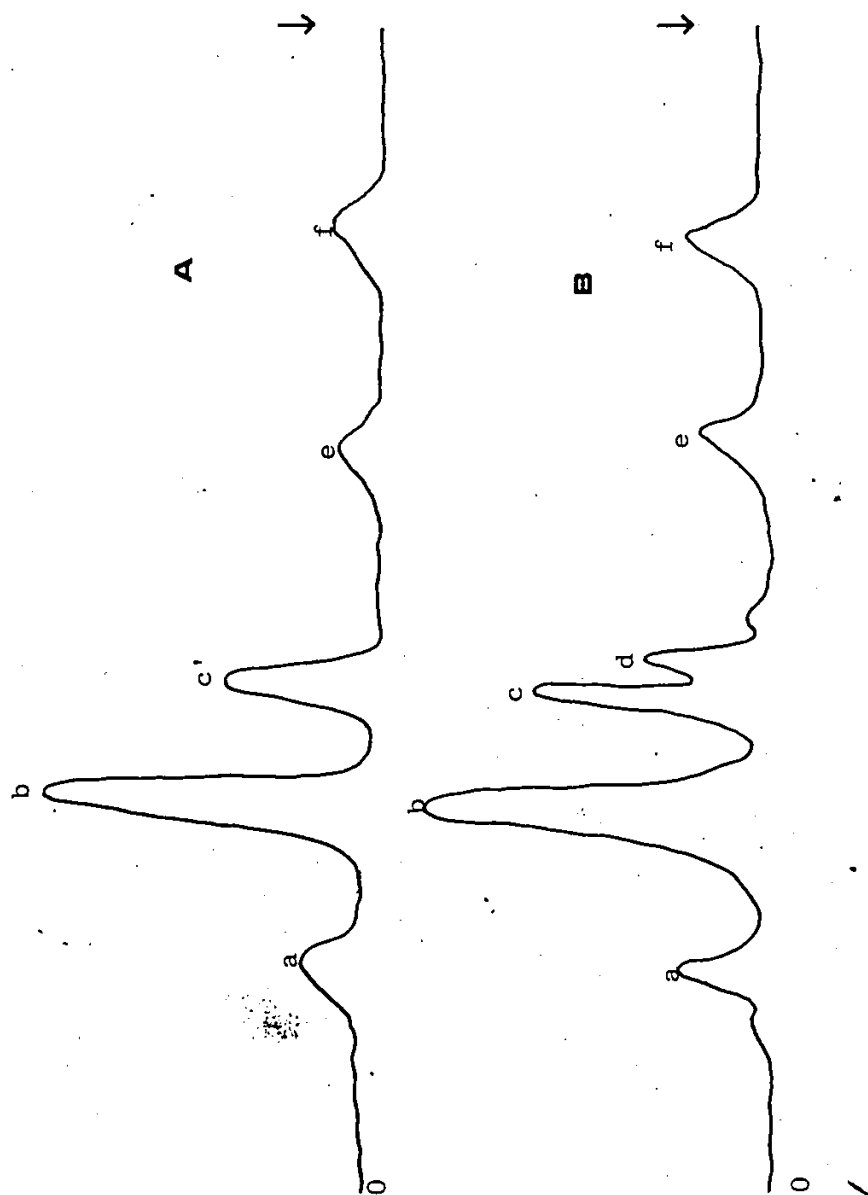
obtained from glucose or succinate grown cells of P. aeruginosa were observed to be about the same (86,90). Proteins D and E, with molecular weights of 50,000 and 45,000, respectively, were induced by growth on glucose, and partially induced by growth on succinate (90). Major differences were only observed when the outer membranes were electrophoresed on gels with concentrations of acrylamide of 14% or greater. Under these conditions protein D was observed as two polypeptides, D1 and D2, of which protein D1 did not appear after growth on succinate (90).

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

Incubation of whole cell suspensions of glucose grown P. putida with 1 mM D-[6-^{3}H]4FG (7.2 mCi/mmol) for 24 h (100% fluoride release) gave an unexpected result. After centrifugation, 70% of the radiolabel used was found in the supernatant with the remainder in the cell pellet. The radiolabeled material in the supernatant was found to be totally dialyzable. After extensive dialysis of the cell

pellet, only about 1% of the total radiolabel used was found to remain cell-associated. If the proposed glycosylation reaction was occurring, it would have been expected that most of the radiolabel would be cell-associated. These results indicate, however, that extensive release of radiolabel may occur as a result of the defluorination reaction.

When the supernatant was reduced to dryness by lyophilization or on a rotary evaporator and the residue suspended to the original volume with distilled water, a 40-50% reduction in the disintegrations per minute (dpm) was observed. Furthermore, a corresponding increase in the dpm was observed in the solvents collected from both processes, indicating the presence of ^3H_2O. To determine more accurately the amount of ^3H_2O present, a borate column (106) was used. This column has been shown to retain a variety of carbohydrates, which are eluted with ammonium tetraborate. Any ^3H_2O would be initially eluted with water (106). Figure 23 illustrates the results of applying 25 µL aliquots of 1 mM D-[U-^{14}C]-glucose, 1 mM D-[6-^{3}H]4FG and supernatant fraction to the borate column. It can be seen that D-[U-^{14}C]glucose and D-[6-^{3}H]4FG were retained by the column and were only eluted with ammonium tetraborate. A large peak corresponding to ^3H_2O was observed by elution of the supernatant fraction with water. Furthermore, a small peak corresponding to a tritiated-carbohydrate(s) ([^{3}H]-carbohydrate) was obtained on elution with ammonium tetraborate. A similar distribution of ^3H_2O and [^{3}H]-carbohydrate was observed in the intra-

Figure 23

Estimation of ^3H_2O and Radiolabeled Carbohydrates on a

Micro-Column of Borate Resin.

Legend:

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

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

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

The preparation of the borate column was as described in

the MATERIALS AND METHODS section. 25 μL of 1 mM D-[U-^{14}C]-

glucose (4.0 mCi/mmol), 1 mM D-[6-^{3}H]4FG (0.3 mCi/mmol) or

supernatant were applied onto the borate column. ^3H_2O

was estimated by elution with 10 x 0.2 mL aliquots of

deionized distilled water. Radiolabeled carbohydrates

were then estimated by elution with 25 x 0.2 mL aliquots

of 0.25 M ammonium tetraborate. Effluents were collected

directly into scintillation vials for radioactivity

determinations. The results are the average of duplicate

trials.

Figure 23

Figure 23
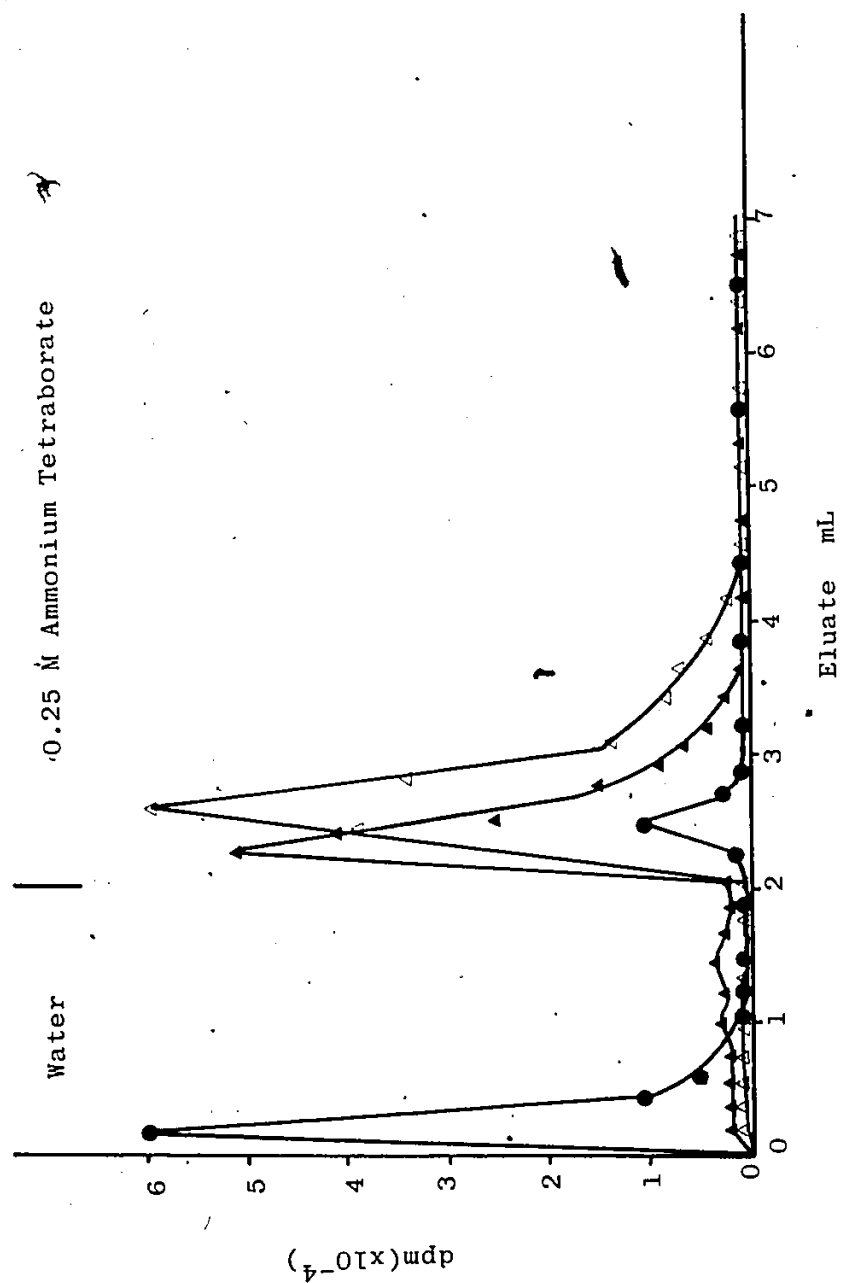
cellular contents prepared from the undialyzed whole cell pellets. Table 10 summarizes the amount and distribution of ^3H_2O and [^3H]-carbohydrate after incubation of whole cells with 1 mM D-[6-^3H]4FG for 24 h. The results indicate that about 67% of the total radiolabel used is released in the form of ^3H_2O. Furthermore, about 20% of the total radio-label used corresponds to a [^3H]-carbohydrate. This distribution of radiolabel may result from the mechanism of fluoride release. The remainder of the radiolabel, except for the 1% that remains cell-associated, may be lost as a result of quenching and/or the assay procedure.

Although the results from the borate column indicated the presence of a [^3H]-carbohydrate in the supernatant, no carbohydrate could be detected by TLC analysis. Concentration of the supernatants, followed by isolation and purification of the radiolabeled material with the use of the borate column, resulted in the appearance of a single radiolabeled spot by TLC analysis (Figure 24). The compound was observed to migrate slower (R_F 0.3) than most of the standard sugars used, indicating a more polar compound. Furthermore, this material gave a positive reaction with aniline hydrogen pthalate (108), indicating that it is a reducing sugar. No fluorine could be detected in the sample by Fourier transform ^{19}F-NMR, consistent with the fact that there was 100% defluorination. Chromatographic detection by the acid-$FeCl_3$-sulfosalicylic acid reagents (109) indicated that the isolated carbohydrate is a sugar-phosphate (Figure 25a).

Table 10

Distribution of Radiolabel from D-[6-^{3}H]4FG in the

Whole Cell Incubation Mixture.*

Fraction	Label present (%)	^3H_2O (%)	[^{3}H]-Carbohydrate (%)
Supernatant	70	50	14
Whole cell pellet[a]	25	17	6
Total	95	67	20

*Whole cell suspensions (24 mg protein) of glucose grown

P. putida in 100 mM potassium phosphate buffer, pH 7.1,

at 30°C were incubated with 1 mM D-[6-^{3}H]4FG (7.2 mCi/mmol),

in a total volume of 4 mL. After 24 h incubation the

suspensions were centrifuged to obtain the whole cell

pellets and supernatants. Results are expressed as per-

centages of the total dpm used.

[a]Results are from undialyzed intracellular contents of

whole cell pellets. 1% of radiolabel was found to remain

cell-bound after extensive dialysis of the whole cell pellets.

Fraction preparations and ^3H_2O and [^{3}H]-carbohydrate

determinations were as described in the MATERIALS AND

METHODS section.

Figure 24

Thin Layer Chromatographic Analysis of the Isolated Radio-
labeled Sample and Various Standard Sugars.

Legend:

S - radiolabeled sample

4FG - 4-deoxy-4-fluoro-D-glucose

GLU - D-glucose

ALL - D-allose

G6P - glucose-6-phosphate

G1P - glucose-1-phosphate

IDO - D-idose

ALT - D-altrose

GAL - D-galactose

2KGA - 2-ketogluconate

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

Figure 24
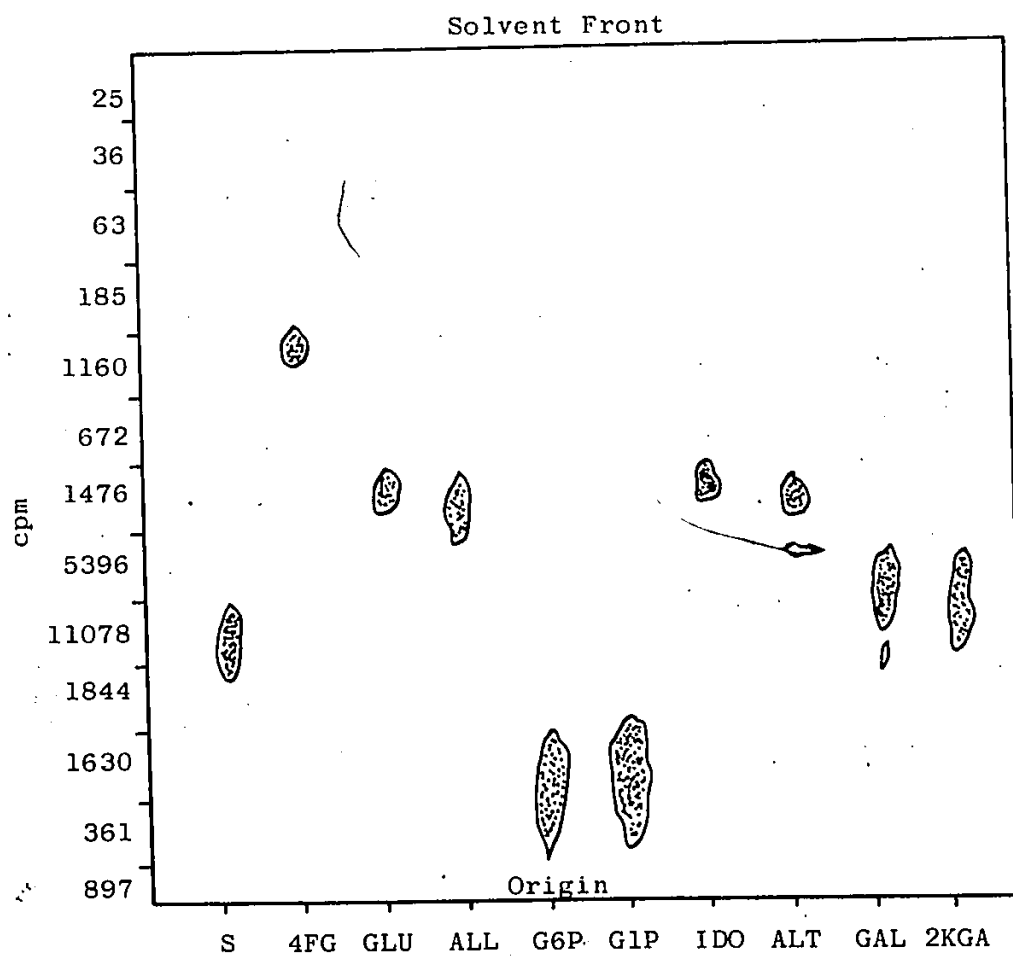
Figure 25a

Chromatographic Analysis of the Isolated Radiolabeled

Carbohydrate by the Sugar-Phosphate Detection Method.

Legend:

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

Sugar-phosphates were detected on Whatman # 1 chromato-

graphy paper as described in the MATERIALS AND METHODS

section. Solvent system; n-propanol: concentrated

ammonia: water (6:3:1).

Figure 25b

Thin Layer Chromatographic Analysis of the Radiolabeled

Sugar-Phosphate, Hydrolysis Product and Various Standard

Sugars.

Legend: .

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

TLC analysis and hydrolysis of the sugar-phosphate was as

described in the MATERIALS AND METHODS section. Solvent

system; ethyl acetate: acetic acid: water (3:3:1). Carbo-

hydrates were detected by sulfuric acid spray.

Figure 25a
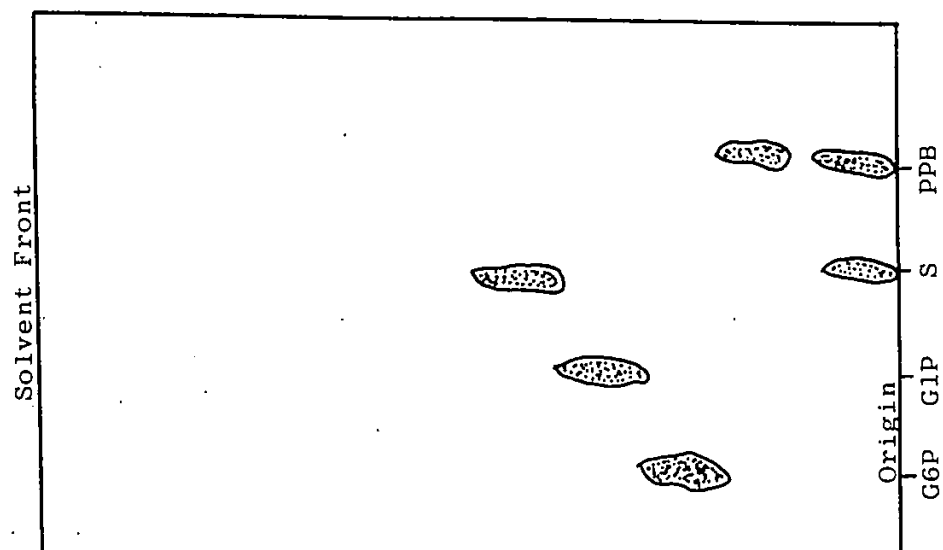
Figure 25b
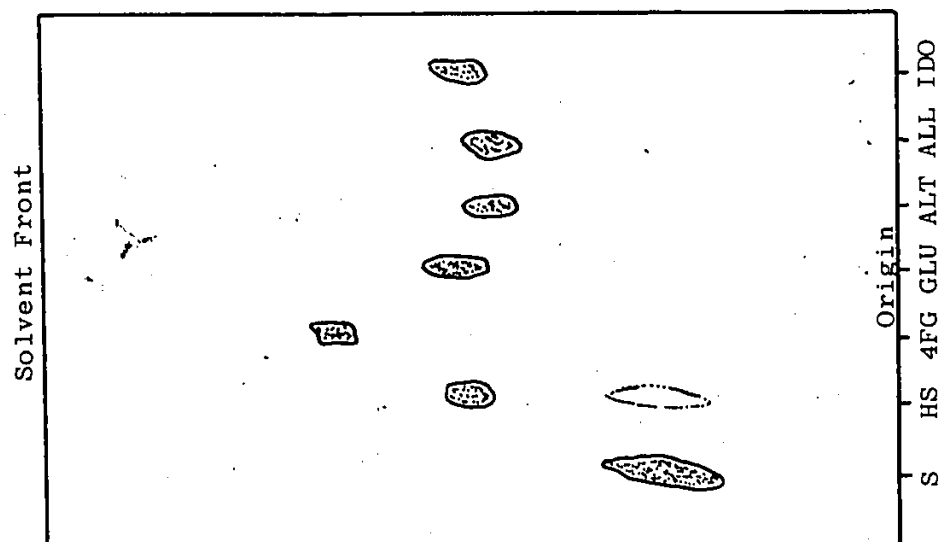
Furthermore, hydrolysis of this sugar-phosphate with 1 M HCl resulted in a new component (R_F 0.5) being produced (Figure 25b). This sugar-phosphate may be formed by diffusion of the defluorinating sugar into the cell and subsequent interaction with a phosphorylating enzyme. Although the identity of the sugar has not yet been deduced, identification may provide evidence for the mechanism of fluoride release.

The amount of ^3H_2O and total [^3H]-carbohydrates in the supernatant and the amount of radiolabel incorporated into the cells were compared to fluoride release from 4FG with respect to time (Figure 26). The formation of ^3H_2O was observed to be virtually linear up to 24 h, although a slight lag is noticeable. On the other hand, the release of fluoride is much more extensive and begins to level off after 4 h. The total [^3H]-carbohydrates, which would be a measurement of both D-[6-^3H]4FG and the defluorination sugar, reaches a minimum value after 4 h and then remains fairly constant. The incorporation of radiolabel into whole cells (presumably by a glycosylation reaction) was found to follow the same profile as the defluorination reaction, reaching a maximum value after 24 h.

Based on the above results, a mechanism for fluoride release, ^3H_2O and [^3H]-carbohydrate formation and protein glycosylation can be proposed (Figure 27). Thus, D-[6-^3H]4FG first binds to a protein (I) (presumably by fluorine-hydrogen bonding) which eventually results in the loss of fluoride. The 4,5-glucopyranoseen (II) may now either

Figure 26

Determination of ^3H_2O, Total Tritiated-Carbohydrates and
Fluoride in the Supernatants and Tritium-Incorporated into
Whole Cells with Respect to Time.

Legend:

     ● - nmol total [^{3}H]-carbohydrates mg protein^{-1}

     △ - nmol ^3H_2O mg protein^{-1}

     ▲ - nmol fluoride mg protein^{-1}

     ■ - nmol [^{3}H]-incorporated into whole cells mg protein^{-1}

Whole cell suspensions (24 mg protein) of glucose grown

P. putida were incubated with 1 mM D-[6-^{3}H]4FG (7.2 mCi/

mmol) for various time intervals in 100 mM potassium

phosphate buffer, pH 7.1, at 30°C and in a total volume

of 3 mL. ^3H_2O, total [^{3}H]-carbohydrates and fluoride in

the supernatants and [^{3}H]-incorporated into whole cells

were determined as described in the MATERIALS AND METHODS

section.

Figure 26
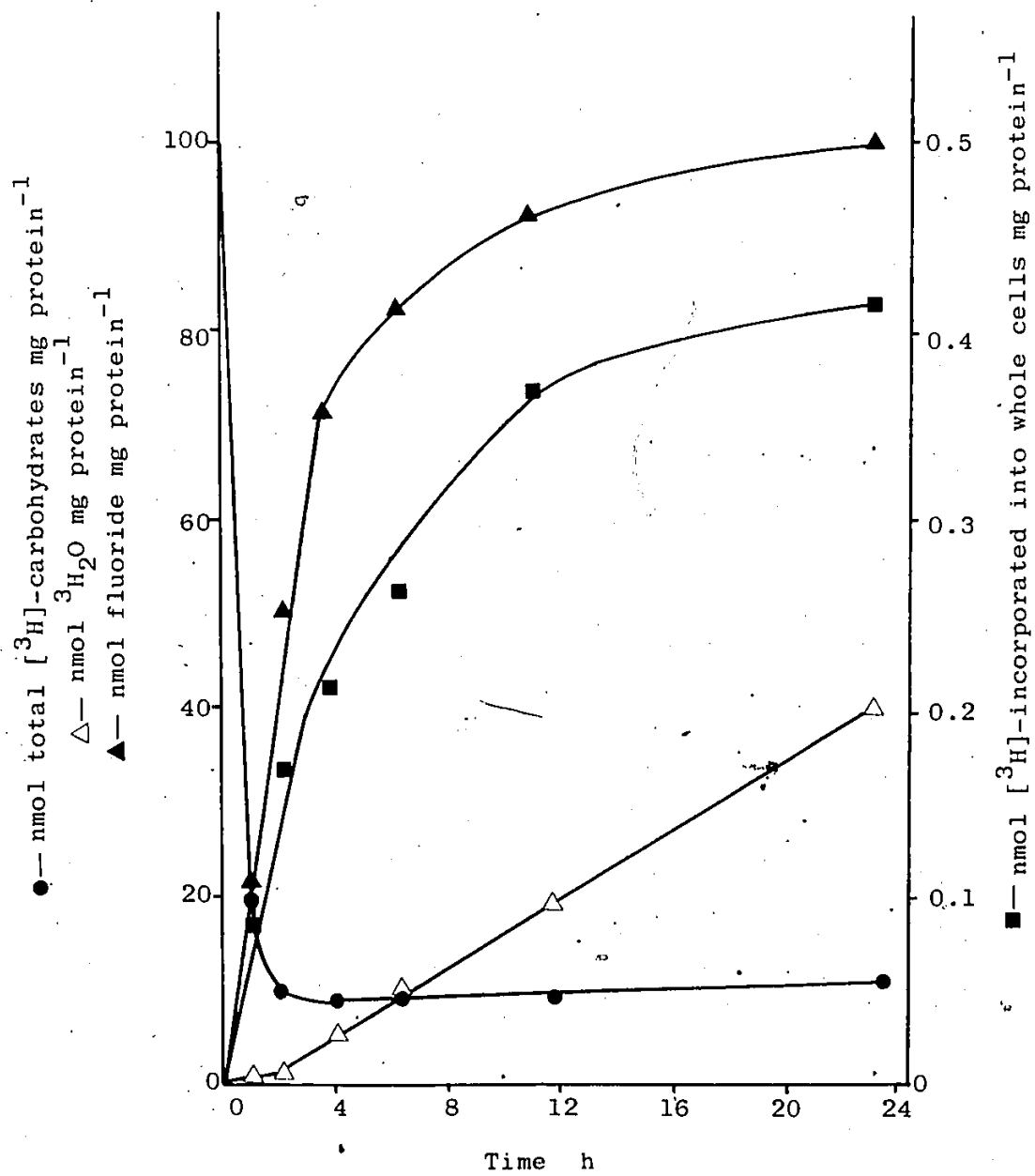
Figure 27

Possible Mechanism of Fluoride Release from D-[6-^{3}H]4FG

and Covalent Attachment of the Defluorination Sugar to

the Protein.

Legend:

HS-●- protein

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

B - ring opening leading to prototropy and
    tritium exchange with water

Figure 27
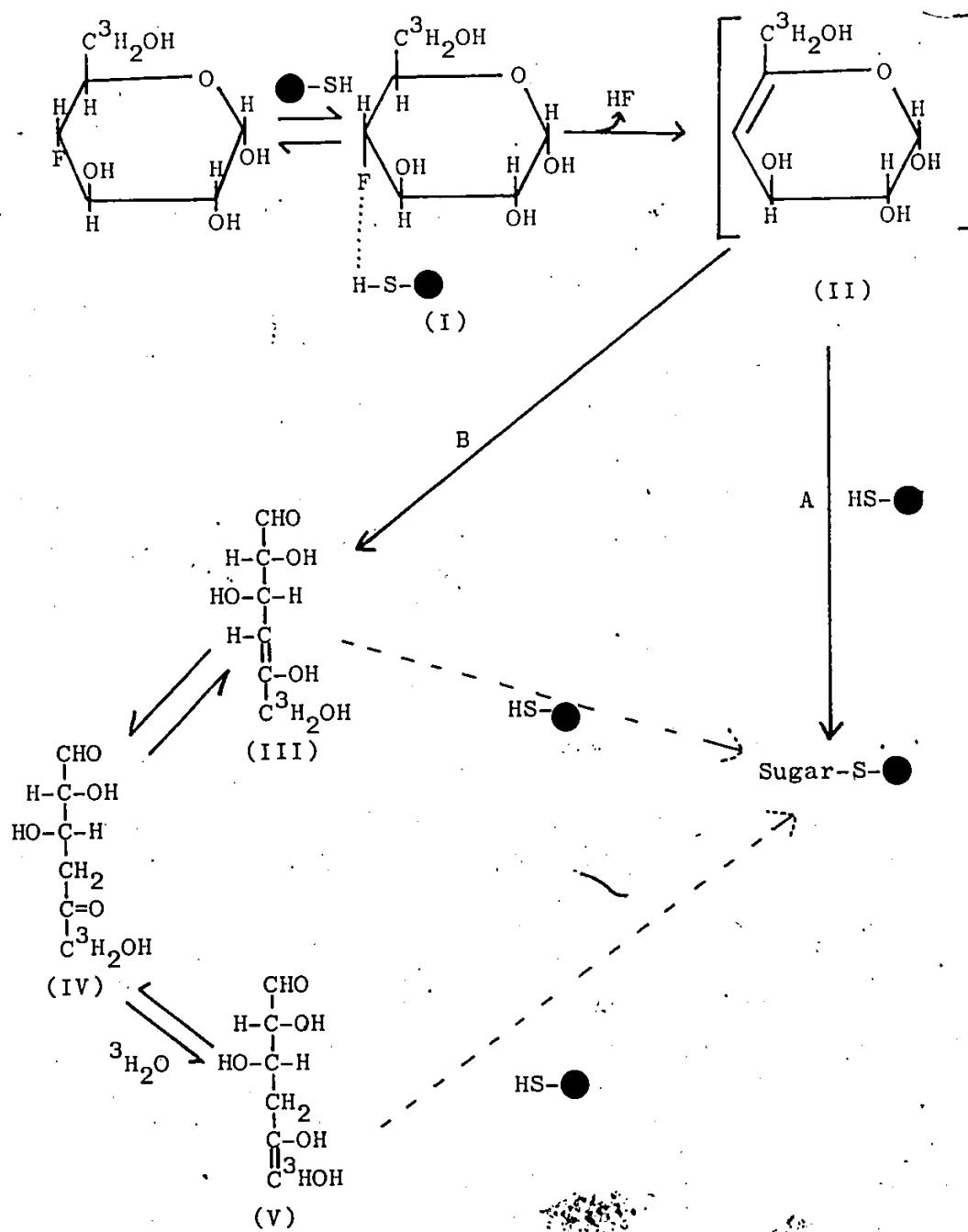
directly react with the -SH group of the protein or in the open chain form (III) undergo prototropy (IV and V) thereby allowing tritium exchange with water to occur. Such a rearrangement may possibly provide alternative substrates (III or V) for nucleophilic attack by the -SH group. The rates and sequence of these reactions will be determined by kinetic factors. In any event the mechanism accounts for fluoride release, the formation of ^3H_2O and $[^3H]$-carbohydrate and suggests that the covalent attachment to the protein may be predominately at the C-4 position of the sugar residue. Recovery of the sugar residue by hydrogenation of the sugar-protein adduct will be required to determine the attachment site(s). To obtain enough sugar-protein adduct for this, $[^{14}C]$-4FG would be required.

In an attempt to isolate the radiolabeled protein(s), dialyzed whole cell pellets containing bound radiolabel were fractionated. As indicated in Table 11, the highest specific activity of radiolabel was obtained in the cell envelope fraction, supporting previous proposals (61). Electrophoretic analysis of the cell envelopes revealed a distribution of about 30 major protein bands. Digestion of 0.5 cm gel slices with H_2O_2 for 24 h at 55°C and subsequent radioactivity determination demonstrated that at least 65% of the radiolabel applied onto the gels was lost. The remainder of the radiolabel was found to be confined within the top 0.5 cm of the gel (Figure 28). Slices of 0.1 cm indicated that the radiolabeled material is at the origin or surface

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Table 11

Distribution of Cell-Bound Radiolabel from D-[6-^{3}H]4FG in

Whole Cells of Glucose Grown P. putida.*

Fraction	nmol radiolabel mg protein^{-1}
Dialyzed whole cells	0.6
Cell-free extracts	0.7
Cell envelopes	2.3
Supernatant of cell envelopes	0.3

*Whole cell suspensions (200 mg protein) of glucose grown

P. putida in 100 mM potassium phosphate buffer, pH 7.1,

were incubated with 1 mM D-[6-^{3}H]4FG (7.2 mCi/mmol) at

30°C, in a total volume of 20 mL. After 24 h the suspensions

were centrifuged and cells fractionated as described in the

MATERIALS AND METHODS section. The amount of radiolabel

bound per mg of protein were determined as outlined in

APPENDIX III. The results are the average of three trials.

Figure 28

Radioactivity Determination in Urea-SDS-Polyacrylamide
Gels of Radiolabeled Cell Envelopes.

Legend:

    A - radiolabeled cell envelopes

Gels were sliced into 0.5 cm sections and each section
placed into scintillation vials. To each vial was added
200 μL of H_2O_2 and the suspensions were then incubated at
55°C for 24 h. 10 mL of liquid scintillation cocktail
was then added to the digested material and radioactivity
determined. Tracing is a reproduction of the densitometer
scans of the urea-SDS-polyacrylamide gels. Gels were
sliced beginning from the origin. The arrows indicate
the position of the tracking dye on the gels. Samples
were prepared and electrophoresis performed as described
in the MATERIALS AND METHODS section. The results are
the average of six trials.

Figure 28

Gel Slice cm

dpm(x10^{-3})
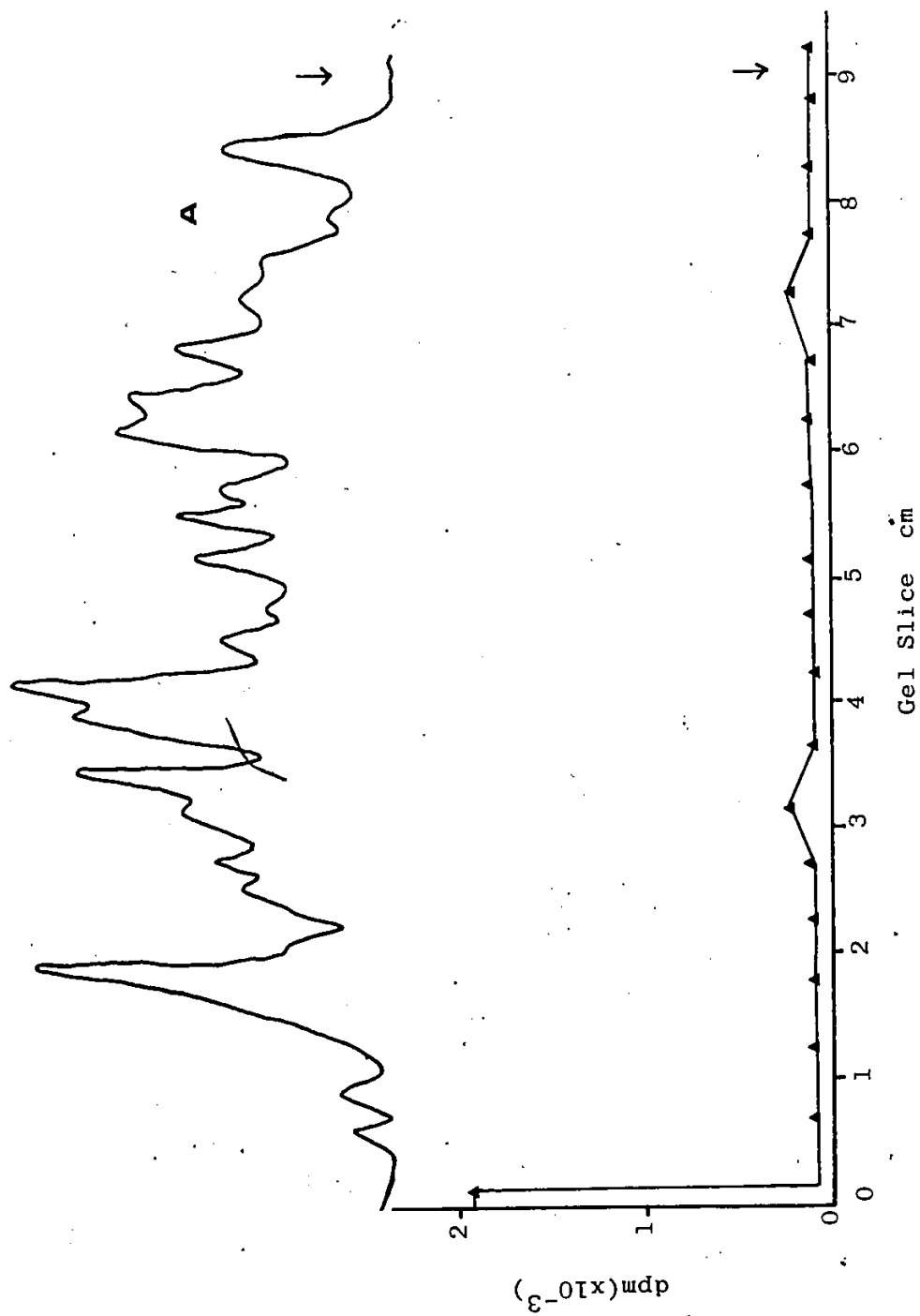
of the gel. This would tend to suggest that the radiolabeled material is a relatively large or uncharged protein. A protein of about 150,000 daltons or more would be expected to remain at the origin in the gel system used (APPENDIX V).

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

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

Figure 29

Elution Profile of Radiolabeled Cell Envelopes on a

Sephadex G-100 Column.

Legend:

BA - bovine albumin (molecular weight of 66,000)

L - lysozyme (molecular weight of 14,300)

V_o - void volume

150 μL of a radiolabeled cell envelope suspension (500 μg

protein, 6,000 dpm) was applied onto a Sephadex G-100

column (0.7 cm x 48 cm bed height). The flow rate was

set at 1.2 mL h^{-1} and 0.6 mL fractions were collected.

0.1 mL was removed from each fraction for the determin-

ation of radioactivity. The remainder of the solutions

were diluted to 1 mL with distilled water and protein

qualitatively measured at an absorbance of 280 nm. The

V_o (6 mL) was determined with blue dextran. Bovine

albumin and lysozyme were used as molecular weight

markers (APPENDIX IV). The radioactive peak corresponds

to a molecular weight of about 38,000. Samples treated with

and gel permeation column chromatography performed in the

presence of SDS as described in the MATERIALS AND METHODS

section.

Figure 29
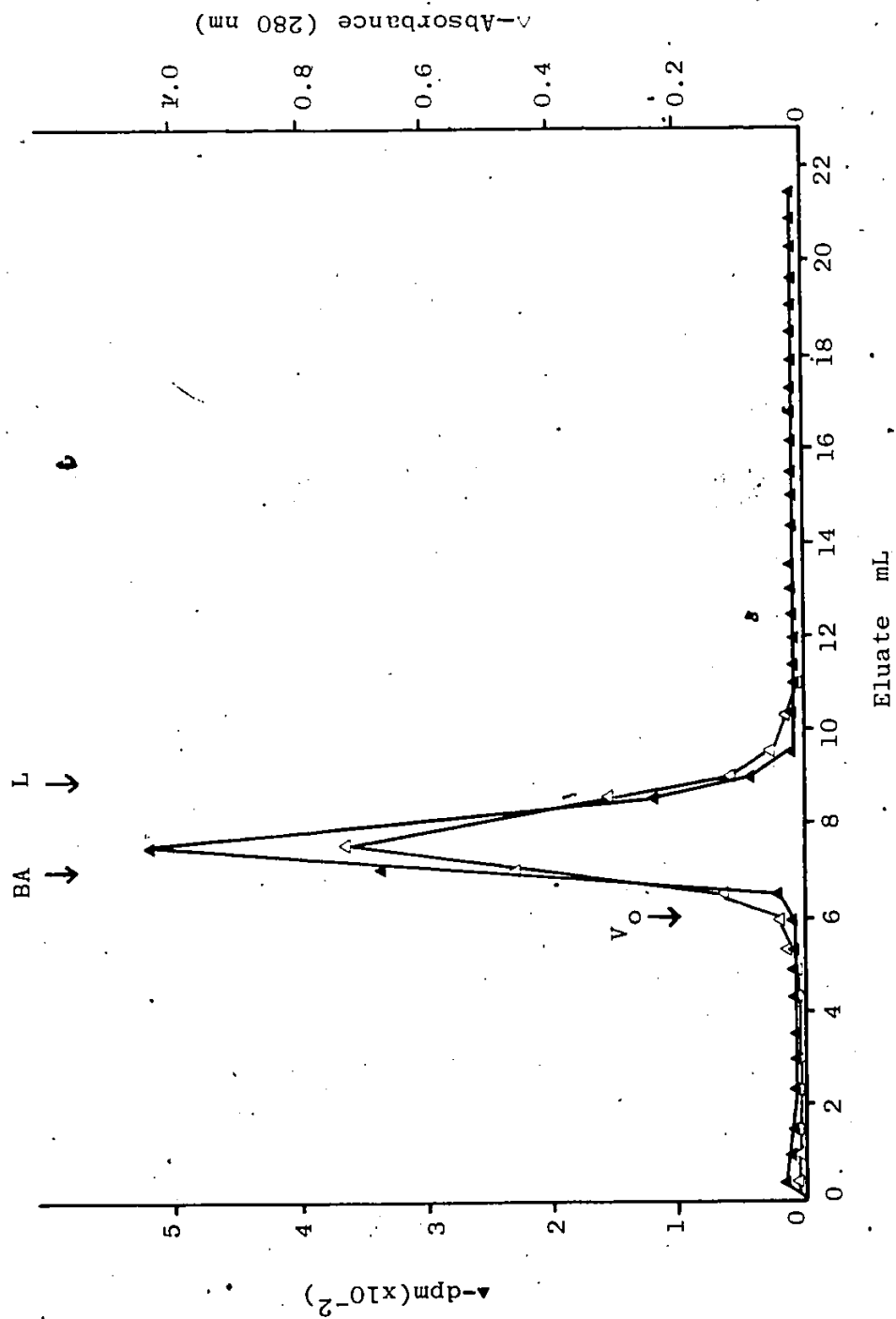
protein. In support of this is the fact that the radio-
labeled material can be precipitated with trichloroacetic
acid. Lyophilization of the fraction containing the highest
radioactivity and subsequent electrophoretic analysis gave
similar results as before. Whereas most of the radiolabel
applied onto the gels was lost, the remainder was found at
the origin of the gels. Although the use of the Sephadex
G-100 column gave an indication as to the presence and
molecular weight of the radiolabeled protein, isolation of
this protein could not be achieved by the column. For the
isolation of this protein further fractionation of the fraction
containing the highest radioactivity would be required.

Alternatively, cell envelopes could be further fraction-
ated by the SDS-extraction method of Mizuno and Kageyama (92).
According to their method, a peptidoglycan-associated protein
complex is obtained by incubation of cell envelopes from
P. aeruginosa with 2% SDS-10% glycerol-10 mM Tris-HCl, pH 7.8,
extraction solution for 60 min at 30°C, followed by centri-
fugation. Similar methods have been used by Heilmann (81)
and Hancock et al. (91) for P. aeruginosa and Lugtenberg
et al. (79) for E. coli. The proteins that remain peptido-
glycan-associated in P. aeruginosa have been shown to
correspond to the outer membrane proteins F, H and I, with
molecular weights of 35,000, 21,000 and 8,000, respectively.
Furthermore, proteins F and H were found to form hydrophilic
aqueous channels in the outer membrane (91,92,100). Indeed,
proteins attached to the peptidoglycan are outer membrane

proteins which may form or be part of the pores or hydrophilic channels through which numerous molecules permeate (121). These proteins remain attached to the peptidoglycan by treatment with SDS at or below 60°C, but are removed by heating above 60°C (92,121). Furthermore, these proteins are released from the peptidoglycan by treatment with NaCl (92) demonstrating that they are not covalently attached to the peptidoglycan, but held together by strong ionic interactions (121). In P. aeruginosa, proteins F, H and I have been shown to be held together by strong ionic interactions (91,92).

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

Analysis of the radiolabeled peptidoglycan-associated protein complex by electrophoresis and column chromatography yielded results similar to the cell envelopes. Although

Table 12

SDS-Extraction of Cell Envelopes and the Distribution

of Radiolabel from D-[6-^{3}H]4FG.

Fraction	Total dpm($\times 10^5$)	Total mg protein	$\dfrac{\text{nmol radiolabel}}{\text{mg protein}}$
Peptidoglycan-associated proteins (SDS-extraction of cell envelopes A at 30°C).	0.5	0.7	5.0
Proteins from supernatant of SDS-extraction of cell envelopes A at 30°C.	0.14	3.0	0.29
Peptidoglycan-associated proteins (SDS-extraction of cell envelopes B at 70°C).	0.005	0.03	0.90
Proteins from supernatant of SDS-extraction of cell envelopes B at 70°C.	0.4	4.5	0.56

Whole cell suspensions (24 mg protein) of glucose grown

P. putida were incubated at 30°C in 100 mM potassium phosphate

buffer, pH 7.1, with 1 mM D-[6-^{3}H]4FG (7.2 mCi/mmol), in a

total volume of 4 mL. After 24 h the suspensions were

centrifuged and cell envelopes prepared by ultrasonication.

SDS-extraction of cell envelopes were as described in the

MATERIALS AND METHODS section. Cell envelopes A and B refer

to cell envelopes prepared from two different whole cell

incubation mixtures under the same conditions. The amount of

radiolabel in cell envelopes A and B were not determined, but

subjected to SDS-extraction at 30°C and 70°C, respectively.

most of the radiolabel applied onto the gels was lost, the
remainder was found at the origin. This may be due to the
high content of peptidoglycan in the sample, although there
is no evidence to support this effect of peptidoglycan on
electrophoresis. An alternative procedure may be to treat
the cell envelopes and peptidoglycan-associated protein complex
with lysozyme before analysis. An apparent molecular weight
of 43,000 could be estimated for the peak of radioactivity
of the peptidoglycan-associated protein complex on the
Sephadex G-100 column. Furthermore, this peak corresponds
to the protein peak.

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

Figure 30

Comparison of the Major Proteins in the Crude Outer

Membranes and Peptidoglycan-Associated Protein Complex

Obtained from Glucose Grown Whole Cells of P. putida.

Legend:

B - crude outer membranes

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

O - represents the origin of the gels

Tracings are reproductions of the densitometer scans

of urea-SDS-polyacrylamide gels. Arrows indicate the

position of the tracking dye. Samples were prepared and

electrophoresis performed as described in the MATERIALS

AND METHODS section.

Outer Membrane Protein Band	Molecular Weight
a	86,000
b	55,000
c	45,000
d	40,000
e	22,000
f	16,000

Figure 30
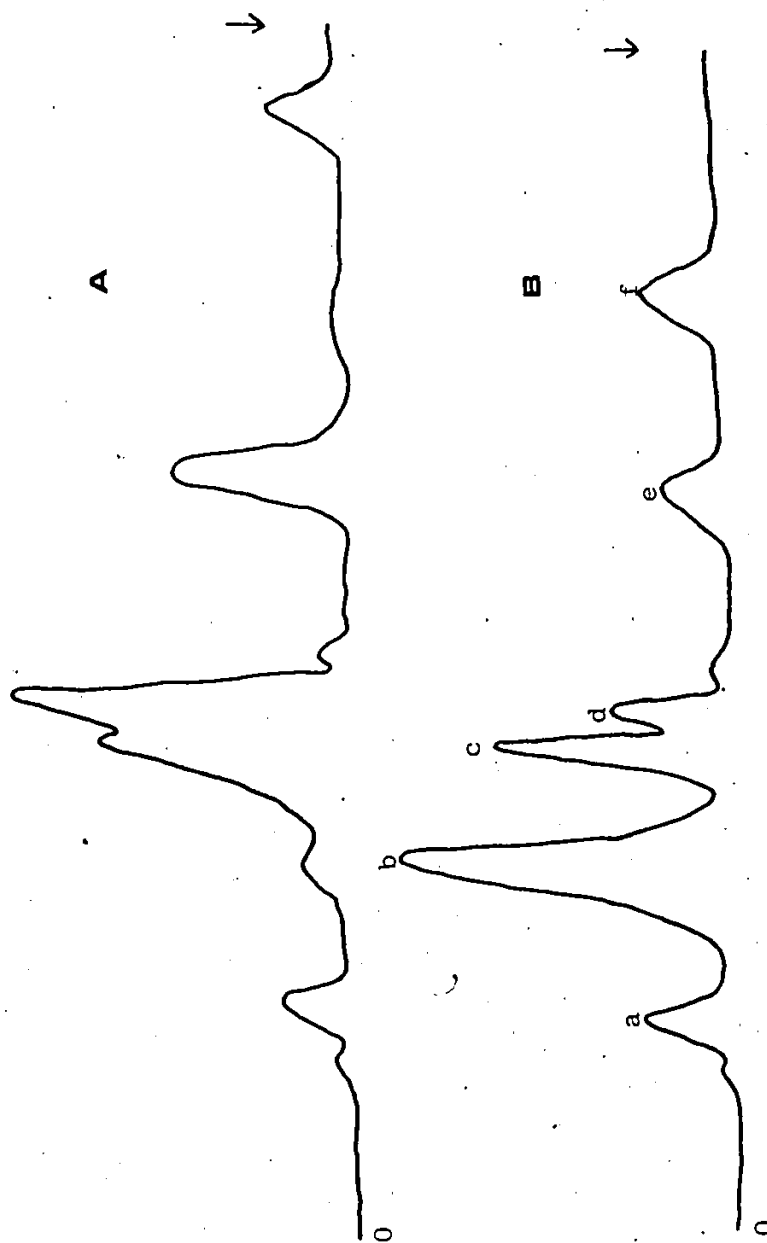
protein complex obtained from succinate grown cells.

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

The above SDS-extraction method may provide a simple and rapid procedure for the isolation and identification of the radiolabeled protein. It may be possible that more than one of the peptidoglycan-associated proteins are radiolabeled. Extraction of the radiolabeled cell envelopes with SDS at $30^{\circ}C$ will yield the peptidoglycan-associated protein complex. This complex may then be treated with the SDS-extraction

Figure 31

Urea-SDS-Polyacrylamide Gel Electrophoretic Comparison of

Fractions Obtained by SDS-Extraction of Cell Envelopes

with the Crude Outer Membranes.

Legend:

    lane 1 - cell envelopes

    lane 2 - proteins from supernatant of SDS-extraction
           of cell envelopes at $30^{\circ}C$

    lane 3 - crude outer membranes

    lane 4 - pellet obtained from SDS-extraction of
           cell envelopes at $30^{\circ}C$

    lane 5 - pellet obtained from SDS-extraction of
           cell envelopes at $70^{\circ}C$

    lane 6 - proteins from supernatant of SDS-treatment
           of peptidoglycan-associated protein complex
           at $70^{\circ}C$

    lane 7 - pellet obtained from 6

    lane 8 - proteins from supernatant of SDS-0.75 M
           NaCl treatment of peptidoglycan-associated
           protein complex at $30^{\circ}C$

    lane 9 - pellet obtained from 8

Protein Band	Molecular Weight
a	86,000
b	55,000
c	45,000
d	40,000
e	22,000
f	16,000
g	9,000

M_r - molecular weight markers used:

    bovine albumin - 66,000
    egg albumin - 45,000
    pepsin - 37,500
    trypsinogen - 24,000
    β-lactoglobulin - 18,400
    lysozyme - 14,300

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

Samples were prepared and electrophoresis performed as

described in the MATERIALS AND METHODS section.

Figure 31

M_r

— 66,000

— 45,000
— 37,500

— 24,000

— 18,400

— 14,300
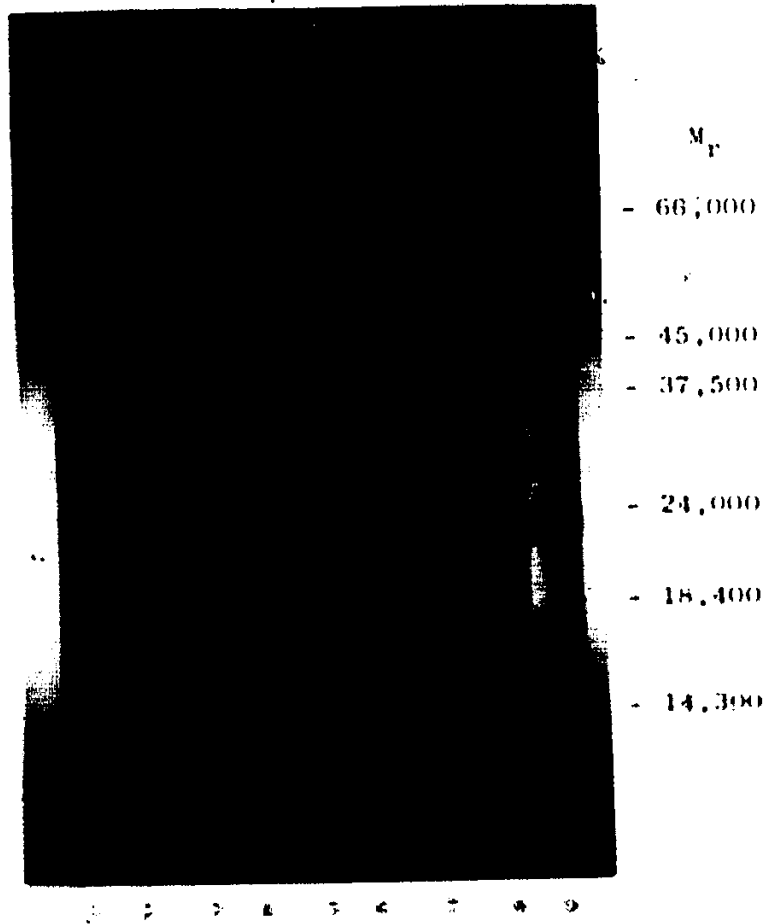
solution for 60 min at $70^{\circ}C$ or with the SDS-extraction solution
supplemented with 0.75 M NaCl for 60 min at $30^{\circ}C$. This will
result in the release of proteins form the peptidoglycan,
which can be recovered by precipitation with 2 volumes ice-
cold acetone. This fraction may then be further fractionated
by ammonium sulfate precipitation, ion-exchange chromatography,
column chromatography or by a combination of these methods.
Isolation of this protein may provide a better understanding
of the mechanism and precise location of fluoride release.

CHAPTER IV

SUMMARY AND CONCLUSIONS

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

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

• This outer membrane protein may form or be part of the
porin channel. Furthermore, the protein may be involved in
the specific translocation of glucose, gluconate or 2-keto-

gluconate across the outer membrane. Such a specific trans-
location system may be envisioned to operate analogous to
the maltose (79) or dicarboxylic acid (66) transport systems
in E. coli.

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

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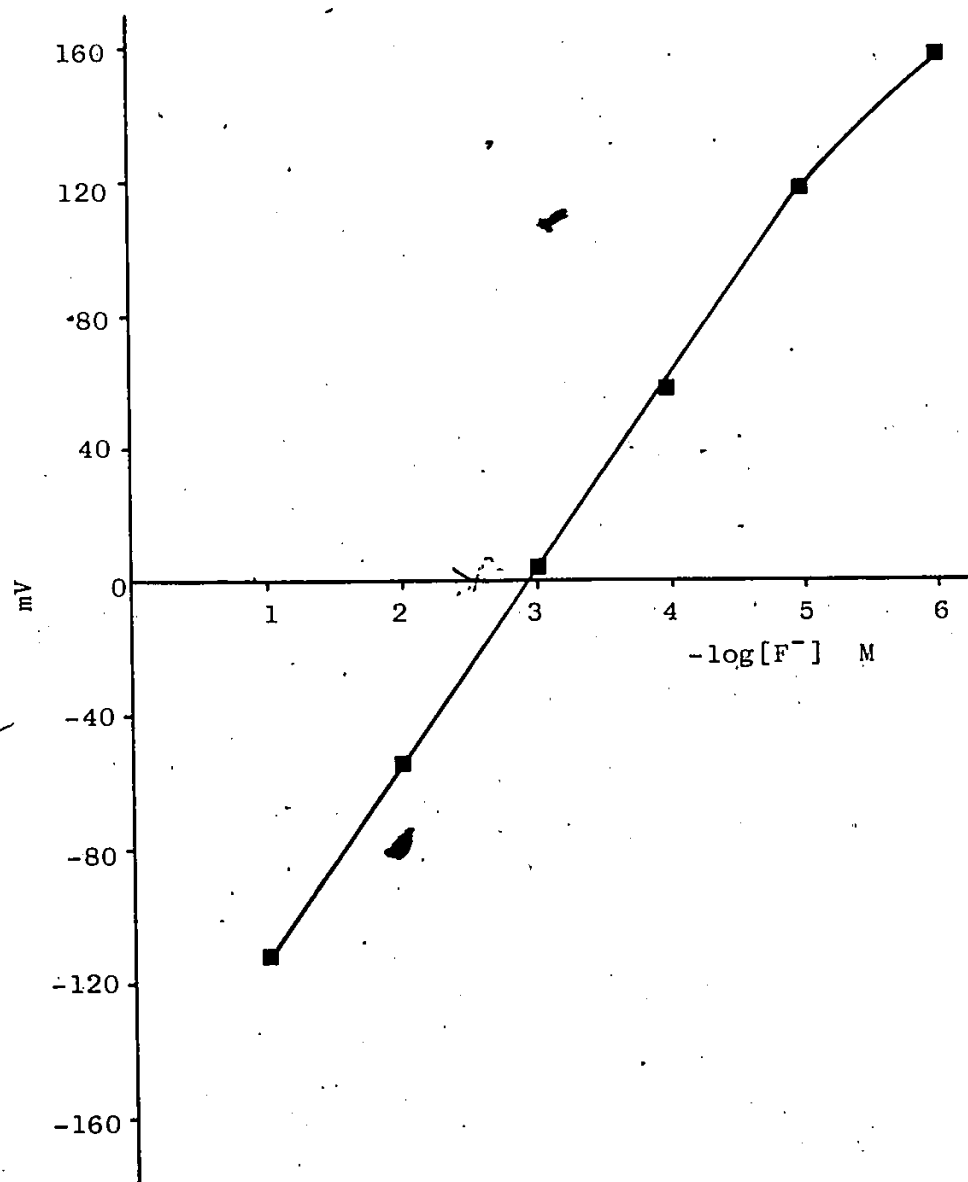
APPENDIX I

Calibration Curve of Voltage Potential Versus Fluoride

Ion Concentration.

Legend:

The calibration curve was constructed with various

concentrations of NaF (10^{-6}–10^{-1} M) in 100 mM potassium

phosphate buffer, pH 7.1. Fluoride was measured with a

fluoride ion specific electrode (Orion Research) as

described in the MATERIALS AND METHODS section.

APPENDIX I

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APPENDIX II

Calculation of Oxygen Consumption

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

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

$$B' \, L(O_2) = B \, L(O_2) \, 273(P-P_w)/(760xT)$$

where P and P_w were the experimental and water vapour pressures, respectively, and T was the experimental temperature (^{O}K).

Therefore, the number of moles of oxygen consumed at STP = $(PxB')/(RxT)$.

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

Therefore, the extent of substrate oxidation

$$= \frac{(PxB')/(RxT)}{A} \qquad \frac{\text{moles oxygen consumed}}{\text{moles substrate}}$$

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

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

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

APPENDIX III

Determination of Radioactivity in Various Samples

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

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

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

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

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

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

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

where B is the mg of protein.

APPENDIX IV

Molecular Weight Calibration Curve for Sephadex G-100

Column.

Legend:

V_e – elution volume

V_o – void volume

The void volume (6 mL) was determined with blue dextran.

The elution volumes were determined as the protein peak

from the elution profile of the molecular weight markers

or from the radioactivity peak of the radiolabeled

samples. The preparation and operation of the column

was as described in the MATERIALS AND METHODS section.

Molecular weight markers used:

bovine albumin – 66,000

lysozyme – 14,300

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

APPENDIX IV
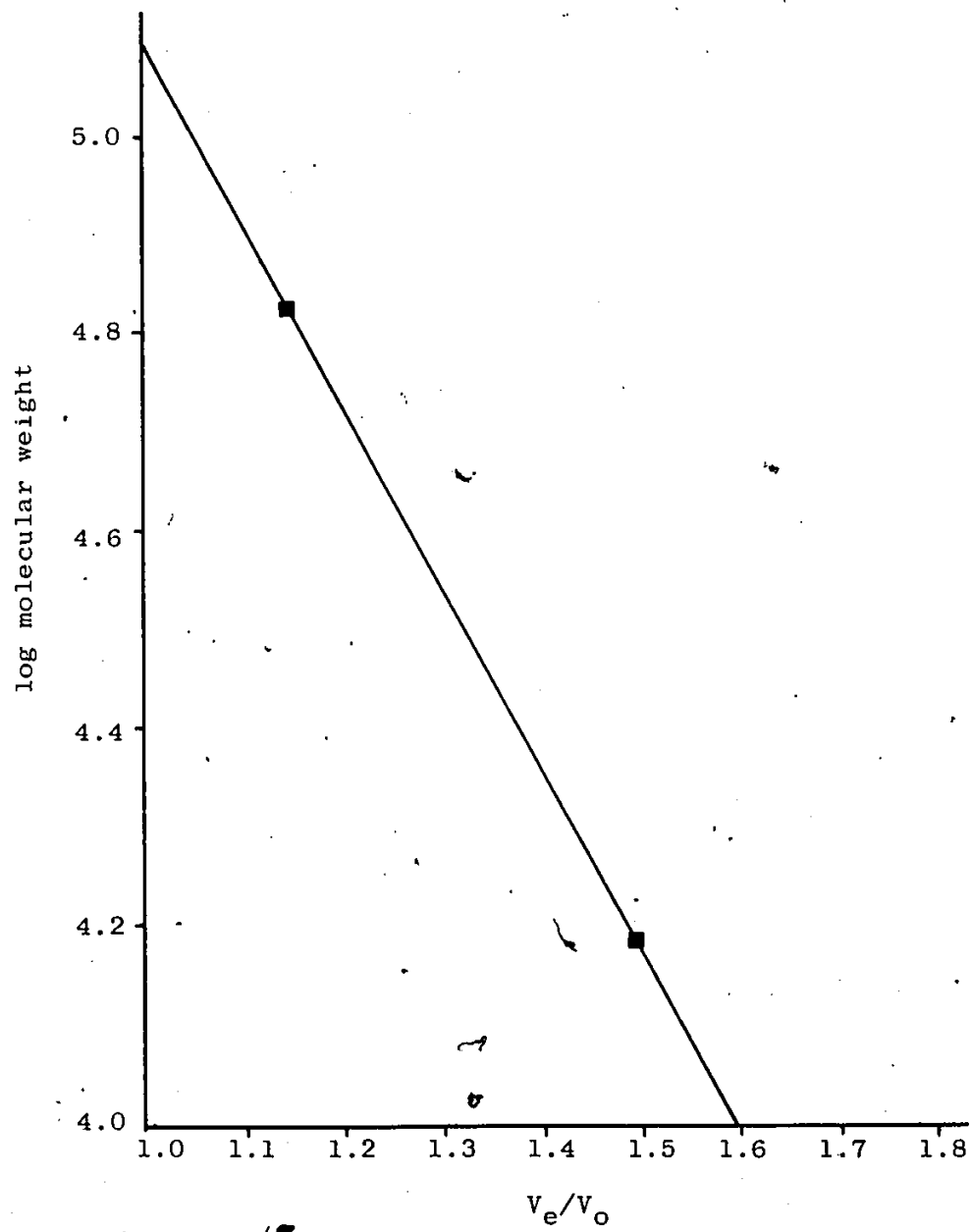
APPENDIX V

Molecular Weight Calibration Curve for Urea-SDS-Poly-

acrylamide Gel Electrophoresis.

Legend:

The relative mobilities were calculated as follows:

$$\text{relative mobility} = \frac{\text{distance of protein band from origin (cm)}}{\text{distance of tracking dye from origin (cm)}}$$

Electrophoresis was performed as described in the

MATERIALS AND METHODS section.

Molecular weight markers used:

        bovine albumin - 66,000

            egg albumin - 45,000

                pepsin - 37,500

            trypsinogen - 24,000

    β-lactoglobulin - 18,400

            lysozyme - 14,300

APPENDIX V
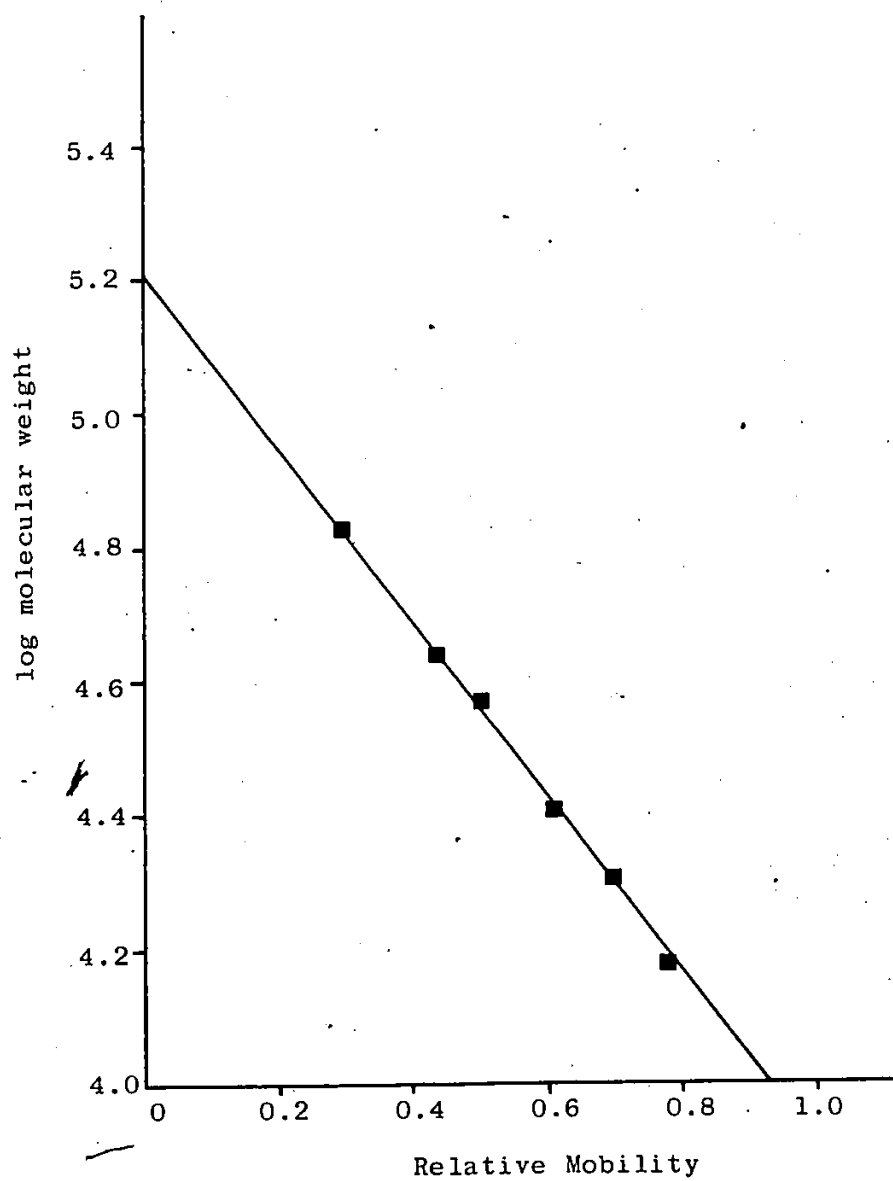
APPENDIX VI

Quench Correction Curve for $[^{14}C]$-Samples.

Legend:

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

APPENDIX VI
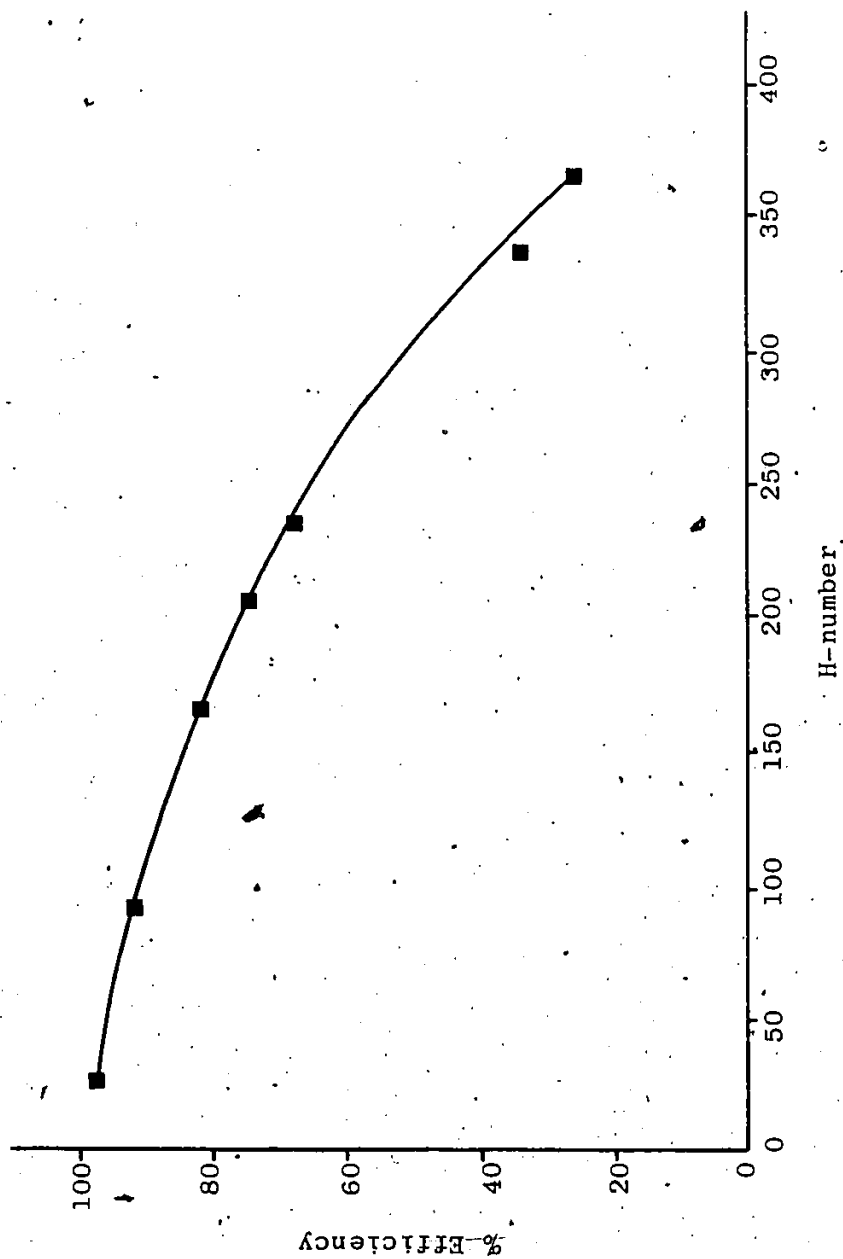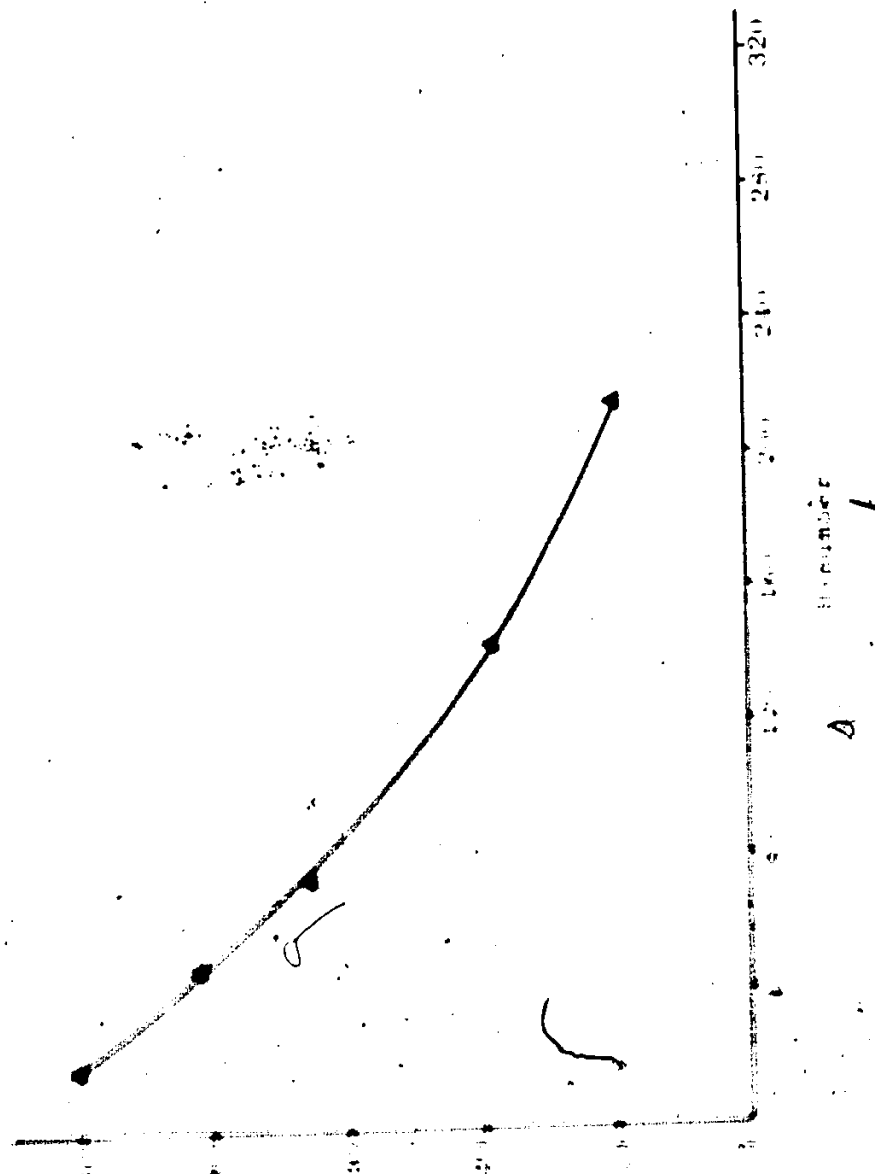
APPENDIX VII

APPENDIX VIII

Calibration Curve for 4FG Concentration.

Legend:

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

APPENDIX VIII
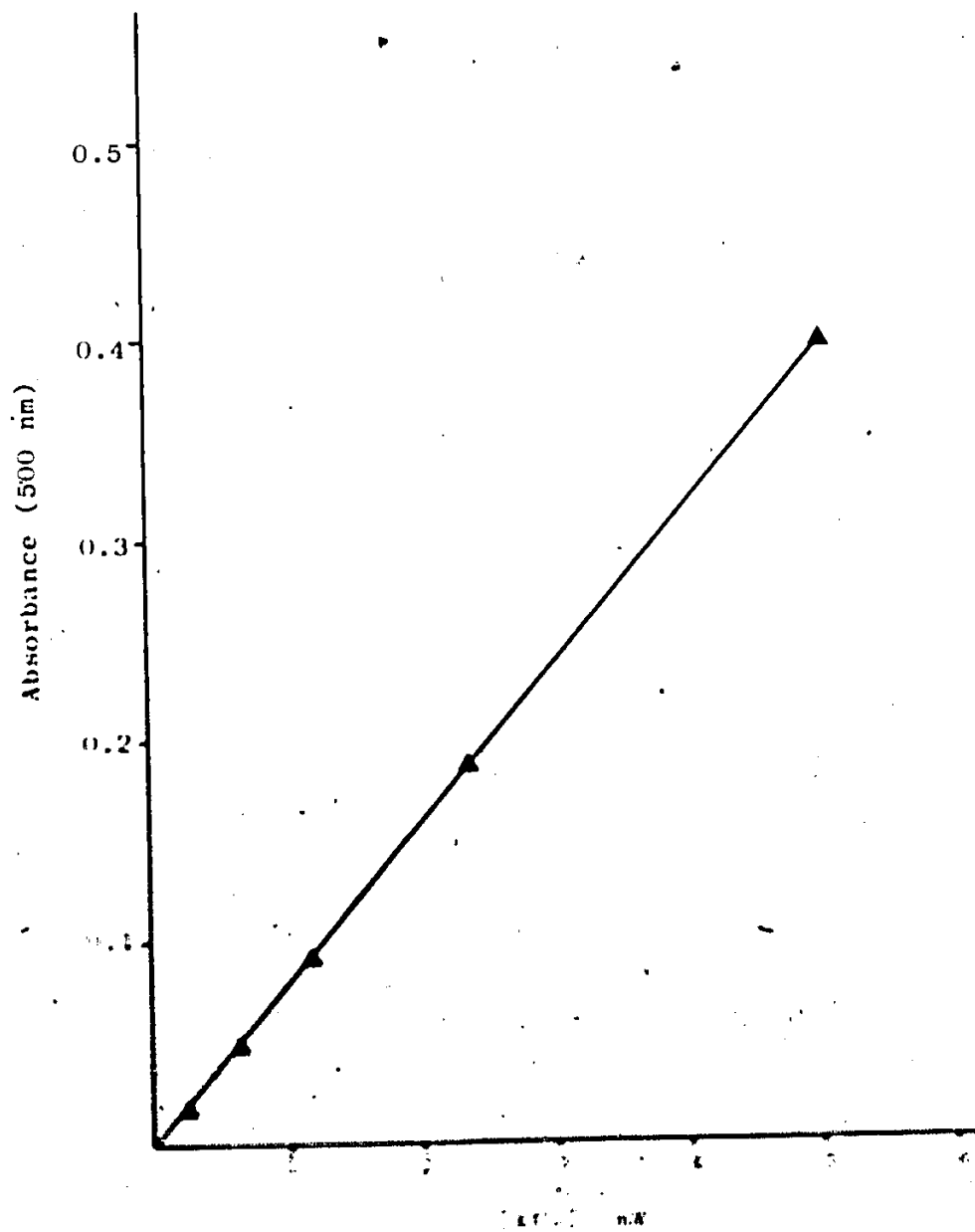
APPENDIX IX

Linear Regression Analysis of the Kinetic Data

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

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

$$m = \frac{\sum xy - \frac{\sum x \sum y}{N}}{\sum x^2 - \frac{(\sum x)^2}{N}}$$

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

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

*The linear regression routine was performed on a Texas Instrument TI-55 programmatic calculator.

APPENDIX X

Maximum Defluorination in Chloramphenicol Treated Cells

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

Therefore, $\dfrac{133 \text{ nmol}}{\text{mg protein}} \times \dfrac{182 \text{ ng 4FG}}{\text{nmol}}$

$= 24,200$ ng 4FG mg protein^{-1}

$= 24$ µg 4FG mg protein^{-1}

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

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