Some biochemical effects of deoxy-fluoromonosaccharides on escherichia coli.

Li-Yu. Louie
University of Windsor

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SOME BIOCHEMICAL EFFECTS OF
DEOXY-FLUOROMONOSACCHARIDES ON
ESCHERICHIA COLI

BY
LI-YU LOUIE

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirement for the Degree of
Master of Science at the
University of Windsor

Windsor, Ontario
1975
ABSTRACT

The natural occurrence and the biochemistry of some carbon-fluorine compounds, with special emphasis on fluoro-carbohydrates and related compounds, is reviewed. A brief description of the glucose effect on lactose utilization by E. coli is also included.

A study of the metabolic effects of 4-deoxy-4-fluoro- and 3-deoxy-3-fluoro-D-glucose on whole cells and cell-free extracts of E. coli is reported. A preliminary series of experiments explores the utilization of 3-deoxy-3-fluoro-β-D-arabinose by whole cells of E. coli (ATCC 11775).

Studies with E. coli, ATCC 11775

(1) 4-deoxy-4-fluoro-D-glucose cannot serve as a source of carbon for the growth of E. coli and reduces the extent of growth on glucose.

(2) Neither whole cells nor cell-free extracts of glucose-grown E. coli metabolize 4-deoxy-4-fluoro-D-glucose to any significant extent.

(3) In frozen and thawed cells of E. coli, 4-deoxy-4-fluoro-D-glucose is tentatively considered to be phosphorylated by the phosphoenolpyruvate dependent phosphotransferase system, and resting cells take up 4-deoxy-4-fluoro-D-glucose to the extent of 0.06 mg/mg dry weight of bacteria.

(4) 3-deoxy-3-fluoro-D-glucose is converted to 3-deoxy-3-fluoro-D-glucose 6-phosphate by the phosphoenolpyruvate
dependent phosphotransferase system in frozen and thawed
cells. Up to 0.029 mg 3-deoxy-3-fluoro-D-glucose is
taken up / mg bacterial dry weight by resting E. coli.

Studies with E. coli K 12, ATCC 14948

(1) The inhibition of β-galactosidase activity in E. coli
by 4-deoxy-4-fluoro-D-glucose is characterized as
uncompetitive.

(2) 4-deoxy-4-fluoro-D-glucose prevents lactose utilization
probably by inhibition of the synthesis and activity of
both β-galactosidase and galactoside permease.

Preliminary experiments indicate that whole cells of
D-arabinose-grown E. coli ( ATCC 11775 ) oxidize 3-deoxy-3-
fluoro-β-D-arabinose without a lag period. Resting whole
cells of E. coli take up 3-deoxy-3-fluoro-β-D-arabinose to
the extent of 0.11 mg/mg dry weight of bacteria during the
first five hours of incubation.
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. N. F. Taylor, my advisor, for initiating my interest in the chemistry and biochemistry of fluorinated sugars, and for the constant help and encouragement received.

I would also like to thank Dr. D. G. Tuck, Head, Department of Chemistry for use of facilities. Particularly, I wish to thank Dr. R. J. Thibert for his precious advice and Dr. G. L. Gagneja for the synthesis of 4-deoxy-4-fluoro-D-glucose.
DEDICATION

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<td>3FG</td>
<td>3-deoxy-3-fluoro-D-glucose</td>
</tr>
<tr>
<td>3FGA</td>
<td>3-deoxy-3-fluoro-D-gluconic acid</td>
</tr>
<tr>
<td>3F2KGA</td>
<td>3-deoxy-3-fluoro-2-keto-D-gluconic acid</td>
</tr>
<tr>
<td>3FG-6-P</td>
<td>3-deoxy-3-fluoro-D-glucose 6-phosphate</td>
</tr>
<tr>
<td>4FG</td>
<td>4-deoxy-4-fluoro-D-glucose</td>
</tr>
<tr>
<td>4FGA</td>
<td>4-deoxy-4-fluoro-D-gluconic acid</td>
</tr>
<tr>
<td>4F2KGA</td>
<td>4-deoxy-4-fluoro-2-keto-D-gluconic acid</td>
</tr>
<tr>
<td>4FG-6-P</td>
<td>4-deoxy-4-fluoro-D-glucose 6-phosphate</td>
</tr>
<tr>
<td>3FA</td>
<td>3-deoxy-3-fluoro-D-arabinose</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic 3', 5'-adenosine monophosphate</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-D-thiogalactoside</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-nitrophenyl galactoside</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>O.D.</td>
<td>optical density</td>
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<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
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CHAPTER I

INTRODUCTION

Section A  The Metabolism of Fluorocarbohydrates and Related Compounds

The biological activity of fluorine is largely determined by whether the fluorine is ionically or covalently bonded. Ionic fluorine produces an entirely different biological response to that of covalently bonded fluorine. There is also a biologically important group of fluorinated organic compounds in which the fluorine is bonded to phosphorus (dialkyl phosphofluoridates\(^1\)), but these are outside the scope of the present work, which is concerned with fluorine covalently bonded to carbon (C-F), and the biological activity of compounds containing this group.

Fluorine is in group VII of the periodic table. But this fact should be considered in light of Pauling's reference to it as a 'superhalogen'\(^2\). Fluorine is more electronegative than the other halogens(F=4.0, Cl=3.0, Br=2.9, I=2.4) and, therefore, is the only halogen unlikely to form a cation\(^3\). The bond energy of the carbon-fluorine bond is among the highest found in natural products and known to be broken enzymatically. The Van der Waal's radius of the fluorine atom (1.35\(\text{\textmu\text{A}}\)) is sufficiently close in size to that of hydrogen (1.26\(\text{\textmu\text{A}}\)) to account for pseudo-substrate activity. The physico-chemical data between C-F and C-OH seem to be closer in a number of respects, such as bond
length, force constant, and more important, electronegativity. These properties of fluorine are of most interest to the biologist, since a knowledge of the size and electronegativity of the atom can be used to make defined alternations in biologically important molecules. Thus, modified biological activity may arise from the presence of a fluorine atom occupying an atomic volume similar to that of a hydrogen atom, or hydroxyl group, and possessing also electronegative character and potential for hydrogen-bonding similar to that of OH. The replacement of OH by F in monosaccharides thus appears to offer attractive possibilities for achieving interesting chemical modification, and at the same time conferring new biochemical properties upon the molecule.

Interest in the biological properties of fluorinated organic compounds was stimulated by Marais in 1944, who discovered that monofluoroacetate, is the toxic principle in the South African Shrub, *Dicapetalum cymosum*. The mechanism of the toxic action has been elucidated mainly by Peters and his colleagues, who have shown that in mammals fluorocacetate is converted enzymatically into monofluorocitrate via fluoroacetyl-CoA as shown in Figure 1. The fluorocitrate produced showed even more profound toxicity, and this has been ascribed to its arrest of the tricarboxylic acid cycle by inhibiting the action of enzyme aconitase.

The role of fluorocitrate on aconitase is complex. The enzyme is multiple, and is believed to consist of
FIGURE 1
PATHWAY FOR THE SYNTHESIS OF FLUOROCITRATE FROM FLUOROACETATE

\[ \text{Fluoroacetate} \xrightarrow{\text{ATP}} \text{FCH}_2\text{COH} \xrightarrow{\text{CoSH}} \text{FCH}_2\text{COSCoA} \xrightarrow{} \text{Oxaloacetate} \]

COOH
C=O
CH₂
COOH

\[ \text{HO-C-COOH} \]
\[ \text{CH}_2\text{COOH} \]

Fluorocitrate enantiomer
two active centres. Fluorocitrate has four isomers. Kun and his colleagues have isolated the active and other isomers and identified the active component as S-threo-fluorocitrate.

Fluoroacetate has now been identified in both the seeds and leaves of a large number of plants. Certain other naturally occurring fluorofatty acids have been reported. The occurrence of ω-fluoro-oleic acid as well as smaller amount of ω-fluoropalmitic, ω-myristic and caproic acids in the *Dicapetalum toxicarum* suggested that fluoroacetate can substitute for acetate, at least partially, in the biosynthesis of long-chain fatty acids. The fact that fluorine occupies the ω-position of the fatty acid is in accord with the scheme for fatty acid biosynthesis which has been demonstrated in animals, plants and microorganisms. In these systems, acetyl-CoA is the two-carbon unit responsible for the elongation of the fatty acid molecule. Yet in the completed, straight-chain fatty acid molecule, only the ω-carbon and the adjacent carbon atom are derived directly from acetyl-CoA; the other acetyl-CoA molecules are carboxylated to form malonyl-CoA and are then decarboxylated in the condensation reaction. Figure 2, which shows the initial cycle of condensation and reduction in the two-carbon elongation of a fatty acid molecule, illustrates the dual role of acetyl-CoA in this process. Acetyl-CoA, which proceeds through malonyl-CoA (shown with the hypothetical X substituent), undergoes reactions not
FIGURE 2

CYCLE IN LONG CHAIN FATTY ACID BIOSYNTHESIS SHOWING A POSSIBLE ROLE FOR FLUOROACETATE

KEY:

ACP—ACYL CARRIER PROTEIN
CoA—COENZYME A
X—H (or ??)
FIGURE 2

CYCLE IN LONG CHAIN FATTY ACID BIOSYNTHESIS SHOWING A POSSIBLE ROLE FOR FLUOROACETATE
required of acetyl-CoA entering the methyl terminal position (shown as fluoroacetyl-CoA). Failure of fluoroacetyl-CoA to proceed through "fluoromalonyl-CoA" would account for the lack of incorporation of fluorine, except at the ω-position of the fatty acid product (Figure 2). Furthermore, since the synthesis of long chain fatty acids in a repetitive process, partial inhibition at any step would be expected to have a large effect on the overall synthesis.

In addition, studies on a wide range of synthetic ω-fluorofatty acids indicated that the acids having even-numbers of carbon atoms are markedly more toxic than those with odd numbers\(^{16,17,18}\). Such findings are in accord with the β-oxidation theory of fatty acid catabolism, proposed by Knoop\(^{19}\), in which the successive loss of two-carbon acetyl-CoA units would yield either the toxic fluoroacetic acid, if there is initially an even number of carbon atoms or the relative non-toxic ω-fluoropropionic acid (or perhaps to the unstable and non-toxic fluoroformic acid) if there is initially an odd number of carbon atoms, in the ω-fluoro fatty acid.

The toxicity of fluoro compounds can indicate whether the biological degradation produces fluoroacetate, and this can often provide a clue to the metabolism of compounds in which the fluorine serves as a marker. For example, in a series of ω-fluoronitriles, \(\text{F(CH}_2\text{)}_n\text{CN}\), those with an odd
number of carbon atoms are toxic, whereas those with an even number are non-toxic. These observations suggest a carbon-cyanide cleavage rather than a hydrolysis or reduction of the nitrile in the metabolism of these compounds. Examples of what might be termed lethal catabolism can also occur in microorganisms. A strain of *Pseudomonas fluorescens* can utilize nicotinate but not 5-fluoronicotinate for growth, presumably due to the accumulation of fluoroacetate and fluorocitrate. Further, studies on the degradation of 2-fluoronitrobenzoate by *Nocardia erythropolis* have made the identification of these toxic products more definitive.

Not all monofluoro compounds are metabolized, however, to toxic end products and a range of interesting biologically active members of this class of substances have been described, including the monofluorocortisone hormones. Among the better known is 9 α-fluorocortisone which has hormonal activities considerably enhanced compared with the parent steroid. Other groups of interesting monofluoro analogues of naturally occurring substances include monofluoroamino acids e.g. 3-fluoro-L-tryrosine, 4-fluoro-L-proline, 3-fluoro-DL-phenylalanine, 4-fluoro-DL-glutamic acid, some of which are activated by bacterial RNA system. Besides, fluorine-containing natural products may also be synthesized by microorganisms. Nucleocidin, an anti-trypanosomal antibiotic produced by a *Streptomyces*, has been assigned the structure of a nucleoside (Figure 3) in which the fluorine atom is attached to the carbohydrate moiety.
FIGURE 3
STRUCTURAL FORMULA OF NUCLEOCIDIN
Most of the examples mentioned so far are cases which a hydrogen atom of the natural substrate is replaced by fluorine giving rise to a molecule capable of deceiving one or more enzymes and thereby leading to inhibition and lethal synthesis. Bergman \(^{27}\) has argued that the similarity in Van der Waals' radii between the elements hydrogen (1.20\(\text{Å}\)) and fluorine (1.35\(\text{Å}\)) is sufficiently close to account for pseudosubstrate activity. This comparison has, however, doubtful relevance, since these two elements hydrogen-bond in the opposite sense, i.e. hydrogen is always at the electropositive end of a hydrogen bonding, while fluorine is electronegative. The physicochemical similarity between C-F and C-OH, however, may be considered closer in bond length, force constant, and more important, electronegativity, i.e. they can hydrogen-bond in the same sense. This suggests that the fluorine atom might act as a hydroxyl analogue in biochemical transformation. Evidence in support of this was found in the close similarity in crystal lattice structure between certain tetriols and pentitols and their monofluoro analogues\(^ {28}\). Since hydrogen bonding is believed to be intimately involved with enzyme-substrate interactions, the replacement of hydroxyl by fluorine in natural substrates or metabolites may bind especially to allosteric enzymes and lead to some interesting metabolism, storage carbohydrates, and nucleic acid biosynthesis.

Arguments such as these have evoked the principle motivation for the synthesis of deoxyfluoro sugars. The
term, deoxy-fluoro sugar, describes carbohydrates in which one or more of the hydroxyl functions, other than at the anomeric position, have been replaced by a fluorine atom. In recent years, quite a number of fluorocarbohydrates with potential biological activity have been synthesized; the role of fluoro analogues as pseudosubstrates or inhibitors in these compounds as models for exploring the nature of interactions of small ligands with biological macromolecules has also been undertaken.

The first reported enzyme studies with fluorinated sugars were those by Helferich, Grünler and Gnüchtel in 1937. The purpose of their work was to examine the enzyme specificity of β-glucosidase to structural changes in substrate. They demonstrated that both phenyl (1) and vanillyl 6-deoxy-6-fluoro-β-D-glucopyranosides (2) would act as

\[
\begin{align*}
(1) & \quad \text{R} = \text{C}_6\text{H}_5 \\
(2) & \quad \text{R} = \text{C}_8\text{H}_7\text{O}
\end{align*}
\]

substrates for this enzyme (as measured by \(k_m\)), which suggested that the aglycone was not critical, providing it had the \(\beta\)-configuration, and that position C-6 was probably not directly involved with the active site. In contrast, Barnett has argued that the C-6 hydroxyl group of \(\alpha\)-D-
glucosides is important in the binding to α-D-glucosidase.

Only a few isolated enzyme studies have been performed with the terminally fluorinated sugars. 6-deoxy-6-fluoro-D-glucose (6FG) has been reported to give the following percentage activities compared with glucose: glucose dehydrogenase, 100; glucose oxidase, 33; and maltose phosphorylase, 80. A cell-free extract of Aerobacter aerogenes has been shown to oxidize 6FG to 6-deoxy-6-fluoro-D-arabino-hexulosonic acid. The effect of 6FG on intact yeast cells has been studied by Blakey & Boyer, who showed that at molar concentrations, comparable to those of the glucose and fructose used, 6FG inhibited the rate of fermentation of intact cells, whereas its effect on cell-free extract was negligible, nor was there any significant effect on yeast hexokinase. In whole cells, the inhibition was competitive with the normal substrates and it was suggested, therefore, that 6FG probably influenced a specific transport process, not hexokinase limited, which controls the rate of entry of glucose and fructose into the cell. Since 6FG cannot undergo enzymic phosphorylation, it might be anticipated in those tissues where the cell entry mechanism is rate limiting. Support for this contention has been provided by a number of workers.

Arita & Matsushima have reported that phenyl-6-deoxy-6-fluoro-α-D-maltoside is a poor substrate compared with phenyl-α-D-maltoside for Taka-amyrase A, which suggests that position C-6 of this substrate is involved in the.
binding to this enzyme. Kissman & Weiss\textsuperscript{42} utilized 5-deoxy-5-fluoro-D-ribose in their attempts to provide suitably modified purine and pyrimidine nucleosides as potential antitumor reagents. Although none of their 5'-fluoronucleosides showed antitumour activity, the inactivity of 6-mercapto-9-(5-deoxy-5-fluoro-\(\beta\)-D-ribofuranosy)-purine (3)

\[
\begin{align*}
\text{(3) } X &= F \\
\text{(4) } X &= OH
\end{align*}
\]

indicated the possibility that the known antitumour activity of 6-mercapturine riboside\textsuperscript{43} involves phosphorylation at the 5'-position of the sugar.

Although no biological data have yet been reported, 6-deoxy-6-fluoromuramic acid has been synthesized\textsuperscript{44} as a potential inhibitor of bacterial cell wall synthesis. Recently, N-fluoroacetyl-\(\alpha\)-D-glucosamine has been used as a molecular probe for lysozyme structure, and this analogue structure could possibly inhibit glycoside transport and bacterial growth\textsuperscript{45}.

Most of the studies mentioned above are confined to extracyclic or terminally substituted fluorohexoses and pentoses. In recent years, quite a number of fluorocarbohydrates and related compounds in which fluorine replaces a secondary hydroxyl group have become available. Such
fluorinated substrates may exhibit interesting metabolic activity since the terminal or primary hydroxyl groups are now available for attack by kinases and dehydrogenases. Various biological studies have been reported with the rac-1-deoxy-1-fluoro-glycerols, which still have free primary hydroxyl groups. Pattison & Norman\textsuperscript{46} showed that rac-1-deoxy-1-fluoroglycerol is toxic to mice, the animal dying after a rather long interval of time, but with the usual signs of fluoroacetate poisoning. Both 1-deoxy-1-fluoro-sn-glycerol (5) (Figure 4) and its enantiomer 3-deoxy-3-fluoro-sn-glycerol (6) have been unambiguously synthesized\textsuperscript{47,48}, and the synthesis of 2-deoxy-2-fluoroglycerol (7) has been improved to obtain more information about the substrate specificity model of glycerol kinase\textsuperscript{49}. Although (5) and (7) are substrates for yeast glycerol kinase (Km values 120mM and 150mM respectively), (6) is not a substrate, presumably because the fluorine atom occupies the phosphorylation site (Figure 4) of sn-glycerol (8). However, (6) is an effective inhibitor of either dihydroxyacetone or glycerol (Ki 5mM). Recently, Eisenthal and his colleagues\textsuperscript{50} have reported that the four diols resulting from replacement of the hydroxyl groups at C-1 or C-2 of sn-glycerol by fluorine or hydrogen are weak substrates for glycerol kinase. On the other hand, similar substitution of the C-3 hydroxyl group gives compounds which act as competitive inhibitors of glycerol or dihydroxy-acetone phosphorylation but show no activity as substrates.
FIGURE 4

STRUCTURES OF SN-GLYCEROL AND FLUOROGLYCEROLS

Site B
OH

Site C
CH₂

Site A
OH

Phosphorylation site

(8)
sn-glycerol

(6)
3-deoxy-3-fluoro-sn glycerol

Site B

CH₂

Site C
HO

Site A
OH

(5)
1-deoxy-1-fluoro-sn-glycerol

(7)
2-deoxy-2-fluoro-glycerol
The behaviour of 3-deoxy-sn-glycerol and 3-deoxy-3-fluoro-
sn-glycerol towards glycerol kinase is consistent with
their orientation in conformation, in which no phosphoryla-
table substituent is available at site (Figure 4). Further,
studies with rac-1-deoxy-1-fluoroglycerol-3-phosphate by
Fondy, Ghangas and Reza\textsuperscript{51} have shown that rac-1-deoxy-1-
fluoroglycerol-3-phosphate can act as a substrate for
L-\(\alpha\)-glycerol phosphate dehydrogenase. They have argued
that it might act, therefore, as a selective toxic agent
to certain human cancer cells, lacking the NAD-linked gly-
cerol-3-phosphate dehydrogenase but possessing an active
phosphatase, thereby allowing the formation of the toxic
1-deoxy-1-fluoro-sn-glycerol.

2-deoxy-2-fluoro-glycerol and rac-2-deoxy-2-fluoro-
glyceraldehyde also cause citrate accumulation in the
heart and kidney of the rat\textsuperscript{52}. Since 2-deoxy-2-fluoro-
glycerol and rac-2-deoxy-2-fluoro-glyceraldehyde are
substrates for alcohol and aldehyde dehydrogenase respectively
and L-fluoroglycemic acid is more toxic than the D-isomer,
Treble & Peters\textsuperscript{53} suggested that fluoroacetate is produced
from 2-deoxy-2-fluoro-glycerate via the enzyme serine
hydroxymethyl transferase. The suggestion by Pattison\textsuperscript{17}
that the fluoroglycerate is oxidized to fluoromalonate
with subsequent decarboxylation to fluoroacetate still
remains a possibility. In the tertitol series, rac-2-deoxy-
2-fluoroerythritol\textsuperscript{54} has been shown to be a potent growth
inhibitor of \textit{Brucella abortus}\textsuperscript{55} for which erythritol is a
growth factor\textsuperscript{56}. The biochemical mode of inhibition has
not yet been established, but the competitive nature of the inhibition can be reversed by addition of erythritol. In recent years a number of irreversible enzyme inhibitors have been found that combine with a functional group at the active site of the enzyme, for example di-isopropylphosphorofluoridate and its reaction with the serine hydroxyl group of esterases and proteases\textsuperscript{57}. In the carbohydrate series 3-fluoro-N-acetylneuraminic acid reacts in a similar manner for its inhibitory effect towards the "in vitro" cleavage of N-acetylneuraminic acid to N-acetylmannosamine and pyruvic acid, by N-acetylneuraminic acid aldolase\textsuperscript{58}. No attempt, however, was made to show either that the inhibitor is covalently bound to the enzyme or that fluoride is liberated.

3-deoxy-3-fluoro-D-glucose (3FG) has been used to study carbohydrate metabolism in several microorganisms. Miles \& Pirt\textsuperscript{59} previously reported that 3FG appears not to undergo any significant metabolism in \textit{Saccharomyces cerevisiae}. Later work of Woodward, Taylor \& Brunt\textsuperscript{60} mainly investigated the effects of 3FG upon the levels of glycolytic intermediates, adenine nucleotides and inorganic phosphate in resting cell suspensions of \textit{S. cerevisiae}. Significant decrease in intracellular levels of uridine diphosphate glucose (UDPG), 2- and 3- phosphoglyceric acid, ATP and inorganic phosphate were detected, whilst increase in ADP and glucose-1-phosphate were observed. This suggests that 3FG may act at least in part as phosphate trap, thereby
occassioning a shift in the energy balance of the cells. Work with *Pseudomonas aeruginosa* has allowed the isolation of a mutant strain which grows on 3FG and 3-deoxy-3-fluoro-D-gluconic acid (3FGA) with the release of fluoride anion during the lag phase.

Recent studies by Miles & Pirt have shown that 3FG appears not to be metabolized in *E. coli*. In frozen and thawed cells, 3FG was converted to 3FG-6-P by the phosphoenolpyruvate-dependent phosphotransferase system, and resting cells took up 3FG to the extent of 0.03 mg/mg dry weight of cells. Uptake of 3FG was not lethal, though 3FG at 0.1 to 10mM completely prevented or severely inhibited utilization of carbon sources other than glucose, and the extent of inhibition is greater than those reported for other non-catabolizable glucose analogues. It prevented lactose utilization by inhibitions of the synthesis and activity of galactoside permease; its inhibitory effect on the utilization of other carbon sources may be related to specific inhibition of the activity and synthesis of the enzymes and permeases involved in their utilization. Also, 3FG-resistant mutants, deficient in the Enzyme II of the phosphotransferase system specific for glucose and for 3FG, were isolated.

3FG has also been used by Kornberg & Smith to isolate mutants deficient in the transport of glucose and glucose analogues in *E. coli*.

In *Pseudomonas fluorescens*, 3FG inhibits the bacterial
growth with glucose as source of carbon. It has been reported that resting whole cell suspensions of Ps. fluorescens metabolize 3FG to 3FGA. Provision of chemically synthesized 3FGA as exogenous substrate allows the further consumption of 1 g atom of oxygen per mole of substrate to yield an oxidation product tentatively considered to be 3-deoxy-3-fluoro-2-keto-D-gluconic acid (3F2KGA). Cell-free extracts, however, oxidize 3FG immediately, to an extent of 2 g atoms of oxygen per mole of substrate and with the retention of the C-F bond to yield presumably 3F2KGA. Recently, Taylor et al. have presented evidence for the characterization of the oxidation product to be 3F2KGA following its isolation from cell-free extracts of Ps. fluorescens. With partially purified enzyme preparation from cell-free extracts of Ps. fluorescens, 3FG and 3FGA are substrates for the bacterial glucose oxidase and gluconate dehydrogenase, with Km values 18.2 mM and 11.8 mM, respectively. Evidence is also obtained that the same enzymes which oxidize glucose and gluconic acid, oxidize 3FG and 3FGA. In the concentration range of 5-50 mM, 3FG and 3FGA are competitive inhibitors of this enzyme preparation for gluconate, with Ki values of 47.5 mM and 14.8 mM, respectively.

In order to study the specificity of 3FG metabolism by Ps. fluorescens, 4FG has been used in this system. Incubation of resting cell suspensions of Ps. fluorescens with 4FG results in a negligible oxidation of the sugar, with the
release of fluoride anion. Incubation of 4FG with crude cell-free extracts results in a rapid oxidation of the sugar, to an extent of 2 atoms of oxygen per molecule of 4FG without the fluoride anion liberation. The stiochiometry of oxidation suggests that 4FG may act as a substrate for glucose oxidase to produce 4FGA which is then oxidized by gluconate dehydrogenase to yield 4F2KGA. The inability of whole cells to oxidize 4FG may be due to the failure of the transport of 4FG to the site of the glucose oxidase in the cell envelope. 4FG can not serve as a carbon source for the growth of Ps. fluorescens and reduces the extent of bacterial growth on glucose. The metabolism of 4FG in Ps. fluorescens may be interpreted by the hypothesis that 4FG is able to act as an alkylating agent and bind to a protein required for glucose transport with the corresponding release of fluorine as fluoride ion. Future investigations with 4FG and Ps. fluorescens might involve the isolation and localization of the 4F2KGA, and establish its metabolic fate by using $^{14}$C or tritium labelled 4FG; hopefully this may lead to the development of new synthetic antibiotics.

Using deoxy and fluorodeoxy glucose analogues to investigate the binding requirements for sugar transport in hamster intestine, Barnett and his coworkers $^{69,70}$ have established the usefulness of these compounds by a comparison of the inhibition constants of the selected sugars for glucose transport. They used this information
to suggest the hydrogen bonding sites between glucose and the carrier protein. Similar studies with human erythrocytes have recently been reported. The findings confirmed the possibility that sugars bind in the pyranose form and a general model for the binding of sugars to human erythrocyte glucose carrier protein is also proposed. Recent work of Riley & Taylor has shown that 3FG is transported across the human erythrocyte membrane by the glucose carrier, and that the binding of the glucose and 3FG to the transport system is equivalent.

The earlier work of Bessell et al. made use of deoxy-fluoro-D-glucopyranoses and related compounds to study the substrate specificity of yeast hexokinase. Their results show that only modification at C₂ of the glucose molecule can be made without loss of binding to the enzyme. Replacement of -OH at C₁ in α or β-D-glucopyranose by a fluorine substituent, however, results in complete loss of binding to the enzyme, this suggests either -OH at C₁ plays a vital role in the binding, or D-glucose does not bind in its pyranose form. They also postulated that -OH at C₃ and -OH at C₄ could have similar roles in substrate–enzyme binding and the hydroxyl groups at position 3, 4, and 6 do not function solely as hydrogen-bond acceptors when located at the receptor site. Recent studies of Bessell and Thomas have involved the investigation of the effect of deoxyfluoro-D-glucose-6-phosphates on yeast glucose phosphatase isomerase. Both 3FG-6-P and 4FG-6-P are substrates
for this enzyme, and in addition the products of this reaction, 3-deoxy-3-fluoro- and 4-deoxy-4-fluoro-D-fructose-6-phosphate respectively, were good substrates for phosphofructokinase. 2FG-6-phosphate is found to be a competitive inhibitor of glucose phosphate isomerase, with a Ki value of 11.3 mM. Although the hydroxyl groups of sugar phosphates are of minor importance in the binding of the substrate to the enzyme\textsuperscript{75}, a 10-fold decrease in binding is observed when -OH at C\textsubscript{3} and -OH at C\textsubscript{4} of the D-glucose-6-phosphate are replaced by a fluorine substituent. Further, experimental data also imply that -OH at C\textsubscript{2} does not play an important role in binding.

\textbf{Section B} The Metabolism and Transport of Glucose By \textit{E. coli} Strain

Since the appearance in 1964 of the classic paper by Kundig, Ghosh & Roseman\textsuperscript{76} on the phosphoenolpyruvate:hexose phosphotransferase system, interest in phosphoenolpyruvate has focused to a large extent on its role in hexose phosphorylation and the transport of sugars into bacterial cells.

The phosphoenolpyruvate:hexose phosphotransferase system from \textit{E. coli}\textsuperscript{76-83} has been resolved into three components, called Enzyme I, Enzyme II and HPr, which participate in the following reactions:

\begin{equation*}
\text{Phosphoenolpyruvate} + \text{HPr} \xrightarrow{\text{Enzyme I}} \text{Phospho-HPr} + \text{Pyruvate}
\end{equation*}
Phospho-HPr + sugar $\xrightarrow{\text{Enzyme II}}$ sugar-6-phosphate + HPr

Mg$^{2+}$

All three components appear to be located at the cell surface and are probably membrane-bound\textsuperscript{80}, although Enzyme I and HPr are readily solubilized\textsuperscript{77,80,82}. HPr is a heat stable protein with a molecular weight of about 10,000\textsuperscript{84}, and has a histidine residue which functions as a phosphoryl carrier during the phosphotransferase reaction\textsuperscript{76}. With the exception of the Enzyme II for glucose and mannose, most Enzymes II are inducible, and differ in their specificites for sugars\textsuperscript{77}.

A transport role for the phosphoenolpyruvate:hexose phosphotransferase system in \textit{E. coli} was originally described by Kundig \textit{et al.}\textsuperscript{77} A more convincing evidence for this system has been obtained by Kaback\textsuperscript{82}, who showed that vesicles formed from isolated membranes of \textit{E. coli} accumulated glucose as its phosphorylated derivative. Incubation of the membrane preparation and phosphoenolpyruvate with $^3$H-glucose in the external medium and $^{14}$C-glucose in the intramembranal pool resulted in the accumulation of $^3$H-glucose-6-phosphate much more rapidly than $^{14}$C-glucose-6-phosphate. This indicates that phosphorylation accompanied migration of glucose from the outside to the inside of vesicles. Membrane preparations from a mutant lacking Enzyme I failed to accumulate glucose either in the presence or absence of PEP. The work of Ghosh & Ghosh\textsuperscript{80} is also in accord with the idea that glucose
is phosphorylated with PEP during transport.

The experimental evidence mentioned above demonstrates almost unequivocally that the transport of glucose is mediated by the PEP : hexose phosphotransferase system. A model for the mechanism of "vectorial phosphorylation" of glucose across a bacterial membrane has been presented in which Enzyme II is complexed with both HPr and Enzyme I in such a manner that the phosphorylation of external sugar results in the passage of this sugar through a diffusion barrier. In this model, shown in Figure 5, Enzyme II is depicted as a horseshoe-shaped structure situated in a porelike region in the membrane. The catalytic site of Enzyme II, located at the apex of the horseshoe, is attached to the wall of the pore, whereas the arms of the structure are free to move. At one extremity of the horseshoe, there is a binding site for HPr. When viewed in cross section, the plane of uncomplexed Enzyme II would be parallel to the wall of the pore (A). In the intact system, in the presence of exogenous HPr and Enzyme I, the plane of the Enzyme II would be tilted towards the inside surface of the membrane (B). In this conformation, when sugar binds to the catalytic site from the outside, it is phosphorylated on the inside surface of the complex, and release to the interior of the cell membrane vesicle. By this means, a vectorial phosphorylation of the sugar would be accomplished producing a group-translocation type of transport mechanism.
FIGURE 5

SCHEMATIC MODEL FOR THE MECHANISM OF VECTORIAL
(i.e. transport) PHOSPHORYLATION OF GLUCOSE
BY ISOLATED BACTERIAL MEMBRANE
PREPARATION

KEY:

A: Stripped Enzyme II

B: Transport: HPr and Enzyme I on inside
FIGURE 5

SCHEMATIC MODEL FOR THE MECHANISM OF VECTORIAL PHOSPHORYLATION OF GLUCOSE BY ISOLATED BACTERIAL MEMBRANE PREPARATION

[caption with diagram showing catalytic site, HPr binding site, and Enzyme I binding site with annotations]

Outside

Inside

(A)

(B)
The metabolism of D-glucose in *E. coli* was described as a combination of the Embden-Meyerhof pathway and the pentose phosphate pathway to the extent of 72% to 28% \(^8\). The significance of these two pathways in *E. coli* had previously been investigated in certain laboratories \(^87,88,89\), and it was concluded that glucose is metabolized primarily via the Embden-Meyerhof pathway and to some extent via the pentose phosphate pathway. Owing to the recent discovery of the methylglyoxal pathway, the pathways of glucose metabolism in *E. coli* have been modified \(^90\) and are as outlined in Figure 6 and Figure 7.

The methylglyoxal pathway for the formation of pyruvate from dihydroxyacetone-P (Figure 6) was first proposed by Cooper & Anderson \(^91\), who found that the triose phosphate isomerase-deficient mutant of *E. coli* formed some methylglyoxal from glucose, or gluconate. The discovery of the methylglyoxal pathway in *E. coli* is particularly interesting in view of the earlier work implicating this compound in glucose metabolism \(^92,93\). Because of the sensitivity of methylglyoxal synthase to inorganic phosphate, Hopper and Cooper \(^94\) proposed that the pathway might normally function when Pi is low and glyceraldehyde-3-phosphate dehydrogenase less active. Nevertheless, the presence of glyoxylase synthase in *E. coli* seems reasonable, in view of the toxicity of methylglyoxal \(^92\).
FIGURE 6

PATHWAYS OF GLUCOSE METABOLISM IN ESCHERICHIA COLI

Abbreviations:

gpd: glucose-6-phosphate dehydrogenase

pgi: phosphoglucone isomerase

pfk: phosphofructokinase

fdp: fructose-1,6-diphosphate phosphatase

fda: fructose-1,6-diphosphate aldolase

tpi: triose phosphate isomerase

pgl: 6-phosphogluconolactonase

gnd: gluconate 6-phosphate dehydrogenase

tkt: transketolase
FIGURE 6

PATHWAYS OF GLUCOSE METABOLISM IN ESCHERICHIA COLI

Glucose → Glucose-6-P → 6-P-Gluconate

↑

Glucose-6-P → Fructose-6-P → Pentose-P

↑

↓

Fructose-6-P → Fructose-1,6-dP → Dihydroxyacetone-P

Fructose-1,6-dP → Glyceraldehyde-3-P

↓

↑

Dihydroxyacetone-P → Pyruvate

↓

↑

Methylglyoxal → Phosphoenolpyruvate

↓

↑

D-Lactate
FIGURE 7

THE NON-OXIDATIVE BRANCH OF THE PENTOSE-PHOSPHATE PATHWAY

Abbreviations:

gnd: gluconate-6-phosphate dehydrogenase

ppe: phosphoketopentose-3-epimerase

rpi: ribose-phosphate isomerase

tkt: transketolase

tal: transaldolase
FIGURE 7

THE NON-OXIDATIVE BRANCH OF THE PENTOSE-PHOSPHATE PATHWAY

Gluconate-6-P

\[ \text{gnd} \]

\[ \text{Ribulose-5-P} \]

\[ \text{ppd} \]

\[ \text{rpi} \]

\[ \text{Xylulose-5-P} \]

\[ \text{Ribose-5-P} \]

\[ \text{Glyceraldehyde-3-P} \]

\[ \text{tkt} \]

\[ \text{Sedoheptulose-7-P} \]

\[ \text{tkt} \]

\[ \text{tal} \]

\[ \text{Fructose-6-P} \]

\[ \text{Erythrose-4-P} \]

\[ \text{Fructose-6-P} \]

(Sum') \[ 3 \text{ Pentose-P} \leftrightarrow 2 \text{ Hexose-P} + 1 \text{ Triose-P} \]
Section C The Metabolism of D-arabinose by E. coli Strain.

The majority of the information gained on the metabolism of D-arabinose in this organism comes from the investigations of LeBlanc & Mortlock\textsuperscript{95,96} working with a strain of E. coli K 12. As a result of these detailed studies, a new pathway of D-arabinose catabolism in E. coli is now established as shown in Figure 8.

The three enzymes in this pathway, i.e. isomerase, kinase, and aldolase, are common to those of the metabolism of L-fucose\textsuperscript{97} by this organism. In figure 8, the dihydroxyacetone phosphate, one of the products of the aldolase formed, might be further metabolized via enzymes of the glycolytic pathway. Glycolaldehyde is most likely oxidized to glycolate by a non-specific aldehyde dehydrogenase, constitutively synthesized by E. coli\textsuperscript{98}. Although pathways for the metabolism of glycolate in E. coli have been described\textsuperscript{99}, no further degradation of the glycolate to glyoxylate was reported by LeBlanc & Mortlock.
FIGURE 8

PATHWAY OF D-ARABINOSE CATABOLISM IN ESCHERICHIA COLI

D-arabinose

\[
\begin{align*}
&\text{CHO} \\
&\text{H-C-OH} \\
&\text{HO-C-H} \\
&\text{H-C-OH} \\
&\text{CH}_2\text{OH}
\end{align*}
\]

D-ribulose

\[
\begin{align*}
&\text{CH}_2\text{O-P} \\
&\text{C=O} \\
&\text{HO-C-H} \\
&\text{H-C-OH} \\
&\text{CH}_2\text{OH}
\end{align*}
\]

D-ribulose-1-phosphate

\[
\begin{align*}
&\text{CH}_3\text{OH} \\
&\text{C=O} \\
&\text{CH}_2\text{O-P}
\end{align*}
\]

Glycolaldehyde

\[
\begin{align*}
&\text{CHO} \\
&\text{CH}_2\text{OH}
\end{align*}
\]

Glycolate

\[
\begin{align*}
&\text{COOH} \\
&\text{CH}_2\text{OH}
\end{align*}
\]
Section D' Glucose Effect on Lactose Utilization in E. coli Strain

It was first noted by Monod\textsuperscript{100} that the growth of wild type E. coli on a medium containing glucose and one of a number of other sugars followed a diauxic growth, in which the utilization of the second sugar did not begin until after the supply of glucose has been exhausted. This is a lag period corresponds to the time required to produce induced levels of enzymes needed for utilization of the second sugar\textsuperscript{101}. This phenomenon of specific inhibition by glucose of inducible enzyme formation, observed also in the case of some inducible degradative enzymes other than those involved in sugar utilization, has become known as the "glucose effect".

Magasnik\textsuperscript{102} has introduced the term 'catabolites repression' to explain the glucose effect. According to Magasnik, catabolites from glucose accumulate in the cell and repress the formation of enzymes whose activity would augment the already large intracellular pool of these compounds. This hypothesis of catabolite repression is supported by certain observations\textsuperscript{103, 104, 105, 106}. Later in 1969, McGinnis & Pagigen\textsuperscript{107} postulated that E. coli has a general regulatory mechanism, termed catabolite inhibition, which controls the activity of early reactions in carbohydrate metabolism, allowing certain substrates to be utilized perferentially. The phenomenon is distinct from catabolite repression in that enzyme activity inhibition is either at
the first metabolic conversion of the substrate or at the
level of entry of the substrate into the cell.

The glycolysis of lactose by \textit{E. coli} has been of
interest in connection with the problem of disaccharide utili-
ization. The enzyme \(\beta\)-galactosidase is responsible for the
utilization of lactose, by which galactose and glucose are
produced inside the cell. It has been established that the
lactose (lac) operon in \textit{E. coli} consists of a group of
linked genes controlling the synthesis of \(\beta\)-galactosidase,
galactoside permease, and thiogalactoside transacetylase\textsuperscript{108, 109}
(Figure 9). The inhibitory effect of glucose on \(\beta\)-
galactosidase synthesis has been described to be a case of
catabolite repression\textsuperscript{110, 111}. Loomis & Magasanik\textsuperscript{112, 113}
have isolated the catabolite repressor-insensitive mutants
for \(\beta\)-galactosidase and pleiotropic for the lac operon,
and evidence for production of a lac repressor protein by
the \(i\) gene\textsuperscript{114} has also been obtained. Further, Kaempfer
and Magasanik\textsuperscript{115} reported that repressor of the lac operon
by the \(i\) gene apparently affects only the synthesis of
specific mRNA. It is visualized that the repressor has an
affinity for a catabolite which serves as the corepressor
and alters repressor binding to the lac operon gene.

Evidence that catabolite repression may be due to
compounds which are not catabolites of glucose has been
presented by Goldenbaum, Broman & Dobrogosz\textsuperscript{116}, who showed
that \(N\)-acetylglucosamine caused catabolite repression of
\(\beta\)-galactosidase synthesis in mutant of \textit{E. coli} able to
FIGURE 9

A SCHEMATIC REPRESENTATION OF THE TRANSCRIPTION AND TRANSLATION OF THE LACTOSE OPERON

Legend

A schematic representation of the transcription and translation of the lactose operon based on Jacob and Monod. The z gene controls the synthesis of β-galactosidase; the y gene that of the permease; and the x gene, that of the galactoside transacetylase. The i gene controls the inducibility of the operon.

Taken from: Alpers & Tomkins.
FIGURE 9

A SCHEMATIC REPRESENTATION OF THE TRANSCRIPTION AND TRANSLATION OF THE LACTOSE OPERON

regulator operator gene gene structural genes
i o z y x

genes

messenger RNA

proteins
galactosidase permease Acetylase
phosphorylate acetylglucosamine but not metabolize it further.

Inhibition of galactoside transport may also lead to inhibition of \( \beta \)-galactosidase and galactoside permease synthesis by lowering the intracellular inducer concentration\(^{117}\). Inhibition of the inducer uptake is another important mechanism in the prevention of galactose utilization by glucose\(^{118,119}\) and as McGinnis & Pagigen\(^{107}\) suggested, may be generally important.
Characterization and Cultivation of Organisms

The organisms used in this study were a neotype strain of *Escherichia coli* (ATCC 11775), and *E. coli* K12 (ATCC 14948). Both species were obtained as freeze-dried samples from the American Type Culture Collection, Rockville, Maryland, U.S.A. The organisms were periodically tested by a variety of morphological and biochemical criteria. Table 1 lists the biochemical criteria used in the periodic characterization of the two organisms.

For the cultivation of organisms the following semi-defined medium of Davis and Mingioli\(^{120}\) was used throughout the studies:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>K(_2)HPO(_4)</td>
<td>7.0</td>
</tr>
<tr>
<td>KH(_2)PO(_4)</td>
<td>3.0</td>
</tr>
<tr>
<td>(NH(_4))(_2)SO(_4)</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO(_4) \cdot 7H(_2)O</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
<td>0.2</td>
</tr>
<tr>
<td>Trace elements solution</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Carbon source (glucose, lactose or D-arabinose)</td>
<td>2.0</td>
</tr>
</tbody>
</table>
CHAPTER II

MATERIALS AND METHODS

CHARACTERIZATION AND CULTIVATION OF ORGANISMS

The organisms used in this study were a neotype strain of *Escherichia coli* (ATCC 11775), and *E. coli* K 12 (ATCC 14948). Both species were obtained as freeze-dried samples from the American Type Culture Collection, Rockville, Maryland, U.S.A. The organisms were periodically tested for purity by a variety of morphological and biochemical criteria. Table 1 lists the biochemical criteria used in the periodic characterisation of the two organisms.

For the cultivation of organisms the following semi-defined medium of Davis and Mingioli\(^1\) was used throughout the studies:

<table>
<thead>
<tr>
<th>Component</th>
<th>Grams/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{K}_2\text{HPO}_4)</td>
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</tr>
<tr>
<td>(\text{KH}_2\text{PO}_4)</td>
<td>3.0</td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4)</td>
<td>1.0</td>
</tr>
<tr>
<td>(\text{MgSO}_4\cdot 7\text{H}_2\text{O})</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
<td>0.2</td>
</tr>
<tr>
<td>Trace elements solution</td>
<td>1.0ml</td>
</tr>
<tr>
<td>Carbon source (glucose, lactose or D-arabinose)</td>
<td>2.0</td>
</tr>
<tr>
<td>Characteristic Biochemical Properties of E. coli</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Type of growth in Hugh &amp; Leifson's medium (1% glucose)</td>
<td>Acid and gas produced throughout both aerobic and anaerobic tubes</td>
</tr>
<tr>
<td>Methyl red</td>
<td>+</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>-</td>
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<tr>
<td>Indole production</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Growth in Kosser's citrate medium</td>
<td>-</td>
</tr>
<tr>
<td>Litmus milk</td>
<td>Acid production with development of gas. (Pink milk)</td>
</tr>
</tbody>
</table>
Solution of trace elements: \[ mg/100\text{ml} \]

- FeSO\(_4\) \cdot 4\text{H}_2\text{O} \quad 40
- MnSO\(_4\) \cdot 4\text{H}_2\text{O} \quad 40
- ZnSO\(_4\) \cdot 7\text{H}_2\text{O} \quad 20
- CuSO\(_4\) \cdot 5\text{H}_2\text{O} \quad 4
- CoCl\(_2\) \cdot 6\text{H}_2\text{O} \quad 4
- KI \quad 30
- Na\(_2\)MoO\(_4\) \cdot 2\text{H}_2\text{O} \quad 5
- CaCl\(_2\) anhyd. \quad 500
- NaCl \quad 1000

The ingredients were dissolved in distilled water and sterilized by autoclaving at 120°C/15 minutes without the carbon source, a 3% (w/v) solution of which was sterilized by autoclaving separately at 120°C/15 minutes to prevent caramelisation, and was aseptically added to the sterilized salts medium to give a final concentration of 0.2% carbon source. Solutions of deoxy, fluoro sugar were sterilized by passage through 0.2 μm membrane filters (Nalgene Sybron Corporation, Rochester, N.Y., U.S.A.)

For the routine maintenance of the organisms, the glucose/mineral salts medium was solidified by the addition of 2.0% (w/v) agar. Alternatively, slopes of nutrient agar (Difco 0001) were used. All the chemicals used were of A.B. grade.
Culture Techniques

For the manometric investigation of washed cell suspensions, the necessary cell yields were obtained by the growth of organisms in 250 ml Erlenmeyer flasks, each containing 75 ml glucose/mineral salts medium. Inocula for these cultures were prepared from 24 hour slope cultures, by washing with sterile distilled water and transferring with the usual aseptic precautions, the appropriate amount to the Erlenmeyer flasks. Cultures were incubated at 30°C in an orbital rotary shaker (Lab-Line Instruments, Inc., Melrose Park, Illinois). Using the above procedure cell yields in the region of 1.0 - 1.5 mg dry weight of cells per ml of the medium were obtained after 10 hours (for E. coli, ATCC 11775) or 13 hours (for E. coli K12, ATCC 14948) of incubation, at which time the cells were entering the stationary phase of growth.

Cells were harvested in a Sorvall superspeed RC2-B centrifuge (Ivan Sorvall Inc., Newtown, Connecticut, U. S. A.) at 4,080 xg for 20 minutes, washed twice in 0.067 M phosphate buffer pH 7.0 and resuspended in buffer of the same molarity and pH to 10-20 mg dry weight/ml. All operations were conducted at room temperature.

For frozen and thawed cells or cell-free extracts, the necessary cell yields were obtained with 6 x 250 ml cultures in 1 litre Erlenmeyer flasks, incubated at 30°C on a rotary shaker. Inocula for these cultures were prepared from 24 hour slope cultures by washing with sterile distilled water.
and transferring, with the usual aseptic precautions, the appropriate amount to the glucose/mineral salts media contained in 1 litre Erlenmeyer flasks. The flasks were aerated by agitation on a rotary shaker at 30°C for 12-14 hours when suitable growth was achieved. The cells were harvested in a Sorvall superspeed RC2-B centrifuge, washed in 0.067M phosphate buffer, and stored at -20°C.

Cell-free Extracts

Cell-free extracts were produced by ultrasonication. Cell breakage was achieved with a Sonic Dismembrator 300. (Artek Systems Corporation, Farmingdale, N.Y.)

A portion of frozen cell paste was thoroughly mixed with 0.067M phosphate buffer pH 7.0 to produce a cell suspension. The bacterial suspension was cooled to 4°C and was subjected to eight, half minute periods of ultrasonic disintegration, using the Artek #300 Sonic Dismembrator operating at 70% of full power output. The temperature during cell breakage being maintained at 0°C - 4°C by an ice/salt mixture. The ruptured cell suspension was centrifuged at 17,000 xg for 15 minutes at 4°C in the Sorvall superspeed RC2-B centrifuge. The straw-colored supernatant from this treatment was drawn off from the sedimented material to produce a cell free extract.

Frozen and Thawed Cells

Frozen and thawed cells were obtained by thawing the
frozen cells which had been kept at $-20^\circ C$ for at least 12 hours, and which were then washed with 0.067M phosphate buffer pH 7.0 and resuspended in the same buffer.

**Manometry**

The oxidation of exogenous substrate by resting whole cell suspensions was followed by the manometric method$^{122}$, using a Warburg respirometer with 15 ml reaction flask (Gilson Medical Electronics, Middleton, Wisconsin) containing 2.0 ml reaction volume. A solution of 0.2 ml 20% KOH, absorbed on a folded paper wick, was placed in each centre well to absorb CO$_2$. The incubation temperature was 30°C.

The manometers were calibrated by using the ferricyanide-hydrazine method which was originally described by Michaelis and Rona$^{123}$.

**Chromatography**

Paper chromatography was performed using Whatman No. 1 chromatography paper, using the ascending technique of development. The system used was butanol–ethanol–water (100:60:36, v/v).

Thin layer chromatography was performed using Silica gel plate. After plate development and removal of solvent, organic compounds were located by spraying with sulphuric acid and heating at 160°C for 10 minutes. Plates were dried at 105°C for 1 hour before used. The system used was ethyl acetate–acetic acid–water (3:3:1, v/v).
Spectrophotometry

All visible absorption spectra and optical density measurements were obtained with a Beckman Acta M-series spectrophotometer.

Estimations

1. Quantitative method of the assay for dry weight of cells: a known volume (1 ml) of the cell suspension was filtered onto a previously dried and weighed milli-pore filter, washed free of suspending buffer and the filter dried to constant weight. The filtration was performed on a Gelman borosilicate filter funnel assembly (Gelman Instrument Company, Ann Arbor, Michigan).

2. Protein was determined by the Lowry Method\textsuperscript{124}. \(\alpha\)-chymotrypsin (Sigma Chemical Company, St. Louis, M.O.) was used to construct a calibration curve. Extinction readings were taken on a Beckman Acta M-series spectrophotometer.

3. 3FG or 4FG was assayed by the 6-toluidine method\textsuperscript{125,126} or by the glucose oxidase method\textsuperscript{127} using the glucostat reagent. In common with many other glucose analogues\textsuperscript{128}, 3FG or 4FG is a substrate for glucose oxidase, but is oxidized at a much lower rate. Samples of solutions to be assayed for the deoxy-fluoro sugar were diluted to 2ml and were incubated for 4 hours in the dark at 30\(^\circ\)C with 8 ml of the glucostat reagent according to the
macro method of regular glucostat. The rate of oxidation of the chromogen dianisidine, measured as increase in absorbance at 400 nm, was directly proportional to the deoxy-fluoro sugar concentration for the tested range: 0.04 mg/2ml - 0.4 mg/2ml.

4. 3FA was assayed by the O-toluidine method\textsuperscript{126}. The yellow-orange colored complex produced was measured as increase in absorbance at 480nm.

5. Fluoride anion determinations were made by coupling a model 94-09 fluoride electrode (Orion Research Inc., Cambridge, Massachusetts, U.S.A.) to a Orion Model 701 digital pH/mv meter.

6. Phosphorylation of 3FG or 4FG: a 2 ml portion of a suspension of organisms or of an extract was added to 0.5 ml of 0.12M MgCl\textsubscript{2} containing the deoxy-fluoro sugar and other substrates where indicated. After up to 3 hours at 37°C, organisms were centrifuged off. The deoxy, fluoro sugar-6-phosphate in the supernatant was demonstrated qualitatively by ascending paper chromatography, using a butanol-ethanol-water solvent (100 : 60 : 36, v/v) and developing with p-anisidine hydrochloride\textsuperscript{129}. The deoxy, fluoro sugar phosphate was determined quantitatively by removal of it and subsequent assay of residual deoxy, fluoro sugar using the O-toluidine method. The deoxy, fluoro sugar-phosphate was removed by ion exchange: 0.5 ml samples were added to 9.5 ml of distilled
water and 5 g of Amberlite IR-45(OH) in a 250 ml Erlen-
meyer flask and the mixture was shaken for 45 minutes
at room temperature. Paper chromatography showed that
removal of the deoxy, fluoro sugar-phosphate was virtually
complete after this time.

7. β-galactosidase activity in the whole organisms was
determined as o-nitrophenyl galactoside (ONPG)
hydrolysis. Organisms were washed and then incubated
in 0.05M sodium phosphate buffer, pH 6.5, containing 2%
(v/v) toluene and 50 μg chloramphenical/ml for 30 minutes
at 37°C. A 0.5 ml sample of toluene-treated organisms
was incubated at 37°C with 4.5 ml of 1.7 mM ONPG in
0.05M sodium phosphate buffer, pH 6.5. After a suitable
period (up to 20 minutes) the reaction was stopped with
2 ml of 1.0 M K₂CO₃ and o-nitrophenol determined as
increase in absorbance at 420 nm. The β-galactosidase
activity of cell-free extracts was similarly determined
by incubation with ONPG in sodium phosphate buffer.

Chemicals

Most of the chemicals were obtained from Sigma
Chemical Company, St. Louis, M.O., and from Fisher Scientific
Company, Fair Lawn, N.J. The chemicals were used as A.R.
grade where appropriate.

Glucostat was obtained from Worthington Biochemical
Corporation, Freehold, New Jersey.
4-deoxy-4-fluoro-D-glucose\textsuperscript{130} was synthesized by Dr. G. L. Gagneja, and its purity was checked by ascending silica gel thin-layer chromatography with ethyl acetate - acetic acid - water (3 : 3 : 1, v/v) and ethyl acetate - ethanol (10 : 1, v/v) developing solvent systems.

\begin{itemize}
  \item 3-deoxy-3-fluoro-D-glucose\textsuperscript{131} and 3-deoxy-3-fluoro-\beta-D-arabinose\textsuperscript{132} were obtained from Dr. N. F. Taylor.
\end{itemize}
CHAPTER III
EXPERIMENTAL RESULTS

THE METABOLIC EFFECTS OF 4-DEOXY-4-FLUORO-D-GLUCOSE ON E. COLI, ATCC 11775.

Growth Studies with E. coli, ATCC 11775

Initial experiments were designed to determine whether E. coli could grow in the mineral salts medium containing 4-deoxy-4-fluoro-D-glucose (4FG) as sole carbon source.

Experiment 1

10 ml aliquots of mineral salts solution were autoclaved at 120°C x 15 minutes in 250 ml Erlenmeyer flasks. A solution of 4FG was sterilized by filtration and was aseptically added to give a final concentration of 5 mM 4FG. A control flask containing mineral salts plus 5mM glucose was also included. The final incubation volumes of all flasks were 15 ml. The flasks were inoculated with 0.2 ml of a culture of E. coli which had been grown in glucose/mineral salts medium with aeration for 12 hours. The flasks were incubated with aeration at 30°C. Growth was measured turbidimetrically on aseptically withdrawn samples at 620 nm in a Beckman Acta M-series spectrophotometer. An optical density reading of 1.0 indicated a dry weight of approximately 0.35 mg per ml. The fluoride ion concentration in the samples withdrawn from the test flask containing 5 mM 4FG was also measured.

The results (Fig. 10a and Fig. 10b) show that 4FG cannot act as a sole source of carbon to support the growth of
FIGURE 10

Growth of *E. coli* on Mineral Salts Medium Supplemented with Glucose and 4FG

**Fig. 10a** Mineral salts + 5 mM glucose

Key:

- ✔ Mineral salts + 5 mM glucose
- ✗ Mineral salts only

**Fig. 10b** Mineral salts + 5mM 4FG

Key:

- ✔ Mineral salts + 5 mM 4FG
- o Molarity $F^-$ in medium
E. coli. After 30 hours of incubation, the concentration of fluoride ion in the medium was $4.75 \times 10^{-5}$ Molar, equivalent to 0.95% release of fluorine.

Since E. coli is unable to grow on 4FG as sole source of carbon, experiments were designed to determine whether 4FG inhibits the growth of E. coli on glucose.

**Experiment 2**

10 ml aliquots of mineral salts solution were autoclaved at $120^\circ C \times 15$ minutes in 250 ml Erlenmeyer flasks. Solutions of 4FG and glucose were sterilized by filtration, and were aseptically added to the flasks to give mixtures of 5 mM glucose + 5 mM 4FG and 10 mM glucose + 5 mM 4FG. Control flasks containing 5 mM glucose and 10 mM glucose were also included. The final volumes of all flasks were 18 ml. The flasks were inoculated with 0.2 ml of a culture of E. coli which had been grown in glucose/mineral salts medium with aeration for 14 hours. The flasks were incubated with aeration at $36^\circ C$. Growth was measured turbidimetrically on aseptically withdrawn samples at 620 nm in a Beckman Acta M-series spectrophotometer. An optical density of 1.0 indicates a dry weight of approximately 0.35 mg per ml. The fluoride ion concentration in the samples withdrawn from the test flasks containing glucose + 5 mM 4FG was also measured.

The results (Figs. 11a, 11b) show that while E. coli does grow on glucose in the presence of 4FG, the final cell yield is less than when grown on glucose alone. After 48
FIGURE 11

Growth of *E. coli* on Glucose/Mineral Salts Medium, and Glucose/Mineral Salts Medium Supplemented with 4FG

**Fig. 11a**

Mineral salts + 5 mM glucose, and

Mineral salts + 5 mM glucose + 5 mM 4FG

**Key:**

- Mineral salts + 5 mM glucose
- Mineral salts + 5 mM glucose + 5 mM 4FG
Figure 11a

Molarity F⁻ in medium, x 10⁻⁵ Molar

O.D. at 620nm

Hours
Fig. 11b  Mineral salts + 10 mM glucose, and

Mineral salts + 10 mM glucose + 5 mM 4FG

Key:

- Mineral salts + 10 mM glucose

- Mineral salts + 10 mM glucose + 5 mM 4FG
hours, chromatographic analyses of the cultures grown in the presence and in the absence of 4FG showed no glucose to be present.

Since a small quantity of $F^-$ was detected in the above experiments, experiments were designed to examine whether $F^-$ inhibits the growth of E. coli on glucose.

**Experiment 3**

10 ml aliquots of mineral salts solution were autoclaved at $120^\circ$C x 15 minutes in 250 ml Erlenmeyer flasks. Solutions of NaF and glucose were sterilized by filtration, and were aseptically added to the flasks to give mixtures of 10 mM glucose + 20 $\mu$M NaF, 50 $\mu$M NaF, 100 $\mu$M NaF. A control flask containing 10 mM glucose was also included. The final volumes of all flasks were 25 ml. The flasks were inoculated with 0.2 ml of a culture of E. coli which had been grown in glucose/mineral salts medium with aeration for 14 hours. The flasks were incubated with aeration at $30^\circ$C. Growth was measured turbidimetrically on aseptically withdrawn samples at 620 nm in a Beckman Acta M-series spectrophotometer. An optical density of 1.0 indicated a dry weight of approximately 0.35 mg per ml.

The results (Fig. 12) showed that fluoride ion concentration even up to 100 $\mu$M did not seem to exert a significant inhibition on the growth of E. coli on glucose.
FIGURE 12

Effect of F⁻ on Growth of *E. coli* on Mineral Salts

Medium Supplemented with 10 mM Glucose

**Key:**

- **□**  ▶ Mineral salts + 10 mM glucose
- **○**  ▶ Mineral salts + 10 mM glucose + 20 μM F⁻
- **○**  ▶ Mineral salts + 10 mM glucose + 50 μM F⁻
- **▲**  ▶ Mineral salts + 10 mM glucose + 100 μM F⁻
Whole Cell Oxidation Studies with Glucose-grown Cells of E. coli, ATCC 11775

Initial experiments were performed to determine whether 4PG could be oxidized by resting cell suspensions, and whether release of fluoride ion occurred during the oxidation.

Experiment 4

6 x 75 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24-hour slope culture of E. coli, and incubated with aeration at 30°C, for 10 hours, to produce early stationary phase cells. The cells were collected by centrifugation at 4,080 xg for 20 minutes, washed twice in 0.067 M phosphate buffer pH 7.0, and resuspended to 15.5 mg dry weight per ml in buffer of the same molarity and pH.

The oxidation of 5 μmole, 10 μmole and 20 μmole amounts of the sugar was followed in the Warburg Apparatus. Fig. 13a and Fig. 13b show the oxygen uptake by these cells when furnished with limited amounts of glucose and 4PG. Glucose oxidation occurred at an immediate high rate, which was approximately linear for the first 45 minutes, and then fell off to proceed subsequently at a much reduced rate, but which again was approximately linear. Oxygen consumption by cells with 4PG as substrate occurred to a negligible extent (Table 2).
FIGURE 13

Oxidation of Glucose and 4PG by Washed Suspensions of
E. coli, ATCC 11775

Warburg conditions: temp. 30° C, reaction volume 2.0 ml,
gas phase = air.

Each flask contained:

main compartment: 1.0 ml 0.067 M phosphate buffer
    pH 7.0, 0.5 ml substrate.
side arm: 0.5 ml cell suspensions (15.5 mg dry weight
    per ml) in 0.067 M phosphate buffer pH 7.0
centre well: 0.2 ml 20% KOH + paper wick.

The reaction was initiated by tipping contents from the
side arm. Endogenous respiration subtracted.

Fig. 13a Oxidation of glucose by washed suspensions of
E. coli

Key: 

x—x 5 /umoles glucose

O—O 10 /umoles glucose

••••• 20 /umoles glucose
Fig. 13b. Oxidation of 4FG by washed suspensions of E. coli

Key:

× ———× 5 μmoles 4FG

▲ ———▲ 10 μmoles 4FG

• ———• 20 μmoles 4FG
FIGURE 13b

UPTAKE OF OXYGEN, µL

MINUTES

0 25 50 75

0 5 10 15 20


<table>
<thead>
<tr>
<th>Substrate oxidized</th>
<th>Net O₂ uptake (μl) (endogenous subtracted)</th>
<th>Moles of O₂ per mole of substrate oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>5μ moles glucose</td>
<td>485</td>
<td>3.902</td>
</tr>
<tr>
<td>5μ moles 4PG</td>
<td>14.3</td>
<td>0.115</td>
</tr>
<tr>
<td>10μ moles 4PG</td>
<td>15.4</td>
<td>0.062</td>
</tr>
<tr>
<td>20μ moles 4PG</td>
<td>17.4</td>
<td>0.035</td>
</tr>
</tbody>
</table>
Experiment 5

6 x 75 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of E. coli and incubated with aeration at 30°C, for 10 hours, to produce early stationary phase cells. The cells were collected by centrifugation at 4,080 xg for 15 minutes, washed twice in 0.067 M phosphate buffer pH 7.0, and resuspended to 17.4 mg dry weight per ml in buffer of the same molarity and pH.

9 Warburg flasks as reaction vessels as well as a Warburg flask as the fluoride control were employed. Each reaction vessel contained 20 Ìmole 4FG, 8.7 mg dry weight cells, and 0.067 M phosphate buffer pH 7.0 to 2.0 ml. The flasks were incubated at 30°C with shaking, and at various times after the start of the incubation the flasks were removed from the bath. The contents of each flask were centrifuged to remove the cells, and a fluoride ion determination was performed. The results (Fig. 14) show that very small oxidation of 4FG occurred, and that F⁻ was released. The concentration of the fluoride ion released into the medium after 5 ½ hours of incubation was 2.16 x 10⁻⁵ Molar, equivalent to 0.2% release of fluoride. No F⁻ was release from 4FG in the absence of cells.
FIGURE 14

Release of $F^-$ from 10 mMolar 4FG by Resting Cells

E. coli, ATCC 11775

Warburg conditions: temp. 30°C, reaction volume 2.0 ml, gas phase = air.

Each reaction flask contained:

- **Main compartment**: 1.0 ml 0.067 M phosphate buffer, pH 7.0, 0.5 ml substrate (20 μmoles 4FG)
- **Side arm**: 0.5 ml cell suspension (17.4 mg dry weight per ml) in 0.067 M phosphate buffer, pH 7.0
- **Centre well**: 0.2 ml 20% KOH + paper wick.

$F^-$ control flask:
- **Main compartment**: as above
- **Side arm**: 0.5 ml distilled water.

The reaction was initiated by tipping contents from side arm.

Key:

- $o-o-o-o-o$ Molarity $F^-$ in medium
- $x-x-x$ Oxidation of 10 mM 4FG by resting cells (endogenous respiration subtracted)
Since a small quantity of F\textsuperscript{-} was released into the medium during the oxidation of 4PG by \textit{E. coli}, experiments were designed to examine whether free F\textsuperscript{-} inhibits glucose oxidation in this system.

\textbf{Experiment 6}

6 x 75 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of \textit{E. coli}, and incubated with aeration at 30\textdegree{}C for 10 hours, at which time they were in early stationary phase. Cells were collected by centrifugation at 4,080 xg for 20 minutes, washed twice and resuspended to 17.4 mg dry weight per ml in 0.067 M phosphate buffer pH 7.0.

The oxidation of mixtures of 10 \textmu{} moles glucose + 1 \textmu{} moles NaF, 5 \textmu{} moles NaF, and 10 \textmu{} moles NaF by the cells were followed in the Warburg Apparatus. Three flasks, each containing 1 \textmu{} mole NaF, 5 \textmu{} moles NaF, or 10 \textmu{} moles NaF were also included to serve as controls.

The results (Fig. 15) indicated that fluoride ion concentration even up to 5 mM did not seem to have any significant, inhibitory effect on glucose oxidation by whole cells \textit{E. coli}.

\textbf{Experiment 7}

6 x 75 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture, and incubated with aeration at 30\textdegree{}C for 10 hours, to
FIGURE 15

Effect of F⁻ on Oxidation of Glucose by Washed suspensions of E. coli

Warburg conditions: temp. 30°C, reaction volume 2.0 ml, gas phase = air.

Each reaction flask contained:

main compartment: 1.0 ml 0.067 M phosphate buffer,
  pH 7.0, 0.5 ml substrate (glucose + 0 μmole NaF, 1 μmole NaF, 5 μmoles NaF, or 10 μmoles NaF).
side arm: 0.5 ml cell suspension (17.4 mg dry weight per ml) in 0.067M phosphate buffer.
centre well: 0.2 ml 20% KOH + paper wick.

Each control flask contained:

main compartment: 1.0 ml 0.067 M phosphate buffer,
  pH 7.0, 0.5 ml distilled water +
  0 μmole NaF, 1 μmole NaF, 5 μmoles NaF, or 10 μmoles NaF.
side arm: as described in reaction flask.
centre well: as described in reaction flask.

The reaction was initiated by tipping contents from side arm.
Endogenous respiration substrated.

Key:

- 10 μmoles glucose
- 10 μmoles glucose + 1 μmole NaF
- 10 μmoles glucose + 5 μmoles NaF
- 10 μmoles glucose + 10 μmoles NaF
FIGURE 15

UPTAKE OF OXYGEN, μL

TIME IN HOURS
produce early stationary phase cells. The cells were collected by centrifugation at 4,080 xg for 20 minutes, washed twice in 0.067 M phosphate buffer pH 7.0, and resuspended to 14.5 mg dry weight per ml in buffer of the same molarity and pH.

The cells were preincubated with glucose and with 4FG in Warburg flasks. Three flasks were employed, each containing 7.25 mg dry weight cells, 10 μmoles 4FG (5 mM 4FG), and 0.067 M phosphate buffer pH 7.0 to 2.0 ml; and three flasks were employed as controls, each containing 7.25 mg dry weight cells, 10 μmoles glucose (5 mM glucose) and 0.067 M phosphate buffer pH 7.0 to 2.0 ml. The flasks were incubated at 30°C with shaking for 13 hours. After this time interval, 5 μmole, 10 μmole, and 20 μmole amounts of glucose, in 0.5 ml volumes were added via the flask side arms to the flasks containing cells preincubated with 4FG and also to the control flasks containing cells preincubated with glucose.

The rates of oxidation of glucose by the 4FG preincubated cells were compared to the rates of oxidation of glucose by glucose preincubated cells. The results (Figs. 16a, 16b, 16c) show that the rates of glucose oxidation by the cells preincubated with 4FG are similar to the rates of glucose oxidation by cells preincubated with glucose, but that the extent of oxygen consumption by the cells preincubated with 4FG is slightly elevated (Table 3).
FIGURE 16

Oxidation of Glucose by Resting Cells E. coli after Preincubation with 4PG

Preincubation

Conditions: temp. 30°C, reaction volume 2.0 ml.

Each Warburg flask contained:

main compartment: 1.0 ml 0.067 M phosphate buffer pH 7.0, 0.5 ml substrate (10 μmoles 4PG or 10 μmoles glucose).

side arm: 0.5 ml cell suspension (14.5 mg dry weight per ml) in 0.067 M phosphate buffer pH 7.0

The preincubation was initiated by tipping contents from side arm. Preincubation time = 13 hours.

Oxidation of added glucose

Warburg condition: temp. 30°C, reaction volume 2.5 ml,

gas phase = air.

After the preincubation period, μmoles quantities of glucose, in 0.5 ml volumes, were added to the flask via the side arms, and the rates of oxidation of the glucose were measured. 0.2 ml volumes of 20% KOH were added to the centre wells to absorb CO₂.

Endogenous respiration subtracted.

Fig. 16a Oxidation of 5 μmoles glucose (2 mMolar)

Key:

--- Cells preincubated with 2.5 mM glucose, 13 hours.

Cells preincubated with 2.5 mM 4PG, 13 hrs.
Fig. 16b  Oxidation of 10 μmoles glucose (4 mMolar)

Key:

● Cells preincubated with 2.5 mM glucose, 13 hours.

×××× Cells preincubated with 2.5 mM 4FG, 13 hours.
Fig. 16c  Oxidation of 20 μmoles glucose ( 8 mMolar )

Key:

- Cells preincubated with 2.5mM glucose, 13 hours.

××× Cells preincubated with 2.5mM 4PG, 13 hours.
**TABLE 3**

Manometric Data on the Oxidation of Glucose by Washed Suspensions of E. coli after Preincubation with 4FG, 13 Hours

<table>
<thead>
<tr>
<th>Preincubation substrate</th>
<th>Substrate oxidized</th>
<th>Net $O_2$ uptake (µl) (endogenous respiration subtracted)</th>
<th>Moles of $O_2$ per mole of substrate oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10 µmoles glucose</td>
<td>5 µmoles glucose</td>
<td>354</td>
<td>2.85</td>
</tr>
<tr>
<td>10 µmoles 4FG</td>
<td>5 µmoles glucose</td>
<td>368</td>
<td>2.96</td>
</tr>
<tr>
<td>2. 10 µmoles glucose</td>
<td>10 µmoles glucose</td>
<td>712</td>
<td>2.86</td>
</tr>
<tr>
<td>10 µmoles 4FG</td>
<td>10 µmoles glucose</td>
<td>769</td>
<td>3.09</td>
</tr>
<tr>
<td>3. 10 µmoles glucose</td>
<td>20 µmoles glucose</td>
<td>1,421</td>
<td>2.86</td>
</tr>
<tr>
<td>10 µmoles 4FG</td>
<td>20 µmoles glucose</td>
<td>1,542</td>
<td>3.10</td>
</tr>
</tbody>
</table>
Cell-free Extract Studies with Glucose-grown Cells of E. coli, ATCC-11775.

Experiments were designed to examine the ability of cell-free extracts to oxidize 4PG, using the Warburg Apparatus.

Experiment 8

A small portion of the frozen cell paste was mixed thoroughly with 0.067 M phosphate buffer pH 7.0, sonicated at 0°C to eight, half minute periods, and centrifuged at 17,000 xg for 15 minutes yielded a straw-coloured supernatant containing 8.34 mg protein per ml. This layer was drawn off from the sedimented material and used immediately in manometric determinations.

Aliquots of the extract were tested for the ability to oxidize 5 μmole, and 20 μmole amounts of 4PG. The oxidation of 5 μmoles, and 20 μmoles glucose by the extract were also followed. The results (Fig. 17 and Table 4) show that the oxygen uptake in the case of glucose as substrate approximated to one molecule of the oxygen per mole of glucose oxidized. Oxidation of limiting amounts of the fluorinated substrate alone did not proceed to any significant extent.
FIGURE 17
Oxidation of 4FG and Glucose by a Cell-free Extract of Glucose-grown E. coli, ATCC 11775

Warburg conditions: temp. 30°C, reaction volume 2.0 ml, gas phase = air.

Each flask contained:
0.5 ml (8.34 mg) protein, 1 mole NAD (95% purity), 0.067 M phosphate buffer pH 7.0, to 1.5 ml in main compartment, 0.2 ml KOH in centre well. Reaction was initiated by tipping substrate(s) in 0.5 ml from side arm.

Endogenous respiration subtracted.

Key:

- - - 5 μmoles 4FG
- - - - 20 μmoles 4FG
△ △ △ 5 μmoles glucose
- - - - - 20 μmoles glucose
TABLE 4

Manometric Data on the Oxidation of 4FG and Glucose by Cell-free Extracts of E. coli

<table>
<thead>
<tr>
<th>Substrate oxidized</th>
<th>Initial rate of oxidation (μ moles O₂/hr/mg protein)</th>
<th>Net O₂ consumption (μl) (endogenous subtracted)</th>
<th>Moles of O₂ per mole of substrate oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μ moles glucose</td>
<td>0.38</td>
<td>124.0</td>
<td>0.998</td>
</tr>
<tr>
<td>5 μ moles 4FG</td>
<td>0.039</td>
<td>6.6</td>
<td>0.053</td>
</tr>
<tr>
<td>20 μ moles 4FG</td>
<td>0.135</td>
<td>12.8</td>
<td>0.026</td>
</tr>
</tbody>
</table>
Uptake and Rate of Phosphorylation of 4FG by Glucose-grown Cells of E. coli, ATCC 11775.

Experiments were designed to study the extent of the uptake of 4FG by resting cells; and the ability of the E. coli cells to phosphorylate 4FG.

Experiment 9

6 x 75 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of E. coli, and incubated with aeration at 30°C for 10 hours, at which time they were in early stationary phase. Cells were collected by centrifugation at 4,080 xg for 20 minutes, washed twice in 0.067 M phosphate buffer pH 7.0, and resuspended in the same buffer pH 7.0 to 17.8 mg dry weight per ml.

8 ml of the bacterial suspension were added to a 250 ml Erlenmeyer flask containing 16 mg 4FG in 8 ml 0.067 M phosphate buffer. The final reaction volume was 16 ml with 4FG at a concentration of 1 mg per ml. The flask was incubated at 30°C with aeration. At intervals samples were withdrawn, the bacteria removed by filtration and the residual 4FG determined by the glucose oxidase method.

Figure 16 shows the uptake of 4FG by resting E. coli. After 5 hours of incubation disappearance of 4FG from the medium had virtually ceased and the uptake of 4FG was limited to 0.06 mg/mg dry weight of bacteria.
FIGURE 18

Uptake of 4FG by Resting E. coli

4FG TAKEN UP (mg/mg dry weight bacteria)

0  50  100  150  200  250  300
MINUTES
Experiment 10

10 x 75 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of E. coli, and incubated with aeration at 30°C for 10 hours, at which time they are in early stationary phase. Cells were collected by centrifugation at 4,080 xg for 20 minutes. The cells were washed twice with 0.067 M phosphate buffer pH 7.0, and divided into two parts. One part forming the 'fresh cells' was resuspended in 0.067 M phosphate buffer to 15 mg dry weight per ml. The other part used for the preparation of a cell-free extract and 'frozen & thawed cells' was kept at -20°C.

A 2 ml portion of a suspension of organisms or extract was added to 0.5 ml of 0.12 MgCl₂ containing 4FG, or mixtures of 4FG and ATP, 4FG and PEP. Initial concentration of 4FG, PEP, and ATP were 10 mMolar. After up to 3 hours at 37°C, organisms were centrifuged off. An aliquot of each supernatant was freeze-dried and subjected to paper chromatography analysis. A new compound, tentatively considered to be 4FG-6-phosphate, in the supernatant was demonstrated qualitatively by ascending paper chromatography, using a butanol-ethanol-water solvent (100:60:36, v/v) and developing with p-anisidine hydrochloride.

The new compound was removed by ion exchange: 0.5 ml samples were added to 9.5 ml of distilled water and 5 g of Amberlite IR-45(OH) in a flask and the mixture was
shaken for 45 minutes at room temperature. Paper chromatography showed that removal of the compound was virtually complete after this time. Subsequent assay of the residual 4FG was performed by using d-toluidine method.

Paper chromatography using butanol-ethanol-water showed that, in the presence of PEP, 4FG (Rf 0.36) was converted to a compound with Rf 0.022. Table 5 showed the rates of phosphorylation of 4FG by the three different types of E. coli cells: fresh cells, cell-free extract, and frozen & thawed cells.

THE EFFECTS OF 3-DEOXY-3-FLUORO-D-GLUCOSE ON GLUCOSE-GROWN CELLS OF E. COLI, ATCC 11775

In order to compare the uptake and phosphorylation of 4FG by E. coli, parallel experiments using 3FG were performed.

Experiment 11

6 x 75 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of E. coli and incubated with aeration at 30°C for 10 hours, at which time they were in early stationary phase. Cells were collected by centrifugation at 4,080 xg for 20 minutes, washed twice in 0.067 M phosphate buffer, pH 7.0 to 12.6 mg dry weight per ml.

10 ml of the bacterial suspension were added to a 250 ml Erlenmeyer flask containing 20 mg 3FG in 10 ml
<table>
<thead>
<tr>
<th></th>
<th>Substrate</th>
<th>Rate of phosphorylation of 4FG (μmoles/h/mg bacterial protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen &amp; thawed cells</td>
<td>4FG:PEP</td>
<td>0.263</td>
</tr>
<tr>
<td></td>
<td>4FG:ATP</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>4FG</td>
<td>0.107</td>
</tr>
<tr>
<td>Fresh cells</td>
<td>4FG:PEP</td>
<td>-</td>
</tr>
<tr>
<td>Cell-free extract</td>
<td>4FG:PEP</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4FG:ATP</td>
<td>0.01</td>
</tr>
</tbody>
</table>
buffer. The final reaction volume was 20 ml with 3FG at a concentration of 1 mg per ml. The flask was incubated at 30°C with aeration. At intervals samples were withdrawn, the bacteria removed by filtration and the residual 3FG determined by the glucose oxidase method. Figure 19 showed the uptake of 3FG by resting E. coli. After 5 hours of incubation disappearance of 3FG from the medium had virtually ceased and uptake of 3FG was limited to 0.029 mg/mg dry weight of bacteria.

**Experiment 12**

10 x 75 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of E. coli and incubated with aeration at 30°C for 10 hours, at which time they are in early stationary phase. Cells were collected by centrifugation at 4,080 xg for 20 minutes. The cells were washed twice with 0.067 M phosphate buffer pH 7.0 and divided into two parts. One part forming the fresh cells was resuspended in 0.067 M phosphate buffer to 12.6 mg dry weight per ml. The other part used for the preparation of cell-free extract and frozen & thawed cells was kept at -20°C for 14 hours.

A 2 ml portion of a suspension of organisms or extract was added to 0.5 ml of 0.12M MgCl₂ containing 3FG, or mixtures of 3FG and ATP, 4PG and PEP. Initial concentration of 3FG, PEP, and ATP were 10 mM. After up to 3 hours at 37°C, organisms were centrifuged off. An aliquot of each super-
FIGURE 19

Uptake of 3FG by Resting E. coli
natant was freeze-dried and subjected to paper chromatography analysis. A new compound, tentatively considered to be 3FG-6-phosphate, in the supernatant was demonstrated qualitatively by ascending paper chromatography, using a butanol-ethanol-water solvent (100:60:36, v/v) and developing with p-anisidine hydrochloride.

The new compound was removed by ion exchange: 0.5 ml samples were added to 9.5 ml of distilled water and 5 g of Amberlite IR-45(OH) in a flask and the mixture was shaken for 45 minutes at room temperature. Paper chromatography showed that removal of this new compound was virtually complete after this time. Subsequent assay of the residual 3FG was performed by using o-toluidine method.

Paper chromatography using butanol-ethanol-water showed that, in the presence of PEP, 3FG (R_f 0.38) was converted to a compound with R_f 0.032. Table 6 showed the rates of phosphorylation of 3FG by the three different types of E. coli cells: fresh cells, cell-free extract, and frozen and thawed cells.

EFFECT OF 4-DEOXY-4-FLUORO-D-GLUCOSE ON LACTOSE UTILIZATION IN E. COLI K12, ATCC 14948

Since β-galactosidase and galactoside permease are always synthesized in a fixed ratio of activities108, only synthesis of β-galactosidase was followed in the following experiments where indicated. Initial experiments were designed to examine whether 4FG inhibits β-galactosidase
### Table 6

**Rates of Phosphorylation of 3FG**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of phosphorylation of 3FG (μ moles/h/mg bacterial protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen &amp; thawed cells</td>
<td></td>
</tr>
<tr>
<td>3FG:PEP</td>
<td>0.26</td>
</tr>
<tr>
<td>3FG:ATP</td>
<td>0.13</td>
</tr>
<tr>
<td>3FG</td>
<td>0.06</td>
</tr>
<tr>
<td>Fresh cells</td>
<td></td>
</tr>
<tr>
<td>3FG:PEP</td>
<td>-</td>
</tr>
<tr>
<td>Cell-free extract</td>
<td></td>
</tr>
<tr>
<td>3FG:PEP</td>
<td>-</td>
</tr>
<tr>
<td>3FG:ATP</td>
<td>0.01</td>
</tr>
</tbody>
</table>
activity in cell-free extracts of *E. coli*.

**Experiment 13**

6 x 50 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of *E. coli* K12 and incubated with aeration at 30°C for 13 hours, at which time they are in early stationary phase. Cells were collected by centrifugation at 4,080 xg for 20 minutes, washed twice with 0.05 M sodium phosphate buffer, pH 6.5, and was kept at -20°C for 14 hours before it was used for the preparation of a cell-free extract.

A 0.1 ml portion of the cell-free extracts was incubated at 37°C with 0.9 ml of 1.7 mM o-nitrophenyl galactoside (ONPG) in 0.05 M sodium phosphate buffer, pH 6.5, containing 4FG or glucose or no sugar. After a suitable period (up to 20 minutes), the reaction was stopped with 0.4 ml 1.0M K₂CO₃ and o-nitrophenol determined as increase in absorbance at 420 nm in a Beckman Acta M-series spectrophotometer.

The results (Table 7) showed that β-galactosidase activity was inhibited by 4FG and by glucose, but 4FG was a more effective inhibitor than glucose.

**Experiment 14**

6 x 50 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of *E. coli* and incubated with aeration at 30°C for 13 hours, at which time they were in early stationary phase. Cells were collected
<table>
<thead>
<tr>
<th>4FG or Glucose (mM)</th>
<th>Inhibition of β-galactosidase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>By 4FG</td>
</tr>
<tr>
<td>25</td>
<td>36.1</td>
</tr>
<tr>
<td>10</td>
<td>25.4</td>
</tr>
<tr>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>1.0</td>
<td>7</td>
</tr>
<tr>
<td>0.1</td>
<td>1</td>
</tr>
</tbody>
</table>
by centrifugation at 4,080 xg for 20 minutes, washed twice with 0.05 M sodium phosphate buffer pH 6.5 and was kept at -20°C.

A small portion of the frozen cell paste was mixed thoroughly with 0.05 M phosphate buffer pH 6.5, sonicated at 0°C to eight, half minute periods, and centrifuged at 17,000 xg for 15 minutes yielded a straw-colored supernatant. This layer was drawn off from the sedimented material to produce a cell-free extract.

A 0.1 ml portion of the cell-free extract was incubated at 37°C with 0.9 ml of ONPG in 0.05M sodium phosphate buffer pH 6.5 with or without 4FG. Rate of ONPG hydrolysis (v) was determined at different concentrations of ONPG as substrate. Four different concentrations of ONPG were employed: 0.5 g/l, 0.2 g/l, 0.1 g/l, and 0.05 g/l. 4FG concentrations used were 1.0 mM, 2.5 mM, 10 mM and 25 mM. After a suitable period (up to 20 minutes), the reaction was stopped with 0.4 ml of 1.0 M K₂CO₃ and o-nitrophenol determined as increase in absorbance at 420 nm in a Beckman Acta M-series spectrophotometer.

The results (Fig. 20) showed that the inhibition produced by 4FG on β-galactosidase is uncompetitive. The double reciprocal plots of 1/v vs. 1/s in the presence of various concentration of 4FG yielded a series of parallel lines.
FIGURE 20

Characterization of Inhibitory Effects of 4PG on β-galactosidase Activity in Cell-free Extracts

Legend

Reaction velocity (v) was determined at differing concentrations of substrate (s). ONPG was used as substrate. 4PG concentrations were: 25mM (■); 10mM (●); 2.5mM (▲); 1.0mM (○) and zero (△).
FIGURE 20

1/v (rate of o-nitrophenyl galactoside hydrolysis in arbitrary units)

1/S (g/l)
With regard to the inhibition of lactose utilization by glucose, an attempt was made to examine whether 4FG is able to bring about effects analogous to those of glucose. Since cyclic 3',5'-adenosine monophosphate (cAMP) should overcome the repression of enzyme synthesis in the presence of inducer, and isopropyl-β-D-galactoside (IPTG) should overcome any repression due to lowering of intracellular inducer concentration which might result from a decreased rate of lactose transport and metabolism; experiments were designed to study the effect on growth and β-galactosidase synthesis of adding 4FG and combination of cyclic AMP and IPTG to bacteria growing exponentially in lactose medium.

Experiment 15

200 ml of lactose/mineral salts medium was inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of E. coli K12 and incubated with aeration at 30°C for 10 hours, at which time it was in exponential phase. This culture growing exponentially in lactose medium was then divided into five parts and the additions of 10 mM 4FG, and combination of 5 mM cAMP and 1.0 mM IPTG were made. The five parts were incubated with aeration at 30°C. At intervals samples were withdrawn aseptically, the effects of the additions on growth and on the rate of β-galactosidase synthesis were followed. Growth was measured turbidimetrically on withdrawn samples at 620nm in a Beckman Acta M-series spectrophotometer. β-galactosidase activity in whole organisms was determined as o-nitrophenyl
galactoside hydrolysis.

Figure 21 and Table 8 showed the effects of 4FG, cAMP and IPTG on growth and on the rate of \( \beta \)-galactosidase synthesis during the first 60 minutes of incubation respectively.

**THE EFFECTS OF 3-DEOXY-3-FLUORO-\( \beta \)-D-ARABINOSE ON:**

(a) GLUCOSE-GROWN CELLS OF *E. coli*, ATCC 11775,
(b) D-ARABINOSE-GROWN CELLS OF *E. coli*, ATCC 11775.

The purpose of this work was to find out whether using the deoxyfluoropentose, 3FA as a substrate for *E. coli* is worth attempting in the future studies with this organism. Preliminary experiments were designed to examine the ability of whole cells to oxidize 3FA, using the Warburg Apparatus.

**Experiment 16**

4 x 75 ml amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture, and incubated with aeration at 30\(^\circ\)C, for 10 hours, to produce early stationary phase cells. The cells were collected by centrifugation at 4,080 xg for 20 minutes, washed twice in 0.067 M phosphate buffer pH 7.0 and resuspended to 10.8 mg dry weight per ml in buffer of the same molarity and pH.

The oxidation of 100 \( \mu \)moles D-arabinose and the oxidation of 100 \( \mu \)moles 3FA by the cells were followed in the Warburg Apparatus. The results (Fig. 22) showed that the oxidation of 3FA or D-arabinose did not proceed to any
FIGURE 21
Effects of cAMP and IPTG on the Growth of *E. coli*
in Lactose Medium with 10mM-4FG

Key:

Additions to lactose medium:

- None
- 4FG
- 4FG : 1.0 mM IPTG
- 4FG : 5 mM cAMP
- 4FG : 1.0 mM IPTG : 5 mM cAMP
FIGURE 21

O.D. AT 620nm

TIME IN HOURS
TABLE 8

Effects of cAMP and IPTG on the Rate of β-galactosidase
Synthesis of E. coli in Lactose Medium with 10 mM 4FG

A culture growing exponentially in lactose medium was divided into five parts and the additions below were made.

<table>
<thead>
<tr>
<th>Additions to lactose medium</th>
<th>Rate of β-galactosidase synthesis, measured up to 60 minutes after the additions were made (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.475</td>
</tr>
<tr>
<td>4FG</td>
<td>0.043</td>
</tr>
<tr>
<td>4FG:1.0mM IPTG</td>
<td>0.247</td>
</tr>
<tr>
<td>4FG:5mM cAMP</td>
<td>0.510</td>
</tr>
<tr>
<td>4FG:1.0mM IPTG:5mM cAMP</td>
<td>1.103</td>
</tr>
</tbody>
</table>
FIGURE 22

Oxidation of D-arabinose and 3FA by Glucose-grown Resting Cells of E. coli, ATCC 11775

Warburg conditions: temp. 30°C, reaction volume 2.0 ml, gas phase = air.

Each flask contained:

- **main compartment**: 1.0 ml 0.067 M phosphate buffer pH 7.0, 0.5 ml substrate.
- **side arm**: 0.5 ml cell suspension (10.8 mg dry weight per ml) in 0.067 M phosphate buffer pH 7.0
- **centre well**: 0.2 ml 20 % KOH + paper wick.

The reaction was initiated by tipping contents from side arm. Endogenous respiration subtracted.

Key:

- 100 μmoles D-arabinose
- 100 μmoles 3FA
significant extent.

**Experiment 17**

A 75 ml D-arabinose/mineral salts medium was inoculated with a suitable dilution in sterile distilled water from a 24 hour slope culture of *E. coli* and incubated with aeration at 30°C for 50 hours. Cells were collected by centrifugation and washed with sterile distilled water and resuspended in sterile distilled water.

1 ml portion of this suspension was aseptically transferred to a 250 ml D-arabinose/mineral salts medium in a one litre Erlenmeyer flask. The flask was incubated with aeration at 30°C for 14 hours, at which time they were in early stationary phase. Cells were collected by centrifugation, washed twice and resuspended to 10.8 mg dry weight per ml in 0.067 M phosphate buffer pH 7.0.

The oxidation of 50 μmole, 100 μmole amounts of 3FA, and 50 μmoles D-arabinose were followed in the Warburg Apparatus. Figure 23 showed the oxygen consumption by the cells when furnished with 50 μmoles D-arabinose. The sugar was immediately oxidized by the cells, and after 7-8 hours, the rate of oxidation of the sugar fell to endogenous oxidation rate. The net oxygen consumption during the oxidation of the sugar was 1.962 μl, equivalent to 157.8 μg atoms of oxygen, giving a stoichiometry of 3.157 g atoms of oxygen per mole of D-arabinose. Under identical experimental conditions, 3FA was oxidized by the cells to an
FIGURE 23

Oxidation of 50 μmoles D-arabinose by D-arabinose-grown Cells of E. coli, ATCC 11775

Warburg conditions: temp. 30°C, reaction volume 2.0 ml, gas phase = air.

Each flask contained:

- **main compartment**: 1.0 ml 0.067 M phosphate buffer pH 7.0, 0.5 ml substrate.
- **side arm**: 0.5 ml cell suspensions (10.8 mg dry weight per ml) in 0.067 M phosphate buffer pH 7.0
- **centre well**: 0.2 ml 20% KOH + paper wick.

The reaction was initiated by tipping contents from side arm. Endogenous respiration subtracted.
FIGURE 23

UPTAKE OF OXYGEN, μL

TIME IN HOURS
extent of 0.159 g atoms of oxygen per molecule of 3FA (Fig. 24 and Table 9).

Experiment 18

2 x 250 ml amounts of D-arabinose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the E. coli cells collected from D-arabinose/mineral salts medium during exponential growth; and incubated with aeration at 30°C for 14 hours, at which time they were in early stationary phase. Cells were collected by centrifugation, washed twice in 0.067 M phosphate buffer pH 7.0 and resuspended to 10.5 mg dry weight per ml.

10 ml of the bacterial suspension were added to a 250 ml Erlehmeyer flask containing 20 mg 3FA in 10 ml distilled water. The final reaction volume was 20 ml with 3FA at a concentration of 1 mg per ml. The flask was incubated at 30°C with aeration. At intervals samples were withdrawn, the bacteria removed by filtration and the residual 3FA determined by the o-toluidine method. After 5 hours of incubation, the uptake of 3FA was 0.11 mg/mg dry weight of bacteria (Fig. 25).
FIGURE 24

Oxidation of 3FA by D-arabinose-grown Cells of

E. coli, ATCC 11775

Warburg conditions: as in Figure 23
Each flask contained: as in Figure 23
The reaction was initiated by tipping contents from side
arm. Endogenous respiration subtracted.

Key:

- - - - - - 50 μmoles 3FA

x - - x 100 μmoles 3FA
FIGURE 9
Manometric Data on the Oxidation of D-arabinose and 3FA
by Resting E. coli, ATCC 11775

<table>
<thead>
<tr>
<th>Substrate oxidized</th>
<th>Net $O_2$ consumption, $\mu$L (endogenous respiration subtracted)</th>
<th>moles of $O_2$ per mole of substrate oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 $\mu$moles D-arabinose</td>
<td>1,962</td>
<td>1.5785</td>
</tr>
<tr>
<td>50 $\mu$moles 3FA</td>
<td>98.7</td>
<td>0.0795</td>
</tr>
<tr>
<td>100 $\mu$moles 3FA</td>
<td>115.2</td>
<td>0.0463</td>
</tr>
</tbody>
</table>
FIGURE 25

Uptake of 3FA by Resting D-arabinose-grown E. coli
CHAPTER IV
DISCUSSION

The ability of a bacterium to utilize a fluorinated organic compound as a source of carbon for growth is largely dependent on its ability to enzymically remove the fluorine atom from the molecule and allow the remaining carbon skeleton to be incorporated into the metabolic pathways. Examples have been reported where the enzymic cleavages of the C-F bond in a fluorinated compound by a bacterium allows the free, non-fluorinated carbon fragment to enter into the metabolic pathways within the cell and serve as a source of carbon and energy for growth.\textsuperscript{133, 134, 135} These probably may be due to the action of an enzyme, which is constitutive with respect to the normal substrate metabolism\textsuperscript{134, 136, 137} or is induced in response to the fluorinated compound\textsuperscript{133, 137}, which cleaves the C-F bond.

The inability of a bacterium to carry out C-F cleavage in a fluorinated organic compound, however, does not necessarily mean that the compound is unable to undergo partial metabolism by the organism\textsuperscript{138, 139}; the fluorinated substrates are only attacked when the bacterium is adapted to metabolizing the parent substrate. This suggests that the same enzyme is probably involved in the oxidation of both the natural compound and its fluoro analogue.

Indeed, the inhibitory effect of fluoro analogues on the bacterial metabolism, or on the bacterial growth may be due to one or more of the following possibilities: (i) the
inability to liberate organic fluorine as fluoride ion; (ii) fluoroacetate production with the subsequent inhibition of the TCA cycle $^{20,21}$; (iii) the inhibition of the induction of the enzymes responsible for the oxidation and assimilation of the parent compound $^{33,138,140}$; (iv) the competition for the key enzymes in a metabolic pathway between the natural substrate and the fluoro analogue, or alternatively a non-inhibitory fluoro analogue may enzymically transformed into a toxic product which retained the C-F bond capable of exhibiting competitively inhibitory effect at the enzymic level with the corresponding natural substrate; (v) the inhibition of the transport of the natural substrate into the cell by the fluoroanalogue as a competitive inhibitor towards the transport molecule $^{33}$.

Because of the various possible fates of fluorinated molecules in bacterial systems, it was of interest to study the biochemical effects of 4FG on E. coli when it is used as a substrate. It is difficult to doubt that the phosphoenolpyruvate:hexose phosphotransferase system is of fundamental biological importance. A transport role for this system in E. coli has been established, and it is believed that glucose-6-phosphate is the initial product of glucose catabolism necessary for further utilization of the substrate. Several analogues of glucose, such as methyl-α-glucoside$^{77}$, 2-deoxyglucose$^{80}$ and 3FG$^{62}$, have been reported to be phosphorylated by this phosphotransferase system through the bacterial cell membrane with their subsequent accumulation as 6-phosphate.
derivatives.

The Effect of 4-DEOXY-4-FLUOPO-D-GLUCOSE on

E. coli, ATCC 11775

E. coli is unable to grow in a mineral salts medium containing 4PG as sole source of carbon, with negligible amounts of fluoride ion is released into the medium (Fig. 10b). The inability of 4PG to act as a carbon source for the growth of E. coli, despite the negligible amount of fluoride ion released into the growth medium, may be due to the accumulation of 4PG-6-P intracellularly, which might inhibit the glycogen degradation.

When E. coli is grown in a glucose/mineral salts medium containing 4PG, the final cell yield is less than the final cell yield obtained when the bacterium is grow in a glucose/mineral salts medium in the absence of 4PG (Figs. 11a, 11b). It has been known that many fluorides, at level of 0.01 M or less, powerfully inhibit a number of phosphotransferases due to the formation of magnesium fluorophosphate, which removed the essential divalent metal, is the underlying cause. Further, the investigation of Pattabiraman suggested that fluorides inhibit hexokinase by competing with the substrates, glucose and ATP. Although low fluoride concentration is detected in the culture containing 4PG, the amount of fluoride ion released (<1%) should not be large enough to cause a significant inhibition (Fig. 12). These results may be interpreted as follows: initially 4PG competes poorly with glucose for transport into the bacterial cells;
as shown in Figs. 11a and 11b, during the first eight hours after the start of the incubation there is no reduction in cell growth compared to the cultures grown on glucose. But, as the glucose concentration is reduced by metabolism 4FG may then be able to enter the cell and accumulate as 4FG-6-P, which may inhibit or reduce the rate of induction of the enzymes responsible for further catabolism of the normal glucose. If the reduced growth were the result of inhibition of enzyme reactions by the accumulated 4FG-6-P, then glucose-6-P isomerase and phosphoglucomutase (which catalyzes the conversion of glucose-1-P to glucose-6-P) are the likely sites. Inhibition of phosphoglucomutase by 4FG-6-P probably may lead to the inactivation of glycogen degradation during the stationary phase, while both glycogen and exogenous glucose are being degraded simultaneously.

4FG is not oxidized to any significant extent by washed whole cell suspensions of *E. coli* (Fig. 13b and Table 2), but the rapid fall of the rate and extent of oxidation of 4FG may be specifically due to the inability of the system to regenerate PEP. On incubation of resting cells of *E. coli* with the fluoro analogue, it appears that the uptake of 4FG is limited to 0.06 mg/mg dry weight of bacteria (Fig. 18).

The rate of glucose oxidation by resting cells of *E. coli* which have been preincubated with 2.5 mM 4FG for 13 hours is similar to the rate of glucose oxidation by the cells preincubated with 2.5 mM glucose, but the extent of glucose oxidation by those cells subsequent to
4FG treatment is slightly elevated (Figs. 16a, 16b and 16c). Due to the very limited uptake of 4FG by whole cells, it is difficult to argue in favour of 4FG occasioning a metabolic block in the intermediary metabolism of this organism. A probable explanation is that 4FG treatment causes a shift in the energy balance of the cell due to ATP consumption. Any lowering of cellular ATP levels would favour a subsequently elevated extent of glucose oxidation to restore the energy status in a manner analogous to increased glucose assimilation by cells depleted of endogenous reserves.

Incubation of 4FG with cell-free extracts prepared from glucose-grown cells shows that only very limited oxygen consumption is involved, and that the addition of ATP does not stimulate enzymic activity (Fig. 17 and Table 4). This may be due to the rupturing of cells by sonication to release very little phosphotransferase activity in the supernatant fractions. Ghosh & Ghosh⁸⁰ have obtained cell-free particulate fractions, presumably membranes, which phosphorylated 2-deoxy-D-glucose with a relatively high specific activity, indicating that all the three components of the phosphotransferase system were obtained in a bound, rather than solubilized form. It appears, therefore, that the disintegration of the components of the membrane-bound phosphotransferase system leads to a high loss of activity.

The ability of the PEP phosphotransferase system to phosphorylate 4FG was investigated in frozen & thawed cells which catalyze the phosphorylation of sugars in the extra-
cellular medium in the presence of PEP<sup>80,62</sup>. Paper chromatography using butanol-ethanol-water showed that, in the presence of PEP, 4FG (R<sub>f</sub> 0.36) was converted to a compound with R<sub>f</sub> 0.022. This new compound is tentatively considered to be 4FG-6-P, though it hasn't be identified due to the unavailability of chemically synthesized 4FG-6-P. In the presence of externally added PEP, 4FG was phosphorylated at a low rate, and the low rate was stimulated to a smaller extent by ATP (Table 6). The origin of the 4FG-6-P under such circumstances is not certain. It may be due to the presence of small amounts of PEP within the organisms, and to stimulation of PEP synthesis by ATP; alternatively it may be due to hexokinase activity. Phosphorylation of 4FG by cell-free extracts in the presence of ATP, but not of PEP was demonstrated chromatographically; so 4FG is possibly a substrate for E. coli hexokinase. However, hexokinase activity would appear of little consequence, since the rate of ATP stimulated phosphorylation of 4FG by cell-free extracts was less than 4% of the rate of PEP-stimulated phosphorylation of 4FG by frozen & thawed organisms. Fresh cells, on the other hand, probably accumulated 4FG-6-P only internally. The relatively high rate of 4FG phosphorylation by frozen & thawed cells may be indicative of an organized state of the phosphotransferase system in E. coli, and freezing presumably destroyed the ability of the cells to accumulate, but not the ability to phosphorylate 4FG (Table 6).
The Effect of 3-DEOXY-3-FLUORO-D-GLUCOSE on E. coli, ATCC 11775

In order to compare the uptake and phosphorylation of 4FG by E. coli, parallel experiments using 3FG were performed. The results show that as a substrate for E. coli, 4FG is better than 3FG (Fig. 19 and Table 7). In the phosphorylation of 3FG, the new compound formed, as detected by paper chromatography using butanol-ethanol-water solvent system, has been reported and identified by Miles & Pirt as 3FG-6-P.

The Effect of 4-DEOXY-4-FLUORO-D-GLUCOSE on Lactose Utilization in E. coli K 12

The inhibition of β-galactosidase activity by glucose has previously been reported, though not characterized. The inhibitory effect of 3FG on β-galactosidase activity has been recently characterized as "uncompetitive." The inhibition produced by 4FG is also found to be uncompetitive (Fig. 20). The comparison between the results on 3FG and the present work on 4FG (Table 7) with reference to glucose, the degree of inhibitory power among the three sugars on β-galactosidase activity increases in the order of glucose, 4FG, 3FG.

While 4FG does not have much effect on the growth of E. coli with glucose, it may affect the utilization of lactose. This may due to inhibition by 4FG at specific steps which are involved in the utilization of lactose rather
than in glucose utilization. The inhibition of lactose utilization by 4FG is similar to that found with glucose and is consistent with the idea that 4FG is a glucose analogue. Such inhibitory reactions are often attributed to catabolites, but the ability of 4FG to bring them about indicates that they are due to 4FG itself, to the sugar phosphate or to some other closely related metabolic product.

With regard to inhibition of lactose utilization by 4FG, both catabolite repression\textsuperscript{102} and catabolite inhibition\textsuperscript{107} seem to be important. Evidence that 4FG may cause catabolite repression is mainly indirect and based on the ability of cyclic AMP to overcome the long term repressive effect of 4FG on lactose utilization. Cyclic AMP should overcome the repression of enzyme synthesis in the presence of inducer, and IPTG should overcome any repression due to lowering of intracellular inducer concentration which might result from a decreased rate of lactose transport and metabolism.

The possibilities that inhibition of galactoside transport may lead to inhibition of \(\beta\)-galactosidase and galactoside permease synthesis by lowering the intracellular inducer concentration\textsuperscript{117} or inhibition of inducer uptake prevents the utilization of galactoside by 4FG, would not appear to be an important mechanism in the effect of 4FG on growth with lactose since, firstly, a high concentration of IPTG did not overcome the inhibition, and secondly, with lactose, 4FG and cAMP together there was sufficient intracellular
inducer to allow a high rate of β-galactosidase synthesis (Table 8). Evidence that cAMP restored the rate of β-galactosidase synthesis (as measured 60 minutes after the additions) in the presence of 4FG as well as the absence, and allowed growth in the presence of 4FG at a constant but lower rate (Fig. 21 and Table 8) indicates that the repression of β-galactosidase and galactoside permease synthesis is a significant factor in 4FG inhibition of growth with lactose. However, 4FG in the presence of cAMP still caused more than 50% reduction in growth, and this presumably due to effects on β-galactosidase and galactoside permease activity.

The inhibition of 3FG on utilization of lactose and other carbon sources has been studied by Miles & Pirt. On the whole, their results with 3FG are similar to the work reported here on 4FG, however, the inhibitory effects of 4FG are less powerful than that of 3FG on lactose utilization.

The Effect of 3-DEOXY-3-FLUORO-β-D-ARABINOSE on E. coli, ATCC 11775

The inability of glucose-grown resting whole cell suspensions of E. coli to oxidize either D-arabinose or 3FA to any significant extent (Fig. 22) is probably due to the lack of the enzymes involved in the metabolism of D-arabinose. A lag phase is thus required to produce induced levels of enzymes needed for the utilization of D-arabinose.
Resting D-arabinose-grown whole cells of \textit{E. coli} oxidize D-arabinose and 3FA immediately without a lag phase (Fig. 23 and Fig. 24). Provision of D-arabinose as exogenous substrate allows the consumption of 3.157 g atoms of oxygen per mole of substrate. Under identical conditions, 3FA was oxidized by the cells to an extent of 0.159 g atoms of oxygen per mole of 3FA (Table 9). After 5 hours, the uptake of 3FA in resting cells was 0.11 mg/mg dry weight of bacteria, and the disappearance of 3FA from the medium apparently would not cease at this time (Fig. 25). Unfortunately, fluoride ion measurement was not performed in this system. Such information would indicate whether oxygen consumption arises from C-F bond cleavage of 3FA or not.

In view of the oxidation and uptake data for the three fluoro-sugars studied here, 3FA is evidently a much better substrate for \textit{E. coli}. Owing to insufficient work done with 3FA, it is still too early to predict the metabolic fate of 3FA in \textit{E. coli}. Nevertheless, the biological activity of 3FA seems interesting, hopefully someone will continue to study its effect in \textit{E. coli}.
REFERENCES


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