SYNTHETIC, ENZYMIC AND METABOLIC STUDIES OF DEOXYFLUORO-MONOSACCHARIDES.

SAMUEL TORTO. SQUIRE

University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation
SQUIRE, SAMUEL TORTO., "SYNTHETIC, ENZYMIC AND METABOLIC STUDIES OF DEOXYFLUORO-MONOSACCHARIDES." (1986). Electronic Theses and Dissertations. 3712.
https://scholar.uwindsor.ca/etd/3712

This online database contains the full-text of PhD dissertations and Masters’ theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email (scholarship@uwindsor.ca) or by telephone at 519-253-3000 ext. 3208.
NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photcopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui sont déjà l'objet d'un droit d'auteur (articles de revue, tests publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.
SYNTHETIC, ENZYMIC AND METABOLIC STUDIES OF
DEOXYFLUOROMONOSACCHARIDES

BY

Samuel Torto Squire

A DISSERTATION

Submitted to the Faculty of Graduate Studies through the
Department of Chemistry and Biochemistry in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy at the
University of Windsor
Windsor, Ontario
1986
Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.
DEDICATION

To my entire family.
ACKNOWLEDGEMENTS

I wish to express my sincere thanks to my supervisor, Dr. N.F. Taylor, for his constant encouragement and valuable guidance throughout this work.

I would also like to thank other members of my committee: Dr. R.J. Thibert, Dr. K.E. Taylor, Dr. A.H. Warner and Dr. B. Mutus (who served on my committee in the absence of Dr. K.E. Taylor) for their cooperation and advice given me throughout my entire work.

The financial assistance from the University of Windsor is also greatly appreciated.

My sincere thanks to Dr. J.M. MacIntosh, Dr. K.G. Rutherford, Dr. B.R. McGarvey for encouragement and useful advice. Thanks also to the entire staff of the Department of Chemistry and Biochemistry of the University of Windsor, not forgetting the late Mr. Ian Grove who passed away during the preparation of this dissertation, and to my fellow students for the help they have given me throughout my stay here.

I would like to thank Sungee John, my host families — the Grahams, the Brush's and the Augustine's and all who have supported me one way or another.

Finally, I would like to thank Miss M.F. Doyle for typing this dissertation.
ABSTRACT

In Part I, a brief review of the various approaches for the introduction of fluorine into carbohydrates is presented. Use of fluorocarbohydrates as biochemical probes and as intermediates for preparation of other compounds is also presented.

An attempt to chemically synthesize 3-deoxy-3-fluoro-D-fructose, by replacement of tolylsulfonyl or mesylsulfonyl groups by fluorine, is made. Neither leaving groups are displaced by fluorine, either by tetrabutylammonium fluoride or other fluoride reagents.

A rationale for the synthesis of 4-deoxy-4-fluoro-D-fructose using a combination of chemical and enzymic methods is proposed and undertaken. The proposal involves the use of 4-deoxy-4-fluoro-D-glucose. This compound is first synthesized by an established route. A new improved synthesis is reported here. The fluorinating agent in this case is diethylaminosulfur trifluoride (DAST). This approach also improves the yield.

Partially purified pyranose-2-oxidase (E.C. 1.1.3.10) is isolated from mycelia of Polyporus obtusus and a study of the enzyme specificity undertaken. Pyranose-2-oxidase is specific for the oxidation of glucose to D-arabinohexos-2-ulose (D-glucosone) with a $K_m$ value of $3.2 \pm 0.08$ mM and a $V_{max}$ of $126 \pm 0.02 \mu$mol min$^{-1}$ mg protein$^{-1}$. 

11
In contrast, 3-deoxy-3-fluoro-D-glucose is not a substrate for the enzyme. 4-Deoxy-4-fluoro-D-glucose, however, does act as a substrate with a $K_m$ value of $5.54 \pm 0.64$ mM and a $V_{\text{max}}$ of $104 \pm 0.07$ μmol min$^{-1}$ mg protein$^{-1}$ and as a competitive inhibitor of glucose with a $K_i$ value of $5.5 \pm 0.02$ mM. Pyranose-2-oxidase is immobilized with activated Sepharose 4B and the product of oxidation of 4-deoxy-4-fluoro-D-glucose isolated and characterized as 4-deoxy-4-fluoro-D-arabino-hexos-2-ulose (1). Catalytic hydrogenation of (1) yields 4-deoxy-4-fluoro-D-fructose (2). Both (1) and (2) are characterized by infrared, $^{19}$F NMR and mass spectrometry. The structure of 4-deoxy-4-fluoro-D-fructose (2) is confirmed by its conversion, by sodium borohydride reduction, to the known 4-deoxy-4-fluoro-D-glucitol.

In Part II, the toxicity of 4-deoxy-4-fluoro-D-glucose in Locusta migratoria is studied and compared with that of 3-deoxy-3-fluoro-D-glucose. 4-Deoxy-4-fluoro-D-glucose is found to be more toxic than 3-deoxy-3-fluoro-D-glucose. $LD_{50}$ for 4-deoxy-4-fluoro-D-glucose is 0.6 mg gm$^{-1}$ locust, compared with 4.8 mg gm$^{-1}$ locust for 3-deoxy-3-fluoro-D-glucose. The formation of metabolites is established by HPLC analysis. One metabolite is identified as 4-deoxy-4-fluoro-D-glucitol. The toxicity of 4-deoxy-4-fluoro-D-glucose is associated with concomitant release of fluoride ion. It is suggested that the defluorination occurs at
the point of action of aldolase, resulting in a glycolytic inhibition. A tentative mechanism for the action of the aldolase on the 4-deoxy-4-fluoro-D-glucose is presented.
TABLE OF CONTENTS

Abstract ........................................................................................................ vi
Acknowledgements ..................................................................................... ix
Table of Contents ....................................................................................... x
Abbreviations ............................................................................................ xii

PART I
INTRODUCTION .......................................................................................... 1
General .......................................................................................................... 1
Some synthetic approaches to fluorocarbohydrates .................................... 12
Replacement of the anomeric and primary hydroxyl groups of carbohydrates ......................................................... 13
Introduction of fluorine at secondary position in the sugar .................... 17
Direct displacement of hydroxy groups of fluorine ................................. 30
Radiolabelled fluorinated carbohydrates .................................................. 36
The Biochemistry of fluorinated carbohydrates ......................................... 38
Objectives ................................................................................................... 42
MATERIALS AND METHODS ................................................................. 43
Chromatography .......................................................................................... 45
Reagents ....................................................................................................... 47
Synthesis of fluoromonosaccharides .......................................................... 48
Isolation and purification of pyranose-2-oxidase from Polyporus obatusus ... 61
Determination of the activity of partially purified solubilized pyranose-2-oxidase (E.C. 1.1.3.10) ................................. 63
Kinetic and inhibition studies with pyranose-2-oxidase .......................... 64
Determination of free fluoride level at end of reaction ........................... 65
Immobilization of pyranose-2-oxidase onto cyanogen bromide activated Sepharose 4B ........................................ 66

RESULTS AND DISCUSSION .................................................................... 75
SUMMARY AND CONCLUSION ............................................................... 122

PART II
INTRODUCTION ......................................................................................... 123
MATERIALS AND METHODS ................................................................... 127
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A°</td>
<td>angstrom</td>
</tr>
<tr>
<td>([\alpha]_D^{23})</td>
<td>specific rotation (D line at 23°C)</td>
</tr>
<tr>
<td>b.p.</td>
<td>boiling point</td>
</tr>
<tr>
<td>c.</td>
<td>concentration (g/100mL)</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter(s)</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>dm</td>
<td>decimeter(s)</td>
</tr>
<tr>
<td>°C</td>
<td>degree(s) celsius</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
</tr>
<tr>
<td>DOPA</td>
<td>dihydroxyphenylalanine</td>
</tr>
<tr>
<td>2-(^{18})FG</td>
<td>[2-(^{18})F]-2-deoxy-2-fluoro-D-glucose</td>
</tr>
<tr>
<td>3-(^{18})FG</td>
<td>[3-(^{18})F]-3-deoxy-3-fluoro-D-glucose</td>
</tr>
<tr>
<td>3FG</td>
<td>3-Deoxy-3-fluoro-D-glucose</td>
</tr>
<tr>
<td>4FG</td>
<td>4-Deoxy-4-fluoro-D-glucose</td>
</tr>
<tr>
<td>4FGS</td>
<td>4-Deoxy-4-fluoro-D-glucosone</td>
</tr>
<tr>
<td>GS</td>
<td>Glucosone</td>
</tr>
<tr>
<td>4FGL</td>
<td>4-Deoxy-4-fluoro-D-glucitol</td>
</tr>
<tr>
<td>4FF</td>
<td>4-Deoxy-4-fluoro-D-fructose</td>
</tr>
<tr>
<td>4FM</td>
<td>4-Deoxy-4-fluoro-D-mannitol</td>
</tr>
<tr>
<td>3FGL</td>
<td>3-Deoxy-3-fluoro-D-glucitol</td>
</tr>
<tr>
<td>3F2KGA</td>
<td>3-Deoxy-3-fluoro-2-keto-D-gluconic acid</td>
</tr>
<tr>
<td>4F2KGA</td>
<td>4-Deoxy-4-fluoro-2-keto-D-gluconic acid</td>
</tr>
<tr>
<td>e.d.</td>
<td>external diameter</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
</tbody>
</table>
GDH  gluconate dehydrogenase
GDP  guanidine diphosphate
GOX  glucose oxidase
Hz   hertz
HPLC high performance liquid chromatography
i.r. infra-red
Ki   inhibitor constant
Km   Michaelis constant
L    litre(s)
M    molar
MHz  megahertz
mCi  millicurie
mg   milligram(s)
min  minute(s)
mL   milliliter(s)
mM   millimolar
mm   millimeter(s)
mMole millimole(s)
m.p. melting point
μM  micromolar
μg  microgram(s)
μMole micromole
M    molar
N    normal
NAD+ nicotinamide-adenine dinucleotide
NADH nicotinamide-adenine dinucleotide; reduced
NADP⁺: nicotinamide-adenine dinucleotide phosphate
NADPH(H⁺): nicotinamide-adenine dinucleotide phosphate, reduced
nm: nanometer(s)
nmr: nuclear magnetic resonance
PETT: positron emission transaxial tomography
pH: -log H⁺ concentration
ppm: parts per million
RF: retardation factor
RT: retention time
TISAB: total ionic strength adjustor buffer
TLC: thin-layer chromatography
Vmax: maximum initial velocity
w/v: weight/volume
λ: wavelength
PART I

SYNTHETIC AND ENZYMIC STUDIES
PART I

INTRODUCTION

1. General

Probably, the first synthesis of an organofluoro compound was in 1896, when Swarts synthesized fluoroacetate. However, there was no significant interest in such compounds at that time. Later the Germans became interested in such compounds to investigate their use as insecticides. This was a further stimulus to the synthesis and study of compounds containing the C-F bond. During the second World War, the United Kingdom became interested in the biological properties of organofluoro compounds as a precautionary measure in case such compounds were used as chemical warfare agents against the Allies. About the same time, the United States of America became interested in the organofluoro compounds for the fractionation of uranium isotopes as the volatile hexafluorides ("Manhattan Project" for the development of atomic bomb).

Such were the scattered and meagre interests in the organofluoro compounds until 1943, when a new interest was reawakened as a result of the work of J.S.C. Marais. In 1943, Marais discovered that the toxic principle of a "Killer Shrub" in South Africa, Dichapetalum cymosum (or gifblaar) was fluoroacetate. Then in a subsequent study on this work, R.A. Peters elucidated the biochemical
mode of toxicity of the fluoroacetate. He found that the toxicity was not due to fluoroacetate itself, but that it was converted in the body of the host, enzymically into another compound, fluorocitrate, which contributed to the toxicity, by inhibiting the enzyme aconitase in mitochondria. Rudolph Peters, therefore, advanced the concept of "Lethal Synthesis". This encompassed the phenomena of the enzymatic transformation of an otherwise non-toxic compound into a toxic product in vivo. This discovery, besides shattering the previous misconception about the specificity of enzymes to their substrates, also provided a stimulus for the design and development of a large number of fluoro-analogs of biological interest. The reversible nature of fluorocitrate inhibition of aconitase prompted further work into the site of action of fluorocitrate. Ernest Kun, investigating this problem found that in contrast to the reversible kinetic data of isolated aconitase, preparations, isolated mitochondria responded to fluorocitrate in a manner which seems to bear more directly on toxicology. His further work with intact mitochondria showed that fluorocitrate irreversibly inhibited a membrane associated carrier protein essential for the transport of citrate. This is now considered to be the main site for the toxicity of fluorocitrate.

A comparison of organofluoro compounds showing biological activity with their natural substrate, shows
that in most of them fluorine has been substituted for a hydrogen atom or hydroxyl group (Table 1). The biochemical rationale for replacement of hydrogen by fluorine and the usefulness of such analogs is that, fluorine and hydrogen have similar Van der Waals radii\(^7,8\) (Table 2) and hence similar size. An even better comparison is seen between the fluoro and the hydroxyl groups. The C-F and C-OH bonds are more similar physicochemically (e.g. bond lengths and polarization). Based on the similarity in size and electronegativity of the fluorine atom and oxygen (Table 2), Taylor and Kent in 1958 rationalized that substitution of oxygen by fluorine might be a useful approach to the design and synthesis of carbon-fluorine compounds of biochemical interest\(^9\). More recently, Barnett\(^10\) has put forth the same argument. Today, there are a wide variety of such compounds (Table 3) and they have found usefulness in a large number of scientific studies including medical research, and clinical application (Table 3). Apart from the synthetic fluoro-analogs, some naturally occurring organofluoro compounds (Table 4) have also found clinical usefulness. An example of this is nucleocidin\(^11\) (1), an antitrypanosomal antibiotic. This compound also exemplifies a naturally
Table 1.
Replacement of hydrogen and hydroxyl group by fluorine in some compounds.

<table>
<thead>
<tr>
<th>NATURAL SUBSTRATE</th>
<th>FLUORO ANALOG</th>
<th>REPLACEMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃COOH</td>
<td>FCH₂COOH</td>
<td>H</td>
</tr>
<tr>
<td>CH₃CONH₂</td>
<td>FCH₂CONH₂</td>
<td>H</td>
</tr>
<tr>
<td>C₆H₁₂O₆</td>
<td>C₆H₁₁O₅F</td>
<td>OH</td>
</tr>
</tbody>
</table>
Table 2. Comparison of physical parameters of some elements covalently bonded to carbon.  

<table>
<thead>
<tr>
<th>Element</th>
<th>Bond length (CH$_3$-X) Å</th>
<th>Van der Waals radius (Å)</th>
<th>Electronegativity</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>1.09</td>
<td>1.20</td>
<td>2.1</td>
</tr>
<tr>
<td>F</td>
<td>1.39</td>
<td>1.35</td>
<td>4.0</td>
</tr>
<tr>
<td>(in OH)</td>
<td>1.43</td>
<td>1.40</td>
<td>3.5</td>
</tr>
<tr>
<td>Cl</td>
<td>1.77</td>
<td>1.30</td>
<td>3.0</td>
</tr>
<tr>
<td>(in SH)</td>
<td>1.82</td>
<td>1.35</td>
<td>2.5</td>
</tr>
</tbody>
</table>
**Table 3.**

*Synthetic organic fluorine compounds of Bio-Medical interest.*

<table>
<thead>
<tr>
<th>Chemical Class</th>
<th>Example</th>
<th>Bio-Medical Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorocarboxylic acids</td>
<td>Fluorocitrate</td>
<td>Biochemical probe</td>
</tr>
<tr>
<td>Fluorotrimidoles</td>
<td>2-fluorohistidine</td>
<td>Antiviral agent</td>
</tr>
<tr>
<td>Fluoropyrimidines</td>
<td>5-fluorouracil</td>
<td>Antineoplastic agent</td>
</tr>
<tr>
<td>Fluorocarbohydrates</td>
<td>3-Deoxy-3-fluoro-D-glucose</td>
<td>Biochemical probe</td>
</tr>
<tr>
<td>Fluorocarbons</td>
<td>Perfluorodecalin</td>
<td>Artificial blood</td>
</tr>
<tr>
<td>Brominated fluorocarbons</td>
<td>Perfluorocetyl bromide</td>
<td>Radiopaque diagnostic agent</td>
</tr>
<tr>
<td>Fluorinated ethers</td>
<td>Fluoroxene</td>
<td>General anaesthetic</td>
</tr>
<tr>
<td>Fluoronucleotides</td>
<td>5-fluoro-2'-deoxy-uridylate</td>
<td>Antineoplastic agent</td>
</tr>
<tr>
<td>Fluorosteroids</td>
<td>9α-fluorohydrocortisone</td>
<td>Anti-inflammatory agent</td>
</tr>
<tr>
<td>Fluoroamino acids</td>
<td>α-monofluoromethyl(DOPA)</td>
<td>Suicide inhibitor</td>
</tr>
<tr>
<td>18F labelled carbohydrates</td>
<td>[2-18F]-2-Deoxy-2-fluoro-D-glucose</td>
<td>In PETT scan</td>
</tr>
</tbody>
</table>
Table 4.

 Naturally occurring carbon-fluorine compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monofluoroacetic acid</td>
<td>Dichapetalum cymosum</td>
</tr>
<tr>
<td>Monofluoroacetone</td>
<td>Arcacia tree</td>
</tr>
<tr>
<td>W-Monofluoropalmitic and oleic</td>
<td>Dichapetalum toxicarum</td>
</tr>
<tr>
<td>acids</td>
<td>Various plant leaves</td>
</tr>
<tr>
<td>Monofluorocitric acid</td>
<td>Mould metabolite</td>
</tr>
<tr>
<td>Nucleocidin</td>
<td>Human plasma</td>
</tr>
<tr>
<td>Perfluorinated fatty acids</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1

1. NUCLEOCIDIN

2. 2-DEOXY-2,2-DIFLUOROARABINOSE

3. 1,1-DIFLUORO-D-RIBITOL
occurring fluorinated carbohydrate in which fluorine has
replaced a hydrogen.

The selective introduction of fluorine into naturally
occurring compounds, especially carbohydrates, has in
general presented the synthetic carbohydrate chemist with
a continuous challenge. An extensive literature on this
topic exists and has been reviewed up to 1981\textsuperscript{12}. The
purpose of this review is to select examples which
illustrate the development of the various synthetic methods
up to the present time. A more comprehensive treatment
is beyond the scope of this Thesis.

In carbohydrates it is usually the hydroxyl groups
that have been replaced by fluorine. In a few instances,
however, fluorine has replaced hydrogen. For example,
2-deoxy-2,2-difluoro-D-arabinose\textsuperscript{13} (2), 2,5-anhydro-1-deoxy-
1,1-difluoro-D-ribitol\textsuperscript{14,15} (3) and the naturally occurring
nucleocidin (1). The biochemical rationale behind the
usefulness of such compounds is that when OH is available,
hydrogen bonding to an enzyme active site is either by
donation of proton or acceptance of proton by the substrate
(Fig. 2). When fluorine replaces the hydroxyl group, it
can act only as an acceptor in hydrogen bond formation.
Also the introduction of fluorine into carbohydrates in
place of hydroxyl uniquely modifies the overall
physicochemical properties of the compound (e.g. the pK\textsubscript{a}
value of adjacent hydroxyl groups) without a significant
Figure 2

Comparison of the hydrogen bonding capabilities of the (a, b) sugar, (c) fluorosugar (d) and deoxy sugar with a protein.
Figure 2

\[ S \text{---O---H} \]
\[ \text{---X} \]
\[ \text{---P} \]

\[ S \text{---O---H} \]
\[ \text{---H} \]
\[ \text{---X} \]
\[ \text{---P} \]

\[ S \text{---F---H} \]
\[ \text{---X} \]
\[ \text{---P} \]

\[ S \text{---H} \]
\[ \text{---H} \]
\[ \text{---X} \]
\[ \text{---P} \]

\[ S = \text{Sugar} \]
\[ P = \text{Protein} \]
\[ X = \text{An electronegative element} \]
change in the overall stereochemistry. This may profoundly affect the biochemical behaviour of the sugar. These features have been used to advantage in biochemical studies.

Other characteristics of the C-F bond, for example, the inductive effect of a C-F₂ group, as in difluoro sugars (2 and 3), has also been used to advantage in biological studies. In the molecule (2), the strong inductive effect of the C-F₂ group increases the acidity of the vicinal C₃-OH. This is believed to be a contributing factor to the observation that this compound is a good substrate for yeast hexokinase₁⁶.

The replacement of the hydroxyl group by fluorine does not impose any gross conformational changes on the molecule. However, the intermolecular hydrogen bonding capabilities of the molecule are changed, and the fluorine can be monitored by ¹⁹F nmr spectroscopic probes³⁻¹⁵. This offers a very sensitive probe in biochemical studies. The biochemical incorporation of 3-deoxy-3-fluoro-D-glucose into glycogen and trehalose in Locusta migratoria has been studied with the aid of ¹⁹F nmr¹⁷.

SOME SYNTHETIC APPROACHES TO FLUOROCARBOHYDRATES

In designing the synthesis of fluorocarbohydrates, the fluorine is introduced into the carbohydrate in place of the anomeric, primary or a secondary hydroxyl group; replacement of hydrogen has been accomplished in some
cases\textsuperscript{11,13-15}. Replacement of the anomeric and primary hydroxyl groups is usually more facile than secondary hydroxyl groups. For this reason, it is necessary to consider the two approaches separately.

(a) Replacement of the anomeric and primary hydroxyl groups of carbohydrates

Replacement of the anomeric hydroxyl groups by fluorine was first achieved by brief treatment of acylated or perbenzoylated aldoses with hydrogen fluoride\textsuperscript{18-20}. This is exemplified by the synthesis of tetra-$\alpha$-acetyl-$\alpha$-$\Omega$-glucopyranosyl fluoride from peracetylated $\alpha$- and $\beta$-glucopyranose\textsuperscript{18-20}. Fluorination at an anomeric carbon may or may not be accompanied by Walden inversion or ring contraction\textsuperscript{21-23}. Apart from hydrogen fluoride as a fluorinating agent, silver fluoride in acetonitrile and silver tetrafluoroborate in ether or toluene has also been used in the synthesis of glycosyl fluorides\textsuperscript{24-27}.

Introduction of fluorine into carbohydrates has been achieved mostly via sulfonyloxy intermediates as leaving groups. With certain exceptions, primary fluorination of carbohydrate derivatives is very facile. Fluoride displacement of sulfonyloxy groups has been achieved by the use of reagents such as potassium or cesium fluoride in 1,2-ethanediol and a variety of other solvents, tetrabutylammonium fluoride (sometimes tetraethylammonium
fluoride) in a aprotic solvent, mostly acetonitrile. Tetrabutylammonium fluoride is more important in fluorination of carbohydrates because it replaces both primary and secondary sulfonyloxy groups. Potassium or cesium fluoride often works with limited success, or extreme conditions must be applied for any displacement to occur, especially with secondary sulfonyloxy groups. The sulfonic ester precursors are usually protected with such normally nonparticipating groups such as cyclic acetal or ethers (methyl or benzyl groups) that are stable to the conditions of fluorination. A few exceptions however, have been encountered with benzyloxy group participation. In previous years, a small number of fluorinated analogs have been prepared without prior protection of the hydroxyl groups. However because of the strong alkali conditions of the reaction, such side reactions as the concurrent formation of the 3,6-anhydro derivatives have been observed. This is the case in the fluorination of methyl 6-0-p-tolylsulfonyl-α-D-glucopyranoside (4) with potassium fluoride in 1,2-ethanediol which yields (5) and the anhydrosugar (6). Sometimes direct replacement, therefore, does not yield the fluorinated product.

Apart from fluoro-aldooses, fluoro-analogs of ketoses have also been reported in literature. Again the fluorine replaces a sulfonyloxy group in such reactions. However,
Figure 3
such reactions as well as that of other sugars have been found not to be always facile. 1,6-Dideoxy-1,6-difluoro-D-fructose was readily obtained from 2,3-0-isopropylidene-1,6-di-0-p-tolysulfonyl-β-D-fructofuranose by treatment with potassium fluoride in 1,2-ethanediol under a stream of carbon dioxide. This reaction should have selectively fluorinated the 6-sulfonyloxy which is more reactive than the 1-sulfonyloxy group. But no selectivity was observed. It was impossible to prepare 1-deoxy-1-fluoro-D-fructose from 2,3:4,5-di-0-isopropylidene-1-0-(methylsulfonyl)(or p-nitrophenylsulfonyl)-β-D-fructopyranoside by treatment with sodium fluoride in 1,2-ethanediol, N,N-dimethylformamide, or formamide at elevated temperatures. This was explained by the fact that nucleophilic displacement with charged nucleophiles at C-1 of 2-hexulopyranose-1-sulfonates is known to be difficult. The difficulty is because the reactivity of the primary hydroxyl group at C-1 is profoundly affected by the presence of the adjacent ketonic function at carbon atom 2 of ketoses. Barnett and Atkins however have reported the synthesis of 1-deoxy-1-fluoro-β-D-fructose, albeit in very low yield, by treating 2,3:4,5-di-0-isopropylidene 1-0-methylsulfonyl-β-D-fructopyranose at 144° with tetrabutylammonium fluoride in N,N-dimethylformamide. The above examples confirm earlier observations that displacement of primary sulfonic esters
is not always facile.\textsuperscript{38,39,41}

(b) Introduction of fluorine at secondary position in the sugar

Epoxide ring cleavage has been used for the preparation of some fluorocarbohydrates. The rings are always cleaved trans-diaxially. The first successful scission in the aldopyranose series was by Taylor et al.\textsuperscript{42} In this procedure methyl-4-O-benzyl-2,3-anhydro-\(\beta\)-L-ribopyranoside was heated (120°C) with anhydrous hydrogen fluoride in 1,4-dioxane to give the 3-fluoro derivative (8) (Fig. 3), in accordance with a trans-diaxial scission of the epoxide (7). Subsequent hydrogenolysis and acid hydrolysis of (8) afforded methyl 3-deoxy-3-fluoro-\(\beta\)-L-xylopyranoside (9). This pioneering work was followed by an improved preparation of the D-enantiomer (11) (Fig. 4) (subsequently hydrolysed to the free sugar) by Taylor et al.\textsuperscript{43}, by treatment of methyl-2,3-anhydro-4-O-benzyl-\(\beta\)-D-ribopyranoside (10) with potassium hydrogen fluoride in 1,2-ethanediol (Fig. 4). 3-Deoxy-3-fluoro-D-glucopyranose (16) has also been prepared by epoxide scission (Fig. 5). Methyl 2,3-anhydro-4,6-di-O-methyl-\(\alpha\)-D-allopyranoside (12) was treated with hydrogen tetrafluoroborate in hydrogen fluoride at 70°C, to yield both the 4,6-di-O-methyl-2-deoxy-2-fluoro-D-altropyranosyl fluoride (13) and 4,6-di-O-methyl-3-deoxy-3-fluoro-D-glucopyranosyl fluoride (14). Hydrolysis of these products (demethylation) afforded the free sugars (15) and (16).
Figure 5

12 \[ \rightarrow \] 13 + 14

13 \[ \rightarrow \] 15 + 16

12 → 13 + 14

13 \[ \text{(1) } \text{HCl-H}_2\text{O} \]

14 \[ \text{(11) } \text{BF}_3-\text{Et}_2\text{O} \]
(Fig. 5), respectively. A similar reaction with the 4,6-diacetate derivative yielded (15) and (16) plus a third product, 2-deoxy-2-fluoro-D-allopyranose44.

The non-specificity of such cleavages is therefore evident. To obtain selective fluorination, a rigid conformation in the epoxide is needed so as to ensure an almost exclusive formation of one of the possible isomers expected from the trans-diaxial ring opening of the epoxide.

Early workers have achieved conformational rigidity by using the 1,6-anhydro-aldohexopyranoses as precursors for the preparation of the epoxide45-47. 4-Deoxy-4-fluoro-D-glucose (18) (Fig. 6), for example has been prepared by the action of potassium hydrogen fluoride in 1,2-ethanediol on 1,6:3,4-dianhydro-2-O-benzyl-β-D-galactopyranose46-47 (17), followed by hydrolysis of the anhydro ring and benzyl group (Fig. 6) to yield (18). 4-Deoxy-4-fluoro-D-glucose has also been prepared by the photolytic removal of tolylsulfonyl group from position 2 of 1,6:3,4-dianhydro-2-O-p-tolylsulfonyl-β-D-galactopyranose (19), and treatment of the product with potassium hydrogen fluoride in 1,2-ethanediol to give the 1,6-anhydro-4-fluoro derivative (20). Opening of the anhydro ring then yielded the free sugar47-48 (Fig. 7).

Nucleophilic displacement of sulfonic esters has been used as an alternative to epoxide ring cleavage for the
Figure 6

17

1,2-Ethanediol

18

[\text{KHF}]

[\text{[H}^+]\text{]}
introduction of fluorine in secondary positions of carbohydrates. This type of displacement reaction is always accompanied by Walden inversion. The approach is far more successful and the success has been attributed to two factors:

i. that displacement occurs under relatively mild conditions when the fluoride source is tetrabutylammonium fluoride in conjunction with an aprotic solvent (tetraethylammonium fluoride has also been used).

ii. that certain factors controlling the reaction, could be judiciously selected to maximize the efficiency of the reaction. Such factors include the geometry (including steric effect) of the molecule, nucleophilicity of the attacking group \(^9\), solubility of the nucleophile \(^9\), polarity and solvation ability of the solvent \(^50\) and the boiling point of the solvent \(^51\). The relative efficiencies of different sulfonates as leaving groups is of secondary importance \(^52\).

As noted in the first factor, mild conditions are used when tetrabutylammonium fluoride is the fluoride source. Use of this reagent in the carbohydrate field was pioneered by its application in the successful preparation of 3-deoxy-3-fluoro-1,2:5,6-di-O-isopropylidene-
α-D-glucofuranose (22) from 1,2:5,6-di-O-isopropylidene-3-O-p-tolylsulfonyl-α-D-allofuranose\textsuperscript{53–55} (21) (Fig. 8). The course of reactions involving the use of tetrabutylammonium fluoride was found to depend upon the quality of the reagent with respect to the fact that it is thermally unstable, and extremely hygroscopic. The successful preparation of the clathrate (Bu\textsubscript{4}NF·32.8H\textsubscript{2}O) of this reagent in the dry form by P.W. Kent and Young\textsuperscript{56} has greatly improved the stability and shelf-life of the reagent such that it is even now available commercially. In a comparative study, fluorination of 1,2:3,4-di-O-isopropylidene-6-O-p-tolylsulfonyl-α-D-galactopyranose in a variety of dipolar aprotic solvents (as well as 1,2-ethanediol) with tetrabutylammonium fluoride as the fluoride source was tried. While 1,2-ethanediol gave no reaction, acetonitrile was found to give the highest proportion of substitution of the sulfonic esters relative to elimination\textsuperscript{53}. Elimination, it must be noted, is the major competing reaction in nucleophilic substitution reactions of this nature, because of the high basicity and low nucleophilicity of the fluoride ion. In another comparative study, tetrabutylammonium fluoride was used in the displacement of the 3-sulfonic esters in the D-allo- and D-gulo-configuration. It was observed that while tetrabutylammonium fluoride successfully fluorinated 1,2:5,6-di-O-isopropylidene-3-O-p-tolylsulfonyl-α-D-
allofuranose (as already stated) to yield, after removal of the blocking groups 3-deoxy-3-fluoro-\(\alpha\)-D-glucose\(^{55,57,58}\) (23) (Fig. 8), substitution in 1,2:5,6-di-\(\beta\)-isopropylidene-3-O-p-tolylsulfonyl-\(\alpha\)-D-gulofuranose (24) yielded some elimination product\(^{59,60}\) (25) (Fig. 9). It was noted that in (24), the 3-p-tolylsulfonyloxy group is trans to H-4, while H-2 is trans to the 3-p-tolylsulfonyloxy group in (21). No elimination occurred by attack at H-2 of (21) probably because the resulting double bond would impose increased strain on the molecule whereas this is not the case in (24)\(^{59,60}\).

Use of potassium fluoride for displacement of secondary sulfonyloxy groups has not always been successful. For example, when 1,2-\(\beta\)-isopropylidene-5,6-\(\beta\)-benzylidene-3-\(\alpha\)-methylsulfonyl-\(\alpha\)-D-glucofuranose (26) (Fig. 10) was fluorinated with potassium fluoride in methanol, only (26) was isolated while displacement of the secondary sulfonyloxy group in (27) with the same reagent afforded the primary fluoride only\(^{61}\) (28) (Fig. 11). However, treatment of 1,2:5,6-di-\(\beta\)-isopropylidene-3-O-p-tolylsulfonyl-\(\alpha\)-D-allofuranose (29) with potassium fluoride in acetamide at 150°C gave (after removing blocking groups), 3-deoxy-3-fluoro-\(\alpha\)-D-glucofuranose\(^{58}\) (30) (Fig. 12).

Judicious selection of leaving group has been known to improve the yield in some cases\(^{52}\). The p-tolylsulfonyloxy group is known to be somewhat more reactive than the
Figure 10

Figure 11
Figure 12

\[
\text{Me}_2\text{C} - \text{O} - \text{CH}_2
\]
\[
\text{O} - \text{CMe}_2
\]
\[
\text{KF} \\
150^\circ \text{C} \\
\text{ACETAMIDE}
\]

\[
\text{Me}_2\text{C} - \text{O} - \text{CH}_2
\]
\[
\text{O} - \text{CMe}_2
\]
\[
\text{[H}^+\text{]}
\]

\[
\text{CHO}_2
\]
\[
\text{OH} - \text{CH}_2
\]
\[
\text{OH}
\]

Number 29

Number 30
methyl sulfonyloxy group in displacement reactions. The paranitrobenzene sulfonate ion, on the other hand, has been found to be superior to p-tolylsulfonate as leaving group. When p-bromobenzene sulfonate was used in place of p-tolylsulfonate in the displacement at C-4 of a D-glucose derivative, the yield was improved and the reaction time shortened. The use of the better leaving group is limited because of resultant extensive decomposition, without any fluorination, and also substitution in the aromatic ring rather than the sugar.

Regarding the solubility of the nucleophile, tetraalkylammonium salts, an example of which is tetrabutylammonium fluorides, have been used to increase the concentration of nucleophile in solution, for displacement of the sulfonyloxy groups by fluoride. An extensive review of such reactions is available.

Replacement of -OH, or leaving groups in carbohydrates by fluorine is a bimolecular nucleophilic substitution (S_N2). Such reactions are in general much faster in aprotic solvents, than in protic solvents. In aprotic solvents most anions are less solvated (especially smaller ions) and presumably more reactive than in protic solvents, whereas larger polarisable charged transition-states are generally more solvated and hence less reactive.
The boiling point temperature of the solvent must be such as not to pose a practical problem of removal from the product after the reaction is finished. As such, where a facile displacement is involved and harsh conditions are not necessary, the use of such lower boiling aprotic solvents as acetonitrile, methylene chloride, butanone or acetone may be advantageous.

DIRECT DISPLACEMENT OF HYDROXY GROUPS BY FLUORINE

Most of the syntheses of fluorinated sugars have been both tedious and time consuming because of the required, protection and deprotection steps. Sulfur tetrafluoride is a well-known reagent for the direct displacement of oxygen functions by fluorine. However, it has the disadvantage that its reaction cannot be carried out at atmospheric pressure and also in glass apparatus, apart from many other disadvantages. These drawbacks of sulfur tetrafluoride are not encountered when the recently introduced fluorinating reagents, dialkylaminosulfur trifluorides are used. They can be used for direct displacement of oxygen functions. Dialkylaminosulfur trifluorides have been shown to achieve the following transformations:

\[ \text{-CO}_2\text{H} \rightarrow \text{-CO(F)} \]

\[ \text{-CHO and C = O} \rightarrow \text{CH(F) and C(F)} \]

\[ \text{-C-OH} \rightarrow \text{C(F)} \]
Such reactions have been exploited in the carbohydrate field especially by Peter Card and his group\(^{72,73}\). Primary, secondary and tertiary alcohols, all react, with high yields of the unrearranged fluoride usually resulting\(^69\). Reactions with dialkylaminosulfur trifluoride can usually be conducted under very mild conditions, so that other groups, including ester groups and other halogens can be present\(^69\). Typically, the alcohol can be added slowly to a solution of the reagent in an inert solvent pre-cooled to \(-50^\circ\text{C}\) to \(-78^\circ\text{C}\)\(^69\). The selection of reaction solvent should be such that the solvents or product could be easily removed after reaction, without destruction to the product. For low-boiling fluorides, diglyme (diethylene glycol dimethyl ether) is a convenient solvent, because the product can be distilled out of the reaction mixture, and the hydrogen fluoride, side product, remains behind complexed with diglyme. For higher-boiling fluorides, lower boiling solvents such as pentane, methylene chloride, or trichlorotrifluoromethane can be used\(^69\). One of these alkylamino fluorides, Diethylaminosulfur trifluoride (DAST) has been used successfully in the direct fluorination of hydroxyl groups\(^{72,73}\). A number of 6-deoxy-6-fluoro aldohexoses have been prepared from pyranoses containing free primary hydroxyl group, and otherwise protected with acetyl groups, by treatment with diethylaminosulfur trifluoride in diglyme\(^69\). DAST has also been found to
be compatible with benzyl protecting groups. Benzene or dichloromethane has been used with this reagent for the reaction of 1,2:3,4-di-\(\alpha\)-isopropylidene-\(\alpha\)-D-galactohexodialdo-1,5-pyranose (31) to give 6-deoxy-6,6-di-fluoro-1,2:3,4-di-\(\alpha\)-isopropylidene-\(\alpha\)-D-galactopyranose (32) (Fig. 13). Treatment of methyl-3,4-\(\alpha\)-isopropylidene-\(\alpha\)-L-erythro-pentopyranoside-2-ulose (33) with DAST in benzene gave the 2,2-difluoro derivative (34) (Fig. 14). Reaction of 1,2:5,6-di-\(\alpha\)-isopropylidene-\(\alpha\)-D-allofuranose with DAST, followed by processing with water afforded the starting material (35) (Fig. 15). The same reaction in the presence of pyridine followed by direct distillation, however, afforded 3-deoxy-3-fluoro-1,2:5,6-di-\(\alpha\)-isopropylidene-\(\alpha\)-D-glucofuranose (37). As with the allofuranose, treatment of 1,2:5,6-di-\(\alpha\)-isopropylidene-\(\alpha\)-D-glucofuranose (38) with DAST in dichloromethane at 0\(^\circ\)C, followed by processing with water, afforded the starting material in excellent yield, whereas the inclusion of two equivalents of pyridine in the mixture followed by direct distillation afforded the alkene (40) (Fig. 16). It is suggested that the gluco- and allo- fluorination proceeds via the intermediates (36) and (39), respectively, (Fig. 15 and 16) via an \(S_N2\) mechanism, rather than an ion pair or a cyclic transition state. The observed difference
Figure 5

31

$\text{Et}_2\text{NSF}_3$
$\text{CH}_2\text{Cl}_2$

32

Figure 14

33

$\text{Et}_2\text{NSF}_3$
$\text{C}_6\text{H}_6$

34
Figure 15
in reaction between the gluco- and allo- furanose derivatives was attributed either to differences in stability of the two alkenes that will be formed by transdialixial elimination, or to the steric effects of the 1,2-O-isopropylidene ring blocking the approach of the nucleophile at the "lower" face of the furanose ring\textsuperscript{75}. Methyl 6-deoxy-6-fluoro-\alpha-D-glucopyranoside, methyl 4,6-dideoxy-4,6-difluoro-\alpha-D-talopyranoside and several other fluorinated intermediates have been prepared by Card, Middleton and others\textsuperscript{69-77}. With DAST fluorination of sucrose has also been achieved\textsuperscript{78}.

**RADICLABELLED FLUORINATED CARBOHYDRATES**

The value of isotopically modified carbohydrates resides in their use in the study of metabolism. This has prompted the introduction of isotopic atoms (e.g. $^{13}$C and $^3$H) into carbohydrates. The radioactive isotope $^{18}$F has been used for the preparation of substituted compounds that have found immense potential in biomedical studies\textsuperscript{79-81}. Diethylaminosulfur $^{[18}$F] trifluoride\textsuperscript{75,82,83}, tetraethylammonium $^{[18}$F] fluoride\textsuperscript{84}, $^{[18}$F$_2$\textsuperscript{95,96} and silver $^{[18}$F] fluoride\textsuperscript{97} have been used as fluorinating agents. 6-deoxy-6-$^{[18}$F]fluoro-\alpha-D-galactopyranoside has been synthesized by treatment of 1,2:3,4-di-O-isopropylidene-6-O-p-tolyl-sulfonyl-\alpha-D-galactopyranoside with tetraethylammonium $^{[18}$F] fluoride in acetonitrile and subsequent deprotection\textsuperscript{98,99}. 3-deoxy-3-$^{[18}$F] fluoro-\alpha-
glucose has been prepared using diethylaminosulfur $[^{18}\text{F}]$-trifluoride as fluorinating agent.\textsuperscript{33} 2-Deoxy-2-$[^{18}\text{F}]$ fluoro-D-mannose\textsuperscript{86} and 2-deoxy-2-$[^{18}\text{F}]$ fluoro-D-glucose\textsuperscript{85,86} have been prepared using $[^{18}\text{F}]_{2}$ as fluorinating agent. 2-deoxy-2-$[^{18}\text{F}]$ fluoro-D-glucose is being used extensively in the PETT (Positron Emission Transaxial Tomography) Scan studies.\textsuperscript{30}

Other isotopes with which fluorinated carbohydrates have been modified include $[^{14}\text{C}]$ as in 2-deoxy-2-$[^{14}\text{C}]$fluoro-D-$[^{14}\text{C}]$glucose\textsuperscript{86}, and recently 4-deoxy-4-fluoro-D-$[^{14}\text{C}]$glucose in our laboratory\textsuperscript{91}. Tritium $[^{3}\text{H}]$ has also been used, as in 2-deoxy-2-fluoro-D-$[^{3}\text{H}]$glucose and mannose\textsuperscript{92}, 4-deoxy-4-fluoro-$[^{3}\text{H}]$glucose\textsuperscript{93} and 3-deoxy-3-fluoro-$[^{3}\text{H}]$glucose\textsuperscript{55}.

The availability of gram quantities of the fluorinated carbohydrates that have been successfully synthesized so far has made it possible for their use as precursors for the synthesis of other analogs and intermediates useful for biochemical studies. Electrolytic oxidation of 3-deoxy-3-fluoro-D-glucose, followed by treatment with acidic resin yielded 3-deoxy-3-fluoro-D-gluconic acid\textsuperscript{94}. 2-Deoxy-2-$[^{18}\text{F}]$ fluoro-D-glucose has been used for the enzymic synthesis of 2-deoxy-2-$[^{18}\text{F}]$ fluoro-D-glucose-6-phosphate\textsuperscript{95}. 6-Deoxy-6-fluoro-\alpha-D-galactopyranosyl phosphate (dipotassium salt) has been synthesized\textsuperscript{96} from methyl 2,3,4-tri-O-acetyl-6-deoxy 6-fluoro-\alpha-D-galactopyranoside by phosphorylation
with silver diphenylphosphate, hydrogenolysis, and finally deacylation to yield the product. Methyl (methyl 4-deoxy-4-fluoro-α-D-glucopyranosid)uronate (42) has been synthesized by reaction of methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (41) with platinum black, amberlite IR 120 (H^+), and subsequent treatment of acid with diazomethane^9^3 (Fig. 17). 4-Deoxy-4-fluoro-2-keto- (44) and 3-deoxy-3-fluoro-2-keto-D-gluconate (43) have been prepared enzymatically^9^7, by the action of gluconate and glucose dehydrogenase on (18) and (16) (Fig. 18). 3-Deoxy-3-fluoro-D-[1-^1^4^C] glucose and 4-deoxy-4-fluoro-D-[1-^1^4^C]glucose have been enzymatically converted to the fluoro-[1-^1^4^C] mannose-1,6-bisphosphate^9^8. The combination of chemical and enzymatic methods for the synthesis of fluorinated sugars hitherto unaccessible by chemical methods alone, promises to be a fruitful approach in the future.

THE BIOCHEMISTRY OF FLUORINATED CARBOHYDRATES

Fluorinated carbohydrates have been used extensively in biological studies, and today the scope has widened greatly with the advent of more sensitive instrumentation, such as the ^19^F nmr probe, high performance liquid chromatography, Fourier transform Infra Red detection systems and Mass Spectroscopy. Barnett has reviewed the biological activities of fluorinated carbohydrates, and their use in the study of binding of sugars to membrane carriers, and as substrates for glycosidases^9^9. Taylor^1^0^0
Figure 17

(I) Pt BLACK/O₂
NaHCO₃

(II) AMBERLITE
IR 120 (H⁺)

CH₃N₂
Figure 18

16

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{1. GoX} \\
\text{F} \quad \text{2. GDH} & \quad \rightarrow \\
\text{OH} \quad \text{43} & \quad \text{HO}
\end{align*}
\]


18

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{1. GoX} \\
\text{F} \quad \text{2. GDH} & \quad \rightarrow \\
\text{OH} \quad \text{44} & \quad \text{HO}
\end{align*}
\]
has also discussed the metabolism and enzymology of fluorinated carbohydrates and related compounds. Only selected examples will therefore be considered. Studies of the metabolism of D-glucose in *Saccharomyces cerevisiae* (ATCC-S288C) have shown that 3-deoxy-3-fluoro-D-glucose inhibits polysaccharide synthesis and glucose uptake in this organism. With galactose metabolism, the fluoro-sugar showed a significant inhibitory effect on respiration and polysaccharide synthesis, but no significant inhibition of galactose uptake was observed. Incubation of *Saccharomyces cerevisiae* (ATCC-S288C) with 4-deoxy-4-fluoro-D-[1-14C]mannose resulted in the formation of 4-deoxy-4-fluoro-D-[1-14C]mannose-1,6-biphosphate, 4-deoxy-4-fluoro-D-[1-14C]mannose-6-phosphate and GDP-4-deoxy-4-fluoro-D-[1-14C]mannose. 2-Deoxy-2-[18F]glucose has been used in the PETT Scan for the early detection of such diseases as stroke. The modified fluoro analogues of glucose, 4-deoxy-4-fluoro and 3-deoxy-3-fluoro-2-keto-D-gluconate have been used in studies involving the transport of 2-keto-D-gluconate in vesicles of *Pseudomonas putida*. 3-Deoxy-3-fluoro-D-[3-3H]glucose has been used for the metabolic studies in *Locusta migratoria* and transport studies in *Pseudomonas putida*.

An interesting report from these laboratories indicates that in some circumstances the carbon-fluorine bond is unstable. Thus, incubation of whole cells of *P. putida*
with 4FG results in complete loss of fluorine as fluoride ion, to yield an unidentified non-fluorinated sugar phosphate. Using $^3$H and $^{14}$C-labelled 4FG, some incorporation of the defluorinated sugar into the peptidylglycan protein associated with the outer membrane component has also been found.

OBJECTIVES:

The objectives of Part I of this dissertation are:

i. to attempt to synthesize 3-deoxy-3-fluoro-D-fructose and 4-deoxy-4-fluoro-D-fructose.

ii. to study the specificity and kinetics of Pyranose-2-oxidase (1:1.3.10) isolated from Polyporus obtusus.
EXPERIMENTAL

MATERIALS AND METHODS

Anhydrous pyridine was prepared by distillation over potassium hydroxide pellets. Anhydrous methanol was prepared by refluxing with magnesium and iodine followed by distillation. Dry acetonitrile was prepared by distillation over phosphorus pentoxide. Alcohol-free chloroform was prepared by washing successively with concentrated sulfuric acid, sodium hydroxide and water, then standing the chloroform over anhydrous sodium carbonate overnight in a brown bottle in the dark. Dry methylene chloride was prepared by distilling over anhydrous magnesium sulfate. Dry acetone was prepared by first drying over anhydrous sodium sulfate then distilling over DRIERITE. Removal of solvent, unless otherwise stated, was carried out under reduced pressure on a Buchi rotavapor-R (Buchi, Switzerland), at a water bath temperature never exceeding 35°C.

All melting point determinations were made on a Fischer-Johns apparatus and are uncorrected. Optical rotations were measured using a manual polarimeter (Rudolph and Sons Inc., N.J., USA; or Atago Polax, Atago Co. Ltd., Japan), with sodium emission D line (λ=589nm), and a 0.5dm tube. IR spectra were recorded over the range 4000-400cm⁻¹ with a Beckman IR-12 spectrophotometer. ²H-
NMR spectra were recorded with a Jeolco-C60HL spectrophotometer. Chemical shifts are expressed downfield from the signal of trimethylsilane as external standard. $^{19}$F NMR spectra were recorded with a Bruker CSD100; $^{19}$F chemical shifts (ppm) are expressed relative to the signal of trifluoroacetic acid. Unless otherwise stated, all HPLC analyses were carried out using Waters M-45 pump equipped with a U6K injector and 401 Refractive Index detector (RI). Aqueous samples were injected into a Bio-Rad Aminex HPX 87H column (300 x 7.8mm). All runs were isocratic and the mobile phase, was 0.007N sulfuric acid. The flow rate was set at 0.5mL min$^{-1}$, at room temperature and the RI detector set at attenuation 16X. The recorder used was a Spectra Physics SP4290 integrator. HPLC grade distilled water was prepared in our Laboratories with a Zenopure Laboratory system (Zenon Environmental Inc., Burlington, Ont. Canada). Filtration of samples for HPLC were done using the following filters: Millex-HV 0.45μm filter unit (3.0cm; e.d.) (Millipore Corporation, Bedford, MA) and a Millipore 0.45μm Filter unit (0.7cm; e.d.) (Millipore, Nihon, Yohezawa, Japan). Solvent filtration during cleaning of solvent was done with a Waters glass filter system composed of a sintered glass funnel top and a buchner filter flask (Waters Associates, Toronto, Ont. Canada), and a 0.45μm Millipore filter (4.7cm; e.d.), (Millipore Corporation, Bedford, MA). All HPLC solvents
were cleaned (degassed) using ultrasonic cleaner (Fisher Scientific Co. USA).

Lyophilizations were done using a Labconco lyophilizer (Labconco Corporation, Kansas City, Missouri, USA) and was set at a temperature of -50°C and a vacuum of 10 microns Hg. All microanalyses reported were determined by Guelph Chemical Laboratories Ltd. Guelph, Ontario, unless otherwise stated.

**Chromatography**

Thin-layer chromatography (TLC) were carried out on a 20x20cm plastic plate, pre-coated with silica gel 60 F254 to a layer thickness of 0.2mm (BDH Chemicals, Toronto, Ont., Canada). Plates were developed in the solvent specified, and the carbohydrates detected by spraying with 50% (v/v) solution of concentrated sulfuric acid in ethanol, followed by heating at 110°C for 5-10 minutes. Reducing sugars were detected by spraying with aniline hydrogen phthalate and heating as for sulfuric acid spray. Preparative thin-layer chromatography was done using 20x20cm glass plates, pre-coated with 2mm layer of silica gel GF (Fischer Scientific, N.J., USA). (Some of these were kind donations from Dr. K.E. Taylor.) The concentrated mixture was dissolved in a minimum amount of a suitable solvent and applied repeatedly as a continuous thin band on the plate, 2 1/2cm away from both edges and from the
bottom (with respect to the point of application) of the plate. The plates were developed in the specified solvent. Another plate 20x4cm was similarly treated. The developed plates were air dried and the 20x4cm plate ("Detector plate") was sprayed with sulfuric acid-ethanol and oven-heated as described for TLC. Bands on the "Detector plate" were used as markers for removing the desired compounds from the 20x20cm plate. The isolated band was eluted with a suitable solvent. Paper chromatography was done using a Whatman No. 1 chromatographic paper.

The following solvent systems were used in all chromatographic work. All ratios are in volumes.

A. Ethyl acetate: Petroleum Ether (35-60°)/3:1
B. Benzene: Methanol/19:1
C. Ethyl acetate
D. Ethyl acetate: Petroleum ether (35-60°)/1:1
E. Ethyl acetate: Petroleum ether (35-60°)/1:2
F. Ethyl acetate: Petroleum ether (35-60°)/1:3
G. Ethyl acetate: Acetic acid: Water/3:3:1
H. Ether: Petroleum ether (35-60°)/3:2
I. Methylene Chloride
J. Ethyl acetate: Ethanol/4:1
K. Ethanol: Water: Ammonium hydroxide/20:4:1

J. Isopropanol: Ethyl acetate: Water: Acetic Acid/83:11:5:1


Reagents

Ruthenium (IV) oxide (RuO₂·XH₂O; H₂O 27.5%) was from Alfa products (Ventron division, MA, USA). Silica gel Grade H (Mesh size 60-200) was from Grace Division Chemicals (Baltimore, Maryland). Celite was from John Mansville Co. (MA, USA). Diethylaminothiourea trifluoride (DAST), tetrabutylammonium hydroxide and 1,1-diphenyl hydrazine hydrochloride were from Aldrich Chemical Co. Inc. (Milwaukee, USA). Malt extract, peptone, agar and yeast extract were from Difco Laboratories (Detroit, Michigan, USA). Cyanogen bromide, Sepharose-4B, O-dianisidine and β-D-glucose were from Sigma Chemicals (Toronto, Ont., Canada). Horseradish peroxidase and catalase were from Boehringer, Mannheim, GmbH (W. Germany). Acetic anhydride was from Baker Chemical Co. (Toronto, Ont. Canada).

All other reagents and chemicals were of ACS grade from Fisher Scientific (Toronto, Ont. Canada) and were used without further purification.
SYNTHESIS OF FLUOROMONOSACCHARIDES

1,2:4,5-DI-O-ISOPROPYLIDENE-D-FRUCTOPYRANOSE (45)

Powdered D-fructose (100g) was shaken with acetone (dry, 1.2L) and zinc chloride (Fused, 140g) for 36 hrs when TLC (Solvent A) showed reaction to be complete. The reaction mixture was allowed to stand for 1 hr, and repeatedly neutralized with freshly prepared sodium hydroxide (7M) until no more precipitation occurred on addition of sodium hydroxide solution. The mixture was evaporated under reduced pressure (30°C) to give a syrup. The resulting syrup was diluted with water (400mL) and extracted with ether (3 x 600mL). The ether extract was dried (anhydrous Na2SO4), filtered, and evaporated under reduced pressure (30°C) to dryness. Crystals of (45) appeared spontaneously. The crude material was recrystallized from ether-petroleum ether (b.p. 30-65°C) to yield the pure title compound (78.3g, 53.4%), m.p. 118-119°C [Lit104,119], Rf 0.75 (Solvent A), [Lit106 0.75].

1,2:4,5-DI-O-ISOPROPYLIDENE-D-ERYTHRO-2,3-HEXODIULO-2,6-PYRANOSE (46)

Potassium metaperiodate (46.0g) and anhydrous potassium carbonate (5.08g) were dissolved in water (266mL). Alcohol-free chloroform (266mL) was added with stirring. Ruthenium dioxide (1.0g) was then added while stirring as continued
until the entire mixture turned yellow. Compound (46) (40g) was added slowly while stirring vigorously (mechanical stirrer). The reaction was allowed to continue for 15 hr when TLC (Solvent B) showed very little starting material. The reaction was quenched by addition of isopropyl alcohol (25mL). The mixture which turned completely black was stirred for 10 more min. and filtered twice through a celite pad. The organic layer was separated, and the aqueous layer extracted with chloroform (3 x 200mL). The combined organic extract was washed once with water (100mL), dried (anhydrous Na₂SO₄) and evaporated under vacuo. White crystals of (46) separated spontaneously. This was recrystallized from n-hexane to give pure title compound (46). (26.1g, 65.5%); m.p. 103⁰C, [Lit¹⁰⁶ 100-102⁰C; 102-109⁰C]; Rᵥ 0.88 (Solvent A) [Lit¹⁰⁶ 0.85] Rᵥ 0.65 (Solvent B) [Lit¹⁰⁶ 0.65].

1,2:4,5-OI-O-ISOPROPYLIDENE-D-PSICOPYRANOSE (47)

Compound (46) (5g) was dissolved in ethanol:water (1:1 v/v) (1000mL). Sodium borohydride (6.3g), dissolved in ethanol:water (10mL) was added gradually (20 min) at 4⁰C with stirring. The reaction was allowed to proceed for 1 hr when TLC (Solvent C) showed reaction to be complete. The reaction mixture was evaporated under vacuo to a third of the initial volume. Water (150mL) was added, and evaporated again to a third of the initial volume. This
was repeated thrice. The mixture was then extracted with ethyl acetate (100mL x 4), and the organic extract evaporated to dryness. The resulting material was taken up in methanol (150mL) and evaporated to dryness. This was repeated three times. The resulting syrupy material was allowed to stand to crystallize at room temperature. Recrystallization from n-hexane afforded the title compound (47) (5.0g; 99%); m.p. 63-64°C [Lit\textsuperscript{107} 62-64°C]; R\_F 0.62 (Solvent C).

1,2:4,5-di-O-isopropylidene-3-O-tolylsulfonyl-D-psicopyranose (48)

1,2:4,5-di-O-isopropylidene-D-psicopyranose (8.26g) was dissolved in (80mL) of dry pyridine. p-Toluene sulphonyl chloride (2 equivalents; 10.83mL) was added dropwise with stirring. Reaction was allowed to proceed for 50 hr at which time TLC of the reaction mixture showed very little starting material. The reaction mixture was poured into ice-cold water with swirling. The fluffy white solid that formed was collected by filtration. The solid was dissolved in chloroform (100mL). The chloroform layer was successively washed with 2N HCl (3 x 100mL), saturated sodium bicarbonate (2 x 100mL) and water (2 x 100mL). The organic extract was dried (anhydrous magnesium sulfate) and evaporated to dryness. The white crystalline material obtained was recrystallized from ethanol:water
(3:1\text{v/v}) to yield the title compound (48) (12.6g, 98%); m.p. (114-115°C), R_F 0.46 (Solvent D) Anal. Calc. for C_{19}H_{28}O_8S; C, 55.07; H, 6.28; S, 7.73. Found: C, 54.88; H, 6.50; S, 7.81.

1,2:4,5-Di-O-Isopropylidene-3-O-Methylsulfonyl-D-
Psicopyranose (49)

1,2:4,5-Di-O-isopropylidene-D-psicopyranose (12.24g) was dissolved in dry pyridine (154.0mL) and the mixture allowed to equilibrate at 4°C. Methane sulfonyl chloride (15.4mL) was added dropwise while stirring and maintaining the temperature at 4°C. Reaction was allowed to continue for 29 hr at which time TLC (Solvents D and E) showed reaction to be complete. The reaction mixture was worked up as for preparation of (48), evaporation under reduced pressure yielded the pure title compound as a syrup (12.9g, 80%), R_F 0.76 (Solvent D) (Alumina coated plastic plates). Anal. Calc. for C_{19}H_{28}O_8S: C, 46.15; H, 6.5; S, 9.46. Found: C, 46.13; H, 6.39; S, 9.72.
METHYL 2,3,6-TRI-O-BENZOYL-α-D-GALACTOPYRANOSIDE⁰⁸ (50)

Methyl galactopyranoside (80g) was dissolved in dry pyridine (1.3L) at -40°C (dry ice acetone bath). Benzoyl chloride (182mL), 4.2 molar ratio, was added dropwise (30-60 min) with exclusion of moisture. The reaction was allowed to go on at -30°C to -40°C for 3 hr, and the temperature gradually raised to 4°C. The reaction was then allowed to go at 4°C for 20 hours, after which time the reaction was allowed to continue for 2 more days at room temperature. At the end of the reaction time when TLC (Solvent E) showed no starting material, the reaction was stopped and evaporated under reduced pressure (35°C). The residue was taken up in chloroform (500mL). The chloroform solution was washed with 2N HCl (5x200mL), then successively with saturated solution of sodium bicarbonate (3x200mL) and water (3x200mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated under reduced pressure to give a syrup which crystallized on standing overnight at room temperature. Recrystallization from 90% aqueous ethanol afforded the title compound as white crystalline needles (153g, 73%) m.p. 140-141°C [Lit¹⁰⁸ 139-140°C], Rf 0.32 (Solvent E) [Lit⁹³ 0.32].
METHYL 2,3,6-TRI-O-BENZOYL-4-O-METHYLSULFONYL-α-D-GALACTOPYRANOSIDE$^{106}$ (51)

To a solution of methyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside (160g) in dry pyridine (640mL) was added methylsulfonyl chloride (80mL). The reaction mixture was protected from moisture and stirred at room temperature for 24 hr at which time TLC (Solvent D) showed reaction to be complete. The reaction mixture was poured into ice-cold water (2L) and extracted with chloroform (3x300mL) after filtration of the fluffy white precipitate. The chloroform extract was washed successively with 2N hydrochloric acid (3x300mL), saturated solution of sodium bicarbonate (3x300mL), water (3x300mL), dried (MgSO$_4$, anhydrous) and evaporated to dryness under vacuo to give a thick syrup which crystallized on standing overnight. Recrystallization of this and the crude precipitate from absolute ethanol afforded the title compound as white crystalline needles (142.2g, 75%) m.p. 141-142°C [Lit 141-142°C]; R$_f$ 0.84 (Solvent D).

METHYL 4-O-METHYLSULFONYL-α-D-GALACTOPYRANOSIDE$^{106}$ (52)

A suspension of methyl 2,3,6-tri-O-benzoyl-4-O-methylsulfonyl-α-D-galactopyranoside (50g) in anhydrous methanol (330mL) was cooled to 4°C. Freshly prepared sodium methoxide (1.7g of sodium in 27mL of anhydrous methanol) was added. The reaction mixture was stirred at 4°C for
4 hr when TLC (Solvent G) showed reaction to be complete. The mixture was neutralized with Amberlite IR-120 (H+) (70mL), filtered immediately and evaporated to dryness under vacuum. The residue was partitioned between equal volumes (180mL) each of water and chloroform, and the water layer extracted. The chloroform layer was washed with water (2x100mL). The combined aqueous extract was washed with chloroform (2x50mL), treated with decolorizing charcoal, and evaporated under reduced pressure to give a solid residue. Recrystallization from absolute ethanol afforded the desired product (17.5g, 75%) m.p. 160-161°C [Lit108 159-160°C]; Rf 0.75 (Solvent G).

**METHYL 2,3,6-TRI-O-BENZYL-4-O-METHYLSULFONYL-α-D-GALACTOPYRANOSIDE**109 (53)

Methyl 4-O-methylsulfonyl-α-D-galactopyranoside (27g) was dissolved in anhydrous dimethyl formamide (234.3mL). Silver oxide (70.2g) was added followed by the addition of benzyl bromide (64.3mL). The reaction mixture was stirred (magnetic stirrer) for three days at room temperature while protecting from moisture. At the end of the reaction, the mixture was filtered and the filtrate washed with chloroform (3x45mL). The filtrate was diluted with chloroform (450mL) and filtered once more. The filtrate was then washed with water (3x360mL). Pyridine (54mL) was added to the chloroform layer and the resulting solution washed successively with water (2x450mL), 2N hydrochlo...
acid (3x180mL) saturated solution of sodium bicarbonate (2x200mL) and finally with water (2x360mL). The chloroform extract was dried (anhydrous MgSO₄) and evaporated under reduced pressure to give a thick light yellow syrup (59g). The syrup was divided into two parts and each part placed on an alumina (Neutral, activity II) column. Elution with petroleum ether (b.p. 35-60°C): ether (5:1 v/v) (1.2L) removed the dibenzyl ether (12g). Elution with ether (1.0L) and evaporation under vacuo afforded the title compound (39.5g, 73.6%) as a colorless syrup; Rf 0.44 (Solvent H).

**2-METHYL 2,3,6-TRI-O-BENZYL-4-DEOXY-4-FLUORO-D-GLUCOPYRANOSIDE**\(^{109}\) (54)

Freshly prepared tetrabutylammonium fluoride was prepared by titration of 40% aqueous tetrabutylammonium hydroxide (64mL) with 50% aqueous hydrofluoric acid to pH 7.0. The solution was concentrated under reduced pressure and the resulting syrup dried overnight by vacuum desiccation over phosphorous pentoxide at 0.1mm.

The tetrabutylammonium fluoride was dissolved in anhydrous acetonitrile (72mL) and methyl 2,3,6-tri-O-benzyl-4-O-methylsulfonyl-D-galactopyranoside (8.0g) was added to it. The reaction mixture was refluxed gently (70-80°C) while protecting the mixture from moisture using a CaCl₂ tube. After 3 days when reaction appeared to be complete,
TLC (Solvent H), the reaction mixture was poured into water (51mL). The aqueous mixture was extracted with ether (3x150mL). The ethereal extract was dried (anhydrous MgSO₄) and concentrated under reduced pressure to give a syrup which was purified by column chromatography (silica gel H, mesh size 60-200, 230g). Elution was with ether: petroleum ether (b.p. 35-60°C) (7:3 v/v). Evaporation of the eluent under reduced pressure yielded the title compound (5.23g, 76.3%) as a clear colorless syrup. Rf 0.64 (Solvent H), 0.68 (Solvent C).

METHYL 4-DEOXY-4-FLUORO-D-GLUCOPYRANOSIDE¹⁰⁹ (55)

A solution of methyl 2,3,6-tri-β-benzyl-4-deoxy-4-fluoro-D-glucopyranoside (5.0g) in ethanol (96mL) was shaken in the presence of hydrogen and 5% palladised charcoal (5.8g) at one atmosphere pressure and room temperature until hydrogen uptake ceased (24 hr). The reaction mixture was filtered through Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure to dryness and the residue dissolved in a minimum amount of methanol then placed on a silica gel (Grade H, Mesh size 60-200, 115g) column. Elution with ethyl acetate and concentration under reduced pressure spontaneously yielded the title compound as white crystals. Recrystallization from ethyl acetate-acetone (1:1 v/v) afforded the desired product as white crystal (1.27g, 60%), m.p. 130-131°C [Lit¹⁰⁹ 129-130], Rf 0.74 (Solvent G).
METHYL 2,3,6-TRI-O-BENZOYL-4-DEOXY-4-FLUORO-D-
GLUCOPYRANOSIDE\textsuperscript{72} \textsuperscript{(56)}

Methyl 2,3,6-tri-O-benzoyl-D-galactopyranoside (5.0g) was dissolved in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (28.25mL), and the mixture cooled to -40\degree C (dry ice-acetone bath). Diethyl aminosulfur trifluoride (DAST, 1.77mL) was added (adding in a single shot was observed to yield better results than dropwise addition). The temperature was maintained between -30\degree C to -40\degree C while the reaction was stirred for 30 min. The temperature was then gradually allowed to increase to room temperature. The reaction mixture was allowed to stir for 24 hr. At the end of 24 hr when TLC (Solvent 1) showed only a trace or no starting material, the reaction was cooled to -20\degree C and quenched by the addition of methanol (10mL). The mixture was poured into a saturated solution of sodium bicarbonate. The mixture was extracted with CH\textsubscript{2}Cl\textsubscript{2} (3x100mL). It was dried, anhydrous magnesium sulfate and evaporated to dryness under reduced pressure to afford the title compound in syrupy form.

The syrupy product was crystallized from ethanol. Recrystallization from the same solvent yielded the title compound as white solid (3.75g, 74.7\%), m.p. 138-140\degree C [Lit\textsuperscript{72} 139-141].
METHYL 4-DEOXY-4-FLUORO-D-GLUCOPYRANOSIDE (55)

Methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-fluoro-D-galactopyranoside (6.47g) was suspended in dry methanol (42.2mL). The mixture was cooled to -10°C. Freshly prepared sodium methoxide (0.48g sodium in 8.3mL dry methanol) was added dropwise. At the end of the addition, the temperature was gradually raised to 4°C. The reaction mixture was stirred at 4°C for 4 hr at the end of which time TLC (Solvent G) showed no starting material. The reaction mixture was immediately neutralized with Amberlite IR 120(H+) (7.0mL) until the evolution of hydrogen ceased. The mixture was filtered and immediately evaporated to dryness under reduced pressure to give a light brown syrup. The syrupy material was partitioned between equal volumes each of chloroform and water (100mL). The chloroform layer was washed with water (3x100mL). The combined aqueous extract was evaporated under reduced pressure to dryness. The residue crystallized spontaneously. Recrystallization from ethyl acetate: acetone (1:1 v/v) or absolute ethanol afforded the title compound as white crystals (2.26g, 75.8%). Rf, 0.74 (Solvent G) m.p. 129-130°C [Lit 10 134.43°C] i.r. (KBr) 3461, 3382 (S,-OH stretch), 1034 (S, C-F stretch) cm⁻¹.

4-DEOXY-4-FLUORO-D-GLUCOSE (18)

A solution of methyl 4-deoxy-4-fluoro-D-glucopyranoside (1.0g) was dissolved in 2M sulfuric acid (100mL) and gently refluxed for 3 hours, after which time TLC (Solvent G)
showed reaction to be complete. Barium carbonate (500g) was suspended in water (1L) and then heated with stirring until it just started to boil. The suspension was cooled briefly and the reaction mixture poured into it. The resulting mixture was stirred overnight after which time the pH became neutral (pH paper). The mixture was filtered through Whatman No. 1 filter paper (3X), then through a sintered glass funnel to remove last traces of barium carbonate. The filtrate was concentrated to dryness under reduced pressure and taken up in absolute ethanol. The ethanol mixture was filtered once more through a sintered glass funnel containing a bed of Kieselguhr and decolorizing charcoal. The resulting solution was evaporated to dryness under reduced pressure to give a syrup which crystallized on standing overnight at room temperature. Recrystallization from ethanol afforded the title compound (0.75g, 81.1%) as white solid, m.p. 189-190°C [Lit\textsuperscript{110} 188-190°C]; [α]\textsubscript{D}\textsuperscript{23} + 26.5° (50° (c 1.5, 24 hr equil., water) [Lit\textsuperscript{109} [α]\textsubscript{D}\textsuperscript{23} + 26.9° (49° (76 hr equil., water)], Ret 0.18 (Solvent J), Ret 0.64 (Solvent G). The compound gave a positive reaction with aniline hydrogen pthalate spray. I.R. (KBr) 3480-3160 (S, -OH stretch), 2980, 2940 (m, C-H stretch), 1360-1350 (S, C-F stretch), 1020 (S, C-O stretch), 908 (m, ring vibration), 898 (S, anemic C-H stretch, θ-anomer) cm\textsuperscript{-1}. Chemical shift (φ) -120ppm. I.R. (KBr) for 3FG; 3390 (S, -OH stretch),
2938 (m, C-H stretch), 1387 (S, C-F stretch),
1082 (m, C-O stretch), 916 (m, ring vibration), 899 (S,
anomeric C-H stretch, β-anomer), 860 (m, anomeric C-H
stretch, α-anomer) cm⁻¹. δ for β-anomer -111ppm, δ for
the α-anomer -116ppm.
ISOLATION AND PURIFICATION OF PYRANOSE-2-OXIDASE FROM
POLYPORUS OBTUSUS

GROWTH OF SLANT CULTURES OF POLYPORUS OBTUSUS (ATCC 26733)

Malt extract (20g), D-glucose (20g), peptone (1g) and agar (20g) were dissolved in 1L of distilled water. The mixture was dispensed into culture tubes (16 x 15mm) and autoclaved (Pelton & Crane, Charlotte, N.C. 28224) at 121°C for 15 min. After autoclaving the mixture was allowed to cool to room temperature. The slants so formed were inoculated with cultures of Polyporus obtusus (ATCC 26733), and the culture allowed to grow at room temperature for 7 days.

GROWTH OF MYCELIA OF POLYPORUS OBTUSUS

Ten seed cultures were prepared by dissolving yeast extract (6g), malt extract (6g), peptone (10g) and glucose (10g) in distilled water (2L), and dispensing the medium into ten 1 litre Erlenmeyer flasks (200mL/1L flask). The seed cultures were autoclaved at 121°C for 15 min. After allowing to cool to room temperature, the ten seed cultures were inoculated aseptically with the slant grown organism. The seed cultures were then grown at room temperature for 9 days while shaking at 250 rpm. At the end of the growth time, mycelia of Polyporus obtusus, small
mushroom shaped product was harvested by vacuum filtering on a Whatman No. 541 filter paper (9 cm diameter). The mycelia was washed twice with 0.05M potassium phosphate buffer, pH 7.0 (300mL), and filtration vacuum maintained for a few additional minutes to dry the mycelia as much as possible. The mycelia thus obtained was stored at -20°C until needed.

**ISOLATION AND PURIFICATION OF ENZYME**

Potassium phosphate buffer (0.05M), pH 7.0 (120mL) and mycelia (4g) were put in a Waring blender, and homogenized at 35 sec. stretches at 4°C until all the mycelia was completely homogenized. The homogenate was dispensed into 50mL plastic centrifuge tubes. The blender was rinsed with a further 20mL of phosphate buffer and added to the homogenate. The homogenate was centrifuged at 7000 rpm for 20 min. at 4°C. The supernatant was removed and placed in a litre Erlenmeyer flask. Polyethylene glycol (Molecular Weight 4,000) was added gradually, with stirring until a final concentration of 10% w/v was obtained. The mixture was allowed to stand at room temperature for 30 minutes, then centrifuged at 7000 rpm for 20 min at 40°C. The supernatant was discarded and the precipitate suspended in 0.05M potassium phosphate buffer (pH 7.0) containing 0.2M sodium chloride (36mL), and vortexed until the precipitate had completely dissolved.
The mixture was allowed to stand at room temperature for 30 minutes then centrifuged at 14,000 rpm for 20 min. at 4°C. The amber-yellow supernatant containing partially purified pyranose-2-oxidase (E.C. 1.1.3.10) was collected and kept at 4°C until needed.

DETERMINATION OF THE ACTIVITY OF PARTIALLY PURIFIED SOLUBILIZED PYRANOSE-2-OXIDASE

Horseradish peroxidase (0.4%, 100 Units/mg), O-dianisidine (1%), β-D-glucose (10%) and sodium phosphate buffer (0.5M), pH 7.0 were all prepared in distilled deionized water. Three sets of reagents were used for the assay.

REAGENT A

This reagent was prepared fresh daily. Into clean dry test tube (triplicate) was pipetted distilled deionized water (690μl), 0.5M sodium phosphate buffer, pH 7.0 (100μl); 0.4% peroxidase (100μl) and O-dianisidine (10μl).

REAGENT B

A solution of 10% β-D-glucose.
Enzyme solution (30μL)

Reagents A and B were incubated at 25°C for 10 min. Various volumes of reagent B (plus distilled deionized water to make 70μL) was added to A. This gave the desired final concentration in a final reaction volume of 1mL. The resulting solution was transferred into a 1.5mL quartz cuvette. Enzyme (30μL) was added and the mixture shaken quickly, put in the cell compartment of the spectrophotometer (Beckman or ACTA MVI), and the change in absorbance at 460nm followed for three minutes. Two types of blanks were used:

(i) an enzyme blank; and 
(ii) a substrate blank.

Both types of blanks gave similar results. Subsequent reactions therefore used only substrate blank. Km, Vmax and Ki values were obtained by the Lineweaver-Burk plots according to the method of least square and the direct linear plots.

KINETIC AND INHIBITION STUDIES WITH PYRANOSE-2-OXIDASE

The assay procedure was the same as already described under general assay for a total reaction volume of 1mL except that different concentrations of glucose, 4FG and 3FG served as reagent B. In each case, the reagents,
except enzyme, were incubated at 25°C for 10 min. before
the assay. Triplicate readings were taken and the mean
of these used, unless otherwise stated. Concentration ranges
used for the sugars were 5, 10, 15, 20, 30 and 40mM.

The procedure was the same as already described. The
reaction mixture was incubated with the effector for 10
min. before the addition of the substrate. The following
effects were studied.

A. Effect on glucose by 4FG and vice versa. Concentrations
   of effector used were 5mM, 10mM and 15mM.

B. Effect on glucose by 3FG. Concentrations of 3FG used
   were 5mM, 10mM and 15mM.

DETERMINATION OF FREE FLUORIDE LEVEL AT END OF REACTION

Fluoride calibration curve was prepared as outlined
in the text. The fluoride level in the reaction mixtures
of 4FG, 3FG and reaction blank was measured on a 'Metrohm'
 meter (Metrohm Herisau Prazisions-pH-meter, Model E 510,
 Switzerland) equipped with a fluoride specific electrode.
The fluoride level in the reaction mixtures and blank
were, measured in millivolt potential, and was converted
into concentration with the aid of the fluoride standard
curve. (See Appendix 6.)
IMMOBILIZATION OF PYRANOSE-2-OXIDASE ONTO CYANOGREN BROMIDE

ACTIVATED SEPHAROSE 4B

Inactive sepharose 4B (30mL) was suspended in cold distilled water (500mL) and the solution stirred for 30 minutes. The suspension was filtered through a coarse sintered-glass disk funnel and the slurry resuspended in cold distilled water (500mL), and filtered again. The washing was repeated several times (3x) until the absorbancy of the filtrate was negligible (from 0.911 down to 0.084) between 230nm to 300nm. The washed sepharose was suspended in 1 volume (30mL) of distilled water and transferred into a 2L beaker placed in a fumehood. The suspension was stirred continuously (magnetic stirrer). The electrode of a pH meter (Prazisions-pH meter #510, Metrohm AG-CH, Switzerland) and a thermometer were introduced into the suspension. Cyanogen bromide (3.5g) was added in a single shot to the suspension, and the pH maintained between 10.8 and 11.2 by the constant titration with 10M sodium hydroxide. Ice was also added to keep the temperature near 20°C. Titration was continued until the release of protons had slowed down, as evidenced by the decreased amount of sodium hydroxide needed to maintain a constant pH. A large amount of ice was added and the suspension quickly transferred to a 2 litre sintered-glass funnel and vacuum filtered. The "activated" sepharose 4B, thus obtained, was washed with ice cold distilled water (2.0L),
then with 0.1M sodium bicarbonate (pH 9.0, 500mL).

The activated sepharose 4B was immediately transferred into a 100mL beaker. Cold sodium bicarbonate (0.1M, pH 8.0, 30mL) was added to the gel. Partially purified Pyranose-2-oxidase (25mL), dialysed overnight against 500mL distilled water, was added to the freshly activated sepharose 4B. The mixture was stirred gently at 25°C for 1.5 hrs. It was then washed successively with 0.1M sodium bicarbonate, pH 8.0 (250mL), 0.05M Tris buffer (250mL), pH 8.0, also containing 0.5M sodium chloride and finally 0.5M sodium formate (250), pH 4.0, also containing 0.5M sodium chloride (250mL). This gave immobilized pyranose-2-oxidase, final wet weight 18g. The solid enzyme was stored in 0.05M potassium phosphate buffer (36mL) pH 7.0, containing sodium chloride (0.2M), at 4°C until needed.

The activity of the immobilized enzyme was checked by the peroxidase-0-dianisidine procedure as already described.
4-DEOXY-4-FLUORO-D-ARABINO-HEXOS-2-ULOSE (57)

4-Deoxy-4-fluoro-D-glucose (18) (350mg), catalase (40 units, from bovine liver), immobilized pyranose-2-oxidase (1.2g wet wt.) and sterile distilled water (100mL) were added to a 300mL Erlenmeyer flask. After shaking at 200 rpm (Lab-line orbit Incubator shaker No. 3595, Lab-line Inc. USA) at 25°C for 28 hr, the mixture was filtered through a Whatmann No. 541 filter paper, then successively through 5-, 0.42-, and 0.20μm filters. The filtrate was lyophilized to yield a pale yellow syrup (241mg, 69.6%), which was stored desiccated (CaCl₂) at 70°C; Rᵢ 0.60 (Solvent L) (cf Rᵢ starting material 0.83) Rᵢ 0.78 (Solvent M). The compound gave a red spot on silica gel TLC plate when sprayed with triphenyltetrazolium chloride. IR (neat) 3600-3140 (s, OH stretch) 1690 (s, C = O stretch) 1350 (s, C-F stretch) 1150-1000 (s, C-O stretch).

HPLC analysis Rᵢ 11.7 min.

¹⁹F NMR (δ)α - 120.4 ppm; (δ)β - 122.5 ppm, [α]D²³ -19.6° (C, 0.46, H₂O). nmr JF,H-4 50 ± 3Hz, JF,H-3 11 ± 3Hz. JF,H-5, 11 ± 3Hz.

4-DEOXY-4-FLUOROFRUCTOSE (58)

4-Deoxy-4-fluoroglucosone (57) (250mg) was dissolved in water (20mL). Palladised charcoal (125mg) was added and the mixture shaken in the presence of hydrogen at
room temperature and 50 psi for 15 hr. The reaction mixture was centrifuged at 30,000 rpm for 30 min. at 4º and the supernatant successively filtered through a Whatmann No. 541 filter paper (9 cm e.d.), twice through 0.45µm membrane filter, then finally through 0.20µm membrane filter (Sartorius membrane filters from Canlab, Toronto, Ontario) placed in a sintered-glass funnel.

Lyophilization of the filtrate yielded a colourless syrup (167mg, 66%), Rf 0.76 (Solvent L), which was stored desiccated at -20ºC. IR (neat) 3440-3080 (S, -OH stretch) 2930 (m; C-H stretch) 1630 (S, C=O stretch) 1300 (S, C-F stretch) 1035 (S, C-O stretch).

HPLC analysis RT 12.1 min.

¹⁹F nmr δ -120.37 ppm (TFA internal standard) [α]D³³, -30.5º (C, 0.83, H₂O), J_F,H-4 39 ± 3Hz, J_F,H-3 12 ± 3Hz, J_F,H-5 12 ± 3Hz.
4-DEOXY-4-FLUORO-D-GLUCITOL (59)

4-Deoxy-4-fluorofructose (400mg) was dissolved in water (20mL) and the mixture stirred at 4°C. A solution of sodium borohydride (200mg) in 10mL water) was added slowly with stirring at 4°C. After stirring for 3 hr, the excess borohydride was destroyed by stirring with Amberlite IR-120 (H⁺), until the evolution of gas ceased. The resulting solution was filtered and the filtrate evaporated under reduced pressure to give a syrup which was repeatedly dissolved in methanol and evaporated to dryness in vacuo to remove borate as the volatile methyl borate. The resulting syrup which failed to crystallize showed two spots on TLC (Solvent J) a major product Rf 0.58 and a minor product Rf 0.65 which was tentatively assigned the structure of the C-2 epimer, 4-deoxy-4-fluoro-D-mannitol. The starting material had Rf 0.76 (Solvent L). The syrup was purified by preparative TLC (Solvent C). The chromatographically pure syrup (319 mg, 79%), failed to crystallize. The compound did not give a positive test with aniline hydrogen phthalate spray. [α]D²³ -3.4 (c, 2.64, methanol) [Lit¹¹¹ [α]D²³ -3.5].

IR. (neat) 3400-3270 (s, -OH stretch; 2947, 2900 (m, C-H stretch); 1098 (s, C-F stretch); 1040 (S, C-O-stretch) cm⁻¹.
4-DEOXY-4-FLUOROFRUCTOSE PHENYLHYDRAZONE (60)

4-Deoxy-4-fluoro-D-fructose (300mg) was dissolved in dry methanol (5mL). Phenylhydrazine dihydrochloride (360mg) was added. The mixture was heated to boiling (water bath). Heating was continued until all the solvent had evaporated. The solid residue was washed with petroleum ether (30-60°C), then ice-cold water. The residue was resuspended in ice water, filtered and washed once more with ice water. The residue was taken up in chilled ether and the resulting solution evaporated to dryness under reduced pressure. The residue was washed twice with Petroleum ether (30°-60°C) and crystallization attempted in Methanol. Purification by column chromatography (Silica gel H, Mesh size 60-200) and evaporation of the eluent to dryness under reduced pressure yielded a syrup which again failed to crystallize. TLC (Solvent F) yielded a single spot Rf 0.75, 309mg (69%) [α]D 27 -20.5 (c 1.0, CHCl₃).

3,5,6-TRI-O-ACETYL-4-DEOXY-4-FLUORO GLUCOSONE-1-DIPHENYL HYDRAZONE (61)

4-Deoxy-4-fluoro-D-glucosone (100mg) was dissolved in 75% aqueous ethanol (1mL) containing 1,1-diphenyl hydrazine (110mg). The reaction mixture was vortexed, then stirred at 25°C for 8 hr. At the end of the reaction, distilled water (3mL) was added, and the mixture partitioned between chloroform (10mL) and water. The aqueous layer was extracted
with chloroform (2x10mL). The combined chloroform extract was dried (anhydrous MgSO₄) and evaporated to dryness under reduced pressure. To the residue was added a 1:1 mixture of dry pyridine and acetic anhydride (1mL), and the mixture stirred at 40°C for 20min. Distilled water (2mL) was added to stop the reaction. The resulting mixture was extracted with ether (3 x 3mL). The ethereal extract was dried (anhydrous MgSO₄) and evaporated under reduced pressure to yield a dark brown syrup which was immediately placed on a Finnigan-MAT CH5 probe and the spectra followed at 200°C and 70ev (electron-impact ionization). The electron impact mass spectrum of the title compound gave the following peaks expressed as m/e (% of relative intensity, using m/e 169 as the base peak). 18(27), 43(80), 61(69), 79(25), 115(5), 169(100), 223(90), 268(19), 322(14), 350(26), 454(8), 472(16), 548(13), 576(32), 638(11).

The above procedure was used for the preparation of phenylhydrazine derivatives of the D-glucosone, prepared using pyranose-2-oxidase, and D-glucose.

1,3,5,6-TETRA-O-ACETYL-4-DEOXY-4-FLUORO-D-FRUCTOPYRANOSIDE (62)

A mixture of dry pyridine (1.4mL) and acetic anhydride (1.0mL) was cooled to 0°C. 4-Deoxy-4-fluoro-D-fructopyranose (100mg) was added. The reaction mixture was stirred until all the sugar dissolved. The reaction temperature was
allowed to increase gradually to room temperature and the reaction allowed to continue at room temperature for 18 hr. The reaction was stopped by the addition of ice cold distilled water (3mL). The aqueous mixture was extracted with anhydrous ether (3x20mL). The ethereal extract was washed successively with 2N hydrochloric acid (3x20mL), saturated solution of sodium bicarbonate (3x20mL), and finally with distilled water (3x20mL). The ether layer was dried (anhydrous MgSO₄) and evaporated under reduced pressure to yield a yellowish syrup. The syrup was taken up in ether and placed on a silica gel column (mesh size 60-200, 2g) and eluted with ether: Petroleum ether (b. p 35-60°C)(3:1 v/v), then ether. Evaporation of the eluent under reduced pressure afforded the title compound (130mg, 68%) as a syrup which gave a single spot on TLC Rf 0.70 (Solvent X). Anal Calc. for C₁₄H₁₉O₉F; C, 48.0; H, 5.4; F, 5.4. Found: C, 46.62; H, 5.35; F, 5.35. The pure syrup was placed on a Finnigan-MAT CH5 Mass spectrometer and probed at 180°C. Probe was started at room temperature and the temperature increased stepwise by 25°C to the final temperature 180°C. The electron impact was 70ev. The electron impact mass spectrum of the title compound gave the following peaks expressed as m/e (% relative intensity, using m/e 43 as the base peak).

18(6.48), 43 (100), 55 (3.16, 61 (32.75), 73 (4.39), 74 (1.31), 84 (10.51), 103 (18.76), 117 (2.11), 129 (0.67),
145 (11.82), 160 (15.79), 174 (0.85), 232 (7.18), 277 (0.50), 290 (1.82), 291 (0.59).
RESULTS AND DISCUSSION

Radiorespirometric and chromatographic studies on the metabolism of 3FG in Locusta Migratoria revealed that one of the products of this metabolic process may be 3-deoxy-3-fluoro-D-fructose. The proposed pathway was as shown (Fig. 19).

It was considered that the 3FF produced may be converted to 3FF-1,6-di-P, the breakdown of which leads to the release of F⁻ (Fig. 19) and death of the insect. In order to further support this proposal, the synthesis of the 3FF was attempted. Such a synthesis was attempted initially by displacement of tolylsulfonyl or mesylsulfonyl leaving groups from 1,2:4,5-di-O-isopropylidene-3-O-tolylsulfonyl(mesylsulfonyl)-psicopyranose using tetrabutylammonium fluoride as the fluoride source (Fig. 20). Neither sulfonic esters underwent displacement by fluoride. The use of several other means including the reagent DAST to achieve displacement of the sulfonic ester groups by fluorine, either in the psico- or fructo-form were attempted without success. The failure to introduce fluorine into the keto-hexose is better understood by looking at the Newman projections in Fig. 21. (48) is represented by (65) and (64) by (67). Both (63) and (64) have permanent dipoles about the anomeric group with those formed as the transition state is developed. The transition state is necessary for fluorination to occur. As the molecules
Figure 19

Proposed pathway for 3FG metabolism in *Locusta migratoria*

Enzymes involved are:

1. Aldose reductase.
2. Sorbitol dehydrogenase.
3. Fructokinase.
4. Phosphofructokinase.
5. Aldolase.
6. Triose phosphate isomerase.
Figure 19. Proposed Pathway for 3FG Metabolism in Locusta Migratoria.
Attempted synthesis of 3-deoxy-3-fluoro-D-fructose.
Nucleophilic displacement of sulfonic esters and unfavourable dipolar repulsion in the transition state (66).
move from the ground state to the transition state, (66), a highly unfavourable dipolar repulsion develops making it impossible to attain the transition state. Also, the bulky rigid isopropylidene groups attached to C-1 and 2 and C-4 and 5 seriously restrain any rotation of the molecule. \( S_N2 \) nucleophilic attack by the fluoride is therefore difficult. Attempts to synthesize fluoroketoses especially 3FF have been documented in literature and although various methods of approach have been employed all the attempts have been unsuccessful.\(^{116-120}\)

As will be discussed later in this thesis, 4FG like 3FG is also toxic to \textit{Locusta migratoria}. As with 3FG, a small but significant release of fluoride ion is also observed. The pathway for 4FG metabolism has yet to be established but by analogy with 3FG (Fig. 19) the formation of 4-deoxy-4-fluoro-D-fructose (4FF) is a distinct possibility. Indirect support for this possibility was suggested by the fact that 4-deoxy-4-fluoro-D-glucitol is known to be a substrate \(^{110}\) for sorbitol dehydrogenase (E.C. 1.1.1.14) with a \( K_m \) value 20.4 mM and a \( V_{\text{max}} \) of 0.061 \( \mu \)M of NADH min\(^{-1}\) mg protein\(^{-1}\).

In view of the difficulties encountered above in our attempts to chemically synthesize 3FF, and that other attempts to synthesize 4FF had been unsuccessful\(^{117}\), it was decided that the strategy for the synthesis of 4FF would be based on the use of a known pre-formed fluorinated
Synthesis of 4-deoxy-4-fluoro-d-glucose.

Figure 22
Figure 23

4-Dexoxy-4-fluoroglucose

Methyl 4-deoxy-4-fluoroglucose

4-Dexoxy-4-fluorofructose
sugar as the starting material. Moreover, a combination of enzymic and chemical methods might be more propitious. With this approach in mind, the following scheme seemed appropriate (Fig. 23).

Initially 4FG (18) was obtained by a longer synthetic method (7 steps from (44)) as previously reported55 (Fig. 22). This synthesis of (18) was much improved (4 steps from (44); Fig. 22), by the direct replacement of the C4-OH group of methyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside (45) by fluorine using the reagent diethylaminosulfur trifluoride (DAST) as developed by P.J. Card72. The fluorination with DAST proceeded very smoothly under mild conditions (-30°C to -40°C; then room temperature) and took only 24 hr instead of 3 to 3½ days with tetrabutylammonium fluoride. Fig. 24 shows the mechanism for replacement of -OH of the partially protected carbohydrate (55) by fluorine using DAST in methylene chloride. The "in situ" formation of a leaving group by DAST as seen in Fig. 24 is an obvious advantage of this reagent over other fluorinating reagents. The diethylamino-difluoro-sulfonyloxy group being a very good leaving group facilitates the fluorination. The S_N2 replacement via the in situ leaving group obviates carbonium ion formation. Carbonium type rearrangements are therefore less likely. Improved yields, have been observed by using DAST in the presence of pyridine 75-121 or 4-(dimethylamino)pyridine
Figure 24

Mechanism for the replacement of -OH of Methyl-
2,3,6-tri-O-Benzoyl-galactopyranoside by fluorine using the
reagent Diethylamino sulfur trifluoride (DAST). Note the in
situ formation of a leaving group by DAST.
(DMAP)\textsuperscript{121}. In our hands a good yield (75\%) has been obtained by adding DAST in "one shot".

Use or absence of a nitrogen atmosphere\textsuperscript{72} did not affect the yield. The use of absolutely dry solvent and glassware was found, however, to be important. Removal of the benzoyl groups was effected by use of freshly prepared sodium methoxide. The reaction time was 4 hours at 40°C. Methyl 4-deoxy-4-fluoro-D-glucopyranoside synthesized by the alternative procedure co-chromatographed with that synthesized by the established route.\textsuperscript{55} Mixed melting point and $[\alpha]_D^{23}$ was identical to the authentic sample. The demethylation product\textsuperscript{19} of the alternative route was identical in $R_F$, melting point and $[\alpha]_D^{23}$ to that of authentic 4FG. The overall yield of the alternative procedure was about eight-fold higher than the established route.

Chemical synthesis of deoxy-fluoro-ketoses have been fraught with difficulty\textsuperscript{34, 106-116-119}, particularly the fluoro-fructoses. Using the pre-formed 4FG, a combination of chemical and enzymic synthesis was the approach of choice. Pyranose-2-oxidase (E.C. 1.1.3.10), isolated from the mycelia of polyporus obtusus oxidizes glucose and other carbohydrates at C-2 to yield the 2-keto derivatives (Fig. 25).\textsuperscript{112, 114, 122-125} The enzyme has been reported as a single protein band on acrylamide gel and consisting of identical subunits, each of molecular weight 68,000 and a total
Figure 25. Oxidation of various sugars by pyranose-6-oxidase.
molecular weight of 220,000$^{125}$. Although no detailed kinetic data was presented, Janssen and Ryelius established the specificity of pyranose-2-oxidase towards glucose and other sugars$^{126}$. They found that the glucose oxidation product was D-arabino-hexos-2-uloside (D-glucosone)$^{112,112,122,123}$. In order to take advantage of this oxidation reaction for synthetic purposes, it became necessary to study the kinetics of the pyranose-2-oxidase with respect to glucose, 4FG and possibly 3FG. Using partially purified pyranose-2-oxidase from *Polyporus obtusus* (ATCC 26733), the unit activity of the enzyme, based on the amount of enzyme producing 1 μmol of H$_2$O$_2$ min$^{-1}$ at 25°C, was found to be 12.8 units mg protein$^{-1}$. The peroxidase-o-dianisidine coupled colorimetric system was used for the assay. As expected, initial rate kinetics with glucose revealed that glucose is a substrate for the enzyme obeying Michaelis-Menten kinetics. A lineweaver-Burk plot, which was estimated by the method of least squares gave a $K_m$ of 3.2 ± 0.08 mM and a $V_{max}$ of 126 ± 0.02 μmol min$^{-1}$ mg protein$^{-1}$ (Fig. 26). The same results for $K_m$ and $V_{max}$ were obtained using the direct linear plot$^{129}$ (Fig. 27). Although a modification of the B-D-gluco configuration at C-2, C-3, and C-4 led to more than 90% decrease in enzyme activity relative to glucose$^{121}$, initial rate kinetics of 4-deoxy-4-fluoro-D-glucose indicated that 4FG is a moderately-good
Figure 26

Lineweaver-Burk plot of initial rate kinetics for glucose oxidation by pyranose-2-oxidase. \( v \), initial rate of hydrogen peroxide production (\( \mu \text{mole min}^{-1} \text{ mg protein}^{-1} \)).

\( S \), [glucose] in mM.

\[ K_m = 3.2 \pm 0.08 \text{ mM} \]

\[ V_{max} = 126 \pm 0.02 \mu \text{moles min}^{-1} \text{ mg protein}^{-1} \]
Figure 27

Direct linear plot of initial rate kinetics for glucose oxidation by Pyranose-2-oxidase. Velocity, initial rate of hydrogen peroxide production (μmole min⁻¹ mg protein⁻¹).

[S], [glucose] in mM.

\[ K_m = 3.2 \pm 0.05 \text{ mM} \]

\[ V_{max} = 125.0 \pm 0.04 \text{ μmole min}^{-1} \text{ mg protein}^{-1} \]
substrate. Plots similar to those of glucose gave a $K_m$ of $5.54 \pm 0.64 \text{mM}$ and a $V_{\text{max}}$ of $104 \pm 0.07 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ (Fig. 28). 4FG was found to affect the oxidation of glucose by pyranose-2-oxidase; and glucose was also found to affect 4FG oxidation. (Figs. 29, 30a,b). These effects were analyzed using the Dixon and Webb relationship$^{140}$ (Appendix II), which showed 4FG to be a competitive inhibitor of glucose oxidation, with a $K_i$ of $5.50 \pm 0.02 \text{mM}$ as determined by the Lineweaver-Burk plots (Fig. 29 and 30a). Glucose on the other hand was found to be a poor competitive inhibitor of 4FG oxidation with a $K_i$ of $24.90 \pm 0.25 \text{mM}$, according to the Lineweaver-Burk plot (30b).

As with 3-O-methyl-D-glucopyranose$^{121}$, the C-3 modified fluoro-analog of D-glucose, 3-deoxy-3-fluoro-D-glucose was found not to be a substrate for the enzyme. Yet it stimulated the reactivity of the enzyme towards glucose. It is of interest to note that a comparison of $^{19}\text{F}$ nmr and infra-red of 4FG and 3FG indicates that the former exists predominantly as the $\alpha$-anomer whilst the latter exists as an equal mixture of $\alpha$- and $\beta$-anomers*. From the Lineweaver-Burk plot (Fig. 31,32), it is seen that the $V_{\text{max}}$ of glucose increases with respect to increasing concentration of 3FG. The pattern displayed by the Lineweaver-Burk plot is also similar to that observed by Krupka$^{127}$ in his studies of acetylcholinesterase inhibition.

* See Appendices 8 and 9
Figure 28

Lineweaver-Burk plot of initial rate kinetics for 4-deoxy-4-fluoro-D-glucose (18) oxidation by pyranose-2-oxidase. $\nu$, initial rate of hydrogen peroxide production ($\mu$ mole min$^{-1}$ mg protein$^{-1}$). $S$, [4FG] in mM

$K_m = 5.54 \pm 0.64$ mM

$V_{max} = 104.00 \pm 0.07$ $\mu$ mole min$^{-1}$ mg protein$^{-1}$
Figure 29

Effect of 4-deoxy-4-fluoro-D-glucose on glucose oxidation by pyranose-2-oxidase.

- glucose only

▼ glucose oxidation in the presence of 15mM 4FG

▼ initial rate of hydrogen peroxide production (μmole min⁻¹ mg protein⁻¹)

S [glucose] in mM

\[ K_i = 5.5 \pm 0.02 \text{mM} \]
Figure 30

Effect of various concentrations of 4-deoxy-4-fluoro-D-glucose on oxidation of glucose by pyranose-2-oxidase.

- Glucose only
- Glucose + 5 mM 4FG
- Glucose + 10 mM 4FG
- Glucose + 15 mM 4FG

Initial rate of hydrogen peroxide production (μmole min⁻¹ mg protein⁻¹)

[S [glucose] in mM]
Figure 30(b)

Lineweaver-Burk plot of the effect of glucose on oxidation of 4-deoxy-4-fluoro-D-glucose (4FG) by the enzyme pyranose-2-oxidase.

- 4FG alone
- 4FG in the presence of 15mM glucose

\[
\text{Initial rate of hydrogen peroxide production (} \mu \text{mole min}^{-1} \text{ mg protein}^{-1} \text{)}
\]

\[S \{4FG\} \text{ in mM}
\]

\[K_i = 24.9 \pm 0.25\]
Figure 31

Effect of 3-deoxy-3-fluoro-D-glucose (3FG) on glucose oxidation by pyranose-2-oxidase. ● Glucose oxidation; □ glucose oxidation in the presence of 160 M 3FG. v, initial rate of hydrogen peroxide production (μmol min⁻¹ mg protein⁻¹). [glucose] in M.
Figure 32

Effect of various concentrations of 3-deoxy-3-fluoro-D-glucose on glucose oxidation by pyranose-2-oxidase.

• Glucose only

○ Glucose oxidation in the presence of 5mM 3FG

★ Glucose oxidation in the presence of 10mM 3FG

☐ glucose oxidation in the presence of 15mM 3FG

ν, initial rate of hydrogen peroxide production (μmole min⁻¹ mg protein⁻¹). S, [glucose] in mM.
by cationic substrates. The increasing maximum velocity with increasing concentration of inhibitor was explained by the presence of a positively charged site in the enzyme some distance away from the catalytic site.\textsuperscript{127} Defluorination of 3FG as a result of some interaction with the enzyme may result in the release of fluoride ion. The presence of a positive site in the enzyme, may result in an interaction between the fluoride ion and such a site. Such interaction may result in a conformational change in the protein causing a possible increase in the affinity of the catalytic site to its natural substrate, glucose. Support for this hypothesis is the finding by earlier workers\textsuperscript{128} that fluoride ion stimulate the accumulation of D-arabino-hexos-2-ulos during the oxidation of glucose by pyranose-2-oxidase. Preliminary experiments in these laboratories have shown increased activity of pyranose-2-oxidase towards oxidation of D-glucose, in the presence of sodium fluoride. Some binding of D-(3-\textsuperscript{3}H)3FG to pyranose-2-oxidase has also been observed in these laboratories. Negligible amounts of fluoride were detected in the reaction mixture for the kinetic studies with 3FG. However, when 79\textmu g of the protein was challenged with 80\textmu moles of 3FG a massive amount of fluoride release (7.8 x 10\textsuperscript{-5} \textmu moles F\textsuperscript{-}, 97\%) occurred. The kinetic data (Fig. 31) was obtained using a maximum concentration of 3FG of 15\textmu moles and 3.5\mu g protein. Theoretically, this
should release $6.92 \times 10^{-8}$ μmoles of F$. This level of fluoride ion is below the limits of detection of the fluoride electrode (10$^{-4}$M).

All the above information suggests that the presence of a positive charged site on this tetrameric protein, at some distance away from the catalytic site is responsible for some form of allosteric interaction. The fact that pyranose-2-oxidase displays non-sigmoidal Michaelis-Menten kinetics is not unusual for a number of allosteric enzymes. That 4FG is a moderately good substrate for the enzyme and also a competitive inhibitor of glucose and that 3FG$^-$ is not a substrate, may indicate a difference in the importance of C-3 and C-4 in binding to the catalytic site of the enzyme.

As it was intended to combine enzymic and chemical synthesis for the preparation of 4FF, and the kinetic studies have shown 4FG to be a substrate for pyranose-2-oxidase, the enzyme was immobilized for use in preparative work. Partially purified pyranose-2-oxidase was immobilized on cyanogen bromide activated Sepharose 4B$^{111,113}$. The immobilized enzyme was incubated with 4FG for 28 hr at room temperature, and the reaction monitored by HPLC. As seen in Fig. 34, the appearance of a new peak (Peak 3) was observed after 3 hr of incubation. Peak 3 had a shorter retention time (11.39 min) compared to the starting material 4FG (Peak 2) which gave a retention
time of 11.80 min. (Figs. 33 and 34). After 28 hr of incubation, only peak 3 was the main product detected on HPLC (Fig. 35). Peak 3 was isolated as a pale yellowish syrup which failed to crystallize, $R_F$ 0.60 (Solvent L). This product, 4-deoxy-4-fluoro-D-glucosone (4FGS) was characterized by infra red analysis which revealed the appearance of a new strong carbonyl stretching vibration at 1690 cm$^{-1}$. This peak was absent from the i.r. of the starting material. The presence of fluorine in the 4FGS was confirmed by a stretching vibration for C-F at 1350 cm$^{-1}$. $^{19}$F nmr analysis of the compound further confirmed the presence of fluorine; (δ)$_{\alpha}$ -120.4 ppm and (δ)$_{\beta}$ -122.5 ppm. The compound had $[\alpha]_D^{23}$ -19.6 (c, 0.5, H$_2$O). The 4FGS is unstable at room temperature and was always stored dessicated at -20°C when it was not being used.

Further confirmation of the identity of 4FGS was achieved by mass spectrometry. A comparative study of the mass spectra of 4FGS, glucosone and D-glucose was done. All the sugars were rendered volatile by converting them to the acetylated hydrazone or osazone (See Experimental). Table 4 shows the major fragments in the study. Derivatized 4FGS showed an intense mass ion peak at m/e 472 (M$^+$, C$_{24}$H$_{25}$N$_2$O$_7$F) and 223 (C$_{14}$H$_{11}$N$_2$O; O=C-CH=N-N-(φ)$_2$) while derivatized D-glucosone showed corresponding intense mass ion peaks at m/e 512 (M$^+$, C$_{26}$H$_{26}$N$_2$O$_9$) and 223 (C$_{14}$H$_{11}$N$_2$O; O=C-CH=N-N-(φ)$_2$). The peak at m/e 223 is diagnostic of
Figure 33.

High pressure liquid chromatographic (HPLC) analysis of 4-deoxy-4-fluoro-D-glucose oxidation by immobilized pyranose-2-oxidase at start of reaction (i.e. time zero). Peak 1 solvent front; peak 2 - 4FG (R_I 11.84); peak 4 unidentified. Assay conditions are described in the Materials and Methods section.
Figure 34

HPLC analysis of the conversion of 4-deoxy-4-fluoro-D-glucose (4FG) into 4-deoxy-4-fluoro-D-glucosone (4FGS) by the action of immobilized pyranose-2-oxidase 3 hours after start of reaction. Peak 2 - 4FG (Rf 11.84 min.); Peak 3 - 4FGS (Rf 11.39 min). Peak 1 is the solvent. Assay conditions are described in the Materials and Methods section.
Figure 35

HPLC analysis of the conversion of 4-deoxy-4-fluoro-D-glucose (4FG) into 4-deoxy-4-fluoro-D-glucosone (4FGS) by the action of immobilized pyranose-2-oxidase 28 hr after start of reaction. Peak 3 - 4FGS (Rₜ 11.39 min); Peaks 4 and 5 - unidentified. Peak 1 is the solvent front.

Assay conditions are as described in Materials and Methods.
D-glucosone\textsuperscript{111,128}. Its presence in the oxidative product of 4FG indicates that the product is indeed a glucosone. The mass ion peak for derivatized D-glucosone is 40 mass units greater than that of derivatized 4FGS. This is confirmatory of the presence of fluorine (m/e 19) in derivatized 4FGS. Because, an acetyl group has a mass of 59 and fluorine a mass of 19. Therefore, replacing an acetyl group by a fluorine will cause a decrease in mass of 40 units in the molecule.

As further evidence for the formation of 4FGS, D-glucosone, D-glucose and 4FGS were co-chromatographed on TLC. The spots were identified by spraying with triphenyltetrazolium chloride. Whereas 4FGS and D-glucosone developed red spots instantly, a red spot for D-glucose only appeared after heating the plate for about 5 to 10 min. at 110°C. Finally, confirmation of the structure of 4FGS was obtained by reducing it to 4-deoxy-4-fluoro-D-fructose by hydrogenation in the presence of palladized charcoal at 50 psi (Fig. 22). The hydrogenated product gave a single spot on TLC $R_f$ 0.76 (Solvent L) (cf, 0.60 starting material). The 4FF was characterized by ir (neat), which revealed a C=O stretch at 1630 (S), a C-F stretching vibration at 1300(S) and a C-O stretching vibration at 1035 (S). The presence of fluorine was also confirmed by $^{19}$F nmr which gave a chemical shift ($\delta$) of -120.37ppm.
Table 4

Comparison of Major Peaks in the Fragmentation Pattern of Derivatized Sugars

Sugars were derivatized by preparing the diphenylhydrazone tetracetate in the case of glucose, and the diphenylhydrazone and osazone tetracetate in the case of glucosone and 4FGS. In the case of 4FF, the derivatized tetracetate was used for mass spectra analysis.

<table>
<thead>
<tr>
<th>Glucose</th>
<th>D-Glucosone</th>
<th>4FGS</th>
<th>4FF</th>
<th>Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>555</td>
<td>512</td>
<td>472</td>
<td>345(M-5)</td>
<td>Mass fragment for hydrazone</td>
</tr>
<tr>
<td></td>
<td>673</td>
<td>636</td>
<td></td>
<td>Mass fragment for osazone</td>
</tr>
<tr>
<td>145</td>
<td>145</td>
<td>145</td>
<td>145</td>
<td>CH₃-CO-C=OCH₃</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OCH₃</td>
</tr>
<tr>
<td>103</td>
<td>103</td>
<td>103</td>
<td>103</td>
<td>CH₃-CO-C=OCH₂</td>
</tr>
<tr>
<td>49</td>
<td>49</td>
<td>49</td>
<td>49</td>
<td>CH₃-CO⁺</td>
</tr>
<tr>
<td>223</td>
<td>223</td>
<td>223</td>
<td></td>
<td>Diagnostic of D-glucosone</td>
</tr>
<tr>
<td>291</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>277</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>232</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>129(M⁺)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>174</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>117</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The 4FF also gave \([\alpha]_D^{23} -30.5\) (C, 0.83, H₂O).

The structure of 4FF was further confirmed by sodium borohydrate reduction to a major product, 4-deoxy-4-fluoro-D-glucitol (Fig. 36), \(R_F 0.58\) (Solvent L). The \(R_F\) value and \([\alpha]_D^{23}\) were identical to that of authentic 4FGL.

A minor product, \(R_F 0.65\) (Solvent L) was also observed on the TLC. This minor product was tentatively assigned the structure of the C-2 epimer, 4-deoxy-4-fluoro-D-mannitol.

Additional confirmation of the structure of 4FF was obtained by mass spectrometric analysis of the tetracetate derivative. The fragmentation pattern (Table 4) was identical to that of other deoxyfluorohexopyranoses, involving loss of acetoxy groups (as acetic acid, ketene, and structurally related moieties).

Four main fragmentation series (Appendix 12) were observed according to the Biemann-Heyns-Chizhov patterns. The first series involved complete loss of acetyl groups with retention of the pyranose ring. Series B which involved the loss of the carbon atom bonded to the ring oxygen, and all groups attached to this carbon resulted in a peak at 232. Series C resulted in a fragment containing \(C_4, C_3, C_5\). The appearance of this fragment is due to the fact that the presence of fluorine atom on \(C_4\) prevents breakage of the \(C_3-C_4\) and \(C_4-C_5\) bonds. This leads to
a fragment containing three-carbon units with two acetyl groups and a fluorine giving a mass of 174. The formation of the B series leading to a mass fragment of 129 is diagnostic of a fructo-configuration. The fourth pathway, or the D series involves the loss of acetyl groups.

The mass ion peaks at m/e 145, 103, and 43 are common to all the derivatized sugars. They are formed by the fragments shown (Table 4) and are diagnostic of fully acetylated monosaccharide.$^{129}$

All attempts to crystallize 4FF or obtain crystalline derivatives have so far failed.
Figure 36

Sodium borohydride reduction of 4-deoxy-4-fluoro-D-fructose to 4-deoxy-4-fluoro-D-glucitol.
4FG

\[ \text{CH}_2\text{-OH} \]
\[ \text{C}=\text{O} \]
\[ \text{HO}-\text{C}-\text{H} \]
\[ \text{H}-\text{C}-\text{F} \]
\[ \text{H}-\text{C}-\text{OH} \]
\[ \text{CH}_2\text{OH} \]

\[ \xrightarrow{\text{NaBH}_4} \]

\[ \text{RM T, 24 HRS.} \]

4FF

\[ \text{CH}_2\text{-OH} \]
\[ \text{H}-\text{C}-\text{OH} \]
\[ \text{HO}-\text{C}-\text{H} \]
\[ \text{H}-\text{C}-\text{F} \]
\[ \text{H}-\text{C}-\text{OH} \]
\[ \text{CH}_2\text{-OH} \]

4F-MANNITOL
SUMMARY AND CONCLUSION

1. An unsuccessful attempt was made to synthesize 3FF. This outcome is similar to the results of other workers.

2. 4FG has been synthesized by an improved procedure, using the fluorinating reagent diethylamino sulfuryl trifluoride. This synthetic procedure is shorter and afforded a higher yield as compared to the previously established route used in these laboratories for the synthesis of 4FG.

3. The kinetics of partially purified pyranose-2-oxidase, isolated from the mycelia of Polyporus obtusus, with glucose and 4FG has been studied. 3FG which appeared not to be a substrate was found to stimulate the activity of the enzyme towards glucose.

4. The partially purified pyranose-2-oxidase has been immobilized on cyanogen bromide activated sepharose 4B and used in conjunction with preformed 4FG for the synthesis of 4FGS and 4FF. The last two compounds have been characterized by infra red, nmr and mass spectrometric techniques.

The combination of chemical and enzymic synthesis as seen in this thesis offers a vital approach to the synthesis of compounds that can otherwise not be obtained by chemical synthesis alone.
PART II

METABOLIC STUDIES WITH LOCUSTA MIGRATORIA
INTRODUCTION

Among the many organo-fluoro compounds that have been studied for toxic action in living organisms, fluorocarbohydrates must be included. Early studies of the toxic action of 3-deoxy-3-fluoro-glucose was done on rats. It was found that this compound displayed several physiological and biochemical effects in the rat\textsuperscript{132}. However, toxicity was not observed even after massive intraperitoneal injection into the rats (5 gram/kg body wt.). They observed that the majority of the fluoro sugar was excreted through the urine, while only small amounts accumulated in the brain and testes. This observation led to the consideration to examine the effect of the fluoro sugar on an organism that had a higher degree of water retention than the rat but small enough to require smaller doses of the drug to effect maximum physiological and biochemical response. The locust suited these requirements. A review of the comparative biochemistry and physiology in locust has been previously reported\textsuperscript{50}.

Recently Taylor and Romaschin\textsuperscript{134} observed that 3FG elicited a toxic action on \textit{Locusta migratoria} and \textit{Schistocerca gregaria}, with an LD\textsubscript{50} of 4.8mg/g locust. Death occurred between three to four days. Qualitative estimation of neutral sugars in three major tissues, the haemolymph, fat body and flight muscle, after 3FG administration revealed that 3FG disappeared from the
tissues, but new metabolites were observed to appear in the haemolymph and fat body. The new metabolite was identified as 3-deoxy-3-fluoro-D-glucitol (3FGL). This observation suggests that 3FG is capable of being metabolized to 3FGL by aldose reductase and possibly to 3-deoxy-3-fluoro-fructose by sorbitol dehydrogenase which may subsequently be phosphorylated and thus enter glycolysis. Using partially purified fat body preparations, Taylor and Romaschin observed that 3FGL was an inhibitor of locust fat body sorbitol dehydrogenase at low concentrations with $K_i$ of 82 mM and as a substrate for the same enzyme at higher concentrations, of $K_m$ 500mM. From the above findings it was rationalized that 3FGL could not possibly be the toxic metabolite. Further experimentation with the intact insect to identify the toxic metabolite led to the following deductions:

i. That the locust pentose phosphate pathway contributed very little to the energetics of 3FG metabolism, and as such is not inhibited by 3FG metabolism. Such a conclusion was also arrived at by Chefurka working with glucose.

ii. That 3FG did not inhibit the tricarboxylic acid cycle.

iii. That 3FG inhibited glycolysis to about 50%. That the onset of inhibition was faster when 3FG was administered intermittently at shorter time intervals.
iv. That 3FG was metabolized as far as the triosephosphate sugars involving the enzymatic action of triosephosphate isomerase—because tritium was observed to be released upon administration of D-[3-3H]-3-deoxy-3-fluoro-D-glucose.

v. That 3FG was significantly defluorinated. This fluoride release may be inhibiting some fluoride sensitive enzyme in the glycolytic pathway (e.g., enolase). This was substantiated by the finding that inorganic fluoride at very low levels was toxic to the insect.

vi. That 3FG administration led to the accumulation of an unidentified phosphorylated fluorinated metabolite. Similar in vitro studies by Taylor and Agbanyo revealed:

i. That locust fat body and flight muscle were capable of metabolizing 3FG but at a rate lower than that for glucose.

ii. That locust fat body and flight muscle showed incorporation of 3FG into the synthesis of fluoro-glycogen and fluoro-trehalose after 3FG administration. It was also found that there was a build up of fructose. That apart from biosynthesis of glycogen and trehalose 3FG was also metabolized along the glycolytic pathway as far as the triose sugars with detritiation and defluorination, the resulting fluoride
may have inhibited enolase as well as other fluoride sensitive enzymes leading to toxicity.

From the foregoing it seems that a metabolite of 3FG was exhibiting a bona fide block somewhere along the glycolytic or other pathways being used for 3FG metabolism, thus resulting in the death of the whole insect. The build-up of fluoroglycogen and fluorotrehalose could be the result of channeling the 3FG into glycogen and trehalose biosynthesis after the inhibition of glycolysis.

These effects observed for 3FG may be quite different if the fluorine atom is substituted in position 4 of the glucose molecule rather than 3.

A comparison of the relative toxicity of 4FG with 3FG may provide an insight into the design of a more potent insecticide.

OBJECTIVES:

The objectives of this part of the dissertation, therefore, are to:

i. establish the comparative toxicity of 4FG and 3FG in the intact locust; determine whether fluoride ion is released during 4FG metabolism.

ii. attempt to identify any metabolites formed in locusts poisoned by 4FG.
EXPERIMENTAL

MATERIALS AND METHODS

All reagents and chemicals were analytical grade meeting ACS specifications, unless otherwise stated. Ethanol was from Fisher. HPLC grade distilled water was used in preparation of all aqueous solutions. D-Glucose, D-fructose, glycogen and sorbitol were from BDH Chemicals, Toronto, Ontario. Trehalose was kindly supplied by Dr. Cotter's Laboratory, Department of Biological Sciences, University of Windsor. Sep PACK units were a gift from Waters, Mississauga, Ontario. Millipore micron filters were from Millipore Filter Corporation, Massachusetts.

EQUIPMENT

All F– measurements were carried out on a Präzision pH meter model 510, (Metrohm AG-CH, Switzerland), which is designed for use with a fluoride specific electrode (Orion Research Cambridge, MA). Centrifugations were all done using a Beckman L8-55 Ultracentrifuge. Tissue homogenizations were carried out with a Polytron tissue homogenizer type PT 10/35. The syringe used for HPLC injections was a Waters U6K syringe. For the injection of locusts, a 50µl Hamilton Syringe from BDH (Toronto, Ontario) was used.
LOCUST REARING CONDITIONS

Locusts were reared under crowded and tropical conditions, in a temperature and humidity controlled environmental room. Temperatures were kept at 34°-35°C and the humidity maintained at 30%. Locust chow consisted of local field grass in the summer, and lettuce head or carrot tops in the winter months. Mixed population of adult locust 10-14 days old after final ecdysis were used for all studies.

THE TOXICITY OF 4-DEOXY-4-FLUORO-D-GLUCOSE IN LOCUSTA MIGRATORIA

Mixed population of locust, 10-14 days old after final ecdysis were starved for three days before 4FG administration. During this period, they were kept in very clean cages and fed only water ad libitum. At the end of the three days, the starved locusts were divided into groups, each containing 10 locusts. Locusts were injected with various concentrations (a particular concentration per group) of 4FG in a total volume of 30μl using a 50μl Hamilton syringe. All injections were made into the haemocoel using a two-inch 24 gauge needle which was inserted to a fixed depth of 5mm through the membranous septum at the base of the frontal thoracic leg. Any locust that was poorly injected as evidenced by leakage of haemolymph was discarded from the study. Control locusts
were similarly injected with distilled water.

After injection locusts were reared in groups according to 4FG concentration in aluminum cages with ventilation at both the top and the bottom. The experimental locusts were fed regular chow after the injection. Toxicity in the locust was observed over a period of three days and the time and number of dead locust per group noted.

LD$_{50}$ was established by plotting % death against $\log_{10}$ dose nmol g$^{-1}$ locust.

**LOCUST TISSUE FLUORIDE MEASUREMENT**

Poisoned locust or control locust (three from each group), were frozen in liquid nitrogen, powdered and homogenized in TISAB (4mL) in a Polytron homogenizer until all the tissue was completely homogenized. Homogenates were centrifuged at 20,000 rpm for 15 min. at 24$^\circ$C. The supernatant was collected and filtered through two layers of cheese cloth to remove the upper lipid layer and tiny debris. The filtrate fluoride level was measured using the "Metrohm" meter equipped with a fluoride electrode. All beakers and other containers used for this study were "Nalgene" plastic ware. A fluoride calibration curve was prepared using standard fluoride solutions. The standard plot was made on a semi-log paper with millivolt potential against fluoride concentration. (See Appendix 5.)
PREPARATION OF LOCUST TISSUE FOR HPLC

Locust tissue were first prepared as described for fluoride measurement. The filtered supernatant was treated with 50% (v/v) ethanol to precipitate protein. The protein which separated as a white precipitate was removed by centrifuging at 15,000 rpm for 30 minutes at 4°C. The resulting solution was allowed to sit in ice for 10 minutes and the supernatant taken and deproteinized again by the same procedure. The supernatant was vacuum desiccated over CaCl₂ to dryness. The residue was taken up in HPLC grade distilled water (500μl) and diluted with an equal volume of absolute ethanol. No protein precipitate was observed. The solution was filtered through a "SEP-PACK" unit. The clear solution obtained was evaporated by vacuum dessication to dryness. The residue was reconstituted in HPLC grade water (200μl), then filtered through 0.45μm millipore filter and the final clear solution used for HPLC analysis.
RESULTS AND DISCUSSION

Mixed populations of *Locusta migratoria* were studied for 4FG toxicity. The initial study employed the established LD$_{50}$ level for 3FG toxicity (4.8 mg g$^{-1}$ locust)$^{135}$. In the case of the 4FG, this level was found to be too high. All ten locusts injected with the fluorocarbohydrate died by 12 hours. Death was preceded with extreme quivering of the whole locust, twitching of the wings and a spin motion of the insect as was observed for rats$^5$. A subsequent study was done using lower concentrations of the 4FG. Table 5 shows the results of the toxicity studies of the 4FG in the *Locusta migratoria*. It is seen that the highest dosage used (2.4 mg gm$^{-1}$ locust) caused death in 80% of the locust in 1$^{1/2}$ days. The studies indicate that the approximate LD$_{50}$ for 4FG is 0.6 mg gm$^{-1}$ locust (Fig. 37). Compared to 3FG (LD$_{50}$: 4.8 mg gm$^{-1}$ locust)$^{135}$, 4FG is seen to be far more toxic than 3FG, with LD$_{50}$ eight-fold higher than that of the 3-deoxy analog of glucose. Table 5 also shows a higher death rate in 4FG poisoned locust than that for 3FG. The LD$_{50}$ for 4FG was observed in two days as compared to three days for 3FG poisoned locust. The higher toxicity of 4FG is therefore apparent. This may indicate that substitution of fluorine on C-4 rather than C-3 affords a more toxic compound. The lower LD$_{50}$ and higher rate of toxicity of 4FG as indicated by this study may well represent differences.
### TABLE 5

<table>
<thead>
<tr>
<th>AMOUNT OF 4FG INJECTED (mg g⁻¹ locust)</th>
<th>nmol 4FG g⁻¹ locust x 10⁻³</th>
<th>% DEATHS</th>
<th>APPROXIMATE TIME OF DEATH (days)</th>
<th>NUMBER OF LOCUST USED</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>13.2</td>
<td>80</td>
<td>1½</td>
<td>10</td>
</tr>
<tr>
<td>1.2</td>
<td>6.6</td>
<td>70</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>0.6</td>
<td>3.3</td>
<td>50</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>0.3</td>
<td>1.65</td>
<td>40</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>0.15</td>
<td>0.825</td>
<td>20</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>0.075</td>
<td>0.413</td>
<td>0</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>0.0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 37

Dose response of *Locusta migratoria* to 4FG and 3FG injections. ▲ 4FG, LD$_{50}$ 0.6mg gm$^{-1}$ locust. ◆ 3FG, LD$_{50}$ 4.8mg gm$^{-1}$ locust.
Figure 37

% Death

Log_{10} Dose, \mu\text{mol gm}^{-1} \text{ locust}

Log_{10} Dose, \text{nmol mg}^{-1} \text{ locust}
in the mode of toxicity between 4FG and 3FG. 4FG may
be exerting its toxic effect at an earlier metabolic point
than 3FG or may be exerting its effect at a greater
number of metabolic steps than 3FG. In spite of the
difference both compounds displayed similar LDDS plots
(Fig. 37).

To understand the mode of toxicity, it became necessary
to establish whether fluoride ion was present in the
poisoned locusts. The release of fluoride in poisoned locust
has been closely associated with 3FG toxicity.\(^{135}\) If 4FG
was metabolized by the glycolytic pathway as far as the
triose sugars, then defluorination may occur at the aldolase
or triosephosphate isomerase steps. (Fig. 38). The presence
of free fluoride at any of the above mentioned points
could lead to a glycolytic inhibition, as has been stated
for 3FG toxicity.\(^{135}\)

As shown in Table 6, a small but significant amount
of fluoride was released in the 4FG poisoned locust. Of
particular interest is the level of fluoride ion in the
first three groups. Locusts injected with the LDDS and
higher dosages of 4FG showed similar levels of fluoride
ion. Although whole locusts were used in this study, in
a previous study with fat body and flight muscle
homogenates, using 3FG, a similar observation was made.\(^{58}\)
Fig. 39 shows the fluoride level against the amount of
4FG injected. It is seen that as the concentration of
Enzymes

1. Glucokinase / hexokinase
2. Phosphoglucoisomerase
3. Phosphofructokinase
4. Aldolase
5. Triosephosphate isomerase

Figure 38. Glycolytic pathway showing step of action of aldolase.
Table 6.

The Fluoride ion level in total tissue homogenates of poisoned locust.

<table>
<thead>
<tr>
<th>Amount of 4FG injected (mg gm⁻¹ locust)</th>
<th>Fluoride ion detected in locust tissue (mg mL⁻¹)</th>
<th>Fluoride ion detected in locust tissue (μmole)</th>
<th>μmol% of fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>5.7x10⁻³</td>
<td>3x10⁻¹</td>
<td>30%</td>
</tr>
<tr>
<td>1.2</td>
<td>5.7x10⁻³</td>
<td>3x10⁻¹</td>
<td>30%</td>
</tr>
<tr>
<td>0.8</td>
<td>5.3x10⁻³</td>
<td>2.8x10⁻¹</td>
<td>28%</td>
</tr>
<tr>
<td>0.3</td>
<td>4.99x10⁻⁴</td>
<td>2.6x10⁻²</td>
<td>2.6%</td>
</tr>
<tr>
<td>0.15</td>
<td>4.75x10⁻⁵</td>
<td>2.5x10⁻³</td>
<td>0.25%</td>
</tr>
<tr>
<td>Control (0)</td>
<td>5.7x10⁻⁶</td>
<td></td>
<td>0%</td>
</tr>
</tbody>
</table>

* Fluoride levels were measured with the fluoride electrode and are the average of three determinations. (See Materials and Methods.)
Figure 39

Plot of level of fluoride ion in locust tissue against amount of 4FG injected.
Figure 39

Amount of 4FG Injected
(mg g⁻¹ locust)
the injected fluorinated sugars increases, the amount of fluoride released increases gradually, attains a point of inflexion as the LD50 dosage is reached and finally reaches a saturation level. It may be deduced that a certain fraction of the injected fluorinated sugar is defluorinated. The remaining portion of the fluorosugar may be channelled to other metabolic pathways. The level of fluoride is in the microgram (or micromolar) range which is known to inhibit enolase136. The present results support the proposal that defluorination is a major factor linked to the toxicity of 4FG and 3FG in Locusta migratoria.

From previous studies135 with 3FG, a direct link was established between the toxicity of the fluorinated sugar and defluorination of the 3FG. It was established that the fluoride ion produced was exerting a bona fide metabolic blockage along the glycolytic pathway. The direct linkage between death of locust and free fluoride level observed in this study is in agreement with these135 findings. It was proposed that the defluorination may take place at the triose phosphate isomerase or aldolase stage. If either of these two enzymes or enolase is inhibited causing the blockage it may be possible that the remaining fluorinated sugar is redirected into other metabolic pathways. Recent metabolic studies with 3FG have revealed redirection of 3FG into biosynthesis of "fluoro-glycogen" or "fluoro-trehalose"17. 4-Deoxy-4-fluoro-glucose may
similarly be channelled into synthesis of "fluoro-trehelose" or "fluoro-glycogen". In the latter case the incorporation of 4FG would be restricted to the terminal positions of glycogen.

The results of the present study with 4FG so far, strongly parallels those of 3FG studies\textsuperscript{17,135}. The difference between the two is that 4FG is more toxic to the locust than 3FG. If glycolytic blockage is the main pathway affected, it may be that the difference in toxicity is a result of 4FG or its metabolites exerting a blockage at a point earlier than the triose phosphate isomerase enzyme. The most attractive target enzyme is aldolase. Based upon the known mechanism for aldolase action\textsuperscript{137}, (Fig. 40) the fluorinated sugar may be irreversibly inactivating this enzyme with the concomitant release of F\textsuperscript{−}. Figure 41 shows a tentative mechanism for inactivation of aldolase by 4FG. The released fluoride at this early target site may then be responsible for subsequent inhibition of enolase.

Analysis of the formation of new metabolites was carried out to further understand the mode of toxicity of 4FG in the insect. Metabolite analysis was carried out using HPLC. Fig. 42 and 43 show the HPLC analytical results for the control and poisoned locust experiments respectively. Two main peaks were observed in the control locust injections, while the injection of the tissue extract
Figure 40

Mechanism of action of Aldolase on Fructose-1,6-di-phosphate, splitting it into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate.
Figure 41

Tentative mechanism for defluorination of 4FG by aldolase, in the Locusta migratoria, after the locust has been injected with 4-deoxy-4-fluoro-D-glucose.
Figure 42

HPLC analysis of control locust extracts. The three peaks in this extract were identified by matching their retention times against the retention times of standards. Peak number 1 ($R_T$ 3.88 min) is the solvent front. Peak 2 ($R_T$ 4.55-4.60) is trehalose. Peak 3 ($R_T$ 5.43-5.48) was identified as glucose.
Figure 43

HPLC analysis of extract from locust poisoned with 4-deoxy-4-fluoro-D-glucose. The various peaks were identified by matching their retention times against those of standards. Peak number 1 ($R_T$ 3.88 min) is the solvent front. Peak 2 ($R_T$ 4.55) is trehalose. Peak 3 ($R_T$ 5.48) is glucose. Peak 4 ($R_T$ 6.4, 6.8) is 4-deoxy-4-fluoro-D-glucitol. Peaks 5 and 6 ($R_T$ 8.02 and 9.82 min; respectively) did not correspond to any of the standards.
from the poisoned locust showed five distinct peaks apart from the solvent peak (Peak 1, $R_T$ 3.88 min). A comparison of the retention times of the peaks from the control and poisoned extract injections on HPLC showed that peaks 2 and 3 appear in both the control and poisoned locust extract. These peaks, $R_T$ 4.55-4.60 min and $R_T$ 5.43-5.48 min corresponded to trehalose ($R_T$ 4.55 min) and glucose ($R_T$ 5.44 min) respectively (Table 7). Peak 4 ($R_T$ 6.4; 6.8 min) which appeared only in the poisoned locust extract corresponded very closely to chemically synthesized 4-deoxy-4-fluoro-D-glucitol ($R_T$ 6.3 min) (Table 7). Peak 4 was, therefore, assumed to be 4-deoxy-4-fluoro-glucitol. No peak was found to correspond to 4-deoxy-4-fluoro-fructose ($R_T$ 6.04 min), in the 4FG poisoned locust extract. The identification of 4FGL in the tissue extract from the poisoned locust is similar to the observation made in previous studies with 3FG. That 4FGL is formed as a result of 4FG toxicity is indicative of the fact that it is being metabolized via aldose reductase and sorbitol dehydrogenase. That no major peak was found to correspond to 4FF ($R_T$ 6.04 min), may be due to the fact that the amount of this sugar produced via the sorbitol pathway may be too small to be detected. Early work in these laboratories with sheep liver sorbitol dehydrogenase has established that 4FGL is a poor substrate for the enzyme. Low levels of 4FF may therefore be expected. Peaks 5
Table 7

Retention times of standard sugars and buffer on HPLC. 5μL injections were made. Flow rate was set at 0.5mL min⁻¹ for all the runs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rₜ(min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris/HCl Buffer (0.05M, pH 7.5)</td>
<td>3.03</td>
</tr>
<tr>
<td>Glucose (10mM)</td>
<td>5.44</td>
</tr>
<tr>
<td>Trehalose (10mM)</td>
<td>4.55</td>
</tr>
<tr>
<td>4FG (5mM)</td>
<td>5.90</td>
</tr>
<tr>
<td>4FGL (5mM)</td>
<td>6.30</td>
</tr>
<tr>
<td>4FF (5mM)</td>
<td>6.04</td>
</tr>
</tbody>
</table>
and 6 (Rf 8.02 and 9.82 respectively) which eluted later from the column did not correspond to any of the standards. Preliminary attempts to identify the presence of any phosphorylated metabolites after 4FG injection into the locust did not reveal any such metabolites. This may be due to the method of preparing samples for the HPLC.

It may be concluded from these preliminary results that 4FG is metabolized in the locust in a manner similar to 3FG. 4FG may be metabolized as far as the enzyme aldolase with a resultant fluoride release which is responsible for the death of the locust. Further work will be needed to understand the interaction of 4FG and the enzyme aldolase and its possible inactivation.
SUMMARY AND CONCLUSION

1. 4FG has been found to be more toxic than 3FG when injected into Locusta migratoria. The LD_{50} for 4FG is 0.6 mg g^{-1} locust compared with LD_{50} of 4.8 mg g^{-1} locust for 3FG. Death occurred faster in 4FG poisoned locust (2 days) compared to 3FG poisoned locust (3 days). In the 4FG poisoned locust death was preceded by loss of motor activity as was the case of rats poisoned with fluoroacetate.  

2. The toxicity of 4FG, like 3FG, was accompanied by fluoride release which increased with increasing amounts of injected 4FG until a saturation level was reached when LD_{50} and higher dosages were injected. This supports the previous proposal on 3FG toxicity studies.

3. As was observed for 3FG, analysis of 4FG poisoned locust extract by HPLC revealed the formation of 4FGL. This indicates that 4FG, may be metabolized in the locust in a manner similar to 3FG. That 4FF was not detected in the extract may be due to the low affinity of sorbitol dehydrogenase for 4FGL.

4. The results of the present study indicate that there may be a metabolic blockage, possibly at a glycolytic step earlier than the triosephosphate isomerase, and that defluorination may occur at that enzymic step.
A possible target enzyme is aldolase. Further work will be needed to establish the interaction of aldolase with 4FG.

4FG is a more potent toxic agent to *Locusta migratoria* than 3FG.
Appendix I

$^{19}$F Fourier transform nuclear magnetic resonance spectrum ($^{19}$F NMR) of 4-deoxy-4-fluoro-D-glucose (18) (Water).

A = (18)

B = trifluoroacetic acid (TFA; external standard)

Conditions used for the scans:

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF (MHz)</td>
<td>84.700</td>
</tr>
<tr>
<td>SW (Hz)</td>
<td>12195.122</td>
</tr>
<tr>
<td>FW</td>
<td>14700.000</td>
</tr>
<tr>
<td>AG</td>
<td>0.336</td>
</tr>
<tr>
<td>NS</td>
<td>605.000</td>
</tr>
<tr>
<td>SI</td>
<td>8.0</td>
</tr>
<tr>
<td>Line broadening (Hz cm$^{-1}$)</td>
<td>2.000</td>
</tr>
</tbody>
</table>

ppm (δ) (relative to TFA), $-120.0$ (doublet of doublets)

$J_{F,H_4} \ 33 \pm 3 \ J_{F,H_3} \ 15 \pm 3 \ J_{F,H_5} \ 15 \pm 3$
Appendix 2

$^{19}$F Fourier transform nuclear magnetic resonance spectrum ($^{19}$F NMR) of 4-deoxy-4-fluoro-arabino-hexos-2-ulose (57) (Water). Trifluoroacetic acid was used as external standard.

Conditions for the scans:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{SF(MHz)}$</td>
<td>84.700</td>
</tr>
<tr>
<td>$\text{SW(Hz)}$</td>
<td>12195.122</td>
</tr>
<tr>
<td>$FW$</td>
<td>14700</td>
</tr>
<tr>
<td>$AQ$</td>
<td>0.3359</td>
</tr>
<tr>
<td>$NS$</td>
<td>154</td>
</tr>
<tr>
<td>$SI$</td>
<td>8.0</td>
</tr>
<tr>
<td>$DW$</td>
<td>22.000</td>
</tr>
<tr>
<td>Line broadening</td>
<td>2.000</td>
</tr>
</tbody>
</table>

($\text{Hz cm}^{-1}$)

$\phi_\alpha$ -120.4 ppm

$\phi_\beta$ -122.5 ppm

$J_{F,H-4}$ 50 ± 3 $J_{F,H-3}$ 11 ± 3 $J_{F,H-5}$ 11 ± 3

The observed resonance spectrum is possibly due to the presence of $\alpha$ and $\beta$ anomeric forms of the 4FGS.
Appendix 3

$^{19}$F Fourier transform nuclear magnetic resonance spectrum ($^{19}$F NMR) of 4-deoxy-4-fluoro-D-fructose (58) (water). Trifluoroacetic acid was used as external standard.

Conditions for scans:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\omega$ (MHz)</td>
<td>84.700</td>
</tr>
<tr>
<td>$\omega$ (Hz)</td>
<td>12195.122</td>
</tr>
<tr>
<td>$\delta$</td>
<td>14700.000</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>0.336</td>
</tr>
<tr>
<td>$\eta$</td>
<td>4852.000</td>
</tr>
<tr>
<td>$\theta$</td>
<td>41.000</td>
</tr>
<tr>
<td>$x$</td>
<td>9K</td>
</tr>
<tr>
<td>Linebroadening</td>
<td>3.000</td>
</tr>
</tbody>
</table>

$\text{ppm (C)}$ -120.37

$J_{F,H_\alpha} 39 \pm 3 J_{F,H_3} 12 \pm 3 J_{F,H_5} 12 \pm 3 \text{ Hz}$. 

Appendix 4

Pathway for fragmentation of tetra-D-acetyl-4-deoxy-4-fluoro-D-fructose (58). Fragmentation series is based on that for acetylated fluorohexoses according to the scheme of Bienheim-Hyers and Chiznov'129-131.
Appendix 5

Calibration curve for fluoride measurements. The curve was prepared by using standard fluoride solution in the concentration range of $10^{-1}$ to $10^{-6}$ M. Fluoride levels were measured as millivolt potential. The millivolt potential was plotted against molar concentrations on a 4 cycle semi-log paper.
Appendix 6

High performance liquid chromatogram of 4-deoxy-4-fluoro-D-fructose (4FF). Peak 2 - 4FF (R<sub>T</sub> 12.11 min).

Peak 3 - unidentified. Peak 1 is solvent. Sample injected 4 µL (10mM); flow rate = 1mL min<sup>-1</sup>; Chart speed 1 cm min<sup>-1</sup>; attenuation = 16X. Samples were run at room temperature.
Appendix 7

Fragmentation peaks for tetra-D-acetyl-4-deoxy-
4-fluoro-D-fructose. The mass spectra was run on a Finnigan-
MAT CH5 mass spectrometer. Probe temperature was 180°C.
Probe was started at room temperature and the temperature
increased stepwise by 25°C to a final temperature of 180°C.
The electron impact was 70 ev.
Appendix 7

FRAGMENTATION PEAKS FOR TETRA-O-ACETYL 4FF

18 (6.48), 27 (1.85), 28 (9.90), 29 (4.69), 30 (1.20),
31 (6.52), 39 (0.69), 41 (1.98), 42 (2.77), 43 (100),
44 (16.56), 45 (4.35), 55 (3.16), 56 (1.53), 57 (1.50),
59 (1.50), 59 (0.62), 60 (3.95), 61 (32.75), 62 (2.86),
67 (0.86), 69 (0.88), 72 (0.75), 73 (4.39), 74 (1.31),
75 (0.63), 77 (1.11), 78 (0.53), 79 (0.80), 81 (1.48),
82 (3.46), 83 (2.89), 84 (10.51), 85 (1.32), 86 (1.01),
91 (5.65), 92 (0.48), 94 (0.94), 96 (1.73), 97 (1.86),
98 (0.51), 99 (2.80), 100 (11.72), 101 (1.33), 102 (0.74),
103 (18.76), 104 (6.12), 105 (1.12), 106 (0.66), 109 (0.51),
117 (2.11), 118 (1.18), 120 (0.98), 121 (1.65), 124 (0.59),
125 (0.79), 126 (0.60), 129 (0.67), 131 (0.61), 136 (0.53),
138 (1.56), 139 (0.62), 142 (1.51), 145 (11.82), 146 (1.31),
147 (1.31), 147 (0.55), 152 (2.16), 159 (0.52), 160 (15.79),
161 (1.03), 172 (0.85), 174 (0.85), 175 (0.84), 186 (0.49),
188 (0.63), 189 (0.63), 198 (0.90), 202 (42.83), 203 (4.63),
204 (13.91), 205 (2.64), 210 (1.02), 214 (0.56), 216 (1.30),
228 (0.56), 230 (5.10), 231 (1.97), 232 (7.18), 233 (1.46),
234 (0.51), 238 (52.12), 269 (14.89), 290 (1.82), 291
(0.59), 316 (1.07), 318 (0.70).
Appendix 8

Infra red pattern for 4-deoxy-4-fluoro-D-glucose (18).

The spectra was run in KBr: 3480-3160 (S,-OH [hydrogen bonded] stretch), 2980,2940 (m, C-H stretch), 1360-1350 (S,C-F stretch), 1020 (S,C-O stretch), 908 (m, ring vibration), 898 (S, anomeric C-H stretch, β-anomer) cm⁻¹.

The anomeric C-H stretch at 898 cm⁻¹ has been assigned to the β-anomer.¹⁴²

The α-anomer will have a C-H stretch at approximately 844 cm⁻¹. This vibrational stretching is not exhibited by the β-anomer.
Appendix 9

Infra red pattern for 3-deoxy-3-fluoro-D-glucose. The spectra was run in KBr disc: 3390 (S, -OH stretch), 2938 (m, C-H stretch), 1387 (S, C-F stretch), 1082 (m, C-O stretch), 916 (m, ring vibration), 899 (S, anomer C-H stretch, β-anomer), 860 (m, anomeric C-H stretch, α-anomer) cm⁻¹.

The anomeric C-H stretch at 860 cm⁻¹ has been assigned the α-anomer.¹⁴²
Appendix 10

$^{19}F$ Fourier transform nuclear magnetic resonance spectrum ($^{19}F$ NMR) of 3-deoxy-3-fluoro-D-glucose.

Trifluoro acetic acid was used as external standard.

Conditions for scan:

| SF (MHz) | 84.700  |
| SW (Hz)  | 20000.000 |
| FW      | 24.000   |
| AQ      | 0.2048   |
| NS      | 1859     |
| DW      | 25.000   |
| SI      | 8K       |
| Linebroadening | 2.000 |

ppm (Φ)$_\beta$ -111

ppm (Φ)$_\alpha$ -116

$J_{F,H-3}$ 29 ± 2 Hz $J_{F,H-2}$ 10 ± 2 Hz $J_{F,H-4}$ 15 ± 2 Hz

The observed resonance spectrum is due to the presence of $\alpha$ and $\beta$ anomeric forms of the 3FG.
Appendix 11

Mathematical analysis of the effect of 4FG on oxidation of glucose by pyranose-2-oxidase and vice versa.
Appendix II

The possibility of competition between 4FG and glucose for the same active site(s) on pyranose-2-oxidase was tested using the Dixon and Webb\textsuperscript{140} relationship (below):

\[
\frac{K_{glu}}{K_{4FG}} = \frac{V_{glu} - V_{glu+4FG}}{V_{glu+4FG} - V_{4FG}} \tag{1}
\]

where \(K_{glu}, V_{glu}, K_{4FG},\) and \(V_{4FG}\) are the \(K_m\) and \(V_{max}\) values for glucose and 4FG, and \(V_{glu + 4FG}\) is the maximum velocity observed with an equimolar mixture of glucose and 4FG. The relationship implies that, theoretically, if the two sugars were competing for the same active site, then when the two substrates are present in equimolar concentrations in the same reaction mixture, the maximum velocity \(V_{glu + 4FG}\) will be less than the sum of the separate rates of reaction \(V_{glu} + V_{4FG}\).

From equation 1:

\[
\frac{K_{glu}}{K_{4FG}} (V_{glu+4FG} - V_{4FG}) = V_{glu} - V_{glu+4FG}
\]

\[
\frac{K_{glu}}{K_{4FG}} (V_{glu+4FG}) + V_{glu+4FG} = \frac{K_{glu}}{K_{4FG}} (V_{4FG}) + V_{glu}
\]

\[
(V_{glu+4FG})(1 + \frac{K_{glu}}{K_{4FG}}) = \frac{K_{glu}}{K_{4FG}} (V_{4FG}) + V_{glu}
\]
The $K_m$ and $V_{\max}$ values for each substrate alone and for the combined substrate reactions were obtained from the initial-rate measurements.

<table>
<thead>
<tr>
<th>Maximum Velocity</th>
<th>Theoretical</th>
<th>Observed</th>
<th>Ratio of Theoretical/Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{glu}} + 4\text{FG}$</td>
<td>118.60</td>
<td>91.78</td>
<td>1.29</td>
</tr>
<tr>
<td>$4\text{FG} + \text{glu}$</td>
<td>118.35</td>
<td>68.70</td>
<td>1.72</td>
</tr>
<tr>
<td>$V_{\text{glu}}$</td>
<td>126.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$4\text{FG}$</td>
<td>104.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{glu}} + 4\text{FG}$</td>
<td>230.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From the table, $V_{\text{glu}} + 4\text{FG} < V_{\text{glu}} + 4\text{FG}$ and $4\text{FG} + \text{glu} < 4\text{FG} + V_{\text{glu}}$ for both observed and theoretical values. A form of competition between the two substrates is therefore indicated.

For the two substrates inhibiting each other competitively, the ratio of theoretical/observed velocities for the joint reaction must be close to unity$^{140}$. From the table, therefore, 4FG is a competitive inhibitor of glucose oxidation. Glucose however, is not a good competitive inhibitor of 4FG oxidation by pyranose-2-oxidase.
**Appendix 12**

Equilibrium dialysis set-up\(^{14}\) for the study of the binding of \([3-^3\text{H}]3\text{FG}\) to pyranose-2-oxidase.

**Conditions for run:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final volume of protein</td>
<td>1900µL</td>
</tr>
<tr>
<td>Final concentration of ([3-^3\text{H}]3\text{FG})</td>
<td>700µM</td>
</tr>
<tr>
<td>Final concentration of cold 3FG</td>
<td>From 200µM to 800µM</td>
</tr>
<tr>
<td>Equilibration buffer—sodium phosphate</td>
<td>((0.5\text{M}, \text{pH 7.5}))</td>
</tr>
<tr>
<td>Total volume in upper chamber</td>
<td>2mL</td>
</tr>
<tr>
<td>Flow rate</td>
<td>(4\text{mL min}^{-1})</td>
</tr>
<tr>
<td>Fraction collected per tube</td>
<td>2mL</td>
</tr>
<tr>
<td>Volume of sample counted</td>
<td>100µL</td>
</tr>
<tr>
<td>Volume of cocktail used</td>
<td>10mL</td>
</tr>
<tr>
<td>Specific activity of ([3-^3\text{H}]3\text{FG})</td>
<td>(6.00\text{µCi µmole}^{-1})</td>
</tr>
</tbody>
</table>
Appendix 12

Upper Chamber: Pyranose-2-oxidase + Radioactive Ligand

Dialysis Membrane

Buffer

Fraction Collector
Appendix 13

Summary of initial rate kinetic results from various plots.
**Appendix 13**

**Initial rate of glucose oxidation by pyranose-2-oxidase**

<table>
<thead>
<tr>
<th>Plot</th>
<th>Correlation</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient</td>
<td>$K_{m}$ (mM)</td>
</tr>
<tr>
<td>Direct Linear</td>
<td>95% confident limit</td>
<td>96% confident limit</td>
</tr>
<tr>
<td>Lineweaver-Burk</td>
<td>0.9998</td>
<td>3.2 ± 0.08</td>
</tr>
<tr>
<td>Hanes-Woolf</td>
<td>1.0</td>
<td>3.2 ± 0.03</td>
</tr>
<tr>
<td>Eadie-Hofstee</td>
<td>0.9995</td>
<td>3.14 ± 0.06</td>
</tr>
</tbody>
</table>

**Initial rate of 4FG oxidation by pyranose-2-oxidase**

<table>
<thead>
<tr>
<th>Plot</th>
<th>Correlation</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient</td>
<td>$K_{m}$ (mM)</td>
</tr>
<tr>
<td>Direct Linear</td>
<td>95% confident limit</td>
<td>96% confident limit</td>
</tr>
<tr>
<td>Lineweaver-Burk</td>
<td>0.9962</td>
<td>5.54 ± 0.64</td>
</tr>
<tr>
<td>Hanes-Woolf</td>
<td>0.9996</td>
<td>5.84 ± 0.16</td>
</tr>
<tr>
<td>Eadie-Hofstee</td>
<td>0.9931</td>
<td>5.59 ± 0.38</td>
</tr>
</tbody>
</table>
REFERENCES


Biochem. J., 128, 199-204.

6(3), 309-315.


27, 2701-2709.


48, 2439-2443.


61. B. Helferich and M. Vock (1941) Ber., 74, 1807-1811.


   125-134.

   Windsor, Ontario, Canada, pp. 52-57.

111. T-N.E. Liu, B. Wolf, J. Geigert, S.L. Neidleman, J.D. 
   151-157.


113. K. Mosbach and B. Mattison (1976) in Immobilized 
   Enzymes, Methods in Enzymology, (K. Mosbach, ed.) 44, 


   Res., 27, 73-77.

116. G.V. Rao, L. Que, Jr., L.D. Hall and T.P. Fondo 

117. C.R. Haylock, L.D. Raelton, K.N. Slessor and A.C. 

   Res., 25, 511.


130. O.S. Chizhov, V.I. Kadentsev and B.M. Zolotarev (1971) 


133. W. Chefurka (1965) in *The Physiol. of Insecta.*, (M. 
New York.

134. A. Romaschin, N.F. Taylor, D.A. Smith and D. Lopes 

59, 262-268.

3895-3902.

137. C.Y. Lai and Horecker (1972) in *Essays in Biochemistry*, 
Vol. 8 (P.N. Campbell and F. Dickens, Eds.), Academic 

56, 658-660.

J.*, 139, 715-720.

140. M. Dixon and E.C. Webb (1963) *The Enzymes* 2nd edition, 

VITA AUCTORIS

NAME: Samuel T право Squire

BORN: September 21, 1953; Accra, Ghana

EDUCATION:

1973-1976 B.Sc. (Hons.) Biochemistry-
Chemistry, University of Ghana
Accra, Ghana

1979-1986 University of Windsor,
Windsor, Ontario, Canada.
Fulfilled all requirements for
the Degree of Doctor of Philosophy
in Biochemistry

WORKING EXPERIENCE:

1976-1978 Instructor (Chemistry)
Kaneshie Secondary Technical School
Accra, Ghana

1979 Research Assistant
University of Ghana Medical School
Accra, Ghana

1979-1986 Teaching Assistant (Chemistry)
University of Windsor
Windsor, Ontario, Canada.

Publications


2. S.T. Squire and N.F. Taylor, Enzymically based
synthesis of deoxy-fluoro sugars. Synthesis of 4-deoxy-
4-fluoro-D-fructose. In progress.