1983

Synthetic and biochemical studies with 4-deoxy-4-fluoro-D-glucose.

John. Samuel
University of Windsor

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THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED
SYNTHETIC AND BIOCHEMICAL STUDIES

WITH

4-DEOXY-4-FLUORO-D-GLUCOSE

by

John Samuel

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

Windsor, Ontario, Canada

1982
ABSTRACT

A review of fluorocarbohydrates as biochemical probes is presented.

A synthetic route for the preparation of [6-^3^H]-4-deoxy-4-fluoro-D-glucose is proposed and undertaken as follows:

A cold synthetic route for the introduction of tritium at C_6 of methyl α-D-glucopyranoside (II) is first established by the following route: platinum black oxidation of (II) to yield methyl α-D-glucopyranosiduronic acid (III), esterification to give methyl (methyl α-D-glucopyranosid)uronate (IV) followed by sodium borohydride reduction to reform (II). This sequence of reactions is repeated with methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (I) using NaB^3^H_4 to yield [6-^3^H]-methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (XV) which on acid hydrolysis gives [6-^3^H]-4-deoxy-4-fluoro-D-glucose (XV), (specific activity 19.88 mCi/mmol). The location of ^3^H is confirmed by the platinum catalyzed selective oxidation of (XV), which resulted in 96.3% loss of ^3^H.

The synthesis of 4-deoxy-4-fluoro-D-glucitol (XIX) by the borohydride reduction of the 4-deoxy-4-fluoro-D-glucose (XII) is described.

Some preliminary enzyme specificity studies on sorbitol dehydrogenase (L-iditol: NAD^+ 5-oxidoreductase

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EC 1.1.1.14) using 4-fluoro-4-deoxy-D-glucitol (XIX) are discussed. The latter compound serves as a substrate at high concentrations ($K_m = 20.4 \text{ mM}$) and an inhibitor at lower concentrations ($K_i = 16.6 \text{ mM}$) for the enzyme. The significance of these observations in relation to the nature of the enzyme substrate binding at C₄ of D-sorbitol is presented.
DEDICATION

to

Vic and Marion Salmons with fond memories of
their unique expressions of love.
ACKNOWLEDGEMENTS

I express my most sincere thanks to:

Prof. N. F. Taylor, D. Phil., for giving me the opportunity to be part of his research group and for providing excellent guidance and constant encouragement throughout the course of these investigations,

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ABBREVIATIONS

$^{23}_{[a]}D$  specific rotation (D line at 23°C)
$A$  angstrom
ADP  adenosine-5'-diphosphate
ATP  adenosine-5'-triphosphate
c  concentration (g/100 ml)
cm  centimeter(s)
cpm  counts per minute
dm  decimeter(s)
dpm  disintegrations per minute
DMF  dimethyl formamide
$2^{-18}_F$  $[2^{-18}F]$-2-deoxy-2-fluoro-D-glucose
$3^{-18}_F$  $[3^{-18}F]$-3-deoxy-3-fluoro-D-glucose
3FG  3-deoxy-3-fluoro-D-glucose
4FG  4-deoxy-4-fluoro-D-glucose
3FGA  3-deoxy-3-fluoro-D-gluconic acid
3FGL  3-deoxy-3-fluoro-D-glucitol
4FGL  4-deoxy-4-fluoro-D-glucitol
3F2KGA  3-deoxy-3-fluoro-2-keto-D-gluconic acid
4F2KGA  4-deoxy-4-fluoro-2-keto-D-gluconic acid
Fig.  figure
g  gram(s)
H #  H-Number: shift in the inflection point of the Compton edge due to quenching
Hz  hertz
i.r.  infra-red
$K_1$  inhibitor constant
$K_m$  Michaelis constant
$k\text{J}$  kilojoules
$M$  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
$\mu\text{M}$  micromolar
$N$  normal
$NAD$  nicotinamide-adenine dinucleotide
$NADH$  nicotinamide-adenine dinucleotide, reduced
$NADP$  nicotinamide-adenine dinucleotide phosphate
$NADPH$  nicotinamide-adenine dinucleotide phosphate, reduced
$nm$  nanometer(s)
$\%$  percentage
$PETT$  positron emission transaxial tomography
$pH$  $-\log H^+$ concentration
$p.p.m$  parts per million
$r\text{CMR}_{\text{glu}}$  regional cerebral metabolic rate for glucose
$R_F$  retardation factor
T.L.C.  thin layer chromatography
UDPG  uridine-5'-diphosphoglucose
v_{max}  maximum initial velocity
w/v  weight/volume
\lambda  wavelength
INTRODUCTION

Despite the synthesis of fluoroacetic acid ($\text{FCH}_2\text{COOH}$) by Swarts in 1896 (1), significant interest in organofluorine chemistry was not initiated until the second quarter of this century, when fluorocarbons were found to be useful in refrigeration. The development of organic fluorine insecticides in Germany during the same period was a further stimulus to the synthesis and the study of compounds containing C-F bond (2, 3). During World War II, a variety of fluorine containing organic compounds were synthesized and their biological properties studied by Saunders et al. (2), in Cambridge (United Kingdom), in view of the precautions to be taken against the possible use of fluorinated compounds against the Allies. At the same time in the United States, fluorinated compounds were used in the fractionation of the isotopes of uranium as the volatile hexafluorides ("Manhattan project" for the development of the atomic bomb) (3).

The discovery by Marais (4), in 1944, that fluoroacetate was the toxic principle of the South African plant * Dichapetalum cymosum*, and the subsequent elucidation of the biochemical mode of its toxicity by Peters et al. (5), widened the biochemical interest in C-F bond. The concept of 'lethal synthesis' (enzymatic transformation of a non-
toxic substrate into toxic product) advanced by Peters (5) besides shattering the previous misconceptions about enzyme specificity, also served to stimulate the design and the development of a large number of fluoro analogues of biological interest.

Many of the fluoro analogues showing biological activity have a fluorine atom replacing a hydrogen atom or a hydroxyl group in the natural substrate. A biochemical rationale for the replacement of hydrogen by fluorine on the basis of the similarities in the Van der Waal's radii of hydrogen (1.20Å) and fluorine (1.35Å) has been proposed by Bergmann (6). Subsequently, Goldman (7) has pointed out the unique characteristics of this 'super halogen' (8) with reference to its physiochemical parameters (e.g., Van der Waal's radii, electronegativity, bond energy to carbon) and its special value in design of analogues (by replacement of H by F) closely approximating the natural biochemical intermediate. More recently, Barnett (9) has drawn attention to the similarities in the size and the electronegativity of fluorine and oxygen (Table 1), and has argued that the substitution of oxygen by fluorine might prove to be another equally useful approach to the design of organic fluorine compounds of interest to biochemists.* Both of these approaches to the preparation of fluoro analogues have been successful, as evident from the wide variety of biologically interesting molecules synthesized (Table 2), some of which are currently being used clinically.

*This rationale was first proposed by N. F. Taylor and P. W. Kent (1958) J. Chem. Soc. 872-875.
TABLE 1

Comparison of Physical Parameters of Some Elements Covalently Bonded to Carbon (9)

<table>
<thead>
<tr>
<th>Element</th>
<th>Bond length ( \text{CH}_3-\text{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>O (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>1.80</td>
<td>3.0</td>
</tr>
<tr>
<td>S (in SH)</td>
<td>1.82</td>
<td>1.85</td>
<td>2.5</td>
</tr>
<tr>
<td>Chemical Class</td>
<td>Example</td>
<td>Bio-medical importance</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>---------------------------------------</td>
<td>-----------------------------------------</td>
<td></td>
</tr>
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<td>Flurocitrate (10)</td>
<td>Biochemical probe</td>
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<tr>
<td>Fluoroimidazoles</td>
<td>2-Fluorohistidine (11)</td>
<td>Antiviral agent</td>
<td></td>
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<td>Fluoropyrimidines</td>
<td>5-Fluorouracil (12-14)</td>
<td>Antineoplastic agent</td>
<td></td>
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<tr>
<td>Fluorocarbohydrates</td>
<td>3-Deoxy-3-fluro-D-glucose (15)</td>
<td>Biochemical probe</td>
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<td>Fluorocarbons</td>
<td>Perfluorodecalin (16)</td>
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<td>Perfluoroctyl bromide (17)</td>
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<td>Fluoroxene (18)</td>
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<td>9-α-fluorohydrocortisone (20)</td>
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<td></td>
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<td>Fluoroamino acids</td>
<td>α-Monofluoromethyl (DOPA) (21)</td>
<td>Suicide inhibitor</td>
<td></td>
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<td>18F labeled carbohydrates</td>
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<td>In PETT scan</td>
<td></td>
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Fluorocarbohydrates as Biochemical Probes

The selective introduction of fluorine into carbohydrates has been done mostly to replace a hydroxyl group of the sugar by fluorine. Such modifications cause only minimal disturbance in the overall stereochemistry of the molecule, as evidenced by the comparative X-ray studies. For example, X-ray studies of methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (I, Fig. 1) (23) show that the molecule exist in the $^4C_1(D)$ conformation, with all the bond lengths identical to those of methyl α-D-glucopyranoside, except that the C$_6$-O bond is shorter in (I). In (I) the C$_6$-O bond is antiperiplanar to the C$_5$-H bond, whereas in (II), C$_6$-O bond is antiperiplanar to the C$_4$-C$_5$ bond.

However, the replacement of OH by F results in significant change in the intermolecular hydrogen bonding capabilities of the molecule. The biological importance of this change has been discussed by Barnett (9). When the substrate binds with the enzyme by hydrogen bond, a hydroxyl group can act either as a donor or as an acceptor (Fig. 2a, and b), whereas fluorine at the same position can only be an acceptor (Fig. 2c) and the corresponding deoxy analogue offers no possibility for hydrogen bonding (Fig. 2d). Therefore, a comparison of the binding affinities of the enzyme or carrier for the substrate with that for the fluoro and deoxy analogues would be expected to be an excellent approach to the study of the
Fig. 1 Comparison of the conformations of methyl 4-deoxy-4-fluor-o-D-glucopyranoside and methyl o-D-glucopyranoside (23, 3).
Fig. 2 Comparison of the hydrogen bonding capabilities of the (a,b) sugar, (c) fluorosugar (d) and deoxy sugar with a protein (9).
nature and location of bonds between the substrates and the enzymes. The presence of fluorine in the substrate further makes it possible to study the nature of these interactions by using \(^{19}\text{F n.m.r.}\) probes (24). A wide variety of fluorinated carbohydrates have been synthesized in view of their potential use in biochemical investigations. The chemistry of these have been extensively reviewed (3, 25). The present discussion draws attention to some typical illustrations of the use of fluorocarbohydrates as biochemical probes.

**Enzyme Binding and Specificity Studies**

Bessel et al. (26) have used a series of deoxyfluoro-D-glucopyranoses and related compounds to study the specificity of yeast hexokinase. The stereospecific binding requirements of the hexokinase isoenzymes were explored in order to investigate the possibility of designing selective inhibitors as antineoplastic agents. 2-Deoxy-2-fluoro-D-glucose, 2-deoxy-2-fluoro-D-mannose, 2-deoxy-2, 2-difluoro-D-arabinohexose and 2-deoxy-D-arabinohexose were found to be good substrates (Table 3), confirming the previous reports that modifications at C\(_2\) of the D-glucose could be made without loss of binding to the enzyme. However, the replacement of an hydroxyl group by fluorine at C\(_3\) and C\(_4\) of the glucose molecule resulted in decreased binding to
### TABLE 3

Kinetic Data for Hexose binding
to yeast hexokinase (26)

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>0.17</td>
<td>1.00</td>
</tr>
<tr>
<td>2-Deoxy-D-arabino-hexose</td>
<td>$0.59 \pm 0.11$</td>
<td>0.85</td>
</tr>
<tr>
<td>2-Deoxy-2-fluoro-D-glucose</td>
<td>$0.19 \pm 0.03$</td>
<td>0.50</td>
</tr>
<tr>
<td>2-Deoxy-2-fluoro-D-mannose</td>
<td>$0.41 \pm 0.05$</td>
<td>0.85</td>
</tr>
<tr>
<td>2-Deoxy-2, 2-difluoro-D-arabino-hexose</td>
<td>$0.13 \pm 0.02$</td>
<td>0.53</td>
</tr>
<tr>
<td>3-Deoxy-3-fluoro-D-glucose</td>
<td>70 $\pm$ 30</td>
<td>0.10</td>
</tr>
<tr>
<td>4-Deoxy-4-fluoro-D-glucose</td>
<td>84 $\pm$ 30</td>
<td>0.10</td>
</tr>
</tbody>
</table>
the hexokinase (Table 3). The similar $K_m$ and $V_{max}$ values suggested that the $C_3$-OH and $C_4$-OH may be playing similar roles in the binding. 6-Deoxy-6-fluoro-D-glucose was shown to be a competitive inhibitor with low binding affinity ($K_i$ 12mM) suggesting that $C_6$-OH is also involved in enzyme substrate binding. As these studies did not involve the use of 3-, 4-, and 6-deoxy analogues, no definite conclusion could be made about the direction of hydrogen bond. Neither of the anomeric glucopyranosyl fluorides served as a substrate or an inhibitor. This led to the postulation that either $C_1$-OH plays a vital rôle in the binding (may be as a hydrogen donor) or that D-glucose does not bind in its pyranose form. A similar approach has been used in investigations of the specificity of other yeast enzymes such as glucose phosphate isomerase (27), glucose-6-phosphate dehydrogenase (28) and galactokinase (29).

The binding of sugars to lysozyme has been studied using fluoroanalogues with the aid of $^{19}$F n.m.r. spectroscopy (24, 30, 31). The addition of lysozyme to methyl-N-fluoroacetyl $\beta$-D-glucosaminide results in a downfield chemical shift in $^{19}$F n.m.r. spectrum (31). This variation of chemical shift was used to determine the binding constant $K_s$, by means of the relationship

$$S_0 = \frac{E \Delta}{\delta} - K_s$$
where \( S_0 \) is the total concentration of the substrate, \( E_0 \) the enzyme concentration, \( \delta \) the observed change in chemical shift and \( \Delta \) the chemical shift of the fully formed complex (24). The binding of the sugar with the enzyme in presence of a paramagnetic ion (\( \text{Gd}^{3+} \)) resulted in an increase in the relaxation time of the fluorine nucleus. Under conditions of fast exchange of the substrate between the free and the bound state, this increase is a function of the distance between the fluorine nucleus and the metal ion (along with the correlation time and the relative concentrations of the free and bound sugar). As the binding site of \( \text{Gd}^{3+} \) in lysozyme was known from X-ray studies, the observed increase in relaxation time could be used for obtaining information about the conformation of the enzyme-substrate complex. The advantage of using fluorine probes in such studies is that fluorine spectrum is simpler than the proton spectrum. Even when the fluorine spectrum is complicated by extensive fluo-
rine proton coupling, it may be simplified by heteronuclear spin decoupling (32).

**Studies on Carbohydrate Metabolism**

The potential of deoxyfluoromonosaccharides to serve as pseudosubstrates for glycolytic enzymes was shown by the extensive biochemical investigations by Taylor et al. using 3-deoxy-3-fluoro-D-glucose (3FG) (33-44). In *Saccharomyces*
cerevisiae (34), the effect of 3PG on glucose metabolism resulted in decreased uptake of the sugar and inhibition of polysaccharide synthesis. The effects on the metabolism on galactose were inhibition of respiration and stimulation of polysaccharide synthesis with no significant change in sugar uptake. These results suggested an inhibitory form of 3PG acting on phosphoglucomutase or uridine diphosphate glucose (UDPG) phosphorylase. The observation that the metabolic effects of 3PG included an increase in ADP concentration, a decrease in ATP concentration and a 20% decrease in inorganic phosphate, suggested the binding of 3PG with inorganic phosphates. This could affect the energy balance of the cell to produce the observed metabolic responses. Another possible explanation could be based on the direct inhibitory effect of 3PG phosphates on glycolytic enzymes. The enzyme studies using synthetic 3PG-1-α-phosphate and 3PG-6-phosphate (37) showed that they were competitive inhibitors of UDPG phosphorylase and phosphoglucomutase respectively. Therefore, the effects of 3PG on glycolysis of yeast cells may be primarily due to inhibitory effect of the phosphate metabolites of 3PG on the glycolytic enzymes, in a manner similar to that proposed for the inhibitory effects of 2-deoxy-D-glucose on yeast cells (45).

In view of the fact that 3PG was a poor substrate for
yeast hexokinase, studies on the effect of 3FG on carbohydrate metabolism were done on *Pseudomonas fluorescens*, since this organism is known to possess oxidative enzymes for hexose catabolism. While the cell free extracts of this organism oxidized 3FG in a two step process to a fluoroketoaldonic acid, 3-deoxy-3-fluoro-α-keto-D-gluconic acid (3F2KGA), the oxidation of 3FG by the whole cells was limited to the formation of the corresponding aldonic acid, 3-deoxy-3-fluoro-D-gluconic acid (3FGA). However, exogenous supply of this oxidation product (3FGA) to the whole cells resulted in further oxidation to 3F2KGA. Based on these experimental observations, a scheme for the oxidation of 3FG by *P. fluorescens* has been proposed (Fig. 3). The limited oxidation by the whole cells was initially explained on the basis that the formation of 3FGA proceeds via the 3-deoxy-3-fluoro-D-gluconic acid-δ-lactone and that the specificity of the normal hydrolase (46) prevents the formation of the free 3FGA. The oxidation of 3FG to 3F2KGA by the cell free preparation suggested that either (i) the same enzymes that oxidize glucose to 2-ketogluconic acid do not possess the necessary specificity at C₃ of glucose and gluconic acid, or that (ii) there are separate enzymes for the 3FG and 3FGA substrates. However, studies on the oxidation rate with glucose/3FG mixture did not show any changes in the $V_{max}$ suggesting that separate enzyme systems
a) Whole cell: 3FG + (6-1actone) → 3FGA → 3F2KGA

b) Cell extract: 3FG → 3FGA → 3F2KGA

Fig. 3 Oxidation of 3FG by P. fluorescens to 3F2KGA: a) by whole cells; b) by cell free preparations (35).
systems do not exist. Based on these it was argued that 3FG serves as a pseudosubstrate for the enzyme that oxidizes glucose to 2-ketogluconic acid due to its lack of specificity for C₃ of glucose and gluconic acid.

The growth studies on *P. fluorescens* showed the inability of the organism to use 3FG as the carbon source, suggesting that the oxidation of 3FG by the organism is not carried to a stage at which the carbon may be assimilated in the cytoplasm by the action of kinases and into the Entner-Doudoroff or hexose monophosphate shunt system known to operate in this organism (47). This was further confirmed by demonstration that with cell free extracts, 3FG, 3FGA or 3P2KGA are not substrates for the gluconokinase present, and that 3FG and 3FGA are competitive inhibitors of the enzyme for gluconate ($K_i$ 47.5 mM and 14.8 mM respectively).

The toxicity studies of 3FG on *Locustia migratoria* and *Schistocerca gregaria* (15, 43, 44) illustrate the use of fluorocarbohydrates as probes for the detection of the previously undetected metabolic pathways. The toxic effect of 3FG on locust has been shown to be associated with its metabolism to 3-deoxy-3-fluoro-D-glucitol (3FGL), which has been isolated from the tissue metabolites. *In vitro* studies on the synthetic 3FGL show that it is a poor substrate for sorbitol dehydrogenase (EC.1.1.1.14), and that it competitively inhibits the enzymatic conversion of
D-glucitol to D-fructose. Based on these results, a scheme has been proposed for the metabolism of D-glucose as well as 3FG in locust (Fig. 4). Further, the poor substrate activity of 3FGL for sorbitol dehydrogenase was taken to indicate stereospecific hydrogen bonding between the hydrogen of the C₃-OH and a receptor group on the protein. A more detailed description of the specificity of this enzyme requires synthetic and enzymatic studies using other deoxy-fluoro analogues of D-glucitol.

Fluoro Analogues in Carbohydrate Transport Studies

The association of the sugar with the carrier in transport systems is believed to be by hydrogen bond. As discussed before, either hydrogen of the hydroxyl group or that of the protein may be used as the bridge hydrogen. Therefore, depending on the existence and the nature of binding at a particular position, fluoro and deoxy analogues of sugars, when used for transport studies, would be expected to show quantitative differences in the transport parameters. The existence and the direction of a hydrogen bond at each position of the sugar may be investigated by systematic comparison of the transport parameters of a series of sugars in which hydroxyl group is substituted by fluorine or hydrogen.

The structural requirements for binding to the active transport system in the hamster intestine have been in-
Fig. 4 Sorbitol Conversion Pathway (15)
vestigated by Barnett et al. (48-51), using the above approach. The initial accumulation data and the $K_i$ values for a series of galactose and glucose derivatives are shown in Table 4 and Table 5. The effects of the modifications at $C_3$ as well as $C_6$ suggest the existence of hydrogen bonds with $C_3$-OH and $C_6$-OH where the protein acts as the donor. The substitution of the ring oxygen by sulphur (Table 5) did not affect the transport significantly showing that no hydrogen bond exists between the carrier and the ring oxygen. The results of the modifications at $C_1$, indicate the presence of another hydrogen bond at this position using protein hydrogen. The relatively poor binding of $\alpha$-D-galactopyranosyl fluoride, may be due to unfavourable steric factors. Very high specificity at $C_2$ hydroxyl group is indicated by the absolute loss of transport as a result of any $C_2$ modification. Since the introduction of fluorine at this position did not result in the restoration of transport, it was argued that the nature of the bond at $C_2$ was different from the rest and was probably covalent (possibly an ester). As the sugars with D-galacto configuration were less well transported than those with D-gluco configuration, existence of a hydrogen bonding with $C_4$-OH of D-glucose was suggested. However, since no 4-deoxy-4-fluoro analogue was available for these studies, no conclusion could be made about the
TABLE 4

Transport Parameters of Monosaccharides in Hamster Intestine (9)

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Initial accumulation (tissue/medium ratio)</th>
<th>$K_i$(mM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Galactose</td>
<td>$8.3 \pm 0.25$</td>
<td>3</td>
</tr>
<tr>
<td>6-Deoxy-D-galactose</td>
<td>$1.5 \pm 0.15$</td>
<td>16</td>
</tr>
<tr>
<td>6-Deoxy-6-fluoro-D-galactose</td>
<td>$4.3 \pm 0.8$</td>
<td>7</td>
</tr>
<tr>
<td>1-Deoxy-D-galactose</td>
<td>$0.4 \pm 0.1$</td>
<td>-</td>
</tr>
<tr>
<td>$\alpha$-D-galactopyranosyl fluoride</td>
<td>$1.2 \pm 0.3$</td>
<td>-</td>
</tr>
</tbody>
</table>

*Using methyl $\alpha$-D-glucopyranoside as substrate.
### TABLE 5
Transport Parameters of Monosaccharides in Hamster Intestine (9)

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Initial accumulation (tissue/medium ratio)</th>
<th>$K_i$ (mM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>$16.4 \pm 0.4^\text{**}$</td>
<td>2.3</td>
</tr>
<tr>
<td>3-Deoxy-D-glucose</td>
<td>$2.1 \pm 0.2$</td>
<td>24</td>
</tr>
<tr>
<td>3-Deoxy-3-fluoro-D-glucose</td>
<td>$26.6 \pm 3.6$</td>
<td>2.5</td>
</tr>
<tr>
<td>5-Thio-D-glucose</td>
<td>$12.5 \pm 0.7^\text{**}$</td>
<td>2.9</td>
</tr>
<tr>
<td>2-Deoxy-D-glucose</td>
<td>$0.2 \pm 0.02$</td>
<td>−</td>
</tr>
<tr>
<td>2-Deoxy-2-fluoro-D-glucose</td>
<td>$0.4 \pm 0.2$</td>
<td>−</td>
</tr>
</tbody>
</table>

*Using D-galactose as substrate
In the presence of 2mM fluoride
direction of the hydrogen bond. A hypothetical model for the binding of D-glucose to the carrier for active transport has been proposed by Barnett (Fig. 5).

Essentially the same approach has been used by Barnett et al. (51, 54) and more recently by Taylor et al. (15, 52, 53) for studying the facilitated transport system for D-glucose in the human erythrocyte. The effects of molecular modifications at various positions of the sugar on transport parameters are summarized (Table 6 and 7). The decrease in the affinity (high $K_1$, Table 7) of the 1-deoxy analogue for the carrier suggested the presence of a hydrogen bond. The considerable restoration of binding by introduction of fluorine atom in $\beta$-configuration and the lack of binding affinity of $\alpha$-D-glucopyranosyl fluoride indicated that the hydrogen bond is to the $C_1$ oxygen of the $\beta$-D-glucopyranose. A similar comparison of the transport parameters ($K_x$, $V_{\text{max}}$ and $K_1$, Tables 6 and 7) for 3-deoxy and 3-deoxy-3-fluoro analogues suggested that $C_3$ oxygen binds with the carrier via a hydrogen bridge derived from the carrier. The involvement of the ring oxygen in a hydrogen bond was indicated by the high $K_x$ value for 5-thio-D-glucose (Table 6). The hydroxyl groups at $C_2$ and $C_6$ were not involved in binding with the carrier was evidenced by the high affinity (low $K_1$, Table 6) of 2-deoxy, 6-deoxy and 6-deoxy-6-fluoro analogues of D-glucose for the carrier (51). Similarly the transport
Fig. 5 Hypothetical model for the binding of D-glucose to the carrier for active sugar transport in hamster intestine (9).
TABLE 6
Exit Transport Parameters of Monosaccharides across the Human Erythrocyte Membrane at 37°C (15, 53)

<table>
<thead>
<tr>
<th>Sugar</th>
<th>$K_x$ (mM)</th>
<th>$V_{max}$ (mmole litre$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>3.9</td>
<td>640</td>
</tr>
<tr>
<td>3-Deoxy-3-fluoro-D-glucose</td>
<td>2.3</td>
<td>600</td>
</tr>
<tr>
<td>3-Deoxy-D-glucose</td>
<td>15.3</td>
<td>795</td>
</tr>
<tr>
<td>4-Deoxy-4-fluoro-D-glucose</td>
<td>4.5</td>
<td>645</td>
</tr>
<tr>
<td>4-Deoxy-D-glucose</td>
<td>4.5</td>
<td>645</td>
</tr>
<tr>
<td>5-Thio-D-glucose</td>
<td>15.0</td>
<td>500</td>
</tr>
</tbody>
</table>

TABLE 7
Inhibition of L-Sorbose Entry into Human Erythrocytes by Glucose Analogues (54)

<table>
<thead>
<tr>
<th>Sugar</th>
<th>$K_l$ (mM, 25°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>7.8</td>
</tr>
<tr>
<td>1-Deoxy-D-glucose</td>
<td>76.0</td>
</tr>
<tr>
<td>$\alpha$-D-glucopyranosyl fluoride</td>
<td>77.0</td>
</tr>
<tr>
<td>$\beta$-D-glucopyranosyl fluoride</td>
<td>15.6</td>
</tr>
<tr>
<td>3-Deoxy-D-glucose</td>
<td>71.5</td>
</tr>
<tr>
<td>3-Deoxy-3-fluoro-D-glucose</td>
<td>6.9</td>
</tr>
<tr>
<td>6-Deoxy-D-glucose</td>
<td>6.7</td>
</tr>
<tr>
<td>6-Deoxy-6-fluoro-D-glucose</td>
<td>1.2</td>
</tr>
<tr>
<td>2-Deoxy-D-glucose</td>
<td>3.2</td>
</tr>
</tbody>
</table>
parameters for 4-deoxy- and 4-deoxy-4-fluoro derivatives (Table 6) showed that C₄ hydroxyl group was also not involved in hydrogen bonding with the carrier. Thus, the oxygen functions located at C₁, C₃ and the ring oxygen at C₅ of β-D-glucopyranose were considered to be necessary for the most effective binding of D-glucose to the transport protein. This model (Fig. 6) proposed by Taylor et al. (15, 53) is in agreement with the detection of three receptor groups (-NH₂, -SH and imidazole on the protein associated with glucose transport (55).

1⁸F Labeled Carbohydrates in Positron Emission Transaxial Tomography (PETT)

PETT is a non-invasive, computer assisted imaging technique involving in vivo use of biologically important compounds labeled with positron emitting nuclides (56, 57). This technique has the unique ability to give quantitative regional information about the metabolism of the tissue or the organ under investigation. When the radionuclides decays, it releases a high energy positron or positive electron. This immediately combines with an electron and both are annihilated with the emission of two gamma rays, which fly off in nearly opposite directions. PETT images (in the form of an axial cross section) are constructed by a computer based on the measurements of this radiation. This represents the three dimensional distribution of the
Fig. 6 Stereospecific binding sites of β-D-glucopyranose in the Cl-conformation to a transport protein. Hydrogen bonds. \( R_1, R_1 \) and \( R_3 \) represent receptor sites on the transport protein (15).
labeled tracer. The biologically important positron emitters most suited for PETT are $^{11}\text{C}(t_{1/2}=20\ min)$, $^{13}\text{N}(t_{1/2}=10\ min)$, $^{15}\text{O}(t_{1/2}=2\ min)$ and $^{18}\text{F}(t_{1/2}=120\ min)$.

$[2-{}^{18}\text{F}]$-2-deoxy-2-fluoro-D-glucose ($2-{}^{18}\text{FG}$) and $[3-{}^{18}\text{F}]$-3-deoxy-3-fluoro-D-glucose ($3-{}^{18}\text{FG}$) have been used for studying the regional metabolic rate for glucose in experimental animals and human subjects (57–62). $2-{}^{18}\text{FG}$ and $3-{}^{18}\text{FG}$ are of special value in studying the regional cerebral metabolic rate for glucose ($r\text{CMR glu}$), since they are effectively trapped in the brain tissue after phosphorylation by hexokinase, and therefore permitting PETT scans even 30–40 minutes after the administration of the radioactive tracer (57). Greenberg et al. have used $2-{}^{18}\text{FG}$ for the metabolic mapping of functional activity by PETT and have reported the effects of different sensory stimuli (tactile, visual or auditory) on the $r\text{CMR glu}$ (60). The regional variations in the cerebral utilization of glucose and its significance in a variety of pathological conditions of brain, such as convulsive disorders, Huntington's chorea, neoplastic conditions and schizophrenia are being explored using the same technique (58).
C-F Bond Cleavage: Fluorocarbohydrates as Potential Affinity Labels

The C-F bond is the strongest single bond formed by carbon [450-485 kJ as compared to ~360kJ for C-O bond (alcohols) and ~427 kJ for C-H bond (methane)]. However, it is known to be cleaved in a number of enzymatic reactions (7, 63, 64).

The cleavage of C-F bond of 4-deoxy-4-fluoro-D-glucose (4FG) by the whole cells of P. putida*, has been reported (15, 65). The cell-free extract of this organism oxidized 4FG with consumption of 2g atoms of oxygen/mole with retention of C-F bond. This is consistent with the formation of 4-deoxy-4-fluoro-2-keto-D-gluconic acid (4F2KGA) in a manner analogous to the established metabolic pathway for 3FG in the P. putida (41). With the whole cells, however, 4FG elicited release of fluoride without any respiration (15, 65). The site of defluorination was identified to be in the outer membrane fraction (65). Despite the C-F cleavage, 4FG fails to act as a carbon source for P. putida (15). The involvement of an endogenous protein of the outer membrane of P. putida in fluoride release was shown by studies on cells pre-incubated with chloramphenicol. The possibility that the protein responsible for defluorination might be serving as a carrier for glucose transport was indicated by

*Previously classified as P. fluorescens.
the protection of fluoride release by D-glucose, D-gluconate, and 2-keto-D-gluconate. Further, the complete protection of fluoride release afforded by N-ethyl maleimide suggested the importance of a protein SH group in C-F bond cleavage. These results are consistent with initial binding of 4FG (by fluorine-hydrogen bonding) to protein, followed by a nucleophilic displacement of fluoride, possibly by SH group participation of protein, which then becomes covalently and irreversibly attached to the sugar (Fig. 7). Thus, 4FG may prove to be an ideal affinity label [as defined by Gorman et al. (66)] for the glucose carrier protein(s) in pseudomonads. However, such detailed biochemical studies await the radiochemical synthesis of 4FG.

Objectives

The objectives of the present work may now be stated.

1. To synthesize [6-3H]-4-deoxy-4-fluoro-D-glucose and locate the position of 3H. This compound is required for the biochemical studies on the glucose transport system in _P. putida_.

2. To synthesize 4-deoxy-4-fluoro-D-glucitol in order to make a preliminary examination of the specificity of sorbitol dehydrogenase.
Fig. 7 Hypothetical mechanism for the interaction of 4FG with an outer membrane protein in *P. putida*.
RESULTS AND DISCUSSION

Synthetic Studies

The Synthesis of [6-\textsuperscript{3}H]-4-deoxy-4-fluoro-D-glucose

Lopes and Taylor have reported a convenient route
for the preparation of 4-deoxy-4-fluoro-D-glucose (4FG)
(53). The present discussion involves investigations
for establishing a modified radiochemical route for the
synthesis of [\textsuperscript{3}H]-4FG, which permit:
a) the introduction of tritium at a position which does
not easily undergo enzymatic oxidation,
b) minimum modifications to the established method for
the synthesis of 4FG, and
c) the incorporation of tritium towards the last steps
in the synthetic sequence.

The methods for incorporation of tritium in sugars
have been extensively reviewed (67-69). The random
labeling methods such as catalytic labeling (70) and the
Wilzbach method (71) are unsuited for the tritiation of
carbohydrates (68). Among the various methods available
for the stereospecific introduction of tritium label
into sugars (Table 8), the most convenient and widely
used is the reduction of a sugar aldehyde, ketone,
### TABLE 8

Methods for Stereospecific Introduction of Tritium into Sugars

<table>
<thead>
<tr>
<th>Method</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Reduction by hydride reagents</td>
<td>[6-³H]-D-glucose (72)</td>
</tr>
<tr>
<td>2. Enzymatic reduction by NADH</td>
<td>[5-³H]-D-fructose (73)</td>
</tr>
<tr>
<td>3. Base catalyzed solvent exchange</td>
<td>[4-³H]-L-arabinose-ascorbic acid (74)</td>
</tr>
<tr>
<td>4. Enzyme catalyzed solvent exchange</td>
<td>[2-³H]-D-glucose (75)</td>
</tr>
<tr>
<td>5. Addition to a double bond</td>
<td>[4-³H]-D-galactose (76)</td>
</tr>
<tr>
<td>6. Conversion from another labeled sugar</td>
<td>[3-³H]-D-arabinose (77)</td>
</tr>
</tbody>
</table>
lactone or ester by a hydride reagent. The introduction of a carbonyl group may be achieved by selective oxidation of a C-OH group of the sugar. Reduction by a hydride reagent (usually sodium borohydride) has been used for the specific introduction of tritium at each of the six carbon atoms in D-glucose (68). Enzymatic reduction by reduced nicotinamide adenine dinucleotide (NADH) allows the reduction of one carbonyl group in the presence of another, as illustrated by the preparation of [5-\(^3\)H]-fructose from D-threo-2,5-hexodiulose (73). The introduction of tritium by base catalyzed solvent exchange is of limited application, since the mildly basic condition required for catalysis may also lead to rearrangement (68). However, such an exchange catalyzed by enzymes is of special value since the incorporation of tritium is stereospecific so that at a methylene group, only one of the two hydrogen atoms becomes labeled. Thus D-glucose-6-phosphate ketol isomerase (E.C.5.3.1.9) incorporates only one tritium atom from tritiated water, giving [1-(R)-\(^3\)H]-D-fructose-6-phosphate (78). The anti-Markownikoff addition of diborane also has been used for the stereospecific introduction of tritium in carbohydrates (76). Sometimes the most convenient method of preparation of the labeled sugar may be chemical or enzymatic modification of a more readily available sugar.
Wolfrom and Anno have reported the sodium borohydride reduction of glycosiduronic acid esters to the corresponding glycosides (79). This may offer a selective method for the introduction of tritium at C₆ in sugars. Tritation at C₆ would be of advantage as C₆-H bonds of 4FG are considered to be stable in many biochemical systems. The preparation of uronic acid esters may be achieved by a two-step reaction. The platinum catalyzed selective oxidation of the glycoside by oxygen (80, 81) will give the corresponding glycosiduronic acid. This may be esterified by using diazomethane (82). Thus a reaction sequence of glycoside → uronic acid → uronic acid ester → glycoside would be expected to provide a general method for the introduction of tritium at C₆ of sugars.

Preliminary studies on the above reaction sequence were carried out using methyl α-D-glucopyranoside (Fig. 8), in order to establish the optimum experimental conditions. The commercially available platinum black was used as the catalyst. The uronic acid produced during the oxidation was prevented from further degradation by conversion to sodium salt (pH was maintained between 7.5 and 8.0 by periodic addition of sodium bicarbonate) and was regenerated after the oxidation using a cation exchange resin (Amberlite IR-120 (H)). The optimum temperature range was noticed to be 40-50°C, beyond which an increase in the degradation
Fig. 8 Synthetic studies using methyl α-D-glucopyranoside.
of the sugar was observed. Methyl α-D-glucopyranosidurononic acid (III) was converted to the methyl ester (IV) using diazomethane. The presence of the ester carbonyl group was confirmed by the strong absorption band around 1740 cm⁻¹ (C = O stretch) in the i.r. spectrum and an n.m.r. signal at δ 3.72 corresponding to C-OCH₃. The sodium borohydride reduction of methyl (methyl α-D-glucopyranosid)uronate (IV) gave methyl α-D-glucopyranoside in 69% yield. The higher yield achieved [as compared to the previously reported yield of 37% (79)] may be due to the greater purity of the uronate used in the present studies. The overall yield of 30.2% for the three steps suggest that this route may successfully be used for the introduction of tritium in carbohydrates

Such a modification applied to the synthesis of 4PG will incorporate only minimum additional steps to the established synthetic route. Besides, the incorporation of ³H will be towards the last steps in the reaction sequence.

As further studies required methyl 4-deoxy-4-fluoro-

α-D-glucoside, its synthesis by the reported route was undertaken (53). The stereoselective replacement of C₄-OH of D-glucose by F involves nucleophilic displacement of the methyl sulfonic ester (53). As this reaction results in Walden inversion (SN₂ mechanism, Fig. 9),
Fig. 9 Nucleophilic displacement ($S_N^2$) of sulphonic esters by fluoride.
the sulphonic ester used for the displacement must be of D-galacto configuration. The protection of the C_{1}-OH may be achieved by its conversion to the highly stable -OCH_{3} group. Other hydroxyl groups (C_{2}-OH, C_{3}-OH and C_{6}-OH) may be protected by benzylation. The advantage of using benzyl groups as protecting groups is due to their stability in a wide variety of reaction conditions and their susceptibility to cleavage by hydrogenolysis over palladium catalysts.

The first four steps in the synthetic route (Fig. 10) are for the preparation of methyl 4-O-methylsulphonyl-α-D-galactopyranoside (IX) (83). The low reactivity of the C_{4} axial OH group, towards benzoylation (due to steric factors) was exploited for the selective introduction of the methylsulphonyl group at C_{4} (Fig. 11). Benzylation (84, 85) of (IX) by benzyl bromide in the presence of silver oxide (weak base) in DMF gave methyl 2,3,6 tri-O-benzyl-4-O-methylsulphonyl-α-D-galactopyranoside (53). The displacement reaction was carried out under relatively mild conditions, using freshly prepared tetrabutyl ammonium fluoride as the fluoride source, in a dipolar, aprotic solvent such as acetonitrile. The methyl 2,3,6-tri-O-benzyl-4-deoxy-4-fluoro-α-D-glucopyranoside thus produced was converted to methyl 4-deoxy-4-fluoro-α-D-glucopyranoside by palladium catalysed hydrogenolysis. The
Fig. 10  Synthesis of 4-deoxy-4-fluoro-D-glucose (53).
\[
\begin{align*}
\text{VIII} & \quad \text{NaOCH}_3 / \text{CH}_3\text{OH} \quad \text{IX} \\
\text{XI} & \quad (C_6\text{H}_5)_4\text{NF} \quad \text{CH}_3\text{CN} \\
\text{X} & \quad \text{H}_2 / \text{Pd} \\
\text{I} & \quad \text{H}_2\text{SO}_4 \\
\text{XII} & \quad \text{Bzl} = \text{C}_6\text{H}_5\text{CH}_2 \\
& \quad \text{DMF} = \text{Dimethyl formamide}
\end{align*}
\]

Fig. 10 (continued)
Fig. 11 The selective introduction of methyl sulphonyl group at C₄ of methyl α-D-galactopyranoside using the low reactivity of C₄-OH towards esterification.
overall yield of the seven steps was 4.81%.

Synthetic studies involving the previously discussed sequence of reactions of oxidation, esterification and reduction were done on methyl 4-deoxy-4-fluoro-α-D-glucopyranoside in order to establish a route for incorporation of tritium (Fig. 12). The experimental conditions used for the synthesis of methyl (methyl 4-deoxy-4-fluoro-α-D-glucopyranosid)uronate (XIV) were the same as those used for the synthesis of methyl (methyl α-D-glucopyranosid)uronate (IV). The presence of an ester carbonyl group was confirmed by the strong absorption band at 1760 cm\(^{-1}\) (C = O stretch) in the i.r. spectrum and by the n.m.r. signal at δ 3.76 (assigned as C-O CH\(_3\)). The \(^{19}\)F n.m.r. chemical shift (δ = 119.5 with reference to trifluoroacetic acid) is comparable with that reported for other deoxyxynonfluoro monosaccharides (86). The geminal \(^{19}\)F-^H coupling constant (\(J_{F,H_4} = 54.5\) Hz) is within the range normally associated with sp\(^3\)-hybridized carbon in a six-membered ring (87). The observed vicinal coupling (\(J = 14.7\) Hz) was assigned to be due to F-H\(_3\) coupling. This is comparable with the \(J_{F,H_3}\) reported for 4-deoxy-4-fluoro-D-glucose (16 Hz) (87). Due to the limited resolution, coupling constants of lower magnitudes such as \(J_{F,H_5}\), \(J_{F,H_6}\), and \(J_{F,H_1}\) could not be determined.

The reduction of the uronic acid ester (XIV) with
Fig. 12 Protocol for the introduction of tritium at C₆ of methyl-4-deoxy-4-fluoro-α-D-glucopyranoside.
sodium borohydride at room temperature resulted in formation of two products. The major component had the same chromatographic mobility as methyl-4-deoxy-4-fluoro-α-D-glucopyranoside and the side product formed had the same chromatographic mobility as the corresponding uronic acid (XIII). The formation of the side product was, therefore, thought to be due to the alkaline hydrolysis of the uronic acid ester. The reduction was repeated at 0-4°, using smaller concentrations of sodium borohydride, in order to exclude the latter possibility. This resulted in slower rate of reduction and considerable reduction in the formation of the side product. However, pure methyl 4-deoxy-4-fluoro-α-D-glucopyranoside could be obtained only after purification by preparative T.L.C. The crystallized product had the same R_p value, melting point, mixed melting point and specific rotation as the reference compound. Further, the acid hydrolysis of this compound gave 4PG which had identical i.r. spectra to that of the reference compound. The overall yield for the three steps (oxidation, esterification and reduction) was 35.63%.

The radiochemical reduction of methyl (methyl 4-deoxy-4-fluoro-α-D-glucopyranosid)uronate by NaB^3H_4 gave [6-^3H]-methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (Fig. 13) in 54% yield (radiochemical yield 23.7%) which on
Fig. 13. Synthesis of $[6-^3\text{H}]-4\text{-deoxy}-4\text{-fluoro-}$
$\alpha\text{-D-glucose.}$
acid hydrolysis gave [6-\textsuperscript{3}H]-4-deoxy-4-fluoro-D-glucose in 59% yield (specific activity, 10.88 mCi/mmole).

The Location of Tritium in [6-\textsuperscript{3}H]-methyl 4-
fluoro-\alpha-D-glucopyranoside

Since the tritiated sugars are usually prepared for use in mechanistic studies with enzymes, the exact location of the radioactive isotope is often required. It is not advisable to rely on the method of synthesis alone, as sometimes the label may be incorporated in an entirely unexpected position (88). Such possibilities could not be ignored in the present studies. Prihar et al. have reported 85% loss of tritium during sodium borohydride reduction of [5-\textsuperscript{3}H]-methyl (methyl \alpha-D-glucopyranosid)-uronate (XVII) (89). This unexpected loss of \textsuperscript{3}H from C\textsubscript{5} during the reduction of the C\textsubscript{6} ester group was explained as due to the base catalyzed enolization of (XVII) during sodium borohydride reduction (Fig. 14). If such tautomeric transformations are associated with the borohydride reduction of methyl (methyl 4-deoxy-4-fluoro-\alpha-D-glucopyranosid)uronate it may result in incorporation of tritium at C\textsubscript{5} to some extent. This necessitated the design of experiments for the localization of tritium. The methods available for locating tritium in carbohydrates include chemical degradation and tritium nuclear magnetic reson-
Fig. 14 Base catalyzed enolization of [5-³H]methyl (methyl-α-D-glucopyranosid)uronate during reduction by NaBH₄ (89).
ance ($^3$H n.m.r) spectroscopy (90-92).

A carrier may be added to the radioactive sample and the molecule may be specifically degraded, often by periodate oxidation, to give each hydrogen atom uniquely located in a crystalline derivative. The dimeredone derivative of formaldehyde and the p-bromophenacyl ester of formic acid have been found to be particularly useful (90). Alternatively, specific removal of tritium by ionization or oxidation also may be used. The location of $^3$H at C$_1$ of aldoses has been confirmed by oxidation and re-isolation of aldonic acids (91). A similar approach could be used for localization of tritium at C$_6$ of sugars by conversion to uronic acids using selective oxidation with platinum catalysts.

The applications of the recently developed technique of $^3$H n.m.r. spectroscopy for the unambiguous localization of tritium in organic molecules have been discussed by Elridge et al. (92). Since the triton chemical shifts are effectively the same as the proton chemical shifts, a comparison of the $^3$H n.m.r. with $^1$H n.m.r. spectra obtained from the same radioactive sample could be used for locating the tritium. The proton decoupling technique may be used as a means for simplifying the spectra as well as for further confirmation of the position of the tritium. Tritium being the nucleus having the highest sensitivity to detec-
tion, monotrinitiated samples of an activity of 1-3 mCi is sufficient to produce a $^3$H n.m.r. spectrum of adequate signal to noise ratio. The non-invasive nature of this technique is another added advantage.

The platinum catalyzed selective oxidation of $C_6$-OH to COOH was chosen as the method for the location of tritium at $C_6$ in the present investigation (Fig. 15), as the experimental conditions for this reaction had already been established for the synthetic work. The high radioactivity of the $[6-^3$H]-methyl 4-deoxy-4-fluoro-$\alpha$-D-glucopyranoside permitted extensive dilution of this compound with the corresponding cold material. The syrupy product obtained after oxidation, retained only 8.7% of the original radioactivity. This activity retained was suspected to be due to a small quantity of the unreacted starting material present in the product. Hence, the product was purified by preparative T.L.C., and its radioactivity was evaluated. The specific activity of the purified oxidation product (uronic acid) was only 3.67% of the specific activity of the starting material. From this, it may be concluded that the incorporation of tritium at position(s) other than $C_6$ (possibly $C_5$) was not more than 3.67% of the overall tritium incorporation in the molecule.
Fig. 15  Selective oxidation of \([6-^3\text{H}]-4\text{-deoxy-4-fluoro}\alpha-L\text{-glucopyranoside}\) for the localization of tritium.
The Synthesis of 4-deoxy-4-fluoro-D-glucitol

4-Deoxy-4-fluoro-D-glucitol (XIX) was synthesized by sodium borohydride reduction of 4-deoxy-4-fluoro-D-glucose (Fig. 16) using the method reported for the synthesis of 3-deoxy-3-fluoro-D-glucitol (43). The syrupy product showed an additional fast moving spot on the T.L.C. The attempted purification by preparative T.L.C. using solvent F resulted in the isolation of a crystalline compound. This crystalline sample did not show fluorine in the $^{19}$F Fourier transform spectrum. The i.r. spectrum was significantly different from that of the syrupy material before the purification. From this information, it was concluded that the preparative T.L.C. procedures resulted in the chemical modification of the compound involving the removal of the fluorine. Hence another solvent system (solvent A) was used for the purification of an additional sample of the borohydride reduction product. The preparative T.L.C. using solvent A resulted in the isolation of the chromatographically pure syrup of the 4-deoxy-4-fluoro-D-glucitol. It was shown to be a non-reducing sugar by its negative reaction to the aniline phthalate spray. The i.r. and the proton n.m.r. spectral patterns were similar to those of D-sorbitol. The $^{19}$F n.m.r. parameters ($\delta$, -131.48, triplet, $J_{F,H}$ 36.6 Hz) were within the range of the values reported for deoxyfluoro-monosaccharides (86, 87). Finally the elemental analysis was consistent with the structure (XIX).
Fig. 16 Synthesis of 4-deoxy-4-fluoro-D-glucitol.
Biochemical Studies

The stereospecific binding requirements for sorbitol dehydrogenase may be investigated by the systematic comparison of the kinetic parameters of a series of deoxy- and deoxy-fluoro-analogues of D-sorbitol (D-glucitol) with those of the D-sorbitol. The present discussion is based on the preliminary studies on the commercially available sheep liver sorbitol dehydrogenase using 4-deoxy-4-fluoro-D-glucitol (4FGL).

The studies on the effect of pH on the reactivity of the enzyme show that the optimum pH for the oxidation of sorbitol is 10.0 and that for the reduction of fructose is 7.0 (95). The present studies were conducted at pH 9.0.

The \( K_m \) for sorbitol as substrate at pH 9.0 was 1.4 mM (Fig. 17). This is comparable with the previously reported value of 1.1 mM at pH 9.6 (95). 4-Deoxy 4-fluoro-D-glucitol (4FGL) served as a poor substrate with \( K_m \) 20.4 mM (Fig. 18). The \( V_{max} \) for sorbitol and 4FGL were 0.509 \( \mu \)M of NADH per min. per mg. and 0.061 \( \mu \)M of NADH per min. per mg., respectively. 4FGL was also found to be a competitive inhibitor with the \( K_i \) of 16.6 mM (Fig. 17). Romaschin et al. have reported the kinetic parameters for 3-deoxy-3-fluoro-D-glucitol (3FGL) for the sheep liver and the locust enzymes (sheep liver enzyme: \( K_m^' \) 8 mM (D-sorbitol), 70 mM (3FGL); locust enzyme: \( K_m \) 60 mM (D-sorbitol), 0.5 M (3FGL); \( K_i \) 82 mM (3FGL)) (43). These
Fig. 17 Kinetic data for sorbitol dehydrogenase illustrating the competitive nature of the 4FGL inhibition. • sorbitol as substrate, $K = 1.4 \text{ mM}$, $V_{\text{max}} = 0.509 \mu\text{M of NADH per min per mg protein}$. ▲ sorbitol in the presence of 10 mM 4FGL, $K_i$; for the 4FGL inhibition = 16.6 mM.
Fig. 17
Fig. 18  Double reciprocal plot for sorbitol dehydrogenase with 4FGL as substrate o.  \( K = 20.4 \text{mM}, \)
\( V_{\text{max}} = 0.061 \mu \text{M of NADH per min. per mg protein.} \)
Fig. 18
studies were conducted at pH 7.5, and therefore it is difficult to make a meaningful comparison of these values with those obtained for 4FGL.

The higher \( K_m \) for 4FGL as compared with sorbitol suggests the possibility that a stereospecific hydrogen bonding between the hydrogen of the hydroxyl group at \( C_4 \) of sorbitol and a receptor group on the enzyme is required for the maximum binding (Fig. 19a). The replacement of the hydroxyl group at \( C_4 \) by fluorine results in the elimination or the change in the direction of the hydrogen bond between the substrate and the enzyme (Fig. 19b). This would reduce the number of the specific binding groups available for the enzyme substrate association. The resultant decrease in the intrinsic binding energy available for the catalytic enhancement may explain the low \( V_{\text{max}} \) value for 4FGL. Similar explanations have been put forward to account for the high \( K_m \) and low \( V_{\text{max}} \) values for 3FGL (43). From these results it may be concluded that the contributions of the hydroxyl groups at \( C_3 \) and \( C_4 \) of D-sorbitol towards the enzyme substrate binding are of similar nature, possibly due to their involvement as hydrogen donors in making hydrogen bonds with the enzyme.
Fig. 19a. Stereospecific hydrogen bonding between $C_4$-OH of D-sorbitol and sorbitol dehydrogenase, b. elimination of the hydrogen bond as result of the replacement of OH by F.
P = Protein

X = An electronegative element

Fig. 19
EXPERIMENTAL

Synthetic Studies

All reagent grade chemicals were used without further purification unless otherwise stated. Anhydrous pyridine was prepared by distillation over potassium hydroxide. Anhydrous methanol was prepared by refluxing with magnesium and iodine followed by distillation. Dimethyl formamide (DMF) and acetonitrile were dried by distillation over phosphorous pentoxide.

The removal of the solvents, unless otherwise stated, was carried out under reduced pressure on a Buchi rotary evaporator, at a water bath temperature below 40°.

All melting points were determined on a Fischer-Johns apparatus and are uncorrected. Optical rotations were measured using a manual polarimeter (Rudolph and Sons Inc., N.J., USA) with sodium emission D line (λ = 589 nm) and a 0.5 dm tube. The micro-analyses reported were determined by Guelph Chemical Laboratories Ltd., Guelph, Ontario.

Chromatography

Thin layer chromatography (T.L.C.) was carried out on 20 x 20 cm plastic plates, precoated with a 0.2 mm layer of silica gel 60 F254 (B.D.H., Toronto, Ont.). The plates were
developed in the solvent specified, and the carbohydrate compounds were detected by spraying with a 50% solution of concentrated sulphuric acid in ethanol, followed by heating at 110° for 5-10 minutes. Reducing sugars were detected by using aniline hydrogen phthalate spray (93).

Preparative thin layer chromatography was done using 20 x 20 cm glass plates, precoated with 2 mm layer of silica gel GF (Fisher, N.J. USA). A concentrated solution of the mixture dissolved in a suitable solvent was applied repeatedly as a continuous thin band on the 20 cm edge of the plate, and the plates were developed in the solvent specified. The developed plates were air dried and the band corresponding to the Rf value of the compound was removed and eluted with a suitable solvent.

The following solvent systems were used in T.L.C. and preparative T.L.C. techniques:

A. Ethyl acetate: Ethanol/9:1
B. Ethyl acetate: Petroleum ether (30-65)/1:1
C. Ethyl acetate: Petroleum ether (30-65)/1:3
D. Ether: Petroleum ether (30-65)/3:2
E. Ethyl acetate: Acetic acid: Methanol/7:2:1
G. Ethanol: Water: Ammonium hydroxide/20:4:1

The column-chromatographic separations were done using silica gel H (Fisher, N.J., USA) or alumina (BDH, Toronto,
Ont.). The details of the separations are described in the appropriate experimental sections.

Spectroscopy

The infra-red (i.r) spectra were recorded over ranges of 4000 - 400 cm\(^{-1}\) using a Beckman IR-12 spectrophotometer. Intensities are coded as follows: \(w\) = weak (100-75% transmission), \(m\) = medium (74-40% transmission), and \(s\) = strong (39-0% transmission). Proton n.m.r. spectra were recorded on a JEOLCO-C.60HL n.m.r. spectrometer. The chemical shifts are expressed in parts per million (\(\delta\)) downfield from the internal standard, tetramethyl silane (T.M.S.). \(^{19}\)F n.m.r. spectra were recorded on a Bruker pulse n.m.r. spectrometer CXD100. \(^{19}\)F chemical shifts were measured relative to trifluoroacetic acid (T.F.A.) as external standard.

Radioactive Counting

Radioactivity measurements were done on Beckman LS7500 liquid scintillation system (Beckman Instruments Inc., Fullerton, CA, USA) using Ready-Solv™MP (Beckman) as a cocktail mixture. The sugars were dissolved in water before the addition of the cocktail.

Methyl (methyl-\(\alpha\)-D-glucopyranosid)uronate (IV)

Methyl \(\alpha\)-D-glucopyranoside (4g), platinum black (0.8g) and sodium bicarbonate (0.42g) in water (200 ml).
were stirred using a mechanical stirrer in a four-necked flask, while oxygen was passed through the solution. The temperature was kept between 40-50°C and the pH was maintained between 7.5 and 8.0 by periodical addition of small quantities of sodium bicarbonate. The progress of oxidation was monitored by T.L.C. The reaction was complete after 3 hours. The solution was filtered, cooled and acidified by stirring with Amberlite IR-120[H]. The resulting solution on evaporation under reduced pressure gave methyl-α-D-glucopyranosiduronic acid as a syrup.

This syrup was dissolved in methanol (120 ml). To this an ethereal solution of diazomethane (Appendix I) was added drop by drop at room temperature, until the reaction was complete as indicated by T.L.C. The solution was boiled with activated charcoal (100 mg) and filtered. The filtrate was evaporated to give a syrupy residue which was submitted to column chromatography over silica gel H (mesh 60-200, 150 g) and eluted with ethyl acetate. The eluent was evaporated under reduced pressure to give the chromatographically pure syrup (2g, 43.7%).

Rf, 0.71 (solvent F); [α]D23° + 122.0° (c, 0.52; water).

i.r (CHCl3) 3500-3300 (s, OH stretch), 1740 (s, C stretch) cm⁻¹.

1H n.m.r. (CDCl3) 6, 3.72 (broad, C-O-CH₃), 3.38 (broad, O-CH₃)
Methyl α-D-glucopyranoside (II) (79)

Methyl (methyl α-D-glucopyranosid)uronate (500 mg) in water (5 ml) was added dropwise to a stirred solution of sodium borohydride (200 mg) in water (3 ml) at room temperature, during a period of 5 minutes. After stirring for an additional 10 minutes, the excess of sodium borohydride was destroyed by stirring with Amberlite IR-120[H] and the boric acid produced was removed by repeated addition of methanol and subsequent evaporation under reduced pressure. The syrupy methyl α-D-glucopyranoside crystallized from ethanol (300 mg, 69%); Rf 0.56 (solvent F); m.p. 163-164°; [α]D23 -157.14 (c 1.12; water) were in agreement with authentic methyl α-D-glucopyranoside.

Methyl α-D-galactopyranoside (VI) (94)

Reagent grade anhydrous D-galactose (200 g) was boiled vigorously with a solution of hydrogen chloride in anhydrous methanol (2% w/v, 1600 ml) until it dissolved, then gently for 7 hours. The cooled solution was neutralized by shaking with lead carbonate (220 g) for 3 hours. Lead salts were removed with the aid of Kieselguhr and washed with methanol (200 ml) and the total filtrate was evaporated to an amber syrup (240 g). The warm syrup was mixed with water (60 ml) resulting in the rapid deposition of the coarse crystals of methyl α-D-galactopyranoside monohydrate.
The crystallization of the title compound was complete after 24 hours at room temperature. The syrupy mother liquor was removed from the crystals by suction and the solid was washed by carefully stirring it under gentle suction, with a small portion of 80% ethanol (chilled to 5°C; total 100 ml), followed by absolute ethanol (2 x 32 ml). The air dried solid (72 g, yield 33.8%) was used for the next reaction without further purification; m.p. 90-97°C [Lit (84) 96-99°C]; RF 0.5 (solvent F).

Methyl 2,3,6-tri-O-benzoyl α-D-galactopyranoside (VII) (83).

A magnetically stirred solution of methyl α-D-galactopyranoside monohydrate (90 g) in anhydrous pyridine (1500 ml) was cooled by means of a solid carbon dioxide-acetone bath at -40°C. Benzoyl chloride (204 ml, 4.2 molar ratio) was added dropwise (30-60 min.) with exclusion of moisture. The bath temperature was kept between -30 to -40°C for 3 hours and then 4°C for 20 hours. The reaction mixture was stirred for two more days at room temperature. The pyridine was removed at reduced pressure below 50°C and the residue was dissolved in chloroform (1000 ml). The chloroform solution was divided into two equal portions and each portion was washed successively with 2N hydrochloric acid (until free from pyridine), a saturated solution of sodium bicarbonate (400 ml) and finally with water (400 ml), and was dried using anhydrous magnesium sulphate. The removal of the
solvent gave a syrupy mixture which crystallized on standing overnight at room temperature. Recrystallization from 90% aqueous ethanol (139 g, 59.2%) as a colourless solid; m.p. 138-140° [Lit (83) 139-140°] Rf 0.32 (solvent C).

Methyl 2,3,6-tri-O-benzoyl-4-O-methylsulphonyl-α-D-galactopyranoside (VIII) (83)

To a solution of methyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside (90 g) in dry pyridine (360 ml) was added methyl sulphonyl chloride (45 ml). The reaction was stirred at room temperature for 24 hours while protected from moisture. Then the reaction mixture was divided into two equal portions. Each portion was slowly poured into 4 litres of ice cold water with stirring, and then allowed to stand. The precipitate formed was filtered and washed repeatedly with water until free from pyridine. The crude solid (105 g) was recrystallized from absolute alcohol (81 g, 77.98%); m.p. 140-142° [Lit (83) 141-142°], Rf 0.84 (solvent B).

Methyl 4-O-methylsulphonyl-α-D-galactopyranoside (IX) (83)

A suspension of methyl 2,3,6-tri-O-benzoyl-4-O-methylsulphonyl-α-D-galactopyranoside (86 g) in anhydrous methanol (560 ml) was cooled to 4°C and a solution of sodium methoxide (2.8 g in 47 ml of anhydrous methanol)
was added. The reaction was stirred for 24 hours at 4°, then neutralized with Amberlite IR-120 (H) (120 ml), and evaporated to dryness \textit{in vacuo}. The residue was partitioned between 300 ml each of chloroform and water. The water layer was washed with 100 ml of chloroform and treated with decolourizing charcoal, and evaporated \textit{in vacuo} to give a solid residue. Recrystallization from absolute alcohol gave 34.2 g (85.38\%) of the product; m.p. 160-162° [Lit (83) 159-160°]; R<sub>p</sub>, 0.75 (solvent F).

\textbf{Methyl 2,3,6-tri-O-benzyl-4-O-methylsulphonyl-α-D-galactopyranoside (X) (53)}

To a solution of methyl-4-O-methylsulphonyl-α-D-galactopyranoside (34 g) in anhydrous dimethyl formamide (295 ml) was added silver oxide (88.4 g) and benzyl bromide (81.6 ml). The reaction mixture was protected from moisture and stirred magnetically for 3 days at room temperature. Then it was filtered and the residue was washed with chloroform (3 x 50 ml).\textsuperscript{9} The filtrate was diluted with chloroform (500 ml) and filtered. The filtrate was divided into two equal portions. Each portion was washed with water (3 x 200 ml). Pyridine (30 ml) was added to each chloroform portion and the solution was washed successively with water (2 x 250 ml), 2N hydrochloric acid (3 x 100 ml) and finally with water (2 x 200 ml). The chloroform extract was dried (Mg SO<sub>4</sub>) and evaporated
to dryness in vacuo. The resulting residue was divided into two parts and column chromatographed over 600 g of alumina (Brockman, activity II). Elution with 5:1 (v/v) petroleum ether (b.p. 30-65°)-ether removed dibenzyl ether. Elution with ether yielded the title compound (47 g, 69.37%) as a colourless syrup. \( R_f \), 0.43 (solvent D).

**Methyl 2,3,6-tri-O-benzyl-4-deoxy-4-fluoro-\( \alpha \)-D-glucopyranoside (XI) (53)**

Tetrabutyl ammonium fluoride was prepared by titration of 40% aqueous tetrabutylammonium hydroxide (68 ml) with 50% aqueous hydrofluoric acid to pH 7.0. The solution was concentrated under reduced pressure and the resulting syrup was dried and stored overnight over phosphorus pentoxide at 0.1 mm.

A mixture of methyl 2,3,6-tri-O-benzyl-4-O-methyl-sulphonyl-\( \alpha \)-D-galactopyranoside (8.5 g), tetrabutyl ammonium fluoride (freshly prepared) and anhydrous acetonitrile was kept under gentle reflux at 70-80°. After 3 days the reaction appeared to be complete and the mixture was poured into water (55 ml) and was extracted with ether (3 x 160 ml). The ethereal layer was dried (MgSO\(_4\)) and concentrated under diminished pressure. The syrupy residue was submitted to column chromatography on silica
gel (grade H, mesh size 60-200, 240 g). Elution with
7:3 (v/v) ether-petroleum ether (b.p. 30-65°) yielded
the title compound (6.5 g 88.9%) as a colourless syrup.
R_f 0.65 (solvent D)

Methyl-4-deoxy-4-fluoro-α-D-glucopyranoside (I) (53)

A solution of methyl 2,3,6-tri-O-benzyl-4-deoxy-
4-fluoro-α-D-glucopyranoside (1.4 g) in ethanol (250 ml)
was shaken in the presence of hydrogen and palladised
charcoal (15 g, 5%) at room temperature until hydrogen
uptake was ceased (24 hours). The reaction mixture was
filtered and concentrated under reduced pressure. The
residue was dissolved in a minimum amount of methyl
alcohol and placed on a column of silica gel (grade H,
mesh size 60-200, 300 g) and eluted with ethyl acetate.
After the removal of the solvent under reduced pressure,
the residue crystallized. Recrystallization from ethyl
acetate-acetone (1:1) gave the title compound as a colour-
less compound (3.2 g, 58.5%). R_f 0.74 (solvent F) m.p.
128-130° [Lit (53) 129-130°]; [α]_D^{23} + 134.43° (c 0.302;
water) [Lit. (53) + 132°].

Methyl (methyl 4-deoxy-4-fluoro-α-D-glucopyranosid)-
uronate (XIV)

Methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (2 g),
platinum black (0.4 g) and sodium bicarbonate (0.21 g) in
water (100 ml) were stirred using a mechanical stirrer in a four necked flask, while oxygen was passed through the solution. The temperature was kept between 40° and 50°. The pH was maintained mild alkaline (7.5 - 8.0) by addition of small quantities of sodium bicarbonate. The progress of oxidation was monitored by T.L.C. The reaction appeared to be complete in 4 hours. The solution was filtered, cooled and acidified by stirring with Amberlite IR-120(H). The resulting solution was evaporated under reduced pressure to give methyl 4-deoxy-4-fluoro-α-D-glucopyranosiduronic acid (XIII).

This syrup was dissolved in methanol (100 ml). To this an ethereal solution of diazomethane (Appendix I) was added drop by drop at room temperature, until the reaction was complete as indicated by T.L.C. The solution was boiled with activated charcoal (~100 mg) and filtered. The filtrate was evaporated to give a syrupy residue which was submitted to column chromatography over silica gel H (mesh 60-200, 300 g) and eluted with ethyl acetate: petroleum ether (35-60) 3:1. The solvent was evaporated under reduced pressure and the chromatographically pure syrupy residue (1.5 g, 65.63%) was dried over P₂O₅ in a vacuum desiccator; Rₚ 0.84 (solvent F); [α]D²³ + 93.83° (c,0.91; water).
i.r. (CHCl₃) 3600-3380 (w, OH stretch), 1760 (s, C stretch cm⁻¹).

¹H n.m.r (CDCl₃), δ, 3.7 (broad, COOCH₃) 3.35 (broad, OCH₃).

¹⁹F n.m.r (CH₃OH) δ, -119.2 (quartet; J₉,F,₃ 54.5 J₉,F,₄ 14.7 Hz).

Anal. calc. for C₆H₁₃O₆F: C, 42.86; H, 5.80; F, 8.48; found: C, 42.85; H, 5.69; F, 6.80.

Methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (I)

Methyl (methyl-4-deoxy-4-fluoro-α-D-glucopyranoside)-
uronate (400 mg) in water (4 ml) was added to a solution of
sodium borohydride (110 mg) in water (1 ml) with stirring at
4°. The reaction was noted to be complete after 1 hour. The
excess sodium borohydride was destroyed by stirring with Ambe-
lite IR-120 (H) and the boric acid produced was removed by re-
peated addition of methanol and subsequent evaporation under
reduced pressure. The syrupy residue was purified by prepara-
tive T.L.C. (solvent E). The chromatographically pure title
compound obtained by elution with ethyl acetate, was crystal-
lized from ethyl acetate: aceton (1:1); m.p. 127-129° [Lit (53)
128-130]; Rₚ, 0.74 (solvent F) [α]D ²³ +129.17° (c, 0.24; water)
were the same as that of the authentic sample.

4-Deoxy-4-fluoro-D-glucose (XII) (53)

A solution of methyl 4-deoxy-4-fluoro-α-D-glucopyra-
noside (II) [180 mg, obtained by sodium borohydride reduction of (XIV) in 2M sulphuric acid (20 ml), was refluxed gently. After 4 hours, the reaction was noticed to be complete. After neutralization with solid barium carbonate, the reaction mixture was filtered and concentrated to dryness under reduced pressure. The residue was taken up in absolute ethanol and the solution was filtered through a bed of kieselguhr and evaporated to dryness and the syrupy residue was crystallized from ethanol (100 mg, 59.82%); m.p. 188-190° [Lit (53) 189-190°]; mixed m.p. with the authentic sample, 187-190°; Rf 0.66 (solvent F), [α]23D + 24.63° 10 min. → + 49.26° (c, 0.406; water) [Lit (53, + 26.5° 10 min. → + 50°]. The i.r. spectrum of the compound was identical to the authentic sample.

[6-3H]-Methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (XV)

Methyl (methyl, 4-deoxy-4-fluoro-α-D-glucopyranosid)uronate (1 g) in water (10 ml) was added drop by drop to a solution of [3H]-sodium borohydride (13 mg, 100 mCi, specific activity 228 mCi/m mole in water (1 ml) with stirring at 4°. After 10 minutes an additional quantity of sodium borohydride (240 mg) was added and stirring was continued for 1 hour. The excess borohydride was destroyed by stirring with Amberlite IR-120(H) (20 ml) and the boric acid produced was removed as methyl borate by the repeated
addition of methanol and subsequent evaporation under reduced pressure. The syrupy residue was purified by preparative T.L.C. Elution with ethyl acetate gave the title product which was crystallised from ethyl acetate: acetone mixture (1:1) (440 mg, specific activity 10.54 mCi/mmole total activity 23.67 mCi, radiochemical yield 23.67%).

\[ {\text{[6-}}^{3}\text{H]}-4\text{-deoxy-4-fluoro-D-glucose (XVI)} \]

A solution of \([6-^{3}\text{H}]-\text{methyl 4-deoxy-4-fluoro-}\alpha-D-\text{glucopyranoside (200 mg) in 2M sulphuric acid (20 ml) was gently refluxed for 4 hours. After neutralization with solid barium carbonate the reaction mixture was filtered and concentrated to dryness under reduced pressure. The residue was taken up in absolute ethanol and the solution was filtered and evaporated to dryness. The syrupy residue crystallized from ethanol (100 mg, 52.75% specific activity 10.88 mCi/mmole. The total radiochemical yield from (XIV) to (XVI) was 12.5%.

Oxidation of \([6-^{3}\text{H}]-\text{methyl 4-deoxy-4-fluoro-}\alpha-D-\text{glucopyranoside (XV) to methyl 4-deoxy-4-fluoro-
\alpha-D-glucopyranosiduronic acid (XIII)} \]

\[ {\text{[5-}}^{3}\text{H]}-\text{Methyl 4-deoxy-4-fluoro-}\alpha-D-\text{glucopyranoside (14 mg) and methyl 4-deoxy-4-fluoro-}\alpha-D-\text{glucopyranoside (96 mg) were dissolved in water (5 ml) and the specific activity of the mixture was determined (1.38 mCi/mmole}} \]
To this, sodium bicarbonate (10 mg) and platinum black (40 mg) were added. The mixture was stirred with a magnetic stirrer, while oxygen was passed through the solution. The temperature was kept between 40-50° and pH was maintained mildly alkaline (7.5 - 8.0) by addition of small quantities of sodium bicarbonate. The reaction was complete after 4 hours. The solution was cooled, filtered and water was evaporated. Addition of water and evaporation was repeated till no more tritium loss to the distillate could be detected. The total radioactivity of the sample was estimated to be 8.67% of the starting material.

The syrup oxidation product was purified by preparative T.L.C. (solvent E). Methyl 4-deoxy 4-fluoro-\(\alpha\)-D-glucopyranosiduronic acid was isolated and the specific activity was determined (0.0507 mCi/mmole). The radioactivity retained, calculated as the specific activity was 3.67% of the starting material.

4-deoxy-4-fluoro-D-glucitol (XIX)

To a solution of 4-deoxy-4-fluoro-D-glucose (400 mg) in water (20 ml) kept at 4°, was added a solution of sodium borohydride (200 mg) in water (10 ml) with stirring. After stirring for 3 hours, the excess borohydride was destroyed by stirring with Amberlite IR-120 (H). The solution was filtered and the filtrate evaporated in vacuo to a syrup which was repeatedly dissolved in methanol
and evaporated to dryness *in vacuo* to remove the boric acid as its methyl ester. The syrup which failed to crystallize was purified by preparative T.L.C. using solvent A. The chromatographically pure syrup failed to crystallize. The compound did not give a positive reaction to aniline phthalate spray.

$R_f$, 0.58 (solvent G); $[\alpha]_D^{23}$ -3.48° (c, 2.64; methanol)

I.r. (neat) 3440-3360 (s, -OH stretch) 2980, 2920 (m, C-H stretch) 1100 (s, C-F stretch) 1050 (s, C=O stretch) cm$^{-1}$

$^1$H n.m.r. (CD$_3$OD) δ, 4.8 [broad, 4H(C$_2$-H, C$_3$-H, C$_4$-H, C$_5$-H)], 4.2-3.7 [complex, 4H (2C$_1$-H and 2C$_6$-H)].

$^{19}$F n.m.r. (CH$_3$OH) δ, -131.48 (triplet; $J_{F,H}$, 36.6 Hz).

Anal. calc. for C$_6$H$_{13}$FO$_5$ · H$_2$O: C, 35.64; H, 8.01; F, 9.40. Found: C, 35.69; H, 7.99; F, 9.51.
Biochemical Studies

Reagents: All the reagents used were of analytical grade or of the highest purity commercially available. Mercaptoethanol, NAD⁺, sorbitol dehydrogenase (L-iditol: NAD⁺ 5-oxidoreductase; sheep liver, EC 1.1.1.14), were obtained from Sigma (St. Louis, Missouri, U.S.A.). D-sorbitol was from BDH (Toronto, Ontario) and glycine from BDH (Poole, U.K.). All the aqueous solutions were prepared using singly distilled deionised water.

Equipment: The pH measurements were performed on an Accumet model 120 pH meter (Fischer; N.J., U.S.A.). All spectrophotometric measurements were done on a Beckman Acta MVI UV-VIS recording spectrophotometer.

Kinetic studies: The kinetic studies were done at room temperature using 1.5 ml matched quartz cuvettes in the presence of NAD⁺ (0.001 molar), mercaptoethanol (0.0005 molar) and glycine NaOH buffer (0.05 molar pH, 9.0) with a final volume of 1.0 ml. The enzyme concentration used was 0.1 mg protein per ml. The reaction velocity was measured by observing the change in absorbance at 340 nm, and is expressed as micromoles of NADH per min. per mg. protein. The kinetic parameters $K_m$, $V_{max}$ and $K_i$ were obtained using Lineweaver-Burk double reciprocal plots (96). The best linear fit was determined by the method of least squares.
APPENDIX I

The Preparation of Diazomethane (97)

A 100 ml distilling flask was charged with a solution of potassium hydroxide (6.6 g) in water 10.6 ml) and ethanol (95%, 34 ml) and connected to a double surface condenser delivering into two receiving flasks in series, both cooled in ice. The second receiver contained ether (30 ml) and the inlet tube was kept dipping below the surface of the solvent. The generating flask was heated to 65° and a solution of p-toluenesulphonylmethylnitrosamide (Diazald) (28.8 g) in ether (260 ml) was added dropwise from a dropping funnel in about 25 minutes. The rate of the distillation was approximately equal to the rate of addition of the Diazald solution. When the dropping funnel was empty, an additional quantity of ether (30 ml) was added slowly and the distillation was continued until the distilling ether was colourless. The combined distillate was expected to contain 4 g of diazomethane.
APPENDIX II

Infra-red (i.r) spectrum of methyl (methyl α-D-glucopyranosid)uronate (IV)(CHCl₃):

3500-3300 (m, -OH [hydrogen bonded] stretch), 1740 (s, C stretch), 1060 (s, C-O stretch) cm⁻¹
APPENDIX III

Proton nuclear magnetic resonance (\(^1\)H n.m.r) spectrum of methyl (methyl \(\alpha\)-D-glucopyranosid)uronate (IV) (CDCl\(\text{3}\), TMS).

p.p.m (\(\delta\)), 3.72 (broad, C-O-CH\(_3\)), 3.38 (broad, O-CH\(_3\)).
APPENDIX IV

Infra-red (i.r) spectrum of methyl (methyl 4-deoxy-4-fluoro-α-D-glucopyranosid)uronate (XIV) (CHCl₃):
3600 (w, OH [free] stretch), 3440-8380 (w, OH [hydrogen bonded] stretch), 1760 (s, C stretch), 1080 (s, C-F stretch), 1060 (s, C-O stretch) cm⁻¹.
APPENDIX V

Proton nuclear magnetic resonance (\(^1\)H n.m.r.) spectrum of methyl (methyl 4-deoxy-4-fluoro-\(\alpha\)-D-glucopyranosid) uronate (XIV) (\(\text{CDCl}_3, \text{TMS}\)):

\[ \delta \text{ p.p.m.} \] 3.7 (broad, C-\(\text{O-CH}_3\)), 3.35 (broad, -\(\text{OCH}_3\)).
APPENDIX VI

$^{19}$F Fourier transform nuclear magnetic resonance spectrum ($^{19}$F n.m.r) of methyl (methyl-4-deoxy-4-fluoro-$\alpha$-D-glucopyranosid)uronate (XIV) (CH$_3$OH).

A = trifluoroacetic acid
B and C = (XIV)

Conditions used for the scans:

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<th>C</th>
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ppm (δ) (relative to TFA), -119.2 (quartet, $J_{F,H_4} = 54.5$ Hz, $J_{F,H_3} = 14.7$ Hz).
APPENDIX VII

Infra-red (i.r) spectrum of 4-deoxy-4-fluoro-α-D-glucopyranoside (XII) prepared by the route for radio-chemical synthesis (KBr):
3440–3280 (s, OH [hydrogen bonded] stretch), 2980, 2940 (m, C-H stretch), 1040 (s, C-F stretch), 1020 (s, C-O stretch).
APPENDIX VIII

Infra-red (i.r) spectrum of 4-deoxy-4-fluoro-D-glucose (XII) prepared by the established route for its synthesis (53) (K Br):

3440-3280 (s, OH [hydrogen bonded] stretch), 2980, 2940 (m, C-H stretch), 1040 (s, C-F stretch), 1020 (s, C-O stretch) cm$^{-1}$.
APPENDIX IX

Quench curve for the Beckman LS 7500 liquid scintillation system generated by plotting the counting efficiencies of \(^3\text{H}\) against H.\# values.
APPENDIX X

Estimation of Specific Activity
SAMPLE CALCULATION

Sample: \([6-^3\text{H}]-\text{deoxy-4-fluoro-D-glucose}\)

\[
\text{Mol. Wt.} = 182
\]

\[
\text{Quantity} = 0.0117 \text{ mc} = 6.4286 \times 10^{-5} \text{ mmole}
\]

\[
\text{cpm} = 527800
\]

\[
\text{H #} = 81
\]

Efficiency \% [estimated by the use of the quench curve (Appendix IX)] = 34

\[
\text{dpm} = \frac{\text{cpm} \times 100}{\text{Efficiency \%}} = \frac{527800 \times 100}{34} = 1552353
\]

Radioactivity = \[
\frac{\text{dpm}}{2.22 \times 10^9} \text{ mCi} = \frac{1552353}{2.22 \times 10^9} = 6.9926 \times 10^{-4}
\]

Specific activity = \[
\frac{\text{Radioactivity (mCi)}}{\text{Quantity of the sample (mmole).}} = \frac{6.9926 \times 10^{-4} \text{ mCi}}{6.4286 \times 10^{-5} \text{ mmole}} = 10.877 \text{ mCi/mmole}
\]
APPENDIX XI

Infra red spectrum of 4-deoxy-4-fluoro-D-glucitol (XIX) (Neat):

3420-3340 (s, OH [hydrogen bonded] stretch), 2960, 2900 (m, C-H stretch), 1090 (s, C-F stretch), 1040 (s, C-O stretch) cm$^{-1}$. 
APPENDIX XII

Proton nuclear magnetic resonance ($^1$H n.m.r) spectrum of 4-deoxy-4-fluoro-D-glucitol (XIX) (CD$_3$OD, TMS):

ppm (δ), 4.8 (broad, 4H [C$_2$-H, C$_3$-H, C$_4$-H, C$_5$-H]), 4.2 - 3.7 (complex, 4H, [2C$_1$-H, 2C$_6$-H]).
APPENDIX XIII

\(^{19}\)F Fourier transform nuclear magnetic resonance (\(^{19}\)F n.m.r.) spectrum of 4-deoxy-4 fluoro-D-glucitol (XIX) (CH\(_3\)OH).

A = trifluoroacetic acid (TFA)

B and C = (XIX)

Conditions used for the scans

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ppm (\(\delta\)) (relative to TFA), -131.48 (triplet \(J_{F,H} = 36.6\) Hz).
REFERENCES


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