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The bacterial metabolism of 3-deoxy-3-flouro-D-glucose.

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THE BACTERIAL METABOLISM OF 3-DEOXY-3-FLUORO-D-GLUCOSE

submitted by F.H.White, B.Sc.

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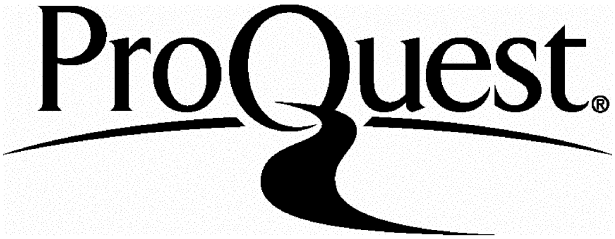

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CONTENTS

ABSTRACT

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The introduction surveys the role of organic fluorine compounds in biology, and it is shown that the disruption of cellular metabolism by these compounds may be due not only to their action as specific enzyme inhibitors but also to their ability to become incorporated into the macromolecules of the cell. The usefulness of covalently-bonded fluorine in the design of analogues is shown to be related to its size, electronegativity, and the stability of the carbon-fluorine bond. The physical similarities between covalently-bonded fluorine and the hydroxyl group are argued to provide a rationale for the synthesis and biochemical investigation of deoxyfluoro sugars. A review of the effects and uses of such compounds in biology is included.

The object of the present investigation has been to examine some of the biological effects of 3-deoxy-3-fluoro-D-glucose (3FG), and for this purpose three saprophytic soil bacteria were chosen. Results may be summarised thus:

1. Neither whole cells nor cell-free extracts of E.coli metabolise 3-deoxy-3-fluoro-D-glucose. Simultaneous glucose oxidation by whole cells is not inhibited by the fluoro analogue, but an elevated extent of glucose oxidation subsequent to treatment with 3-deoxy-3-fluoro-D-glucose is found. Possible explanations for this are discussed. Preincubation with 3-deoxy-3-fluoro-D-glucose inhibits the subsequent growth of E.coli on glucose.

2(a). The effect of 3-deoxy-3-fluoro-D-glucose on glucose-grown Ps.fluorescens at two different buffer concentrations has been examined. In 0.067M buffer starting concentrations of analogue varying from 5-30 mM are oxidised with the consumption of one oxygen atom per mole of substrate and concomitant acid production. At this buffer molarity with substrate

concentrations in excess of 30 mM the acidic oxidation product sufficiently lowers the buffer pH as to completely inhibit further cellular oxidations and to cause cell lysis and death. In 0.67M buffer all concentrations of 3-deoxy-3-fluoro-D-glucose examined are oxidised with the consumption of one oxygen atom per mole of substrate, and without any inhibition of subsequent cellular oxidations. An explanation is proposed for the increased release of E_{260}- absorbing material during oxidation of the analogue at this buffer molarity, which occurs without a corresponding drop in cell viability.

(b) Cell-free extracts of Ps.fluorescens oxidise 3-deoxy-3-fluoro-D-glucose with the consumption of one mole of oxygen per mole of substrate and the reduction of cytochrome pigments. Explanations for the limitation of whole cell oxidation of 3-deoxy-3-fluoro-D-glucose in the light of the extent of oxidation by cell-free extracts are discussed.

(c) Chromatographic and manometric evidence favours 3-deoxy-3-fluoro-D-gluconic acid (3FGA) as the product of 3-deoxy-3-fluoro-D-glucose oxidation.

(d) Whole cells and extracts of glucose-grown Ps.fluorescens oxidise 3-deoxy-3-fluoro-D-gluconate with the consumption of one atom of oxygen per mole of substrate, and the formation of a reducing compound tentatively considered to be 3-deoxy-3-fluoro-2-keto-D-gluconic acid (3F2KGA).

(e) Though 3-deoxy-3-fluoro-D-gluconate is not oxidised by asparagine-grown cell suspensions of Ps.fluorescens, it inhibits the formation of the gluconate-oxidising system in these cells.

(f) 3-Deoxy-3-fluoro-D-glucose does not itself support the growth of Ps.fluorescens and inhibits the growth of this organism on glucose.

3. Whole cell suspensions of Ps.saccharophila do not oxidise 3-deoxy-3-fluoro-D-glucose nor is glucose metabolism outwardly affected by the analogue.

The differing oxidative abilities of the three organisms towards 3-deoxy-3-fluoro-D-glucose, outlined above, is discussed with reference to their differing enzymic complements.

The appendices review synthetic routes to deoxyfluoro sugars and detail the synthesis of 3-deoxy-3-fluoro-D-gluconic acid.

INTRODUCTION

INTRODUCTION

The biological activity of fluorine is largely determined by whether the fluorine is ionically or covalently bonded. Ionic fluorine (F^-) produces an entirely different biological response to that of covalently bound fluorine - the fluoridation of water supplies as a means of preventing dental caries is a controversial topic. There is also a biologically important group of organic fluorine compounds in which the fluorine is bonded to phosphorus, (dialkyl phosphofluoridates[1]), but these are outside the scope of the present work, which is concerned with fluorine covalently bonded to carbon (C-F), and the biological activity of compounds containing this group.

Fluorine is in group VII of the periodic table, but this fact should be considered in light of Pauling's reference to it as a 'superhalogen'[2]. Table 1 shows that the properties of the other halogens place them in a group distinct from fluorine:

TABLE 1

Some properties of the halogens[2]

Halogen	Van der Waals' radius (A)	Electro-negativity	Bond Energy	
			To Carbon	To Hydrogen
F	1.35	4.0	107	147.5
Cl	1.80	3.0	66.5	102.7
Br	1.95	2.8	54.0	87.3
I	2.15	2.4	45.5	71.4

Fluorine is much more electronegative than the other halogens and, therefore, is the only halogen unlikely to form a cation[3]. The bond energy of the carbon-fluorine bond is among the highest found in natural products and known to be broken enzymatically. The Van der Waals' radius of the fluorine atom (1.35 angstroms) is closest in size to that of hydrogen (1.2 angstroms). Most of the other substituent groups used to replace hydrogen in analogues are much larger (see Table 1 for sizes of the other halogens; methyl, trifluoromethyl and phenyl groups are also large). Thus fluorine is of unique value in the design of analogues which can very closely approach the natural biochemical intermediate. Good analogues of this type can be used therapeutically, but they can also be very valuable in defining critical sizes that contribute to structural considerations in enzyme-substrate interactions.

These properties of fluorine are of most interest to the biologist, since a knowledge of the size and electronegativity of the atom can be used to make defined alterations in biologically important molecules. Studies based on these properties of fluorine are governed often by the characteristics of the C-F bond which determine the reactivity of such molecules in biological systems.

Interest in the biological properties of organic fluorine was first stimulated by the work of Marais[4], who isolated the active principle of the highly toxic South African shrub Dichapetalum cymosum, and identified it as monofluoroacetic acid. This discovery not only explained the toxicity of these plants to grazing cattle, but initiated a period of research on the actions of fluoroacetate[5] which has had many ramifications

in the development of biochemical pharmacology.

Studies in the laboratories of Peters and Martius made it clear that fluoroacetate toxicity in mammalian tissues was related to an inhibition of citrate metabolism, the inhibition being caused by a factor distinct from fluoroacetate which could be isolated after tissues had been incubated with fluoroacetate. This inhibitory factor proved to be fluorocitrate[6], which acts as an inhibitor of the T.C.A. cycle. Fluorocitrate is synthesised from fluoroacetate by a pathway analogous to that of the synthesis of citrate from acetate (Fig.1). Fluoroacetate itself, was shown, using 'in vitro' studies, to be without significant effect upon a large number of enzyme-catalysed reactions. Only in systems containing both the T.C.A. cycle enzymes, and intermediates, and the necessary coenzyme A, to activate the fluoroacetate, did its lethal effect become apparent. Peters demonstrated, that in such systems, fluoroacetate was 'mistaken' for acetate by the cell.

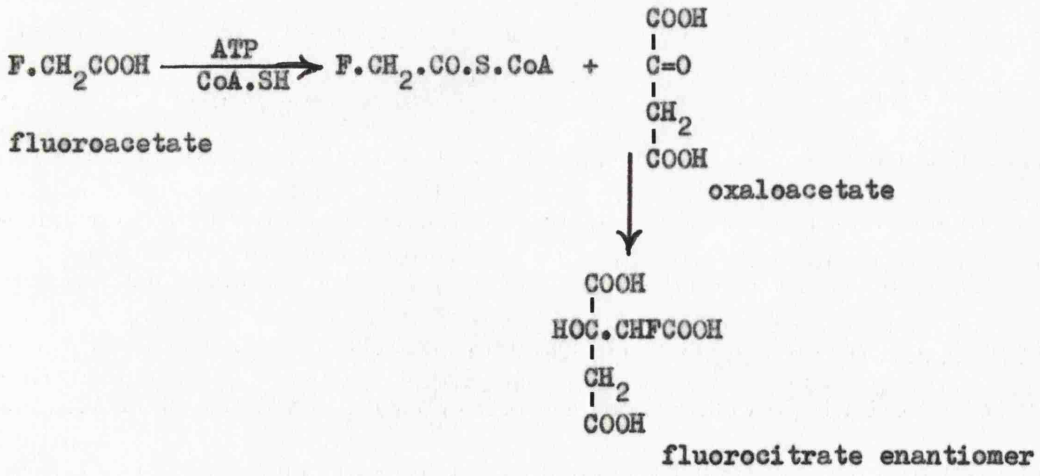


Fig.1. Pathway for the synthesis of fluorocitrate from fluoroacetate

Fluorocitrate competes with normal citrate for the active sites of the enzyme aconitase, which catalyses the next step in the T.C.A. cycle - the conversion of citrate to isocitrate through cis-aconitate. One of the four isomers of fluorocitrate appears to have very much greater affinity for this enzyme than citrate, with the result that only a very small amount of fluorocitrate is required to block the enzyme. Once formed, the enzyme-fluorocitrate complex is stable and does not break down, so that the T.C.A. cycle is blocked at this point, leading to the accumulation of citrate, which is characteristic of fluoroacetate poisoning. This was the original definitive example of what Peters has called 'lethal synthesis' ie. a process by which a foreign molecule, harmless in itself, is converted by a series of reactions within the cell to a toxic intermediate. Such a mode of action has been found in a number of pharmacologically active agents, and is of fundamental importance in the design of new therapeutic agents.

The role of fluorocitrate as an inhibitor of the T.C.A. cycle is complicated, as indicated by Fanshier et al[7]. These workers have extended the evidence that only one of the four isomers of fluorocitrate made by organic synthesis is inhibitory; as expected, this corresponds to the isomer made enzymatically by the pathway of Fig.1. Furthermore, they have shown that succinic dehydrogenase as well as aconitase is inhibited by fluorocitrate. Aconitase seems to undergo competitive inhibition only if initial rates are examined, since there is also progressive time-dependent inactivation of the enzyme by fluorocitrate[7].

Recently Kirk and Goldman[7a] have demonstrated the selective degradation, by a species of Pseudomonas, of one of the enantiomers of DL-erythro-fluorocitrate. The degradation of this enantiomer was followed by the release of fluoride and, although the nature of the product

has not yet been established, it has been demonstrated by these workers that the enantiomorph of fluorocitrate degraded is the active inhibitor of aconitase. This work will allow, therefore, the absolute assignment of configuration to the fluorocitrate stereoisomer responsible for the inhibition of aconitase, since the mirror image, which is inactive to aconitase and is not degraded by the pseudomonad, remains in the culture medium and can be isolated in sufficient quantities for physico-chemical studies.

The complex interaction of fluorocitrate with aconitase may indicate an additional value of fluoroanalogues, ie. in the elucidation of the detailed relation between proteins and small molecules. Such a possibility is suggested by the finding that only one of the four isomers of fluorocitrate seems to react with the enzyme; data on the properties of these isomers might provide exact knowledge of the features of citrate which are required for the interaction, and, by inference, some notion of the configuration of the protein at the reactive site.

In contrast to the studies with aconitase are studies on the allosteric activation of acetyl coenzyme A (acetyl CoA) carboxylase by citrate. In this system, fluorocitrate (containing all four isomers) is as effective as citrate[8]; this indicates that different structural aspects of citrate are responsible for its interaction with this protein.

The occurrence of ω-fluoropalmitate and ω-fluorooleate in the seeds of Dichapetalum toxicarium[9] suggests that fluoroacetate can substitute for acetate, at least partially, in the biosynthesis of long-chain fatty acids. The fact that fluorine occupies the ω-position of the fatty acid is in accord with the scheme for fatty acid biosynthesis which has now been

demonstrated in animals, plants and microorganisms[10]. In all of these systems, acetyl-CoA is the 2-carbon unit responsible for elongation of the fatty molecule. Yet in the completed straight-chain fatty acid molecule, only the ω -carbon and the adjacent carbon atom are derived directly from acetyl-CoA; the other acetyl-CoA molecules are carboxylated to form malonyl-CoA and are then decarboxylated in a condensation reaction. Figure 2, shows the initial cycle of condensation and reduction in the 2-carbon elongation of a fatty acid molecule and illustrates the dual role of acetyl-CoA in this process. Acetyl-CoA, which proceeds through malonyl-CoA (shown with the hypothetical X substituent), undergoes reactions not required of acetyl-CoA entering the methyl terminal position (shown as fluoroacetyl-CoA). Failure of fluoroacetyl-CoA to proceed through 'fluoromalonyl-CoA' would account for the lack of incorporation of fluorine except at the ω -position of the fatty acid product. The findings of fluorine exclusively at the ω-position may also occur because a substituent at the 2-position in the condensed product (Fig.2) interferes with the reductive steps required after the condensation reaction. Furthermore, since the synthesis of long-chain fatty acids is a repetitive process, partial inhibition at any step would be expected to have a large effect on the overall synthesis.

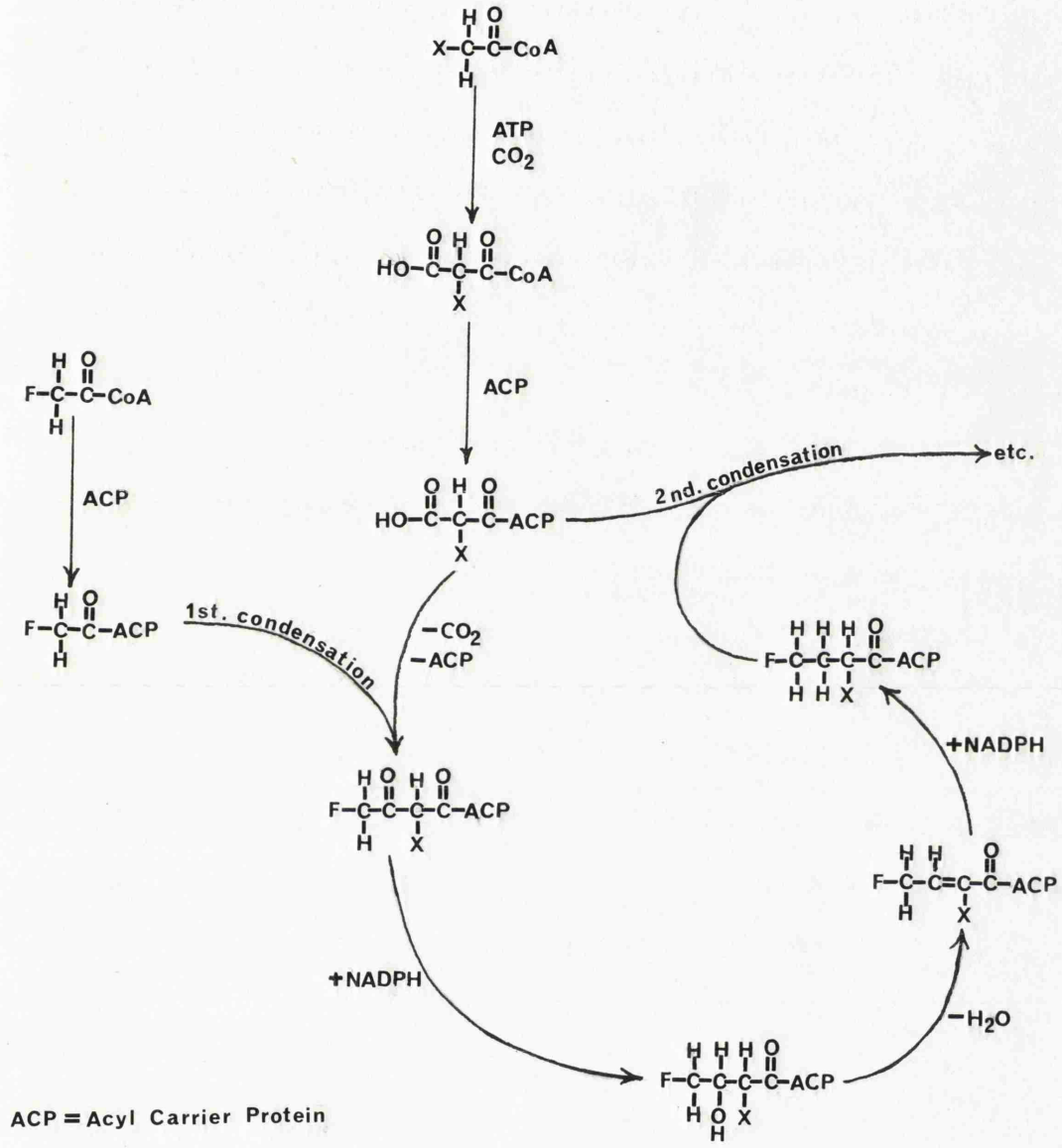


Fig.2. Cycle in long-chain fatty acid biosynthesis showing a possible role for fluoroacetate

The acceptability of fluoroacetyl-CoA in the initial condensation reaction is in accord with results of studies on fatty acid synthesis in an enzyme system obtained from adipose tissue. In this enzyme system a number of acyl-CoA compounds can substitute for acetyl-CoA in the initiation of fatty acid synthesis[11]. Such a substitution for acetyl-CoA yields a product which differs from the natural one simply by a change in the acyl group substituted for acetate at the ω-position. In other words, the subsequent reactions with malonyl-CoA seem not to be greatly affected by the acyl group initiating the condensation. Considerations of this kind might explain the finding of ω-fluoro fatty acids in the seeds of Dichapetalum toxicarium, and the apparent small incorporation of fluoroacetate into the lipids of rats fed sublethal doses of this compound[12].

Saunders, Pattison and their coworkers have shown that ω-fluoro fatty acids containing an even number of carbon atoms have a toxicity to rats comparable to that of fluoroacetate, whereas ω-fluoro acids with an odd number of carbon atoms are strikingly less toxic[13]. Such findings are in accord with the β-oxidation theory of fatty acid catabolism, proposed by Knoop[14], in which the successive loss of 2-carbon acetyl-CoA units would yield either the toxic fluoroacetyl-CoA, if there is initially an even number of carbon atoms or the relatively non-toxic 3-fluoropropionate, if there are initially an odd number of carbon atoms, in the ω-fluoro fatty acid.

The toxicity of fluoro compounds can indicate whether the biological degradation produces fluoroacetate, and this often can provide a clue to the metabolism of compounds in which the fluorine serves as a marker.

For example, in a series of ω-fluoro-nitriles, $F(CH_2)_nCN$, those with an odd-number of carbon atoms are toxic, whereas those with an even-number are nontoxic[15]; these observations suggest a carbon-cyanide cleavage rather than a hydrolysis or reduction of the nitrile in the metabolism of these compounds.

Examples of what might be termed lethal catabolism can also occur in microorganisms. The failure of many fluorine-substituted aromatic and heterocyclic compounds to support the growth of bacteria which oxidise them has often been attributed to their breakdown to fluoroacetate with the subsequent poisoning of the T.C.A. cycle. Hughes in 1955[16] showed that a strain of Ps. fluorescens capable of oxidising nicotinic acid could also oxidise some halogeno-nicotinic acids. This organism could not utilise the halogenated analogues as a sole carbon source although it grew readily upon nicotinic acid. Similar observations were reported by Behrman and Stanier[17], who attributed the failure of 5-fluoronicotinate to support bacterial growth to its enzymic conversion to fluoroacetate with a resulting blockage of terminal respiration. Smith and Cain[18] suggested that Nocardia erythropolis, after growth on benzoate or p-nitrobenzoate, oxidised the corresponding 2-fluoro analogues to fluoroacetate, and further studies[19] have confirmed the presence of this toxic product in extracts of cells from such incubations, also the ability of the citrate synthetase from N. erythropolis to condense fluoroacetyl-CoA with oxaloacetate to produce fluorocitrate[19].

The principles which have emerged in the study of fluoroacetate as a selective metabolic inhibitor have inspired the development of other fluoro analogues. In Bergmann's laboratory a systematic effort has been made to synthesise intermediates of the glycolytic pathway and T.C.A. cycle with a

fluorine substituent[20]. One of the most biologically-important of these compounds is 3-fluoropyruvate[21]. The potent antimetabolic properties expected of this compound were refuted by the outcome of pharmacological trials, which showed a low toxicity of fluoropyruvate for mammals, and showed that the compound was not metabolised 'in vivo' via fluoroacetate[22,23]. However, fluoropyruvate proved 20-50 times more effective than fluoroacetate in inhibiting, albeit temporarily, bacterial growth[24], and has been shown to inhibit competitively the enzymes lactate dehydrogenase[25] and pyruvic oxidase[26] in 'in vitro' studies.

The non-toxicity of fluoropyruvate, which (as stated) applies only to mammals, may well be due to the lability of its C-F bond. In fact, it has been found that it reacts immediately with SH-compounds liberating hydrofluoric acid. The compounds formed are characterised by their spectra, which are different if the SH compound has a free amino group in the β-position or not. In the former case, eg. cysteine, the product absorbs at 295-300 mμ ; in the latter, eg. glutathione, at 265-275 mμ , but this maximum is also shifted to 295-300 mμ by the addition of borate[25,27,28]. Judging from existing data in the case of p-hydroxyphenylpyruvic acid[29], the absorption at 295-300 mμ is due to the enolic form of an α-keto acid, and this enolisation is promoted by borate. If an NH_2 group is present in the β-position to the mercapto group, the product is already largely enolised without borate. Fluoropyruvic acid, is, however, not transformed quickly enough by this reaction to be without any biological effect at all. It causes a swelling of the mitochondria, and produces, indirectly, the biochemical effects connected with this phenomenon. Here, too, the

reaction may be due to an SH-group which is linked normally to the ATP-magnesium complex; this link is destroyed by the acid[30,31]. However, the most interesting effect of fluoropyruvate is the differentiation of non-glycolysing (eg. chorio-allantoic membrane) and glycolysing (eg. tumours and chick fibroblasts) cell systems. Both are completely inhibited by 10^{-3}M of fluoropyruvate, but in tumours and fibroblasts this inhibition is overcome by the addition of glucose, which permits a rapid reduction of fluoropyruvate to the nontoxic fluorolactate, thus preventing any irreversible damaging effect of fluoropyruvate. In support of this explanation, the glucose effect is abolished by 30×10^{-2}M pyruvic acid, which competes successfully with the fluoropyruvic acid as hydrogen acceptor. In principle, therefore, fluoropyruvate could be used for the selection of cells of desired glycolytic activity[32].

The determination of the effects of exchanging one or more hydrogen atoms by fluorine is well known enzyme substrate molecules on their reactivity with purified enzymes has been used by Kun et al. as an approach to the study of control mechanisms of enzyme activity in complex systems. To this end a number of specific enzyme inhibitors have been synthesised and characterised by Kun and his co-workers, who have studied their effects on certain key enzymes in complex biological systems.

As well as characterising and studying the mechanism of action of the enzyme-inhibitory isomer of monofluorocitrate[7], (see page 4) the enzyme regulatory mechanisms of the entry of glutamic acid into metabolic pathways in kidney tissue, and the mode of action of monofluorolactate on heart muscle lactate dehydrogenase, have also been studied by these workers[33,34].

A determination of the inhibitory effects of the mono- and difluoro-oxaloacetates, fluoroglutarate, and amino-oxyacetate on the 'in vitro' metabolism of glutamate by rat kidney mitochondria and homogenates showed that each inhibitor alone was capable of almost completely inhibiting glutamate metabolism. To explain these findings a sequential enzymatic mechanism was proposed, involving sequential coupling of glutamic dehydrogenase and glutamate/aspartate and glutamate/alanine transaminases[33].

Working with the L-(+)-stereoisomer of ß-monofluorolactic acid, Ayling and Kun[34] showed that this compound is a specific inhibitor of lactic acid dehydrogenase. The inhibition is competitive with respect to L-(+)-lactate, uncompetitive with NAD and pyruvate, and noncompetitive with NADH. Fluorolactate in the forward reaction can combine only with the enzyme-NAD complex, while in the reverse direction the prerequisite for inhibition is the formation of an enzyme-pyruvate-NADH complex.

It is perhaps in the field of nucleic acid metabolism that the greatest volume of current work on fluorinated natural products is being done. This stems directly from the work of Heidelberger and his co-workers, who realised that fluorinated pyrimidines (synthesised chemically) might provide a means of interfering with the cellular synthesis of nucleic acids[35]. This approach to blocking nucleic acid synthesis as a method of cancer chemotherapy seemed particularly attractive since uracil utilisation is greater in tumours than in normal tissues. Of the compounds developed in this approach, the most useful have been 5-fluorouracil and 5-fluoro-2-deoxyuridine, both of which depend for their effectiveness on metabolic transformation within the cell to an active form, namely, 5-fluorodeoxyuridine monophosphate

(F-dUMP, Fig.3). In this form the fluorinated pyrimidine becomes an inhibitor of thymidylate synthetase[36,37,38], an enzyme responsible for the methylation of deoxyuridine monophosphate to yield thymidine monophosphate, which is required as a precursor in the synthesis of DNA (Fig.3).

This example of lethal synthesis is analogous to that of fluoroacetate, since 5-fluorouracil is converted to its active form by a normal metabolic pathway, in this case that responsible for the conversion of uracil to deoxyuridine monophosphate. In the form of 5-fluorodeoxy uridine monophosphate the fluorine of fluorouracil becomes responsible for blocking a step in the overall pathway leading to the synthesis of DNA. Tumours resistant to 5-fluorouracil can have a low level of an enzyme on the pathway from uracil to deoxyuridine monophosphate or a resistant thymidylate synthetase, and are thus protected from the formation or action of the active chemotherapeutic agent[39]. Fortunately for the usefulness of these compounds, the metabolic degradation of 5-fluorouracil 'in vivo' yields the relatively nontoxic α-fluoro- β-alanine (Fig.4) rather than a fluorine compound with other toxic properties[35].

The unique value of fluorine in the design of analogues is illustrated by the fact that 2-fluoroadenosine is strikingly more toxic for cultured cells than other 2-halogenated adenosines. The basis for this difference apparently lies, at least in part, in the specificity of adenosine kinase, which phosphorylates 2-fluoroadenosine more readily than adenosine, but is unreactive with the other 2-halogenated adenosines[40].

Another aspect of the toxicity of 2-fluoroadenosine might be its ability to conform to the geometric restrictions inherent in the structure of polynucleotide helices, in contrast to homologous compounds with

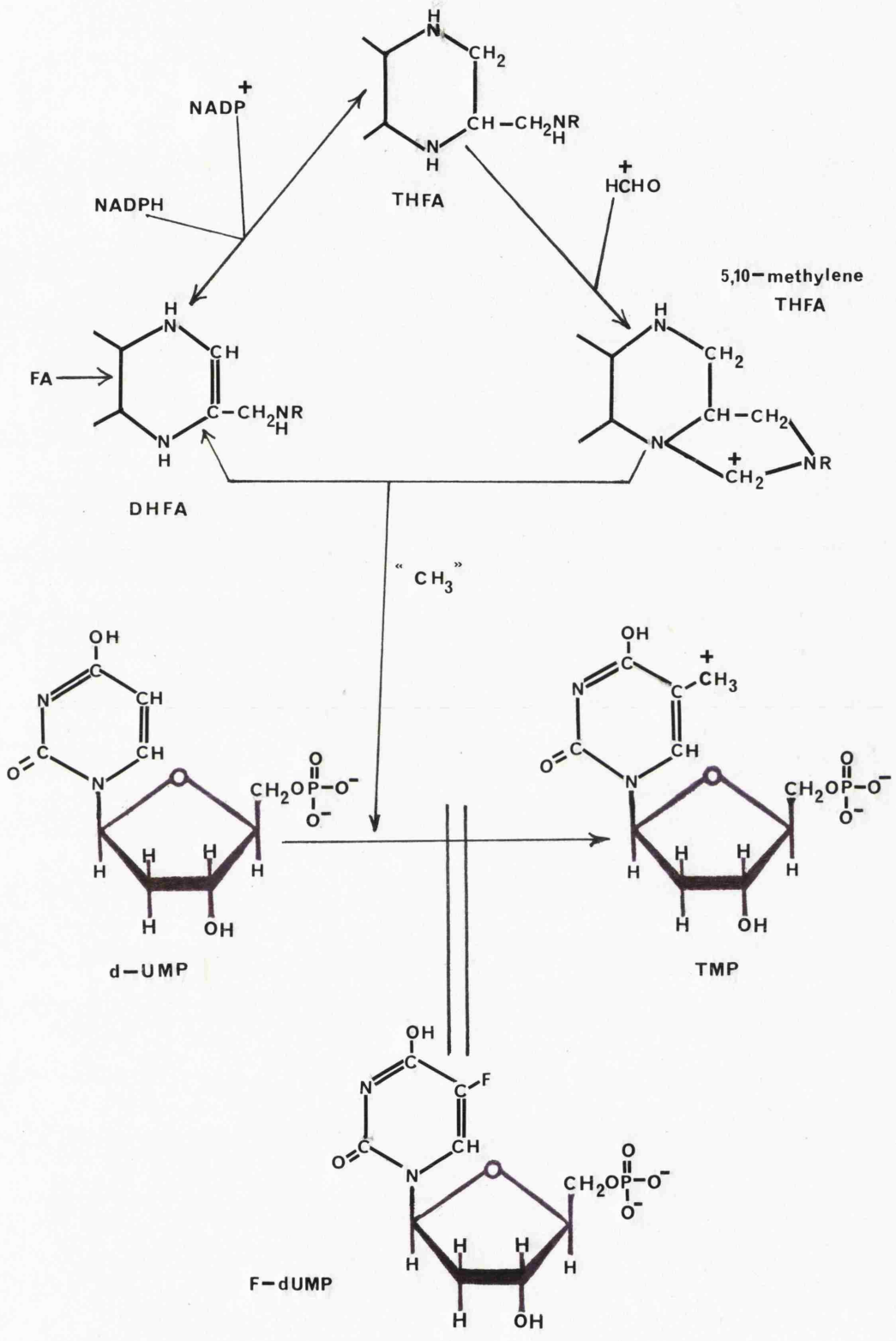


Fig.3. Thymidylate synthetase[37]

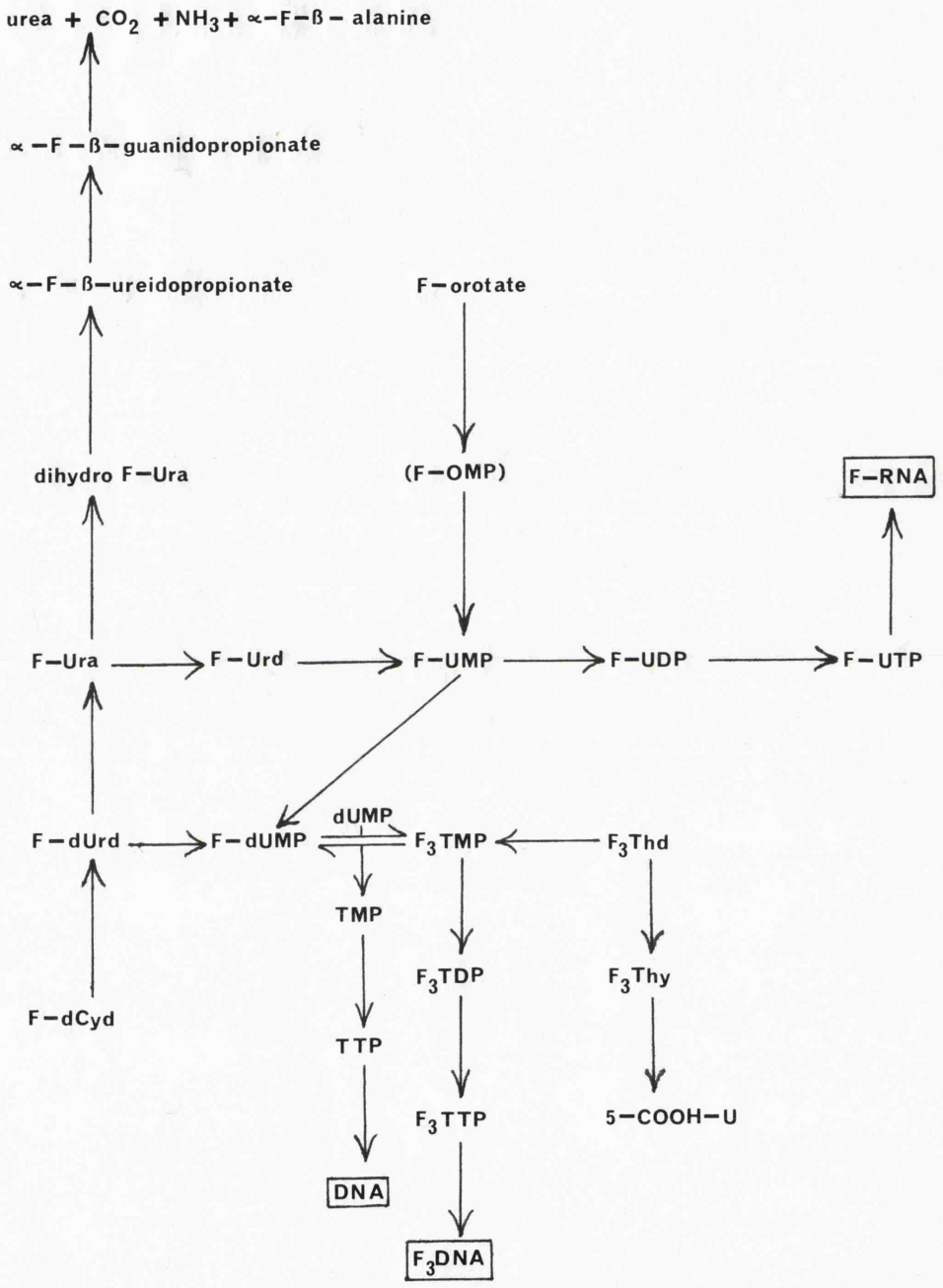


Fig.4. Summary of the metabolism of fluorinated pyrimidines[35]

bulkier substituents, such as bromine or iodine, which would not meet these criteria. Studies based on these structural considerations, by Acs[41], have shown that a number of 8-substituted purines (including 8-bromoadenosine), which are nontoxic for fibroblasts, are also not substrates for the adenosine kinase. However, the toxic analogue 8-aminoadenosine is phosphorylated, but not incorporated, into RNA or DNA by polymerases 'in vitro'. On the basis of model building studies, it is uncertain whether the moderately small amino substituent could conform to the restrictions at the 8-position of the purine imposed by the DNA structure. Kapuler and Reich[41], have shown that 8-bromo and 8-keto guanosine triphosphate are not incorporated into polymers by replicating enzymes; however, they note that the smaller fluorine atom at the 8-position of the purine might satisfy the conformational requirements necessary for incorporation. The fluorinated compound would, therefore, be of great value in defining the restrictions imposed by the helical structures and in establishing criteria for toxicity. If the 8-fluoro purines were incorporated by polymerases, the fluorine substituent could resemble the hydrogen atom sufficiently to permit the substitution to occur without toxicity.

Arguments such as these emphasise the particular value of fluorinated analogues in providing insight into biological systems.

In addition to their action as inhibitors of specific enzymes, fluoro analogues can also disrupt cellular function by being incorporated into the macromolecules of the cell. In determining the role of macromolecules in cellular functions, changes in cell activity as the result of analogue administration can be attributed to alterations in the particular macromolecule known to be affected by the analogue.

Among the fluorinated amino acids incorporated into proteins are 5',5',5'-trifluoroleucine, 2-fluorophenylalanine, 3-fluorophenylalanine, 4-fluorophenylalanine, 3-fluorotyrosine and 4-fluoroproline[42-45]; the fluorophenylalanines causing inhibition of substrate-induced enzyme formation[42], and fluoroproline undergoing enzymic hydroxylation[44].

The use of these analogues in elucidating the role of macromolecules in the cell is illustrated by work in which tissue culture cells are infected with virus in the presence of 4-fluorophenylalanine. The limitation of viral infection when the analogue is present suggests that new proteins, which are nonfunctional because of analogue incorporation, must be synthesised early in the infectious cycle[46]. These experiments serve to emphasise the intimate relationship between RNA synthesis and protein sythesis.

The physiological effect of altered proteins after analogue incorporation is suggested by experiments on certain E.coli mutants which are induced to form capsular polysaccharide by the presence of 4-fluorophenylalanine in the growth medium, and on mutants with temperature-sensitive alkaline phosphatase repressors, which are derepressed at a lower temperature when 4-fluorophenylalanine is added to the medium. The change in capsular polysaccharide has been shown to be phenotypic, and due to an increase in the enzymes involved in capsular polysaccharide synthesis. The results suggest that 4-fluorophenylalanine is incorporated into the protein product of the repressor gene, and alters it sufficiently to allow derepression of several enzymes. The explanation for the derepression of the temperature-sensitive gene is that 4-fluorophenylalanine is incorporated into the genetically altered repressor protein, which suffers a further critical loss in its function[47].

In addition to inhibiting DNA synthesis, fluorouracil can be incorporated into the RNA of mammalian cells[48], bacteria[49], viruses[50] and 'phage[51]. Both the messenger RNA[52] and transfer RNA[53] of E.coli seem to incorporate the analogue. The consequence of this can be lower infectivity of a virus[50a,51], altered protein synthesis as in the case of bacterial enzymes[52,54,55], or a change in the phenotype of a bacteriophage[56]. It has been suggested that these types of alterations are a consequence of the incorporation of fluorouracil into RNA in place of cytosine during transcription or viral replication, and that fluorouracil, so incorporated, would be read in translation as if it were uracil instead of cytosine. However the transcription errors that would be predicted when 5-fluorouridine triphosphate is substituted for uridine triphosphate have not been demonstrated experimentally[57]. It has been demonstrated that transfer RNA undergoes changes in its physical properties as the result of the incorporation of fluorouracil and that this altered transfer RNA does not function normally in the amino acid-incorporating system under the direction of phage R17 RNA[53]. Results suggest that altered transfer RNA may contribute to the altered cell function arising from the incorporation of fluorouracil into RNA.

Changes in the physical properties of RNA have also been found when fluorouracil is incorporated biosynthetically into the RNA phage MS2. Although the RNA particles containing fluorouracil are not infectious, they are more active than normal MS2 RNA in directing the synthesis of phage-specific proteins in cell extracts. Results indicate that RNA fragments containing fluorouracil represent approximately $^{2}/_{3}$ of the 5'-end of the phage RNA molecule and direct the synthesis of phage coat protein but not RNA synthetase[58].

Another aspect of the relation of fluoro analogues to macromolecules comes from the studies of Rennert which indicate that 5',5',5'-trifluoroleucine can be incorporated into the protein of mouse embryos but not into that of the mature mouse. A difference in amino acid-activating enzymes and in transfer RNA seem responsible for the difference[59]. The altered response to analogues of the immature tissue may be of importance in the understanding of the biochemical basis of differentiation. This study also illustrates the value of analogues in the elucidation of biochemical differences between tissues.

In the steroid field a wide range of fluorinated analogues have been synthesised[60], and an interest in fluorination of the steroid molecule has increased knowledge of the chemistry of fluorination reactions and also given additional insight into the features of the steroid molecule which are responsible for its biological activity. With these advances has come the development of steroids which are more active chemotherapeutic agents. The usefulness of fluorine in this field of medicinal chemistry can be illustrated by one example from the studies of Fried[61]. Hydrocortisone (Fig.5) requires an 11 ß-hydroxyl group for its biological activity. This activity, which can be measured as an effect on increasing liver glycogen in the rat, can be correlated with the nature of various substituents at the 9∝-position, as shown in Table 2. If the activity of the substituent were related to its size, one would expect the fluorine substituent to have about the same effect as the hydroxyl or hydrogen group, and similarly chlorine to have the same effect as the methoxyl substituent. Since this is clearly not the case, the electronegativity of the substituent (demonstrated by the effect of the substituent in increasing the acidity of substituted acetic acids) is also included in Table 2. In this series, the greater the constant Ka, the more readily the substituent withdraws electrons and the more easily a proton is released from the neighbouring carboxyl group.

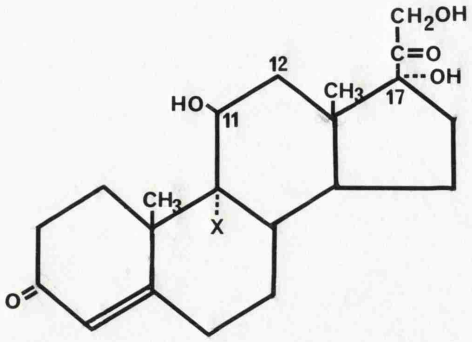


Fig.5. Hydrocortisone with X indicating the substituent at the 9∝-position

TABLE 2

Relation of adrenal cortical activity to the electronegativity of the substituent at the 9∝-position of hydrocortisone[61]

Substituent	Glycogenic activity of 9∝-X-hydrocortisone	Ka of XCH_2COOH ($X10^5$)
Fluorine	10.7	217
Chlorine	4.7	155
Bromine	0.3	138
Iodine	0.1	75
Hydroxyl	0.2	16
Methoxyl	< 0.2	33
Hydrogen	1.0	1.82

The data indicate the biological activity of the steroid molecule correlates quite well with the electronegativity; this correlation suggests that biological activity may be related to the acidity of the 11ß-hydroxyl group caused by the substituent. This prediction, of increased biological activity due to increased acidity of the 11ß-hydroxyl group, is further supported by studies of a family of compounds in which biological activity and the acidity of the hydroxyl group are altered by substitutions at the 12-position of the molecule[61].

Because of its electronegativity, a fluorine substituent can stabilise more labile adjacent carbon-halogen bonds in a molecule. The use of CF_3I, containing I^{131} as a radioactive inert gas, for the measurement of local cerebral blood flow[62] illustrates this, since the parent compound, CH_3I, is quite unstable in biological systems. A field in which systematic substitution of a fluorine atom in various compounds has been helpful in elucidating relations between structure and activity is the pharmacology of general anaesthetic agents[63].

In general, the usefulness of fluorine in the development of interesting pharmacological agents has been related either to its size or its electronegativity, which affects the acidity or stability of neighbouring groups. Implicit in the importance of fluorine for these purposes is the extraordinary stability of the carbon-fluorine bond in biological systems. Indeed, because of the difficulty encountered by the analytical chemist in cleaving this bond, it has been questioned whether the bond could be broken enzymatically. Fluoroacetate illustrates this stability since fluoride is released only slowly from this compound when it is refluxed in 20% sodium hydroxide or heated at 100°C in conc. sulphuric acid. Complete release of fluoride occurs only in sodium fusion at 400°C or after refluxing in 30% sodium hydroxide[64].

Subsequently, two examples of the enzymatic cleavage of the carbon-fluorine bond were found. The first was the action of horseradish peroxidase on 4-fluoroaniline[65]. Cleavage of the carbon-fluorine bond in this case is not unique, since chloro, iodo, or methoxy substituents at the 4-position of an aniline compound are displaced in analogous reactions by the enzyme.

The removal of fluoride from another aromatic compound occurs by the action of phenylalanine hydroxylase on 4-fluorophenylalanine. Several possible schemes have been advanced by Kaufman[66] to explain this reaction, which yields stoichiometric amounts of fluoride and tyrosine from 4-fluorophenylalanine at the expense of excessive NADPH and presumably molecular oxygen. Aside from the abnormal stoichiometry of the reaction with 4-fluorophenylalanine, there is nothing to suggest that this reaction differs from the normal reaction of the enzyme with phenylalanine. It is not clear how any known mechanism can explain the cleavage of the carbon-fluorine bond by these two enzymes.

Since fluoroacetate is a natural product[67], Goldman and his co-workers argued that, in spite of its great stability[64], there might be enzymes capable of degrading it, and, indeed, an enrichment culture containing fluoroacetate as the sole source of carbon was successful in yielding a pseudomonad containing an enzyme capable of breaking the carbon-fluorine bond in this compound[68]. Studies with the partially-purified enzyme indicated that fluoride was released from fluoroacetate according to the following equation:-

$$FCH_2.COO^- + H_2O \longrightarrow HOCH_2.COO^- + HF$$

The enzyme was specific for the dehalogenation of monohalogenated acetic acids and was therefore named haloacetate halidohydrolase[68]. A consideration of the other substrates which react with the fluoroacetate-splitting enzyme shows that, in spite of its status as a 'superhalogen', fluoride is liberated by a mechanism identical to that by which the other halides are released from the analogous compounds. This similar release of halides, in spite of the higher bond energy of the carbon-fluorine bond, has been related to the hydrogen-halogen bond which has been postulated[69] to be formed during the

reaction. A summary of these bond energies (Table 1) indicates that, in general, the energy of the hydrogen-halogen bond correlates with that of the carbon-halogen bond. This relation suggests that the energy lost in the cleavage of the particular carbon-halogen bond may be gained during the reaction by the formation of the corresponding hydrogen-halogen bond. The net energy change during the reaction with the carbon-fluorine bond would then be about the same, or even slightly more favourable, than that for the other carbon-halogen bonds. A mechanism which invokes protonation of the halide leaving group, as suggested by Goldman[69], would explain why fluorine is a better leaving group than iodine in this reaction, but steric factors may also be involved, since 2-fluoropropionate (differing from fluoroacetate in the introduction of a bulky methyl substituent) is not dehalogenated.

Goldman et alia, using dichloroacetate as sole carbon source in an enrichment culture, isolated another pseudomonad, which had the ability to produce two additional halidohydrolases; one induced during growth on dichloroacetate (halidohydrolase II) and the other during growth on chloroacetate (halidohydrolase I)[70]. These two enzymes have different physical properties and attack the same substrates but at different rates. The range of substrates attacked overlaps with that of haloacetate halidohydrolase, the enzymes differing in this regard only in their ability to attack fluoroacetate and certain 2-substituted halogenated acids. These three enzymes also share a common pH optimum of about 9.2[68,70].

The reactions catalysed by the two additional halidohydrolases can be summarised by:

$$\text{L- RCHX.COO}^- + \text{H}_2\text{O} \longrightarrow \text{D- RCHOH.COO}^- + \text{HX}$$

where X = Cl or I, and R = H, CH_3, or $CH_3.CH_2$.

As in the reactions of haloacetate halidohydrolase, these halidohydrolases displace the halide with a hydroxyl group, containing oxygen derived from water[70,71]. This is an important distinction between the cleavage of the carbon-halogen bond by the halidohydrolase, and that in 4-fluorophenylalanine by phenylalanine hydroxylases. Although formally in each reaction the halide is displaced by a hydroxyl group, in the hydroxylase-catalysed reaction the oxygen is derived from molecular oxygen rather than from water[72].

Similarities among the three halidohydrolases indicate that these enzymes can be considered to belong to the same class. Halidohydrolase I and II, however, by virtue of being reactive with optically reactive substrates, provide some additional insight into the mechanisms involved in this type of reaction. The fact that a Walden inversion occurs during the hydroxyl displacement of the halide indicates that the enzyme catalyses a bimolecular nucleophilic substitution reaction. This is the mechanism involved in the nonenzymic hydroxyl displacement of a halide which occurs when these compounds are treated with sodium hydroxide[73].

The increased use of fluorinated compounds in enzymology has revealed other examples of the enzymatic hydrolysis of the carbon-fluorine bond. Barnett et al. tested α-D-glucosyl fluoride as a reagent which might form a covalent bond to the postulated carrier for sugar transport in intestinal cells. They found, however, that α-D-glucopyranosyl fluoride was hydrolysed enzymatically to glucose and fluoride, apparently by the enzyme α-D-glucosidase[74]. Here the fluorine atom must take the place of the alkoxy radical in the initial protonation stage. Similar results have been obtained with α- and β-galactopyranosyl fluoride[75] and their

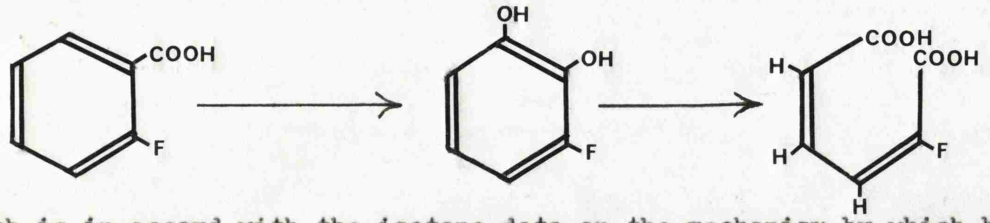
respective enzymes. These results have been confirmed and extended to ß-D-glucopyranosyl, ∝-D-mannopyranosyl, and ∝-D-xylopyranosyl fluorides with the respective glycosidases[76]. The reaction seems to be a general one obeying the same specificity rules as those for more normal glycosidase substrates. The rates of enzymic cleavage are high and the fluorides seem to be useful substrates for the polarimetric determination of the initial product (∝-D or ß-D anomer of the parent sugar) of the action of glycosidases[76].

A similar chance discovery of a defluorinating reaction was made by Henkart et al. during an attempt to modify carbonic anhydrase by the Sanger reagent, 1-fluoro-2,4-dinitrobenzene. They found that the enzyme catalyses the hydrolysis of this compound, presumably with the formation of 2,4-dinitrophenol and fluoride[77].

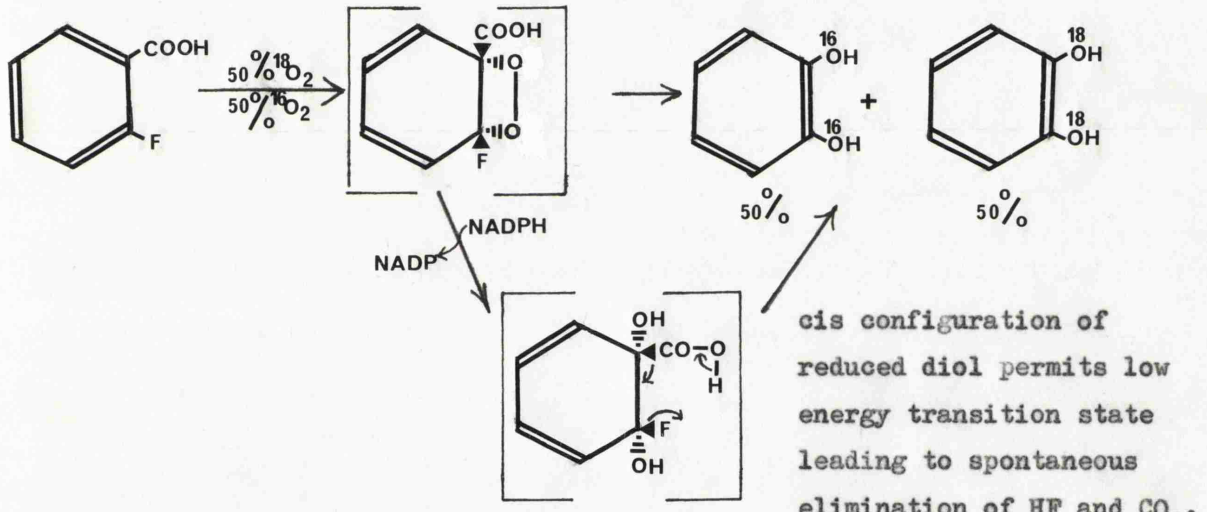
Fluoride is released from 2-fluorobenzoic acid in a reaction involving molecular oxygen that has been found in a pseudomonad isolated from an enrichment culture on that substrate[78]. The enzyme for this reaction has not been characterised, but in whole cells the catechol derived from 2-fluorobenzoic acid has been isolated. Furthermore, when the incubation of the cells is carried out in an atmosphere containing oxygen labelled with both oxygen-16 and oxygen-18, it can be demonstrated that both oxygen atoms in the catechol are derived from a single molecule of oxygen[79]. Although other explanations are possible, this finding suggests that the reaction proceeds through a cyclic peroxide (Fig.6) such as has been postulated for the analogous oxidation of anthranilic acid to catechol[80].

Although over 80% of the fluorine in 2-fluorobenzoic acid is liberated as fluoride, 3-fluorocatechol and 2-fluoromuconic acid were isolated from the

growth medium[78], suggesting the following metabolic pathway:



which is in accord with the isotope data on the mechanism by which benzoic acid undergoes oxidative decarboxylation and ring cleavage to catechol and cis- cis- muconate. In this organism, therefore, there are two possible fates for 2-fluorobenzoic acid - either oxidative decarboxylation to 3-fluorocatechol by a benzoate oxidase, or defluorination,



cis configuration of reduced diol permits low energy transition state leading to spontaneous elimination of HF and CO_2.

Fig. 6. The microbial metabolism of 2-fluorobenzoate in an atmosphere which is 50% O^{16}-labelled O_2 and 50% O^{18}-labelled O_2 [79]

Defluorination by a mixed function oxidase is known for mammalian systems[66,81], and suggests the possibility that 2-fluorobenzoate can be hydroxylated at both positions ortho to the carboxy group by the same enzyme, resulting in catechol, if hydroxylation is at the 2-position, and 3-fluorocatechol, if at the 6-position.

The microsomal hydroxylating system from rabbit liver which catalyses the conversion of 4-fluoroaniline to 4-hydroxyaniline is another example of carbon-fluorine bond cleavage which seems to require molecular oxygen[81].

Except for the reaction catalysed by peroxidase, all the reactions of carbon-fluorine bond cleavage involve the replacement of the fluorine by a hydroxyl group. These reactions differ however depending on whether the oxygen in the hydroxyl is derived from water or molecular oxygen. Oxygen has been shown to come from water in the reaction catalysed by haloacetate halidohydrolase, and oxygen almost certainly comes from water in the reactions catalysed by α-glycosidase and carbonic anhydrase, where water is the usual reactant with the enzyme. On the other hand, oxygen comes from molecular oxygen in the reaction by which catechol arises from 2-fluorobenzoic acid, and almost certainly in the reaction of phenylalanine hydroxylase on 4-fluorophenylalanine and in the reaction with 4-fluoroaniline catalysed by the microsomal enzyme. Presumably the defluorination of 4-fluoroproline which occurs in a collagen-synthesising system[44] also requires molecular oxygen.

The specificity of the enzyme haloacetate halidohydrolase indicates that the carbon-fluorine bond can be cleaved by mechanisms which are applicable to the fission of comparable bonds involving the other halogens. On the other hand, in aromatic compounds undergoing reactions with hydroxylases, the fate of fluorine can be different from that of the other halogens. In the conversion of 4-fluorophenylalanine to tyrosine by phenylalanine hydroxylase, fluoride is released; whereas in the reaction of the same enzyme with 4-chlorophenylalanine, the major product is 3-chlorotyrosine[82]. The difference in the fate of the two halogens does not necessarily imply any fundamental difference in the enzyme mechanism

whereby the two substrates are hydroxylated. Fluorine and chlorine substituents do not necessarily behave differently during hydroxylation since, in the microsomal system, 4-hydroxyaniline is the product of the reaction with both 4-fluoro- and 4-chloroaniline[81].

Although considerable insight has been gained into the enzymatic mechanisms of carbon-fluorine cleavage, the biochemistry of carbon-fluorine bond formation remains unknown. By developing an 'in vitro' system for fluoroacetate formation in homogenates of Acacia georginae, Peters and his colleagues have begun to attack this problem[83].

The liberation of fluoride ion during the oxidation of 2-fluorobenzoic acid by a pseudomonad observed by Goldman[78], has also been demonstrated for a Vibrio sp.[84] utilising 4-fluorobenzoic acid, and the inability of certain organisms to use halogenated analogues for growth can often be attributed, at least in part, to their failure to liberate the halogen and to carry the oxidation to a stage at which carbon may be assimilated.

The fluorobenzoates inhibit the oxidation of benzoic acid by adapted cells of both Ps. fluorescens[85] and Nocardia erythropolis[18]. Using N. erythropolis, Smith and Cain[18] observed that the respective halogen-analogues acted as non-metabolisable enzyme inducers for benzoic acid and p- nitrobenzoic acid oxidation, and also accelerated induction of the organism to benzoate but retarded enzyme induction in the case of p- nitrobenzoate. In contrast, Hughes[85], working with a Ps. fluorescens strain, found that the halogenobenzoates competitively inhibited the formation of the benzoate-oxidising system induced by benzoic acid and, though the organism did not adapt to oxidise the halogenobenzoates when these

were used as inducers the fluorobenzoates reduced the lag period taken to form the benzoic acid - oxidising system. In contrast with the effect of 5-fluoronicotinate, which was able to induce systems oxidising both itself and nicotinate[16], unless the cells were completely adapted to oxidise benzoate they could not oxidise the halogen analogues. The inhibition of the induction of enzymes responsible for the oxidation of the parent compound can be considered another reason for the inability of this species of pseudomonad to use halogen analogues for growth.

In most of the above examples it is a hydrogen atom which is replaced by fluorine, giving rise to a molecule able to 'deceive' one or more enzymes into accepting it as a substrate and leading to the observed effects of inhibition, lethal synthesis, etc. This is thought to be indicative of a close similarity between the natural substrate and the fluorinated analogue, and Bergmann[20] has rationalised this by drawing attention to the similarity in Van der Waals' radii of the two elements, that of hydrogen being 1.20Å, fluorine 1.35Å. However, this comparison has doubtful relevance, since Van der Waals' radii, in this context, represent hydrogen-bonding distances, and these two elements hydrogen-bond in the opposite sense, ie. hydrogen is always at the electro positive end of a hydrogen bond, while fluorine is strongly electro negative.

A stronger case can be made out in favour of a similarity between hydroxyl and fluorine. Thus the Van der Waals' radii are of the same order (hydrogen 1.20Å, fluorine 1.35Å, oxygen 1.40Å, hydroxyl 1.76Å) and, more important, hydroxyl and fluorine are similarly electro negative and therefore hydrogen-bond in the same sense. Comparison of the covalent radii (H 0.30Å, F 0.64Å, O 0.74Å, OH 1.04Å) is suggestive of a similarity between fluorine and hydroxyl, and evidence in support of this is found in the close

similarity in crystal lattice structures between certain tetritols and pentitols and their monofluoro-analogues[86]. Since hydrogen-bonding is thought to be intimately involved with enzyme-substrate interactions, it seemed likely to certain workers that replacement of hydroxyl by fluorine in metabolites would lead to interesting effects. Thus replacement in carbohydrates could have repercussions in intermediate carbohydrate metabolism, storage carbohydrates and nucleic acid biosynthesis.

Arguments such as these have provided the principle motivation for the synthesis of deoxyfluoro sugars*. Until recently, and in spite of considerable efforts,[87] deoxyfluoro sugars as a class of carbohydrate derivative were poorly exemplified, and, in the case of secondary fluorides having the gluco-configuration, they are still relatively inaccessible synthetically. However, during the last few years synthetic methods have been developed by various groups, notably at Oxford, Bath, London and New York which now permit the replacement of hydroxyl groups at positions 1,2,3,4 and 6 of D-glucose.

As the object of the present work has been to examine some of the biochemical effects of one such deoxyfluoro sugar, the synthetic and structural aspects of introducing fluorine into carbohydrates and related compounds is reviewed in the Appendix.

Work on fluorinated carbohydrates and their derivatives has shown that, in the main, these compounds act by direct competitive inhibition

* The term deoxyfluoro sugars connotes carbohydrates in which one or more of the hydroxyl functions, other than at the anomeric position, have been replaced by a fluorine atom.

in those cases where antimetabolic effects exist. Thus Blakley and Boyer[88] demonstrated that, at molar concentrations comparable to those of glucose or fructose used, 6-deoxy-6-fluoro-D-glucose inhibited the rate of fermentation by intact yeast. The effect on yeast extract fermentation was very small, nor was there any significant effect on hexokinase 'per se'. The inhibition of intact yeast fermentation was competitive, and with glucose or fructose was overcome by increasing the concentration of these sugars. It was postulated that 6-deoxy-6-fluoro-D-glucose influences a specific process, not hexokinase limited, controlling the rate of entry of glucose and fructose into the yeast cell.

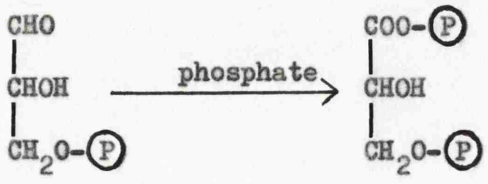
Subsequently Serif and Wick[89] reported the competitive inhibition of glucose and fructose oxidation by 6-deoxy-6-fluoro-D-glucose in rat kidney slices with no effect on the oxidation of lactate or acetate. Using specifically labelled glucose they showed that inhibition occurs before glucose-6-phosphate formation. These authors also found an anomalous response to increasing concentrations of the inhibitor and suggested that 6-deoxy-6-fluoro-D-glucose inhibits a specific pathway of glucose oxidation to carbon dioxide but that alternative unaffected pathways could exist. Similar observations have been made in rat epididymal adipose tissue and rat diaphragm muscle[90], and more recently evidence has been presented[91] in further support of 6-deoxy-6-fluoro-D-glucose inhibiting a glucose cell entry mechanism in those tissues in which cell entry is rate limiting[92].

6-Deoxy-6-fluoro-D-glucose has been found to give the percentage activity indicated compared with D-glucose, using the following enzymes: glucose dehydrogenase[93], 100, glucose oxidase[88], 3, maltose phosphorylase[94], 80.

It is oxidised by Aerobacter aerogenes extracts to 6-deoxy-6-fluoro-D-arabino-hexulosonic acid[95]. 6-Deoxy-D-glucose is also a useful substrate for those enzymes for which the 6-fluoro compound is effective.

Varying biological activity has been shown by (±)-2-deoxy-2-fluorotetritols and 2-deoxy-2-fluoroglyceraldehyde[96]. Thus O'Brien and Peters[97] showed that 2-deoxy-2-fluoroglyceraldehyde (FGA) was lethal to rodents, probably by being metabolised to fluorocitrate and causing citrate accumulation. On extending this work, liver aldehyde dehydrogenase and, to a lesser extent, xanthine oxidase were found capable of oxidising FGA to the corresponding acid[98] and, since (-)-2-deoxy-2-fluoroglycerate (probably L) caused more citrate to accumlate in guinea-pig kidney particles than its stereoisomer or FGA, 2-deoxy-2-fluoroglycerate was proposed as an intermediate between FGA and fluorocitrate. It was suggested that its conversion to fluoroacetate was catalysed by L-serine hydroxymethyltransferase, 2-deoxy-2-fluoroglycerate acting as a structural analogue of serine in this respect. Thus FGA can be metabolised to bring about the lethal synthesis of fluorocitrate by the action in turn of four enzymes, aldehyde dehydrogenase, serine hydroxymethyltransferase, acetate-activating enzyme and condensing enzyme[98].

The effect of (±)-2-deoxy-2-fluoroglyceraldehyde-3-O-phosphate on the reaction:



catalysed by rabbit muscle triose phosphate dehydrogenase, was found to be insignificant, but this was thought to be due to the fluoroglyceraldehyde being a racemic mixture.[87]

Nevertheless inhibitory action has been displayed by fluorinated sugars which probably consisted of mixtures of optical isomers. Thus (±)-2-deoxy-2-fluoroerythritol, prepared by Taylor and Kent[96], has been shown to be a potent inhibitor of the growth of Brucella abortus, for which erythritol is a growth factor[100]. The specific mode of inhibition has not been determined, but the competitive nature of the inhibition was illustrated by its reversal on the addition of erythritol. Transport into the bacterial cell seems to be inhibited by the analogue.

The binding of an enzyme and substrate can be explored by finding the apparent affinity, K_m, of modified substrates for the enzyme. If the modified analogue is not a substrate, but an inhibitor, the apparent affinity for the enzyme is given by the inhibitor constant, K_i.

In recent years there has been developed a series of enzyme inhibitors that combine the features of an alkylating agent with specificity for the active site of an enzyme, thus permitting alkylation and identification of a group at or near the active centre of an enzyme, or a particular enzyme to be specifically inactivated. Thus a 1-chloro-4-phenyl-3-p-toluenesulphonamido-2-butanone inactivates chymotrypsin (which cleaves a peptide bond adjacent to an aromatic residue[101]), and 7-amino-1-chloro-3-p-toluenesulphonamido-2-heptanone inhibits trypsin (which cleaves a peptide bond adjacent to lysine[102]). In both cases a histidine residue at the active site is alkylated, and neither inhibitor will inhibit the other enzyme at low concentrations.

This type of reagent requires a specific substrate having a good leaving-group, which can be provided in the sugars by a halogen adjacent to a carbonyl group. Although it was not recognised as such, it seems probable that one such reagent already exists in the sugar series. Thus 3-fluoro-N-acetylneuraminic acid (3-fluorosialic acid, Fig.7) inhibits the 'in vitro' cleavage of N-acetylneuraminic acid to N-acetylmannosamine and pyruvic acid by N-acetylneuraminic acid aldolase:

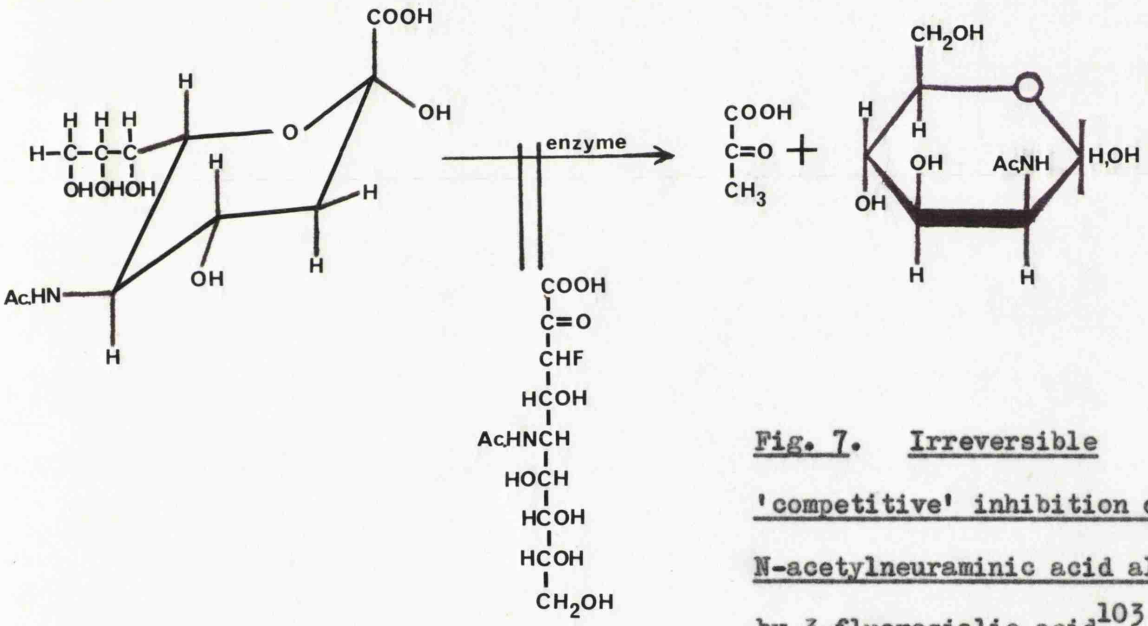


Fig. 7. Irreversible 'competitive' inhibition of N-acetylneuraminic acid aldolase by 3-fluorosialic acid[103]

Inhibition is competitive at zero time, and the enzyme is irreversibly inhibited by the reagent[103]. Unfortunately no attempt was made to show either that the inhibitor is covalently bound to the enzyme or even that fluoride ion is produced during the process.

The importance of each hydroxyl group of the carbohydrate molecule in binding may be investigated by forming the deoxy or fluoro analogue. If these analogues are substrates, having a K_m similar to that of the normal substrate, the group is inactive in binding and the steric requirements of the enzyme site can be investigated by increasing the size of the substituent as in the series F, Cl, Br, I. The use of halogenated sugars in this way was pioneered by Helferich and coworkers[104] in their 'in vitro' studies on ß-glucosidase, using phenyl and vanillyl 6-deoxy-6-fluoro- ß-D-glucopyranosides (Fig.8) as members of a series of halogenated glucosides. The aim of their work was to correlate the activity of ß-glucosidase, a hydrolytic enzyme, to structural changes in the substrate. The phenyl and vanillyl glycosides were used to show the insensitivity of the enzyme to the aglycon, so

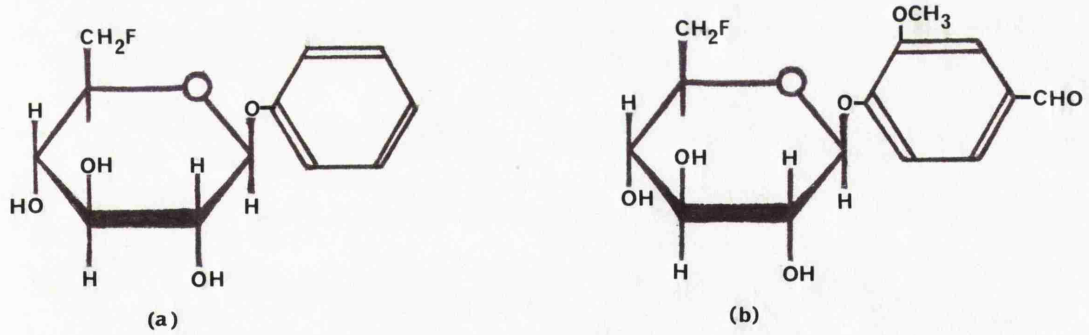


Fig. 8. (a) Phenyl and (b) vanillyl 6-deoxy-6-fluoro- ß-D-glycopyranosides[104]

long as the ß-configuration was present, thus demonstrating that the specificity of the enzyme, intimately related to the sites of enzyme-substrate interaction, was directed principally toward the carbohydrate portion of the molecule. Using suitable analogues Helferich demonstrated the absolute specificity of the enzyme for the ß-glucose configuration at C_1-C_4 of the sugar.

Furthermore hydrolysis was prevented if the hydroxyl groups at C_2, C_3, or C_4 were substituted. Substitution at C_6 however did not halt the reaction, and, using the phenyl and vanillyl glycosides and the 6-chloro-, bromo-, and iodo-glucosides, Helferich established a correlation between enzyme activity and the size of the substituent at C_6 of the glycoside: thus enzyme activity was found to be inversely proportional to the atomic volume of the substituent. In contrast, the use of halogenated sugars in the investigation of the properties of ∝-D-glucosidases, has shown the importance of the C_6 hydroxyl group of the D-glucoside in the binding of the enzyme[76].

Certain tumours apparently have more extensive glycolytic activity[105] than do corresponding normal tissues, and considerable interest has been aroused in glycolysis inhibitors[106] and their effect on tumour growth. Since the hexokinase step appears[107] to be rate-limiting in the glycolytic sequence, Foster et al.[108] have begun the search for hexokinase inhibitors, with the dual purpose of evaluating gross anti-tumour activity 'in vivo' and establishing structure-activity relationships from studies of isolated isozymes[109] of normal and tumour cells.

The affinity of derivatives of D-glucose for hexokinase (as indicated by K_m values) indicates[107] that positions 1, 3, 4 and 6 are importantly involved in the active site, since removal of the hydroxyl groups or inversion of configuration variously at these positions diminishes the affinity for the enzyme. Position 2 is apparently exceptional since D-mannose and 2-deoxy-D-arabino-hexose have low K_m values. It is not known whether the hydroxyl groups at positions 1, 3, 4 and 6 in D-glucose are involved in proton donation or acceptance in hydrogen-bonding with the enzyme-substrate complex, and it may be possible to segregate these effects by replacing the hydroxyl groups by chlorine or fluorine atoms since they can

function only as proton acceptors. (Both functions would be eliminated in deoxy sugars). Additionally, the electronic effect of halogen atoms, especially fluorine could affect the acidity of neighbouring hydroxyl groups and thereby the hydrogen-bonding characteristics and the formation of an enzyme-substrate complex. In this connection the 2,2-dihalogeno derivatives of D-glucose are of particular importance[108a].

Halogenated sugars have also been used in the study of carrier-mediated carbohydrate transport across the intestinal membrane[76], which appears to be of two types: (i) active transport, in which the sugar is transported against a concentration gradient, and (ii) facilitated transport, in which the sugars are selectively transported down a concentration gradient. In each type there are specific structural requirements for the sugars which can be transported[110], and these are not the same for the two types of transport. In a mixture of sugars, the presence of a transported sugar depresses the rate of transport of another transported sugar, indicating that they compete for the same site[110].

The series D-galactose, 6-deoxy-D-galactose, 6-deoxy-6-fluoro-D-galactose, 6-deoxy-6-chloro-D-galactose, and 6-deoxy-6-iodo-D-galactose has been tested for active transport using everted slices of hamster intestine. These substances are transported in the following order of the substituent group at C-6: OH > F >> Cl, H > I, which seems to implicate hydrogen-bonding at C-6 in the active transport process, since only fluorine and oxygen form such bonds readily. The iodo compound is not actively transported, probably because of the size of the iodine atom[76]. In the mammalian intestine, 6-deoxy-6-fluoro-D-glucose[111] and 6-deoxy-6-fluoro-D-galactose[76] are actively transported and compete with D-glucose for the transport site,

thus diminishing the rate at which D-glucose is absorbed. Similarly, in facilitated transport systems, 6-deoxy-6-fluoro-D-glucose[112] interferes with D-glucose transport, and 6-deoxy-6-fluoro-D-galactose shows counterflow with D-galactose in red-blood corpuscles[76].

A more complicated phenomenon is, however, observed in rat diaphragm, which is sensitive to insulin, a hormone that increases the rate of transport of D-glucose. Although 6-deoxy-6-fluoro-D-glucose enters this tissue in a facilitated fashion, and inhibits the entry of D-glucose, the transport is not stimulated by insulin[113]. This may indicate the presence of two types of facilitated transport site in such tissue, viz. (i) an 'insulin-sensitive site' that will not transport 6-deoxy-6-fluoro-D-glucose, and (ii) a 'normal site' that will transport the compound.

Analogues of D-glucose modified at C-3 have been tested for active accumulation by everted segments of hamster intestine[114]. D-Glucose, 3-deoxy-3-fluoro-D-glucose, 3-deoxy-3-chloro-D-glucose and, to a smaller extent, the 3-deoxy-3-bromo-derivative bound, and were transported, more strongly than 3-deoxy-D-glucose and other sugars not containing an electronegative atom in the gluco configuration at C-3. From these results, has been inferred the presence of a hydrogen-bond from the carrier to the hydroxyl group at C-3 of D-glucose[114].

As well as the studies reported in the present work, current biochemical studies in these laboratories have shown that 3-deoxy-3-fluoro-D-glucose (3FG) becomes involved with the intracellular metabolism of Saccharomyces cerevisiae. Preincubation of resting cell suspensions of S. cerevisae produces contrasting effects on the subsequent metabolism of glucose or of galactose. Thus, whilst with glucose the respiration rate was only

transiently affected, an inhibition of glucose uptake and of polysaccharide synthesis was detected. In contrast, with galactose, long term respiration was inhibited, sugar uptake unaffected and polysaccharide synthesis stimulated[115]. Although such results suggest that more than one site of action for 3FG may exist, the effects on respiration and polysaccharide synthesis indicate the possibility of an inhibitory form of the analogue acting on either phosphoglucomutase or UDPG pyrophosphorylase, in a similar fashion to that suggested for 2-deoxy-D-glucose[116]. In an attempt to localise possible sites of action, studies of the effect of 3FG on the resting levels of glycolytic intermediates, adenine nucleotides and inorganic phosphate in resting cell suspensions of S. cerevisiae have been made[117]. Significant decreases in the levels of UDPG, of 2- and 3-phosphoglyceric acid, of ATP and of inorganic phosphate were detected, whilst the levels of ADP, glucose-1-phosphate and fructose-6-phosphate were increased. Changes in ATP, ADP, and glucose-1-phosphate became apparent after the other changes had become established.

A comparison of these effects with those seen in parallel experiments with 2-deoxy-D-glucose in yeast[116, 118] and in rat kidney[119] show the effects of 3FG to be qualitatively similar, and there are indications that 3FG acts, at least in part, at similar sites to those suggested for 2-deoxy-D-glucose. The results are argued to be consistent with 3FG acting as a phosphate trap and thereby occasioning a shift in the energy balance of the cell[117].

Finally, mention must be made of recent studies with certain fluorinated nucleosides in which the 2'-hydroxyl of ribose (or the hydrogen of 2-deoxy-ribose) has been replaced by fluorine[120,121]. Wright, Wilson and Fox[121] have

examined the susceptibility of 1-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl) cytosine and related fluoro sugar cytosine nucleosides to partially purified cytidine deaminase, together with the susceptibility of several corresponding fluoro sugar adenine nucleosides to adenosine deaminase. While the 2'- or 3'-fluoro-substituted pyrimidine nucleosides were either poor substrates or nonsubstrates for cytidine deaminase, the fluoro-substituted adenine nucleosides were even better substrates for calf intestine adenosine deaminase than adenosine. It was demonstrated that in both the arabino and xylo series, the presence of a 2'- or 3'-OH (respectively) is not necessary for deamination by this enzyme. Preliminary screening studies of the fluoro sugar cytosine nucleosides against L1210 mouse leukaemia suspension cultures, revealed that 1-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl) cytosine (2'-F-ara-C) has a growth inhibitory effect comparable with that of ara-C and ara-FC (1-ß-D-arabinofuranosyl-5-fluorocytosine)[121]. The results of future experiments with these and related compounds are awaited with interest.

MATERIALS AND METHODS

MATERIALS AND METHODS

Characterisation and cultivation of organisms

The organisms used in this study were a strain of *E.coli* obtained from the microbiology department of this university, *Pseudomonas saccharophila*[122] (NCIB 8570 = ATCC 9114), and *Pseudomonas fluorescens*, strain A, 3.12 (NCIB 9494 = ATCC 12633) first isolated by Stanier[123], and subsequently proposed by him as the neotype strain for *Pseudomonas putida*, biotype A[124]. This particular strain has been used over the past 20 years in many different biochemical and physiological studies, and there is consequently a large body of information about its metabolism and physiology. Both pseudomonad species were obtained as freeze-dried samples from the NCIB (Dept. Scientific and Industrial Research, Torry Research Station, Aberdeen). All three organisms were periodically tested for purity by a variety of morphological and biochemical criteria. Table 3 lists the biochemical criteria used in the periodic characterisation of the three organisms used in these studies.

For the cultivation of organisms the following semi-defined medium was used throughout these studies (Davis and Mingioli, 1950[125]):

	grams/litre
K_2HPO_4	7
KH_2PO_4	3
$(NH_4)_2SO_4$	1
$MgSO_4$	0.1
yeast extract (Oxoid)	0.2
trace elements solution	1 ml.
carbon source (glucose or asparagine)	20

TABLE 3

Some characteristic biochemical properties of Ps. fluorescens, Ps. saccharophila and E. coli.

	E. coli.	Ps. saccharophila	Ps. fluorescens A, 3.12.
Type of growth in Hugh and Leifson's medium (1% glucose)	acid and gas produced throughout both aerobic and anerobic tubes	acid production only at top of aerobic tube	acid production only at top of aerobic tube
methyl red	+	-	-
Voges Proskauer	-	-	-
gelatin liquefaction	-	-	-
indole production	+	-	-
ammonia from arginine	-	+ after 1 week	+
oxidase	-	+	+
catalase	+	+	+
growth in Koser's citrate medium	-	+	+

Solution of trace elements (Barnett and Ingram, 1955[126]):

	mg./100 ml.
$FeSO_4.4H_2O$	40
$MnSO_4.4H_2O$	40
$ZnSO_4.7H_2O$	20
$CuSO_4.5H_2O$	4
$CoCl_2.6H_2O$	4
KI	30
$Na_2MoO_4.2H_2O$	5
$CaCl_2$ anhyd.	500
NaCl	1000

The ingredients were dissolved in tap water (3 parts) and distilled water (1 part), proportions which supply extra mineral salts and, at the same time, avoid precipitation of phosphates, and the pH adjusted to 7.0 before dispensing. The medium was sterilised by autoclaving at 120°/15 min. without the carbon source, a 30% (w/v) solution of which was sterilised by filtration (Sartorius membrane filters, V.A.Howe and Co. Ltd., London, S.W.6) and the appropriate amount added aseptically when the rest of the medium had been autoclaved. Large volumes of culture medium (10 litres), for quantities of cells suitable for the preparation of cell-free extracts, were sterilised by autoclaving at 120°/40 min. For the routine maintenance of cultures, this medium was solidified by the addition of 2.0% (w/v) Oxoid Ion Agar No.1. All the chemicals used were of A.R. grade (B.D.H., Lab. Chemicals Div'n., Poole, Dorset).

Cultural techniques

For the manometric investigation of washed whole cell suspensions, the necessary cell yields were achieved by the growth of organisms in 250 ml. Ehrlenmeyer flasks containing 75 ml. amounts of glucose/mineral salts medium. Inocula for these cultures were prepared from 24 hr. slope cultures, by washing with sterile distilled water and transferring, with the usual aseptic precautions, the appropriate amount to the Ehrlenmeyer flasks. Cultures were incubated at 30° in a Gallenkamp orbital incubator (A.Gallenkamp and Co. Ltd., Technico House, London, E.C.2). Using the above procedure cell yields in the region of 1- 1.5 mg. dry weight of cells per ml. of medium were obtained after 18-20 hr. incubation, at which time the cells were entering the stationary phase of growth.

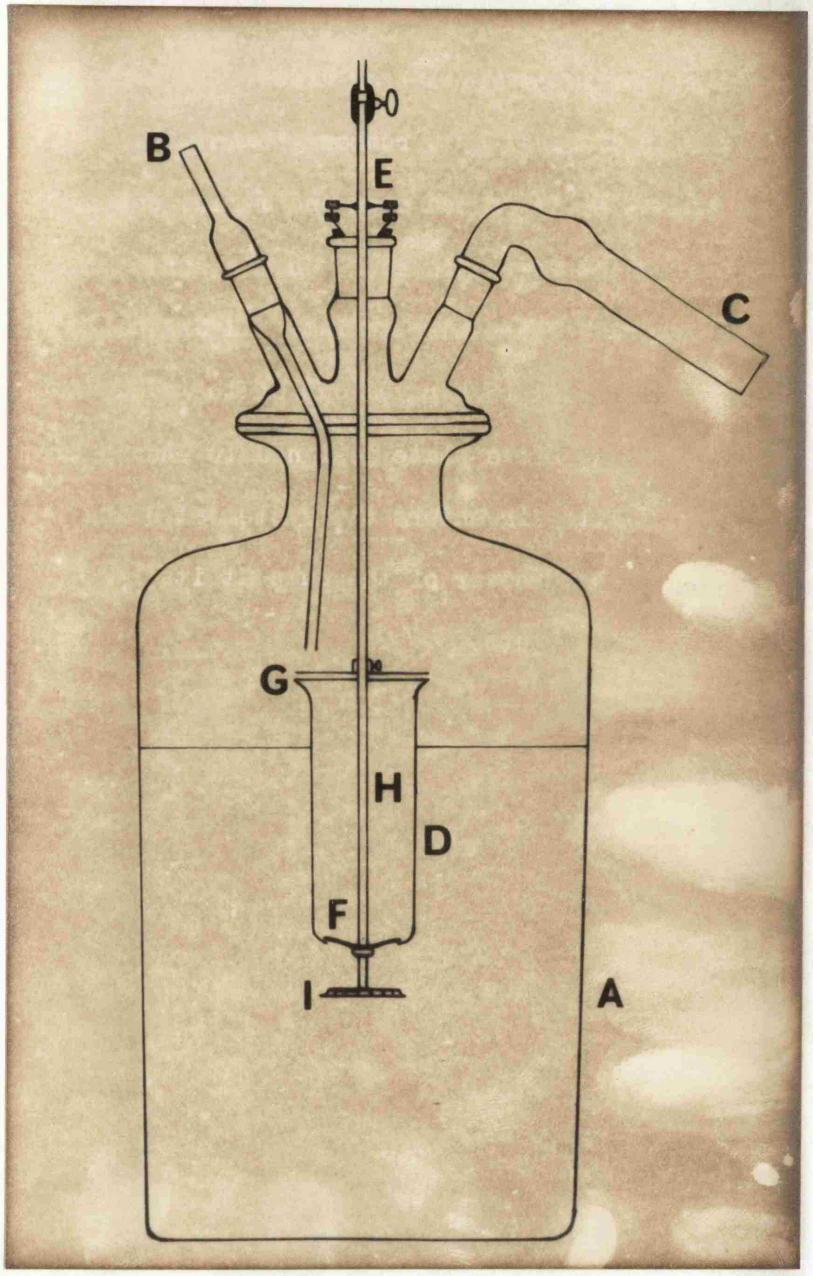
Cells were harvested in a B.T.L. bench centrifuge (Baird and Tatlock (london) Ltd.) with a 50 ml. angle head at 4040xg for 10 min., washed twice with either 0.067 M or 0.67 M K_2HPO_4 - KH_2PO_4 buffer, pH 7.0, and resuspended to approximately 20 mg. dry weight/ml. in phosphate buffer of the same molarity and pH, all operations being carried out at room temperature. Cell yields necessary for cell-free preparations were achieved initially with 8x250 ml. cultures in 1 litre Ehrlenmeyer flasks, incubated at 30° on a rotary action shaker. However, this method was superceded by the use of a 10 litre fermentation vessel. Inocula for these cultures were prepared from 24 hr. slope cultures by washing with sterile distilled water and transferring, with the usual aseptic precautions, to 2x125 ml. amounts of glucose/mineral salts medium contained in 1 litre Ehrlenmeyer flasks. The

flasks were aerated by agitation on a rotary shaker (Orbital Shaker Mark V, L.H. Engineering Co. Ltd., Stoke Poges, Bucks.) at 30° for 18 hr., when suitable growth was achieved. Aeration for large scale culture vessels was supplied by a Vibromischer electrical drive (Vibromischer, Chemap. A.G. Zurich) with a type S.U.B. pump attached (Fig.9). The advantage of this method is the spraying of the growth medium from the top to the sides of the containing vessel as a thin sheet, exposing a large surface area to the air and, at the same time, preventing excessive foam production. A counter-current of air under positive-pressure from a pump and sterilised by filtration, was supplied through an air lead to the top of the vessel. Using this apparatus, high oxygen tensions throughout the medium were obtained with rapid, high growth yields.

For the isolation of the product(s) of bacterial catabolism of 3FG, large scale incubations of buffer-washed, resting, whole cell suspensions with 3FG were carried out. The cells were prepared by incubating 8x125 ml. amounts of glucose/mineral salts medium in 1 litre Ehrlenmeyer flasks by aseptic transfer from 24 hr. slope cultures, and incubating at 30° on an orbital shaker (Orbital Shaker Mark V, L.H. Engineering Co. Ltd.) for a period of 20 hr. Cells were harvested in a 'Mistral 6L' centrifuge at 6,200xg for 15 min., (Measuring and Scientific Equipment Ltd., Buckingham Gate, London, S.W.1) washed twice with 0.067 M K_2HPO_4 - KH_2PO_4 buffer, pH 7.0, and resuspended in phosphate buffer of the same molarity and pH. They were incubated with 1-2 gram amounts of 3FG in a total liquid volume of 200 ml. in a 1 litre Ehrlenmeyer on a rotary shaker at 30°. The progress of the incubation was followed by noting the gas exchange from a 2 ml. sample

Fig.9. Large scale culture vessel

The fermentation vessel, A, is equipped with a lift pump, D, attached to a rapidly reciprocating drive. A hermetic seal against contamination is provided by a rubber grommet, E. On the down-stroke the rubber valve, F, is forced open and medium enters the barrel, H. On the up-stroke the valve closes. On the subsequent down-stroke the valve opens and admits more medium. H Gradually fills and, at equilibrium, the medium is sprayed out of the adjustable gap at G. I Is a reciprocating sieve plate designed to ensure circulation of the medium. Sterile air enters the system at B and leaves by the guard tube C. The turnover of the pump at its largest stroke is about $3\frac{1}{2}$ litres per minute.


B
E
C
G
H
D
F
I
A

in the Warburg apparatus. After a known time period the cells were separated from the supernatant by centrifugation in the 'Mistral 6L', and the clear supernatant immediately freeze-dried in a 'Speedivac' Model 30P2 centrifugal freeze dryer (Edwards High Vacuum Ltd., Crawley, Sussex). All subsequent isolation procedures were carried out on the freeze-dried supernatant.

Cell-free extracts

Cells were harvested with a continuous-flow Sharples Super centrifuge separator at 25,000 r.p.m. (Sharples Centrifuges Ltd., Camberley, England). The concentrated suspension was spun in a 'Mistral 6L' centrifuge at 6,200xg for 15 min., (M.S.E. Ltd.) the cells washed twice with 0.067 M phosphate buffer, pH 7.0, and a thick paste of cells in phosphate buffer, pH 7.0, made. Cell breakage was effected using one of the following methods:

1) Hughes' press. Pseudomas fluorescens A, 3.12 could be broken by one passage through a Hughes' press[127] (Shandon Scientific Co. Ltd., London, N.W.10) previously cooled to -20°. Since the crushes obtained were highly viscous, due to unbroken DNA strands, they were diluted with an equal volume of buffer and treated at room temperature with a few crystals of deoxyribonuclease (B.D.H. Ltd.). Any unbroken cells remaining in the crush were removed by centrifugation in a M.S.E. 'super-speed 50' centrifuge at 20,000xg for 15 min. at 5°, (M.S.E. Ltd). The supernatant from this treatment contained soluble and particulate enzymes which were used as the crude cell-free preparation.
2) Eaton press (N.R. Eaton, 1962[128]), a modified Hughes' press was used in a similar manner.

3) French press. A small percentage of Ps. fluorescens could be broken only after repeated passage through an Aminco French press at 3-4,000 p.s.i. and 5° (American Instrument Co. Inc., Silver Spring, Maryland).

4) Ball-mill. Repeated shaking with glass beads (0.11 - 0.012 mm) in a Braun ball-mill (B.Braun Apparatebau, Melsungen) for varying time periods, using solid CO_2 as a coolant, produced negligible disruption of Ps. fluorescens.

5) Ultrasonication. Ultrasonic cell breakage was carried out with a magnetostrictive transducer unit (Radyne Ltd., Wokingham, Surrey) driven at 20 Kc/sec. by a 200 watt power amplifier, built by Mr.V.Gott of the department of electrical engineering from a circuit supplied by Dr. N.J. Cartwright and Dr. M.H. Jeynes of the department of bacteriology, Birmingham University. The transforming stub was of titanium and therefore of low heat conductance, and the temperature during the break was kept below 10° by a salt-ice mixture. Pseudomonas fluorescens A,312 suspended in one volume of 0.067 M phosphate buffer, pH 7.0, was homogenised and ultrasonicated for 3x2 min. periods with a short break to prevent heating. The ruptured cell suspensions obtained by this method were less viscous than crushed preparations, no DNA ase treatment was necessary, and centrifugation in the 'super-speed 50' at 20,000xg for 15 min. removed whole cells.

Chromatography

Paper chromatography was carried out on Whatman No.1 paper previously washed in 2N-acetic acid and distilled water[129]. Free sugars and aldonic acids were separated by downward elution in methanol-pyridine-0.88-ammonia-water

(5:2:1:2 v/v)[87], and detected by the benzidine-periodate method[130].

Thin layer chromatography (t.l.c.) was carried out on 20x20 cm. plates using Silica gel G according to Stahl (Anderman and Co. Ltd., London, S.E.1). After the solvent was removed from the developed plate it was sprayed with sulphuric acid/ethanol (60:40 v/v) and heated to 100° for 10 min. Aldonic acid lactones and esters were located by the formation of their hydroxamic acid derivatives[131]. All preparative thin layer plates (40x20 cm.) were prepared with Silica gel PF 254 (Shandon Scientific Co. Ltd.). After the solvent had been removed from the developed plate it was examined under u.v. (254 mμ) and the different zones eluted. The solvent systems used were ethyl acetate - acetic acid -water (3:3:1 v/v) and ethyl acetate - ethanol (4:1 v/v).

Column chromatography was carried out using Kieselgel 0.05 - 0.20 mm. Silica gel for chromatography (Anderman and Co. Ltd.). Samples were applied in ethanol and eluted with a mixture of ethyl acetate - ethanol (9:2 v/v).

The freeze-dried supernatants, obtained from incubations of washed, resting cell suspensions of Ps. fluorescens with 3FG, were applied to paper or t.l.c. plates in aqueous solution. In the case of preparative t.l.c., or column chromatography, the freeze-dried residue was extracted with cold ethanol, concentrated 'in vacuo', and applied to the plate (as a band), or the column.

Manometry

Oxygen uptake was followed by the manometric method (Umbreit, Burris, Stauffer, 1949[132]) in a Warburg respirometer (Warburg Apparatus, Model V, B.Braun, G.m.b.H Melsungen, W.Germany). 20% (w/v) KOH Was used to absorb CO_2 and Warburg conditions maintained at 30° and pH 7.0. Acid production

from 3FG by Ps. fluorescens was determined manometrically by measuring the CO_2 release from bicarbonate buffer in an atmosphere of 5% CO_2, observed values being corrected for accompanying respiratory gas exchange determined by simultaneous measurements on cells in phosphate buffer.

Estimations

1) Quantitative method of assay for dry weight of cells: a known volume (1 ml.) of the suspension was filtered onto a previously dried and weighed membrane filter (Sartorius membrane filters, V.A. Howe and Co. Ltd., London, S.W.6), washed free of suspending buffer, and the membrane and contents dried to constant weight.
2) Protein was estimated by the Lowry method[133] with bovine serum albumin (Sigma London Chemical Co. Ltd., London, S.W.6) as a protein standard. Measurements were made using a SP 500 (Unicam Instruments Ltd., Cambridge).
3) Total carbohydrate determinations on both the suspending fluid of cells and trichloroacetic acid cell extracts were made by the phenol-sulphuric acid method[134]. The phenol-sulphuric acid reagent was also found to react quantitatively with 3FG and thereby provided a means of assay for 3FG in those systems otherwise devoid of carbohydrate material. Range: 0.05-0.5 μ moles.
4) Glucose was determined by the Glucostat reagent (F. Hoffmann - La Roche and Co. Ltd., CH-4133 Schweizerhalle/Basle, Switzerland) Range: 0.05-0.5 μ moles/ml.
5) Orthophosphate was determined by the procedure of Fiske and Subbarow[135] with Elon (p-methylaminophenol sulphate) as the reducing agent.
6) Fluoride anion determinations[136] were made by coupling a model 96-09-00 fluoride electrode (Orion Research Inc., Cambridge, Massachusetts, U.S.A.) to a Radiometer Model 26 pH meter (Radiometer, Copenhagen NV Denmark) and reading the electrode potential on the

mV scale using a 10-fold expansion mode. Sample bottles were of 50 ml. capacity and made of low density polythene with screw caps of the same material (Arnold R. Horwell Ltd., London, N.W.6). They were routinely washed in a 2% solution of Decon 75 (Medical and Pharmaceutical Developments Ltd., Shoreham, Sussex), followed by two separate rinsings in distilled water. As well as measuring fluoride ion 'per se', organically bound fluorine, in both cell suspending fluids and perchloric acid cell extracts, could be estimated by the release of fluoride anion produced on alkaline hydrolysis. The procedure adopted for the estimation of organically bound fluorine was as follows.

To a 1 ml. sample of the fluorinated material in a polythene bottle 1 ml. of 10N KOH was added. After equilibration in a shaking water bath at 90°, the polythene bottle was sealed with a screw cap and the sample left to digest for 6 hours, at which time the bottle was allowed to cool to room temperature, and then shaken to ensure that any condensate at the top of the bottle was included in the sample. The bottle was opened, 1 ml. acetate-citrate buffer added, (58 gm. NaCl, 57 ml. glacial acetic acid, 0.3 gm. Na citrate, in 600 ml. of distilled water, the pH adjusted to 5.5 with 4N NaOH and the resultant solution made up to 1 litre with distilled water) and the contents adjusted to pH 5.5 by the addition of a recorded volume of 'analar' conc. hydrochloric acid. (This sequence ensured that the contents did not become strongly acid with the possible loss of hydrogen fluoride). After cooling, the sample was ready to be estimated for F^- using the electrode. Sodium fluoride was used to obtain a standard curve of mV against log, final F^- ion concentration. The advantages of this method include lack of interference by anions such as phosphate and sulphate, as well as the simple quick, digestion procedure. Effective range: 10^{-2}-10^{2} μ moles F^-/ml.

3FG and 3-Deoxy-3-fluorogluconic acid, as the calcium salt, were synthesised by Dr. N.F. Taylor and Mrs. B. Hunt, and their purity checked either by ascending silica-gel t.l.c. with ethyl acetate-acetic acid-water (3:3:1 v/v) as solvent, or by descending paper chromatography with methanol-pyridine-0.88-ammonia-water (5:2:1:2 v/v)[87] as solvent. D-Gluconic acid (potassium salt grade 111) and D-gluconic acid lactone (glucono-δ-lactone grade IX) were obtained from the Sigma London Chemical Company Ltd. Coenzymes NAD (87% purity) and NADP (85% purity) were obtained from the Boehringer Corporation (London) Ltd., London, W.5.

RESULTS

E. coli whole cell suspensions

Once standard conditions for growth and respirometric studies with this organism had been determined, preliminary experiments using a 3FG syrup supplied by Prof. A.B. Foster and Mr. R. Hems compared the rates and extents of oxygen consumption by washed, log-phase, cell suspensions respiring limiting amounts of glucose and 3FG syrup.

EXPT.1. 4x75 ml. Amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture, and incubated, with aeration at 30°, for 14 hours. Cells were collected by centrifugation at 4040xg for 10 minutes, washed once in 0.067M phosphate buffer, pH 7.0, and resuspended in phosphate buffer of the same molarity and pH to 20 mg. dry weight per ml. Fig.10 shows the oxygen uptake by these cells when furnished with limiting amounts of glucose and 3FG syrup. Glucose oxidation occurred at an immediate high rate, which was approximately linear for the first 30 minutes, and then fell off to proceed subsequently at a much reduced rate, but which again was approximately linear. Oxygen consumption by E. coli with 3FG as substrate occurred to a negligible extent (Table 4).

TABLE 4

Manometric data on the oxidation of glucose and 3FG syrup by washed suspensions of glucose-grown E. coli. (see Fig.10)

substrate oxidised	initial rate of oxidation (μ moles O_2/mg.dry wt./hr.)	net O_2 uptake (μl.) (endogenous subtracted)	moles of O_2 per mole of substrate oxidised
5 μ moles glucose	2.10	360	3.22
10 μ moles glucose	2.52	610	2.72
15 μ moles glucose	2.54	780	2.32
5 μ moles 3FG	0.26	30	0.27
10 μ moles 3FG	0.34	50	0.22
15 μ moles 3FG	0.41	46	0.14

Fig.10. Oxidation of glucose and 3FG syrup by washed suspensions of E. coli.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air

Each flask contained: 5, 10 or 15 μ moles substrate, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. 20% KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (10 mg. dry weight) from side arm.

Endogenous respiration subtracted.

Key:

—○——○——○— 15 μ moles glucose

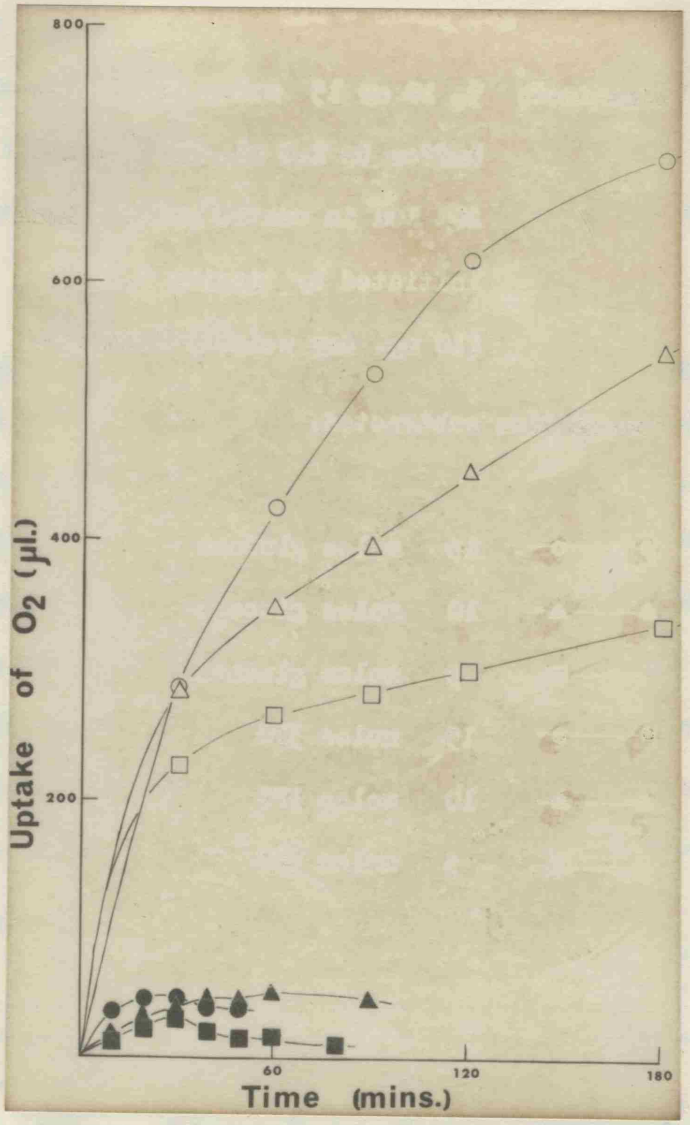
—△——△——△— 10 μ moles glucose

—□——□——□— 5 μ moles glucose

—●——●——●— 15 μ moles 3FG

—▲——▲——▲— 10 μ moles 3FG

—■——■——■— 5 μ moles 3FG


800
600
400
200
Uptake of O_2 (µl.)
60
120
180
Time (mins.)

An examination of total carbohydrate levels in cold trichloroacetic acid extracts of cells, before and after incubation with 3FG, showed that there was a drop in total cellular carbohydrate during the period of incubation parallel to that found in control cells respiring endogenously for the same period. However, in contrast to the controls, addition of glucose to cell suspensions previously treated with 3FG did not restore the level of cellular carbohydrate. Increases in total cellular carbohydrate were apparent when cells respired glucose normally (Table 5).

TABLE 5

Carbohydrate levels in cold trichloroacetic acid extracts of E. coli cell suspensions before and after incubation with 3FG syrup and/or glucose. Cells were grown in glucose/mineral salts medium, collected, washed and incubated in the Warburg apparatus with 10 μmole amounts of either 3FG or glucose for 3 hours. At this point either 1 ml. samples were removed, filtered and the cells extracted with trichloroacetic acid, or an additional 10 μ moles of glucose were added, and the incubation continued for a further 3 hours before the determination of cellular carbohydrate levels.

substrate(s)	starting level of cellular carbohydrate (μ moles glucose/ 10 mg. dry wt. of cells)	final level of cellular carbohydrate (μ moles glucose/ 10 mg. dry wt. of cells)
——————— 6 hrs.,	6.7 μ moles	5.7 μ moles
——— 3 hrs., glucose, 3 hrs.,	"	5.9
3FG, 3 hrs.,	"	5.7
3FG, 3 hrs., glucose, 3 hrs.,	"	5.6
glucose, 3 hrs.,	"	7.6
glucose, 3 hrs., glucose, 3 hrs.,	"	9.6

The effect of preincubation with 3FG syrup on subsequent glucose oxidation by washed cell suspensions is shown in Fig.11. When glucose was added, immediate maximum rates of oxygen consumption were observed with both 3FG-pretreated cells and control cells previously respiring with no added substrate. With the controls however, the initial linear rate of oxygen uptake quickly declined after 40-60 minutes, presumably due to the preferential assimilation of glucose by these starved cells into their depleted storage product (glycogen[137]). Cell suspensions preincubated with 3FG syrup oxidised glucose at a rate comparable to that of the controls; however, despite the fact that during the preincubation period total carbohydrate in 3FG-treated cells fell to the same level as that in endogenously respiring cells, the extent of subsequent glucose oxidation was greater than that observed with the controls by approximately one mole of oxygen per mole of substrate oxidised (Table 6).

TABLE 6

Manometric data on the oxidation of glucose by E. coli suspensions preincubated with 3FG syrup.

Cells, grown for 14 hours with aeration at 30° in glucose/mineral salts medium, were collected, washed once and resuspended in 0.067M phosphate buffer, pH 7.0, to 20 mg. dry weight per ml. After incubation in the Warburg apparatus with varying amounts of 3FG for 120 minutes, glucose (10 μ moles) was tipped from the side-arm and the subsequent oxygen uptake measured (Fig.11).

preincubation substrate	substrate oxidised	rate of oxidation (μmoles O_2/ mg. dry wt./hr.)	net O_2 uptake (μl.) (endogenous subtracted)	moles of O_2 per mole of substrate oxidised
———	10 μ moles glucose	2.37	430	1.92
5 μ moles 3FG	"	2.58	671	3.00
10 μ moles 3FG	"	2.40	660	2.95
15 μ moles 3FG	"	2.26	649	2.90

Fig.11. Glucose oxidation by washed suspensions of E. coli after incubation with 3FG syrup.

Washed cells, suspended to 20 mg. dry weight per ml. in 0.067M phosphate buffer, pH 7.0, were incubated in the Warburg apparatus with the additions shown below. After 120 minutes 10 μ moles of glucose were added and the subsequent oxygen consumption measured.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air

Each flask contained: 5, 10 or 15 μ moles 3FG, 10 mg. dry weight of cells, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 10 μ moles of glucose from side-arm after 120 minutes.

Endogenous respiration subtracted.

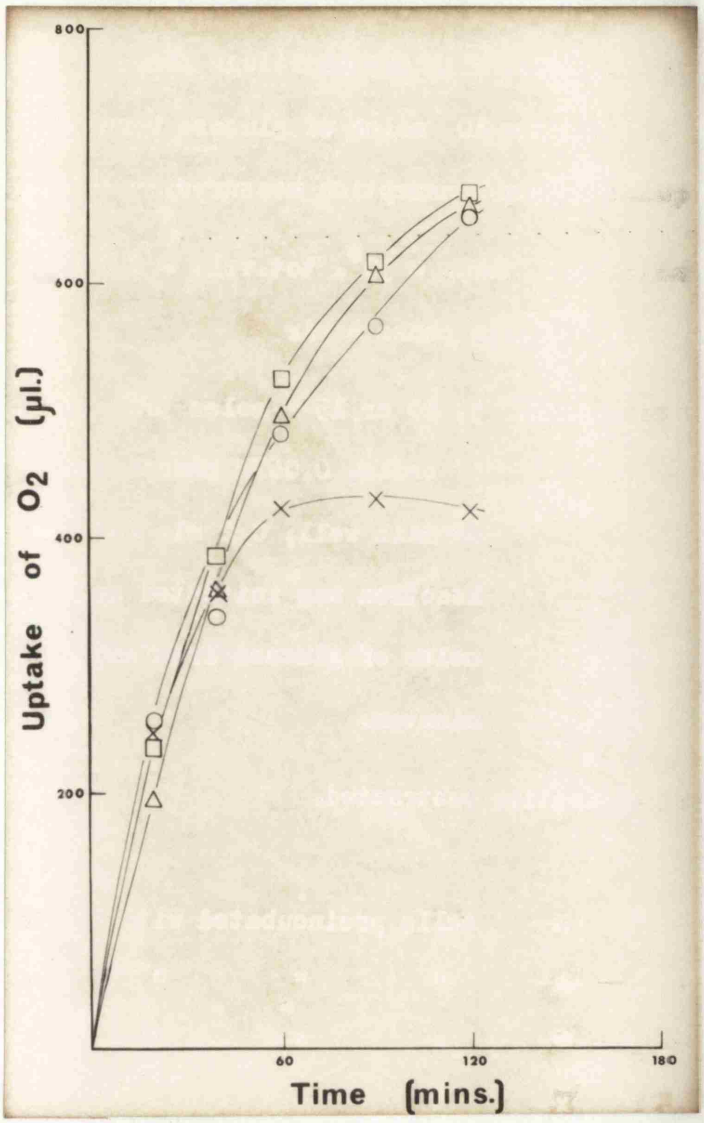
Key:

—□——□——□— cells preincubated with 5 μ moles 3FG

—△——△——△— " " " 10 μ moles 3FG

—○——○——○— " " " 15 μ moles 3FG

—×——×——×— " " endogenously


800
600
400
200
Uptake of O_2 (µl.)
60
120
180
Time (mins.)

Working with crystalline 3FG synthesised by Dr. N.F. Taylor and Mrs. B. Hunt, it was decided to examine the oxygen uptakes by washed resting cell suspensions of E. coli when given 3FG alone and 3FG at the same time as glucose.

EXPT.2. 4x75 ml. Amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture of E. coli, and incubated with aeration at 30° for 20 hours, at which time they were in early stationary phase. Cells were collected by centrifugation at 4040xg for 10 minutes, washed twice and resuspended to 22 mg. dry weight per ml. in 0.067M phosphate buffer, pH 7.0. Fig.12 shows the oxygen consumption by these cells when furnished with limiting amounts of 3FG alone and 3FG with glucose. Though there was no oxygen uptake when 3FG alone was substrate, significant increases in oxygen consumption were apparent when the 3FG, glucose mixtures were ultilised as substrate, compared with the oxygen uptake when that amount of glucose in the mixture was respired alone (Table 7). It appeared, therefore, that, either glucose was oxidised to a greater extent in the presence of 3FG, or that 3FG was capable of undergoing partial oxidation when present with glucose. However, the differences in total oxygen uptake, between cell suspensions oxidising 3FG, glucose mixtures and suspensions respiring glucose alone, were not proportional to the amounts of 3FG taken up by cells during the incubation period (as determined by the differences between starting and final carbohydrate levels in the incubation filtrates (Table 7).

Fig.12. Oxidation of glucose, 3FG, and glucose/3FG mixtures by washed suspensions of E. coli.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air

Each flask contained: 10, 50 or 100 μ moles 3FG alone or with 10 μ moles glucose, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (11 mg. dry weight) from side-arm.

Endogenous respiration subtracted.

Key:

-□—□—□- 10 μ moles glucose, 100 μ moles 3FG

-○—○—○- 10 μ moles glucose, 50 μ moles 3FG

-▲—▲—▲- 10 μ moles glucose, 10 μ moles 3FG

-△—△—△- 10 μ moles glucose

-●—●—●- 10, 50, 100 μ moles 3FG

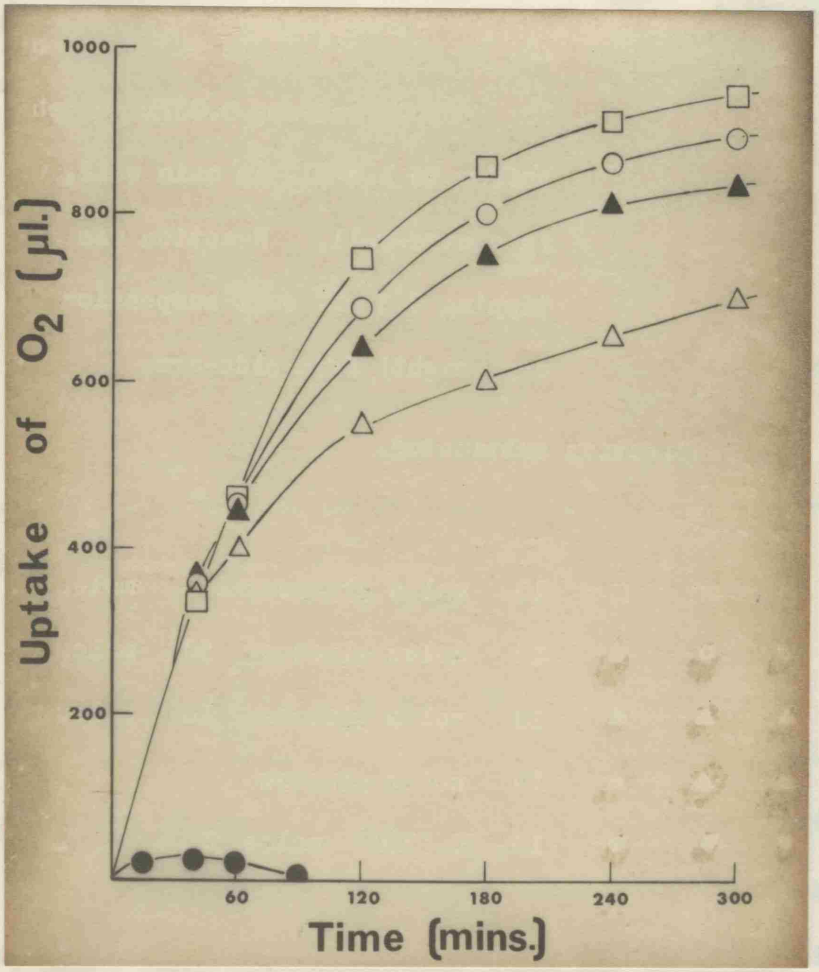

1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
240
300
Time (mins.)

TABLE 7

Manometric data on the oxidation of 3FG, glucose mixtures by washed suspensions of E. coli. (see Fig.12).

Washed cell suspensions, suspended to 22 mg. dry weight per ml. in 0.067M phosphate buffer, were furnished with limiting amounts of glucose and 3FG, simultaneously, in the Warburg apparatus. When oxidation had gone to completion (5 hours), flask contents were filtered and 1 ml. filtrate samples assayed for total carbohydrate and glucose. The differences were assumed to be a measure of residual exogenous 3FG.

substrate starting level	final level of exogenous substrate	net O_2 uptake (μl.) (endogenous subtracted)	moles of O_2 per mole of glucose oxidised
10 μmoles glucose	0.00	700	3.12
10 μmoles, 10 μmoles 3FG	9.05 μmoles 3FG	830	3.70
10 μmoles, 50 μmoles 3FG		890	3.97
10 μmoles, 100 μmoles 3FG	91.50 μmoles 3FG	940	4.20

The subsequent oxidation of glucose by cells incubated with limiting amounts of 3FG for 3 hours, and by cells respiring endogenously for the same period, is shown in Fig.13. Similar linear rates of oxygen consumption were apparent with all cell suspensions, the differences being in the total oxygen uptakes, which were greater by approximately one mole of oxygen per mole of glucose oxidised in the 3FG-treated cells (Table 8). Since these experiments were carried out in the presence of residual exogenous 3FG, cells were separated from their preincubation substrate by centrifugation, washed once in 0.067M phosphate buffer and resuspended in buffer of the same

Fig.13. Glucose oxidation by washed suspensions of E. coli after incubation with 3FG.

Washed cells, suspended to 22 mg. dry weight per ml. in 0.067M phosphate buffer, pH 7.0, were incubated in the Warburg apparatus with the additions shown below. After 180 minutes glucose (10 μ moles) was added, and subsequent oxygen consumption measured.

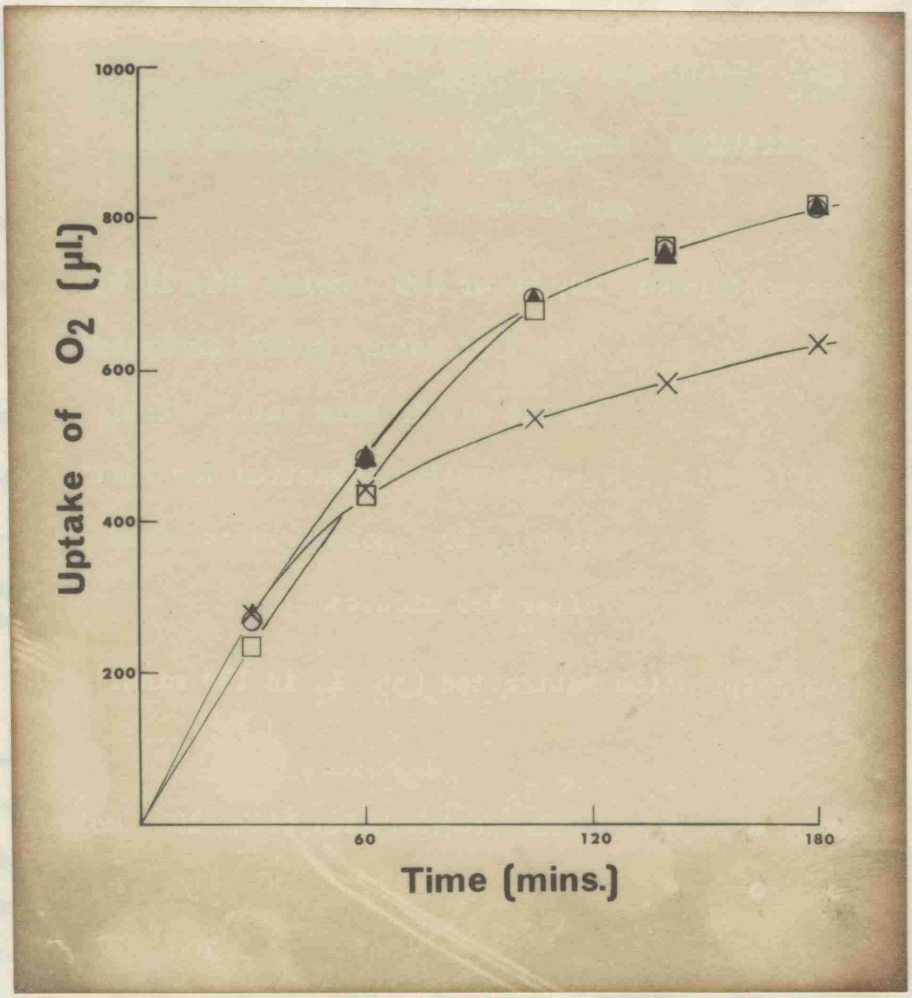
Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air

Each flask contained: 10, 50 or 100 μ moles 3FG, 11 mg. dry weight of cells, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 10 μ moles glucose from side-arm after 180 minutes.

Endogenous respiration subtracted (55 μl. in 180 mins.)

Key:

–▲——▲——▲–	cells preincubated with			10 μ moles 3FG
–o——o——o–	"	"	"	50 μ moles 3FG
–□——□——□–	"	"	"	100 μ moles 3FG
–x——x——x–	"	"	endogenously	


1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
Time (mins.)

molarity and pH, prior to the addition of glucose. However, after this treatment, the extents of subsequent glucose oxidation by the 3FG-treated cells were still greater than that by the endogenous controls by approximately one mole of oxygen per mole of glucose oxidised, and it was therefore concluded that elevated glucose oxidation was not dependent on the presence of exogenous 3FG.

TABLE 8

Manometric data on the oxidation of glucose by resting cell suspensions of E. coli preincubated with 3FG.

Cells, grown for 20 hours with aeration at 30° in glucose/mineral salts medium, were collected, washed twice and resuspended to 22 mg. dry weight per ml. in 0.067M phosphate buffer, pH 7.0. After 180 minutes incubation in the Warburg apparatus with limiting amounts of 3FG, glucose (10 μ moles) was tipped from the side-arm and the resulting oxygen uptake determined (Fig.13).

preincubation substrate	substrate oxidised	rate of oxidation (μmoles O_2/ mg. dry wt./hr.)	net O_2 uptake (μl.) (endogenous subtracted)	moles of O_2 per mole of substrate oxidised
———	10 μ moles glucose	1.80	630	2.81
10 μ moles 3FG	"	1.96	810	3.62
50 μ moles 3FG	"	1.96	810	3.62
100 μ moles 3FG	"	1.77	810	3.62

Total carbohydrate levels in cold trichloroacetic acid extracts of cells incubated with 3FG for 2 hours again showed a drop parallel with that found in cells incubated with no exogenous substrate for the same period. However, in contrast with controls, the cellular carbohydrate level in 3FG-treated cells remained the same throughout subsequent glucose metabolism (Table 9). This, together with the finding that 3FG uptake by E. coli was minimal for substrate concentrations of 5-50 mM (Table 9), suggested that cell suspensions were in the main respiring endogenously during incubation with 3FG. However, in contrast to the high degree of assimilation always observed when washed suspensions of E. coli were starved for 2-6 hours and subsequently given glucose, after exposure to 3FG for similar time periods glucose metabolism was predominately oxidative and no assimilation into the depleted carbohydrate reserves of the cell was observed.

Work with cell-free extracts of E. coli was confined to a study of whether or not this organism exhibited a cryptic response to 3FG, and the effect on glucose oxidation.

EXPT.3. 125 mls. Of glucose/mineral salts medium, suitably inoculated and incubated with aeration at 30° for 20 hours, were used to inoculate 5 l. of the same medium in a 6 l. bolt-head flask. The latter was incubated at 30° for 20 hours, aeration being provided by the Vibromischer drive unit attached to a lift pump. Cells were collected in a Sharples centrifugal separator at 25,000 r.p.m., washed twice in 0.067M phosphate buffer, pH 7.0, and resuspended in one volume of buffer of the same molarity and pH. A crude enzyme extract was prepared by subjecting the suspension to ultrasonic vibrations for 5x2 minute periods, and centrifugation at 20,000xg for 15 minutes yielded a straw-coloured supernatant containing approximately 20 mg. protein per ml. This layer was drawn off from the sedimented material and used immediately in manometric determinations.

TABLE 9

Carbohydrate levels in cold trichloroacetic acid extracts of E. coli cell suspensions before and after incubation with 3FG, and in the filtrates at the end of such incubations.

Cells were grown in glucose/mineral salts medium, collected, washed and incubated in Warburg flasks (10 mg. dry weight of cells per flask) with either 3FG or glucose for 2 hours. At this time either 1 ml. samples were removed, filtered, trichloroacetic acid cell extracts made, and both filtrates and cell extracts assayed for carbohydrate, or 10 μ moles of glucose were added and the incubations continued for a further 2 hours prior to filtration and carbohydrate determinations.

substrate (starting levels)	final level of exogenous carbohydrate	starting level of cellular carbohydrate (μmoles glucose/ 10 mg. dry wt.)	final level of cellular carbohydrate (μmoles glucose/10 mg. dry wt.)
———, 4 hrs.,	0.18 μ moles	6.7 μ moles	5.7 μ moles
——2 hrs., glucose, 2 hrs.,	0.00	6.7 μ moles	6.0 μ moles
10 μ moles 3FG, 2 hrs.,	9.05 μ moles 3FG	6.7 μ moles	5.6 μ moles
10 μ moles 3FG, 2 hrs., 10 μ moles glucose, 2 hrs.,	9.05 μ moles 3FG	6.7 μ moles	5.0 μ moles
20 μ moles 3FG, 2 hrs., 10 μ moles glucose, 2 hrs.,	19.05 μ moles 3FG		
30 μ moles 3FG, 2 hrs., 10 μ moles glucose, 2 hrs.,	29.00 μ moles 3FG	6.7 μ moles	5.6 μ moles
100 μ moles 3FG, 2 hrs., 10 μ moles glucose, 2 hrs.,	91.50 μ moles 3FG		
10 μ moles glucose, 2 hrs.,	0.00	6.7 μ moles	7.2 μ moles

Fig.14 shows the oxygen consumption by a cell-free extract of E. coli furnished with limiting amounts of glucose and 3FG, both separately and as mixtures of the natural and fluorinated substrate. Glucose alone and equimolar amounts of 3FG and glucose were oxidised at similar rates, and the total oxygen uptake in both cases approximated to one molecule of oxygen per mole of glucose oxidised (Table 10). Oxidation of limiting amounts of the fluorinated substrate alone did not proceed to any significant extent and oxygen uptakes quickly declined to control values. The addition of ATP did not stimulate oxidation of the fluoro analogue and it was concluded that cell entry was not the only contributing factor in the non-metabolism of 3FG by E. coli.

TABLE 10

Manometric data on the oxidation of 3FG and glucose by cell-free extracts of E. coli. (see Fig.14)

substrate oxidised	rate of oxidation (μmoles O_2/ mg. protein/ hr.)	net O_2 consumption (μl.) (endogenous subtracted)	moles of O_2 per mole of substrate oxidised
10μ moles glucose,	0.58	246	1.10
10μ moles glucose, 10μ moles 3FG	0.70	257	
50μ moles 3FG	0.43	116	0.10
20μ moles 3FG	0.21	73	0.16
10μ moles 3FG	0.18	44	0.20

Fig.14. Oxidation of 3FG and glucose by a cell-free extract of glucose-grown E.coli.

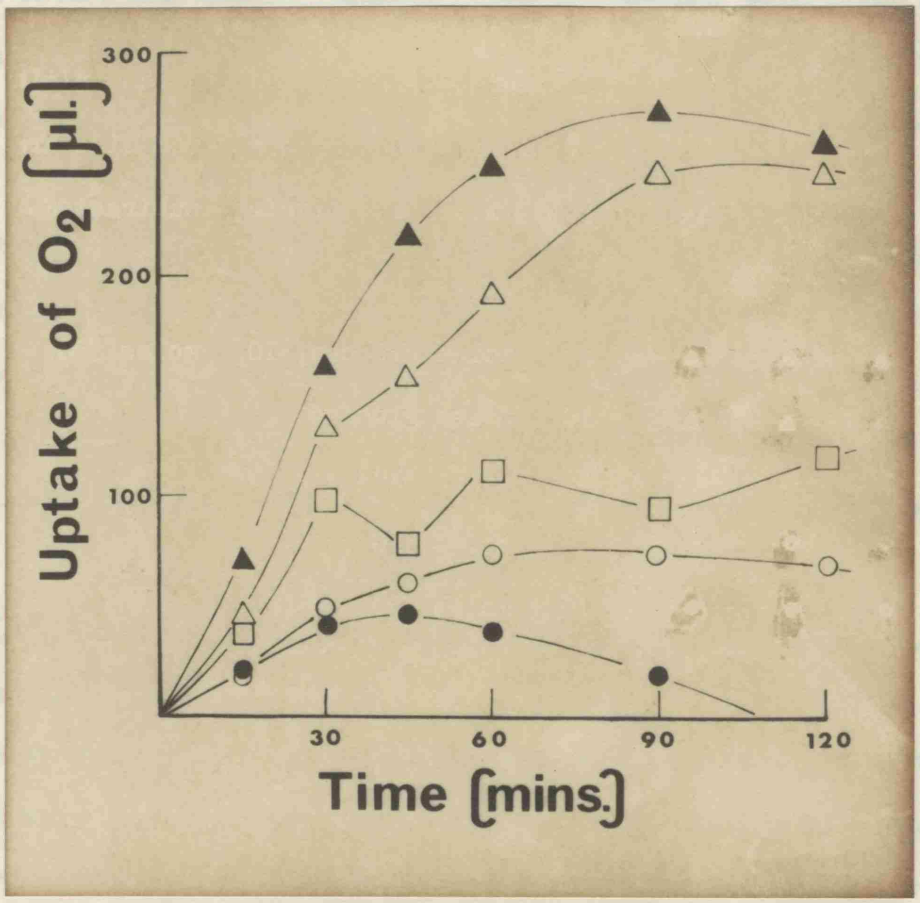
Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air

Each flask contained: 1 ml. (20 mg.) protein, 1 μ mole NAD (87% purity), 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping substrate(s) from side-arm.

Endogenous respiration (639 μl. in 140 minutes) subtracted.

Key:

–▲——▲——▲–	10 μ moles glucose, 10 μ moles 3FG
–△——△——△–	10 μ moles glucose
–□——□——□–	50 μ moles 3FG
–○——○——○–	20 μ moles 3FG
–●——●——●–	10 μ moles 3FG


Uptake of O_2 [µl.]
300
200
100
30
60
90
120
Time [mins.]

The possible effects of 3FG on the growth of E. coli in glucose/mineral salts medium were investigated by preincubation of E. coli cell suspensions for set time periods in liquid medium containing 3FG as carbon source, and comparing the subsequent growth of such cells on glucose with that by cells preincubated for the same time period with no carbon source.

EXPT.4. The growth from a 24 hour slope culture, suspended in sterile distilled water, was used to inoculate 2x25 ml. amounts of mineral salts medium in 250 ml. Ehrlenmeyer flasks, one containing 10mM 3FG as carbon source, the other with no carbon source. Both flasks were incubated with aeration at 30° for 120 minutes, and the cells, collected by centrifugation at 4040xg for 10 minutes and resuspended in one volume of 0.067M phosphate buffer, used to inoculate 2x75 ml. amounts of glucose/mineral salts medium. Growth was followed turbidimetrically at 620 mμ in a Unicam SP600. From Table 11 it can be seen that preincubation of cell suspensions with 3FG inhibited both the rate and final cell yield of subsequent growth on glucose.

TABLE 11

Effect of preincubation with 3FG on the subsequent growth of E.coli in glucose/mineral salts medium.

Growth media and measurements of growth were as described in the text. An O.D. reading of 1.0 indicates a dry weight of approximately 0.38 mg. per ml.

preincubation medium (carbon source)	growth in glucose/mineral salts (O.D. at 620 mμ)							
	2 hrs.	3 hrs.	4 hrs.	8 hrs.	9 hrs.	11 hrs.	13 hrs.	21½ hrs.
10mM 3FG	0.13	0.29	0.42	1.71	1.89	2.40	2.85	2.85
none	0.24	0.45	0.96	2.63	3.34	3.45	3.42	3.48

3FG did not support the growth of E. coli when present in liquid medium as the sole carbon source, and the addition of 3FG to a medium containing glucose as carbon source had negligible inhibitory effect on the subsequent growth pattern; on the contrary slight elevations in O.D. readings were sometimes apparent in 3FG-containing medium (Table 12).

TABLE 12

The growth of E. coli in glucose/mineral salts medium containing 3FG.
The mineral salts medium used was the same as that for bulk growth. 25 ml. Portions were sterilised in 250 ml. Ehrlenmeyer flasks, and the carbon sources, sterilised separately by filtration, were added aseptically afterwards. Growth was measured turbidimetrically on aseptically removed samples at 620 mμ in a Unicam SP600. Inocula were grown for 24 hours on slope cultures, resuspended in sterile distilled water, and one drop added to each of the test flasks which were then incubated with aeration at 30°.

carbon source		growth (O.D. at 620 mμ)						
conc'n. of glucose (mM)	conc'n. of 3FG (mM)	2 hr.	4 hr.	6 hr.	8 hr.	10 hr.	12 hr.	24 hr.
10	0	0.29	0.70	1.34	1.63	1.84	1.86	3.90
7.5	0	0.22	0.80	1.34	1.44	1.37	1.46	2.67
7.5	2.5	0.26	0.85	1.60	1.77	1.63	1.63	3.04
5.0	0	0.24	1.46	1.25	1.33	1.34	1.24	1.26
5.0	5.0	0.24	0.80	1.19	1.22	1.17	1.28	1.10
2.5	0	0.27	0.71	0.67	0.75	0.69	0.67	0.62
2.5	7.5	0.25	0.76	0.76	0.68	0.71	0.79	0.75
0	10	0.04	-	-	-	0.03	-	0.06

Since 3FG uptake by both growing cultures and resting cell suspensions of E. coli occurred to a negligible extent during the time period of the above studies, the only metabolic effect observed being one on subsequent glucose oxidation, it was decided to discontinue work with this organism and to concentrate on parallel studies with pseudomonad species, which were yielding more obvious indications of 3FG metabolism.

Ps. fluorescens A, 3.12, glucose-grown whole cell suspensions

Once the optimum conditions for growth and manometric studies with this organism had been established, preliminary experiments tested the ability of washed resting cell suspensions to oxidise 3FG.

EXPT.5. 4x75 ml. Amounts of glucose/mineral salts medium were inoculated with a suitable dilution in sterile distilled water of the growth from a 24 hour slope culture and incubated with aeration at 30° for 18 hours, at which time the cultures were in early stationary phase. Cells were collected by centrifugation at 4040xg for 10 minutes, washed twice in 0.067M phosphate buffer, pH 7.0, and resuspended to 36 mg. dry weight per ml. in buffer of the same molarity and pH. Fig.15 shows the oxygen consumption by these cells when furnished with limiting amounts of 3FG and glucose. Both substrates gave rise to an immediate uptake of oxygen the rate of which increased with substrate concentration but, in the case of 3FG, quickly declined when that value was reached which was theoretically required (one atom per mole of substrate) for a one step oxidation of the molecule (Table 13).

TABLE 13

Manometric data on the oxidation of 3FG by washed suspensions of Ps. fluorescens (see Fig.15)

substrate oxidised	rate of oxidation (μmoles O_2/ mg.dry wt./hr.	net O_2 uptake (μl.) (endogenous subtracted)	moles of O_2 per mole substrate oxidised
10 μ moles 3FG	0.28	145	0.65
20 μ moles 3FG	0.40	225	0.50
30 μ moles 3FG	0.81	360	0.53
10 μ moles glucose	1.65	870	3.88

Fig.15. Oxidation of 3FG by washed suspensions of Ps. fluorescens

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 10, 20 or 30 μ moles substrate, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (18 mg. dry weight) from side-arm.

Endogenous respiration subtracted.

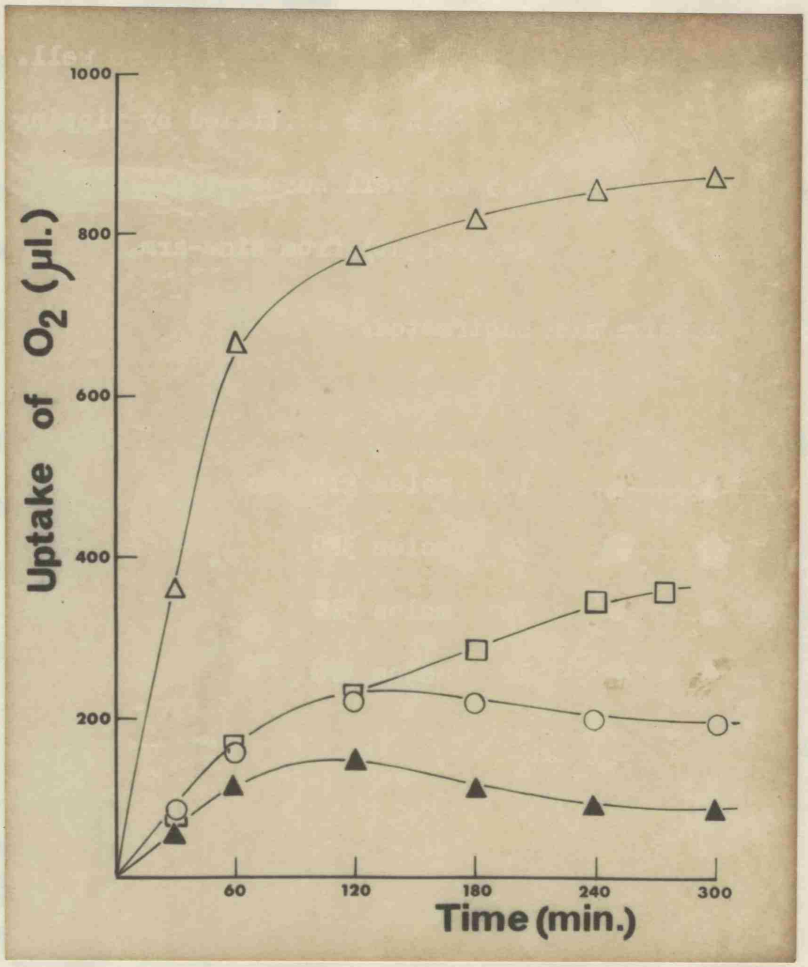
Key:

—△——△——△— 10 μ moles glucose

—□——□——□— 30 μ moles 3FG

—o——o——o— 20 μ moles 3FG

—▲——▲——▲— 10 μ moles 3FG


Uptake of O_2 (µl.)
1000
800
600
400
200
60
120
180
240
300
Time (min.)

An examination of the total carbohydrate levels in cold trichloroacetic acid extracts of cells showed that during 3FG oxidation the cellular carbohydrate content increased by an amount comparable with that in cells respiring glucose for the same period, and continued to rise when cells respired glucose subsequent to 3FG oxidation (Table 14).

TABLE 14

Carbohydrate levels in cold trichloroacetic acid extracts of Ps. fluorescens cell suspensions after 3FG and glucose oxidation.

Cells, grown in glucose/mineral salts medium, collected and washed, were incubated in the Warburg apparatus (10 mg. dry weight per flask) with 10 μ moles of 3FG or glucose. When oxidation was complete (2 hrs.) either 1 ml. samples were removed, filtered, trichloroacetic acid cell extracts made, and both extracts and filtrates assayed for carbohydrate, or additional glucose (10 μ moles) added and the incubations continued for a further 2 hours, before filtration and carbohydrate determinations.

substrate(s)	starting level of cellular carbohydrate (μmoles glucose/ 10 mg. dry wt.)	final level of cellular carbohydrate (μmoles glucose/ 10 mg. dry wt.)	final level of exogenous substrate(s)
——— 4 hrs.,	5.5 μ moles		0.0
——— 2 hrs.; glucose, 2 hrs.;	"	7.2 μmoles	0.0
glucose, 2 hrs., glucose, 2 hrs.,	"	6.6 μmoles	0.3 μmoles
glucose, 2 hrs., 3FG, 2 hrs.,	"	7.1 μmoles	0.7 μmoles
3FG, 2 hrs., glucose, 2 hrs.,	"	6.5 μmoles	4.0 μmoles
30 μmoles 3FG, 2 hrs., glucose 2 hrs.,	"	7.9 μmoles	1.4 μmoles 3FG
	"	8.3 μmoles	4.0 μmoles 3FG

Fig.16 shows a comparison of the oxidation of 10 μ moles amounts of glucose by cell suspensions which had previously oxidised to completion varying amounts of 3FG, with that by cells previously respiring endogenously, and also glucose oxidation by cells which had already respired 10 μ moles of glucose. The oxidation of 3FG had only slight effect on the rate of subsequent glucose oxidation, but there was a decrease in the total oxygen consumed directly related to the concentration of 3FG previously oxidised, which also occurred when gluconic acid (Fig.17) or pyruvic acid were added subsequent to 3FG oxidation (Table 15).

TABLE 15

Manometric data on the oxidation of glucose and gluconate by Ps. fluorescens cell suspensions after 3FG oxidation.

Cells, grown for 20 hours with aeration at 30° in glucose/mineral salts, were washed twice and resuspended to 20 mg. dry weight per ml. in 0.067M phosphate buffer, pH 7.0, and their ability to oxidise varying amounts of 3FG tested manometrically. When the rate of oxygen consumption had fallen to that of control cells (180 minutes), 10 μ moles of either glucose or gluconate were tipped from the side-arm, and the subsequent oxygen uptake determined (Figs.16 and 17).

preincubation substrate	substrate oxidised	rate of oxidation (μ moles O_2/ mg. dry wt./hr.)	net O_2 uptake (μl.) (endogenous subtracted)	moles of O_2 per mole of substrate oxidised
———	10 μ moles glucose	1.21	675	3.01
10 μ moles 3FG	"	1.22	630	2.81
20 μ moles 3FG	"	1.08	600	2.68
30 μ moles 3FG	"	0.93	530	2.37
———	10 μ moles gluconate	1.41	625	2.79
10 μ moles 3FG	"	1.18	590	2.63
20 μ moles 3FG	"	0.94	520	2.32
30 μ moles 3FG	"	0.79	415	1.85

Fig.16. Glucose oxidation by washed cell suspensions of Ps. fluorescens subsequent to 3FG oxidation

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 10, 20, or 30 μ moles 3FG, 10 mg. dry weight of cells, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 10 μ moles glucose from side-arm after 180 mins.

Endogenous respiration subtracted.

Key:

	preincubation substrate
—△——△——△—	10 μ moles glucose
—▲——▲——▲—	10 μ moles 3FG
—o——o——o-	20 μ moles 3FG
—□——□——□-	30 μ moles 3FG
—X——X——X-	endogenous control

Fig.17. Gluconate oxidation by washed cell suspensions of Ps. fluorescens subsequent to 3FG oxidation

Warburg conditions: as above

Each flask contained: as above. Reaction was initiated by tipping 10 μ moles gluconic acid from side-arm after 180 mins.

Key:

	preincubation substrate
—△——△——△—	10 μ moles glucose
—▲——▲——▲-	10 μ moles 3FG
—o——o——o-	20 μ moles 3FG
—□——□——□-	30 μ moles 3FG
—X——X——X-	endogenous control

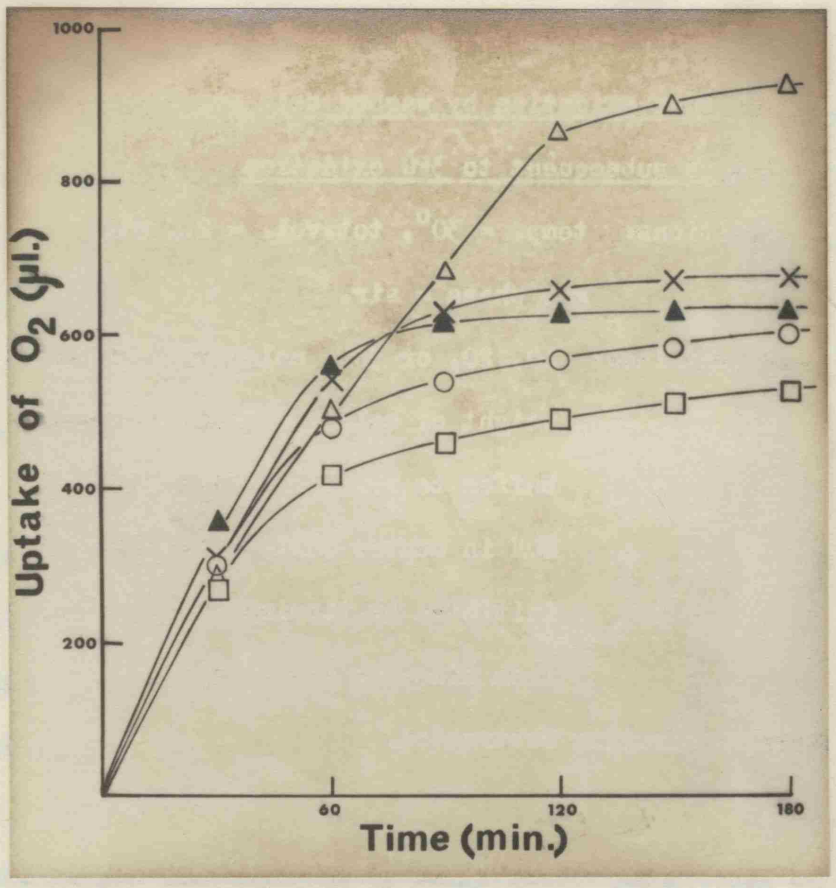

1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
Time (min.)

Fig.16

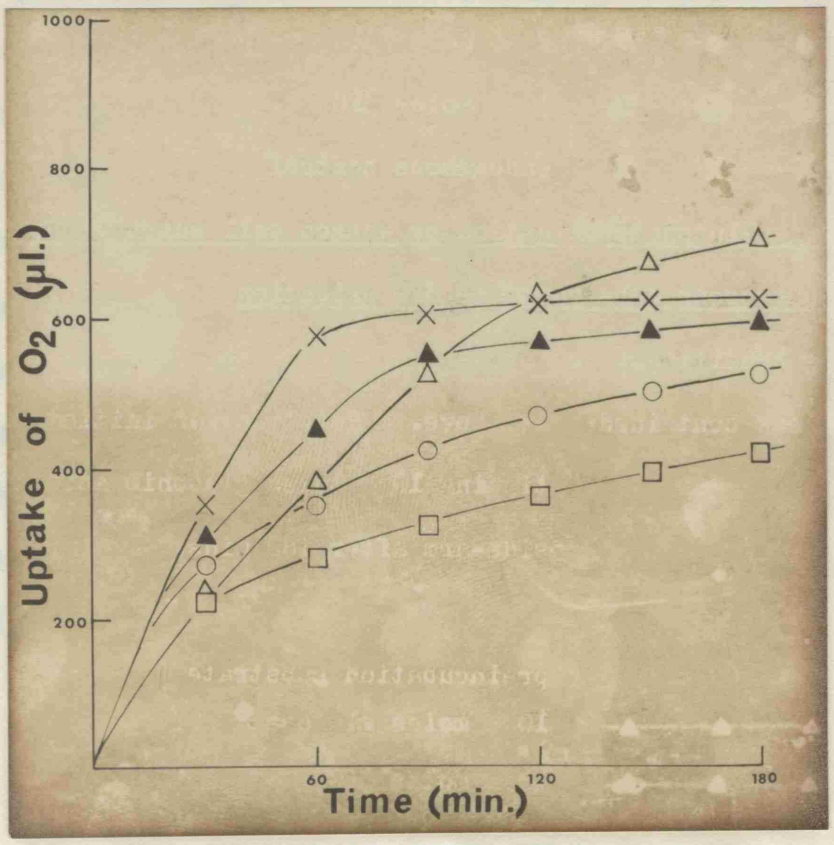

1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
Time (min.)

Fig.17

The reduction in the extent of subsequent glucose and gluconate oxidation by cell suspensions after 3FG oxidation prompted an investigation of the oxygen consumption by washed suspensions respiring 3FG and glucose, and 3FG and gluconate, together.

EXPT.6. 4x75 ml. Amounts of glucose/mineral salts medium, suitably innoculated from a 24 hour slope culture, were grown with aeration at 30° for 20 hours. Cells were collected by centrifugation at 4040xg for 10 minutes, washed twice and resuspended to 18 mg. dry weight per ml. in 0.067M phosphate buffer, pH 7.0. Figs. 18 and 19 show the oxygen uptake by cells furnished with limiting amounts of glucose, 3FG and gluconate, 3FG mixtures respectively. In contrast to the reductions in extents of glucose and gluconate oxidation subsequent to 3FG oxidation shown in Figs.16 and 17, there were marked increases in the total oxygen consumptions by cells respiring glucose or gluconate at the same time as 3FG, compared with the total oxygen uptakes for those amounts of glucose or gluconate respired alone. From Table 16 the increase in oxygen consumption can be seen to approximate very closely the theoretical value required for a one step oxidation of the 10 and 50 μ mole amounts of 3FG (indeed the oxygen uptake for 10 μ moles of 3FG when present with an equal amount of glucose approached a value corresponding to one mole of oxygen per mole of the fluoro analogue). However, when 100 μ moles of 3FG were present the initial rapid rate of oxidation levelled off after approximately 3 hours, and then swiftly declined to a value well below endogenous control levels. The increased extent of oxidation at this concentration of 3FG never reached a value corresponding to one atom of oxygen per mole of substrate (Table 16).

Fig.18. Oxidation of 3FG, glucose mixtures by glucose-grown washed cell suspensions of Ps. fluorescens.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 10 μ moles glucose, 10, 50 or 100 μ moles 3FG, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (9 mg. dry weight) from side-arm.

Endogenous respiration subtracted (526 μ l. in 5 hr.)

Key:

—□——□——□— 10 μ moles glucose, 100 μ moles 3FG

—o——o——o— 10 μ moles glucose, 50 μ moles 3FG

—▲——▲——▲— 10 μ moles glucose, 10 μ moles 3FG

—△——△——△— 10 μ moles glucose

Fig.19. Oxidation of 3FG, gluconate mixtures by glucose grown washed cell suspensions of Ps. fluorescens

Warburg conditions: as above

Each flask contained: 10 μ moles gluconic acid, 10, 50 or 100 μ moles 3FG, and as above

Endogenous respiration subtracted

Key:

—□——□——□— 10 μ moles gluconate, 100 μ moles 3FG

—o——o——o— 10 μ moles gluconate, 50 μ moles 3FG

—▲——▲——▲— 10 μ moles gluconate, 10 μ moles 3FG

—△——△——△— 10 μ moles gluconate

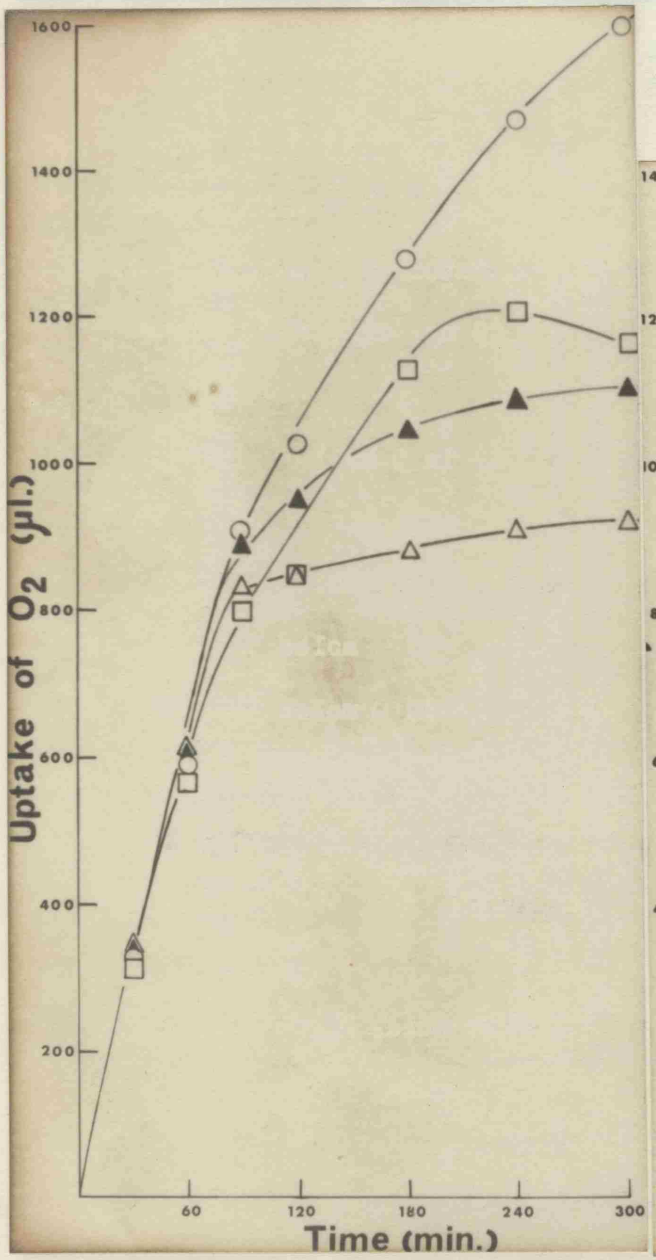

Uptake of O_2 (µl.)
1600
1400
1200
1000
800
600
400
200
60
120
180
240
300
Time (min.)

Fig. 18

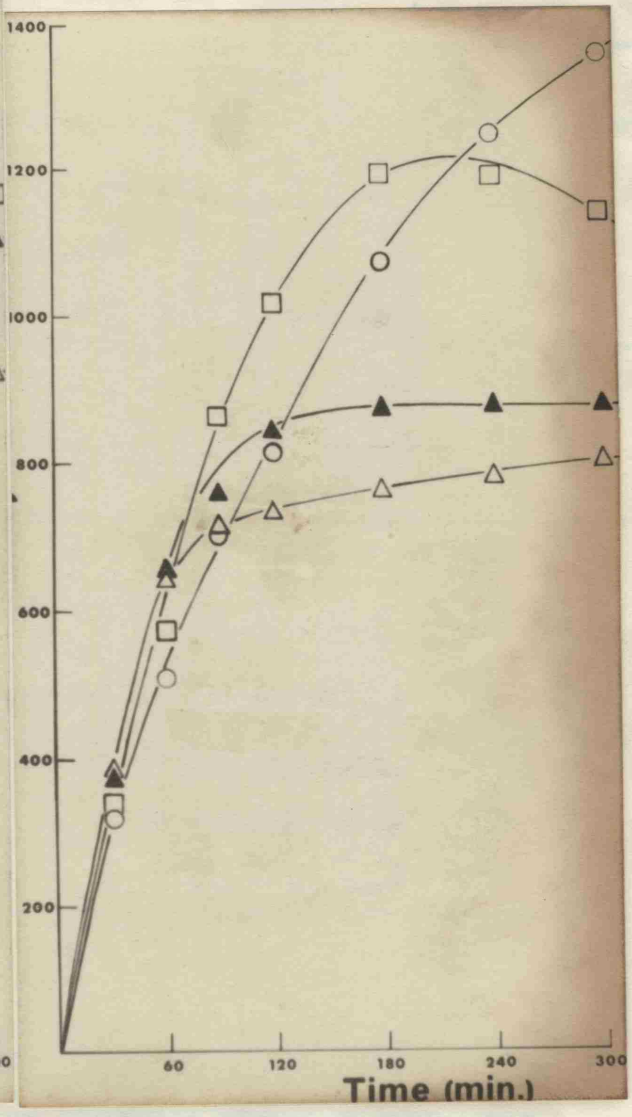

1400
1200
1000
800
600
400
200
60
120
180
240
300
Time (min.)

Fig. 19

TABLE 16

Manometric data on the oxidation of 3FG, glucose and 3FG, gluconate mixtures by washed suspensions of Ps. fluorescens. (see Figs.18 and 19)

	observed value (µl.)	moles of O_2 per mole of substrate oxidised
endogenous O_2 uptake	526	
*net O_2 uptake 10 µ moles glucose	920	4.10
*net O_2 uptake 10 µ moles glucose, 10 µ moles 3FG	1090	
*net O_2 uptake 10 µ moles glucose, 50 µ moles 3FG	1550	
*net O_2 uptake 10 µ moles glucose, 100 µ moles 3FG	1200	
•net O_2 uptake 10 µ moles 3FG	170	0.76
•net O_2 uptake 50 µ moles 3FG	630	0.56
•net O_2 uptake 100 µ moles 3FG	285	0.13
*net O_2 uptake 10 µ moles gluconate	800	3.57
*net O_2 uptake 10 µ moles gluconate, 10 µ moles 3FG	875	
*net O_2 uptake 10 µ moles gluconate, 50 µ moles 3FG	1355	
*net O_2 uptake 10 µ moles gluconate, 100 µ moles 3FG	1210	
°net O_2 uptake 10 µ moles 3FG	75	0.335
°net O_2 uptake 50 µ moles 3FG	555	0.50
°net O_2 uptake 100 µ moles 3FG	410	0.18

* endogenous O_2 uptake subtracted.

• endogenous O_2 uptake and O_2 uptake on 10 µ moles glucose subtracted.

° endogenous O_2 uptake and O_2 uptake on 10 µ moles gluconate subtracted.

It appeared therefore that there was an upper limit to the concentration of 3FG capable of being utilised as substrate by Ps. fluorescens, and so, returning to respirometric studies with 3FG alone, the ability of washed cell suspensions to oxidise concentrations of 3FG between 0.01M and 0.05M was tested.

EXPT.7. Cells, grown for 20hours with aeration at 30° in glucose/mineral salts medium, were collected by centrifugation at 4040xg for 10 minutes, washed twice in 0.067M phosphate buffer, pH 7.0, and resuspended in buffer of the same molarity and pH to 20 mg. dry weight per ml. Their ability to oxidise 3FG was determined manometrically. Fig.20 shows that, although for all concentrations there was an immediate maximum rate of oxidation which increased with substrate concentration, the total oxygen uptake by cells respiring 0.05M 3FG was below that by cells oxidising 0.04M 3FG, and for 3FG concentrations above 0.03M the total oxygen consumption no longer approached a value approximating to one atom of oxygen per mole of substrate (Table 17). It was also evident that once the maximum level of oxygen uptake had been reached for concentrations of 3FG above 0.03M, there was a cessation not only of exogenous substrate oxidation but also of endogenous respiration.

Glucose oxidation subsequent to the oxidation of 0.01-0.05M 3FG by washed cell suspensions is shown in Fig.21, and it is to be noted that prior oxidation of 0.04M and 0.05M 3FG totally supressed subsequent glucose oxidation (Table 18). After filtration, the suspending buffer from such incubations was assayed for both glucose and total carbohydrate, and the differences between the two values taken as a measure of the amount of residual 3FG.

Fig.20. Oxidation of 3FG by glucose-grown washed cell suspensions of Ps. fluorescens.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 20-100 μ moles substrate, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (10 mg. dry weight) from side-arm.

Key:

—X—X—X— 100 μ moles 3FG (endogenous respiration not substrated)

—▲—▲—▲— 100 μ moles 3FG*

—□—□—□— 80 μ moles 3FG*

—o—o—o— 60 μ moles 3FG*

—△—△—△— 40 μ moles 3FG*

—●—●—●— 20 μ moles 3FG*

*endogenous respiration subtracted (450 μl. in 4 hrs.).

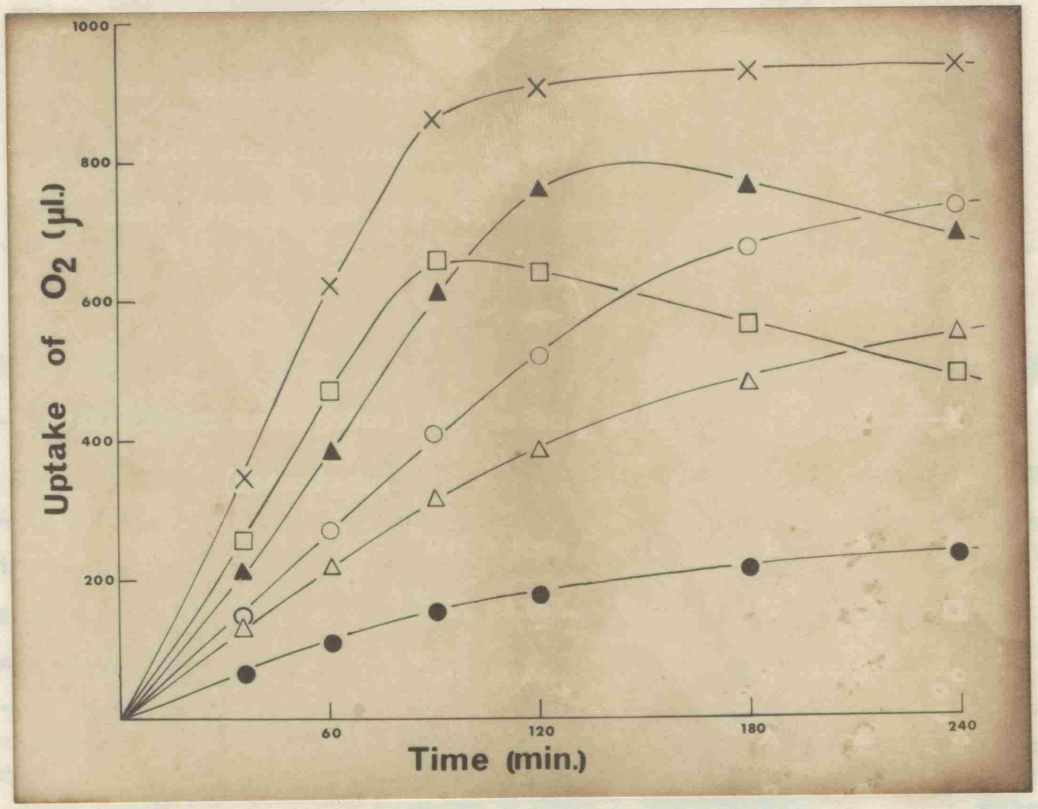

1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
240
Time (min.)

TABLE 17

Manometric data on the oxidation of 0.01M-0.05M 3FG by washed suspensions of Ps. fluorescens. (see Fig.20)

substrate oxidised	rate of oxidation (μmoles O_2/mg.dry wt./hr.)	net O_2 uptake (μl.)^2(endogenous subtracted)	moles of O_2 per mole of substrate oxidised
20 μ moles 3FG	0.45	233	0.52
40 μ moles 3FG	0.93	550	0.51
60 μ moles 3FG	1.22	735	0.55
80 μ moles 3FG	1.81	787	0.44
100 μ moles 3FG	1.945	655	0.29

TABLE 18

Manometric data on the oxidation of glucose by Ps. fluorescens cell suspensions after 3FG oxidation.

Cells grown in glucose/mineral salts medium, collected and washed as before in 0.067M phosphate buffer were resuspended to 24 mg. dry weight per ml. and their ability to oxidise 3FG determined manometrically. When substrate had been oxidised to completion (3 hours), 10 μ moles of glucose were added from the side-arm, and the subsequent oxygen consumption determined (Fig.21).

preincubation substrate	substrate oxidised	rate of oxidation (μmoles O_2/mg. dry wt./ml.)	net O_2 uptake (μl.)^2(endogenous) subtracted)	moles of O_2 per mole of substrate oxidised
———	10 μ moles glucose	3.05	890	3.97
20 μ moles 3FG	"	2.40	840	3.75
40 μ moles 3FG	"	2.23	790	3.53
60 μ moles 3FG	"	1.83	690	3.08
80 μ moles 3FG	"	0	95	0.43
100 μ moles 3FG	"	0	0	0

Fig.21. Glucose oxidation by Ps. fluorescens cell suspensions after oxidation of 20-100 μ moles 3FG.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml.
gas phase = air.

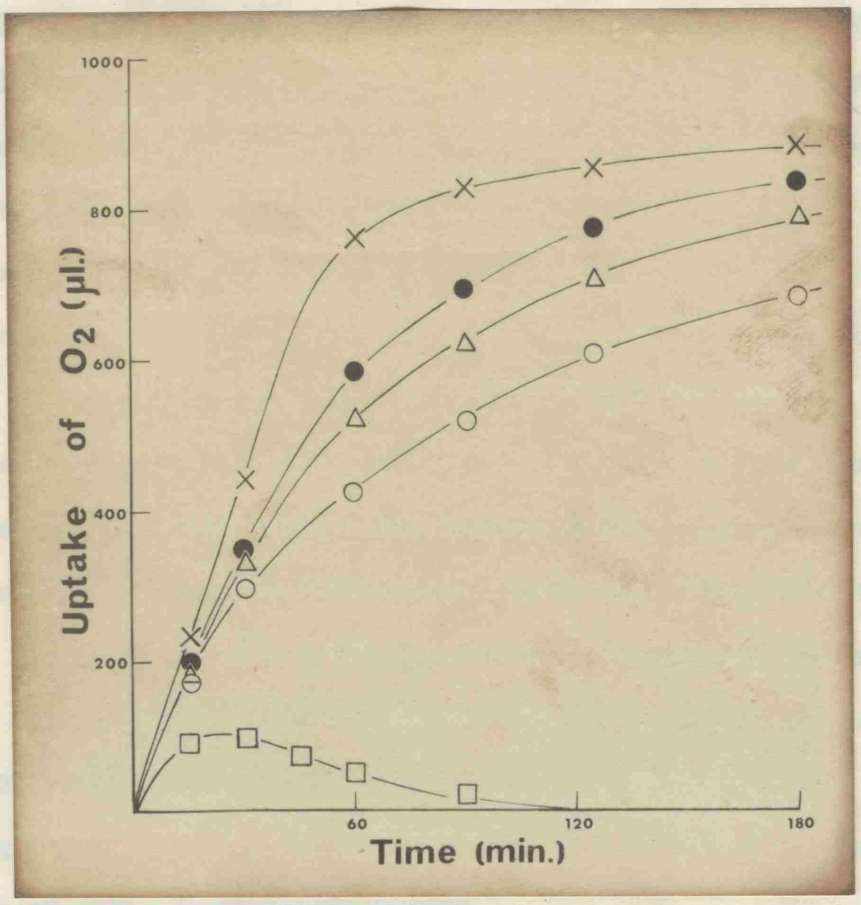
Each flask contained: 20-100 μ moles 3FG, 12 mg. dry weight of cells, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 10 μ moles glucose from side-arm when 3FG oxidation had gone to completion (180 mins.).

Endogenous respiration subtracted (335 μl. in 3 hrs.).

Key:

	preincubation substrate
—●——●——●—	20 μ moles 3FG
—△——△——△—	40 μ moles 3FG
—o——o——o—	60 μ moles 3FG
—□——□——□—	80 μ moles
—X——X——X—	nothing

no subsequent glucose oxidation by cells preincubated with 100 μ moles 3FG


1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
Time (min.)

From Table 19 it can be seen that when the exogenous 3FG remaining in the suspending buffer after bacterial oxidation was subtracted from the amount of fluorinated substrate originally present, and the difference taken to represent the true amount of substrate taken up by cell suspensions, then for all starting concentrations of 3FG the extent of oxidation by Ps. fluorescens approximated one atom of oxygen per mole of substrate.

Since 3FG oxidation by Ps. fluorescens occurred with an immediate maximum rate of oxygen uptake (Figs.15 and 20), and no adaptive lag was observed with glucose-grown cell suspensions, it was assumed that 3FG oxidation occurred by virtue of the constitutive system of hexose metabolism in this organism i.e. a direct oxidation by particle-bound enzymes of the cytoplasmic membrane to aldonic acid and ketoaldonic acid without the intervention of phosphorylated intermediates[138,139]. Because the total oxygen consumption by cell suspensions oxidising 3FG indicated that the analogue was oxidised only to the level of an aldonic acid, it was decided to look for the presence of acid in the suspending buffer of cells incubated with 3FG. Acid production was determined manometrically by measuring the CO_2 released from bicarbonate buffer, observed values being corrected for the accompanying respiratory gas exchange, which was determined by simultaneous measurements on cells in phosphate buffer.

TABLE 19

Carbohydrate levels in Warburg filtrates after 3FG and glucose metabolism by washed suspensions of Ps. fluorescens.

Cells grown in glucose/mineral salts medium, collected and washed, were incubated in Warburg flasks (10 mg. dry weight per flask) with 20-100 μ moles 3FG for 3 hour periods. After this time either, 1 ml. samples were removed, filtered and the filtrates assayed for both glucose and total carbohydrate or, additional glucose was added and the incubations continued for a further 3 hour period, prior to filtration and carbohydrate assay.

substrate(s) starting levels	period of incubation	net O_2 consumed (μl.) (endogenous subtracted)	final level(s) of exogenous substrate(s) (μ moles)	cell-bound substrate (μmoles per 10 mg. dry wt.)	moles of O_2 per mole of substrate utilised
————	6 hours	0	0	0	0
————	3 hours	0	0	0	0
10 μ moles glucose	3 hours	890	0.50	9.50	4.18
20 μ moles 3FG	3 hours	166	5.35	14.65	0.51
20 μ moles 3FG	3 hours	144	1.98	18.02	0.36
10 μ moles glucose	3 hours	842	0.20	9.80	3.84
40 μ moles 3FG	3 hours	332	11.72	28.28	0.52
40 μ moles 3FG	3 hours	441	2.35	37.65	0.52
10 μ moles glucose	3 hours	792	0.20	9.80	3.62
60 μ moles 3FG	3 hours	625	12.82	47.18	0.59
60 μ moles 3FG	3 hours	652	8.00	52.00	0.56
10 μ moles glucose	3 hours	687	0.40	9.60	3.20
80 μ moles 3FG	3 hours	712	14.00	66.00	0.48
80 μ moles 3FG	3 hours	792	18.00	62.00	0.57
10 μ moles glucose	3 hours	95	1.90	8.10	0.52
100 μ moles 3FG	3 hours	661	34.00	66.00	0.45
100 μ moles 3FG	3 hours	935	21.60	78.40	0.53
10 μ moles glucose	3 hours	0	8.10	1.90	0

EXPT.8. Cells, grown for 18 hours in glucose/mineral salts medium with aeration at 30°, were collected by centrifugation at 4040xg for 10 minutes and divided into two equal portions. One batch was washed twice and resuspended to 20 mg. dry weight per ml. in 0.067M phosphate buffer, pH 7.0, and the other portion was similarly treated in 6.6×10^{-3}M bicarbonate buffer.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml.,
gas phase = air or 5% (v/v) CO_2,

Each flask contained: 30 μ moles substrate, and either: (a) 6.6×10^{-3}M bicarbonate buffer to 2.0 ml. in main well, (b) 0.067M phosphate buffer to 2.0 ml. in main well, or (c) 0.067M phosphate buffer to 1.8 ml. in main well and 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension, (10 mg. dry weight) in appropriate buffer, from side-arm.

It is evident from Table 20 that, although the total oxygen consumption by cells respiring 3FG was very close to one atom per mole of substrate, the equivalent of acid produced per mole of 3FG oxidised was short of the value needed to satisfy the stoichiometry of the reaction:

$$3FG + \tfrac{1}{2} O_2 \longrightarrow \text{3-deoxy-3-fluoroaldonic acid}$$

However, since only acid released into the suspending buffer was assayed by this method, it seemed reasonable to assume that not all of the product was being detected and that the remainder was either intracellular or bound to the cytoplasmic membrane.

TABLE 20

Manometric data on the oxidation of 3FG by glucose-grown washed cell suspensions of Ps. fluorescens.

3FG oxidised : 30 μ moles (see text for experimental conditions)

	observed value (μl.)	moles of O_2 per mole of 3FG oxidised
O_2 uptake, endogenous	527	
O_2 uptake with substrate	885	
Net O_2 uptake with substrate	358	0.53
CO_2 output, endogenous	270	
CO_2 output with substrate	272	
Net CO_2 output with substrate	2	0.00
Acid formation with substrate	251	0.37

It has already been noted from Fig.20 that, for concentrations of 3FG approaching 0.05M, once a critical level of oxygen uptake had been reached, not only further oxidation of the exogenous substrate, but also endogenous respiration, ceased and there was no futher oxygen consumption by cell suspensions. Since acid was now known to be produced as a result of 3FG oxidation by Ps. fluorescens, the pH of the suspending buffer was monitored during oxidation of the fluoro analogue.

It is evident from Fig.22 that, for values of 3FG above 60 μ moles in a total liquid volume of 2.0 ml. (0.03M), the amount of acid released as a result of substrate oxidation by washed cell suspensions was sufficent to produce a drop in pH of 0.067M phosphate buffer, originally at pH 7.0, to pH 4.0. That this drop in the external pH was sufficient to account for the subsequent drastic changes in the oxidative ability of cell suspensions, was shown by simultaneous measurements of oxygen uptake by washed cells respiring glucose at various values of pH, when a complete cessation of both exogenous and endogenous oxygen consumption was observed at pH 4.0 (Table 21).

Fig.22. Changes in pH of the suspending buffer in Warburg vessels during the course of 3FG oxidation by washed suspensions of Ps. fluorescens

Cells grown in glucose/mineral salts medium with aeration at 30° for 21 hrs., collected washed twice, and resuspended to 19 mg. dry weight per ml. in 0.067M phosphate buffer, pH 7.0.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 20-100 μ moles 3FG, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (9.5 mg. dry weight) from side-arm.

After 4 hrs. the pH of flask contents was determined and plotted against starting levels of 3FG.

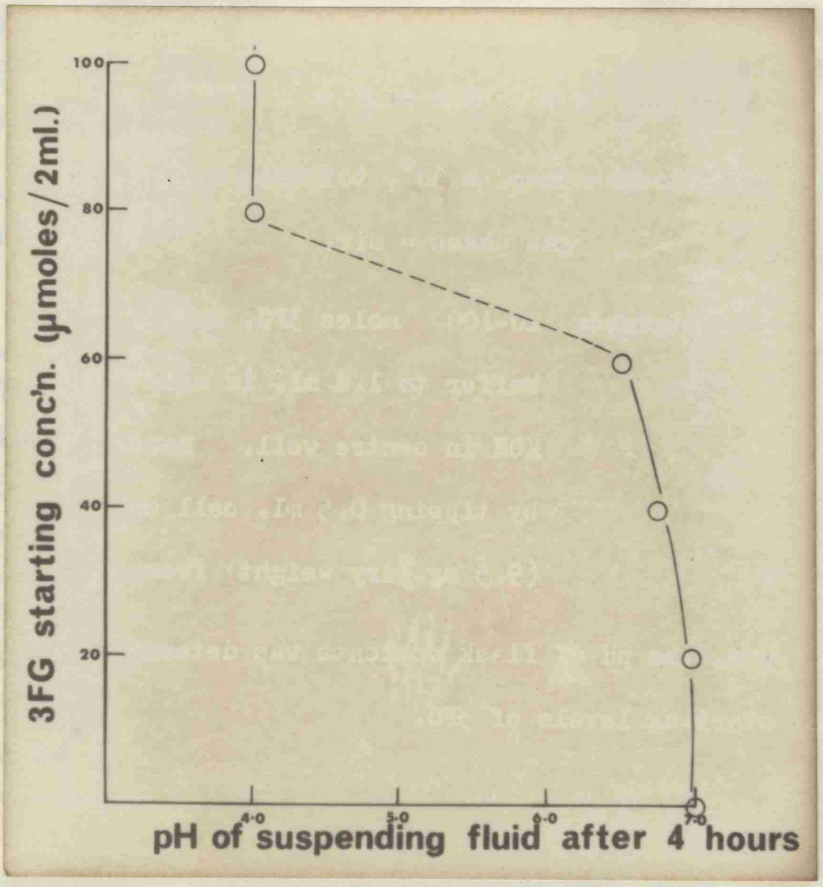

3FG starting conc'n. (µmoles/2ml.)
100
80
60
40
20
4·0
5·0
6·0
7·0
pH of suspending fluid after 4 hours

TABLE 21

Effect of external pH on glucose oxidation by washed suspensions of Ps. fluorescens.

Cells, grown for 20 hours in glucose/mineral salts medium with aeration at 30°, were collected by centrifugation and divided into four equal pellets, each of which was washed twice and resuspended to 16 mg. dry weight per ml. in either 0.067M or 0.67M phosphate buffer of the appropriate pH. Their ability to oxidise glucose was estimated manometrically.

glucose oxidised : 10 μ moles

buffer molarity	pH	O_2 uptake, endogenous (μl./mg.dry wt./hour)	O_2 uptake with substrate (μl./mg.dry wt./hour)	net O_2 uptake with substrate (μl./mg.dry wt./hour)	inhibition (per cent)
0.067M	7.0	6.95	21.33	14.38	0.0
	6.0	6.73	21.47	14.73	0.0
	5.0	5.73	20.33	14.60	0.0
	4.0	0.00	0.00	0.00	100
0.67M	7.0	1.85	11.80	9.95	47.5
	6.0	3.08	21.80	18.73	0.0
	5.0	1.72	2.61	0.89	95.0
	4.0	1.07	1.14	0.08	99.5

Because of the acid conditions produced in incubation vessels containing high 3FG concentrations, cell damage and possible lysis and death were looked for, by both release of UV-absorbing material into the suspending buffer, and colony counts on serial dilutions of bacterial suspensions after 3FG oxidation.

EXPT.9a. Cells, grown for 20 hours with aeration at 30° in glucose/mineral salts medium from a standard inoculum, were collected by centrifugation at 4040xg for 10 minutes, washed twice and resuspended, as before, in 0.067M phosphate buffer, pH 7.0, to 20 mg. dry weight per ml., prior to incubation in the Warburg apparatus.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml.,
gas phase = air

Each flask contained: 20-100 μ moles 3FG, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (10 mg. dry weight) from side-arm.

For the measurement of UV-absorbing material, flask contents were filtered to remove cells at selected times during the incubation, and the filtrates examined spectrophotometrically in a Unicam SP800 against a water-blank. Figs.23 and 24 show typical absorption spectra of filtrates from both endogenous and 3FG-respiring incubations. Both sets of filtrates showed the presence of material with a maximum absorption at 260 mμ, a minimum absorption at 240 mμ, and which reacted positively with the orcinol reagent. This material was therefore concluded to consist of RNA, or RNA degradation products, or both. The presence of UV-absorbing material in the suspending fluid of endogenously respiring pseudomonads has been observed by previous workers and was concluded to be mostly the result of intracellular metabolism rather than of cell lysis i.e. directly related to endogenous respiration[140]. The increased quantities of this material in the suspending buffer of 3FG-oxidising cell suspensions compared with that from endogenously respiring cells over the same time period, and the correlation between the

Fig.23. E260-Absorbing material in the suspending fluid of washed suspensions of Ps. fluorescens after 3FG metabolism.

For Warburg conditions and flask contents see text. After 4 hrs. undiluted flask filtrates were examined in a Unicam SP800 against a water blank.

Key:

—▲———▲— 100 μ moles 3FG

—□———□— 80 μ moles 3FG

—o———o— 60 μ moles 3FG

—△———△— 40 μ moles 3FG

—●———●— 20 μ moles 3FG

—X———X— endogenous control

Fig.24. Increase of E260-absorbing material with time in the suspending fluid of 3FG-metabolising and endogenously respiring Ps. fluorescens cell suspensions.

For Warburg conditions and flask contents see text. Undiluted flask filtrates were examined in a Unicam SP800 against a water blank.

Key:

—△———△— 100 μ moles 3FG, 4 hrs.

—□———□— 100 μ moles 3FG, 2 hrs.

—o———o— 100 μ moles 3FG, 0 hrs.

—▲———▲— endogenously respiring 4 hrs.

—■———■— endogenously respiring 2 hrs.

—●———●— endogenously respiring 0 hrs.

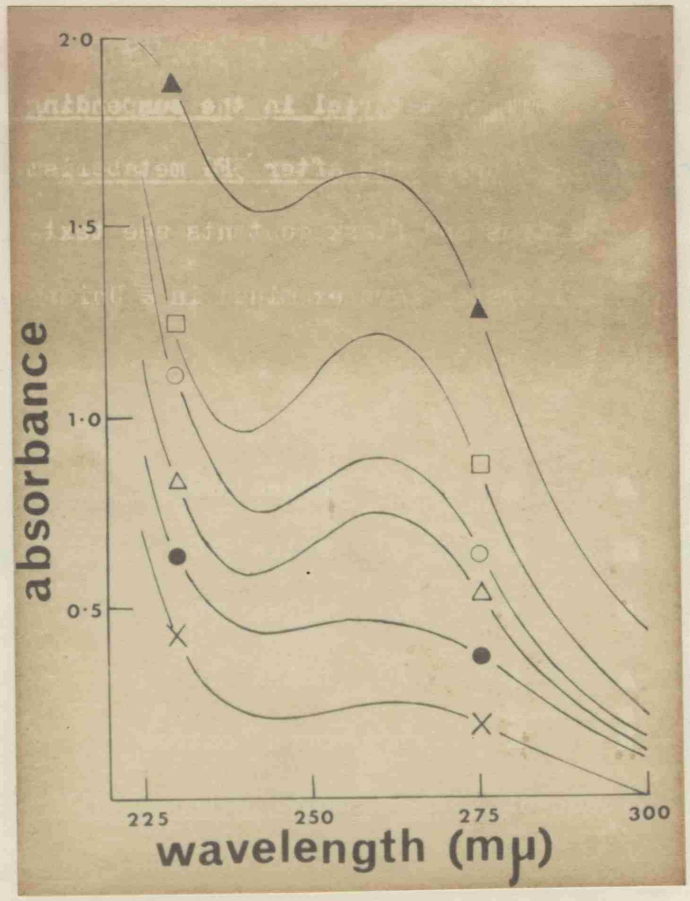

2·0
1·5
1·0
0·5
absorbance
225
250
275
300
wavelength (mμ)

Fig.23

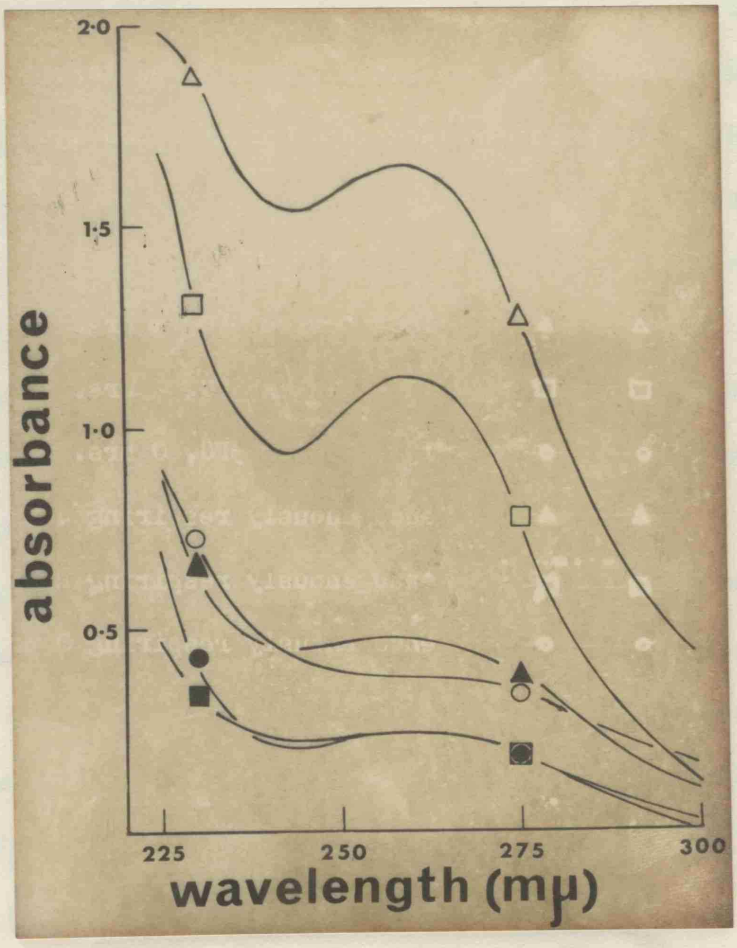

2·0
1·5
1·0
0·5
absorbance
225
250
275
300
wavelength (mμ)

Fig24

amount released, the quantities of 3FG oxidised, and the period of incubation indicated by Figs.23 and 24, suggested that the release of this material from such cells was the direct result of 3FG oxidation.

To determine whether in fact lysis occurred in cell suspensions during 3FG oxidation, viable counts on bacterial populations exposed to 3FG in Warburg vessels were made.

EXPT.9b. Cells were grown, collected and washed as in expt.9a. Warburg conditions were the same as used previously and each flask contained 20-100 μ moles 3FG, 0.067M phosphate buffer to 1.8 ml. in the main well and 0.2 ml. KOH in the centre well. Reaction was initiated by tipping 0.5 ml. of cell suspension (10 mg. dry weight) from the side-arm. At zero time 1 ml. of suspension was removed from an endogenous control flask, serially diluted in sterile distilled water, and 1 ml. aliquots of the appropriate dilutions mixed with molten nutrient agar (Oxoid Ltd.) in a petri dish and allowed to solidify. This procedure was followed for substrate flasks and a further control flask after 4 hours incubation in the Warburg apparatus. Plates were incubated at 30° for 48 hours before colonies were counted, and from these the number of viable bacteria per ml. in the incubation vessels calculated (Table 22). From Fig.25 it can be seen that the drop in cell viability during 3FG oxidation was directly related to the amount of 3FG oxidised, and a comparison of Figs.22 and 25 shows a direct relationship between pH change and cell viability.

Fig.26 shows the results of viable counts made at different time periods during the oxidation of 0.05M 3FG by washed suspensions of Ps. fluorescens. It can be seen that the drop in cell viability did not start until approximately 2 hours after substrate oxidation had begun, confirming that it was not the presence of 3FG itself which was responsible for cell lysis, but rather that lysis occurred as a result of 3FG catabolism by the cell.

Fig.25. Effect of 3FG metabolism on the viability of washed suspensions of Ps. fluorescens.

For Warburg conditions and incubation mixtures see text. Viable counts made by serial dilution of Warburg contents after 4 hr. incubation period.

Fig.26. Viability changes with time of 3FG-metabolising Ps. fluorescens cell suspensions.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 100 µ moles 3FG, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (9.5 mg. dry weight) from side-arm.

One substrate and one control flask were removed from the Warburg apparatus at hourly intervals, and 1 ml. samples serially diluted in sterile distilled water. 1 ml. Aliquots withdrawn from the appropriate dilutions, mixed with molten nutrient agar in petri dishes and allowed to solidify, were incubated at 30° for 48 hrs.

Key:

—X——X— endogenously respiring cells

—o——o— cells metabolising 100 µ moles 3FG

Fig.25

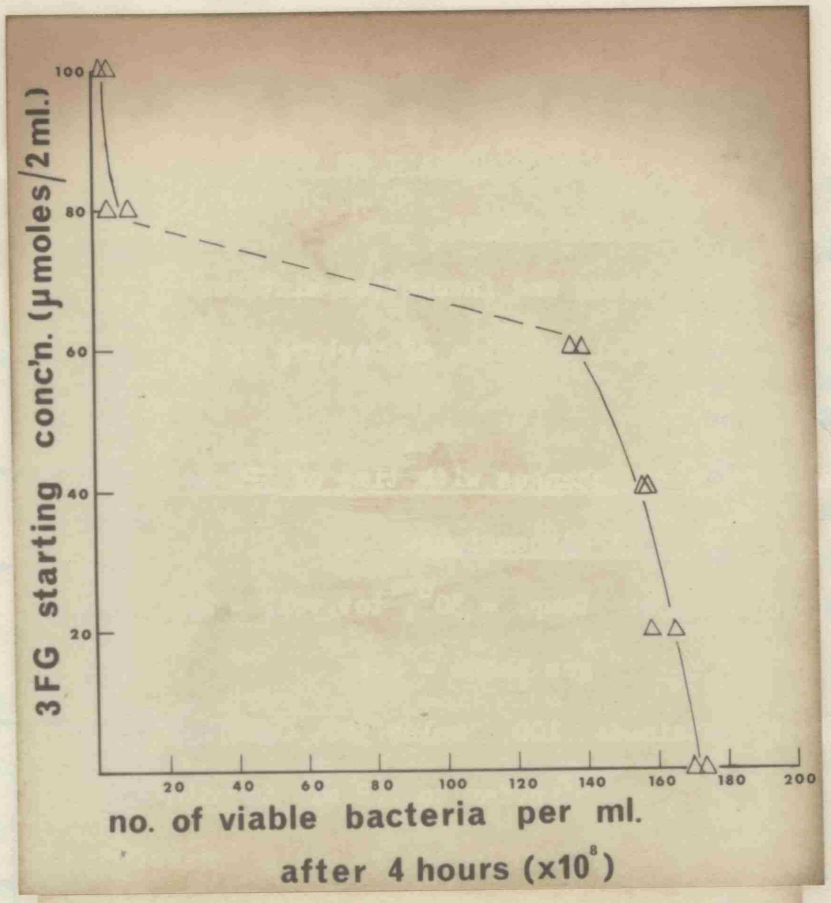

3 FG starting conc'n. (µmoles/2 ml.)
100
80
60
40
20
20 40 60 80 100 120 140 160 180 200
no. of viable bacteria per ml.
after 4 hours (x10⁸)

Fig.26

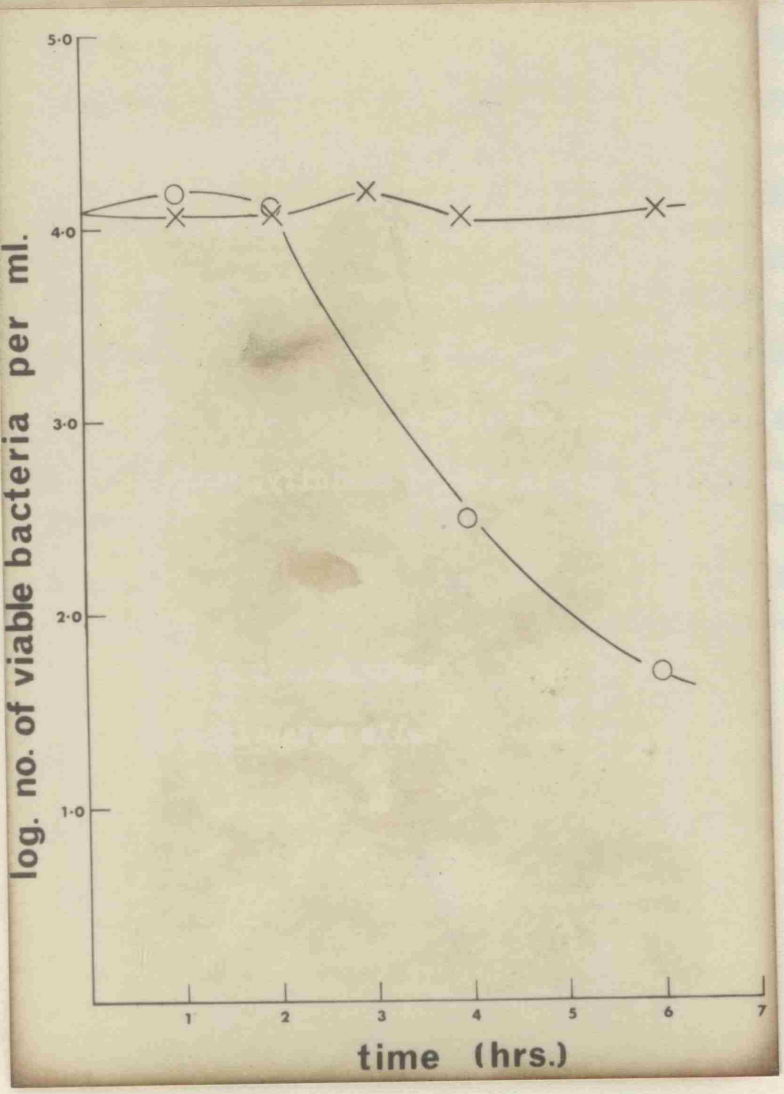

log. no. of viable bacteria per ml.
5·0
4·0
3·0
2·0
1·0
1 2 3 4 5 6 7
time (hrs.)

TABLE 22

Effect of 3FG metabolism on the viability of washed suspensions of Ps. fluorescens.

(see text for experimental conditions)

substrate	period of incubation	dilution	number of viable bacteria per ml.	viable bacteria per ml. in incubation vessel
-	0 hours	10^{-8}	170	170×10^8
-	4 hours	2×10^{-8}	340	170×10^8
-	4 hours	10^{-8}	173	173×10^8
10mM 3FG	4 hours	2×10^{-8}	330	165×10^8
"	4 hours	10^{-8}	158	158×10^8
20mM 3FG	4 hours	2×10^{-8}	313	156×10^8
"	4 hours	10^{-8}	157	157×10^8
30mM 3FG	4 hours	2×10^{-8}	278	139×10^8
"	4 hours	10^{-8}	136	136×10^8
40mM 3FG	4 hours	2×10^{-8}	19	10×10^8
"	4 hours	10^{-8}	4	4×10^8
50mM 3FG	4 hours	2×10^{-8}	4	2×10^8
"	4 hours	10^{-8}	4	4×10^8

Preliminary chromatographic attempts to characterise the product of 3FG oxidation by Ps. fluorescens were made on the suspending fluid from Warburg flasks, after 3FG oxidation had been followed to completion, using the method of Norris and Campbell (1949)[141]. This involved vacuum distillation, under nitrogen, of the clear supernatant obtained after cells had been removed by centrifugation, and descending paper chromatography in methanol-ethanol-water (45:45:10 v/v). The dried chromatograms were sprayed with either aniline diphenylamine or ammoniacal silver nitrate. However, this method was superceded by the freeze-drying of supernatants, and the separation of free sugars and aldonic acids by, either descending paper chromatography using methanol-pyridine-0.88-ammonia-water (5:2:1:2 v/v) with benzidine/periodate as locating agent, or silica gel G t.l.c. in ethyl acetate-acetic acid-water (3:3:1 v/v) with spot location by the sulphuric acid/ethanol (60:40 v/v) spray reagent.

EXPT.10. Cells, grown for 20 hours with aeration at 30°, collected by centrifugation at 4040xg for 10 minutes, washed twice and resuspended to 20 mg. dry weight per ml. in 0.067M phosphate buffer, were incubated in the Warburg apparatus with either glucose or 3FG. Warburg conditions were as used previously and each flask contained: 30 μ moles substrate, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (10 mg. dry weight) from side-arm. When substrate oxidation had gone to completion (3 hours), flask contents were filtered and the clear supernatants immediately freeze-dried.

Uninoculated control flasks were similarly treated. Freeze-dried residues, resuspended in minimum volumes of water, were applied to t.l.c. plates. In ethyl acetate-acetic acid-water glucose and gluconic acid were found to migrate with R_fs of 0.45 and 0.35 respectively, while 3FG and chemically synthesised 3-deoxy-3-fluoro-D-gluconic acid*(3FGA), the latter applied as an aqueous solution of its calcium salt, occupied positions at R_f 0.52 and 0.38 respectively.

From the flask containing glucose as substrate no glucose spot was found after oxidation was complete, but a spot corresponding to gluconate was visible. A similar result was obtained with the flask originally containing 3FG; a 3FG spot was no longer visible but a spot with identical R_f to 3FGA marker was visible. No spot was found after chromatography of the reaction mixture without substrate. The chromatograms are illustrated in Fig.27.

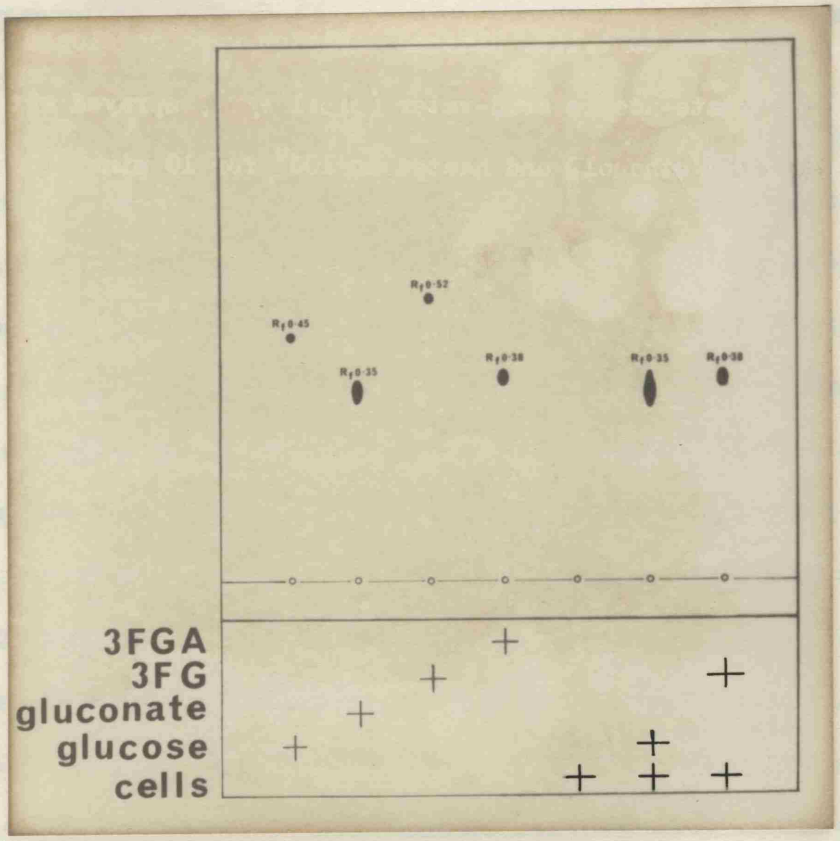
Repeated attempts have been made to isolate the product of 3FG oxidation by Ps. fluorescens in characterisable amounts. Since work with synthetic 3FGA has been unsuccessful in obtaining crystalline derivatives of the acid**, extraction procedures from culture supernatants have looked at ways of isolating the free acid or one of its salts. These have involved the incubation of large batches of washed cells with 0.05M 3FG in 200 ml. volumes of phosphate buffer, for varying time periods at differing buffer molarities, and subsequent freeze-drying of both the clear supernatants and cell washings when substrate oxidation had gone to completion. A variety of solvents have been used to extract fluorinated material from the freeze-dried residues,

* see Appendix-2 for synthesis of 3-deoxy-3-fluoro-D-gluconic acid.

** crystalline 3-fluoro-3-deoxy-D-gluconic acid has now been prepared.

Fig.27. Formation of 3-deoxy-3-fluorogluconic acid by 3FG oxidation.

When substrate oxidation had gone to completion, flask contents were filtered, concentrated by freeze-drying, and applied in aqueous solution to t.l.c. plates. Plates were developed in ethyl acetate-acetic acid-water (3:3:1 v/v), sprayed with sulphuric acid/ethanol, and heated to 100° for 10 minutes.


R_f 0·52
R_f 0·45
R_f 0·35
R_f 0·38
R_f 0·35
R_f 0·38
3FGA
3FG
gluconate
glucose
cells

and the subsequent separation of organic material from contaminating phosphate buffer effected by either preparative chromatographic, or desalting, techniques, or a combination of both. In all cases acidic material has been found which behaved in an identical manner to synthetic 3FGA, and spots corresponding to the acid lactones have been located on chromatograms by the formation of their hydroxamic acid derivatives.

EXPT.11. The growth from 8x125 ml. amounts of suitably inoculated glucose/mineral salts medium, incubated with aeration at 30° for 21 hours, was collected by centrifugation at 6,200xg for 15 minutes, washed twice, and resuspended in 0.067M phosphate buffer, pH 7.0, to 40 mg. per ml. To 50 ml. of the bacterial suspension in a 1 litre Ehrlenmeyer flask was added 10m moles of 3FG in 50 ml. of water, 50 ml. 0.067M phosphate buffer, pH 7.0, and 50 ml. of 0.67M phosphate of the same pH (the latter to prevent production of an acid pH during the incubation with subsequent cessation of bacterial metabolism). The resulting mixture was incubated with aeration at 30° for 13 hours, at which time oxidation was complete, and the clear supernatant obtained by centrifugation immediately freeze-dried. The cell pellet was washed once in 50 ml. of distilled water and the clear washings also freeze-dried. Samples of the freeze-dried residues from both supernatant and washings, taken up in minimum volumes of water and applied to t.l.c. plates, chromatogrammed as one spot with an R_f of 0.38 in ethyl acetate-acetic acid-water.

Ethanolic extraction of the acidified residues from both freeze-dried supernatant and washings was effected at room temperature and, in order to separate fluorinated organic material from inorganic phosphate, the concentrated extracts were applied to silica gel columns. Columns were eluted with 400 ml. of ethyl acetate-ethanol (9:2 v/v) and the concentrated eluents chromatogrammed. Material was now present which migrated as

three spots at R_fs 0.53, 0.65, 0.70 in the above solvent system and, since synthetic 3FGA chromatogrammed in a similar manner after elution off a silica gel column, lactone formation was assumed to have occurred. Evidence of this suggestion came from the finding that the two fast moving spots at R_fs 0.65 and 0.70 could be detected by the hydroxylamine/ferric chloride reagent which is specific for the $-\overset{\overset{O}{\|}}{C}-O-R$ grouping.

The concentrated eluents were evaporated to dryness 'in vacuo', the residues taken up in minimum volumes of water, and the acidic aqueous solutions thus obtained titrated to pH 10.0 using 0.1M calcium hydroxide. Excess ethanol was added to the concentrated solutions, which resulted in the formation of white precipitates. The latter were removed by centrifugation, the supernatants again concentrated to small volumes, and more ethanol added. Again white precipitates were formed, removed by centrifugation, and the procedure repeated a further six times. All precipitates were combined, but absorbed moisture on exposure to air and formed a syrup. This material ran with an R_f of 0.53, when applied in ethanolic solution to a t.l.c. plate and developed with ethyl acetate-acetic acid-water, but was always in insufficient quantities for further characterisation.

An anylsis of the various fractions obtained from a similar large scale incubation for organically-bound fluorine revealed that, out of a total of 11, 110 μ moles of 3FG added at the start of the incubation, 10, 850 μ moles could be accounted for (97.5%) and the distribution was as follows:

8,320 μ moles (76.7% of total recovered) in the supernatant,

2,500 μ moles (23.0% of total recovered) in the cell washings, and

30 μ moles (0.3% of total recovered) in perchloric acid cell extracts.

However, neither treatment at room temperature nor overnight refluxing of the acidified freeze-dried residues with ethanol could extract above 10% of the total organic fluorine present. Only by refluxing the residues with 5% (w/w) ethanolic HCl for one hour could significant quantities of organic material be liberated into solution but, since this treatment also resulted in cleavage of the C-F bond, it was unsuitable for the extraction of fluorinated substrates. Similar results have been obtained with other solvent extraction procedures- at no time have sufficient quantities of the fluorinated metabolite been isolated for unambiguous characterisation. Attempts to remove HPO_4^{--} and $H_2PO_4^-$ ions preferentially to 3-deoxy-3-fluorogluconate anion by passage down an anion exchange resin also failed to separate sufficient quantities of the fluoro analogue for characterisation, though again material was present which chromatogrammed in an identical manner to synthetic 3FGA. As time was pressing these studies had to be discontinued and it is hoped that future work will be successful in characterising the product of bacterial oxidation of 3FG.

Returning to manometric studies with 3FG, it was decided to examine the ability of washed cell suspensions of Ps. fluorescens to oxidise the analogue under conditions where concomitant acid production was not limiting. Accordingly the molarity of the suspending buffer was increased ten-fold, and this concentration maintained throughout subsequent manometric studies with synthetic 3FGA (free acid) thereby affording a direct comparison between 3FG and 3FGA oxidation by cell suspensions, and obviating the necessity to neutralise solutions of the free acid before their use as substrates for manometric studies.

EXPT.12. Cells, grown for 20 hours with aeration at 30° from a standard inoculum, were collected by centrifugation at 4040xg for 10 minutes, washed twice in 0.67M phosphate buffer, pH 7.0, and resuspended in buffer of the same molarity and pH to 16 mg. dry weight per ml. Fig.28 shows the oxygen consumption by washed suspensions of Ps. fluorescens in 0.67M phosphate buffer when furnished with limiting amount of 3FG. In most cases the rate reached maximum within 20 minutes, though delays of upto 90 minutes were noted in some experiments with the lower concentrations of substrate. Subsequently it was approximately linear, rates increasing with substrate concentration, but the total oxygen consumption by cells for all concentrations of 3FG still approximated to one atom of oxygen per mole of substrate (Table 23).

TABLE 23

Manometric data on the oxidation of 3FG by washed suspensions of Ps. fluorescens in 0.67M phosphate buffer. (see Fig.28)

substrate	rate of oxidation (μmoles O_2/mg. dry wt./hr.)	net O_2 uptake (μl.) (endogenous subtracted)	moles of O_2 per mole of substrate oxidised
20 μ moles 3FG	0.27	230	0.51
40 μ moles 3FG	0.53	432	0.48
60 μ moles 3FG	0.87	675	0.50
80 μ moles 3FG	1.13	922	0.51
100 μ moles 3FG	1.57	1332	0.60

The oxidation of limiting amounts of 3FGA by a twice washed cell suspension of Ps. fluorescens in 0.67M phosphate buffer is shown in Fig.29. With all concentrations there was an immediate rapid rate of oxygen uptake which increased with substrate concentration and, after an approximately linear

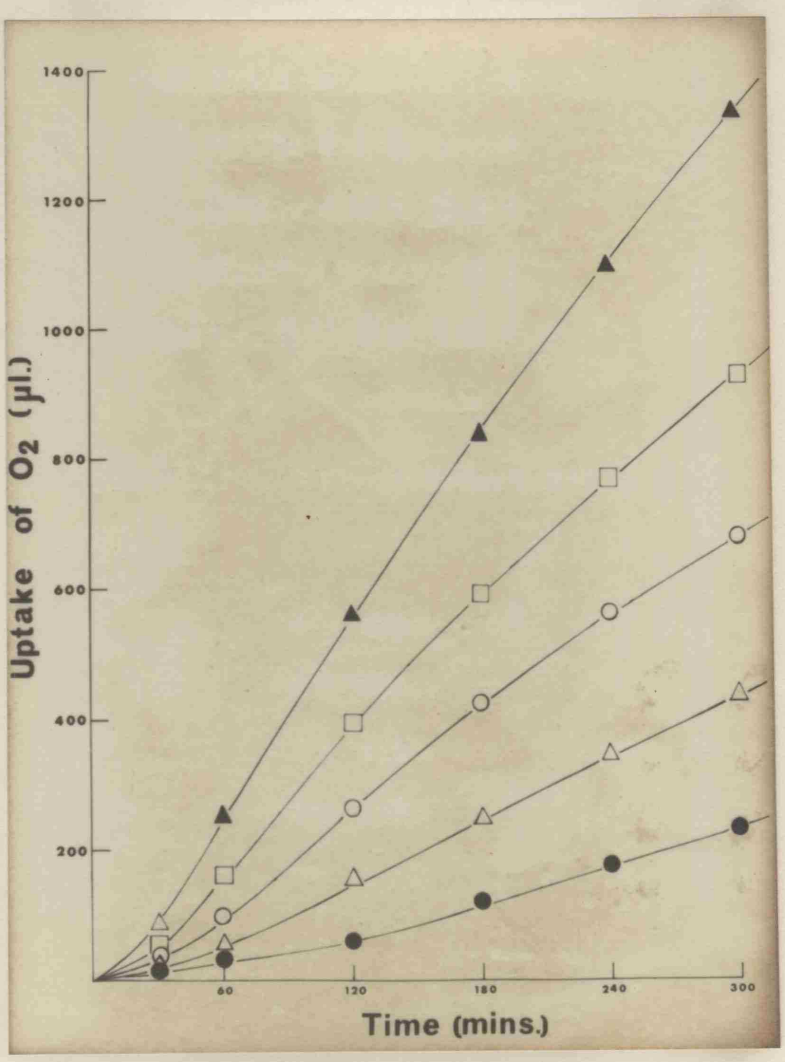

1400
1200
1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
240
300
Time (mins.)

Fig.29. Oxidation of 3FGA by a washed resting cell suspension of Ps. fluorescens.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 20-100 μ moles substrate, 0.67M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (15 mg. dry weight) from side-arm.

Endogenous respiration subtracted (144 μl. in 5 hrs.).

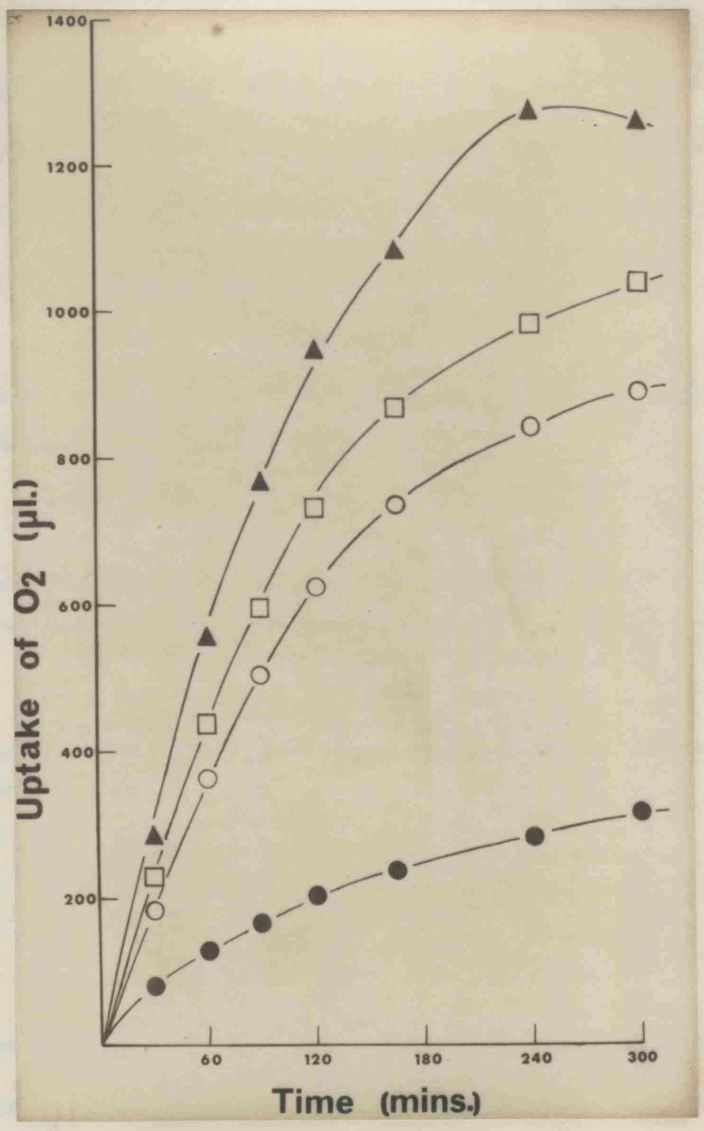
Key:

—▲——▲——▲— 100 μ moles 3FGA

—□——□——□— 80 μ moles 3FGA

—o——o——o— 60 μ moles 3FGA

—●——●——●— 20 μ moles 3FGA


1400
1200
1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
240
300
Time (mins.)

period of 120-180 minutes, fell off progressively to that of controls without added substrate. The total oxygen consumption approximated a value corresponding to one atom of oxygen per mole of substrate (Table 24). (The apparent anomaly of the proposed product of 3FG oxidation by Ps. fluorescens being capable of oxidation by cell suspensions only when present as the primary substrate and not when it accumulates as the product of 3FG oxidation will be discussed later in the light of results obtained with cell-free extracts of this organism.)

TABLE 24

Manometric data on the oxidation of 3-deoxy-3-fluoro-D-gluconic acid by washed suspensions of Ps. fluorescens.

Cells grown for 20 hours with aeration at 30° were collected, washed twice and resuspended in 0.67M phosphate buffer to 30 mg. dry weight per ml. Their ability to oxidise 3FGA was determined manometrically (Fig.29).

substrate	rate of oxidation (μmoles O_2/mg. dry wt./hr.)	net O_2 uptake (μl.) (endogenous subtracted)	moles of O_2 per moles of substrate oxidised
20μ moles 3FGA	0.48	315	0.71
60μ moles 3FGA	1.10	890	0.66
80μ moles 3FGA	1.38	1035	0.58
100μ moles 3FGA	1.68	1255	0.57

The effects of 3FG and 3FGA oxidation by cell suspensions in 0.67M phosphate buffer on subsequent glucose oxidation are shown in Figs. 30 and 31 respectively. Increased initial rates of oxidation by suspensions which had previously respired limiting amounts of either 3FG or 3FGA were observed, compared with glucose oxidation by cell suspensions previously respiring endogenously. The increased rates were approximately linear for 40-60 minutes and then fell off progressively; in contrast the rate of subsequent glucose oxidation by endogenously respiring controls was approximately linear over 180 minutes (Table 25). No glucose remained in any of the incubation filtrates after 180 minutes.

Fig.30. Glucose oxidation by Ps. fluorescens cell suspensions in 0.67M phosphate buffer after 3FG oxidation.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 20-100 μ moles 3FG, 8.5 mg. dry weight of cells, 0.67M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 10 μ moles glucose from side-arm after 360 mins.

Endogenous respiration subtracted (78 μl. in 150 mins.).

Key:

	preincubation substrate
—▲——▲——▲—	100 μ moles 3FG
—□——□——□—	80 μ moles 3FG
—△——△——△—	40 μ moles 3FG
—●——●——●—	20 μ moles 3FG
—X——X——X—	endogenous control

Fig.31. Glucose oxidation by Ps. fluorescens cell suspensions in 0.67M phosphate buffer after 3FGA oxidation.

Warburg conditions: as above

Each flask contained: 10-100 μ moles 3FGA, 8.5 mg. dry weight of cells, 0.67M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 10 μ moles glucose from side-arm after 225 mins.

Endogenous respiration subtracted (75 μl. in 165 mins.).

Key:

	preincubation substrate
—▲——▲——▲—	100 μ moles 3FGA
—o——o——o—	10 μ moles 3FGA
—X——X——X—	endogenous control

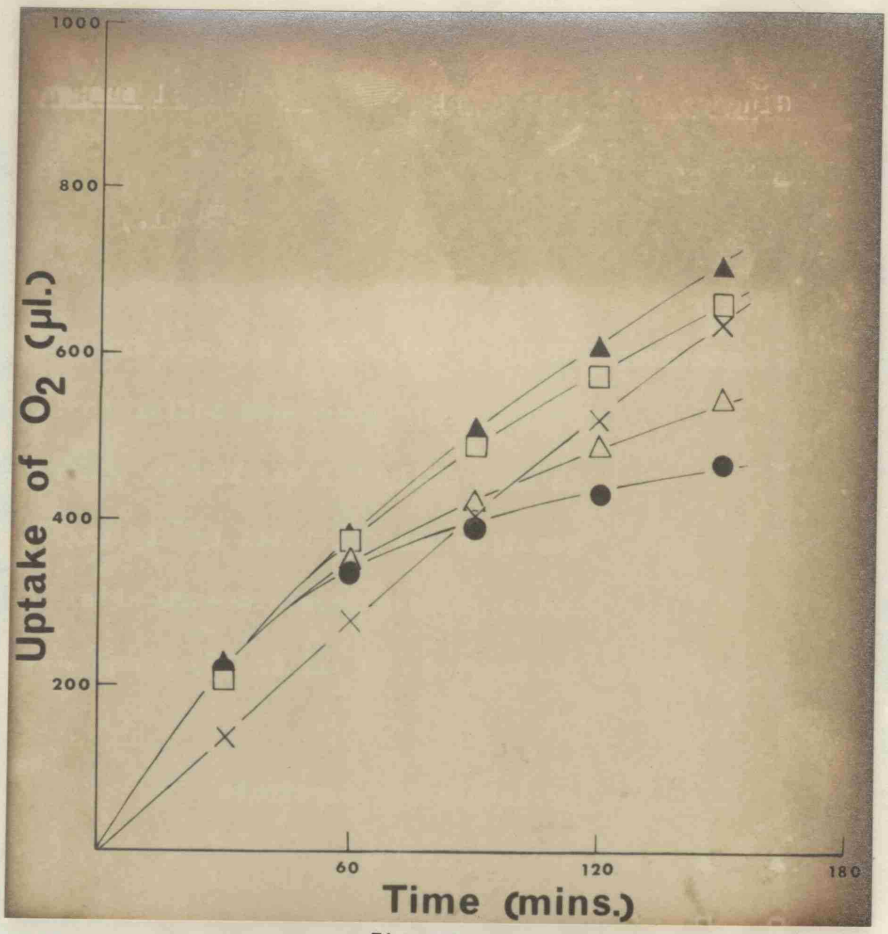

1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
Time (mins.)

Fig. 30

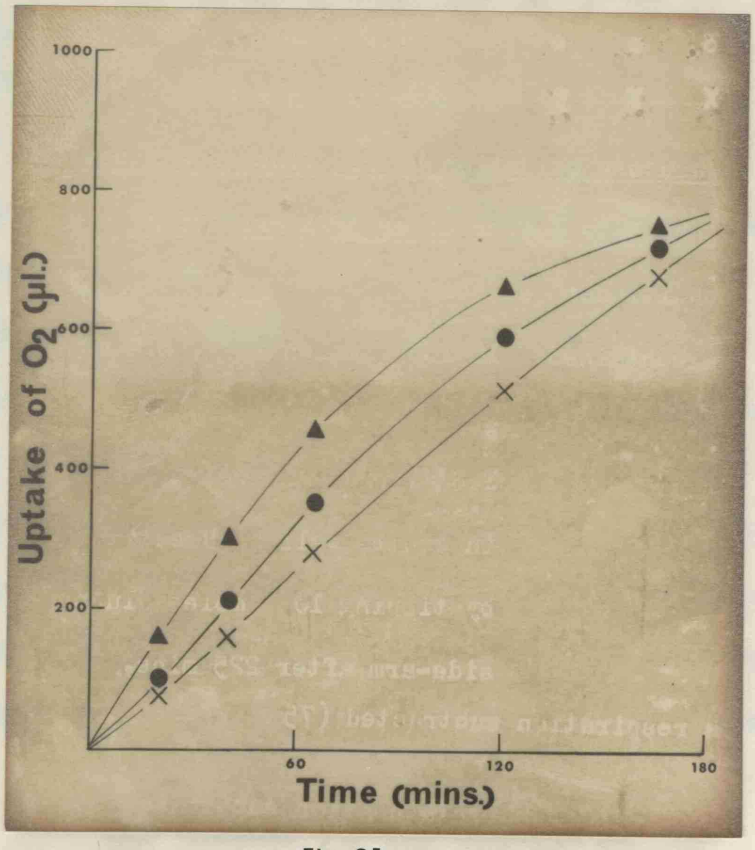

1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
Time (mins.)

Fig. 31

TABLE 25

Manometric data on the oxidation of glucose by Ps. fluorescens cell suspensions previously respiring either 3FG or 3FGA in 0.67M phosphate buffer.

Cells, grown for 20 hours in glucose/mineral salts medium, collected, washed and resuspended to 17 mg. dry weight per ml., were incubated in the Warburg apparatus with limiting amounts of 3FG or 3FGA. When the rate of oxygen uptake had fallen to a level comparable with that of control cells (6 hours), 10 μ moles of glucose were added from the side-arm and the subsequent oxygen consumption measured (Figs. 30 and 31).

preincubation substrate	substrate oxidised	rate of oxidation (μmoles O_2/ mg. dry wt./hr.)	net O_2 uptake (μl.) (endogenous subtracted)	moles of O_2 per mole of substrate oxidised
———	10 μ moles glucose	1.41	637	2.84
20 μ moles 3FG	"	1.38	471	2.11
40 μ moles 3FG	"	1.48	545	2.43
80 μ moles 3FG	"	1.71	662	2.96
100 μ moles 3FG	"	1.78	707	3.16
10 μ moles 3FGA	"	1.57	724	3.23
100 μ moles 3FGA	"	1.75	761	3.40

The oxygen uptake by washed cell suspensions of Ps.fluorescens furnished with limiting amounts of glucose and 3FG together is shown in Fig.32. Immediate maximum rates of oxygen uptake, which increased with substrate concentration and were approximately linear for 120-180 minutes, were observed for all ratios of the fluorinated and natural substrates. The extent of oxidation of the 3FG present (assumed to be the difference between the total oxygen uptake by suspensions respiring both 3FG and glucose and the oxygen consumed by cells respiring that amount of glucose in the mixtures alone) was equivalent to one atom of oxygen per mole of 3FG when 50 or 100 μ mole

Fig.32. Oxidation of glucose, 3FG mixtures by Ps. fluorescens cell suspensions in 0.67M phosphate buffer.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 10 μ moles glucose, 10-100 μ moles 3FG, 0.67M phosphate buffer to 1.8 ml. in main well. Reaction was initiated by tipping 0.5 ml. cell suspension (8 mg. dry weight) from side-arm

Endogenous respiration subtracted (287 μ l. in 420 mins.).

Key:

—□——□——□— 10 μ moles glucose, 100 μ moles 3FG

—o——o——o— 10 μ moles glucose, 50 μ moles 3FG

—▲——▲——▲— 10 μ moles glucose, 10 μ moles 3FG

—△——△——△— 10 μ moles glucose

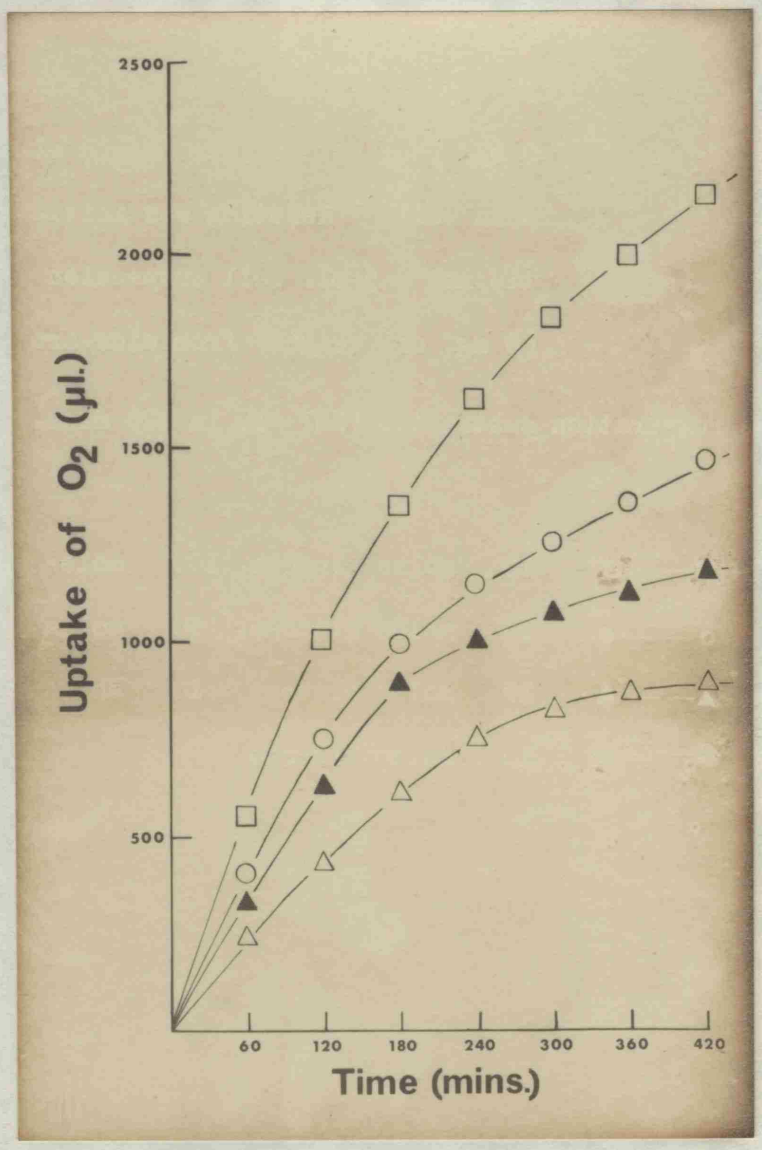

2500
2000
1500
1000
500
Uptake of O_2 (µl.)
60
120
180
240
300
360
420
Time (mins.)

Fig.28. Oxidation of 3FG by Ps. fluorescens cell suspensions in 0.67M phosphate buffer.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 20-100 μ moles substrate, 0.67M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (8 mg. dry weight) from side-arm.

Endogenous respiration subtracted (260 μl. in 6 hrs.).

Key:

—▲——▲——▲— 100 μ moles 3FG

—□——□——□— 80 μ moles 3FG

—o——o——o— 60 μ moles 3FG

—△——△——△— 40 μ moles 3FG

—●——●——●— 20 μ moles 3FG

amounts of 3FG were present, and one mole of oxygen per mole of 3FG when 3FG and glucose were present in equimolar amounts (Table 26). Only when 3FG was oxidised in the presence of an equimolar amount of glucose did the extent of its oxidation by whole cell suspensions reach this value.

TABLE 26

Manometric data on the oxidation of 3FG and glucose together by washed suspensions of Ps. fluorescens in 0.67M phosphate buffer.

Cells, grown for 20 hours with aeration at 30° in glucose/mineral salts medium from a standard inoculum, were collected by centrifugation at 4040xg for 10 minutes, washed twice in 0.67M phosphate buffer, pH 7.0, and resuspended in buffer of the same molarity and pH to 16 mg. dry weight per ml. Their ability to oxidise glucose, 3FG mixtures was tested manometrically (Fig.32).

	observed value (µl.)	moles of O_2 per mole of substrate oxidised	rate of oxidation (µmoles O_2/mg. dry wt./hr.)
O_2 uptake, endogenous	287		
* Net O_2 uptake, 10µ moles glucose	892	3.98	1.21
* Net O_2 uptake, 10µ moles glucose 10µ moles 3FG	1175	2.63	1.76
* Net O_2 uptake, 10µ moles glucose 50µ moles 3FG	1453	1.08	2.08
* Net O_2 uptake, 10µ moles glucose 100µ moles 3FG	2138	0.87	2.79
** Net O_2 uptake, 10µ moles 3FG	283	1.26	
** Net O_2 uptake, 50µ moles 3FG	561	0.50	
** Net O_2 uptake, 100µ moles 3FG	1246	0.57	

* endogenous O_2 uptake subtracted

** endogenous O_2 uptake and O_2 uptake on 10µ moles glucose subtracted

From Figs. 30, 31 and 32 it would appear that 3FG or 3FGA oxidation has very little effect on glucose oxidation by washed cell suspensions in 0.67M phosphate buffer. The simultaneous oxidation of 3FG and glucose caused no noticeable inhibition of glucose oxidation, and the differences between glucose oxidation subsequent to oxidation of either of the fluoro analogues and that subsequent to incubation with no exogenous substrates were not consistent, and probably due to the oxidation of residual exogenous fluorinated material. (When cell suspensions were washed once and resuspended in 0.67M phosphate buffer after 3FG or 3FGA oxidation, subsequent glucose oxidation did not differ significantly from that of control cells).

A comparison of the suspending fluid of cell suspensions oxidising 3FG or 3FGA in 0.67M phosphate buffer with that of endogenously respiring controls in buffer of the same molarity and pH still showed the presence of increased amounts of uv-absorbing material, again with a maximum absorbance at 260 mμ, a minimum at 240 mμ, and which reacted positively with the orcinol reagent (Figs.33 and 34). This again suggested some degree of cell damage as a result of fluoro analogue oxidation, although there was no pH change in such a strongly buffered system. Accordingly viable counts were made on bacterial populations during 3FG oxidation in 0.67M phosphate buffer.

EXPT.13. Cells, grown for 20 hours with aeration at 30° from a standard inoculum, collected by centrifugation at 4040xg for 10 minutes, and washed twice and resuspended in 0.67M phosphate buffer, pH 7.0, were incubated for 5 hours in the Warburg apparatus with 0.01M-0.05M 3FG.

Fig.33. Release of E260 - absorbing material into the suspending fluid by washed suspensions of Ps. fluorescens metabolising 3FG in 0.67M phosphate buffer.

Cells grown for 20 hrs. with aeration at 30°, collected, washed and resuspended as before, in 0.67M phosphate buffer, pH 7.0, to 17 mg. dry weight per ml., were incubated for 6 hrs. in the Warburg apparatus with 20-100 μ moles 3FG per flask. Flask contents were then filtered, diluted 1:3 with distilled water and examined in the SP800 (Unicam Instruments Ltd.) against a water blank.

Key:

—▲——▲— 100 μ moles 3FG

—□——□— 80 μ moles 3FG

—o——o— 60 μ moles 3FG

—△——△— 40 μ moles 3FG

—●——●— 20 μ moles 3FG

—X——X— endogenous

Fig.34. Release of E260 - absorbing material in the suspending fluid by washed suspensions of Ps. fluorescens metabolising 3FGA in 0.67M phosphate buffer.

Cells grown, collected, washed and resuspended, as above in 0.67M phosphate buffer, pH 7.0, were incubated in the Warburg apparatus for 4 hrs. with 20-100 μ moles 3FGA per flask. Flask contents were then filtered, diluted 1:3 with distilled water, and examined spectrophotometrically against a water blank.

Key:

—▲——▲— 100 μ moles 3FGA

—□——□— 80 μ moles 3FGA

—△——△— 40 μ moles 3FGA

—●——●— 20 μ moles 3FGA

—X——X— endogenous

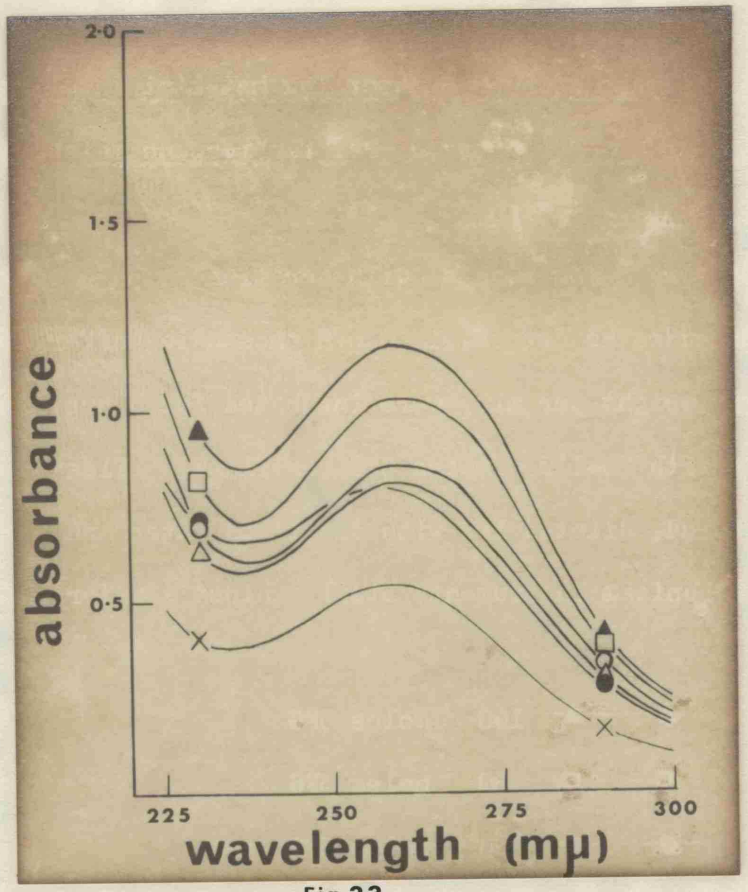

2·0
1·5
1·0
0·5
absorbance
225
250
275
300
wavelength (mμ)

Fig. 33

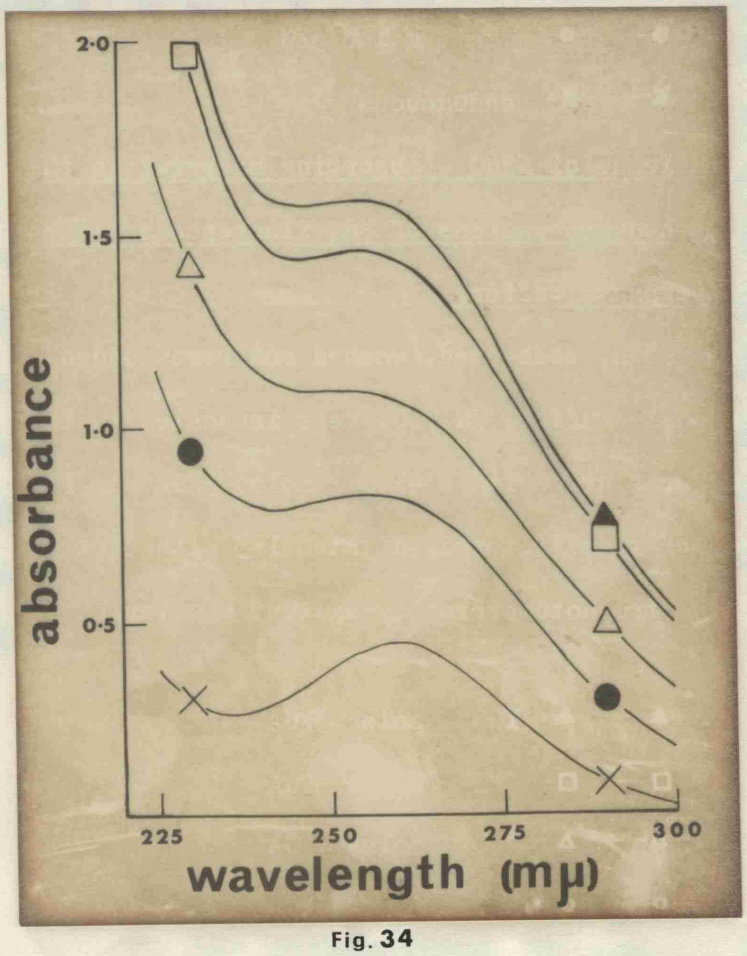

2·0
1·5
1·0
0·5
absorbance
225
250
275
300
wavelength (mμ)

Fig. 34

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml.,
gas phase = air.

Each flask contained: 20-100 μ moles 3FG, 0.67M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (8 mg. dry weight) from side-arm.

At zero time 1 ml. of suspension was removed from an endogenous control flask, serially diluted in sterile distilled water, and 1 ml. aliquots of the appropriate dilutions mixed with molten nutrient/agar and allowed to solidify. This procedure was followed for substrate flasks and a further control flask after 5 hours incubation. Table 27 shows the viable counts.

Although there were differences in the counts obtained these were not related to substrate concentrations as was the release of E_{260}-absorbing material, and at no time was the drop in viable count during 3FG oxidation greater than that observed in controls without added substrate. Therefore, to explain the release of E_{260}-absorbing material from cell suspensions oxidising 3FG without a concomitant drop in cell viability, a mechanism similar to that invoked to explain the release of this material without cell lysis during endogenous respiration by Ps. fluorescens must be proposed i.e. that the release of uv-absorbing material from cell suspenions oxidising 3FG in 0.67M phosphate buffer is a direct result of an intracellular effect of the fluoro analogue, whereby increased breakdown of RNA or ribonucleoprotein occurs with subsequent release into the suspending buffer.

TABLE 27

Effect of 3FG metabolism on the viability of washed suspensions of Ps. fluorescens in 0.67M phosphate buffer.

(see text for experimental conditions)

substrate	period of incubation	dilution	number of viable bacteria per ml.	viable bacteria per ml. in incubation vessel
	0 hours	10^{-6}	433	35×10^7
	"	10^{-7}	26	
	5 hours	10^{-6}	53	5×10^7
	"	10^{-7}	4	
10mM 3FG	"	10^{-6}	233	18×10^7
"	"	10^{-7}	13	
20mM 3FG	"	10^{-6}	44	7×10^7
"	"	10^{-7}	10	
30mM 3FG	"	10^{-6}	74	5×10^7
"	"	10^{-7}	3	
40mM 3FG	"	10^{-6}	285	29×10^7
50mM 3FG	"	10^{-6}	250	25×10^7

A brief study of the product of 3FGA oxidation by washed suspensions of Ps. fluorescens was made by confirming the presence of reducing material in the reaction vessel after 3FGA oxidation had gone to completion, and by chromatographic analysis of an aqueous solution of the concentrated supernatant after substrate oxidation. On t.l.c. plates developed in ethyl acetate-acetic acid-water no new chromatographically distinquishable material could be detected after 3FGA oxidation. However, after overnight treatment with an equal volume of 10% (w/v) phenylhydrazine hydrochloride, material could be located at R_fs 0.4 and 0.7 on thin layer chromatograms of oxidised 3FGA developed in the above solvent system; but due to a lack of suitable markers this material could not be further characterised. Both 3FGA and gluconic acid were unaffected by phenylhydrazine treatment.

Table 28 shows the results of tests for reducing material in reaction flasks after 3FGA and gluconate oxidation. The parallel results obtained with these two substrates (the weak positive reaction obtained as a result of gluconate oxidation is assumed to be due to subsequent metabolism of the 2-ketogluconate produced) together with the finding that the product of 3FGA oxidation is affected by phenylhydrazine treatment, suggested the possibility of ketoaldonic acid formation as a result of 3FGA oxidation and, by analogy with glucose and gluconate oxidation in this organism[138], 2-keto-3-deoxy-3-fluorogluconic acid is the most probable oxidation product.

With the dual purpose of determining whether the C-F bond in 3FG or 3FGA underwent any appreciable enzymic cleavage during the time period of the above incubations with Ps. fluorescens and also of localising and quantifying intracellular organically-bound fluorine, a series of fluoride determinations, on both the suspending fluid of buffered suspensions of resting cells and on the alkali hydrolysates of perchloric acid-cell extracts, were made after the oxidation of either 3FG or 3FGA, using the fluoride electrode.

TABLE 28

Formation of reducing material from 3FGA oxidation by washed suspensions of Ps. fluorescens.

Cells, grown for 19 hours with aeration at 30° on glucose/mineral salts medium, collected, washed twice, and resuspended in 0.067M phosphate buffer to 20 mg. dry weight per ml., were incubated in the Warburg apparatus with either gluconate or 3FGA. When oxidation had gone to completion (3½ hours), cells were removed by centrifugation, and 1 ml. samples from the supernatants mixed with 1 ml. of Benedict's reagent and heated in a boiling water bath for 10 minutes. The presence of reducing material was shown by the production of a coloured precipitate of cuprous oxide.

substrate oxidised	time (hrs.)	reducing material
5mM gluconate	0	-
"	3½	±
25mM 3FGA	0	-
"	3½	+

(no reducing material was produced in the absence of cells)

EXPT.14. 6x75 ml. Amounts of glucose/mineral salts medium, suitably inoculated from a 24 hour slope culture, were incubated with aeration at 30° for 20 hours. Cells were collected by centrifugation at 4040xg for 10 minutes, washed twice and resuspended in either 0.067M or 0.67M phosphate buffer, pH 7.0, to 40 mg. dry weight per ml., and incubated in the Warburg apparatus.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml.,
gas phase = air.

Each flask contained: 20-100 μmoles 3FG or 3FGA, 0.067M or 0.67M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (20 mg. dry weight) from side-arm.

Control flasks were incubated without cells.

When substrate had been oxidised to completion (3-5 hours), flask contents were filtered to remove cells, and a known volume of filtrate, in a polypropylene centrifuge tube, made up to 2.0 ml. with distilled water. 2 ml. Acetate-citrate buffer, pH 5.5, were added, the fluoride electrode inserted, and the electrode potential determined. The concentration of free F^- ion present in the filtrates at the end of the incubation period was then calculated. It is evident from Table 29 that the concentration of fluoride anion present in the suspending fluid after 5 hours incubation of washed cell suspensions with between 10 and 50mM of either 3FG or 3FGA, accounted for a maximum of 0.7% of the fluorine present in the substrate. Cleavage of the C-F bond in either 3FG or 3FGA was therefore assumed to occur to a negligible extent during such incubations.

In order to localise and quantify fluorinated material after bacterial oxidation, the incubation mixtures were filtered, and cells pellets washed once with buffer before being extracted with perchloric acid. The level of fluoride anion in alkali-hydrolysed samples from the supernatant, washings, and cell extract fractions was then determined.

TABLE 29

Concentration of fluoride anion in the suspending fluids from buffered incubations of Ps. fluorescens with either 3FG or 3FGA.

(see text for experimental details)

substrate starting conc'ns.	period of incubation	suspending buffer molarity	free F^- ion present at end of incubation (μmoles/ml.)	F^- ion release (per cent)
25mM 3FG*	3 hours	0.067M	0.016	0.06
25mM 3FG	"	"	0.060	0.24
0	5 hours	0.67M	0.058	
10mM 3FG	"	"	0.048	0.48
20mM 3FG	"	"	0.104	0.52
30mM 3FG	"	"	0.152	0.50
40mM 3FG	"	"	0.128	0.32
50mM 3FG	"	"	0.152	0.30
50mM 3FG*	"	"	0.058	0.12
10mM 3FGA	"	"	0.072	0.72
20mM 3FGA	"	"	0.064	0.32
30mM 3FGA	"	"	0.088	0.29
40mM 3FGA	"	"	0.024	0.60
50mM 3FGA	"	"	0.248	0.50

* bacteria-free controls

EXPT.15a. Cells, grown for 20 hours with aeration at 30°, collected by centrifugation at 4040xg for 10 minutes, washed twice and resuspended to 16 mg. dry weight per ml. in 0.67M phosphate buffer, pH 7.0, were incubated in the Warburg apparatus with varying amounts of 3FG and 3FGA.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml.,
gas phase = air

Each flask contained: 20-100 μ moles 3FG or 3FGA,
0.67M phosphate buffer to
1.8 ml. in main well, 0.2 ml.
KOH in centre well. Reaction
was initiated by tipping
0.5 ml. cell suspension
(8 mg. dry weight) from
side-arm.

When oxidation had neared completion (3½ hours), flask contents were filtered, the filtrates retained, and cells washed once with 0.67M phosphate buffer, pH 7.0. The cell washings were immediately freeze-dried, and the residues so obtained resuspended to a known volume in distilled water. After filtration, cells were subsequently extracted for 30 minutes with a known volume of 0.5N perchloric acid, which was then neutralised with a measured amount of ION KOH. Samples from the neutralised cell extracts, cell washings, and suspending fluids were hydrolysed with equal volumes of ION KOH at 90° for 6 hours, and the resulting hydrolysates, after adjustment to pH 5.5 with a known volume of 'analar' hydrochloric acid, assayed for fluoride ion. Table 30 shows the distribution of organic fluorine between the three fractions, calculated from the fluoride ion concentrations found in the hydrolysates. The level of fluorinated material in the acid extracts

of washed cells was a very small proportion of the starting level of substrate, but large amounts of fluoride ion were released on hydrolysis of the cell washings, accounting for between 25-50% of the total fluoride determined. The presence of such quantities of fluorinated substrates in the fraction obtained by one washing of cell suspensions suggested that oxidation of 3FG and 3FGA by Ps. fluorescens occurs on, or in, the cytoplasmic membrane by the enzymes localised there, and that further passage into the cell occurred to a very limited extent. Fluoride ion was never found in cell extracts without prior hydrolysis thus confirming the retention of the carbon-fluorine bond during metabolism.

TABLE 30

Distribution of fluoride ion in alkali hydrolysates of the fractions obtained from buffered incubation of Ps. fluorescens cell suspensions with either 3FG or 3FGA. (see text for experimental details)

Substrate starting levels	perchloric acid cell extracts		cell washings (0.67M phosphate buffer)		incubation filtrates	
	μ moles F^- ion	% total found	μ moles F^- ion	% total found	μ moles F^- ion	% total found
20 μ moles 3FG	1.93	8.1%	11.00	46.4	10.80	45.5
60 μ moles 3FG	1.00	1.6	15.80	25.8	44.65	72.6
100 μ moles 3FG	2.18	2.1	41.90	39.7	61.30	58.2
20 μ moles 3FGA	0.21	1.0	8.00	38.6	12.50	60.4
60 μ moles 3FGA	0.35	0.6	21.85	37.1	36.70	62.3
100 μ moles 3FGA	3.70				48.90	

In a further series of experiments, the distribution of fluorinated metaboli tes between cells and suspending fluid was determined at varying times during the incubation period.

EXPT.15b. Cells, grown for 21 hours at 30° with aeration, collected by centrifugation at 4040xg for 10 minutes, washed twice and resuspended to 20 mg. dry weight per ml. in 0.67M phosphate buffer, pH 7.0, were incubated in the Warburg apparatus with either 3FG or 3FGA.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml.,
gas phase = air

Each flask contained: 50 μ moles substrate, 0.67M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (10 mg. dry weight) from side-arm.

At selected intervals of time during the incubation, flasks were removed and 1 ml. samples of the contents filtered. The unwashed cells were extracted as before with a predetermined volume of 0.5N perchloric acid for 30 minutes, which was then neutralised with a measured volume of 10N KOH. Samples from the flask filtrates, and neutralised perchloric acid cell extracts, were then hydrolysed with 10N KOH at 90° for 6 hours, the pH adjusted to 5.5, and the mV of the resulting solution determined with the fluoride electrode. From Table 31 it can be seen that, when 3FG was substrate for resting cell suspensions of Ps. fluorescens, there was a slow increase in the amount of cell-bound organic fluorine with time and a corresponding decrease in the level of fluorinated substrate in the suspending fluid. When 3FGA was the substrate however, the change in extracellular and intracellular levels of fluorinated metabolites with time was not so apparent. The

intracellular fluorine level never rose above 12 μ moles per 10 mg. dry weight of cells and, after an initial drop, the level of fluorinated material in the suspending fluid remained constant. Since these values represented the total levels of fluorinated 'metabolites', it was not possible to say what proportion of substrate or of its oxidised product were represented or, once substrate oxidation has occurred, to what extent the product passes back into the suspending fluid, thus maintaining the level of extracellular fluorinated substrate(s).

TABLE 31

Fluoride concentrations in alkali hydrolysates of perchloric acid cell extracts and of suspending fluids from buffered incubations of Ps. fluorescens with either 3FG or 3FGA. (see text for experimental details)

substrate starting level	period of incubation	F^- ion in hydrolysed cell extracts (μmoles/10 mg. dry wt.)	F^- ion in hydrolysed suspending fluid (μmoles/1.8 ml.)
50 μ moles 3FG	0 minutes	1.50	50.40
"	30 minutes	5.00	
"	1 hour	10.05	35.40
"	2 hours	11.80	34.77
"	3 hours	13.60	33.16
"	4 hours	15.10	32.50
"	5 hours	19.30	29.70
50 μ moles 3FGA	0 hour	1.84	
"	1 hour	11.30	38.80
"	2 hours	11.58	39.20
"	3 hours	11.42	39.50
"	4 hours	12.01	39.20
"	5 hours	11.35	43.90

Cell-free extracts of glucose-grown Ps. fluorescens A, 3.12.

An early investigation of cell-free activity towards 3FG was made using the cell crush obtained by one passage through a Hughes' press at -20°, of the buffer-washed cell paste from 8x125 ml. amounts of suitably inoculated glucose/mineral salts medium, incubated for 20 hours, with aeration, at 30°. After treatment, at room temperature, with a few crystals of DNAase, and dilution in one volume of 0.067M phosphate buffer, pH 7.0, the extract was spun at 40,000xg for 15 minutes, and the straw-coloured supernatant (15 mg. protein per ml.) so obtained used as a crude enzyme preparation. Tested manometrically, it was found that the preparation was able to oxidise 3FG, albeit at approximately half the initial rate of oxygen uptake on a similar amount of glucose, with a total oxygen consumption approaching 1 atom of oxygen per mole 3FG.

However, because of subsequent damage to the Hughes' press, alternative methods of cell disruption had to be sought. These involved the use of a Braun ball-mill, the Eaton press, and a French press, all of which proved unsatisfactory for obtaining cell-free extracts of Ps. fluorescens. It was not until a 200 watt amplifier, driving a 20 kc. oscillator and harnessed to a magnetostrictive transducer unit with a titanium transforming stub attached, had been constructed that bacterial extracts rich in protein could be readily prepared, and it was by use of this apparatus that all further cell-free extracts were prepared.

EXPT.16. The growth from 2x125 ml. amounts of suitably inoculated glucose/mineral salts medium, incubated with aeration and at 30° for 18 hours, was used to inoculate 9 litres of the same medium in a 10 litre fermentation vessel. aeration for the large scale incubation being provided by the Vibromischer reciprocating drive unit attached to a lift pump.

After 20 hours growth at 30°, cells were harvested in a Sharples centrifugal separator at 25,000 r.p.m., washed twice in 0.067M phosphate buffer, pH 7.0, and the cell paste stored at -20°. A crude enzyme extract was prepared by resuspending a portion of the melted cell pastein one volume of 0.067M phosphate buffer, pH 7.0, and subjecting the suspension to ultrasonic vibrations for 5x2 minute periods. After this treatment, centrifugation at 20,000xg for 15 minutes yielded an opalescent, reddish supernatant and a pink particulate layer.

Tested manometrically, a mixture of supernatant and particulate layer oxidised glucose, gluconate, 3FG and 3FGA rapidly, and without the addition of accessory hydrogen carriers such as phenazine dyes or methylene blue (Fig.35). 10 μMole amounts of substrate were oxidised at an approximately linear rate for 20-30 minutes and oxygen consumption then quickly declined to that of the control without added substrate. Calculated from the near-linear period, the rate of 3FG oxidation proceeded at approximately half that of the same amount of glucose, whereas both gluconate and 3FGA oxidation proceeded at rates comparable to one another (Table 32). The net oxygen consumption per mole of substrate added approximated to 2 atoms per mole for both glucose and 3FG, and 1 atom per mole for gluconate and 3FGA (Table 32). These are the theoretical values required for the conversion of sugars and aldonic acids to ketoaldonic acids, and tests on the suspending fluid from Warburg flasks at the end of glucose, 3FG, gluconate and 3FGA oxidation revealed the presence of reducing material which reacted positively with Benedict's reagent. This material was undoubtedly 2-ketogluconate in supernatants from the glucose-and

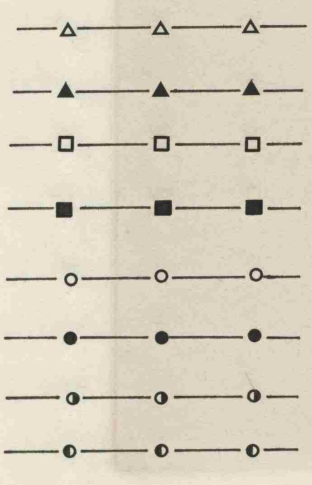
Fig.35. Oxidation of 3FG and 3FGA by cell-free extracts of glucose-grown Ps. fluorescens.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air

Each flask contained: 1 ml. (48.5 mg.) protein, 1 µmole NAD (87% purity), 0.67M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 10 or 20 µmoles substrate from side-arm.

Endogenous respiration subtracted (278 µl. in 180 mins.).

Key:



20 µmoles glucose
20 µmoles 3FG
10 µmoles glucose
10 µmoles 3FG
20 µmoles gluconate
20 µmoles 3FGA
10 µmoles gluconate
10 µmoles 3FGA

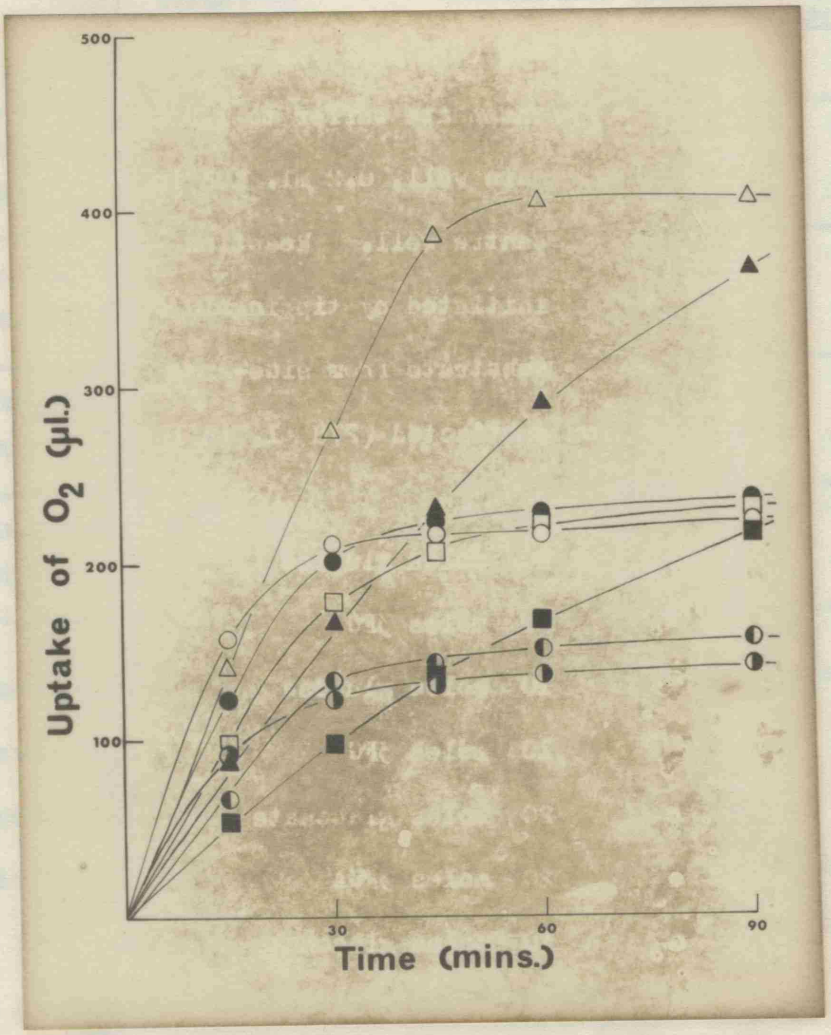

500
400
300
200
100
Uptake of O_2 (µl.)
30
60
90
Time (mins.)

TABLE 32

Manometric data on the oxidation of 3FG and 3FGA by cell-free extracts of glucose-grown Ps. fluorescens. (see Fig.35)

substrate oxidised	rate of oxidation (μ moles O_2/mg. protein/hr.)	net O_2 consumption (μl.) (endogenous subtracted)	moles of O_2 per mole of substrate oxidised
20 μ moles glucose	0.52	410	0.92
20 μ moles 3FG	0.29	410	0.92
10 μ moles glucose	0.36	235	1.05
10 μ moles 3FG	0.20	260	1.16
20 μ moles gluconate	0.57	225	0.50
20 μ moles 3FGA	0.45	235	0.53
10 μ moles gluconate	0.34	145	0.65
10 μ moles 3FGA	0.31	155	0.69

gluconate-containing flasks, and most likely, by analogy, 2-keto-3-fluorogluconate from 3FG and 3FGA oxidation though time did not permit any further characterisation of this latter oxidation product.

A preliminary examination of the Pseudomonas extracts indicated that the 3FG and 3FGA oxidising systems had similar characteristics to those for glucose and gluconate. Neither NAD nor NADP stimulated oxygen uptake with the crude extracts, and oxidation was considered to proceed without prior substrate phosphorylation, since neither the 3FG nor the 3FGA oxidation rates were dependent upon the addition of ATP. The particulate nature of the 3FG and 3FGA oxidising system was demonstrated by its ability to be separated from the soluble proteins by high speed centrifugation

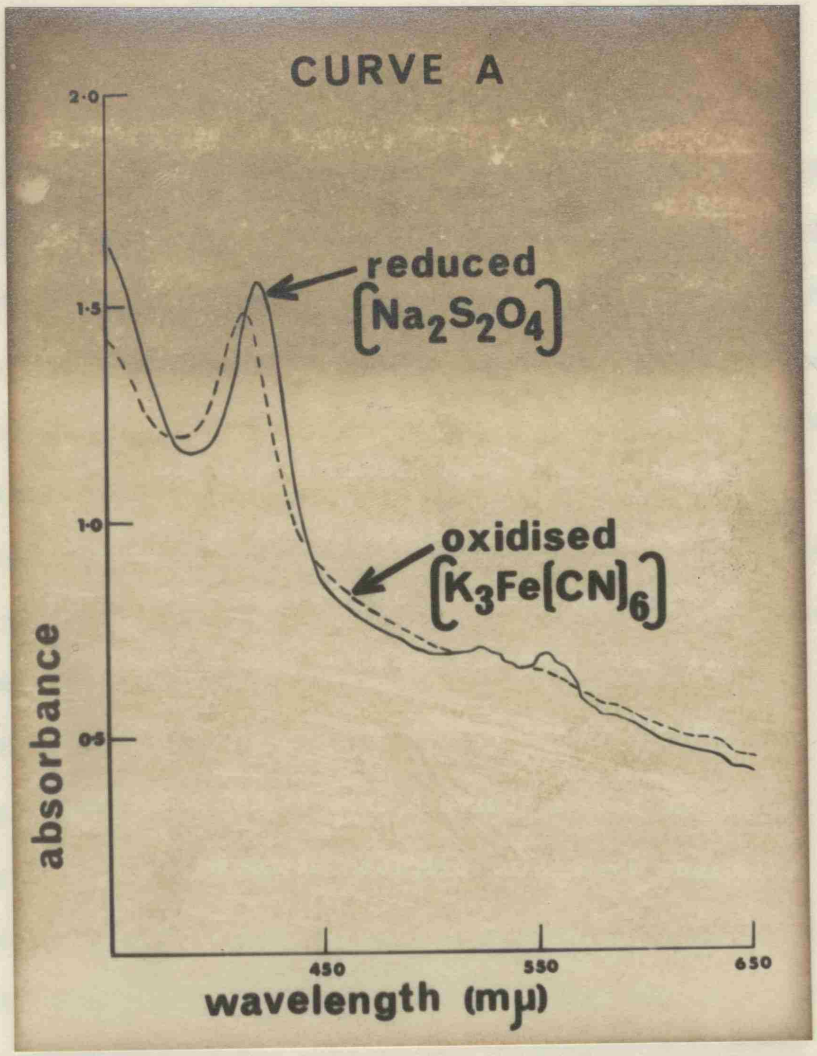
(140,00xg for 15 minutes). Although only a 33⅓% inhibition of 3FG oxidation was observed in the presence of 10^{-2} M cyanide, a similar low level of inhibition of glucose oxidation was also observed and did not eliminate the cytochrome pigments as carriers in 3FG oxidation. Spectroscopic studies of the respiratory catalysts gave more direct evidence implicating the cytochromes. Crude cell-free extracts showed a strong absorption below 450 mμ and distinct bands between 520 and 570 mμ. The latter could be discharged with ferricyanide but reformed almost immediately, presumably due to the oxidation of endogenous substrates. The endogenous oxidation of cytochrome pigments still persisted in both the particulate and supernatant material resulting from high speed centrifugation (140,000xg for 15 minutes). However, the supernatant obtained by spinning, at 20,000xg for 15 minutes, a cell extract prepared immediately after centrifugation and washing of a freshly grown batch of cells, contained an intact system for 3FG and 3FGA oxidation, and, when sparged with oxygen, did not develop the reduced cytochrome bands endogenously. This preparation was subsequently used to study the effect of added substrates.

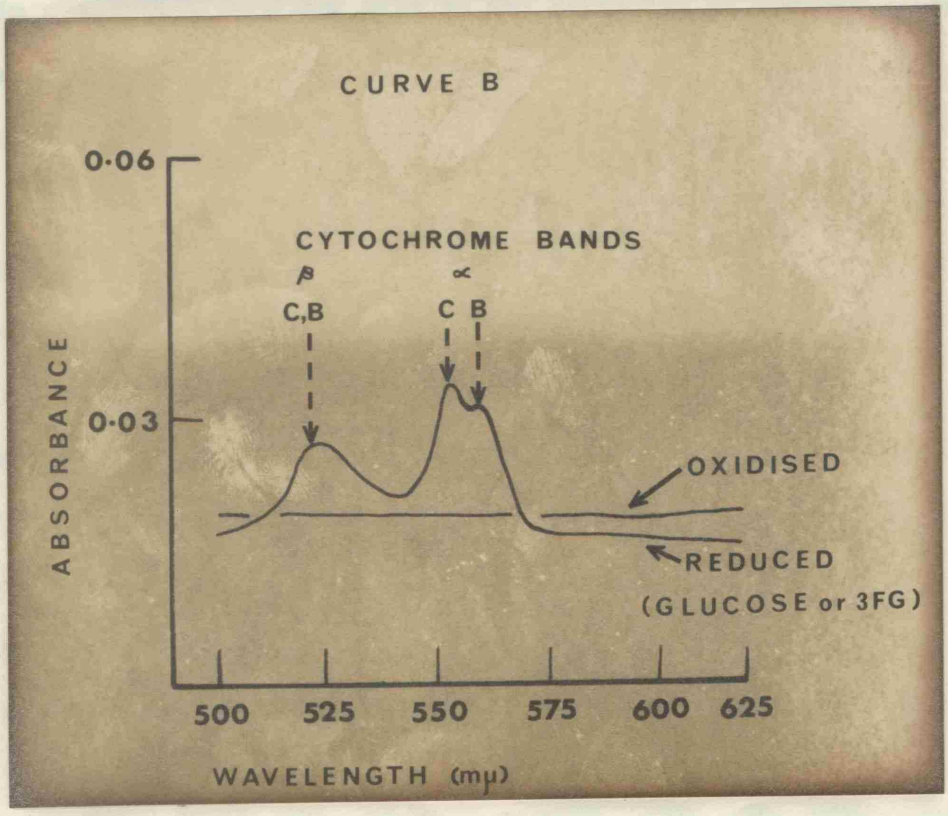
The addition of glucose, 3FG, and dithionite caused the formation of a distinct band at 550-560 mμ and a second band, less distinct between 560-565 mμ. Another band was detected in the region of 520-530 mμ, but nothing was visible in the region of 600 to 620 mμ. These bands could be discharged by oxygen and ferricyanide and corresponded approximately to the reduced α-and β-peaks of cytochromes c and b1. Fig.36a shows the absorption spectrum of a crude extract when sparged with oxygen and when reduced with

Fig.36. Cytochrome absorption spectra of cell-free extracts of Ps. fluorescens.

Curve A: cuvettes contained 0.5 ml. extract (15 mg. protein), together with 2.5 ml. of 0.067M phosphate buffer, pH 7.0, and were oxidised by either sparging with oxygen or by the addition of 0.05 ml. of 1% (w/v) potassium ferricyanide. A few crystals of sodium dithionite, glucose, or 3FG were added as indicated. The absorption was measured with a Unicam SP1800 against an air blank.

Curve B was the same as Curve A except that absorption was measured against a blank containing oxidised extract.


CURVE A
2·0
1·5
1·0
0·5
absorbance
reduced
[Na2S2O4]
oxidised
[K3Fe[CN]6]
450
550
650
wavelength (mμ)


CURVE B
0·06
0·03
ABSORBANCE
CYTOCHROME BANDS
β
C,B
∝
C
B
OXIDISED
REDUCED
(GLUCOSE or 3FG)
500
525
550
575
600
625
WAVELENGTH (mμ)

dithionite. With a small amount of oxygenated extract (0.5 ml. in 3 ml.), the peak of the oxidised cytochromes at approximately 415 mμ was detectable. When a small amount of sodium dithionite was added, the α-peak of cytochrome c at approximately 555 mμ, the β-absorption at 520-530 mμ, and the γ-peak at 425 mμ were readily observed. The absorption curves with glucose and with 3FG were identical with one another and with that of dithionite.

More detailed measurements after 3FG addition to a protein extract, made between 500 and 600 mμ, established the cytochrome c α-peak at 555 mμ and another component, as evidenced by the dissymetry of the peak, at 560 mμ, the latter corresponding to the α-peak of cytochrome b1. The β-absorption peaks of reduced cytochromes b1 and c 520-530 mμ could not be resolved (Fig.36b). For these measurements the instrument was set with both test and blank cuvettes containing aliquots of the same oxygenated preparation to obtain a horizontal base line; thus subsequently observed changes in absorption were due only to the formation of reduced components as a result of substrate additions to the test cuvette. The cytochrome spectra observed after 3FG addition are in essential agreement with those recorded by Wood and Schwerdt[139] for the glucose-oxidising system in Ps. fluorescens. Moreover the absorption observed when glucose was added to the test cuvette could be cancelled by the addition of 3FG to the blank mixture and vice versa.

A preliminary attempt to determine the rates of transphosphorylation from ATP to gluconate and 3-deoxy-3-fluorogluconate by gluconokinase in the crude extracts was made by coupling the transphorylation reaction to NAD production via phosphoenolpyruvate, pyruvate and lactate and following the rate of optical density change at 340 mμ. However, the considerable

adenosinetriphosphatase (ATPase activity) in the extracts (0.035μ moles of ADP produced per minute per mg. of protein at 30° and pH 7.4) masked any possible gluconokinase activity and no phosphorylation of either 3FGA or gluconate could be detected. Unfortunately time did not permit any further fractionation of crude extracts to separate gluconokinase from ATPase, so it is not known whether 3FGA can be phosphorylated by extracts of Ps. fluorescens or not.

Ps. fluorescens A. 3.12, asparagine-grown whole cell suspensions.

After growth on asparagine, Ps. fluorescens is unadapted to oxidise gluconic and 2-ketogluconic acids but can immediately oxidise glucose[138]. Studies with asparagine-grown cells tested (a) the ability of washed suspensions to oxidise 3FG and 3FGA; (b) the effect of 3FG and 3FGA on induction of the gluconate-oxidising system by gluconate; and (c) whether or not 3FG or 3FGA could act as enzyme inducers for washed cell suspensions.

EXPT.17. Cells, grown for 21 hours with aeration and at 30° in asparagine/mineral salts medium, were collected by centrifugation at 4040xg for 10 minutes, washed twice, resuspended to 16 mg. dry weight per ml. in 0.067M phosphate buffer, pH 7.0, and their ability to oxidise 3FG and 3FGA tested manometrically. Fig.37 shows the oxygen uptakes by asparagine-grown cell suspensions furnished with limiting amounts of 3FG, 3FGA, glucose and gluconic acid. 3FG Oxidation occurred at an approximately linear rate, 20-25% of that observed with glucose-grown cells under identical conditions, but with the same eventual oxygen uptake corresponding to one oxygen atom per mole of 3FG oxidised. However, during a 6 hour period of exposure to varying concentrations of 3FGA, there was no significant increase in oxygen consumption by cells over that of control cells without added substrate (Table 33). It appeared, therefore, that for 3FGA oxidation by Ps. fluorescens

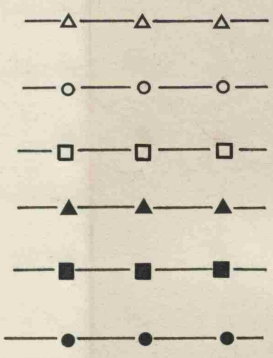
Fig. 37. Oxidation of glucose, gluconic acid, 3FG, and 3FGA by washed suspensions of asparagine-grown Ps. fluorescens.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 10 or 50 μ moles substrate, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (8 mg. dry weight) from side-arm.

Endogenous respiration (660 μ l. in 4 hours) subtracted.

Key:



10 μ moles glucose

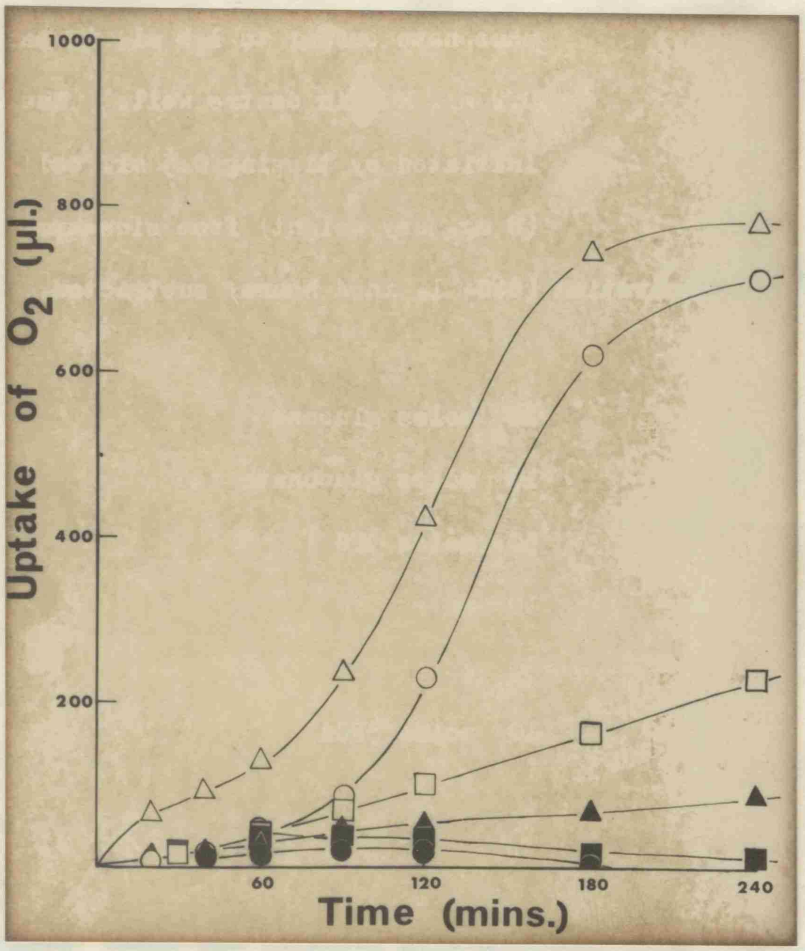
10 μ moles gluconate

50 μ moles 3FG

10 μ moles 3FG

50 μ moles 3FGA

10 μ moles 3FGA


1000
800
600
400
200
Uptake of O_2 (µl.)
60
120
180
240
Time (mins.)

to occur, cells had to be adapted to oxidise gluconate. Asparagine-grown cells oxidised gluconate only after a lag period of 60-90 minutes (Fig.37) and, in experiments to determine the effects of 3FG or 3FGA on the formation of induced enzymes in washed suspensions, a concentration of 1mM gluconate was used in the induction mixture. After incubation at 30° for 90-120 minutes, the rate of oxidation of 5mM gluconate by cells was determined. To avoid any possible interference of the fluoro analogues with gluconate oxidation, experiments were conducted as follows.

TABLE 33

Manometric data on the oxidation of 3FG and 3FGA by washed suspensions of asparagine-grown Ps. fluorescens. (see Fig.37)

	observed value (µl.)	moles of O_2 per mole of substrate oxidised
endogenous O_2 uptake	660	
net O_2 uptake 10µ moles 3FG	90	0.40
net O_2 uptake 10µ moles 3FGA	20	0.09
net O_2 uptake 10µ moles glucose	780	3.48
net O_2 uptake 10µ moles gluconate	720	3.22

EXPT.18a. Cells, grown for 20 hours with aeration at 30° in asparagine/mineral salts medium, were collected by centrifugation at 4040xg for 10 minutes, washed twice in 0.067M phosphate buffer, pH 7.0, and resuspended in buffer of the same molarity and pH to 4 mg. per ml. 5 ml. Aliquots of this suspension, in 25 ml. Ehrlenmeyer flasks containing 0.01M gluconate (1 ml.)

and 0.01M or 0.1M 3FG or 3FGA (1 ml.), were shaken at 30°. Meanwhile 1 ml. from a flask containing gluconate but no fluorinated substrates, and 1 ml. from a control flask containing no exogenous substrates were shaken in the Warburg apparatus. When oxygen uptake in the Warburg flask containing gluconate began to increase over that in the control flask, indicating that enzymic induction was almost complete (120 minutes), the cells in the Ehrlenmeyer flasks were collected by centrifugation, washed once in 0.067M phosphate buffer, resuspended in 2 ml. of the same buffer, and their ability to oxidise gluconate tested manometrically. Under these conditions there was an immediate oxidation of substrate when gluconate was present either alone or with 3FG for the whole of the induction period. In the control, to which no inducing gluconate was added, the substrate was oxidised after a further 60 minutes. The length of the lag period before gluconate oxidation was increased by the addition of 3FGA (0.01M to 1mM) at the same time as inducer, and this increase was greatest with the highest concentration of 3FGA and least with the lowest (Fig.38, Table 34). Thus, as judged by its effect on the length of the induction period, 3FGA inhibited the formation of the gluconate-oxidising system induced by gluconate. In no experiment was the length of the lag period before the oxidation of gluconate decreased by replacing glucose or gluconate normally used as inducer by 3FG or 3FGA.

EXPT.18b. Cells grown for 22 hours in asparagine/mineral salts medium were collected by centrifugation at 4040xg for 10 minutes, washed twice in 0.067M phosphate buffer, pH 7.0, and resuspended in buffer of the same molarity and pH to 4 mg. dry weight per ml. 5 ml. Aliquots of this suspension, in 25 ml. Ehrlenmeyer flasks containing either 0.02M or 0.1M 3FG or 3FGA (1 ml.) were incubated with shaking at 30°. Meanwhile 1 ml. from a flask containing glucose but no fluorinated substrates, and 1 ml. from a control flask containing no exogenous substrates were shaken in the Warburg apparatus.

Fig.38. Adaptation to gluconate and inhibition of adaptation by 3FGA in washed suspensions of Ps. fluorescens.

Cells were grown with asparagine as carbon source, collected, washed, and incubated with the additions shown below to adapt the cells. Their ability to oxidise gluconate was followed manometrically after cells had been separated from induction mixture by centrifugation, washing, and resuspension.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml.,
gas phase = air.

Each flask contained: 1 ml. cell suspension (10 mg. dry weight),
0.067M phosphate buffer to 1.8 ml.
in main well, 0.2 ml. KOH in centre well.
Reaction was initiated by tipping
10 µ moles gluconate from side-arm.

Endogenous respiration (670 µl. in 435 minutes) subtracted.

Key:

	induction mixture:
—△——△——△—	10 µ moles gluconate
—○——○——○—	10 µ moles gluconate, 10 µ moles 3FG
—●——●——●—	10 µ moles gluconate, 100 µ moles 3FG
—□——□——□—	10 µ moles gluconate, 10 µ moles 3FGA
—■——■——■—	10 µ moles gluconate, 100 µ moles 3FGA
—▲——▲——▲—	no addition

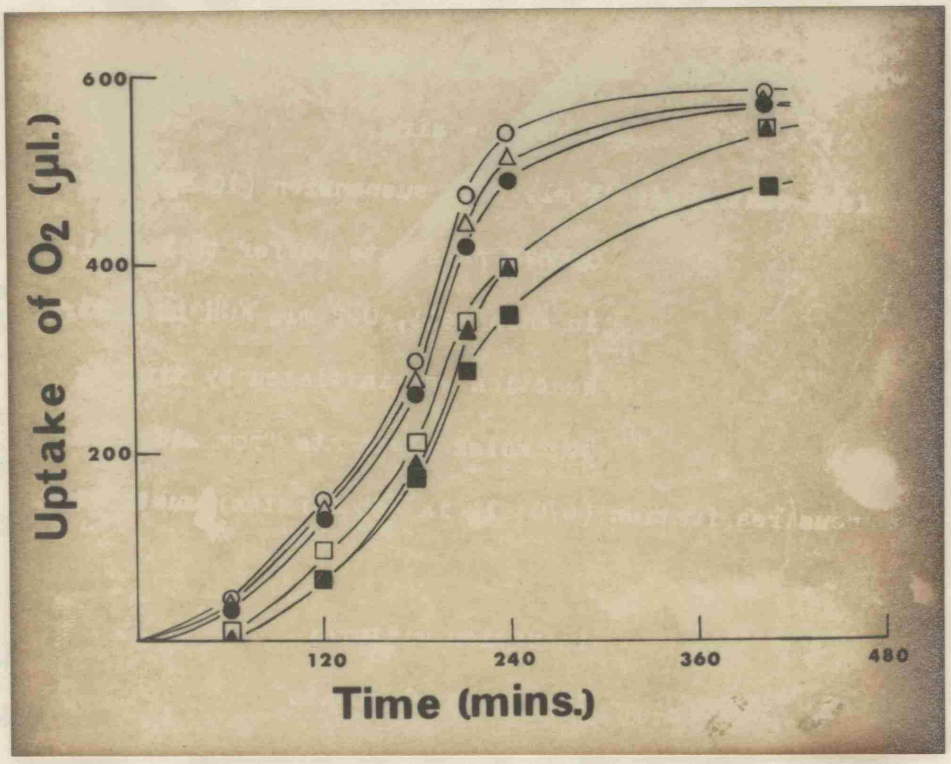

600
400
200
Uptake of O_2 (µl.)
120
240
360
480
Time (mins.)

TABLE 34

The effect of 3FG and 3FGA on induction of the gluconate-oxidising system by gluconic acid.

Cells were grown in liquid medium containing asparagine as carbon source, collected, washed, and incubated at 30° in 25 ml. Ehrlenmeyer flasks with 1mM gluconate as inducer and additions shown below. After 120 minutes cells were collected, washed once, resuspended to 10 mg. dry weight per ml. and incubated in Warburg flasks with 5mM gluconate. The time taken for the cells to oxidise gluconate at the maximal rate was measured (Fig.38).

addition to induction mixture	time at which gluconate oxidation was initiated (mins.)	rate of substrate oxidation (μmoles of O_2/mg. dry wt./hr.)		
		time 60 mins.	240 mins.	435 mins.
none	15	0.19	0.50	0.35
10μ moles 3FG	15	0.20	0.52	0.36
100μ moles 3FG	15	0.16	0.46	0.35
10μ moles 3FGA	45	0.05	0.38	0.33
100μ moles 3FGA	70	0.00	0.32	0.29
control (with no inducer)	60	0.00	0.37	0.33

After 3 hours incubation with the fluorinated substrates, at which time gluconate induction had already occurred in the cells exposed to glucose, flask contents were centrifuged, cells washed once and resuspended to 10 mg. dry weight per ml. in 0.067M phosphate buffer, and their ability to oxidise glucose and gluconate determined manometrically (Fig.39 a and b). As from Fig.37 it

Fig.39a and b. Effect of 3FG and 3FGA as inducers for gluconate oxidation by washed suspensions of Ps. fluorescens.

Washed suspensions of asparagine-grown cells were incubated for 3 hours with the additions shown below, in Ehrlenmeyer flasks, and their ability to oxidise (a) glucose and (b) gluconate determined manometrically, after separation from the induction mixture by centrifugation and washing.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 1 ml. cell suspension (10 mg. dry weight), 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 10 µ mole amounts of either glucose or gluconate from side-arm.

Endogenous respiration (505 µl. in 480 minutes) subtracted.

Key:

	inducer
—△——△——△—	20 µ moles glucose
—●——●——●—	100 µ moles 3FG
—○——○——○—	20 µ moles 3FG
—■——■——■—	100 µ moles 3FGA
—□——□——□—	20 µ moles 3FGA
—▲——▲——▲—	no additions

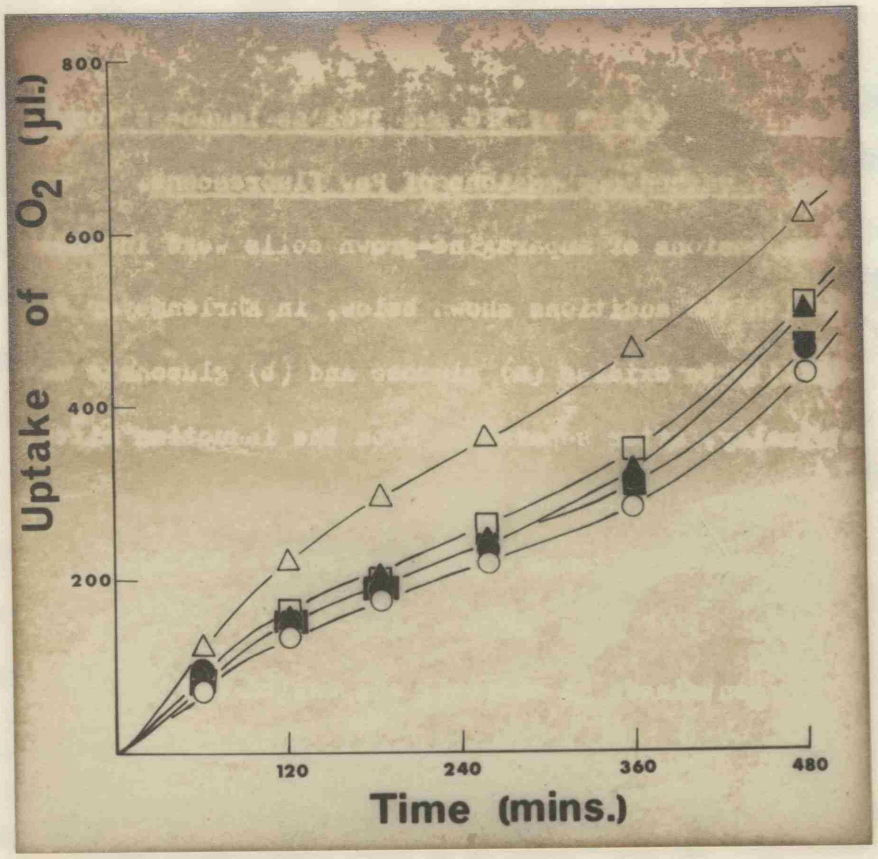


Fig.39a

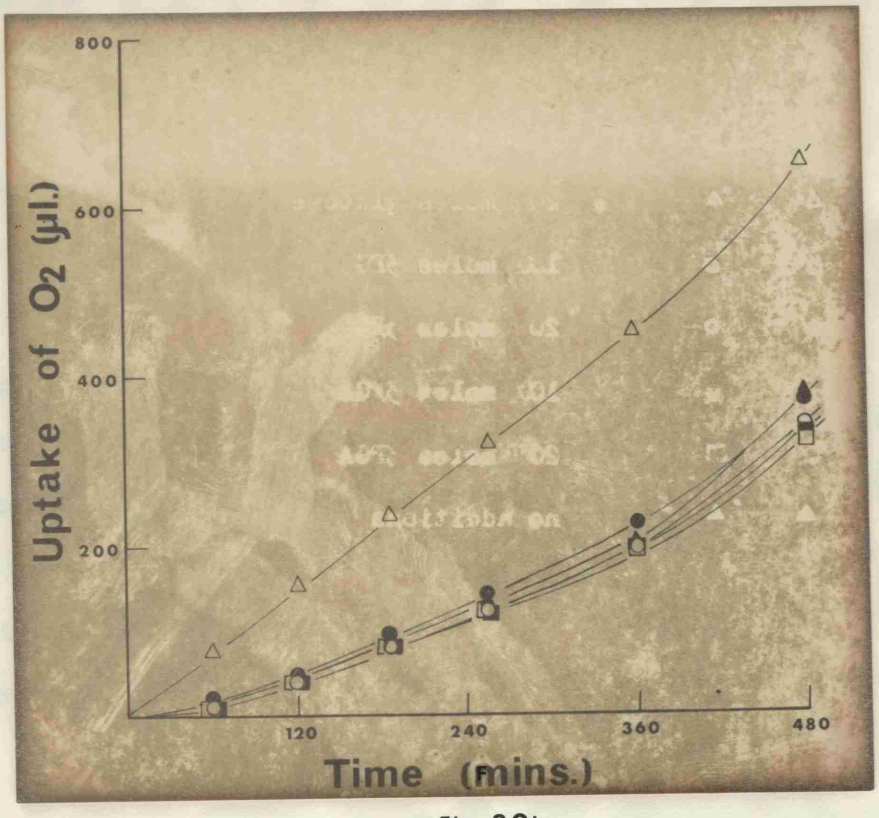


Fig.39b

was evident that cells did not adapt to oxidise 3FGA when 3FGA alone was present, so from Fig.39 a and b it is evident that neither 3FG nor 3FGA reduced the lag period taken to form the gluconate-oxidising system; on the contrary, pretreatment of cells with 3FGA slightly increased the time for subsequent adaptation to gluconate (Table 35).

TABLE 35

Effect of 3FG and 3FGA as inducers of the gluconate-oxidising system.

Washed cells (20 mg. dry weight) were incubated for 3 hours with the additions shown below, centrifuged, washed, and resuspended and their ability to oxidise gluconate (10 μ moles) measured manometrically (Fig.39b).

addition	rate of oxidation (μ moles of O_2/mg. dry wt./hr.)		
time (mins.)	60	180	480
none	0.07	0.13	0.21
20 μ moles 3FG	0.05	0.12	0.19
100 μ moles 3FG	0.06	0.14	0.21
20 μ moles 3FGA	0.04	0.12	0.18
100 μ moles 3FGA	0.03	0.11	0.18
20 μ moles glucose	0.33	0.35	0.36

The effect of 3FG on the growth of Ps. fluorescens A, 3.12.

At concentrations of glucose between 0.01-0.05M the growth of strain A, 3.12 reached a maximum in 18-48 hours. At higher concentrations (0.1M) the time taken for maximum growth was anything up to 4 days. Below 0.01M glucose the final yield was limited by the available carbon source since more growth was obtained when asparagine was also present (Table 37).

3FG Did not support growth when added as the sole carbon source, nor did it increase the final yield when added to a medium containing asparagine (Tables 36 and 37). The addition of 3FG to a medium containing glucose as carbon source reduced growth, the reduction being greates in the highest concentration of 3FG and the least in the lowest (Table 36). This suggests that 3FG could act as a competitor of glucose. Examination of flask filtrates after 4 days revealed no significant amounts of fluoride anion.

Twelve soil samples from widely differing regions were homogenised in water and samples incubated at 30° with shaking in the mineral medium containing 3FG (0.1% w/v), but no organisms capable of growing with 3FG as sole carbon source were ever isolated.

TABLE 36

Effect of 3FG on the growth of Ps. fluorescens with glucose as carbon source.

Growth tests were carried out in the same mineral salts medium as used for bulk growth. Portions (25 ml.) were placed in 100 ml. Ehrlenmeyer flasks and sterilised by autoclaving at 15 p.s.i. for 15 minutes. The carbon sources, sterilised by filtration, were added aseptically in the concentrations shown. Inocula were grown for 24 hours on solid medium, resuspended in sterile distilled water, and 0.1 ml. of a suitable dilution added to each of the test flasks, which were then incubated with aeration at 30°. Growth was measured turbidimetrically on aesptically removed samples at 620 mμ in a SP600. An optical density reading of 1.0 indicates a dry weight of 0.35-0.40 mg. per ml.

substance added		growth (optical density at 620 mμ)				
concn. of glucose (mM)	concn. of 3FG (mM)	12 hrs.	18 hrs.	24 hrs.	36 hrs.	48 hrs.
100	0	1.07	2.57	2.67	2.73	2.81
50	0	1.08	3.24	4.05	4.98	5.16
40	0	1.40	3.09	3.96	4.95	4.65
40	10	1.07	3.00	3.00	4.02	4.65
40	20	0.05	1.78	2.93	3.44	3.66
40	30	0.00	0.46	2.31	2.76	2.75
30	0	1.44	3.33	4.20	3.87	3.21
30	10	0.36	2.90	3.47	3.81	3.75
30	20	0.14	2.85	2.84	2.75	3.39
30	30	0.00	0.02	1.67	3.00	3.48
20	0	1.00	3.20	3.15	3.14	1.19
20	10	0.38	1.97	2.49	2.79	1.42
20	20	0.08	0.24	2.13	2.52	2.48
20	30	0.07	0.10	0.33	2.14	2.49
10	0	0.65	1.74	1.36	1.09	1.25
10	10	0.23	0.43	1.34	1.54	1.37
10	20	0.09	0.14	0.41	1.14	0.82
10	30	0.00	0.08	0.35	0.66	0.86
5	0	0.47	0.79	0.95	0.88	0.87
5	10	0.18	0.74	0.80	0.93	0.98
5	20	0.06	0.44	0.41	0.87	0.80
5	30	0.00	0.02	0.23	0.66	0.48

TABLE 37

Effect of 3FG on the growth of Ps. fluorescens with asparagine as carbon source

Growth media and measurements of growth were as described for Table 36. Each flask contained 25 ml. of mineral salts medium and 10mM asparagine plus the additions shown below. Inoculations were made from a suspension in sterile distilled water of the growth from a 24 hour asparagine slope culture. An optical density reading of 1.0 indicates a dry weight of 0.35-0.40 mg. per ml.

addition	concn. of addition (mM)	growth (optical density at 620 mμ)				
		12 hrs.	18 hrs.	24 hrs.	36 hrs.	42 hrs.
none		1.58	0.42	0.46	0.26	0.26
		1.52	0.40	0.49	0.28	0.29
glucose	50	3.00	5.01	6.00	5.85	5.58
	30	2.99	4.50	4.62	4.29	4.11
	20	3.12	2.60	3.48	2.94	2.73
	10	2.69	2.13	2.01	1.76	1.51
3FG	50	1.34	0.59	1.17	0.97	0.92
	30	1.42	1.00	1.20	1.02	0.99
	20	1.41	0.97	1.13	1.04	1.02
	10	1.25	0.99	0.98	0.80	0.74

Ps. saccharophila, glucose-grown whole cell suspensions.

In view of the unusual enzymic machinery of this organism whereby dehydrase action converts gluconic acid exclusively to 2-keto-3-deoxygluconic acid before the formation of 3-carbon fragments, a brief study of the respiration rates with 3FG and 3FGA and the effect of these compounds on glucose catabolism was made using washed glucose-grown cell suspensions. EXPT.19. After 22 hours aerated growth at 30° in glucose/mineral salts medium, cells were collected by centrifugation at 30°, washed twice in 0.067M phosphate buffer, pH 7.0, resuspended to approximately 28 mg. dry weight per ml. in buffer of the same molarity and pH, and their ability to oxidise 3FG, 3FGA, glucose and gluconate, both separately and as mixtures of the fluorinated and natural substrates determined manometrically (Fig.40). Oxygen consumption by cells furnished with limiting amounts of 3FG and 3FGA proceeded initially at a rate approximately 25% of that achieved by cells respiring 10 μ moles glucose, and independent of the amount of fluoroanalogue present. In most cases, however, the initial rate declined rapidly after 45-60 minutes and oxygen consumption subsequently fell below the level of control cells respiring without exogenous substrate. Only with 10 μ moles amounts of 3FGA could an oxygen uptake approaching that necessary for a single stage oxidation of the molecule be attained, and this after a 60 minute lag following on the initial oxygen uptake (Fig.40). However, it was noted from carbohydrate measurements of incubation filtrates that upto 60% of a 5 mM solution of 3FG could be taken up by washed cell suspensions. The oxidation of equimolar amounts of 3FGA and glucose together was increased in both rate and extent compared with the oxidation of the glucose in the mixture alone, the differences in total oxygen uptake approximating the value theoretically required for one atom of oxygen per mole of 3FGA (Table 38). However, the oxidation of equimolar amounts of 3FG and glucose added together, did not differ significantly

Fig.40. Oxidation of 3FG, 3FGA, glucose and gluconate by washed suspensions of Ps. saccharophila.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 10 or 50 μ moles substrate, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well. Reaction was initiated by tipping 0.5 ml. cell suspension (14 mg. dry weight) from side-arm.

Endogenous respiration (1200 μl. in 300 minutes) subtracted.

Key:

—□——□——□— 10 μ moles glucose, 10 μ moles 3FGA

—o——o——o— 10 μ moles glucose, 50 μ moles 3FG

—▲——▲——▲— 10 μ moles glucose, 10 μ moles 3FG

—△——△——△— 10 μ moles glucose

—■——■——■— 10 μ moles gluconate

—●——●——●— 10 μ moles 3FGA

—x——x——x— 50 μ moles 3FGA, 50 μ moles 3FG, 10 μ moles 3FG

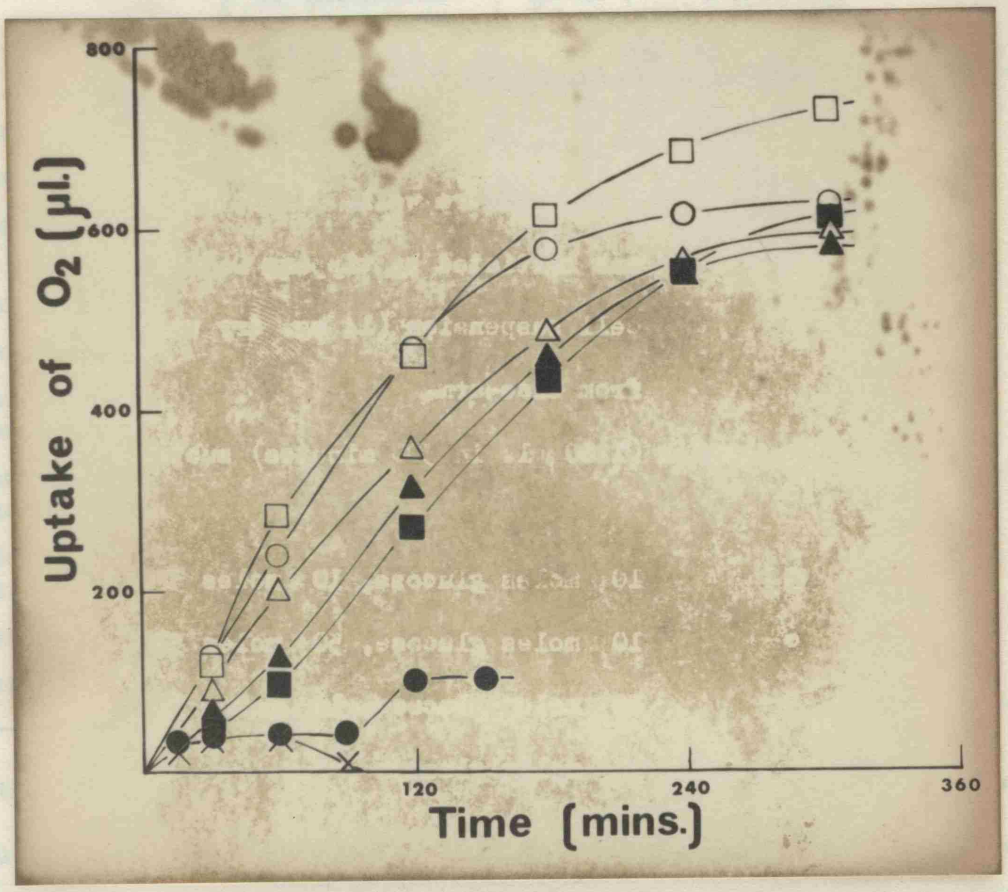

800
600
400
200
Uptake of O_2 (µl.)
120
240
360
Time (mins.)

TABLE 38

Manometric data on the oxidation of fluorinated and natural substrates by washed suspensions of Ps. saccharophila. (see Fig.40).

substrate(s)	maximal rate of oxidation (μmoles O_2/mg. dry wt./hr.)	net O_2 consumption (μl.)^2(endogenous subtracted)	moles of O_2 per mole of substrate oxidised
10 μ moles 3FG	0.16	37	0.17
50 μ moles 3FG	0.17	39	0.17
10 μ moles 3FGA	0.17 and 0.38	102	0.46
50 μ moles 3FGA	0.15	36	0.03
10 μ moles glucose	0.56	591	2.64
10 μ moles glucose, 10 μ moles 3FG	0.50	573	
10 μ moles glucose, 50 μ moles 3FG	0.74	624	
10 μ moles glucose, 10 μ moles 3FGA	0.73	724	
10 μ moles 3FGA		133	0.59
10 μ moles gluconate	0.53	605	2.70

from the oxidation of the glucose alone. Only by raising the ratio of 3FG to glucose, was any increase in the oxygen consumption by cells apparent, but the increment was never enough to satisfy the stoichiometry required for oxidation of the fluoro analogue (Fig.40).

Subsequent oxidation of glucose by washed cells incubated with either 3FG or 3FGA did not differ significantly from that of control cells preincubated without any exogenous substrate, though a slight elevation in the total oxygen uptake was always apparent (Fig.41, Table 39). At no time was an inhibition of glucose respiration by either 3FG or 3FGA observed.

Fig.41. Glucose oxidation by washed suspensions of Ps. saccharophila preincubated with 3FG or 3FGA.

Warburg conditions: temp. = 30°, tot.vol. = 2.0 ml., gas phase = air.

Each flask contained: 10 or 50 μ moles 3FG or 3FGA, 14 mg. dry weight of cell suspension, 0.067M phosphate buffer to 1.8 ml. in main well, 0.2 ml. KOH in centre well, Reaction was initiated by tipping 10 μ moles of glucose from side-arm after 150 minutes.

Endogenous respiration (370 μ l. in 210 minutes) subtracted.

Key:

—o——o——o— cells preincubated with 50 μ moles 3FG

—▲——▲——▲— " " " 10 μ moles 3FG

—●——●——●— " " " 50 μ moles 3FGA

—x——x——x— " " endogenously

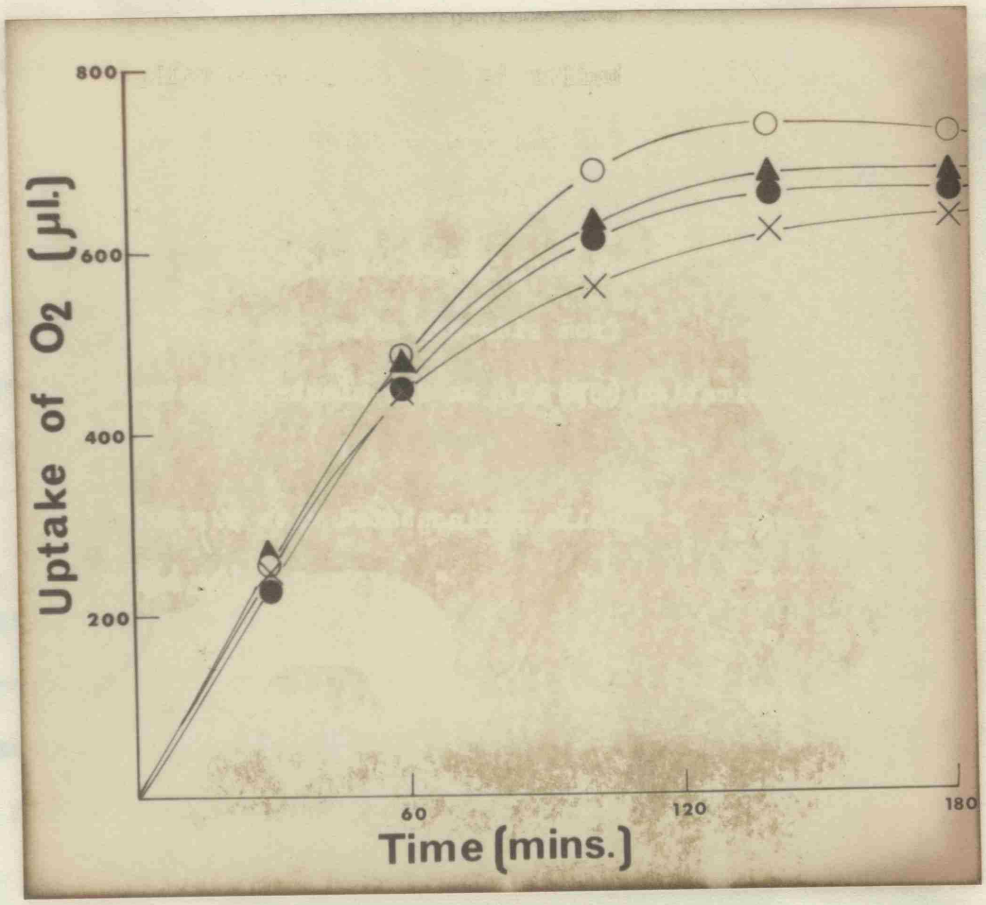

800
600
400
200
Uptake of O_2 (µl.)
60
120
180
Time (mins.)

TABLE 39

Manometric data on glucose oxidation by washed suspensions of Ps. saccharophila after incubation with 3FG or 3FGA.

Washed suspensions of Ps. saccharophila suspended to 28 mg. dry weight per ml. were incubated in the Warburg apparatus with the additions shown below. After 150 minutes 10 μ moles of glucose were added from the side-arm, and the subsequent oxygen uptake determined (Fig.41).

preincubation substrate	substrate oxidised	rate of oxidation (μmoles O_2/ mg. dry wt./hr.)	net O_2 consumed (μl.)^2(endogenous subtracted)	Moles of O_2 per mole of substrate oxidised
———	10μmoles glucose	1.41	653	2.92
10 μ moles 3FG	"	1.51	681	3.04
50 μ moles 3FG	"	1.53	728	3.25
50 μ moles 3FGA	"	1.43	689	3.07

A cell-free extract of glucose-grown Ps. saccharophila, prepared by ultasonic disruption of buffer-washed cell suspension and suspended to approximately 30 mg. of protein per ml., did not oxidise 10 mM 3FG when supplemented with either NAD and/or NADP; nor did any significant utilisation of the fluoro analogue occur in the presence of 5 mM ATP.

DISCUSSION

DISCUSSION

Emphasis has already been placed on the unique value that its size and electronegativity confer on fluorine in the design of analogues which can very closely approach the natural biochemical substrate. That these analogues can be used not only therapeutically, and as selective enzyme inhibitors, but also enable the definition of critical sizes contributing to structural considerations in biochemically important molecules, has undoubtedly inspired the synthesis of fluoroanalogues in the steroid[60], carboxylic acid[13] and heterocyclic[37] fields.

However, whereas fluoro analogues of many biologically active substances have been used effectively for many years, the use of analogues of glucose or its immediate metabolic products is a comparatively new field of study. The recognition of glucose as the universal source of energy for living cells coupled with the fact that anaerobic glycolysis is perhaps one of the most vital processes in the metabolism of neoplastic tissues, has given great impetus to the development and use of glucose analogues in cancer research. It is only recently, however, that fluoropentoses[142] and fluorohexoses[176] with fluorine in other than anomeric[143] or terminal[144] positions of monosaccharides have become available.

The amazing versatility shown by bacteria in their colonisation of most terrestrial environments is exemplified by the ability of many saprophytic soil bacteria to utilise not only aromatic and heterocyclic molecules as a sole source of carbon but also to degrade the fluoro analogues of these molecules. That fluorinated substrates are only attacked when the bacterium is adapted to metabolise the parent substrate suggests that the same enzyme oxidises both the natural compound and its analogue[85]. In certain instances fluoroacetate has been characterised as the end product of fluoro analogue catabolism by bacteria, with subsequent poisoning of terminal respiration[17,18,19],

but other inhibitory effects of fluoro analogues in bacterial systems, including the inhibition of the induction of the enzymes responsible for the oxidation and assimilation of the parent compound, have also been observed[85]. In addition to the metabolism of fluorinated substrates 'in toto', certain bacteria, isolated by suitable enrichment techniques, are capable of the enzymic cleavage of the carbon-fluorine bond in fluorinated molecules, with the subsequent release of fluoride anion into the suspending medium, and of utilising the remaining organic portion of the molecule for growth[68,78]. Indeed, a lack of means to liberate organic fluorine as fluoride ion is one of the main reasons for the inability of most bacteria to utilise fluorinated substrates for growth; other reasons may be cited as: (i) fluoroacetate production with subsequent inhibition of the TCA cycle; (ii) the oxidation of analogues not being carried to a stage at which carbon may be assimilated; (iii) the inhibition of the induction of the enzymes responsible for the oxidation and assimilation of the parent compound.

Glucose, because of its wide distribution and importance in animal metabolism, has served as the initial substrate for most studies of product formation and the mechanism of carbohydrate metabolism in bacteria. However, the only previous mention of the bacterial metabolism of a fluorinated analogue of glucose is the oxidation of 6-deoxy-6-fluoro-D-glucose to 6-deoxy-6-fluoro-2-ketogluconic acid by a cell-free preparation from Aerobacter aerogenes[95]. Studies with both this analogue and 2-deoxy-D-glucose show that the structure of D-glucose at C-2 and C-6 may be changed without destroying its activity as a substrate in this system.

Because of the variety of possible fates of fluorinated molecules in bacterial systems, it was of interest to study the metabolic fate and biochemical effects of 3-deoxy-3-fluoro-D-glucose when utilised as substrate

by microorganisms, and because of the known manner in which they respire glucose, the following organisms were chosen for this study.

(i) E.coli, which ferments glucose and a wide variety of carbohydrates and related compounds with the formation of acid and gas, both aerobically and anaerobically. The percent pathway participation in glucose metabolism (E.M. 72%, H.M.P. 28%)[145] resembles that of S.cerevisiae (E.M. 88%, H.M.P. 12%) - which organism was thought initially to oxidise 3PG by means of the glycolytic sequence[115a].

(ii) Ps. fluorescens A, 3.12 and Ps. saccharophila, both obligate aerobes which attack glucose and other carbohydrates oxidatively. Ps. saccharophila respires glucose exclusively[145] by the Entner-Doudoroff pathway, which involves an initial phosphorylation of glucose at carbon-6 and subsequent oxidation of glucose-6-phosphate followed by dehydrase action before the production of three-carbon fragments[146]. In Ps. fluorescens A, 3.12 the glycolytic sequence is virtually absent due to the lack of both hexokinase and phosphofructokinase[147a], but the complete system for the shunt and Entner-Doudoroff pathway is present[147b]. (An idea of the percent pathway participation in glucose catabolism by this organism can be gained from a comparison with Ps. aeruginosa and other pseudomonad species (E.D. 71%, H.M.P. 29%)[145,148]). Glucose is oxidised directly to gluconic acid and 2-ketogluconic acid without a prior phosphorylation. These latter oxidations are considered to occur in part on the cytoplasmic membrane and the whole system can be represented as follows:

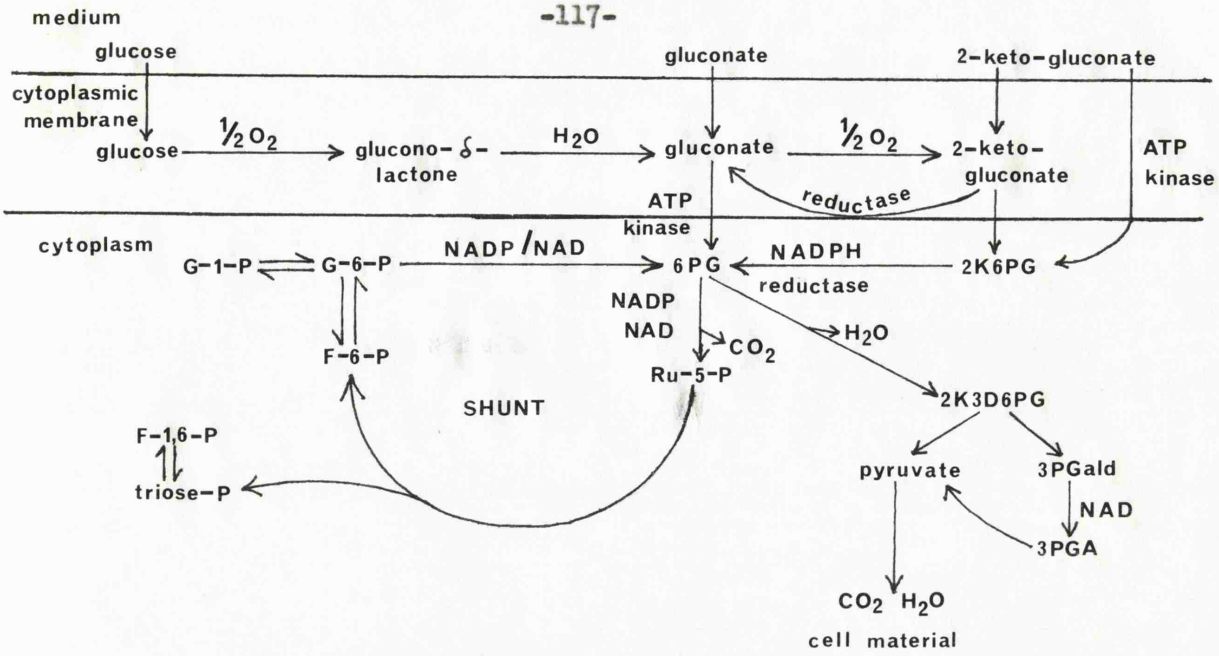


Fig.42. Carbohydrate metabolism in Ps. fluorescens and a proposal for the localisation of enzymes in this organism.

In this organism glucose is metabolised by two pathways, in one of which it is rapidly converted into gluconate and 2-ketogluconate by enzymes of the cytoplasmic membrane, and in the other more slowly by way of kinases into the shunt or Entner-Doudoroff scheme. After all the glucose has disappeared, most of it has accumulated as 2-ketogluconate which can then be fed back into the shunt either by the system 2-ketogluconokinase and 2-keto-6-phosphogluconoreductase or by the system 2-ketogluconoreductase and gluconokinase[149].

Thus the essential differences between the primary catabolic reactions of glucose degradation in E.coli, Ps. fluorescens, and Ps. saccharophila are:

(i) whether or not glucose-6-phosphate is the initial product of glucose catabolism whose formation is necessary for further utilisation of the substrate, as in E. coli and Ps. saccharophila;

(ii) if formed, whether glucose-6-phosphate is metabolised via hexosediphosphate, in the Emden-Meyerhof glycolytic scheme, or the fermentative and oxidative hexosemonophosphate pathways.

1) E.coli.

3FG is not oxidised to any significant extent by washed suspensions of E.coli (Fig.10, Table 4), and from measurements of the carbohydrate levels in the suspending buffer after the incubation of cell suspensions with the fluoro analogue (Tables 7 and 9) it was concluded that 3FG uptake by cells was minimal during the time period of such experiments. At the end of the incubation period the level of intracellular carbohydrate had fallen by the same amount as that of control cells incubated for the same time without an exogenous carbon source (Table 5 and 9), presumably due to the metabolism of endogenous carbohydrate reserves. However, in contrast with the endogenously respiring control cells, the level of carbohydrate in the 3FG-treated cells is not restored by the subsequent addition of glucose, rather glucose is oxidised to an extent greater than that of subsequent glucose oxidation by the controls by approximately one mole of oxygen per mole of glucose oxidised (Figs.11 and 13, Tables 6 and 8). This increase is apparently independent of the concentration of 3FG present during the preincubation period, and whether or not residual exogenous 3FG is present during the subsequent glucose oxidation.

When 3FG and glucose are given simultaneously to cell suspensions, the rate of oxygen uptake is the same as for that amount of glucose in the mixture when given alone as substrate, but the extent of oxidation is elevated by an amount which increases with the concentration of 3FG present (Fig.12, Table 7). However, the differences in total oxygen uptake between cells furnished with limiting amounts of glucose and 3FG, and cell suspensions exposed to glucose alone, do not correlate with the amounts of 3FG entering the cells, and it cannot be said from these results alone whether the

elevated oxygen consumption is due to (partial) oxidation of the fluoro analogue, possibly facilitated by the presence of an exogenous energy source, or an increased extent of glucose oxidation in the presence of 3FG, similar to the effect of 3FG on subsequent glucose oxidation. At no time was an inhibition of glucose oxidation by 3FG observed.

The glucose transport system in E.coli, as measured by α-methylglucoside accumulation, has been shown to possess the properties of a 'permease' system consisting of at least three separate reactions; an energy-requiring entrance reaction, an energy-requiring exit reaction, and an exit reaction which acts by a diffusion mechanism[150]. In addition other constituents of the permeation system, not possessing the same stereospecificity as the true permeases, have been demonstrated. Isolated membranes from glucose-grown E.coli take up and phosphorylate at carbon-6 α- and β-methylglucoside, glucose,2-deoxyglucose, fructose, galactose and 3-O-methylglucose in the presence of phosphoenolpyruvate. Thus the phosphoenolpyruvate phosphotransferase system provides a mechanism for the passage of certain sugars through the bacterial cell membrane with their subsequent accumulation as phosphorylated derivatives[151].

The inability of glucose-grown E.coli to accumulate, and therefore metabolise, 3FG may well be due to the as yet undefined absolute stereospecificity of the sugar permease systems in this organism. However, studies with cell-free preparations of E.coli show that not only does the limited oxygen consumption by extracts furnished with 3FG occur immediately (Fig.14, Table 10), and is therefore more likely to be due to oxidation of the fluoro analogue itself than oxidation of a (phosphorylated) intermediate but also that the addition of exogenous ATP does not stimulate enzymic activity. It was therefore concluded that phosphorylation of 3FG occurred to a negligible extent during these studies, and since the presence

of an equimolar amount of 3FG in no way inhibited enzymic activity towards glucose, it was also concluded that 3FG has a very much reduced affinity for E.coli hexokinase and that this is the main reason for its non-metabolism by this organism.

The elevated extent of glucose oxidation by whole cell suspensions of E.coli subsequent to 3FG treatment, together with the failure of subsequent glucose respiration to restore the level of intracellular carbohydrate, suggest an inhibition by 3FG in the assimilatory pathways from glucose - similar to that reported in 3FG-treated S.cerevisiae[115b]. However, due to the very limited uptake of 3FG by E.coli, it is difficult to argue in favour of 3FG occasioning a metabolic block in the intermediary metabolism of this organism. A more probable explanation is that 3FG treatment causes a shift in the energy balance of the cell due to ATP consumption in either the limited 3FG uptake which occurs, or the active transport of 3FG out of the cell. Any lowering of cellular ATP levels would favour a subsequently elevated extent of glucose oxidation to restore the cells energy status in a manner analogous to increased glucose assimilation by cells depleted of endogenous reserves.

The effect of preincubation with 3FG on the subsequent growth of E.coli in glucose/mineral salts medium suggests that the fluoro analogue may under these circumstances be acting in a competitive manner towards glucose, since both the rate of the subsequent logarithmic phase of growth and the final cell density achieved on glucose were retarded by pretreatment with the fluorinated substrate (Table 11). An alternative explanation is that preincubation with 3FG causes a similar effect in growing cells to that suggested to account for its effect on resting cells i.e. a lowering of cellular energy levels. 3FG exerted an inhibitory effect only

when cell suspensions were incubated with the analogue prior to their addition to the glucose medium. No significant inhibition of E.coli growth in glucose medium containing 3FG was observed (Table 12).

2) Ps. fluorescens A, 3.12.

Glucose-grown, washed cell suspensions of strain A, 3.12 oxidise 3FG at an immediate maximum rate which increases with substrate concentration and continues until the total oxygen consumption approximates to one atom per mole of substrate, when it declines to control level (Figs. 15, 20 and 28, Tables 13, 17 and 23). Thus the 3FG-oxidising system is constitutive in glucose-grown cells, but oxidation does not proceed further than one step (Table 19). The product which accumulates in the suspending buffer is acidic (Table 20), and chromatographically identical to chemically synthesised 3-deoxy-3-fluoro-D-gluconic acid (Fig.27). Although the total oxygen uptake by cell suspensions furnished with limiting amounts of 3FG is sufficient to satisfy the stoichiometry of the reaction:

$$3FG + \tfrac{1}{2}\,O_2 \longrightarrow 3FGA$$

the amount of acidic material detected in the suspending fluid is short of that theoretically expected from a one to one conversion. It must be concluded therefore that, either part of the fluorinated product is being transported into the cytoplasm, or is remaining bound at the site of 3FG oxidation (which, from studies with cell-free extracts has been shown to be of a particulate nature and is most probably on the cytoplasmic membrane). The determinations of fluoride anion concentrations in hydrolysates of the various fractions obtained from the incubation of 3FG with washed cell suspensions (Table 30) support the latter conclusion i.e. that a considerable portion of the product of 3FG oxidation remains bound to the cytoplasmic membrane, with only a very limited transportation of fluorinated material into

the cytoplasm.

When chemically synthesised 3FGA is the primary substrate for washed suspensions of Ps. fluorescens it is oxidised at an immediate maximum rate (Fig.29, Table 24), higher than that observed for the oxidation of 3FG under the same conditions (compare Tables 23 and 24), which increases with increased substrate concentration, and quickly declines to control levels when the total oxygen uptake reaches a value corresponding approximately to one atom per mole of substrate oxidised. The product of 3FGA oxidation has been shown to be a reducing compound (Table 28) chromatographically similar to 3FGA, and from manometric data (Table 24) is known to be at the oxidation level of a ketoaldonic acid. The absence of any adaptive lag before bacterial oxidation of the fluoro analogue suggests that oxidation of 3FGA, like that of 3FG, occurs by means of a constitutive enzymic process. However, studies with cell-free preparations of Ps.fragi show that the enzymes oxidising sugar acids have different properties to those of aldose dehydrogenases, being soluble, formed by induction and highly specific for the inducing substrate[152]. Because asparagine-grown pseudomonad suspensions are unable to oxidise either gluconic acid or 3FGA unless they have been induced to form the oxidising system by glucose or gluconic acid itself, it seems reasonable to assume that the same enzyme oxidises both gluconic acid and its analogue. 3FG oxidation by asparagine-grown cells, like that of glucose, occurs by a constitutive system, although at a reduced rate to that of its oxidation by glucose-grown cells.

Evidence for the particulate nature of the 3FG-oxidising system in strain A, 3.12 comes from studies with cell-free preparations of the glucose-grown organism. 3FG is oxidised to the same extent as glucose i.e. one mole of oxygen is consumed per mole of substrate, while 3FGA oxidation follows an identical pattern to that of gluconate, both substrates being oxidised at similar rates and with the same total oxygen uptake corresponding to one atom per mole of substrate (Fig.35, Table 32). Moreover, it was shown that the 3FG- and 3FGA-oxidising system is not a flavoprotein oxidase, since it was sensitive to cyanide, and does not involve a NAD- or NADP-specific dehydrogenase. The complete system, including carriers, is localised in particles which can be separated from the soluble proteins by centrifugation. The role of cytochrome carriers in the oxidation is supported by the formation of absorption peaks at 555 and 560 mμ in the presence of 3FG which can be discharged by oxygen or ferricyanide. These correspond to the bands of reduced cytochrome c1 and b1 respectively (Fig.36).

It is tempting to speculate on the identical nature of the 3FG- and glucose-oxidising systems in this organism. In Ps.fragi evidence has been found of a single particulate enzyme responsible for the oxidation of various sugars in the furanose form with the hydroxyl group at C-2 in the D-configuration, yielding the γ-lactone[152]. In contrast, the existence of separate enzyme systems for the oxidation of 2-deoxy-D-glucose and D-glucose has been demonstrated in cell-free extracts of A.aerogenes[95] and Ps. aeruginosa[153]. In Fig.18 it can be seen that the rates of oxidation of mixtures of glucose and 3FG are very similar to that of the most rapidly oxidised substrate (glucose), until the concentration of that substrate falls to such a level that rate is no longer maximal. This suggests that the two substrates may be oxidised by the same enzyme system. However, from

Fig.32, which shows the oxygen uptakes by cell suspensions furnished with mixtures of the two sugars in 0.67M phosphate buffer, it appears that mixing the two substrates results in a more rapid rate of oxidation than either substrate alone. A comparison of the rates of oxidation of the glucose, 3FG mixtures in Table 26 with the sum of the rates of glucose oxidation alone (Table 26) and 3FG oxidation under the same conditions (Table 23, from which, by extrapolation, the rate of oxidation of 10 and 50 μ mole amounts of 3FG approximates to 0.13 and 0.70 μ moles of O_2/mg.dry wt./hr. respectively) shows that the increased oxidation rate of the mixtures approximates very closely to the sum of the rates for each substrate alone. Since increasing the concentration of 3FG to equal the sum of the two substrates results in a comparatively small increase in the oxidation rate (Table 23), these results might suggest that the increased rates observed in the presence of the mixed substrate are due to the action of separate enzymes acting on each substrate. It is hoped that further work with cell-free extracts of this organism will help to clarify the exact relationship between the 3FG- and glucose-oxidising systems.

As already stated, the extents of oxidation of 3FG and 3FGA by extracts of Ps. fluorescens are those theoretically required for the oxidation of both sugar and aldonic acid to a ketoaldonic acid:

cell extracts $\quad 3FG \xrightarrow{\frac{1}{2}O_2} 3FGA \xrightarrow{\frac{1}{2}O_2} 3FGKA\ (?)$

$\qquad\qquad\qquad\qquad 3FGA \xrightarrow{\frac{1}{2}O_2} 3FKGA\ (?)$

However, with whole cell suspensions of this organism the amount of oxygen consumed at the expense of substrate 3FG corresponds to one atom of oxygen per mole of substrate - the amount theroretically required for oxidation of sugar to aldonic acid. When 3FGA is the primary substrate for whole cell oxidation, again one atom of oxygen per mole of substrate is consumed:

$$3FG \xrightarrow{\frac{1}{2}O_2} 3FGA$$

whole cell suspensions

$$3FGA \xrightarrow{\frac{1}{2}O_2} 3FKGA\ (?)$$

There are two possible explanations for the apparent anomaly of whole cell oxidation of 3FG proceeding only as far as aldonic acid formation, although 3FGA is itself oxidised by cell suspensions when present as the primary substrate:

(i) assuming that 3FGA has a low binding affinity for its specific carrier protein (presumably an aldonic acid - specific permease), or that the aldonic acid dehydrogenase reaction has a high K_m then, due to the slow (relative to glucose) rate of 3FG oxidation there is an insufficient concentration of 3FGA present at any one time to either bind to any significant extent to the carrier protein and/or to be oxidised at a detectable rate by the dehydrogenase enzyme. Because of the low concentration of 3FGA, what 3FGA/protein complex is formed, dissociates and rereleases 3FGA which is free to pass out into the suspending buffer. The concentrations of 3FGA given to cell suspensions as primary substrate varied from 10mM to 0.05M, and it is assumed that at these concentrations the equilibrium of the reaction:

$$3FGA + CP \rightleftharpoons 3FGA\text{-}CP$$

(CP = carrier protein)

is pushed over to the right, and that V_{max} for both the binding and subsequent dehydrogenase reaction are reached. However, the concentrations of substrate 3FG in the manometric experiments also varied from 10mM to 0.05M and the subsequent rates of oxidation (= the rates of 3FGA production) varied from 3.0 - 15.7 μ moles of oxygen consumed per hour per 10 mg. dry weight of cells in 0.67M buffer (Table 23), and in 0.067M buffer, for concentrations of 3FG

oxidised without the subsequent cessation of cellular respiration, the rate of 3FG oxidation varied from 4.5 - 12.2 μ moles of oxygen consumed per hour per 10 mg. dry weight (Table 17). Theoretically, therefore, after one-two hours of 3FG oxidation by cell suspensions in Warburg vessels, there should be a sufficient concentration of 3FGA for binding and subsequent oxidation. Since this does not occur, other factors must be involved:

(ii) the binding and transportation of 3FG and 3FGA across the cytoplasmic membrane is energy-requiring. It is now generally accepted that the entry into a bacterial cell of most of the organic nutrients which it is able to metabolise is mediated by specific permeation systems[154], ATP regenerating a high energy donor:

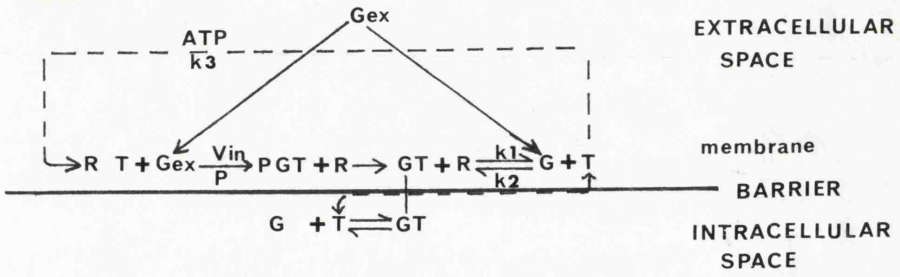


Fig.43. Model of permease mechanism[155].

The external galactoside has free access to the membrane enzyme permease P which catalyses the reaction R~T + G $\longrightarrow$ GT + R, forming the galactoside - transporter compound by transfer from the high energy donor. In parentheses is the enzyme-substrate complex. ATP regenerates R~T. This reaction is not the rate-limiting step for uptake in normal conditions. However, when the substrate being transported is a fluoro analogue, whose subsequent oxidation is not carried to a stage where energy is derived from the process, in an organism which requires the oxidation of sugars to ketoaldonic acids by

enzymes on the cytoplasmic membrane before phosphorylation and subsequent transportation into the cytoplasm can occur, and which contains neither endogenous carbohydrate nor fatty acid reserves, all ATP may be utilised in the binding of the primary substrate and insufficient be available for the further transportation of its oxidation product. Under these conditions the initial product of 3FG or 3FGA oxidation will accumulate at the site of oxidation and, if not further utilised by the cell, will pass out into the suspending buffer. (That fluorinated material does in fact accumulate on the cytoplasmic membrane of cells during 3FG and 3FGA oxidation is shown by the fluoride anion concentrations in hydrolysates of unwashed cell extracts determined at intervals during such incubations (Table 31)).

Evidence in support of the energy limitation of 3FG oxidation by whole cells can be found in the manometric data for the oxidation of equimolar amounts of 3FG and glucose added together to washed cell suspensions. Under these conditions the total oxygen consumed at the expense of the fluorinated component of the mixture approximates to one mole of oxygen per mole of 3FG (Figs. 18 and 32, Tables 16 and 26). The fact that throughout these studies a two stage oxidation of the deoxyfluoro sugar was only ever reached by whole cell suspensions when exogenous glucose was also present implies that the natural substrate overcomes the limitation of analogue oxidation. It is to be noted from Fig.19 (Table 16) however, that no elevated extent of oxidation of the fluoro analogue occurs when gluconate replaces glucose in the substrate mixture.

When both substrates are given simultaneously to washed whole cell suspensions of strain A, 3.12, 3FG has no inhibitory effect on glucose oxidation (Figs.18 and 32). Only when glucose is respired by cells in

0.067M phosphate buffer subsequent to 3FG oxidation is its catabolism noticeably impaired (Figs.16 and 21, Tables 15 and 18). The primary effect is a reduction of the total oxygen uptake rather than of the rate of oxidation. A similar effect is observed with subsequent gluconate oxidation (Fig.17, Table 15), and was also noted with subsequent pyruvate respiration. The inhibition increases with the amount of 3FG oxidised upto a critical level, when both further 3FG oxidation and the oxidation of alternative exogenous and endogenous substrates cease (Figs.20 and 21, Tables 17 and 18). Subsequently it was shown that acid release into the suspending buffer as a result of 3FG oxidation by cell suspensions (Fig.22) produces a sufficient lowering of the pH to account for the subsequent cessation of oxidative processes by the organism (Table 21). The correlation between the amount of u.v.-absorbing material released, the concentration of substrate 3FG, and the length of the incubation period (Figs.23 and 24), together with the drop in cell viability observed in 0.067M buffered systems, which is directly related to the amount of 3FG oxidised (Fig.25, Table 22) and the incubation time (in Fig.26 there is a lag period of 2 hours from the time of exposure to 3FG before a drop in viable counts can be detected) confirm that it is not 3FG itself, rather the product of 3FG oxidation, which is responsible for the observed effects.

In 0.67M buffered systems glucose oxidation by whole cell suspensions after 3FG or 3FGA oxidation differs from that of control cells previously respiring endogenously by an increased initial rate of oxidation which subsequently declines after 40-60 minutes to a much reduced rate, and a total oxygen uptake which increases with the concentration of fluorinated substrate originally present (Figs.30 and 31, Table 25). The latter finding suggested that the observed differences may be due to the oxidation of residual

exogenous fluorinated material, and further evidence in support of this conclusion came from the marked reduction in oxygen uptake differences when cell suspensions oxidising the fluoro analogues were separated from them by centrifugation and washing prior to glucose oxidation. The release of E_{260}-absorbing material into 0.67M buffer by washed cell suspensions oxidising either 3FG or 3FGA increases with substrate concentration (Figs.33 and 34) and is apparently the direct result of an intracellular effect of the fluoro analogues since it occurs without a parallel increase in cell lysis (Table 27). It can only be speculated whether or not the energy-deficient state of the cells suggested to result from the transport and oxidation of the fluoro analogues enforces an increased endogenous metabolism to maintain an energy supply so long as exogenous primary substrate is available. That the presence of exogenous substrates can enhance the endogenous respiration of certain Gram-negative bacteria has been shown by Dietrich and Burris[156], and would account for the subsequent proportional increases in the amount of u.v.-absorbing material released.

3FG is oxidised by asparagine-grown Ps. fluorescens A, 3.12 at an immediate maximum rate, approximately 25% of that by glucose-grown cells (Fig.37). The reduction in the oxidation rate of the fluoro analogue suggests that, though asparagine-grown cells contain the enzyme system necessary to oxidise glucose to gluconic acid, it is not as susceptible to the fluorinated substrate as the oxidising system in glucose-grown cells. Possibly sugar uptake proceeds initially at a reduced rate in asparagine-grown cells and is not sufficiently stimulated by the fluoro analogue to operate at the maximum rate. 3FGA is not oxidised by

asparagine-grown cell suspensions, when present as sole exogenous substrate over a period of six hours (Table 33). Since the oxidation of the fluoro analogue appears to be dependent on the cells ability to oxidise the parent substrate it is assumed that the same oxidising system is necessary for the oxidation of both substrates.

Neither 3FG nor 3FGA induce the gluconate- or 3FGA- oxidising system (Fig.39, Table 35). 3FG is without effect on the induction of the gluconate-oxidising system by gluconate, but the presence of 3FGA either during or prior to the inducing period inhibits induction (Fig.38, Tables 34 and 35). These results are in contrast to studies of the effect of the fluorobenzoates on the induction of the benzoate-oxidising system by a strain of Ps. fluorescens. Though the fluorobenzoates competitively inhibited induction to benzoate when present with the inducing substrate, and though incapable of oxidation when the cells were unadapted to benzoate, nevertheless the fluorobenzoates reduced the lag-period taken to form the benzoate-oxidising system when the analogues were used as inducers[85]. In contrast yet again, the halogenbenzoates also acted as non-metabolisable enzyme inducers for benzoate and p-nitrobenzoate oxidation by Nocardia erythropolis, but were observed to accelerate enzymic induction of the organism to the parent substrate in the case of benzoic acid, and to retard enzyme induction in the case of p-nitrobenzoic acid[18].

The inhibition of enzymic induction to gluconate by 3FGA is most likely directly concerned with the induction process itself. An inhibition of the gluconate-permease system, or its formation, by the fluoro analogue, or an inhibition of the depression by gluconate, could explain the present results. However, again in contrast to results obtained with the fluorobenzoates and benzoic acid oxidation by bacteria, the gluconate-oxidising

system once induced is not inhibited by 3FGA.

Though strain A, 3.12 of Ps. fluorescens oxidises 3FG, it cannot utilise it for growth when present as a sole carbon source. Similar results have been found with halogenonicotinic[16] and halogenobenzoic acids[18,85]. 3FG does not increase the final cell yield when added to a medium containing asparagine (Table 37), and the addition of the fluoro analogue to a medium containing glucose as carbon source reduces growth (Table 36). The latter suggests that 3FG could in these circumstances be acting as a competitor of glucose. The inability of the organism to grow on 3FG appears to be due to two main causes: (i) the oxidation of the analogue is not carried to a stage at which the carbon may be assimilated; (ii) the inability of the organism to liberate organic fluorine as fluoride ion.

No fluoride ion has been detected during the oxidation of 3FG or 3FGA by either whole cells (Table 29) or cell-free extracts during the present work. However, liberation of free fluoride from p-fluorobenzoate by a presumably constitutive enzyme of a Vibrio species grown on benzoate[84], and the adaptive formation of specific carbon-fluorine bond-cleaving enzymes in pseudomonad species[68,78], have been demonstrated. Nevertheless, the occurrence of organisms in the soil capable of growing on 3FG is rare as judged by the failure to isolate such organisms from selective cultures inoculated with differing types of soil.

3) Ps. saccharophila. Washed cell suspensions of glucose-grown Ps. saccharophila do not oxidise 3FG to any significant extent (Fig.40, Table 38). The simultaneous oxidation of glucose is not inhibited by either 3FG or 3FGA, nor is the total oxygen uptake by cells furnished with limiting amounts of 3FG and parent substrate elevated sufficiently to suggest any oxidation of the fluoro analogue (Fig.40, Table 38). Glucose oxidation subsequent to 3FG- or 3FGA-treatment occurs to a slightly greater

extent compared with that by control cells previously respiring without an exogenous carbon source (Fig.41, Table 39). This may be due either to preferred assimilation of glucose by the starved controls (this organism stores poly-β-hydroxybutyrate as an endogenous reserve), or preferred oxidation of glucose by the possibly energy-depleted cells exposed to 3FG or 3FGA. Carbohydrate assays of the suspending buffer from such incubations suggest that 3FG is taken up by this organism, albeit in limited amounts, but this in no way inhibits (subsequent) glucose uptake. Since a suitably supplemented cell-free extract of Ps. saccharophila was also unreactive towards 3FG and there was no stimulation of enzymic activity by the presence of ATP, it was concluded that the inability of this organism to oxidise the fluorinated sugar was probably due to the reduced affinity of 3FG for the pseudomonad hexokinase.

Any attempt to correlate the differing modes of glucose degradation by the three organisms used in this study with their differing responses towards 3FG is of necessity arbitrary, since, in the main, studies were confined to whole cell suspensions, and the differing abilities and specificities of the sugar uptake systems undoubtedly affect these results. Nevertheless, certain correlations between the differing enzymic complements of the three organisms and their ability to utilise 3FG can be drawn.

3FG is readily taken up and oxidised by strain A, 3.12 of Ps. fluorescens, an organism in which glucose respiration proceeds by at least one, usually two, oxidation steps before phosphorylation and further utilisation of the sugar. E.coli and Ps.saccharophila, both of which phosphorylate glucose at carbon-6 as the first catabolic step, do not oxidise 3FG. In none of the studies with the above three organisms was a specific inhibition of glucose oxidation

by 3FG observed. This suggests that the fluoro analogue differs from glucose by a very much reduced affinity for the sugar uptake systems and for the hexokinases of E.coli and Ps.saccharophila. 3FG oxidation by the fluorescent pseudomonad proceeds at only a fraction of the rate of glucose oxidation, and when added with glucose, the fluoro analogue has no inhibitory action towards utilisation of the parent substrate. On the contrary, the presence of glucose appears to facilitate 3FG oxidation by this organism. Oxidation of the fluoro sugar proceeds apparently independent of product accumulation, until the external pH is sufficiently acid as to terminate all subsequent cellular respiratory activity.

That 3FG can compete with glucose under certain conditions is suggested by its inhibition of the growth of Ps. fluorescens on glucose, and the retardation of the subsequent growth of E.coli on glucose after preincubation with the fluoro analogue. Competition between fluoro analogue and parent substrate is also suggested by the inhibition of the gluconate-oxidising system in asparagine-grown Ps. fluorescens by 3FGA. It is hoped that future studies will ascertain whether 3FG can compete with glucose at an enzymic level.

APPENDIX

APPENDIX - 1

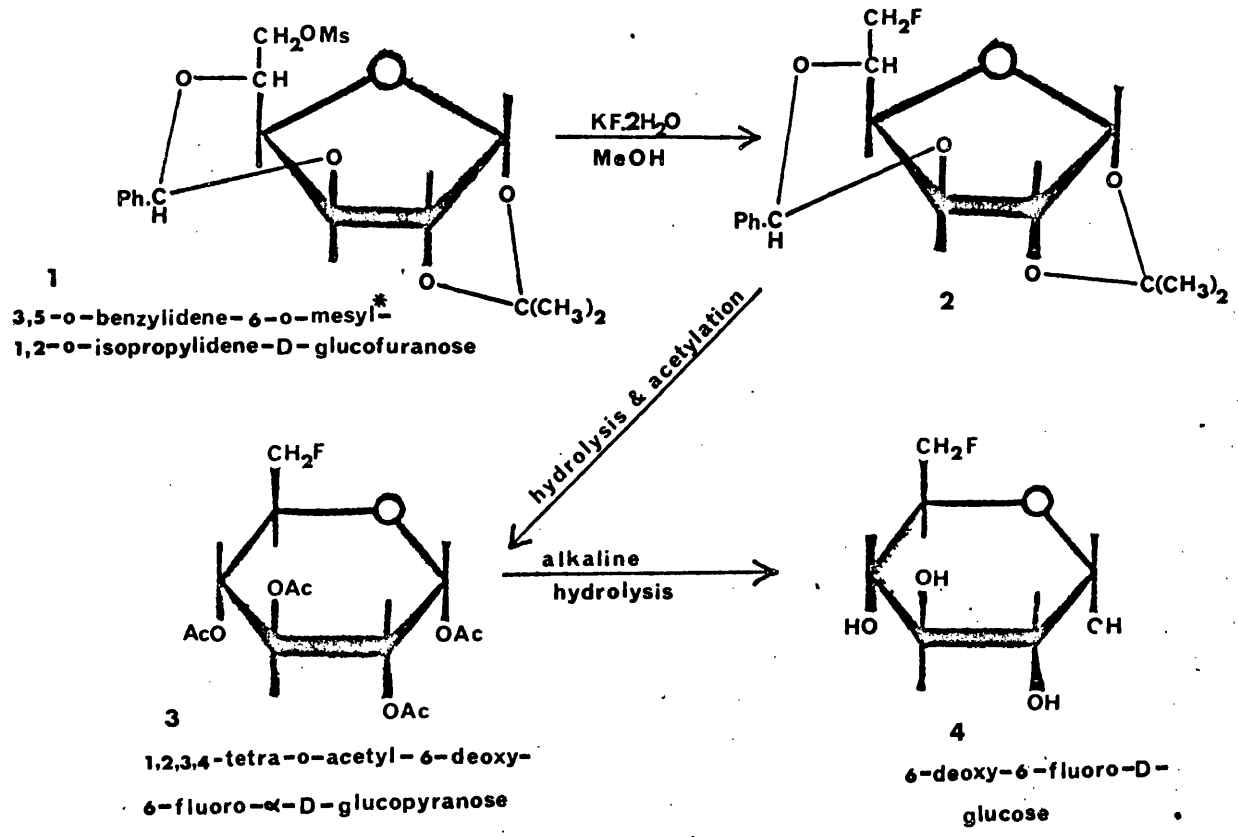
The chemical synthesis of fluorinated carbohydrates

Comprehensive reviews on carbohydrates halogenated at C-1[157], glycosyl fluorides[143], halogenated inositol analogues[158], and monosaccharides monohalogenated at positions other than C-1[76] are available.

Primary deoxyfluoro sugars are readily obtained by nucleophilic displacement of suitably substituted sugar derivatives by fluoride salts in such solvents as ethylene glycol. In this way derivatives of 6-deoxy-6-fluoro-D-glucose[159] (4), 6-deoxy-6-fluoro-D-galactose[144,160] (10) and 5-deoxy-5-fluoro-D-ribose[144] (7) have been prepared.

Work on the structure and reactions of 6-deoxy-6-fluoro-D-galactose has shown that the fluorosugar behaves normally in those reactions where the carbon atom substituted with fluorine is not involved, and is not dehalogenated[161], illustrating the carbon-fluorine bond stability.

a)



1
3,5-o-benzylidene-6-o-mesyl*-1,2-o-isopropylidene-D-glucofuranose

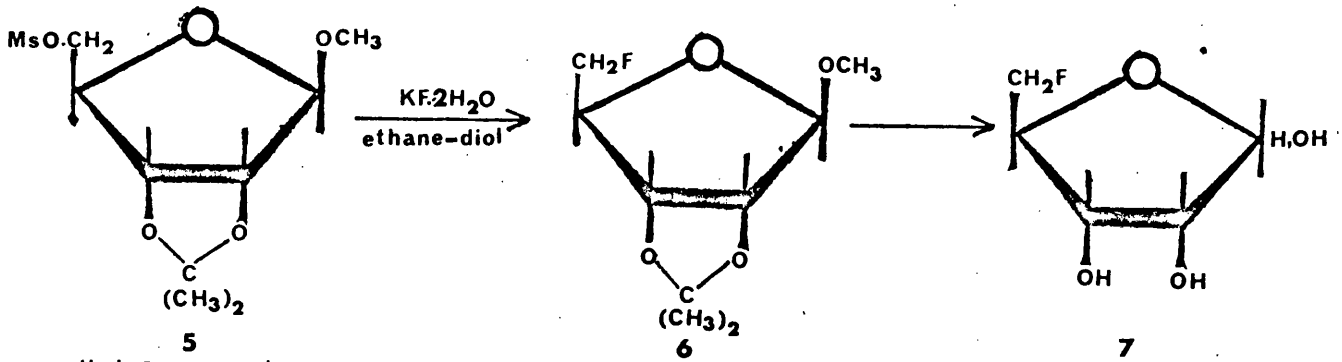
2

3
1,2,3,4-tetra-o-acetyl-6-deoxy-6-fluoro-α-D-glucopyranose

4
6-deoxy-6-fluoro-D-glucose

* mesyl = Ms = methanesulphonyl

b)

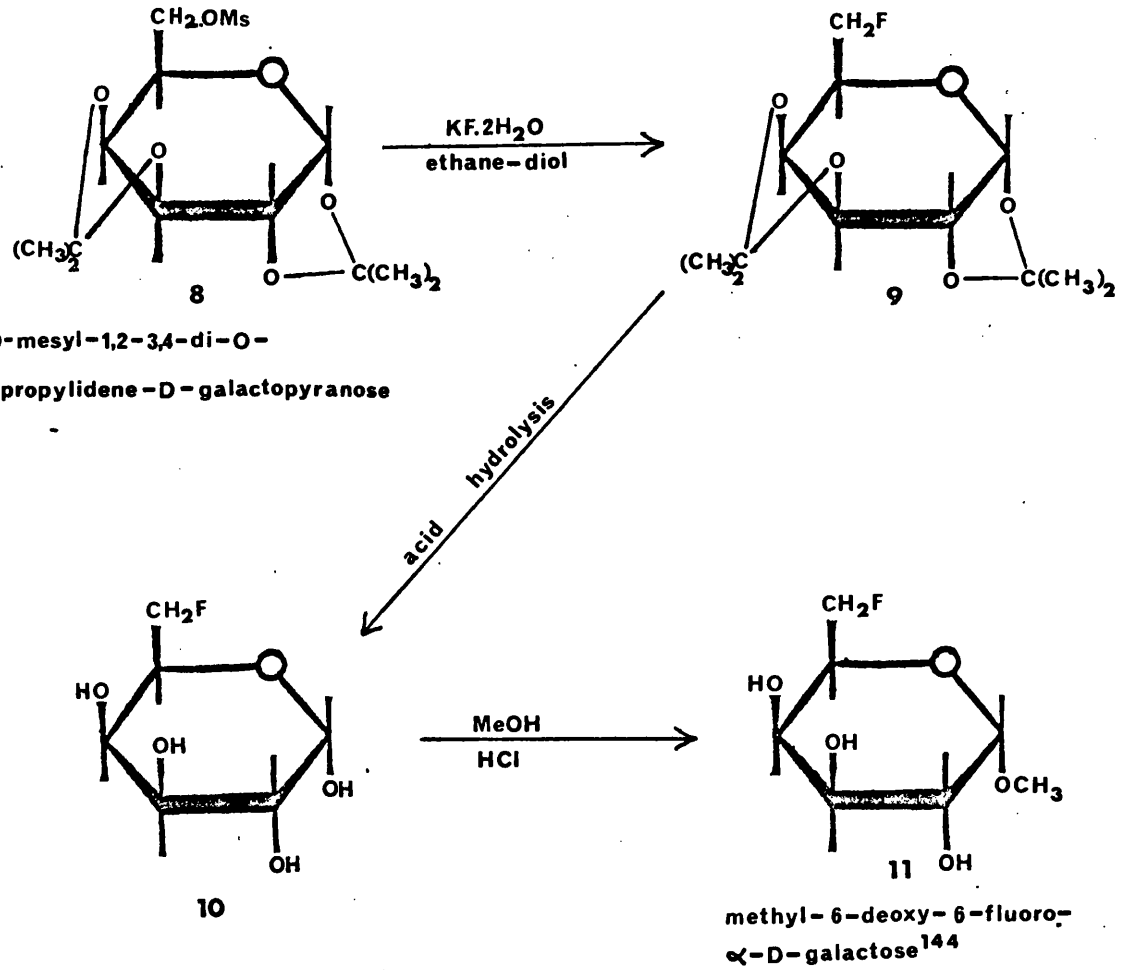


5
methyl-5-o-mesyl-
2,3-o-isopropylidene-
β-D-ribofuranoside

6

7
5-fluoro-5-deoxy-D-
ribose[144]

c)



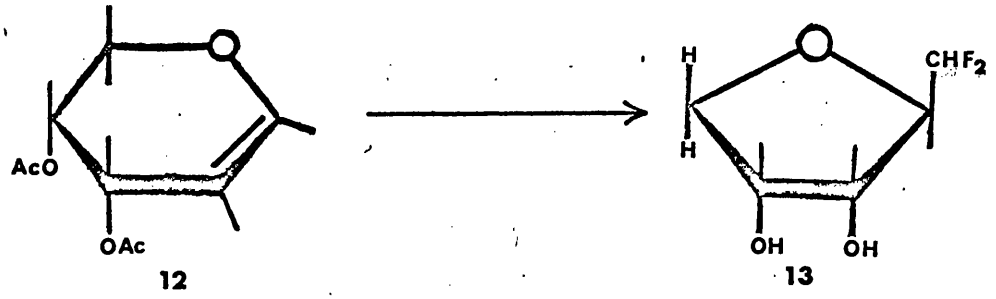
8
6-O-mesyl-1,2-3,4-di-O-
isopropylidene-D-galactopyranose

9

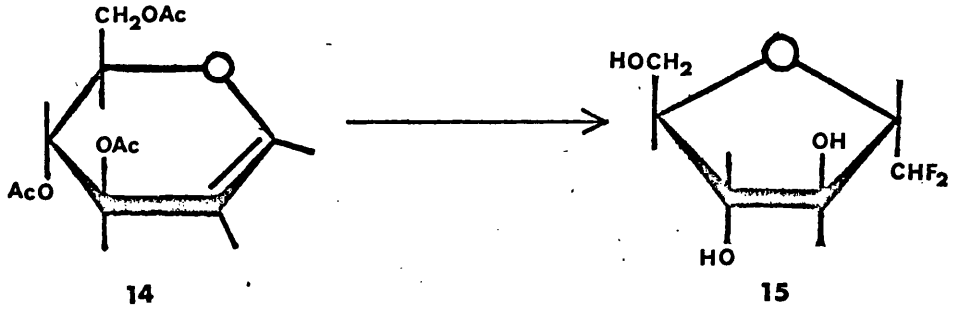
10

11
methyl-6-deoxy-6-fluoro-
α-D-galactose[144]

The only examples of carbohydrate geminal difluorides, 2,5-anhydro-1-deoxy-1,1-difluoro-D-ribitol (13) and 2,5-anhydro-1-deoxy-1,1-difluoro-D-mannitol (15) were obtained by treatment of 3,4-di-O-acetyl-D-arabinal (12) and 3,4,6-tri-O-acetyl-D-glucal (14) respectively with lead tetra-acetate-hydrogen fluoride followed by deacetylation[162], a reaction which has been used in the steroid field to bring about stereospecific cis addition of fluorine to olefins[163].

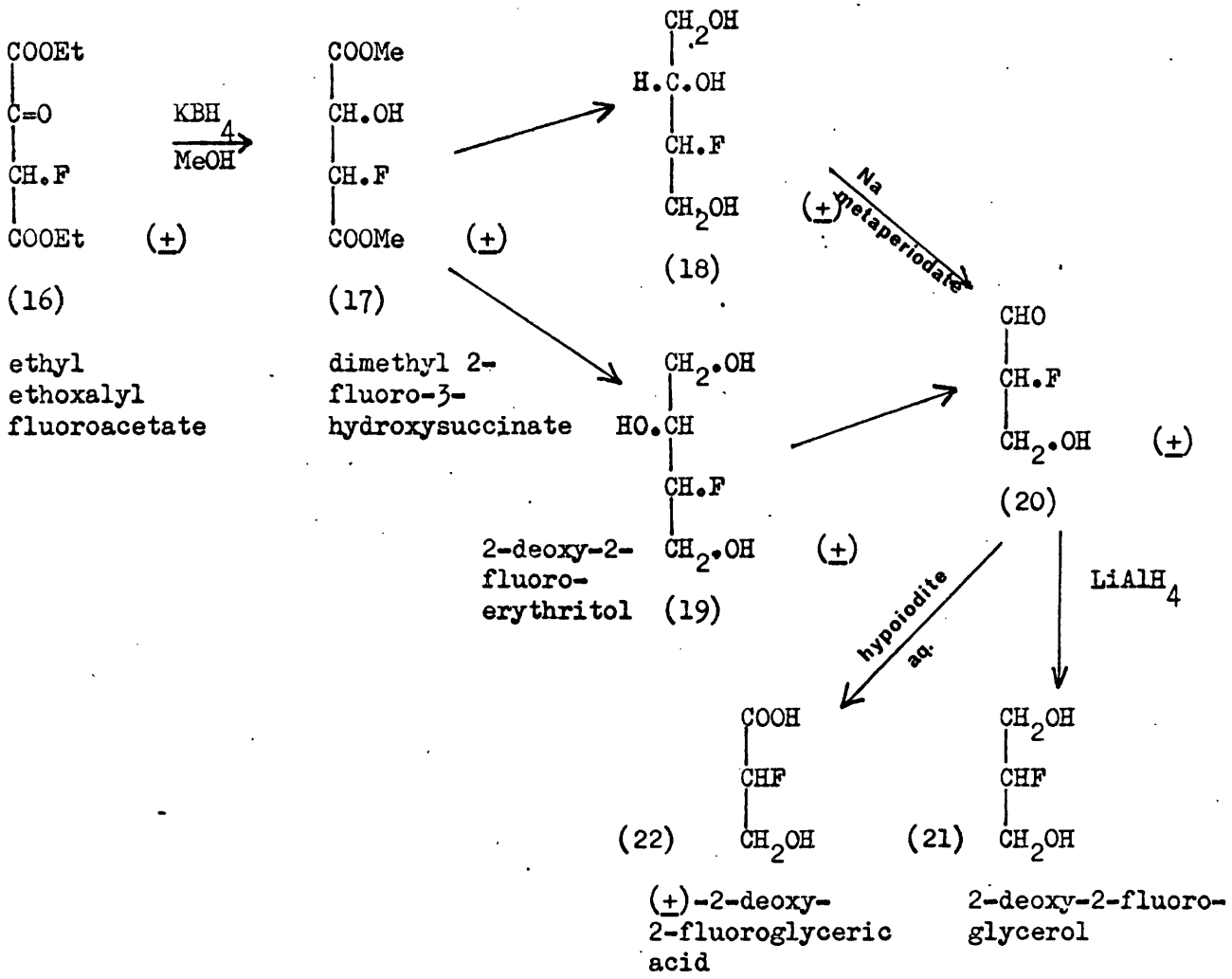


12 13

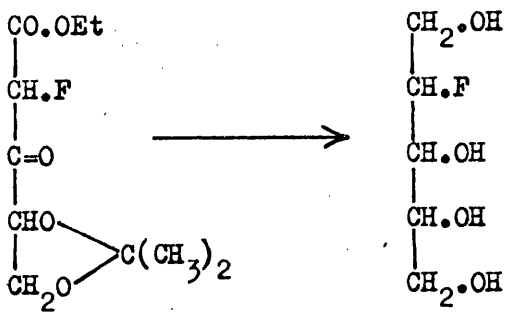


14 15

There have been several approaches to the synthesis of carbohydrate secondary fluorides. Total synthesis from simple aliphatic precursors has given (±)-2-deoxy-2-fluoroglyceraldehyde[87,96](20) and 2-deoxy-2-fluoro-DL-ribitol[164] (24) but this approach is limited because of the formation of racemic products.

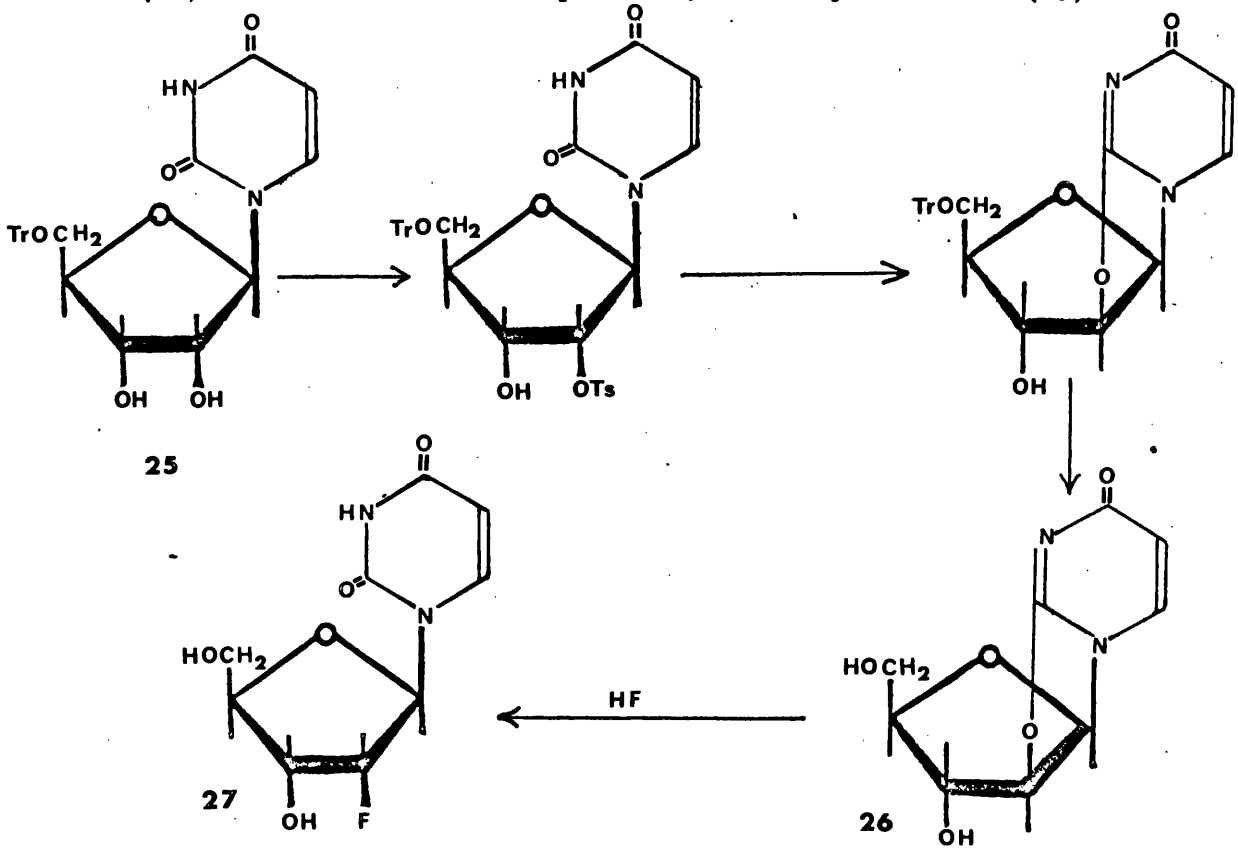


The synthesis of (±)-2-deoxy-fluoropentitols was achieved by carrying out a Claisen condensation between ethyl fluoroacetate and methyl 2,3-O-isopropylidene-DL-glycerate, followed by borohydride reduction of the resulting isomeric ethyl 2-deoxy-2-fluoro-3-oxo-4,5-O-isopropylidene-(±)-pentonates[164] (23):



(±)-2-deoxy-2-fluororibitol -shown by X-ray crystallography to have a close spatial similarity to ribitol, in keeping with earlier observations on erythritol and its (±)-2-deoxy-2-fluoro-analogue[86].

The first compound synthesised containing fluorine in a secondary position of a carbohydrate, as distinct from a polyol moiety (excepting (±)-2-deoxy-2-fluoroglyceraldehyde (20)), was reported in 1961 by Fox et al.[165] who prepared 2'-deoxy-2'-fluoro-uridine (27) by the action of anhydrous hydrogen fluoride in dioxan on 2'2-anhydro-1-(β-D-arabinofuranosyl) uracil (26) obtained in three steps from 5'-O-trityl*-uridine (25)[166]:

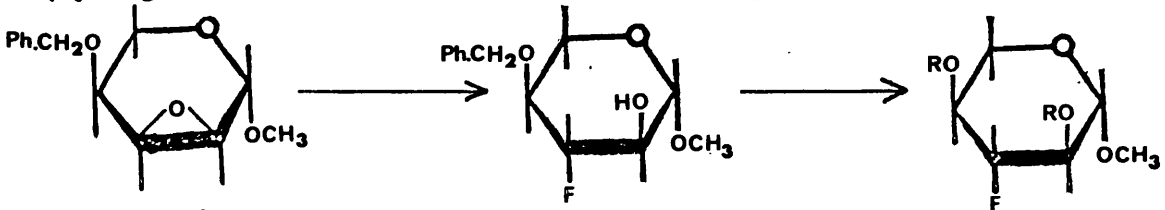


As well as preparing the 2'-chloro- and 2'-bromo-derivatives of (27) Fox et alia went on to synthesise 2'-deoxy-2'-halogeno derivatives of thymidine, deoxyuridine, 5-fluorouridine[167], and cytidine[168].

* trityl = Tr = triphenyl-methyl

In 1966 the sugar moiety 2-deoxy-2-fluoro-D-ribose was isolated from the nucleoside (27) by catalytic hydrogenation and glycosidic cleavage[169].

A closely related route to the above synthetic scheme, viz. epoxide ring scission, has found wide application particularly in the steroid field.[60] The cleavage of suitably protected sugar epoxides with hydrogen fluoride has permitted the synthesis of 3-deoxy-3-fluoro-L-xylose[170] (hydrogen fluoride-p-dioxane at 120°), 3-deoxy-3-fluoro-D-arabinose[142] (potassium hydrogen fluoride in ethylene glycol), 3-deoxy-3-fluoro-D-xylose[171] (34) (potassium hydrogen fluoride-diethylene glycol, potassium fluoride-molten acetamide), and 2-deoxy-2-fluoro-D-altrose and 3-deoxy-3-fluoro-D-glucose[172] (hydrogen fluoride-boron trifluoride at -70°):



(28) methyl 2,3-anhydro-4-O-benzyl-β-L-ribo-pyranoside.

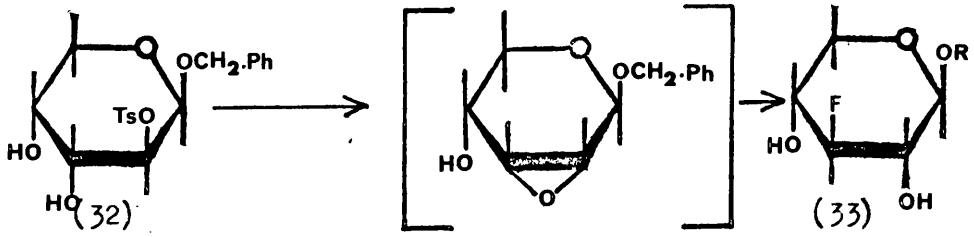
(29) methyl 4-O-benzyl-3-deoxy-3-fluoro-β-L-xylopyranoside.

(30) R=H

(31) R= Ts

(29) underwent catalytic hydrogenation to the glycoside (30) which was characterised as the crystalline 2,4-di-O-tosyl* derivative[170] (31).

Cohen, Levy, and Bergmann[171] circumvented epoxide scission by utilising the attack of the fluoride ion on the secondary tosyl ester (32):



(32) benzyl 2-O-tosyl-β-D-arabinopyranoside.

epoxide intermediate

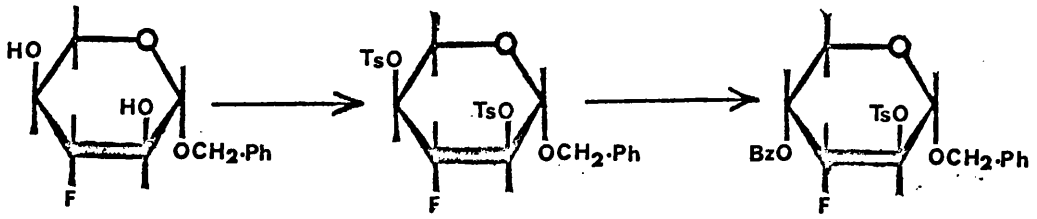
(33) benzyl 3-deoxy-3-fluoro-β-D-zylopyranoside.

(34) R = H

R = CH_2.Ph

* tosyl = Ts = toluene-p-sulphonyl

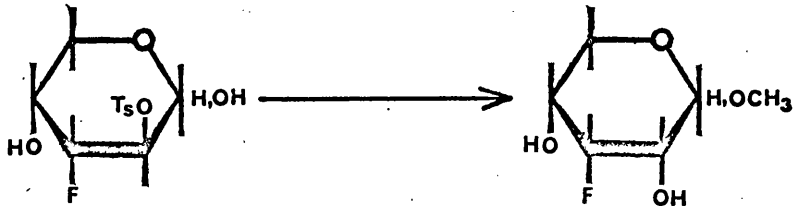
A similar sequence of reactions in the L-arabinose series led to benzyl 3-deoxy-3-fluoro-β-L-xylopyranoside (35), the di-O-tosyl ester of which (36) underwent exchange with benzoate at C-4 only, with inversion, yielding benzyl 4-O-benzoyl-3-deoxy-3-fluoro-2-O-tosyl-α-D-arabinoside (37), thus effecting entry into the D-arabino series:



(35) (36) (37)

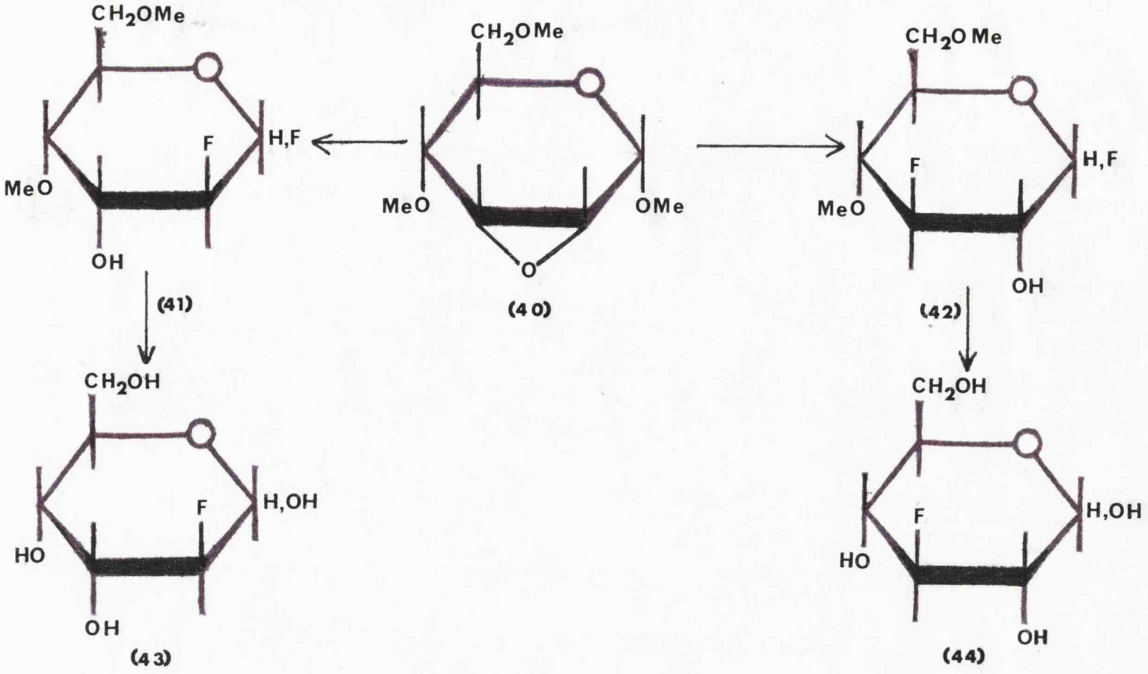
$B_z = C_6H_5CO$ = benzoyl

From (37), by hydrolysis and hydrogenation, 3-deoxy-3-fluoro-2-O-tosyl-D-arabinose (38) was prepared, and treatment of this with cold sodium methoxide in methanol gave a syrup which Bergmann tentatively identified as methyl 3-deoxy-3-fluoro-αβ-D-riboside (39):



(38) (39)

Johansson and Lindberg[172] described the opening of a hexose epoxide, methyl 2,3-anhydro-4-di-O-methyl-α-D-allopyranoside (40) using boron trifluoride in hydrogen fluoride at -70° to give a mixture of monodeoxy-monofluoro-di-O-methyl-hexosyl fluorides (41,42) from which by hydrolysis and demethylation the two reducing sugars 2-deoxy-2-fluoro-D-altrose (43) (predominating) and 3-deoxy-3-fluoro-D-glucose (44) were obtained:



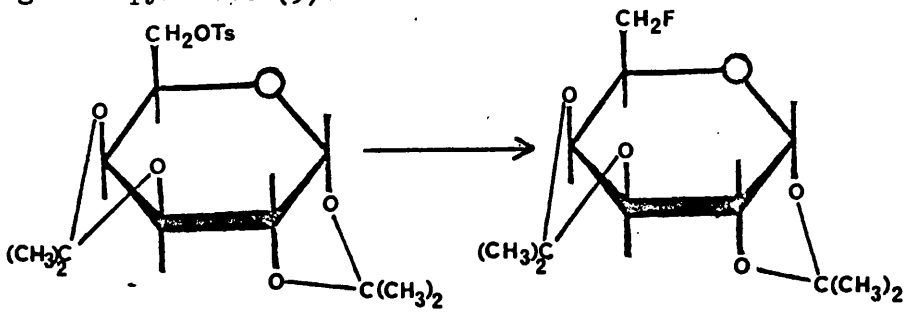
The same free fluorosugars were arrived at by proceeding from methyl, 4,6-di-O-acetyl-2,3-anhydro-α-D-allopyranoside. However, during removal of the acetyl blocking groups, partial epimerisation occurred and some 2-deoxy-2-fluoro-D-allose was formed.

Johansson and Lindberg's route gave a fair yield of 2-deoxy-2-fluoro-D-allose (24% based on the epoxide), but a poor yield (6% based on (40)) of the biologically more interesting 3-deoxy-3-fluoro-D-glucose (44).

Several methods that have been used successfully in the synthesis of aliphatic secondary fluorides failed when applied in the carbohydrate field[173]. Although direct displacement of carbohydrate primary sulphonates with fluoride ion proceeds readily[144], the method has not been successfully applied to secondary sulphonates[160]. Because of solvation, fluoride ion in a protic solvent is a relatively poor nucleophile, but, in dipolar aprotic solvents, only cations are strongly solvated[174] and the nucleophilicity of fluoride ion is significantly enhanced. Thus, Henbest and Jackson[175]

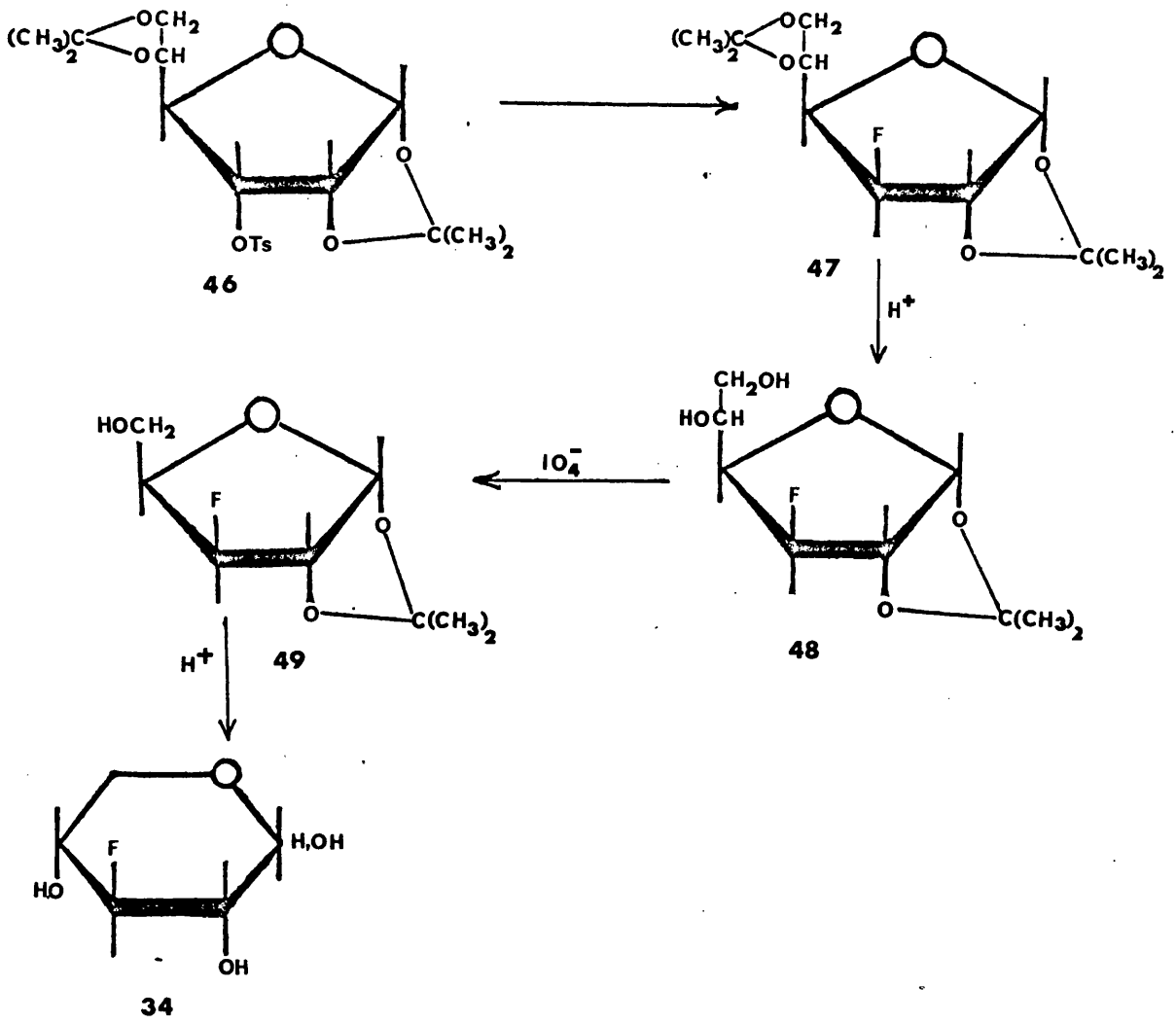
have shown that sulphonates attached to five-membered carbocyclic compounds (testosterone toluene-p-sulphonate) and axially or equatorially to six-membered rings (cholestan-3-α- and 3-β-yl toluene-p-sulphonate) can be converted into fluorides in good yield, with inversion of configuration, by treatment with tetrabutylammonium fluoride in acetone or ethyl methyl ketone.

Foster, Hems and Webber [176] applied this route, using a variety of dipolar aprotic solvents, to the carbohydrate field. Thus, using 1,2-3,4-di-O-isopropylidene-6-O-tosyl-α-D-galactopyranose (45) as a model, they obtained yields of upto 69% of 6-deoxy-6-fluoro-1,2-3,4-di-O-isopropylidene-D-galactopyranose (9):

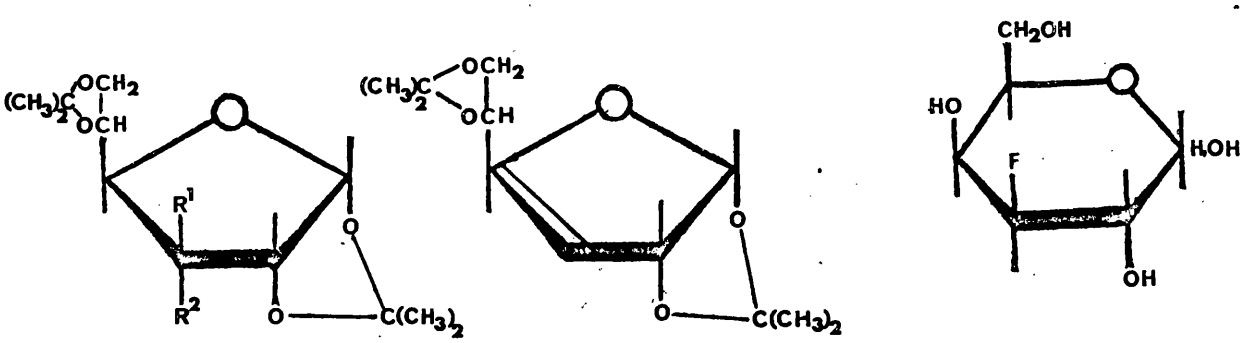


(45) (9)

On treatment of 1,2-5,6-di-O-isopropylidene-3-O-tosyl-α-D-allofuranose (46) with tetrabutylammonium fluoride in acetonitrile a 74% yield of 3-deoxy-3-fluoro-1,2-5,6-di-O-isopropylidene-α-D-glucofuranose (47) was obtained, (the structure of (47) being established by unambiguous degradation to the known[171] 3-deoxy-3-fluoro-D-xylose (34) and its derived 2,5-dichlorophenylhydrazone[142]). Acid hydrolysis of (47) gave 3-deoxy-3-fluoro-D-glucose (44).



This route has also been used by Foster and his coworkers[177] to prepare 3-deoxy-3-fluoro-D-galactose (53), starting from 1,2-5,6-di-O-isopropylidene-3-O-tosyl-α-D-gulofuranose (50) and treating it with tetrabutyl ammonium fluoride to give a mixture of 3-deoxy-3-fluoro-1,2-5,6-di-O-isopropylidene-α-D-galactofuranose (51) and 3-deoxy-3-fluoro-1,2-5,6-di-O-isopropylidene-α-D-erythro-hex-3-enofuranose (52):



(50) R^1 = H, R^2 = OTs

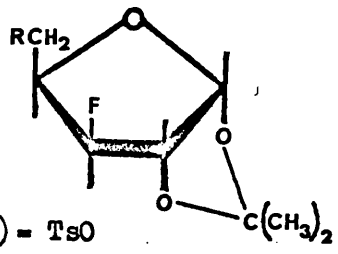
(51) R^1 = F, R^2 = H

52

53

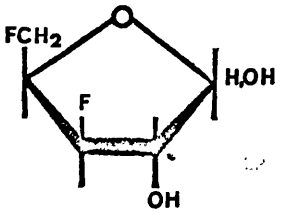
Acid hydrolysis of (51) gave the fluorodeoxy sugar (53).

The availability of 3-deoxy-3-fluoro-1,2-5,6-di-O-isopropylidene-α-D-glucofuranose (47) and its ready conversion to 3-deoxy-3-fluoro-1,2-O-isopropylidene-α-D-glucofuranose (48) and 3-deoxy-3-fluoro-1,2-O-isopropylidene-α-D-xylofuranose (49) has opened a synthetic route to di- and poly-fluorinated sugar derivatives, and by treating 3-deoxy-3-fluoro-1,2-O-isopropylidene-5-O-tosyl-α-D-xylofuranose (54) with anhydrous KF and hydrolysing the resulting difluoroacetal (55), Foster et al.[178] have prepared 3,5-dideoxy-3,5-difluoro-D-xylofuranose (56):



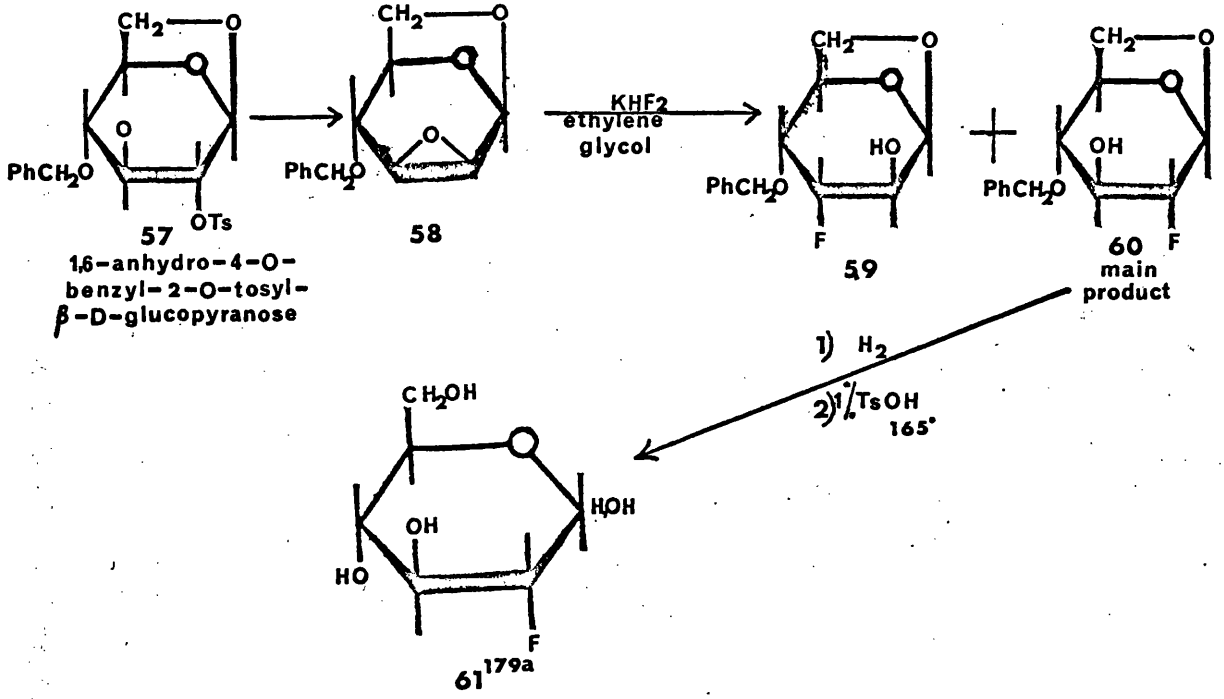
(54) = TsO

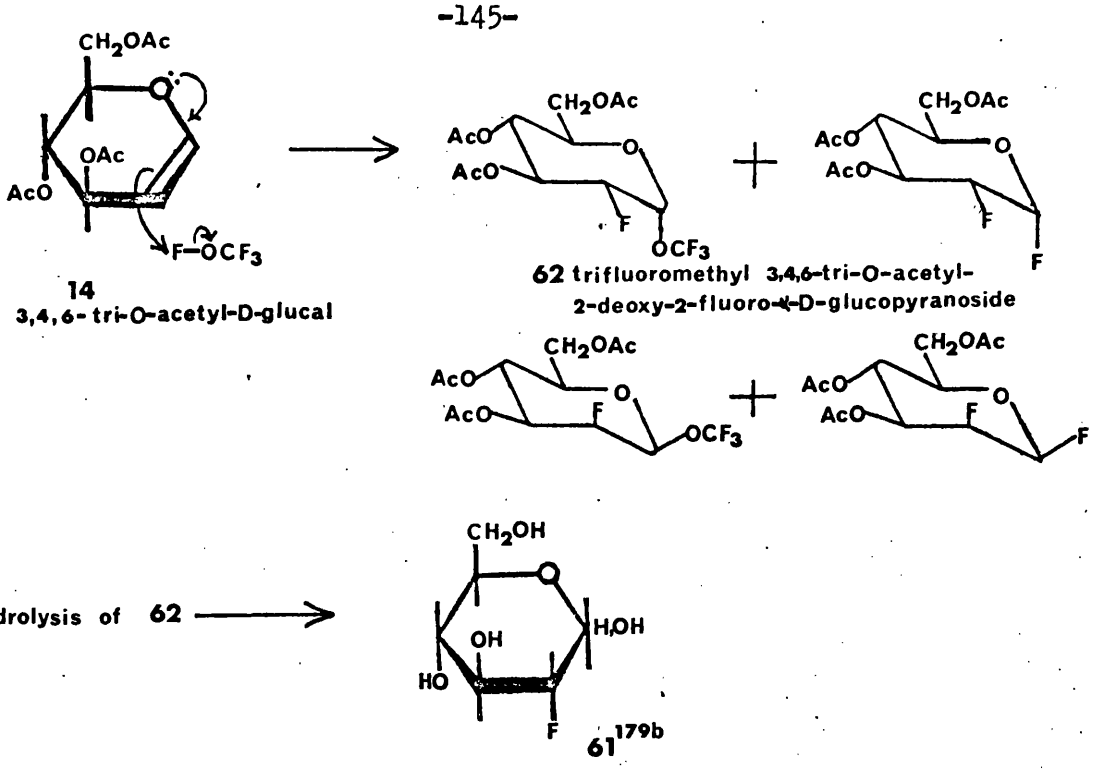
(55) R = F



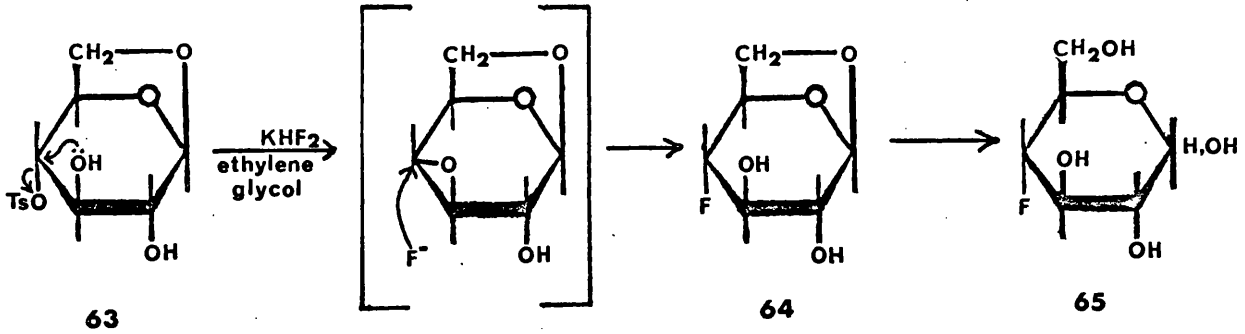
(56)

Within the last year, the remaining two members of the deoxyfluoro-D-glucopyranose derivatives, 2-deoxy-2-fluoro- and 4-deoxy-4-fluoro-D-glucose have been synthesised, the former compound by two independent routes:





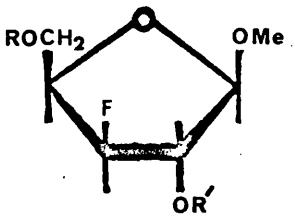
1,6-Anhydro-4-O-tosyl-β-D-glucopyranose (63) has been used as the starting compound for a synthesis of 4-deoxy-4-fluoro-D-glucose[180] (65) which proceeds via 1,6-anhydro-4-deoxy-4-fluoro-β-D-glucopyranose (64):



However, the low yield in the conversion of 1,6-anhydro-D-glucose into the 4-tosyl derivative largely deprives the above synthesis of convenience as a route for the large-scale preparation of 4-deoxy-4-fluoro-D-glucose, and alternative synthetic approaches are being investigated.

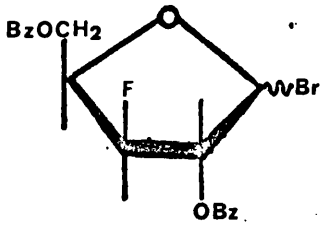
Until the last ten years work on the introduction of fluorine into nucleosides had been based on the replacement of hydrogen by fluorine in the heterocyclic moiety (eg. 2-fluoroadenosine[181] and 5-fluorouridine[182]). In the early 1960s, as part of a prgramme of synthesis of nucleosides with biochemical and chemotherapeutic potential[183], Fox and his coworkers reported the syntheses of 2'-deoxy-2'-fluoro-analogues of uridine[165], 5-fluorouridine, ribothymidine[167], and cytidine[168] by reaction of the 2,2'-anhydro nucleosides with HF in dioxan. However, since this method appeared to be limited to pyrimidines having an oxygen function at the 2-position of the heterocycle, Wright and Taylor[120a], and Wright, Taylor and Fox[120b] have since utilised conventional condensation of preformed deoxyfluoro sugar derivatives with either 6-benzamidopurine or 2,6-dichloropurine to obtain crystalline fluoronucleosides.

Methyl 5-O-benzyl-3-deoxy-3-fluoro-β-D-xylofuranoside (66) and methyl 5-O-benzyl-3-deoxy-3-fluoro-α-D-arabinofuranoside (71) were converted, via the 2,5-di-O-benzoyl derivatives (67) and 72), into the corresponding α β-D-glycosyl bromides (68) and (73). Condensation of the latter compounds with 6-benzamidopurine yielded the fluorinated nucleosides, 6-benzamido-9-(2,5-di-O-benzoyl-3-deoxy-3-fluoro-β-D-xylofuranosyl) purine (69) and 6-benzamido-9-(2,5-di-O-benzoyl-3-deoxy-3-fluoro-α-D-arabinofuranosyl) purine (74) respectively. Alkaline hydrolysis yielded the crystalline fluoronucleosides (70) and (75)[120a];



66 R = CH_2Ph, R' = H

67 R = R' = Bz



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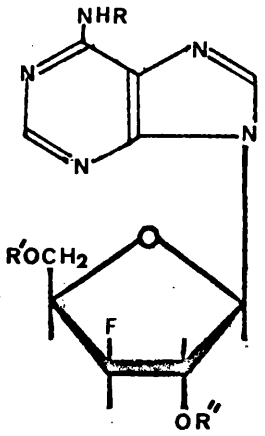
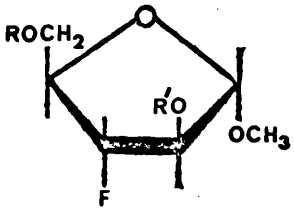


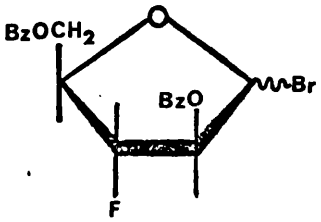
69 R = R' = R'' = Bz

70 R = R' = R'' = H

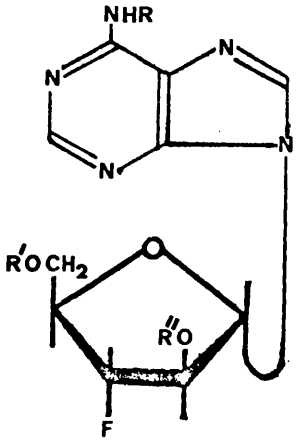


71 R = CH_2Ph, R' = H

72 R = R' = Bz



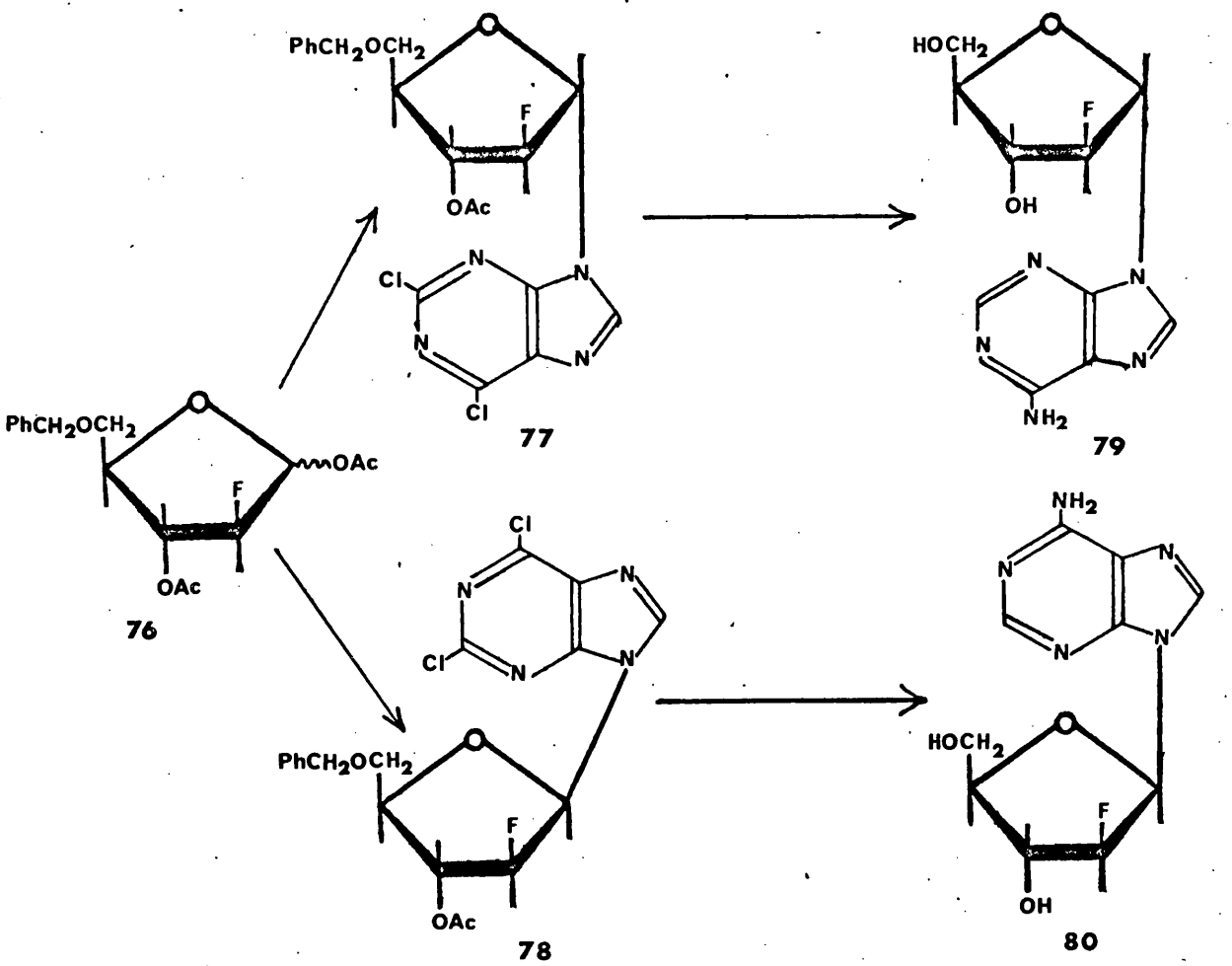
73



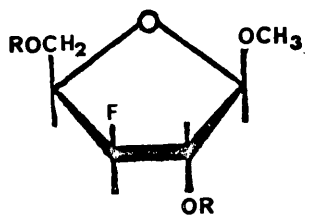
74 R = R' = R'' = Bz

75 R = R' = R'' = H

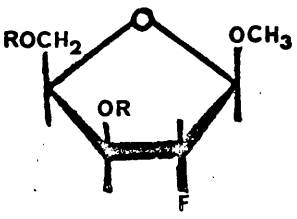
In later work[120b], nucleophilic attack of KHF_2 on methyl 2,3-anhydro-5-O-benzyl-α-D-riboside was shown to produce methyl 5-O-benzyl-2-deoxy-2-fluoro-α-D-arabinoside, and condensation of the 1,3-di-O-acetyl derivative (76) with 2,6-dichloropurine gave an anomeric mixture of α- and β-9-glycosylpurine nucleosides (77,78) which were converted into 9-(2-deoxy-2-fluoro-α- and -β-D-arabinofuranosyl) adenines (79) and (80):



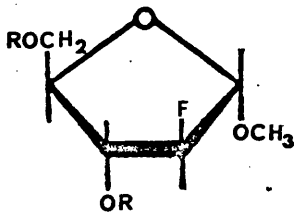
The latest work in this area of fluorinated nucleoside chemistry reports the syntheses of 1-(3-deoxy-3-fluoro- and 2-deoxy-2-fluoro-β-D-xylofuranosyl) cytosines, (90) and (91) respectively, and 1-(2-deoxy-2-fluoro-α- and -β-D-arabinofuranosyl) cytosines, (92) and (93), from their corresponding suitably protected halogenoses[121];



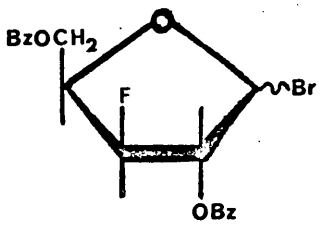
81 R=H
84 R=Bz



82 R = H
85 R = Bz



83 R=H
86 R=Bz



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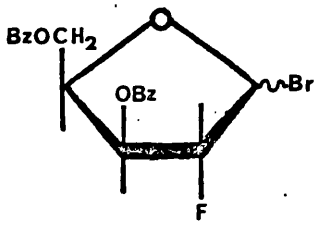

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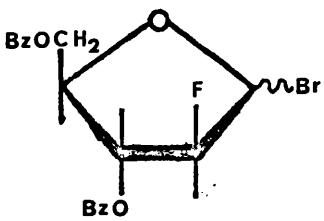
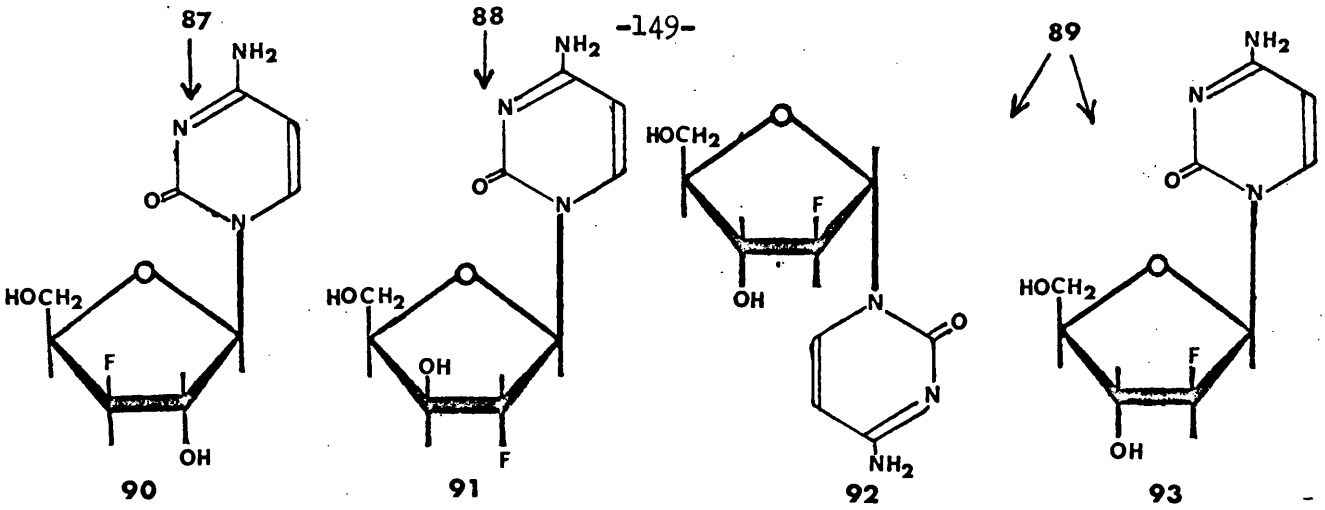


89



The preliminary account of the biological properties of these and other fluorodeoxy sugar nucleosides has been reviewed in the introduction (p.39).

APPENDIX - 2

(a) The synthesis of calcium 3-deoxy-3-fluoro-D-gluconate

(adapted from Frush and Isbell, 1964[184a]).

The oxidation was conducted in a crystallising dish 9 cm. in diameter and 6 cm. in height fitted with a lid in which there were three holes 3 cm. apart. The dish was fitted with an efficient stirrer and the two external holes fitted with graphite electrodes 2 mm. in diameter. To the dish was added 3-fluoro-3-deoxy-D-glucose (syrup 3 gm.), calcium bromide (1.26 gm. of 85% $CaBr_2$), calcium carbonate (1.8 gm.), and water (67 ml.). A steady current of 40 mA was passed through the well-stirred solution for 40 hours, the polarity of the current being reversed at intervals to prevent the build up of calciferous deposits on the electrodes. The electrolysed solution was then filtered through filter aid and concentrated under reduced pressure to a thin syrup. Ethanol was added to incipient turbidity and the solution left to crystallise. The product was recrystallised from water. As the product was expected to be hydrated, tests were carried out using a vacuum drying pistol, and it was shown that the crystals contained three molecules of water of crystallisation.

Yield: 3 gm. = 77%

Melting point: 198°-200° with decomposition.

R_f Value: spotted in aqueous acetic acid onto t.l.c. plates and developed in ethyl acetate-acetic acid-water (3:3:1 v/v) the calcium salt occupied positions at R_f 0.38, 0.53, 0.65.

Analysis:

	C	H
Found	29.58%	5.13%
$Ca(C_6H_{10}O_6F)_2 \cdot 3H_2O$ requires	29.71%	5.32%

(b) The synthesis of 3-deoxy-3-fluoro-gluconic acid.

An aqueous solution of calcium 3-deoxy-3-fluoro-D-gluconate (3 gm.) was passed down a cation exchange column (2 cm.x40 cm. Amberlite IR-120H). The column was eluted with water, and the fractions collected were evaporated under reduced pressure (bath temperature $< 35^{\circ}$). The resulting syrup was lyophilysed. Thin layer chromatography revealed a mixture of three components:

R_f 0.38 title compound predominant

0.53

0.65 assumed to be δ and γ lactones of above

Yield: 2.45 gm. = 100%

If, instead of lyophilisation, the column eluent was concentrated to dryness 'in vacuo' crystalline free acid was obtained. Recrystallised from dioxan/ether this compound had the following constants:

Melting point: 119° (uncorrected)

R_f Value: 0.38 in ethyl acetate-acetic acid-water (3:3:1 v/v) when applied as an aqueous solution or in dilute acetic acid, 0.53 when applied in methanol.

An R_f of 0.65 was obtained on chromatography of the solution obtained by refluxing the crystalline acid for one hour in glacial acetic acid containing one drop of conc. HCl (method for the preparation of D-aldonic acid-1,4-lactone from Methods In Carbohydrate Chemistry, vol.II[184b]).

Specific Rotation: $[\alpha]_D^{24}$ -6°

Analysis:

	C	H	F
Found	36.32%	5.58%	9.01%
$C_6H_{11}O_6F$ requires	36.30%	5.56%	9.60%

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