FLUOROCARBOHYDRATES

PART XXIV[1]. CHEMICAL AND ENZYMIC REDUCTION AND PHOSPHORYLATION OF 6-DEOXY-6-FLUORO-α-D-GALACTOSE*

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ABSTRACT

6-Deoxy-6-fluoro-α-D-galactose (**1**) was converted by conventional methods into crystalline 2,3,4-tri-*O*-acetyl-6-deoxy-6-fluoro-α-D-galactosyl bromide (**2**). Treatment of **2** with methanol–silver carbonate gave the methyl β-glycoside and reaction with silver diphenyl phosphate, followed by hydrogenolysis and deacetylation, gave 6-deoxy-6-fluoro-α-D-galactosyl dipotassium phosphate (**6**). A product chromatographically identical with **6** resulted when **1** was incubated with galactokinase and ATP at 37° and pH 6.8. Reduction of **1** with sodium borohydride gave 1-deoxy-1-fluoro-L-galactitol (**7**) which was also formed, by the action of polyol dehydrogenase, when rabbit-lens capsules were incubated with **1**. Biological reduction of **1** proceeds ~3 times faster than that of D-galactose.

INTRODUCTION

Recent investigations have shown that fluoromonosaccharides may be transformed by living cells in a number of ways. Partial utilization of 3-deoxy-3-fluoro-D-glucose by enzymes of the glycolytic pathway has been reported in yeast[2], and in *Staph. aureus*, fluoroacetate has been found[3] to be incorporated into the cell-wall structures. In mammalian epithelial tissues, some fluoro sugars appear to inhibit glycoprotein formation in surviving mucosal preparations[4]. It is of interest that 2-deoxy-2-fluoro- and 6-deoxy-6-fluoro-D-galactose, as well as 2-deoxy-2-fluoroacetamido-D-glucose[5], appear to influence glycolipid biosynthesis in the spontaneously transformed, mouse epithelial-cell line T-AL/N, resulting in the alteration of amino–sugar metabolism without evident toxicity to the cells[5]. Detailed studies of the specific binding of 2-deoxy-2-fluoroacetamido-D-glucose and related derivatives to enzymes, *e.g.* hen-egg lysozyme, have been made by using ^{19}F n.m.r. techniques[6–8].

The metabolic fate of 6-deoxy-6-fluoro-D-galactose thus becomes relevant to its biological activities, especially in mammalian cells. Earlier investigations have shown that the fluoro sugar was neither used by, nor was inhibitory toward, D-galactose-adapted yeasts. The present paper is concerned with the enzymic reduction and

*Dedicated to Professor M. Stacey, C.B.E., F.R.S., in honour of his 65th birthday.

phosphorylation of the fluoro sugar into products obtained independently by chemical synthesis.

Other fluoro sugar phosphates already investigated include 2-deoxy-2-fluoro-DL-glyceraldehyde 3-phosphate[9], and 6- and 3-deoxy-3-fluoro-D-glucosyl phosphates[10].

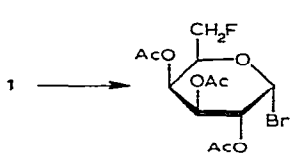
METHODS

Chromatographic techniques. — Paper chromatography was carried out on Whatman No. 4 paper by downward elution with *A* ethyl acetate–pyridine–water (2:1:2), *B* butanone–acetic acid–saturated aqueous boric acid (9:1:1), *C* 2-methoxyethanol–butanone–3M ammonia (7:2:3), *D* 2-methoxyethanol–pyridine–ammonia–water (5:2:1:2), *E* 1-butanol–pyridine–water (4:1:1), *F* 1-butanol–acetic acid–water (40:9:20). Reducing sugars were detected with silver nitrate–potassium hydroxide or aniline hydrogen phthalate. Alditols were detected with periodate–benzidine[11]. For phosphate analysis, all chromatographic papers were pre-washed in 0.5M acetic acid and water, and the components were detected with molybdate–benzidine[12]; ~0.2 μg of P (as dipotassium hydrogen phosphate) could be detected by this means. Chromatographic methods for phosphates have been reviewed by Lederer[13].

Thin-layer chromatography (t.l.c.) was performed on plates (4 × 20 cm) coated with Kieselguhr G (200 μm), using ethyl acetate–benzene (3:7). Sugar components were detected with a sulphuric acid spray. Mobilities (R_B) are measured relative to that of penta-*O*-acetyl-β-D-glucose. Isopropylidene derivatives could also be detected by reverse phase chromatography[14] with upward elution in methanol–water (6:4).

Charcoal column chromatography involved columns (2-cm diameter) prepared[17] from activated charcoal (Ultrasorb SC 120/140 British Carbo Norite Union Ltd.) pre-washed with distilled water [~1 litre per 20 g (dry weight) of charcoal]. Inorganic salts were eluted with water, monosaccharides and their phosphates by 5–20% aqueous ethanol, and nucleotides by 50% aqueous ethanol containing 1.5% of ammonia. The loading of the column did not exceed 1 mg of fluoro sugar phosphate per g (dry weight) of charcoal.

Rates of hydrolysis. — Acid hydrolyses of 6-deoxy-6-fluoro-α-D-galactosyl phosphate (**6**) were conducted at 37 and 60° and followed by determination of the liberation of inorganic phosphate[15,16].



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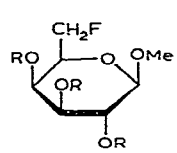

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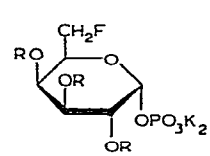

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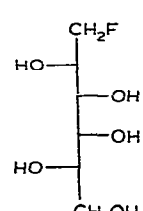

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2,3,4-Tri-O-acetyl-6-deoxy-6-fluoro-α-D-galactosyl bromide (**2**). — 6-Deoxy-6-fluoro-D-galactose (**1**, 1 g) dissolved in dry pyridine (7 ml) was mixed slowly at 2° with dry acetic anhydride (5 ml). After 12 h, the solution was poured into water and the tetra-acetate was extracted with chloroform in the usual way to give a syrup (1.6 g, 84%), $[\alpha]_D^{20}$ +57° (*c* 1.2, chloroform) which consisted chiefly of the β-tetra-acetate. A syrupy product having $[\alpha]_D^{23}$ +28° (*c* 1.5, chloroform) was obtained when **1** was acetylated by acetic anhydride–sodium acetate.

The foregoing syrupy tetra-acetate (1 g) was shaken with a 45% solution (1 ml) of HBr in acetic acid. After 20 min, when crystallisation was observed, further acetic acid (2 ml) was added and shaking was continued for 2 h. The product was collected and recrystallised from chloroform–light petroleum (b.p. 40–60°) to give **2**, m.p. 145–146°, $[\alpha]_D^{20}$ +231 ±2° (*c* 1.8, chloroform), R_B 1.74 (t.l.c.) (Found: C, 38.9; H, 4.1; Br, 20.9; F, 5.1. $C_{12}H_{16}BrFO_7$ calc.: C, 38.8; H, 4.3; Br, 21.5; F, 5.1%).

The glycosyl bromide **2** was also synthesized directly from **1** (1.6 g) by treatment with a mixture of acetic anhydride (6 ml) and 72% perchloric acid (0.05 ml) at 35 ±2°. Cold chloroform (25 ml) was added to the mixture and the acetylated sugar was isolated in the usual manner. The syrupy product was shaken for 10 min with a 45% solution (3.5 ml) of hydrogen bromide in acetic acid and the product **2** (1.7 g, 51%), which separated and was recrystallised as described above, had m.p. 143–144°. The recrystallised product was stable when stored in a vacuum desiccator over sodium hydroxide at 2°.

6-Deoxy-6-fluoro-α-D-galactosyl dipotassium phosphate (**6**). — A solution of the galactosyl bromide **2** (1.66 g) in warm dry benzene (12 ml) was refluxed with dry silver diphenyl phosphate (1.45 g) for 30 min. The inorganic salts were then collected and washed with benzene, and the combined washings and filtrate were evaporated. The resulting syrup [1.5 g, R_B 1.34 (t.l.c.)] was hydrogenated at room temperature and pressure over Adams' catalyst, until the calculated quantity of hydrogen had been taken up (~2 h). The resulting (acidic) material was catalytically deacetylated by the addition of potassium methoxide (1.2 g) dissolved in methanol (15 ml). The white, gelatinous product was centrifuged and crystallised from aqueous ethanol (1:1.7). The crude product **6** (786 mg, 47%), which had $[\alpha]_D^{21}$ +62 ±1° (*c* 0.5, water), was free from reducing sugar, and contained 8.56% of ester P and 2.8% of inorganic P. The compound was further purified either on a charcoal column (100 g, Ultrasorb S. C. 120/140) by elution first with 5% aqueous ethanol (1.5 l) and then 20% aqueous ethanol, or by fractional crystallisation of the magnesium ammonium salt[19]. The crude phosphate **6** (75 mg) was dissolved in 5% aqueous ammonia (1 ml) and a solution (0.5 ml) of hydrated magnesium acetate (16 mg) was added. After storage at 2° for 5 h, the precipitated inorganic phosphate was removed and the solution was freeze-dried. The remaining solid was fractionally crystallised from aqueous ethanol to give **6**, $[\alpha]_D^{18}$ +81 ±1° (*c* 0.2, water) R_{G-1-P} 0.95 (solvent *C*), 1.0 (solvent *D*) (Found: P, 8.1. $C_6H_{10}FK_2O_8P \cdot 2H_2O$ calc.: P, 8.3%).

The phosphate **6** (70 mg) was dissolved in an aqueous solution (1.5 ml) of carrier-free dipotassium hydrogen phosphate-^{32}P (1 μCi). Cold ethanol (2.5 ml, 2°)

was slowly added and the crystals (Fraction 1) which separated after 24 h were filtered off and washed with fresh solvent. The product was recrystallised twice from the same solvent, giving fractions 2 and 3, respectively. The specific activities (c.p.m./mg) of fractions 1, 2, and 3 were 1278 ±20, 1057 ±9, and 990 ±20, respectively.

Phosphorylation of 6-deoxy-6-fluoro-D-galactose by galactokinase. — The fluoro sugar **1** (5 μmoles) was incubated in 0.1M Tris buffer (pH 6.8, 1 ml) containing 0.1mM $MgCl_2$, ATP (10 μmoles), and galactokinase (100 μg, Sigma) at 37°. At measured intervals up to 120 min, samples were withdrawn and the amount of reducing sugar remaining was determined[20]. Control experiments were performed omitting ATP, and enzyme in turn. The products of the enzyme reaction were identified chromatographically by using the incubated, complete reaction mixture. The sugar components were separated by elution from a charcoal column [Ultrasorb SC 120/140, 100 g (dry weight) of charcoal] with 20% aqueous ethanol as described above. The eluate was concentrated at low temperature and examined by paper chromatography on Whatman No. 4 paper by downward elution in solvent *D*.

Methyl 6-deoxy-6-fluoro-β-D-galactopyranoside (**4**). — A solution of the galactosyl bromide **2** (0.3 g) in methanol (6 ml) was boiled in the presence of dry silver carbonate (0.32 g) for 1 h. Removal of the solvent from the filtered solution gave methyl tri-*O*-acetyl-6-deoxy-6-fluoro-β-D-galactopyranoside **3** (0.25 g) which, after recrystallisation from ether–light petroleum (b.p. 40–60°), had m.p. 108°, $[\alpha]_D^{22}$ −11.0 ±0.2° (*c* 0.5, chloroform), R_B 1.34 (t.l.c.) (Found: C, 48.2; H, 6.2; F, 5.9; OMe, 10.0. $C_{13}H_{19}FO_8$ calc.: C, 48.4; H, 5.9; F, 5.9; OMe, 9.6%).

Catalytic deacetylation of **3** (0.1 g) by barium methoxide (0.1M, 3 ml) and methanol (5 ml) at 0° for 24 h gave methyl 6-deoxy-6-fluoro-β-D-galactopyranoside (**4**), m.p. 117–118° (from acetone–ether), $[\alpha]_D^{21}$ −1.3 ±0.3° (*c* 0.9, water), R_F 0.55 (solvent *E*) (Found: F, 9.8. $C_7H_{13}FO_5$ calc.: F, 9.7%).

Treatment of methyl 6-deoxy-6-fluoro-α-D-galactopyranoside [R_F 0.58 (solvent *A*)] with acetic anhydride and pyridine gave the tri-acetate, m.p. 74°, $[\alpha]_D^{20}$ +152 ±3° (*c* 1, chloroform), R_B 1.45 (t.l.c.).

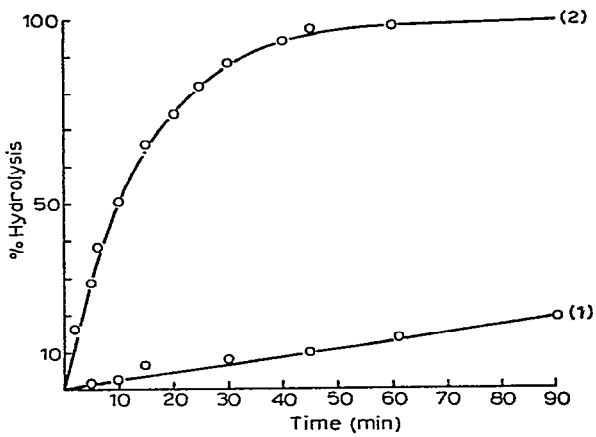
1-Deoxy-1-fluoro-L-galactitol (*6-deoxy-6-fluoro-D-galactitol*) (**7**). — 6-Deoxy-6-fluoro-D-galactose (0.5 g) dissolved in a mixture of ethanol (25 ml) and methanol (15 ml) was added dropwise to a stirred solution of potassium borohydride (0.2 g) in ethanol (25 ml) at 0° over a period of 45 min. After 4 h, 15% methanolic hydrogen chloride (3 ml) was added and the solution was distilled, with repeated additions of methanol, until all the methyl borate had been removed. After neutralisation ($PbCO_3$), the filtered solution was concentrated to a syrup which crystallised from methanol to give **7** (0.22 g), m.p. 173–174°, $[\alpha]_D^{21}$ +4.2 ±0.3° (*c* 0.5, water), R_F 0.58 (solvent *A*), 0.41 (solvent *C*) (Found: C, 39.2; H, 7.4; F, 10.1. $C_6H_{13}FO_3$ calc.: C, 39.1; H, 7.1; F, 10.3%).

Enzymic reduction of 6-deoxy-6-fluoro-D-galactose by rabbit-lens capsules (in collaboration with Dr. Ruth van Heyningen). — Each incubation was carried out at 35° in 95% air–5% CO_2 in a medium containing sodium hydrogen carbonate (16 ml, 2.52%), D-glucose (0.124 ml, 10%), and incubation fluid TC 199 (440 mg) to which

streptomycin (6.2 mg) and penicillin (6.2 mg) had been added, the whole being diluted to 56 ml with water. Whole lenses from rabbits (animals aged 3 months) were incubated for 3 days in the above medium (10 ml) to which aqueous D-galactose (2 ml, 20 μmoles) and aqueous 6-deoxy-6-fluoro-D-galactose (1 ml, 10 μmoles) had been added. The complete medium was changed daily. In control experiments, incubations were conducted with 3 ml of added, aqueous D-galactose (30 μmoles), the fluorogalactose being omitted. Each lens was extracted with trichloroacetic acid (2 ml, 10%) and the solution was shaken with chloroformic methyldi-*n*-octylamine. Sugars were estimated chromatographically in the resulting neutralised solution, samples (5 μl and 10 μl) being eluted on Whatman No. 52 paper in solvent *F*. Standard amounts (20, 10, 5, and 2.5mM, 10 μl) of D-galactose, 6-deoxy-6-fluoro-D-galactose, 1-deoxy-1-fluoro-L-galactitol, and galactitol were run simultaneously. The concentration of D-galactose in lens tissue was 0.0318 μmole/mg wet wt., and after incubation the amount of galactitol was 0.15 μmole/mg. In the presence of D-galactose (0.0203 μmole/mg) and 6-fluoro-6-deoxy-D-galactose (0.0122 μmole/mg), the incubated lens contained both galactitol (0.045 μmole/mg) and 1-deoxy-1-fluoro-L-galactitol (0.134 μmole/mg).

DISCUSSION

2,3,4-Tri-*O*-acetyl-6-deoxy-6-fluoro-α-D-galactosyl bromide (**2**) is a useful intermediate from which to obtain numerous derivatives of 6-deoxy-6-fluoro-D-galactose (**1**). Acetylation of **1** either with acetic anhydride and pyridine or sodium acetate gave non-crystalline products which, on optical rotatory evidence, were



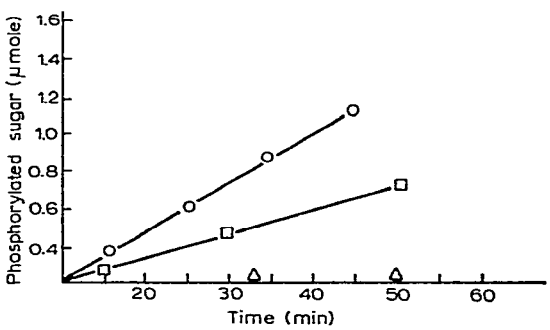


Fig. 1. Acid hydrolysis of 6-deoxy-6-fluoro-α-D-galactosyl dipotassium phosphate (**6**) in 0.25M HCl (1) 37°, k 3.1×10^{-3} min^{-1}; (2) 60°, k 69.0×10^{-3} min^{-1}.

Fig. 2. Phosphorylation of 6-deoxy-6-fluoro-D-galactose by galactokinase. Complete incubation mixture (total vol. 1 ml) contained enzyme (0.1 mg) in 0.1M Tris buffer (pH 6.8), monosaccharide (5 μmoles), ATP (10 μmoles), and $MgCl_2$ (5 μmoles). Incubation was at 37°. ○, Galactose; □, 6-deoxy-6-fluoro-D-galactose; △, 6-deoxy-6-fluoro-D-galactose, ATP-free.

mixtures of α and β isomers in different proportions. Conventional treatment of each of these products with hydrogen bromide in acetic acid gave the crystalline fluorogalactosyl bromide **2** from which the tri-*O*-acetyl-β-glycoside **3** could be easily obtained by the action of methanol and silver carbonate. The methyl β-glycoside **4**, obtained by catalytic deacetylation of **3**, was hydrolysed to 6-deoxy-6-fluoro-D-galactose in 3 h by M sulphuric acid at 100°.

Phosphorylation of the fluorogalactosyl bromide **2** was achieved by reaction with silver diphenyl phosphate, thus minimising Walden inversion[21,22]. Catalytic hydrogenation of the product was accomplished without loss of fluorine but with the formation of some inorganic phosphate (as has been noted in certain similar cases[22]). 6-Deoxy-6-fluoro-α-D-galactosyl phosphate (**6**) was obtained as the dipotassium salt (dihydrate) of 94% purity after chromatography. The removal of inorganic phosphate from **6** was examined by isotopic dilution by recrystallisation of **6** after a single addition of carrier-free $K_2H^{32}PO_4$. Even after three recrystallisations, 28% of the added radioactivity was retained by the product, and it is concluded that co-crystallisation occurs or that the inorganic phosphate is strongly absorbed by the

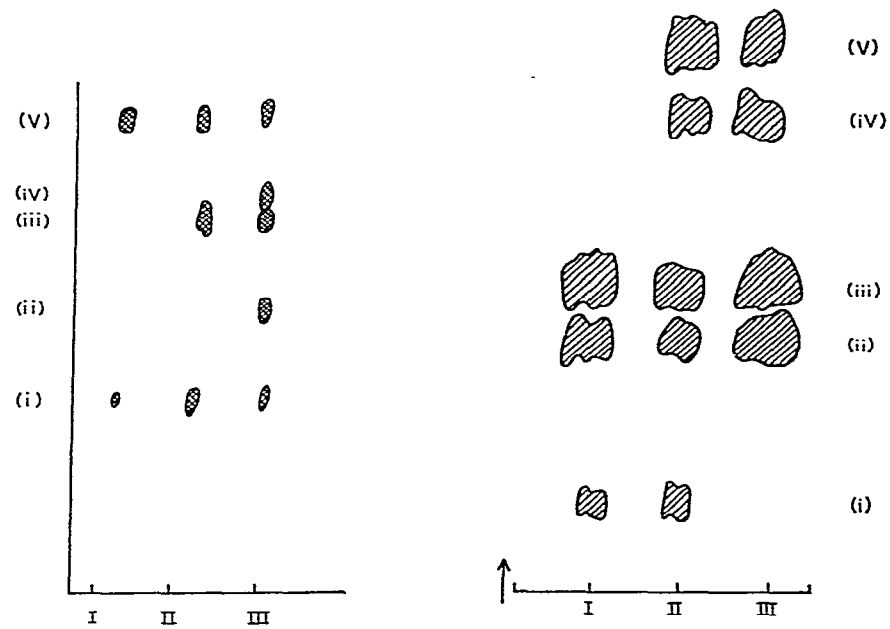


Fig. 3. Chromatographic separation of 6-deoxy-6-fluoro-α-D-galactosyl phosphate (**6**) formed by galactokinase action (*cf.* Fig. 4). Downward elution on Whatman No. 4 paper in solvent *D*. I, ATP-free; II, complete incubation mixture; III, standards: (*i*) inorganic phosphate (R_F 0.41) (*ii*) D-galactose 6-phosphate (R_F 0.71), (*iii*) 6-deoxy-6-fluoro-α-D-galactosyl phosphate (R_F 2.1), (*iv*) D-glucosyl phosphate (R_F 1.0), (*v*) 6-deoxy-6-fluoro-D-galactose (R_F 0.95).

Fig. 4. Enzymic reduction of 6-deoxy-6-fluoro-D-galactose by rabbit lens-capsule (see Discussion). Downward elution on Whatman No. 52 paper in solvent *F*. Substrates: I, galactose; II, D-galactose + 6-deoxy-6-fluoro-D-galactose; III, standards: (*i*) inositol, (*ii*) D-galactose, (*iii*) galactitol, (*iv*) 6-deoxy-6-fluoro-D-galactose, (*v*) 1-deoxy-1-fluoro-L-galactitol.

carbohydrate phosphate. The phosphate **6** was readily hydrolysed by 0.25M hydrochloric acid to the reducing sugar (Fig. 1). The rate of hydrolysis of **6** at 37° was 3.1×10^{-3} min^{-1}, whereas α-D-glucosyl dipotassium phosphate had a k value 3.0×10^{-3} min^{-1}. D-Galactosyl phosphates, on the other hand, were more readily hydrolysed under these conditions, k for the α-isomer being 13.6×10^{-3} min^{-1} and for the β-isomer 12.9×10^{-3} min^{-1}. Similar differences have been reported[23] for the rates of acid hydrolysis of methyl α-D-galactoside and its 6-fluoro analogue. The fluorogalactose phosphate **6** was obtained when **1** was incubated with yeast galactokinase and ATP, at pH 6.8 and 37°, though the rate of esterification was markedly less than that of the parent sugar (Fig. 2). The tentative identity of the product was made on chromatographic evidence (Fig. 3).

In common with other fluoromonosaccharides, 6-deoxy-6-fluoro-D-galactose (**1**) was reduced to the corresponding crystalline fluorohexitol **7** by potassium borohydride at low temperatures[24]. Biological experiments with rabbit lens, a tissue well-known to enzymically reduce D-galactose to galactitol, showed that, under organ culture conditions, a similar transformation was brought about with **1**. Incubations in which both D-galactose and the 6-fluoro sugar were present showed that the latter compound was reduced approximately three times as fast as the parent sugar (Fig. 4). The fluoro sugar thus both penetrates the tissue and is an effective substrate for its enzymes.

Some difficulty was encountered in the analysis of fluoro sugar phosphates due to intereference of P in the fluorine analytical procedure devised by Belcher, Leonard, and West[25]. Whereas, as the authors state, free phosphate does not interfere with colour formation, there is interference when phosphate is present at the combustion stage. Combustion of phosphate alone was found to give colours similar to that given by fluorine. The procedure of Greenhalgh and Riley[26], however, successfully overcame these difficulties.

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