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Fluorine as a Hydroxy Analogue

STEREOSPECIFIC PHOSPHORYLATION OF 2-DEOXY-2-FLUOROGLYCEROL BY GLYCEROL KINASE

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Glycerol kinase catalyses the phosphorylation of the symmetrical substrate, 2-deoxy-2-fluoroglycerol, by ATP to an asymmetric product, 2-deoxy-2-fluoro-*sn*-glycerol 3-phosphate. The stereospecificity of the enzymic reaction was established by unambiguous chemical synthesis of 2-deoxy-2-fluoro-*sn*-glycerol labelled with ^2H at C-1, followed by glycerol kinase-catalysed phosphorylation and isolation of the labelled phosphate. The configuration of the ^2H-labelled phosphate was determined by n.m.r. spectroscopy. This enzymic phosphorylation of 2-deoxy-2-fluoroglycerol is absolutely stereospecific in the same sense as that of glycerol, with fluorine replacing the C-2 hydroxy group. The behaviour of fluorine as a hydroxy analogue in directing the stereospecific course of the enzyme reaction is relevant to the use of the fluorine atom of fluoro analogues of substrates as a reporter group for hydroxy-binding sites of enzymes.

Substrate analogues, in which fluorine replaces hydrogen or hydroxy groups, have considerable potential in the study of enzyme–substrate interactions (Ciba Foundation Symposium, 1972). Because of its small size, the presence of the fluoro substituent does not result in gross steric distortion of the parent molecule. In addition fluorine can act as a reporter group, as the ^{19}F n.m.r. signal of enzyme-bound analogues should reflect the electronic environment of the fluorine atom. However, interpretation of the n.m.r. data in terms of interactions at specific subsites depends on knowledge of the orientation of the fluorinated analogue at the active site.

The interaction of glycerol kinase with the monodeoxymonofluoroglycerols has been examined by steady-state kinetics (Eisenthal *et al.*, 1972). In this series of compounds each of the three hydroxy groups at C-1, C-2 and C-3 of *sn*-glycerol is in turn replaced by fluorine. The behaviour of 1-deoxy-1-fluoro-*sn*-glycerol as a substrate and of 3-deoxy-3-fluoro-*sn*-glycerol as a competitive inhibitor allowed reasonably precise location of the fluorine atom of these analogues within the active site. However, a distinction between the two alternative orientations of the fluorine atom at C-2 of the substrate 2-deoxy-2-fluoroglycerol could not be made on the basis of kinetic evidence. We now report that phosphorylation of 2-deoxy-2-fluoroglycerol catalysed by glycerol kinase leads to an asymmetric product. The configuration of the phosphorylated product can be rationalized in terms of the behaviour of the fluorine atom as a hydroxy analogue in its interaction with the active site.

Experimental

Materials and general methods

Glycerol kinase was purchased from Boehringer Corp. Ltd. (London W.5, U.K.) as a suspension in 2.4M-$(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ and other low-molecular-weight material were removed by loading a sample of the commercial preparation on a column of Sephadex G-25. The enzyme was eluted with 1mM-triethanolamine–HCl buffer containing dithioerythritol (2mM), tetramethylammonium chloride (0.1mM) and EDTA (disodium salt) (0.1mM) and adjusted to pH7.5 with NaOH. Unless otherwise specified, laboratory reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

T.l.c. was performed by upward irrigation on glass plates coated with silica gel G (E. Merck, Darmstadt, W. Germany), or cellulose powder CC 41 (Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) Phosphates were detected by means of the ammonium molybdate–H_2S spray (Hanes & Isherwood, 1949); other compounds were detected by charring after spraying with 40% (w/v) H_2SO_4.

^{14}C radioactivity was measured by using a Phillips liquid-scintillation counter, model PW 4510/01. The scintillant contained Triton X-100–toluene (2:1, v/v) and 0.4% PPO (2,5-diphenyloxazole); the efficiency of counting was about 90%.

N.m.r. spectra were measured with a JEOL PS-100 spectrometer at 100MHz. Concentrations were carried out under reduced pressure.

Chemical syntheses

1,2-*O*-*Isopropylidene*-*sn*-[3-^2H]*glycerol* (*IIa*). $LiAl^2H_4$ (2g) (⩾99 atom% ^2H; Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) was added portionwise to a stirred solution of 2,3-*O*-isopropylidene-D-glyceraldehyde (I) (12g) [prepared by lead tetra-acetate oxidation of 1,2:5,6-di-*O*-isopropylidene-D-mannitol as described by Baer & Fischer (1939)] in dry ether (100ml). The suspension was stirred at 22°C for 1h and finally boiled under reflux for 15min. Ethyl acetate (10ml) and water (4ml) were successively added dropwise to the stirred, cooled reaction mixture. The resulting precipitate was removed by filtration and washed well with ether. Filtrate and washings were combined and concentrated. The residue (12g) was taken up in chloroform (100ml), washed with water (3×100ml), dried (over $MgSO_4$) and reconcentrated to a syrup which was distilled to give 1,2-*O*-isopropylidene-*sn*-[3-^2H]-glycerol (IIa) (6.36g, 53%), b.p. 93.5–94.5°C/3kPa (22mmHg), $[\alpha]_D^{22}$ +15.1±0.4° (in substance).

The unlabelled compound (IIb) prepared by $NaBH_4$ reduction of the aldehyde (I) as described by Wickberg (1958) had b.p. 93–94°C/3kPa (22mmHg), $[\alpha]_D^{22}$ +15.0±0.4° (in substance).

sn-[3-^2H]*Glycerol* (*IIIa*). A solution of the isopropylidene ketal (IIa) in 1M-HCl (15ml) was stirred at 22°C for 16h and neutralized by passage down a column (30ml) of Amberlite IR-45 resin (OH^- form). The eluate was concentrated to a syrup which was dried in vacuum over P_2O_5 to give *sn*-[3-^2H]glycerol (IIIa) (3.4g, 98%).

1,3-*Di*-*O*-*triphenylmethyl*-*sn*-[3-^2H]*glycerol* (*IVa*). *sn*-[3-^2H]Glycerol (IIIa) (3.3g) in dry pyridine (25ml) was stirred with triphenylmethyl chloride (20g) at 105°C for 3h. The cooled reaction mixture was stirred into ice–water (250ml) and the resulting precipitate removed by filtration and recrystallized from benzene–light petroleum (b.p. 60–80°C) (1:5, v/v) to give 1,3-di-*O*-triphenylmethyl-*sn*-[3-^2H]glycerol (IVa) (10g, 49%) (R_F 0.3; silica with benzene solvent), m.p. 179–182°C. Preparative layer chromatography [silica gel 60PF_{254} (E. Merck) with benzene solvent] of the mother liquors gave a further yield of chromatographically pure product (1.3g, 6%).

The unlabelled compound (IVb) prepared from glycerol (IIIb) by the above procedure had R_F 0.3 (silica, benzene solvent) and m.p. 178–180°C. Helferich *et al.* (1923) quote 185–186°C and Zerban & Sattler (1942) give 174–175°C.

2-*O*-*Toluene*-*p*-*sulphonyl*-1,3-*di*-*O*-*triphenylmethyl*-*sn*-[3-^2H]*glycerol* (*Va*). A solution of the di(triphenylmethyl) ether (IVa) (11.3g) and toluene-*p*-sulphonyl chloride (18g) in dry pyridine (50ml) was left at 22°C for 3 days. The reaction mixture was poured into ice–water (500ml) and the crystalline precipitate removed by filtration, washed with water and dissolved in chloroform (100ml). The chloroform solution was washed with water, dried (over $MgSO_4$) and concentrated to give a solid residue which was recrystallized from ethyl acetate to give 2-*O*-toluene-*p*-sulphonyl-1,3-di-*O*-triphenylmethyl-*sn*-[3-^2H]glycerol (Va) (9.5g, 65%) (R_F 0.6; silica, benzene solvent), m.p. 158–160°C.

The unlabelled compound (Vb) prepared from 1,3-di-*O*-triphenylmethylglycerol (IVb) by the above procedure had R_F 0.6 (silica, benzene solvent) and m.p. 162–163°C; Zerban & Sattler (1942) quote m.p. 155–156°C.

2-*Deoxy*-2-*fluoro*-1,3-*di*-*O*-*triphenylmethyl*-*sn*-[1-^2H]*glycerol* (*VIa*). Treatment of the toluene-*p*-sulphonate (Va) with tetra-*n*-butylammonium fluoride in acetonitrile, as previously described for the unlabelled compound (Vb) (Lloyd & Harrison, 1971) gave 2-deoxy-2-fluoro-1,3-di-*O*-triphenylmethyl-*sn*-[1-^{2}H]glycerol (VIa) (R_F 0.65; silica, benzene solvent), m.p. 174–178°C.

The unlabelled compound (VIb) (Lloyd & Harrison, 1971) had R_F 0.65 (silica, benzene solvent) and m.p. 172–175°C.

2-*Deoxy*-2-*fluoro*-*sn*-[1-^2H]*glycerol* (*VIIa*). Hydrolysis of the di(triphenylmethyl) ether (VIa) in acetone–HCl as described for the unlabelled compound (VIb) (Lloyd & Harrison, 1971) gave 2-deoxy-2-fluoro-*sn*-[1-^{2}H]glycerol (VIIa), m.p. 30–35°C, which crystallized without distillation.

The unlabelled compound (VIIb) (Lloyd & Harrison, 1971) had b.p. 96–97°C/400Pa (3mmHg), m.p. 39–40°C.

2-*Deoxy*-2-*fluoro*-[2-^{14}C]*glycerol*. [2-^{14}C]Glycerol (50μCi, sp. radioactivity 10.5mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was diluted with unlabelled glycerol (21g) and converted into 2-deoxy-2-fluoro-[2-^{14}C]glycerol (3.7g, 6.3μCi), b.p. 92–93°C/133Pa (1mmHg), m.p. 38–39°C, as described for the conversion of *sn*-[3-^{2}H]glycerol (IIIa) into 2-deoxy-2-fluoro-*sn*-[1-^{2}H]glycerol (VIIa).

rac-2-*Deoxy*-2-*fluoroglycerol phosphate* (*VIIIb*) *dicyclohexylammonium salt*. Treatment of 2-deoxy-2-fluoroglycerol (VIIb) with dibenzylphosphoryl chloride followed by catalytic hydrogenation as described by Lloyd & Harrison (1974) gave chromatographically pure crystalline *rac*-2-deoxy-2-fluoroglycerol phosphate (VIIIb) dicyclohexylammonium salt [R_F 0.70; cellulose, propan-2-ol–acetic acid–water (3:1:1, by vol.) as solvent], m.p. 167–168°C.

Enzymic synthesis

Phosphorylation of 2-*deoxy*-2-*fluoroglycerol by ATP* (*glycerol kinase*). Into a thermostatically controlled reaction vessel was placed 1ml of an aqueous solution of ATP (disodium salt) (300μmol) and $MgCl_2$ (300μmol). The pH was adjusted to 7.5 with 2M-NaOH, and 700μg of glycerol kinase in about 3ml of elution buffer (see under 'Materials and general

methods') was added. After 10min, during which no adenosine triphosphatase activity was detected, the reaction was started by addition of 2-deoxy-2-fluoro-[2-^{14}C]glycerol (50mg, 532μmol), giving a final volume of 6.5ml. The reaction proceeded for 21h at 30°C under a blanket of CO_2-free N_2. The pH was maintained at pH7.5 throughout the reaction by continuous titration of released protons with 0.1M-NaOH by using a pH-stat (titrator TTT 11, pH meter 26; Radiometer Corp., Copenhagen, Denmark).

The reaction mixture was then stirred with freshly washed Bio-Rad AG 50W-X8 resin (H^+ form) to remove cations. The resin was removed by filtration, washed with water, and the combined filtrates (diluted to 120ml) were fractionated by ion-exchange chromatography. The presence of adenine nucleotides in the eluate was monitored by absorption at 254nm; these were eluted in the order AMP, ADP, ATP (Bartlett, 1959).

P_i was assayed by the method of Fiske & Subba-Row (1925). Unchanged and phosphorylated 2-deoxy-2-fluoroglycerol were detected by ^{14}C radioactivity. The chromatographic elution pattern is shown in Fig. 1. Unchanged 2-deoxy-2-fluoroglycerol was completely eluted in the first seven fractions. Virtually all of the radioactivity in the initial 2-deoxy-2-fluoroglycerol was accounted for in fractions 1–7 (containing non-adsorbed material) and fractions 17–19 (containing the phosphorylated product together with some P_i). Fractions 17 and 18 were concentrated and freeze-dried to a syrup (20mg). Water (0.5ml) was added to the syrup followed by an ice-cold solution of cyclohexylamine (1.0ml) in ethanol (1.5ml). The residue remaining after removal of the solvent was recrystallized from hot ethanol to give 2-deoxy-2-fluoro-*sn*-[2-^{14}C]glycerol 3-phosphate dicyclohexylammonium salt (20mg, 10.5%). The melting point, chromatographic behaviour and n.m.r. spectrum of the product were identical with those of the dicyclohexylammonium salt of the chemically synthesized phosphate (VIIIb).

Phosphorylation of 2-deoxy-2-fluoro-*sn*-[1-^{2}H]-glycerol (VIIa) was carried out in an analogous manner. Fractions (10ml) from the ion-exchange column were collected; the fractions eluted between AMP and P_i were pooled and concentrated as described above. From 50mg of compound (VIIa) was obtained 10mg (21%) of 2-deoxy-2-fluoro-*sn*-[1-^{2}H]-glycerol 3-phosphate (VIIIa) dicyclohexylammonium salt; chromatographic behaviour on cellulose was identical with that of the dicyclohexylammonium salt of compound (VIIIb), m.p. 164–166°C.

Results and Discussion

The chemically synthesized racemic 2-deoxy-2-fluoroglycerol phosphate (VIIIb) has a well-defined n.m.r. spectrum in which the signals corresponding to the methylene protons at the phosphorylated and non-phosphorylated ends of the molecule are clearly separated. Unambiguous assignment of the peaks in the spectrum can be made on the basis of the splitting patterns and by comparison with the spectra of the synthetic precursors (Table 1). Enzymic phosphorylation of 2-deoxy-2-fluoro-*sn*-[1-^{2}H]glycerol (VIIa) catalysed by glycerol kinase gave the crystalline phosphate (VIIIa) which behaved identically with the unlabelled phosphate (VIIIb), but had a different n.m.r. spectrum. The methylene proton signals of phosphates (VIIIa) and (VIIIb) are shown in Fig. 2. The

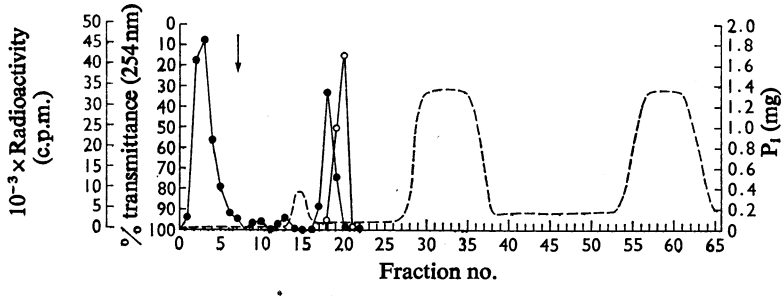


Fig. 1. *Anion-exchange chromatography of the reaction mixture from phosphorylation of 2-deoxy-2-fluoro-[2-^{14}C]glycerol by ATP catalysed by glycerol kinase*

The decationized reaction mixture (120ml) was applied to a column (1.3cm×15cm) of Bio-Rad AG1-X8 resin (formate form; 100–200 mesh). The column was eluted with 120ml of water followed by a linear (0–3.6M-formate) gradient generated from a mixing chamber containing 1000ml of water and a limit buffer of 0.9M-ammonium formate in 2.7M-formic acid. The gradient was started after fraction 6 (indicated by arrow); flow rate, 40ml/h; fraction size, 20ml. Eluate flow was continuously monitored at 254nm (LKB Uvicord) and portions (0.5ml) were taken for radioactivity assay. No radioactivity was detected in the eluates after fraction 19. Further details are given in the Experimental section. ●, ^{14}C radioactivity; ○, P_i; ----, % transmittance (254nm).

Table 1. *N.m.r. data for glycerol protons of 2-deoxy-2-fluoro-sn-[1-^2H]glycerol 3-phosphate, its precursors and corresponding unlabelled compounds*

Numbers of protons giving rise to signals specified in columns H-1, H-2 and H-3 are indicated in parentheses after the chemical shift. For details of H-1, H-2 and H-3 protons see the text and Fig. 2.

Compound	Solvent	Chemical shifts (p.p.m. from tetramethylsilane) H-2	H-1	H-3	Coupling constants (Hz)
IIa	CCl_4	3.6–4.2 (3) multiplet		3.4–3.6 (1) multiplet	$J_{2,3} = 4.5$
IIb	CCl_4	3.6–4.2 (3) multiplet		3.50 (2) doublet	
IVa	$C^2HCl_3 + {}^2H_2O$	3.88 (1) multiplet	3.22 (3), multiplet		$J_{1,2} = J_{2,3} = 5.5$
IVb	$C^2HCl_3 + {}^2H_2O$	3.90 (1) quintet	3.25 (4), doublet		$J_{1,2} = J_{2,3} = 5.5$
Va	C^2HCl_3	4.55 (1) quartet	3.28 (3), multiplet		$J_{1,2} = J_{2,3} = 5$
Vb	C^2HCl_3	4.57 (1) quintet	3.28 (4), multiplet		$J_{1,2} = J_{2,3} = 5$
VIa	C^2HCl_3	4.70 (1) octet	3.32 (3), quartet		$J_{1,2} = J_{2,3} = 5$; $J_{1,F} = J_{3,F} = 21$; $J_{2,F} = 48$
VIb	C^2HCl_3	4.70 (1) decet	3.32 (4), quartet		$J_{1,2} = J_{2,3} = 5$; $J_{1,F} = J_{3,F} = 21$; $J_{2,F} = 48$
VIIa	^2H_2O	5.10 (1) two multiplets	4.18 (3), 'quartet'*		$J_{1,2} = J_{2,3} = 5$; $J_{1,F} = J_{3,F} = 25$; $J_{2,F} = 48$
VIIb	^2H_2O	5.10 (1) decet	4.18 (4), 'quartet'*		$J_{1,2} = J_{2,3} = 5$; $J_{1,F} = J_{3,F} = 25$; $J_{2,F} = 48$
VIIIa	^2H_2O	5.20 (1) two multiplets	4.23 (1) two multiplets	4.35 (2) octet	$J_{2,3} = 5$; $J_{1,F} = 27$; $J_{3,F} = 23$; $J_{2,F} = 48$; $J_{3,P} = 6$
VIIIb	^2H_2O	5.20 (1) two multiplets	4.23 (2) 'quartet'*	4.35 (2) octet	$J_{1,2} = J_{2,3} = 5$; $J_{1,F} = 25$; $J_{3,F} = 23$; $J_{2,F} = 48$; $J_{2,P} = 6$

* The third peak in the 'quartet' is further split into a doublet.
† Two 'quintets', the second of which is further split.

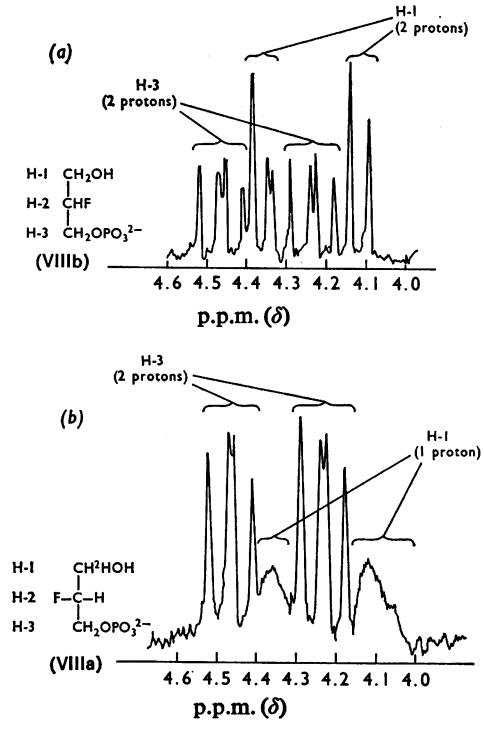
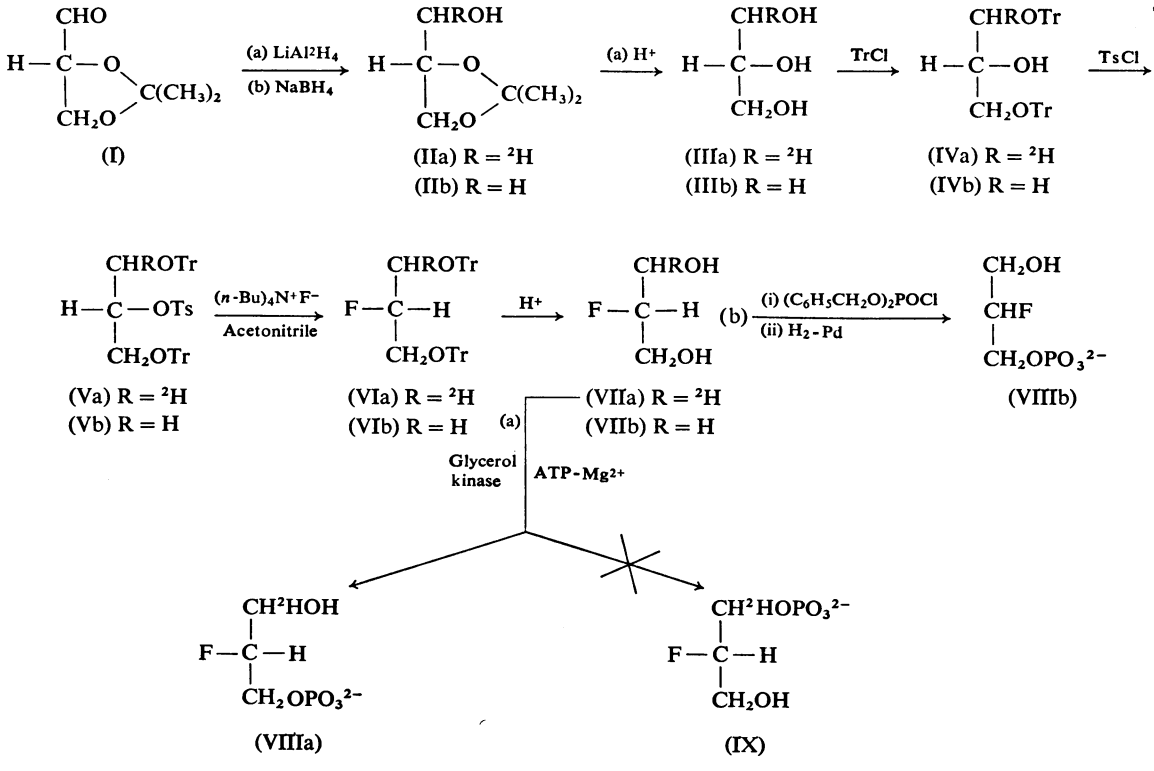


Fig. 2. *N.m.r. spectra of the methylene protons of (a) rac-2-deoxy-2-fluoroglycerol phosphate and (b) 2-deoxy-2-fluoro-sn-[1-^2H]glycerol 3-phosphate*

Experimental details are as given in the Experimental section and in Table 1.

methylene protons of the non-phosphorylated end of compound (VIIIb) (H-1) give rise to a 'quartet' (2 protons) and those at the phosphorylated end (H-3) to an octet (2 protons). This octet, again integrating for two protons, appears unchanged in the spectrum of the ^2H-labelled enzymically prepared phosphate (VIIIa). However, the sharp peaks corresponding to the non-phosphorylated end of compound (VIIIb) are replaced by two complex multiplets in compound (VIIIa), which integrate for one proton only. This shows that enzymic phosphorylation has occurred specifically at the carbon atom which bears two hydrogen atoms. Repeated integration of the peaks corresponding to the protons at the non-phosphorylated and phosphorylated ends of the ^2H-labelled molecule (VIIIa) gave a ratio of $1:2.04 \pm 0.07$ ($\pm$S.D., five determinations) showing that stereospecificity was 100%. Complete absence of stereospecificity would have given a ratio of 1:1.

The proton at the ^2H-labelled carbon atom gives rise to a complex spin–spin splitting pattern caused partly by ^2H–proton coupling. As ^2H was introduced

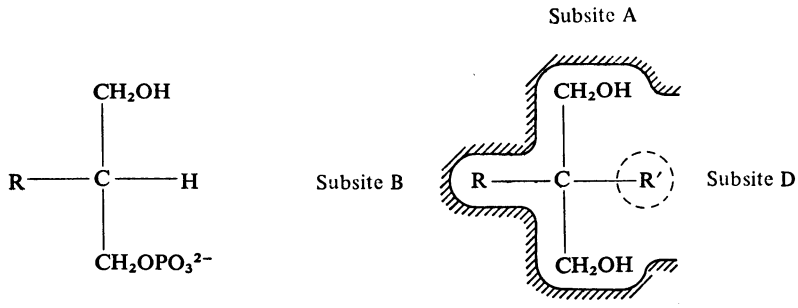


Scheme 1.

For details see the text. Prefixes (a) and (b) denote the agent used to synthesize the labelled and unlabelled compounds respectively. Tr, Triphenylmethyl, Ts, toluene-*p*-sulphonyl; $(n\text{-Bu})_4N^+F^-$, tetra-*n*-butylammonium fluoride.

by a non-specific chemical ($LiAl^2H_4$) reduction of an aldehyde, this proton will be evenly distributed between the pro-*R* and pro-*S* configurations, thus further complicating the peak pattern. A similar situation occurs in the n.m.r. signal of the analogous H-3 proton of 1,2-*O*-isopropylidene-*sn*-[3-^{2}H]glycerol (IIa), where a complex multiplet integrating for one proton replaces the simple doublet shown by the two H-3 protons of the unlabelled compound (IIb). Comparison of the spectrum of the synthetic unlabelled phosphate (VIIIb) with that of the enzymic ^{2}H-labelled phosphate (VIIIa) shows that increased multiplicity caused by geminal ^{2}H is only observed in the signal corresponding to the non-phosphorylated methylene proton. This is consistent with absolute stereospecificity of the enzymic phosphorylation.

The evidence from n.m.r. data is therefore unambiguous in placing the phosphate group of the enzymically prepared fluoroglycerol phosphate (VIIIa) solely at the non-^{2}H-labelled end of the molecule. The stereochemical purity of the phosphate requires not only that the enzymic phosphorylation be 100% stereospecific but also that the substrate (VIIa) be likewise stereochemically pure. There are a number of implications regarding the synthetic reactions (Scheme 1) leading to the fluoroglycerol (VIIa) arising from this conclusion. First, no racemization at C-2 can have occurred during the synthesis as a result of reactions at the primary carbon atoms. Moreover, the introduction of fluorine by displacement of the toluene-*p*-sulphonyloxy group of compound (Va) must have occurred either with full retention or complete inversion of configuration at C-2. It is unlikely that the triphenylmethyl ether groups of the toluene-*p*-sulphonate (Va) will be involved in neighbouring group participation leading to retention of configuration. Further, it is known that displacements of sulphonyloxy groups attached to monosaccharide structures normally proceed by an S_N2 mechanism accompanied by Walden inversion (Foster & Westwood, 1973). It appears, therefore, that the F^- ion displacement reaction involves total inversion of configuration. Since no other reaction in the sequence (I)–(VIIa) is likely to affect the steric orientation at C-2, the configuration of fluoroglycerol (VIIa) will be that shown in Scheme 1 and the product of its



(Xa) R = OH
(Xb) R = F

(XIa) R = OH; R′ = H
(XIb) R = F; R′ = H
(XIc) R = H; R′ = F

Scheme 2. *Possible orientation of glycerol (XIa) and of 2-deoxy-2-fluoroglycerol (XIb and XIc) at the active site of glycerol kinase leading, to the products (Xa) and (Xb) respectively*

For details of subsites A, B, C and D see the text.

enzymic phosphorylation must be 2-deoxy-2-fluoro-*sn*-[1-^{2}H]glycerol 3-phosphate (VIIIa) rather than the epimeric 2-deoxy-2-fluoro-*sn*-[1-^{2}H]glycerol 1-phosphate (IX).

Since C–^{2}H bonds are neither made nor broken during the enzymic phosphorylation, the possibility of a primary isotope effect influencing the steric course of the reaction can be ruled out. Secondary isotope effects might well be present but these are likely to be of negligible significance (Thomson, 1963). Unlabelled 2-deoxy-2-fluoroglycerol would therefore be enzymically phosphorylated in the same steric sense as the ^{2}H-labelled compound leading specifically to 2-deoxy-2-fluoro-*sn*-glycerol 3-phosphate (Xb).

Glycerol is stereospecifically phosphorylated by glycerol kinase to give *sn*-glycerol 3-phosphate (Xa). A schematic illustration (Scheme 2) of the orientation of glycerol at the active site is shown in structure (XIa) in which 'subsites' A, B and C correspond to the positions on the enzyme associated with the hydroxy groups at C-1, C-2 and C-3 respectively of *sn*-glycerol. Phosphorylation of the hydroxy group at subsite C gives rise to the observed product (Xa) (Gancedo *et al.*, 1968; Eisenthal *et al.*, 1972). Since the spatial relationships of the fluorine atom of 2-deoxy-2-fluoroglycerol and of the C-2 hydroxy group of glycerol to their respective phosphorylated hydroxymethyl groups are the same (compounds Xa and Xb), productive binding of 2-deoxy-2-fluoroglycerol is likely to result from orientation of this substrate as in structure (XIb); i.e. the fluorine atom is at the same subsite (B) as that occupied by the C-2 hydroxy group of glycerol.

It is unlikely that binding of 2-deoxy-2-fluoroglycerol could occur in the alternative orientation (structure XIc) in which hydrogen occupies subsite B and fluorine subsite D (normally associated with the hydrogen at C-2 of glycerol). Phosphorylation is known not to be prevented by steric bulk at subsite D since C-2 substituted analogues of glycerol, i.e. 2-methylglycerol and dihydroxyacetone hydrate (Eisenthal *et al.*, 1974), are substrates of glycerol kinase. Nor is phosphorylation prevented by the presence of a hydrogen atom at subsite B, since 2-deoxyglycerol (propane-1,3-diol) is also a substrate (Eisenthal *et* al., 1972). There is no reason to suppose therefore that binding in orientation (XIc) would be non-productive. The consequence of productive binding in this orientation would be the phosphate (compound IX) which is not observed. It appears therefore that 2-deoxy-2-fluoroglycerol is oriented at the active site exclusively as in structure (XIb).

The absolute stereospecificity of glycerol kinase is determined by the nature of the interactions of substituents at C-2 of the substrate with the active site. 2-Deoxy-2-fluoroglycerol binds to the active site of glycerol kinase in a glycerol-like conformation in which the fluorine atom occupies the position normally associated with the C-2 hydroxy group of the natural substrate. Occupancy of this position by fluorine rather than by hydrogen must arise from properties common to the C–F and C–OH bonds and not shared by the C–H bond. For instance, the bond length (Sutton, 1965) and polarity (Pauling, 1963*a*) of the C–F bond resemble those of the C–OH bond more than do those of C–H. Moreover, the covalent van

der Waals radii of fluorine and oxygen are similar (Pauling, 1963*b*) and covalently bound fluorine has the ability to accept a hydrogen bond (Buckley *et al.*, 1968). Any of these factors may be implicated in interactions of fluorine with the enzyme which lead to its specific orientation at the active site. Conversely it is unlikely that hydrogen-bond donation from substrate to enzyme plays a major role in determining the orientation of the natural substrate, glycerol, at the active site.

Further information concerning the hydroxy-binding site and its interactions with substrate might be obtained from ^{19}F n.m.r. studies. It may be noted that the interactions involved in the specific binding of 2-deoxy-2-fluoroglycerol to glycerol kinase are not reflected by any gross differences between the K_m and V values of 2-deoxy-2-fluoroglycerol and those of the deoxy analogue propane-1,3-diol (Eisenthal *et al.*, 1972). This serves to underline the caution that must be exercised in interpreting steady-state kinetic parameters in terms of specific enzyme–substrate interactions, especially in view of the complex mechanisms that are known to exist for multisubstrate enzymes such as glycerol kinase (Thorner & Paulus, 1973).

Replacement of a hydroxy group of a natural metabolite by fluorine often results in analogues possessing biological activity (Taylor, 1972). Comparisons of the properties of deoxyfluoro analogues with those of the corresponding hydroxy compounds have been made in studies of membrane transport (Barnett, 1972; Riley & Taylor, 1973) and isolated enzyme systems (Lloyd & Harrison, 1974; Thomas *et al.*, 1974) using steady-state kinetics. In only a few instances have systematic comparisons (e.g. with the corresponding deoxy analogues) indicated that the deoxyfluoro compound is acting as a hydroxy analogue. In most of the above cases the apparent activity of a fluoro substituent as a hydroxy group can be explained in terms of the profound effects that fluorine is known to have on neighbouring substituents. Since 2-deoxy-2-fluoroglycerol is a symmetrical molecule, any inductive effects of fluorine will equally influence the reactivity of each hydroxymethyl group. Thus the stereospecific phosphorylation of 2-deoxy-2-fluoroglycerol by glycerol kinase constitutes an unambiguous demonstration of the activity of fluorine as a hydroxy analogue in its specific interaction with a hydroxy-binding site.

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