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Activity of Fluoro and Deoxy Analogues of Glycerol as Substrates and Inhibitors of Glycerol Kinase

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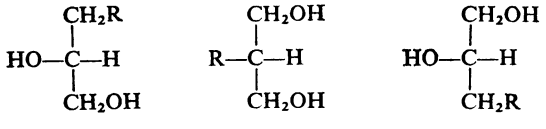
Analogues of glycerol in which each of the three hydroxy groups is successively replaced by fluorine or hydrogen have been examined as substrates or inhibitors of glycerol kinase (*Candida mycoderma*) to assess the ability of fluorine to mimic a substrate hydroxy group in enzyme–analogue interactions. The four diols resulting from replacement of the hydroxy groups at C-1 or C-2 of *sn*-glycerol by fluorine or hydrogen are weak substrates. Similar substitution of the C-3 hydroxy group gives compounds which act as competitive inhibitors of glycerol or dihydroxyacetone phosphorylation but show no activity as substrates. Comparison of the steady-state kinetic parameters of the corresponding analogues shows that replacement of a hydroxy group by either fluorine or hydrogen leads to compounds with similar activity in this system. A convenient synthesis of (+)-propane-1,2-diol is described.

Since the discovery of the lethal synthesis of fluorocitrate from fluoroacetate there has been continuing interest in the synthesis of organic fluorine compounds with potential biological activity; the role of fluoro analogues as pseudo-substrates or inhibitors in metabolic studies is now well established (Ciba Foundation Symposium, 1972). The use of these compounds as models for exploring the nature of interactions of small ligands with biological macromolecules is beginning to receive attention. Thus the n.m.r. of ^{19}F nuclei allows the fluorine atom to act as a reporter group for transmitting information concerning the micro-environment of the binding site. Such use of fluorine as a molecular probe has so far been restricted to substrate analogues in which fluorine has been substituted for covalent hydrogen (Dwek *et al.*, 1971; Raftery *et al.*, 1972).

In recent years a number of fluorocarbohydrates, in which fluorine replaces a hydroxy group, have been synthesized in these and other laboratories (Wright *et al.*, 1969; Barford *et al.*, 1971) and reports of their biological activity have appeared (Barnett *et al.*, 1970; Taylor *et al.*, 1972; Kent & Wright, 1972). As fluorocarbohydrates have potential as enzyme probes, it is important to know the extent to which fluorine can substitute for a replaced hydroxy group in enzyme–analogue binding. A systematic comparison of the fluoro analogues with the corresponding hydroxy and deoxy compounds is therefore required both at the metabolic and at the isolated enzyme level. Direct comparison of the activities of fluoro-



(I) (II) (III)

(*a*) R = F (*b*) R = H (*c*) R = OPO_3^{2-}

carbohydrates with those of their deoxy analogues has been reported in a limited number of biological systems (Smith *et al.*, 1965; Barnett *et al.*, 1969), but in no case has an isolated enzyme system been examined in this respect.

We now report the behaviour of the deoxyfluoroglycerols and their deoxy analogues with glycerol kinase. 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 (I*a*, II*a*, III*a*) or by hydrogen (I*b*, II*b*, III*b*). Together with glycerol these compounds constitute a simple model system in which to compare the contribution of corresponding hydroxy, fluorine and hydrogen groups to enzyme–ligand binding.

* To clarify the interrelationships involved, the deoxyfluoroglycerols and propanediols (deoxyglycerols) are named as glycerol derivatives. Stereospecific numbering as recommended by the IUPAC–IUB Commission, 1967, is used for glycerol and these derivatives throughout.

A preliminary account of some of these findings has been published (Eisenthal *et al.*, 1970).

Materials and Methods

Chemicals and enzymes

Glycerol (AnalaR grade), dihydroxyacetone and 2-deoxyglycerol (propane-1,3-diol) were obtained from BDH Chemicals, Poole, Dorset, U.K. Glycerol was dried by the method of Vogel (1957). Dihydroxyacetone was kept under vacuum over phosphorus pentoxide for 24h before use. 2-Deoxyglycerol was distilled, b.p. 95°C/266N/m² (2mmHg).

1-Deoxy-*sn*-glycerol [(−)-propan-1,2-diol] was prepared by fermentative reduction of acetol as described by Levene & Walti (1943); it had b.p. 71°C/119N/m² (0.9mmHg), $[\alpha]_D^{22}$ −16.1±0.5° (in substance).

3-Deoxy-*sn*-glycerol [(+)-propane-1,2-diol] was prepared from 1-deoxy-*sn*-glycerol by successive toluene-*p*-sulphonylation, benzoate exchange with inversion of configuration at C-2, and methanolysis as described below.

Toluene-*p*-sulphonyl chloride (30.4g) in dry pyridine (75ml) was added dropwise to a cooled (0°C) stirred solution of 1-deoxy-*sn*-glycerol (3g) in dry pyridine (25ml). The reaction mixture was kept at room temperature for 2 days, shaken with water (2ml) to destroy excess of toluene-*p*-sulphonyl chloride, and poured into ice–water (500ml). The crystalline precipitate was removed by filtration, well washed with water, and recrystallized from methanol to give 1-*deoxy*-2,3-*di*-*O*-*p*-*toluenesulphonyl*-*sn*-*glycerol* (11g, 72%) which had m.p. 69–70°C, $[\alpha]_D^{22}$ +20±0.4° (*c*5.0 in chloroform), Found: C, 53.1; H, 5.4; S, 16.8; $C_{17}H_{20}O_6S_2$ requires C, 53.1; H, 5.2; S, 16.7%).

A solution of 1-deoxy-2,3-di-*O*-*p*-toluenesulphonyl-*sn*-glycerol (8g) and sodium benzoate (15g) in dry *NN*-dimethylformamide (70ml) was boiled under reflux overnight. The cooled reaction mixture was partitioned between chloroform and water, and the chloroform layer washed with water, dried (magnesium sulphate) and concentrated under reduced pressure. The syrupy residue was purified by preparative layer chromatography on silica gel PF_{254} (Merck; supplied by Anderman and Co. Ltd., London S.W.1, U.K.) in benzene–methanol (5:1, v/v) and distilled to give 1,2-di-*O*-benzoyl-3-deoxy-*sn*-glycerol (3.5g, 59%) b.p. 200°C/133N/m² (1mmHg), $[\alpha]_D^{22}$+10±0.4° (*c* 2.7 in chloroform). Heim & Poe (1944) quote b.p. 232°/1.60kN/m² (12mmHg) for the racemic compound.

A solution of 1,2-di-*O*-benzoyl-3-deoxy-*sn*-glycerol (3g) in methanolic hydrogen chloride (5%, w/w; 50ml) was boiled under reflux overnight. The reaction mixture was concentrated under reduced pressure and the bulk of the hydrogen chloride removed by repeated addition and evaporation of methanol. The residual syrup was freed from last traces of acid by dissolving in methanol and stirring with Deacidite FF resin (CO_3^{2-} form) (BDH Chemicals Ltd., Poole, Dorset, U.K.). Filtration and evaporation yielded a syrup which was taken up in water (50ml), washed well with chloroform, concentrated under reduced pressure and dried over phosphorus pentoxide under vacuum overnight to give 3-deoxy-*sn*-glycerol (0.46g, 58%), $[\alpha]_D^{22}$ +15.4±0.2° (*c*7.7 in water). Gas chromatography (at 90°C) of the trimethylsilyl derivative showed that it contained less than 0.1% impurity and gave a trace identical with that of its enantiomer.

The monodeoxymonofluoroglycerols were synthesized as described by Lloyd & Harrison (1971).

Glycerol kinase (*Candida mycoderma*), pyruvate kinase, lactate dehydrogenase, hexokinase, phosphoenolpyruvate, ATP and NADH were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K.

Kinetic Determinations

In all experiments initial rates of enzyme reactions were followed by coupling the rate of ADP formation to the oxidation of NADH, by using phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase. Decrease in E_{340} was measured in a Unicam SP.800 spectrophotometer at 30°C. Reaction mixtures (final pH7.5) contained 43.3mM-triethanolamine–HCl buffer (adjusted to pH7.6 with 2M-NaOH), 0.81mM-ATP, 0.13mM-NADH, 3.04mM-phosphoenolpyruvate, 2.6mM-$MgSO_4$, 0.87mM-KCl, 43.3μg of pyruvate kinase, 8.66μg of lactate dehydrogenase, 0.5–2.5μg of glycerol kinase and various concentrations of substrates and inhibitors in a total volume of 1.005ml. Sufficient ATP and magnesium were present in this reaction mixture to saturate the enzyme at all times. Reactions were started by addition of substrate. Control experiments were carried out to ensure that the rate-limiting step was that catalysed by glycerol kinase and also that the fluoro and deoxy analogues had no effect on the coupling enzymes. For the latter control, the reaction mixture was as described above except that glucose and hexokinase replaced substrate and glycerol kinase respectively. None of the analogues used affected the rate of NADH oxidation in this system. A standard assay for glycerol kinase activity, with 0.2mM-glycerol as substrate, was performed before and after each series of experiments to check the stability of the enzymes. One unit of glycerol kinase activity is defined as that amount of enzyme catalysing the transformation of 1μmol of glycerol/min under the conditions of the standard assay.

Values of K_m' (apparent K_m at 0.81mM-ATP), V and K_i and their standard errors were calculated from plots of [S]/v versus [S] by the method of least squares by using an ICL450 computer.

Results

1-Deoxy-1-fluoro-*sn*-glycerol (I*a*), 2-deoxy-2-fluoroglycerol (II*a*) and the corresponding 1- and 2-deoxy analogues (I*b* and II*b*) all behave as substrates of glycerol kinase and show Michaelis–Menten kinetics. The low substrate activity of these compounds is reflected in both their K'_m and V values. The K'_m values (Table 1) are an order of magnitude greater than that of dihydroxyacetone and three orders of magnitude greater than that of glycerol. The corresponding V values are approximately 30 times smaller than those of dihydroxyacetone or glycerol (Table 1). Although caution must be exercised in interpretation of relative K'_m values, the structural similarity of the diol substrates coupled with the small variation in both K'_m and V suggests that no gross differences in binding or mechanism exist within this series of analogues.

Neither 3-deoxy-3-fluoro-*sn*-glycerol (III*a*) nor 3-deoxy-*sn*-glycerol (III*b*) showed detectable substrate activity at concentrations up to 300mM. However, both compounds effectively inhibited glycerol kinase with either glycerol or dihydroxyacetone as substrate. In all cases the ordinate intercepts of Lineweaver–Burk plots were independent of inhibitor concentration (Fig. 1) indicating competitive inhibition. K_i values are presented in Table 2. For both inhibitors, K_i versus glycerol was about 2.5 times greater than K_i versus dihydroxyacetone. Dependence of K_i on the nature of the substrate might imply partial competitive inhibition. However, the linearity of the secondary plots (Fig. 2) indicates the strict competitive nature of the inhibition; the variation in K_i may therefore reflect different kinetic pathways for dihydroxyacetone and glycerol. The similarity of the K_i values for the two inhibitors with a given substrate suggests that similar enzyme–inhibitor binding results when the C-3 hydroxy group of glycerol is replaced by either fluorine or hydrogen.

The observed substrate activity of 1-deoxy-*sn*-glycerol does not conflict with the previous findings of Gancedo *et al.* (1968), who did not detect substrate activity of racemic propane-1,2-diol with a similar assay system. On the basis of our values of K'_m for the (−)-propane-1,2-diol (1-deoxy-*sn*-glycerol) and K_i for the inhibitory (+)-propane-1,2-diol (3-deoxy-*sn*-glycerol), the predicted velocity of reaction of the racemic substrate would not be detectable at any substrate concentration in this assay system.

Discussion

The phosphorylation of glycerol by ATP, catalysed by glycerol kinase, yields exclusively *sn*-glycerol 3-phosphate (III*c*) (Bublitz & Kennedy, 1954). The strict stereospecificity of glycerol kinase implies a high degree of restriction on the mutual orientation of the

Table 1. *Substrate activity of glycerol analogues with glycerol kinase*

Details are as described in the Materials and Methods section. Values for glycerol and dihydroxyacetone are included for comparison. Values are means ±S.E.M.

Substrate	[S] (mM)	K'_m (mM)	V (nmol of NADH/min per glycerol kinase unit)
1-Deoxy-1-fluoro-*sn*-glycerol (I*a*)	53.8–269	165 ± 32	29 ± 7
1-Deoxy-*sn*-glycerol (I*b*)	40–500	45 ± 3	22 ± 2
2-Deoxy-2-fluoroglycerol (II*a*)	113–250	100 ± 3	35 ± 1
2-Deoxyglycerol (II*b*)	64–330	88 ± 4	14 ± 1
3-Deoxy-3-fluoro-*sn*-glycerol (III*a*)	Up to 300	—	<1.7†
3-Deoxy-*sn*-glycerol (III*b*)	Up to 300	—	<1.7†
Glycerol	0.035–0.2	0.050 ± 0.007	1200 ± 200
Dihydroxyacetone	8.0–40	7.5 ± 0.6	850 ± 90

† Calculated assuming $K_m \simeq K_i$.

Table 2. *Kinetic constants for competitive inhibition of glycerol kinase by 3-deoxy-3-fluoro-sn-glycerol and 3-deoxy-sn-glycerol*

K_i values (±S.E.M.) were calculated from the horizontal intercept of the secondary plots (Fig. 2) assuming competitive inhibition.

Substrate	Inhibitor	K_i (mM)
Glycerol	3-Deoxy-3-fluoro-*sn*-glycerol	8.6 ± 0.7
Glycerol	3-Deoxy-*sn*-glycerol	4.6 ± 1.2
Dihydroxyacetone	3-Deoxy-3-fluoro-*sn*-glycerol	3.6 ± 0.8
Dihydroxyacetone	3-Deoxy-*sn*-glycerol	1.2 ± 0.04

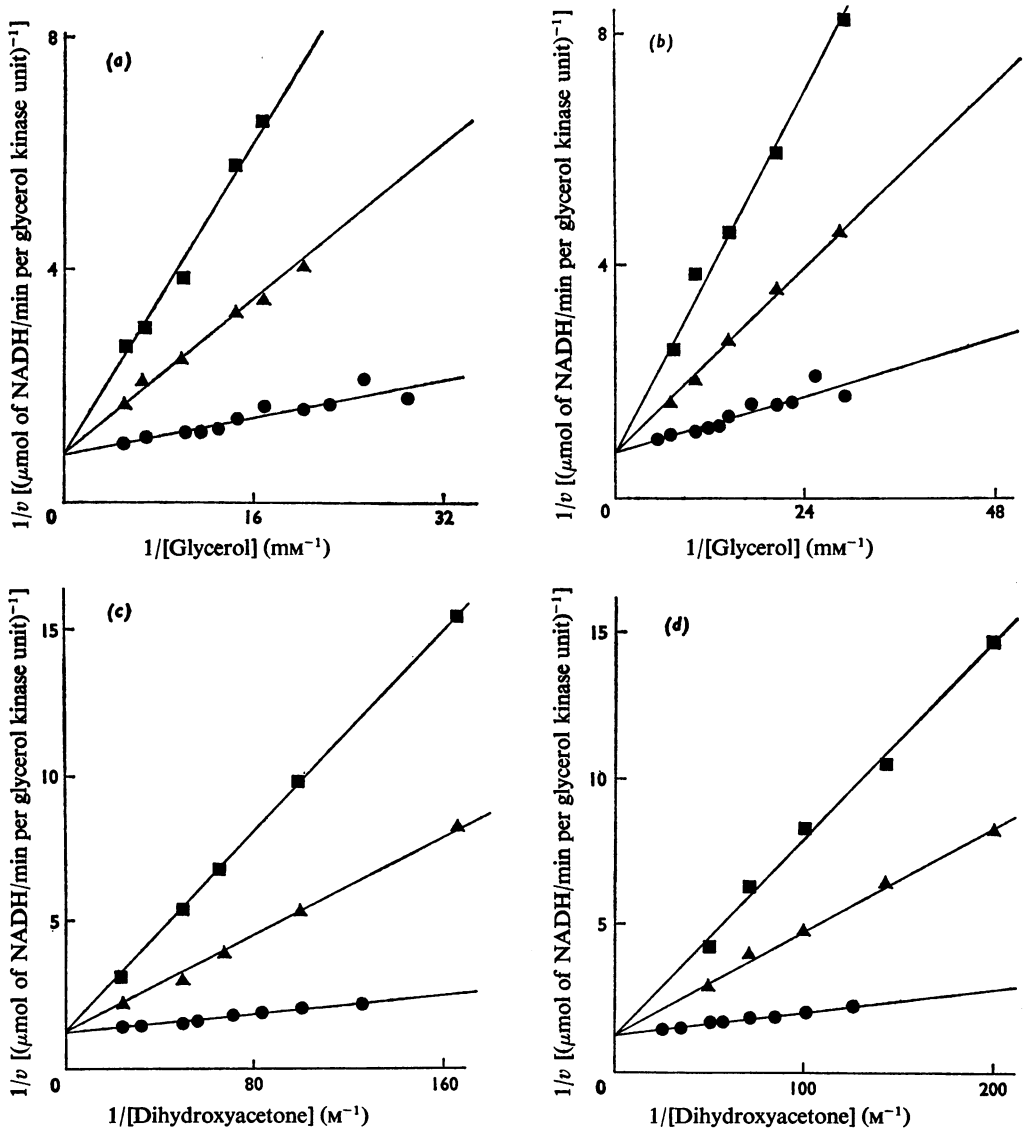


Fig. 1. *Lineweaver–Burk plots for the inhibition of glycerol kinase by 3-deoxy-3-fluoro-sn-glycerol and 3-deoxy-sn-glycerol*

Reaction conditions were as described in the Materials and Methods section with the addition of inhibitor as indicated. (*a*) Inhibition of glycerol phosphorylation by 3-deoxy-3-fluoro-*sn*-glycerol. Concentration of inhibitor: ●, uninhibited; ▲, 21.8mM; ■, 54.9mM. (*b*) Inhibition of glycerol phosphorylation by 3-deoxy-*sn*-glycerol. Concentration of inhibitor: ●, uninhibited; ▲, 10mM; ■, 27mM. (*c*) Inhibition of dihydroxyacetone phosphorylation by 3-deoxy-3-fluoro-*sn*-glycerol. Concentration of inhibitor: ●, uninhibited; ▲, 11.8mM; ■, 39.0mM. (*d*) Inhibition of dihydroxyacetone phosphorylation by 3-deoxy-*sn*-glycerol. Concentration of inhibitor: ●, uninhibited; ▲, 4.6mM; ■, 11.5mM.

three hydroxy groups of glycerol in the enzyme–substrate complex. A schematic illustration of such an orientation is shown by structure (IV*a*) in which 'sites' A, B and C correspond to loci at the active centre of the enzyme associated with the hydroxy groups at C-1, C-2 and C-3 respectively of *sn*-glycerol.

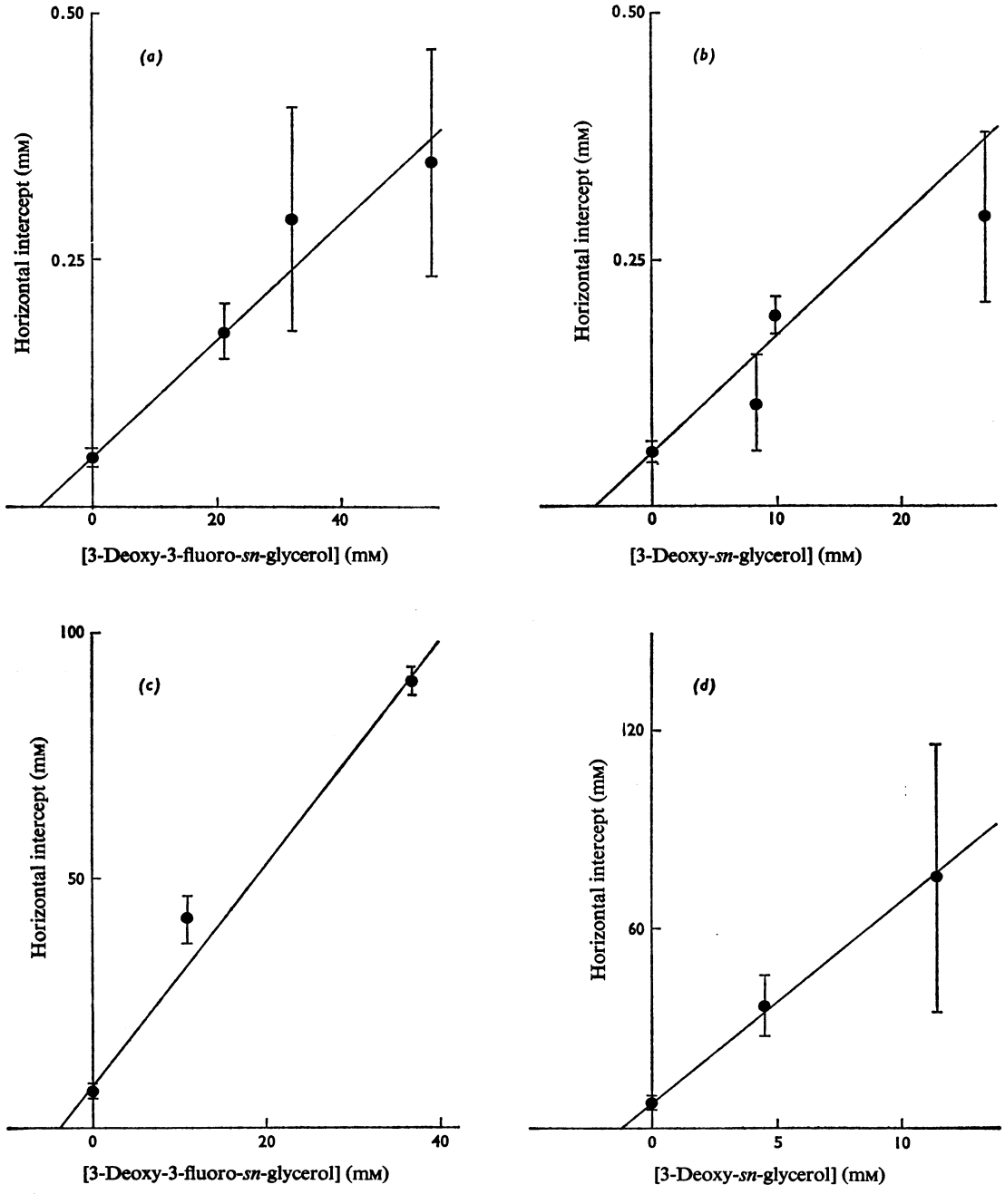
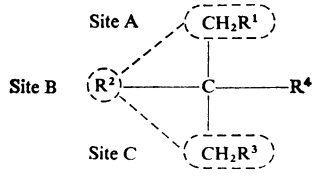


Fig. 2. *Plot of horizontal intercepts of* [*S*]/*v versus* [*S*] *plots for inhibition of glycerol kinase against inhibitor concentration*

(*a*) Inhibition of glycerol phosphorylation by 3-deoxy-3-fluoro-*sn*-glycerol. (*b*) Inhibition of glycerol phosphorylation by 3-deoxy-*sn*-glycerol. (*c*) Inhibition of dihydroxyacetone phosphorylation by 3-deoxy-3-fluoro-*sn*-glycerol. (*d*) Inhibition of dihydroxyacetone phosphorylation by 3-deoxy-*sn*-glycerol. Reaction conditions are as described in Fig. 1. Bars represent the S.E.M.

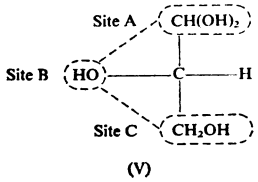
Phosphorylation of the hydroxy group at site C gives rise to the observed product. Gancedo *et al.* (1968) have proposed that effective binding of a substrate to glycerol kinase requires the presence of three hydroxy groups capable of orientation in a 'glycerol-like' conformation. The behaviour of dihydroxyacetone and L- and D-glyceraldehyde as substrates was explained by assuming that these carbonyl compounds interact with the enzyme in their hydrated forms (structures IV*b*, V and VI respectively).

The observed behaviour of the diol analogues as substrates or inhibitors of glycerol kinase is consistent with orientation of these compounds in the enzyme–analogue complex in a 'glycerol-like' conformation, which provides for maximum interaction of the hydroxy groups with sites A, B and C. Such orientation of 1-deoxy-*sn*-glycerol (IV*c*), 1-deoxy-1-fluoro-*sn*-glycerol (IV*d*), 2-deoxyglycerol (IV*e*) and 2-deoxy-2-fluoroglycerol (IV*f* or IV*g*) results in the juxtaposition of a primary hydroxy group and site C, the phosphorylation position, and accounts for the substrate activity of these compounds. The behaviour of 3-deoxy-*sn*-glycerol and 3-deoxy-3-fluoro-*sn*-glycerol as competitive inhibitors of glycerol kinase, together with their lack of substrate activity, is consistent with their orientation as shown in structures (IV*h*) and (IV*i*) respectively, in which no phosphorylatable substituent is available at site C. Thus in the fluoro compounds (IV*d*, IV*f* and IV*i*) and the deoxy compounds (IV*c*, IV*e* and IV*h*) a fluorine or hydrogen respectively occupies, or is very close to, the enzyme site normally associated with the replaced hydroxy group of glycerol. A comparison of the relative abilities of fluoro and hydrogen substituents to act as a hydroxy analogue in an isolated enzyme system is therefore possible.

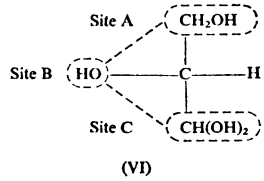


(IV)

(*a*)	Glycerol	$R^1 = R^2 = R^3 = OH$; $R^4 = H$
(*b*)	Dihydroxyacetone	$R^1 = R^2 = R^3 = R^4 = OH$
(*c*)	1-Deoxy-*sn*-glycerol	$R^1 = R^4 = H$; $R^2 = R^3 = OH$
(*d*)	1-Deoxy-1-fluoro-*sn*-glycerol	$R^1 = F$; $R^2 = R^3 = OH$; $R^4 = H$
(*e*)	2-Deoxyglycerol	$R^1 = R^3 = OH$; $R^2 = R^4 = H$
(*f*)	2-Deoxy-2-fluoroglycerol	$R^1 = R^3 = OH$; $R^2 = F$; $R^4 = H$
(*g*)	2-Deoxy-2-fluoroglycerol	$R^1 = R^3 = OH$; $R^2 = H$; $R^4 = F$
(*h*)	3-Deoxy-*sn*-glycerol	$R^1 = R^2 = OH$; $R^3 = R^4 = H$
(*i*)	3-Deoxy-3-fluoro-*sn*-glycerol	$R^1 = R^2 = OH$; $R^3 = F$; $R^4 = H$



(V)



(VI)

That covalent fluorine might prove a better hydroxy analogue than hydrogen in enzyme–analogue interactions is suggested by the similarity in electronegativity and size of the fluoro and hydroxy groups. Moreover it has been demonstrated that covalently bound fluorine, like a hydroxy group, can accept a hydrogen bond (McDaniel & Brown, 1955; Pavlath & Leffler, 1962; Martin, 1965; Buckley *et al.*, 1968). All these physicochemical factors have been implicated in an explanation of the close resemblance in crystal structure between 2-deoxy-2-fluoroerythritol and erythritol itself (Bekoe & Powell, 1959). Direct comparison has shown the effectiveness of the fluoro substituent as a hydroxy analogue in two physiological systems. Smith *et al.* (1965) reported that 2-deoxy-2-fluoroerythritol inhibited the growth of *Brucella abortus* whereas the deoxy analogue 2-deoxyerythritol was inactive. Barnett *et al.* (1969) found that 3-deoxy-3-fluoro-D-glucose and 6-deoxy-6-fluoro-D-galactose were actively accumulated by everted segments of hamster intestine more rapidly than the deoxy analogues.

In the glycerol kinase system comparison of kinetic parameters can be made to test whether analogues resulting from replacement of each hydroxy group in turn by fluorine exhibit enhanced binding or substrate activity over the corresponding deoxy compounds. Our results show that such enhancement is not kinetically detectable within the pairs of analogues resulting from replacement of the C-1 (compounds I*a* and I*b*) or the C-2 (compounds II*a* or II*b*) hydroxy groups of *sn*-glycerol. Loss of either hydroxy group leads to a very large increase in K'_m and decrease in V value, each to a value which is similar whether the replacement atom is fluorine or hydrogen. The similar K_i values of the inhibitors (III*a* and III*b*) likewise show no evidence of increased binding by the fluoro compound compared with the deoxy analogue.

The behaviour of the three monodeoxymonofluoroglycerols as substrates or inhibitors allows reasonably precise location of the fluorine atom within the active site, which may aid interpretation of future n.m.r. studies with ^{19}F as a probe. In the case of 2-deoxy-2-fluoroglycerol two alternative locations of the fluorine atom are possible, depending on whether the analogue is oriented as in structure (IV*f*)

or structure (IV*g*). That both orientations are sterically possible is consistent with the substrate activity of dihydroxyacetone as its hydrate (IV*b*). Formation of an asymmetric phosphorylated product from 2-deoxy-2-fluoroglycerol could be evidence for preferential binding of fluorine over hydrogen in the enzyme–ligand complex.

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