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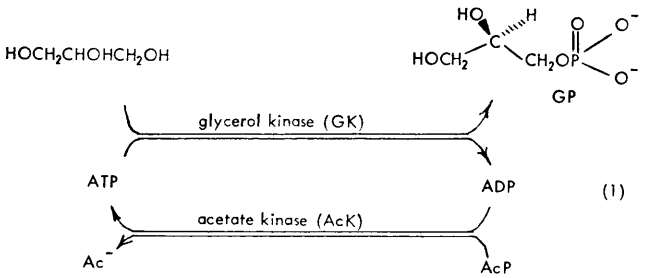
Enzymatic Synthesis of *sn*-Glycerol 3-Phosphate[1]

Sir:

We describe here a practical procedure for the synthesis of *sn*-glycerol 3-phosphate (GP, L-glycerol 3-phosphate) based on enzymatic phosphorylation of glycerol using ATP and glycerol kinase (E.C. 2.7.1.30) (eq 1). The ATP is regenerated



using the recycling system described previously,[2] with acetyl phosphate (AcP) as the ultimate phosphorylating agent.[3] GP is an important intermediate in syntheses of phospholipids.[4] Present preparations of chiral glycerol derivates are based on isolation from natural sources,[5] or on cleavage of the C-3–C-4 bond of derivatives of mannitol.[6] Both types of procedure are capable of generating substantial amounts of materials, but require several steps. The enzymatic synthesis described here requires only a single step, and provides what is probably the most practical method presently available for the preparation of quantities of enantiomerically pure GP.

A representative synthesis was carried out in a 5-L round-bottomed flask equipped with a pH electrode and containing a magnetic stirring bar and 5 g of nylon beads to facilitate stirring. The flask was charged with 1 L of doubly distilled water containing glycerol (110 mmol), ATP (2 mmol), $MgCl_2 \cdot 6H_2O$ (4 mmol), and dithiothreitol (DTT, 10 mmol).[7] The solution was adjusted to pH 7.6 (no buffer). Polyacrylamide gel particles containing immobilized glycerol kinase (42 U, 30 mL of gel) and acetate kinase (E.C. 2.7.2.1, 42 U, 7 mL of gel) were added to the mixture.[8] An aqueous solution (60 mL, 1 M, pH 7.6) containing 120 mmol of diammonium acetyl phosphate[3] was added continuously to the stirred reaction mixture over 48 h at a rate of 2.5 mL/h (2.5 mmol/h).[9] The mixture was maintained between pH 7.4 and 7.8 by addition of 1.5 M NH_4OH, using an automatic pH controller. Reaction was carried out at ambient temperature, and the reaction mixtures and all reagent solutions were deoxygenated before use and maintained under argon. After addition of 120 mmol of AcP over 48 h, enzymatic assay[10] indicated that 100 mmol of GP had been formed. Stirring was stopped and the gel suspension allowed to settle for 6 h at ambient temperature. The supernatant was decanted under positive argon pressure using a stainless-steel cannula. The reactor was then reloaded with glycerol, ATP, DTT, and $MgCl_2$ and the addition of AcP continued for another 48 h. Three consecutive reactions (134 h of operation) generated a total of 318 mmol of GP. The combined supernatant from these reactions (3810 mL) was adjusted to pH 3.0 with concentrated HCl and concentrated under vacuum (10 Torr, 60 °C) to a volume of 40 mL. This concentrate was adjusted to a pH between 0.0 and 0.5 with concentrated HCl and 120 mL of methanol was added. The mixture was allowed to stand for 20 min at 4 °C, the precipitate (mainly inorganic phosphate) separated by filtration, and the filtrate treated with 2 equiv of cyclohexylamine (63 g, 636 mmol).[11] Any precipitate which formed at this point was separated by filtration and discarded. The mixture was poured slowly into 1000 mL of acetone with vigorous stirring. The resulting white, fluffy precipitate was filtered and washed with acetone (2 × 500 mL) and anhydrous ether (500 mL). The precipitate (115 g) was dried over Drierite for 12 h under vacuum: it contained 95% di(cyclohexylammonium) GP (238 mmol, 79% based on AcP, 76% based on glycerol). The activities of GK and AcK in the recovered gel after these three consecutive runs were 98 and 51%, respectively, of the activities of the original immobilized preparations.

This same enzymatic system has been used to prepare *sn*-glycerol-2-d_1 3-phosphate in 0.5-mol scale (213 g of the dicyclohexylammonium salt) and *sn*-glycerol-3-d_1 3-phosphate in 30-mmol scale.[12]

This synthesis illustrates the practicality of synthesizing chiral intermediates by enzymatic reactions which require ATP. The requirement for substantial amounts of isotopically and chemically substituted phospholipids in membrane biochemistry and the prospect that large quantities of enantiomerically pure phospholipids may be needed if liposomes prove useful as drug delivery systems justify the development of this synthesis of *sn*-glycerol 3-phosphate. A facile preparation of GP should also prove useful in syntheses of other substances (triglycerides, trichoic acids, cardiolipins)[5,13] derived from it biosynthetically.

References and Notes

(1) Supported in part by the National Institutes of Health, GM 26543, and by the Exxon Research Foundation.
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(7) The equilibrium constant for formation of glycerol phosphate from glycerol and ATP is $>10^3$. The relevant Michaelis constants at pH 7.5 are K_m(MgATP) = 1.0, K_m(glycerol) = 0.03 mM. Cf Grunnet, N.; Lundquist, F. *Eur. J. Biochem.* **1967**, *3*, 78.
(8) Enzymes were obtained from Boehringer–Mannheim and used without purification. They had specific activities (μmol min^{-1} mg^{-1}, after treatment with DTT): GK, 46 U, AcK, 70 U. Assays followed procedures described: (a) Wieland, O. *Biochem. Z.* **1957**, *329*, 313. (b) Holz, G. In "Methoden der Enzymatischen Analyse", H. U. Bergmeryer, Ed.; Verlag Chemie; Weinheim, 1970; Vol. 2, p 1486. Enzymes were immobilized in 91% yield (GK) and 60% yield (AcK) in polyacrylamide gels: Pollak, A.; Baughn, R. L.; Adalsteinsson, O.; Whitesides, G. M. *J. Am. Chem. Soc.* **1978**, *100*, 302.
(9) The acetyl phosphate solution was prepared each day and maintained at 4 °C to minimize hydrolysis. The rate of addition of AcP was maintained at a rate sufficiently low that it was overall rate limiting for formation of glycerol phosphate. A low steady-state concentration of AcP minimized its hydrolysis.
(10) Hohorst, H.-J. In ref 8b, p 1379 ff.
(11) Friedkin, M. *J. Biol. Chem.* **1950**, 184, 449.
(12) Glycerol-2-d_1 was prepared by Dr. Richard Wittebort of the M.I.T. National Magnet Laboratory by reduction of dihydroxy acetone with $NaBD_4$; *sn*-glycerol 3-d_1 was prepared by similar reduction of optically pure (*R*)-glyceraldehyde. After purification by distillation, these glycerol samples contained traces of a borate which strongly inhibited GK. This inhibition was overcome by carrying out the reaction in the presence of 3 mM triethanolamine.
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