gel) and Hepes buffer (0.1 M, pH 7.6, 70 mL). Potassium ferricyanide (3.3 g, 10 mmol) was added and the mixture was stirred under an argon atmosphere. The pH was maintained at 7.0 by automatic addition of 2.5 N KOH. Aliquots (0.5 mL) of the reaction were removed periodically and centrifuged, a portion of the supernatant was diluted in a 1:20 ratio, and the absorbance was measured at 420 nm. After 6 h, 6.3 mL of 2.5 N KOH (15.8 mmol, 3.2 equiv) had been added. Figure 4 shows a plot of the addition of base vs. time for the reactor and a plot of the ratio of the absorbance to initial absorbance at 420 vs. time. The mixture was centrifuged to separate the gel. The activitites of the recovered enzymes were 60 U for GlcDH (140%) and 32 U for diaphorase (150%). The turnover number for NAD was 125. The product of the oxidation, gluconate, was not isolated.

**Determination of Partition Coefficients of (+)-(1*R*,2*S*)-*cis*-8-Oxabicyclo[4.3.0]nonan-7-one between Hexane and Water.** A solution of the lactone (55 mg, 0.39 mmol, 78 mM) and octadecane (30 mg, 0.12 mmol, 24 mM) in hexane (5 mL) was analyzed by GLC, (10% Carbowax). The response factor, $R_f$, was found to be 1.91 ($R_f$ = [mmol of lactone/mmol off octadecane]/[area of lactone/area of octadecane]). A 2-mL portion of the hexane solution was equilibrated by shaking with a 2-mL aliquot each of glycine buffer (0.25 M, pH 9.0) and of distilled water. The hexane layer was analyzed by GLC using octadecane as the internal standard. The partition coefficients ($\sigma$) with glycine buffer and distilled water were 1.0 and 1.9, respectively.

**Decomposition of Oxaloacetate.** A stock solution of oxaloacetate (100 mM in Hepes buffer, pH 7.6) was prepared by combining oxalacetic acid (132 mg, 1 mmol), NaOH (1.8 mL of a 1 N standard solution), and Hepes buffer (8.2 mL). Three vials (10 mL) were prepared: the first contained Hepes (0.1 M), glycine (0.1 M, pH 7.6, 4.5 mL), and oxalacetate (0.5 mL); the second contained HLADH (5 mg), Hepes (0.1 M, pH 7.6, 0.9 mL), and oxalacetate (0.1 mL); the third contained Hepes (0.1 M, pH 7.6, 4.5 mL) and oxalacetate (0.5 mL). Aliquots (50 $\mu$L) were removed and assayed for oxalacetate.[18] Figure 1 shows a plot of the first-order decomposition.

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**Registry No. 1**, 15753-50-1; **2**, 65376-02-5; NAD, 53-84-9; NADH, 58-68-4; MB, 61-73-4; MV, 1910-42-5; FMN, 146-17-8; DCIP, 956-48-9; PMS, 299-11-6; $O_2$, 7782-44-7; $Fe(CN)_6^{3-}$, 13408-62-3; $CH_3CHO$, 75-07-0; EtOH, 64-17-5; $K_3Fe(CN)_6$, 13746-66-2; glutamate dehydrogenase, 9029-12-3; diaphorase, 9001-18-7; D-glucose, 50-99-7; D-gluconate, 526-95-4; alcohol dehydrogenase, 9031-72-5; glucose dehydrogenase, 9028-53-9; oxidoreductase, 9055-15-6; dehydrogenase, 9035-82-9; cyclohexanone, 108-94-1; cyclohexanol, 108-93-0; malate dehydrogenase, 9001-64-3; glycerol dehydrogenase, 9028-14-2; lactate dehydrogenase, 9001-60-9; malic enzyme, 9028-47-1; 3-hydroxybutyrate dehydrogenase, 9028-38-0; isocitrate dehydrogenase, 9001-58-5; 2-oxoglutarate, 328-50-7.

# Glycerol Kinase: Substrate Specificity[1]

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**Abstract:** Glycerol kinase (E.C. 2.7.1.30, ATP: glycerol-3-phosphotransferase) catalyzes the phosphorylation of 28 nitrogen, sulfur, and alkyl-substituted analogues of glycerol. A survey of 66 analogues has defined qualitatively the structural characteristics necessary for acceptability as a substrate by this enzyme. This survey was conducted using an assay based on $^{31}P$ NMR spectroscopy which detected products produced even by slow reactions. These studies indicate that glycerol kinase accepts a range of substituents in place of one terminal hydroxyl group (that which is not phosphorylated), and that the hydrogen atom at C-2 can be replaced by a methyl group. Replacement of the second terminal hydroxyl group (that which is normally phosphorylated) by other nucleophilic centers usually results in loss of activity. Glycerol kinase is a useful catalyst for the synthesis of chiral organic substances, especially starting materials for the preparation of phospholipids and analogues. Comparisons of kinetic constants for enzymes from four microorganisms (*Candida mycoderma*, *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus stearothermophilus*) indicate little variation among them. All phosphorylated products have stereochemistry analogous to that of *sn*-glycerol-3-phosphate.

## Introduction

We and others are developing practical synthetic methods based on enzymatic catalysis for the selective functionalization of organic molecules.[2-5] Much of the work concerned with enzymes as catalysts, both in mechanistic enzymology and in catalytic organic synthesis, has centered on reactions involving naturally occurring substances. As a result, enzymes have developed the reputation of being "highly specific", that is, by implication, of limited utility for reactions requiring transformations of "unnatural" substrates (substrates not encountered in vivo or not considered as primary substrates for the enzymes of interest). Recent work on enantioselective transformations involving esterases (especially lipase and hog liver acylase[6-9]) and oxoreductases (especially horse liver alcohol dehydrogenase[10]) has demonstrated that certain enzymes can accept a broad range of substrate structures and still retain catalytic rates and enantioselectivities useful in organic synthesis. These enzymes are, however, believed to function in vivo as broad-spectrum catalysts, and it is perhaps neither unexpected nor representative that they accept a range of substrates. The work reported in this and the following paper[11] was intended to provide complementary information concerning the substrate specificity of a representative enzyme (glycerol kinase) chosen from the group of enzymes believed, on the basis of assigned in vivo function (reactions in major metabolic pathways), to have

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(10) Takemura, T.; Jones, J. B. *J. Org. Chem.* **1983**, *48*, 791-6, and references therein.
(11) Crans, D. C.; Whitesides, G. M., *J. Am. Chem. Soc.*, following paper in this issue.

0002-7863/85/1507-7008$01.50/0 

*narrow* specificity.[12] The objectives of this work were to establish the range of unnatural substrates accepted by this enzyme, and to determine if synthetically useful differences in substrate selectivity characterized enzymes derived from different sources.

We selected glycerol kinase (E.C. 2.7.1.30, ATP: glycerol-3-phosphotransferase) for four reasons.

1. Glycerol kinases derived from four microbial sources (*Candida mycoderma*, *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus stearothermophilus*) are commercially available. This enzyme is inexpensive and stable when immobilized. It has high specific activity.

2. The assigned function of glycerol kinase[13]—enantiospecific phosphorylation of glycerol and generation of *sn*-glycerol-3-phosphate[14]—provides a route to an intermediate important in the synthesis of phospholipids. The ability to synthesize chiral analogues of *sn*-glycerol-3-phosphate would be useful in the preparation of new phospholipids.[15]

3. Organic phosphates are relatively inconvenient to synthesize using classical chemical methods, especially if enantiomerically enriched materials are required. Synthetic methods based on glycerol kinase do not duplicate existing synthetic methods.

4. The supporting technology required to use glycerol kinase—especially that for in situ regeneration of ATP and for immobilization of the enzyme—is already fully developed.[2,3] The successful use of glycerol kinase in kilogram-scale synthesis of *sn*-glycerol-3-phosphate and dihydroxyacetone phosphate has previously established the practicality of synthetic procedures based on this enzyme.[16-18]

Glycerol kinases from *C. mycoderma*,[19-24] *E. coli*,[12,25,26] and *B. stearothermophilus*[27] have been reported to accept a total of eight compounds as substrates. Gancedo et al.[20] and Eisenthal et al.[23] have proposed stereochemical models for the active site(s) of these enzymes. We have confirmed the results from these prior studies and examined a number of additional potential substrates. Based on these examinations, we were able to define the limits of synthetic utility of glycerol kinase and propose a simple model which summarizes the structural features required for activity as a substrate. Comparison of substrate selectivity for the four enzymes derived from different sources indicates that only minor variation in substrate selectivity is observed and that, with certain exceptions, kinetic characteristics of these enzymes are similar. We conclude from these studies that glycerol kinase should be useful in the preparation of a number of analogues of *sn*-glycerol-3-phosphate, and that the most useful enzyme for synthetic applications is presently that from *S. cerevisiae*.

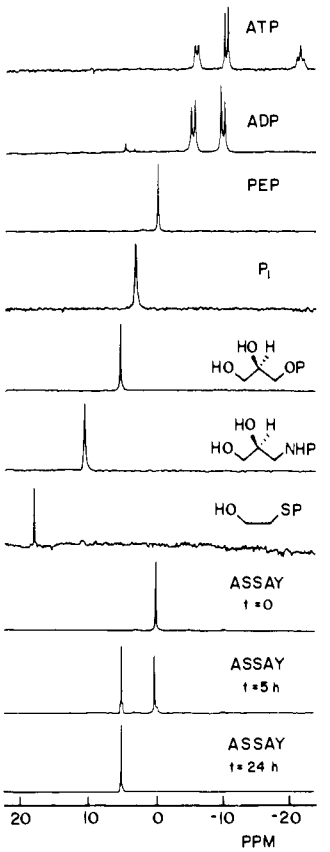


**Figure 1.** Proton-decoupled $^{31}P$ NMR spectra of phosphorus-containing species. All spectra were recorded in deuterium oxide at pH 7.5, except that for the phosphoramidate which was recorded at pH 9.5. The spectra labeled "assay" were obtained using DL-3-chloropropane-1,2-diol as substrate for glycerol kinase with PEP as the phosphoryl donor for the ATP regeneration system. The assay was carried out using triethanolamine as the buffer at pH 7.5.

Glycerol kinase consists of four similar or identical subunits. The enzyme from *E. coli* has a molecular weight of 217 000,[26] and that from *C. mycoderma* has a molecular weight of 251 000.[19] Glycerol kinase has been obtained in crystalline form from both *E. coli*[26] and *C. mycoderma*,[19] but to our knowledge no crystal structure is available. Glycerol kinase contains sulfhydryl groups and is stable only in oxygen-free solution.[12] Glycerol is essential for the stability of glycerol kinase in solution.[12] Immobilization of glycerol kinase in PAN gel[28] markedly increases its stability; immobilized enzyme (from either *E. coli* or *S. cerevisiae*) lost no activity on storage for 6 months at 4 °C, and lost only 30% of its activity on use for 1 month at 25 °C.

High specific activity of enzymes used as catalysts in practical-scale organic synthesis permits the construction of compact reactors; low cost for the enzyme is critical when poor substrates are being transformed and when large quantities of enzyme must be employed. Glycerol kinases from the four commercial sources examined have specific activities of 50–100 U/mg (measured with glycerol as substrate) and cost $50–125 per 1000 U (1 U = 1 μmol of product formed per min; 700 U produces ~1 mol of product per day).

## Results

**Qualitative Studies of Substrate Reactivity.** Previous studies of the substrate specificity of glycerol kinase have relied on en-

(12) Thorner, J. W.; Paulus, H. In "The Enzymes"; Boyer, P. D., Ed.; Academic Press: New York, 1973; Vol. 8, pp 487–508.

(13) In higher organisms the primary role of glycerol kinase is to salvage the glycerol released upon lipolysis. In microorganisms the primary function of the enzyme is to utilize glycerol as a carbon source: Lin, E. C. C. *Annu. Rev. Biochem.* **1977**, *46*, 765–95.

(14) The IUPAC chemical name for the compound is D-propane-1,2,3-triol-1-phosphate. It is known as *sn*-glycerol-3-phosphate (or L-glycerol-3-phosphate) in the biochemical literature. The analogues of *sn*-glycerol-3-phosphate will be referred to by their systematic chemical names in this work. We use DL nomenclature rather than *RS* nomenclature to indicate absolute configuration. The names of the following substrates for glycerol kinase illustrate our choice of nomenclature system: D-propane-1,2-diol ((*R*)-propane-1,2-diol), D-3-fluoropropane-1,2-diol ((*S*)-3-fluoropropane-1,2-diol), D-3-aminopropane-1,2-diol ((*R*)-3-aminopropane-1,2-diol), and D-3-mercaptopropane-1,2-diol ((*S*)-3-mercaptopropane-1,2-diol).

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(16) Rios-Mercadillo, V. M.; Whitesides, G. M. *J. Am. Chem. Soc.* **1979**, *101*, 5828–9.

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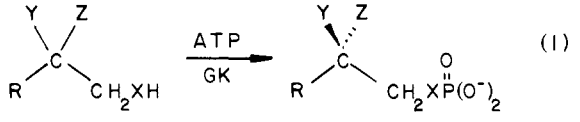
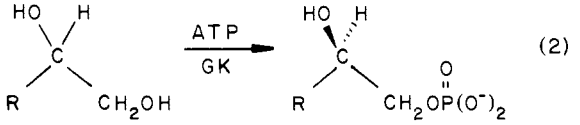
zymatic assays.[19-27] Enzymatic assays are unreliable for poor substrates because these assays may not function reliably in the concentrated solutions of reactants required to achieve measurable rates, and because they do not distinguish a slow reaction due to a poor substrate from a slow reaction due to low concentrations of reactive impurities in a nonsubstrate. In this work we have relied on a semiquantitative procedure based on $^{31}P$ NMR spectroscopy to follow the rates of phosphorylation of substrates and to identify the structures of the phosphorylated products. Phosphorus NMR spectroscopy is particularly well suited to follow enzyme-catalyzed phosphorylation reactions for several reasons. First, enzymatic activity in phosphorylation of substrate is clearly differentiated from ATPase activity. Second, the several types of phosphorus compounds of interest in these studies (nucleoside phosphates, inorganic phosphate, phosphoenolpyruvate (PEP), organic phosphates, amidates, and thiophosphates) have distinct and characteristic chemical shifts.[29] Third, $^{31}P$ NMR assays are applicable to studies in the concentrated solutions of interest in organic synthesis because small concentrations of contaminating reactive substrates are easily identified. These NMR assays can also be carried out at high or low values of pH. Fourth, other types of spectroscopic information (phosphorus–proton coupling constants, changes in chemical shift with changes in pH) can be helpful in assigning structure. The central advantage of the $^{31}P$ NMR assay is, however, that it unambiguously follows the formation of the product and is not misled by competing side reactions.

Figure 1 summarizes $^{31}P$ NMR spectra of a number of compounds important in these assays, together with spectra observed during one representative assay procedure. We emphasize that the results obtained using this assay procedure are qualitative; it is possible to differentiate between substrates that react rapidly, those that react slowly, and those that do not react under the conditions employed. Quantitative comparison of rates depends upon classical kinetic techniques reported in a subsequent section. Table I summarizes the results of these survey experiments carried out by $^{31}P$ NMR spectroscopy. Triethanolamine was used as buffer at pH 7–8; glycine was used at pH 9–10.[30] All potential substrates containing nitrogen were examined both at pH 7.5 and pH 9.5–10 in order to test for reactivity of protonated ($RNH_3^+$) and unprotonated ($RNH_2$) materials. Certain of the substrates listed in this table which show little or no activity were relatively impure (impurity ≥90%). We believe that the low activities are real, but we note explicitly that they might, in principle, reflect inhibition of the enzyme by impurities.

The entries in Table I are classified relative to the structure and reactivity of glycerol (eq 1 and 2). The structural features




(1)




(2)

listed in the table are those that differ from those of glycerol. An entry $r$ = +++ indicates that the reactivity of the indicated substance is >10% that of glycerol; $r$ = ++ indicates that the reactivity is 10% > $r$ > 0.1% that of glycerol; $r$ = + indicates that the reactivity is <0.1% that of glycerol but still detectable. A reactivity of 0 indicates that no detectable reaction occurred over 72 h. The products included under the group $r$ = + were characterized only by $^{31}P$ NMR spectroscopy and were not isolated or otherwise identified. All of the compounds listed in Table I, except D-propane-1,2-diol,[23] D-3-fluoropropane-1,2-diol,[23] and 2-fluropropane-1,2-diol,[23] were examined in this work; certain of them have also been examined previously by others.[19-27] The results of our studies and these previous reports are in good qualitative agreement.

The most important conclusion from Table I is that a number (28) of compounds other than glycerol and dihydroxyacetone are substrates for glycerol kinases. Because the enzyme is inexpensive, even relatively unreactive substrates ($r$ = ++) can be considered for practical-scale (>10 g) preparations using this enzyme. A second observation is that the phosphorylation of substrates containing nucleophilic substituents (nitrogen and sulfur) is not dictated solely by the nucleophilicity of the substituent. DL-3-Aminopropane-1,2-diol is phosphorylated on nitrogen even at pH 7.5, at which pH 99% of the amino groups are protonated.[31] DL-3-Mercaptopropane-1,2-diol is phosphorylated on the hydroxyl group even at pH 10.5, at which pH 90% of the thiol groups are deprotonated.[31] A third observation concerns the greater flexibility in substrate structures that are accepted by the glycerol kinase from *S. cerevisiae* relative to that from *E. coli*. Both enzymes show high substrate activity with glycerol, DL-3-mercaptopropane-1,2-diol, and DL-3-methoxypropane-1,2-diol. The inactivity ($r$ = 0) of glycerol kinase from *E. coli* with compounds having R groups larger than $CH_2OCH_3$ contrasts with the greater tolerance of the glycerol kinase from *S. cerevisiae* for substituents in this position. The *S. cerevisiae* enzyme accepts DL-3-thiomethylpropane-1,2-diol ($r$ = +++), DL-3-ethoxypropane-1,2-diol ($r$ = ++), DL-3-thioethylpropane-1,2-diol ($r$ = ++), DL-butane-1,2-diol ($r$ = ++), and DL-*N*-acetyl-3-aminopropane-1,2-diol ($r$ = +). The glycerol kinase from *E. coli* does, however, selectively phosphorylate DL-butane-1,2,3-triol at C-1, while the enzyme from *S. cerevisiae* appears to be less selective. A fourth observation concerns the greater flexibility in substrate structures at C-2 accepted by the glycerol kinase from *C. mycoderma* relative to that of *E. coli* and *S. cerevisiae*. Substrate activity of DL-2-methylpropane-1,2,3-triol with glycerol kinase from *C. mycoderma* has previously been reported[24] (and confirmed by our $^{31}P$ NMR assay). We observed no substrate activity with glycerol kinase from *E. coli* and *S. cerevisiae*.

Certain of the observations in this table require brief explanation. First, a racemic mixture of propane-1,2-diol is inactive even though the pure D enantiomer is phosphorylated.[23] The inactivity of the racemic material reflects the weak substrate activity of the D enantiomer and the strong inhibitory capability of the L enantiomer.[23] The substrate activity ($r$ = ++) of DL-butane-1,2-diol contrasts with the inactivity of DL-propane-1,2-diol with glycerol kinase from *S. cerevisiae*, and probably reflects the fact that L-butane-1,2-diol does not bind as strongly to the enzyme as does L-propane-1,2-diol. Activities reported for dihydroxyacetone and derivatives require caution in interpretation: dihydroxyacetone exists in aqueous solution primarily as its hydrate;[33] α-hydroxy ketones are also hydrated. The actual substrate for the enzymatic reaction is probably the hydrated form of these compounds,[20] although this mechanistic hypothesis has not been proven experimentally.

A number of other substances not fitting conveniently into the organization of Table I were also established not to act as substrates for glycerol kinase: *cis*- and *trans*-cyclohexane-1,2-diol, *cis*- and *trans*-cyclohexane-1,3-diol, 2,2-bis(hydroxymethyl)-1-

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(31) The p$K_a$ values for protonated DL-3-aminopropane-1,2-diol and for DL-3-mercaptopropane-1,2-diol were determined from their titration curves to be respectively 9.4 and 9.6.

(32) Eisenthal et al.[23] determined the $K_m$ for L-propane-1,2-diol, L-3-fluoropropane-1,2-diol, and 2-fluoropropane-1,3-diol to be respectively 45, 165, and 100 mM. They also determined $K_i$ for D-propane-1,2-diol and D-3-fluoropropane-1,2-diol using glycerol as substrate to be respectively 4.6 and 8.6 mM (using dihydroxyacetone as substrate, the $K_i$ values are 1.2 and 3.6 mM). Their work was carried out using glycerol kinase from *C. mycoderma*. We confirmed with the $^{31}P$ NMR assay that glycerol kinase from *S. cerevisiae* and *E. coli* catalyzed the phosphorylation of glycerol in the presence of DL-propane-1,2-diol (10 mM).

(33) Bell, R. P. *Adv. Phys. Org. Chem.* **1966**, *4*, 1–29.

Table I. Qualitative Substrate Activities of Compounds Having the Structure $XCH_2CYZR$ (Eq 1) for Glycerol Kinase from *S. cerevisiae* and *E. coli*[a]

| X | Y | Z | R | activity | notes | ref |
|---|---|---|---|---|---|---|
| OH | OH | H | $CH_2OH$ | +++ | | 17–24 |
| | | | H | ++ | ATPase[b] | |
| | | | $CH_3$ | + | (D only)[c] | 21 |
| | | | $CH_2F$ | + | (D only)[c] | 21 |
| | | | $CH_2Cl$ | +++ | | 19, 33 |
| | | | $CH_2Br$ | +++ | | |
| | | | $CH_2SH$ | +++ | | 24 |
| | | | $CH_2NH_2$ | +++[d] | | |
| | | | $CH_2OCH_3$ | +++ | | |
| | | | $CH_2OCOCH_3$ | +++(0)[e] | | |
| | | | $CH_2OCH_2CH_3$ | +++(0)[e] | | |
| | | | $CH_2SCH_3$ | +++(0)[e] | | |
| | | | $CH_2SCH_2CH_3$ | ++(0)[e] | | |
| | | | $CH_2NHCH_3$ | 0[d] | | |
| | | | $CH_2N(CH_3)_2$ | 0[d] | | |
| | | | $CH_2NHCOCH_3$ | +(0)[e,f] | | |
| | | | CH=O | ++ | (D ATPase)[g] | 18, 19 |
| | | | $CH(OCH_3)_2$ | 0[h] | | |
| | | | COOH | + | ATPase[b] | 24 |
| | | | $CH_2CH_3$ | ++(0)[e] | ATPase[b] | |
| | | | $CH_2CH_2OH$ | ++ | | |
| | | | $CH(CH_3)OH$ | +(++)[e] | | |
| | | | $CHOHCH_2OH$ | 0 | | |
| | | | $CH_2CN$ | ++ | | |
| | | | $CH_2CH_2CH_3$ | 0 | | |
| | | (=O) | | +++ | | 18, 19, 21 |
| | | (=O) | H | 0 | | |
| | | (=O) | $CH_3$ | + | ATPase[f] | |
| | | (=O) | $CH_2CH_2OH$ | 0 | | |
| | | ($OCH_2$) | H | + | ATPase[f] | |
| | H | | H | 0 | | |
| | H | | $CH_3$ | 0 | | |
| | H | | | ++ | | 18, 19, 21 |
| | H | | $CH_2Cl$ | 0 | | |
| | H | | $CH_2Br$ | 0 | | |
| | H | | $CH_2NH_2$ | 0[d] | | |
| | H | | $CH_2OCH_2CH_3$ | 0 | | |
| | H | | $CH_2CH_3$ | 0 | | |
| | H | | $CH_2CH_2OH$ | 0 | | |
| | H | | $CH(CH_3)OH$ | 0 | D,L all 0 | |
| | H | | $CH_2CH_2CH_3$ | 0 | | |
| | | $CH_3$ | | 0[i] | | 22 |
| | | $CH_2CH_3$ | | 0[i] | | |
| | $CH_2OH$ | $CH_2OH$ | | 0 | | |
| | $CH_2OH$ | $CH_2CH_3$ | | 0 | | |
| | F | | | ++[j] | | 21 |
| | SH | | H | 0 | | |
| | SH | | $CH_2SH$ | 0 | | |
| | $NH_2$ | | | 0[d] | | |
| | $NH_2$ | | H | 0[d] | | |
| | $NH_2$ | | $CH_3$ | 0[d] | D,L all 0 | |
| $NH_2$ | | | | +++[d] | | |
| $NH_2$ | | | H | 0[d] | | |
| $NH_2$ | | | $CH_3$ | 0[d] | D,DL all 0 | |
| $NH_2$ | | | $CH_2NH_2$ | 0[d] | | |
| $NH_2$ | | | $CH_2Cl$ | 0[d] | | |
| $NH_2$ | | | $CH_2OCH_3$ | 0[d] | ATPase[f] | |
| H | | | $CH(CH_3)OH$ | 0 | | |

[a] The structural features listed are those that *differ* from glycerol. Unless indicated otherwise, the activities were indistinguishable using the glycerol kinases from *S. cerevisiae* and *E. coli*. An entry $r$ = +++ indicates that the reactivity of the substance is $r > 10\%$ that of glycerol; $r$ = ++ indicates that the reactivity is $10\% > r > 0.1\%$ that of glycerol; $r$ = + indicates that the reactivity is $r < 0.1\%$ that of glycerol or that the reactivity is accompanied by significant side reactions. See footnotes *c*, *f*, or text for detailed information on specific substrates; $r$ = 0 indicates that no detectable reaction occurred over 72 h. Unless indicated otherwise, the reactions were carried out under these conditions; triethanolamine buffer (0.3 M, pH 7.5, 30 °C), magnesium chloride (12.5 mM), PEP (65 mM), ATP (6.5 mM), and substrate (250 mM). The solution contained $D_2O$ as internal NMR lock. [b] The activity in substrate phosphorylation was accompanied by ATPase activity (<40% of total activity). [c] The unreactive enantiomer (L) is such a potent inhibitor that only the pure D enantiomer is phosphorylated. Racemic mixtures do not show substrate activity. These studies were carried out using glycerol kinase from *C. mycoderma*.[21] The inactivity of racemic propane-1,2-diol was confirmed for glycerol kinase from both *S. cerevisiae* and *E. coli*. [d] The reactions were carried out at pH 7.5 (described in footnote *a*) and pH 9.5 in order to examine the substrate activity of both the protonated and nonprotonated forms. At pH 9.5 the reaction solution contained the following: glycine (0.3 M), magnesium chloride (12.5 mM), PEP (65 mM), ATP (6.5 mM), and substrate (250 mM). [e] The activity of the indicated substance was found to be different with glycerol kinase from *S. cerevisiae* and *E. coli*. The reactivity indicated in parentheses is that with glycerol kinase from *E. coli*. [f] Hydrolysis of ATP was a substantial side reaction (>40% of total activity). The ATPase activity may be induced by the substance or may reflect rapid hydrolysis of reaction products. [g] Only ATPase activity was observed. [h] The activity was determined in the presence of hydrazine (0.4 M) in order to remove residual D-glyceraldehyde. [i] 2-Methylpropane-1,2,3-triol and 2-(hydroxymethyl)butane-1,2-diol (2-ethylpropane-1,2,3-triol) show substrate activity with glycerol kinase from *C. mycoderma*, but not with glycerol kinases from *E. coli* or *S. cerevisiae*. [j] The activity of 2-fluoropropane-1,3-diol was previously reported for glycerol kinase from *C. mycoderma*. The activity of this compound was not examined with glycerol kinase from *E. coli* or *S. cerevisiae*.

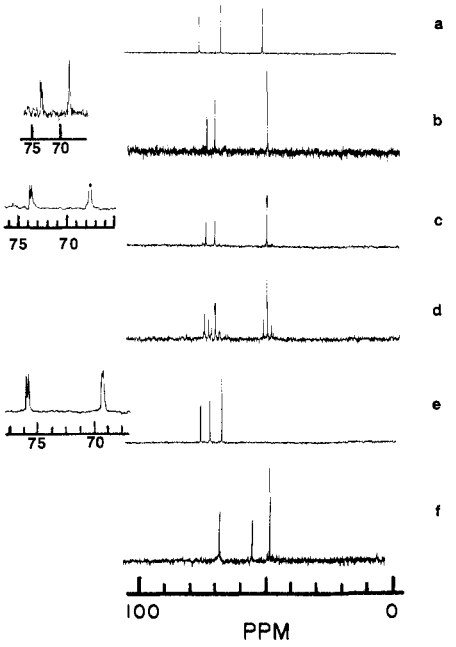



**Figure 2.** Proton-decoupled $^{13}C$ NMR spectra of D-3-chloropropane-1,2-diol-1-phosphate and related species: (a) DL-3-chloropropane-1,2-diol, (b) D-3-chloropropane-1,2-diol-1-phosphate prepared by phosphorylation catalyzed by glycerol kinase, (c) authentic DL-3-chloropropane-1,2-diol-1-phosphate, (d) off-resonance decoupled spectrum of (b), (e) authentic *sn*-glycerol-3-phosphate, (f) authentic DL-glycidol-1-phosphate.

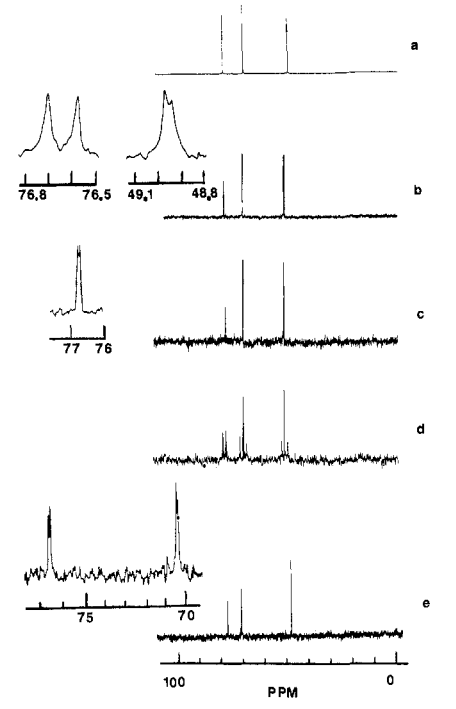



**Figure 3.** Proton-decoupled $^{13}C$ NMR spectra of D-3-aminopropane-1,2-diol-3-phosphate and related species: (a) DL-3-aminopropane-1,2-diol, (b) D-3-aminopropane-1,2-diol-3-phosphate prepared by phosphorylation catalyzed by glycerol kinase, (c) DL-3-aminopropane-1,2-diol-1-phosphate prepared chemically (eq 4), (d) off-resonance decoupled spectrum of (b), (e) L-3-aminopropane-1,2-diol-1-phosphate prepared chemically (eq 5).

butanol (2-ethyl-2-hydroxymethylpropane-1,3-diol), D-ribose, D-arabinose, DL-3-mercaptopropane-1,2-diol disulfide, and DL-*S*-acetyl-3-mercaptopropane-1,2-diol. DL-*S*-Acetyl-3-mercaptopropane-1,2-diol was not recovered after the reaction; its inactivity may reflect acetyl migration from sulfur to oxygen under the reaction conditions.

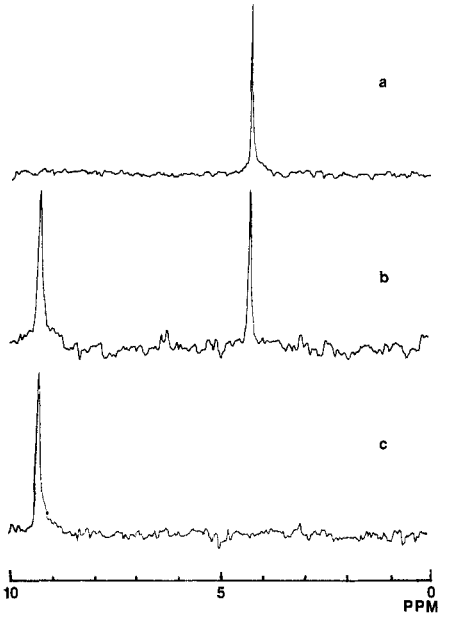
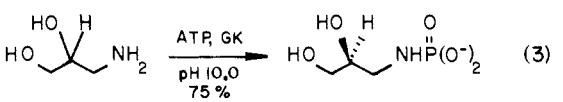



**Figure 4.** Comparison of proton-decoupled $^{31}P$ NMR spectrum of the phosphoramidate and the phosphate ester of DL-3-aminopropane-1,2-diol: (a) D-3-aminopropane-1,2-diol-3-phosphate, (b) D-3-aminopropane-1,2-diol-3-phosphate and D-3-aminopropane-1,2-diol-1-phosphate, (c) D-3-aminopropane-1,2-diol-1-phosphate.

**Identification of the Structures of Phosphorylated Products.** For many of the substrates listed in Table I, it was possible to establish the structures of the phosphorylated products directly by examination of $^{31}P$ NMR spectra. For others, more detailed examination was required. In many cases, even though the structure of a product could be deduced directly from knowledge of the structure of the substrate and from the $^{31}P$ spectra, the phosphorylated product was isolated and examined using other techniques. Carbon-13 NMR spectroscopy proved particularly useful in this context. Specific characterizations follow.

The isolation and identification of the phosphorylated product from DL-3-chloropropane-1,2-diol are of particular interest, since this substance is currently in use as an antifertility agent for rats.[34,35] The activity of this compound as a substrate for glycerol kinase has previously been ascribed to glycerol impurities.[36] Glycerol is present as an impurity in DL-3-chloropropane-1,2-diol,[36] and it is also formed in aqueous solutions under basic conditions,[36] presumably via glycidol as an intermediate. It is therefore difficult to distinguish whether activity detected by enzymatic assay is due to DL-3-chloropropane-1,2-diol or to the product of hydrolysis. Figure 2 summarizes relevant $^{13}C$ NMR spectra. The assignment of the structure 3-chloropropane-1,2-diol-1-phosphate to the phosphorylated product rests on two pieces of information. First, the $^{13}C$ NMR spectrum is indistinguishable from that of authentic DL-3-chloropropane-1,2-diol-1-phosphate.[37] Second, both $^{13}C$ and $^{31}P$ spectra of this material are clearly distinct from those of authentic *sn*-glycerol-3-phosphate (or DL-glycerol-3-phosphate) and DL-glycidol-1-phosphate.

The compound isolated following phosphorylation of DL-3-aminopropane-1,2-diol at pH 10.0 (eq 3) was assigned the unusual

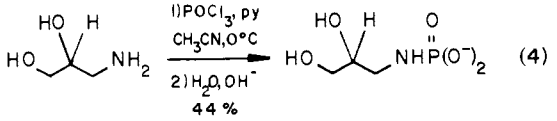



(3)

structure of the corresponding phosphoramidate based on five pieces of evidence. First, the $^{31}P$ and $^{13}C$ spectra (Figures 3 and 4) of this material were indistinguishable from those of authentic

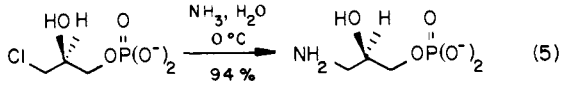
(34) Lobl, T. J. *Clin. Androl.* **1980**, *5*, 109–22.
(35) Jones, A. R. *Aust. J. Biol. Sci.* **1983**, *36*, 333–50.
(36) Brooks, D. E. *J. Reprod. Fertil.* **1979**, *56*, 593–9.
(37) Zetzsche, F.; Aeschlimann, F. *Helv. Chim. Acta* **1926**, *9*, 708–14.

racemic material (eq 4).[38] Second, the observation of a $^2J_{PNC}$



(4)

= 1.6 Hz (±0.3) for the carbon attached to the nitrogen in the $^{13}C$ NMR spectrum is compatible with N-phosphorylation.[39] Third, this substance is stable in basic solution and decomposes rapidly in acidic solution.[40] No decomposition was observed for chemically prepared DL-3-aminopropane-1,2-diol-1-phosphate after 2 weeks at ambient temperature at pH <1. Phosphoramidates are base stable and acid labile.[41] Fourth, the $^{31}P$ and $^{13}C$ NMR spectra of D-3-aminopropane-1,2-diol-1-phosphate prepared chemically (eq 5) are distinct from those of the product from the



(5)

phosphorylation catalyzed by glycerol kinase (Figures 3 and 4). Fifth, the D-3-aminopropane-1,2-diol-1-phosphate prepared chemically does not transfer a phosphoryl group to nitrogen to form D-3-aminopropane-1,2-diol-3-phosphate in aqueous solutions (pH 1–14, ambient temperature). The phosphoramidate isolated from the enzymatic reaction is thus not formed by rearrangement of an initially formed phosphate ester.

Phosphorylation of DL-3-mercaptopropane-1,2-diol was confirmed to be on the C-1 hydroxyl group by $^{13}C$ NMR spectroscopy: in particular, the two-bond P–C coupling constant was that expected ($^2J_{POC}$ = 3.7 Hz).[42] In addition, this substance oxidized to the corresponding disulfide on standing overnight in solution at pH 13 in contact with air. Addition of sodium borohydride to a solution of the disulfide regenerated the thiol quantitatively (as observed by both $^{13}C$ and $^{31}P$ NMR spectroscopy).

The site of phosphorylation of DL-butane-1,2,4-triol was assigned as C-1 on the basis of its $^{13}C$ spectra (Figure 5). The most important observation is that of a three-bond P–C coupling to the carbon atom bearing the secondary hydroxyl group expected for phosphorylation at C-1 ($^3J_{POCC}$ = 9.2 Hz).

The site of phosphorylation of DL-butane-1,2,3-triol was assigned to the C-1 hydroxyl on the basis of $^{31}P$ spectroscopy; a triplet is observed in the proton-coupled $^{31}P$ NMR spectrum. The $^{13}C$ NMR spectra confirmed that the phosphorylation took place at C-1; in particular, the two-bond P–C coupling constant was that expected ($^2J_{POC}$ = 3.7 Hz).

The assignment of structures to other compounds listed in Table I is based on similar, although less complex, $^{31}P$ and $^{13}C$ NMR data. These data are summarized in the Experimental Section (Table IV).

**Assignment of Absolute Configuration.** Assignment of absolute configuration to the products obtained from phosphorylations catalyzed by glycerol kinase was based on activity of these substances as substrates for glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8, *sn*-glycerol-3-phosphate: NAD 2-oxidoreductase). This assay depends upon the stereospecific oxidation of *sn*-glycerol-3-phosphate and structural analogues (D-3-fluoropropane-1,2-diol-1-phosphate) to dihydroxyacetone phosphate (and analogues).[43,44] The lack of substrate activity of the enantiomer

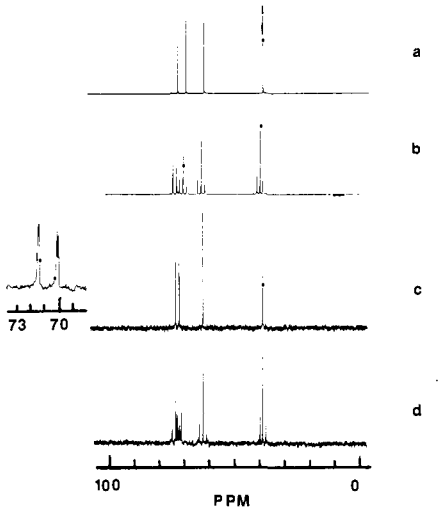


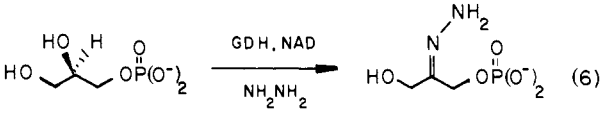

**Figure 5.** Proton-decoupled $^{13}C$ NMR spectra of D-butane-1,2,4-triol-1-phosphate and related species: (a) DL-butane-1,2,4-triol, (b) off-resonance spectrum of (a), (c) D-butane-1,2,4-triol-1-phosphate prepared by phosphorylation catalyzed by glycerol kinase, (d) off-resonance decoupled spectrum of (c).

**Table II.** Estimates of Enantiomeric and Chemical Purities of the Analogues of *sn*-Glycerol-3-phosphate ($^{-2}O_3PXCH_2CYZR$, eq 1) Prepared by Glycerol Kinase Catalyzed Phosphorylation as Determined with Glycerol-3-phosphate Dehydrogenase (GDH, eq 6)[d] and Quantitative $^{31}P$ NMR Spectroscopy[b]

| | | | | assay | |
|---|---|---|---|---|---|
| X | Y | Z | R | GDH(%)[a] | $^{31}P$ NMR(%)[b] |
| OH | OH | H | $CH_2OH$ | >97 | >95 |
| | | | $CH_2Cl$ | >97 | >95 |
| | | | $CH_2Br$ | 95 | >95 |
| | | | $CH_2OCH_3$ | 94[c] | >95[c] |
| | | | $CH_2SH$ | 95[d,e] | >95[e] |
| $NH_2$ | | | | >97 | >95 |
| | | | $CH_2CH_2OH$ | *f* | >95 |

[a] The enantiomeric purity of the organic phosphates was determined by enzymatic assay using *sn*-glycerol-3-phosphate dehydrogenase. The assay conditions are described in the Experimental Section. [b] The chemical purity of the organic phosphates was determined by quantitative $^{31}P$ NMR. Sodium phosphate was used as the internal standard. The recording parameters are described in the Experimental Section. [c] Inorganic phosphate (4%) was observed by quantitative $^{31}P$ NMR. [d] Dithiothreitol (DTT, 1 mM) was added to the assay. [e] Inorganic phosphate (2%) was observed by quantitative $^{31}P$ NMR. [f] D-Butane-1,2,4-triol-1-phosphate was not a substrate for *sn*-glycerol-3-phosphate dehydrogenase.

having the opposite configuration at C-2 is well documented.[43,45] The assay is carried out in the presence of hydrazine in order to drive the equilibrium (eq 6).[46]



(6)

Our results are summarized in Table II. The close agreement between estimates of enantiomeric and chemical purities of the samples indicates high enantioselectivity for the reactions. For example, for 3-chloropropane-1,2-diol-1-phosphate, >97% of the sample was oxidized by NAD/GDH, and is, as a result, assigned the structure D-3-chloropropane-1,2-diol-1-phosphate; >95% of the sample contained, by $^{31}P$ NMR, 3-chloropropane-1,2-diol-1-

(38) Horner, L.; Gehring, R. *Phosphorus Sulfur* **1981**, *11*, 157–76.
(39) Under these conditions no coupling was observed for the carbon attached to the primary hydroxyl group.
(40) The half-lives for DL-3-aminopropane-1,2-diol-3-phosphate at various pH are as follows (pH, $t_{1/2,h}$): 7.0, 5; 8.0, 18; 9.0, 51; 10.0, 336; 11.0, 960; 12.0, >5000.
(41) Fluck, E.; Haubold, W. In "Organic Phosphorus Compounds"; Kosolapoff, G. M., Maier, L. Eds.; Wiley-Interscience: New York, 1973; Vol. 6, Chapter 16, pp 580–831.
(42) McFarlane, W.; *Proc. R. Soc. London, Ser. A* **1968**, *306*, 185–99. Curzon, E. H.; Hawkes, G. E.; Randall, E. W.; Britton, H. G.; Fazakerley, G. V. *J. Chem. Soc., Perkin Trans. 2* **1981**, 494–9. For reference, the coupling constants for *sn*-glycerol-3-phosphate are respectively (Hz): $^2J_{COP}$ = 5.5, $^3J_{CCOP}$ = 7.3 (pH ~1); $^2J_{COP}$ = 3.5, $^3J_{CCOP}$ = 6.2 (pH ~12).
(43) Michal, G.; Lang, G. In "Methods of Enzymatic Analysis", 2nd ed.; Bergmeyer, H. U., Ed.; Verlag Chemie: Weinheim, 1974; Vol. 3, pp 1415–8.
(44) Ghangas, G. C.; Fondy, T. P. *Biochemistry* **1971**, *10*, 3204–10.
(45) Fondy, T. P.; Pero, R. W.; Karker, K. L.; Ghangas, G. S.; Batzold, F. H. *J. Med. Chem.* **1974**, *17*, 697–702.
(46) Fondy, T. P.; Ghangas, G. S.; Reza, M. J. *Biochemistry* **1970**, *9*, 3272–80.

Table III. Apparent Values of $K_m$ and Relative Values of $V_{max}$ for Phosphorylation of Compounds Having the Structure $XCH_2CYHR$ Catalyzed by Glycerol Kinase[a]

| | | | *C. myco.* | | *S. cere.* | | *E. coli* | | *B. stearo.* | |
|---|---|---|---|---|---|---|---|---|---|---|
| X | Y | R | $K_m$ (mM) | $V_{max}$[b] (%) | $K_m$ (mM) | $V_{max}$[b] (%) | $K_m$ (mM) | $V_{max}$[b] (%) | $K_m$ (mM) | $V_{max}$[b] (%) |
| OH | OH | $CH_2OH$ | 0.044 | 100 | 0.041 | 100 | 0.042 | 100 | 0.054 | 100 |
| | | | 0.049 | 107[c] | | | 0.047 | 107[c] | | |
| | | $CH_2Cl$ | 8.0 | 84 | 12.9 | 103 | 5.7 | 72 | 7.2 | 87 |
| | | $CH_2Br$ | 5.5 | 66 | 12.1 | 87 | 8.7 | 74 | 3.4 | 74 |
| | | $CH_2NH_3^+$[d] | 5.0 | 30 | 2.2 | 43 | 2.9 | 65 | 1.4 | 38 |
| | | $CH_2NH_2$[e] | | | | | 1.1 | 30 | | |
| | | $CH_2SH$ | 1.2 | 97 | 1.4 | 110 | 2.3 | 101 | 2.1 | 95 |
| | | $CH_2OCH_3$ | 1.7 | 104 | 4.0 | 95 | 2.9 | 104 | 1.5 | 92 |
| | H | | 86 | 0.5 | 51 | 1 | 21 | 0.5 | 80 | 3 |
| | | $CH_2CH_2OH$ | | | 6.4 | 2 | 7.6 | 0.6 | | |
| | | H | | | | | 38 | 0.04 | | |

[a] Reactions were carried out under these conditions unless indicated otherwise: pH 7.6, 25 °C, [ATP] = 5 mM, [$Mg^{2+}$] = 15.6 mM, [triethanolamine] = 50 mM, [NADH] = 0.16 mM, [$K^+$] = 1.03 mM, pyruvate kinase = 6 U/mL, and lactate dehydrogenase = 12 U/mL. Both the $K_m$ and $V_{max}$ values are in most cases reproducible within 10%. In the case of DL-3-chloropropane-1,2-diol, DL-3-bromopropane-1,2-diol and the weak substrates, the $K_m$ and $V_{max}$ values are reproducible within 20%. [b] Values of $V_{max}$ are relative to $V_{max,glycerol}$ = 100 as determined by reduction of NAD at pH 9.8 and 37 °C catalyzed by *sn*-glycerol-3-phosphate dehydrogenase. Absolute values of $V_{max,glycerol}$ obtained (as described in footnote *a*) were: 0.500 for *C. mycoderma*, 0.203 for *S. cerevisiae*, 0.823 for *E. coli*, and 0.491 for *B. stearothermophilus*. [c] [ATP] = 1 mM. [d] The predominant form of the amine group at pH 7.6 is indicated ($pK_a$ = 9.4). [e] pH 9.5; The triethanolamine buffer (50 mM) was substituted with glycine buffer (50 mM). Assay conditions are otherwise as described in footnote *a*.

phosphate (configuration undetermined). These two numbers agree within experimental error and suggest that the sample contains only one (±~2%) enantiomer of the organic phosphate.

Most of the organic phosphates examined were substrates for glycerol-3-phosphate dehydrogenase and were, as a result, assumed to have the D configuration. In two cases (3-chloropropane-1,2-diol-1-phosphate and 3-aminopropane-1,2-diol-3-phosphate), we established explicitly that the opposite (presumably L) enantiomer was not accepted by glycerol-3-phosphate dehydrogenase by demonstrating that a racemic mixture of L and D enantiomers was oxidized only to the extent of 50% by the enzyme. In one case (D-butane-1,2,4-triol-1-phosphate) the organic phosphate was not a substrate for glycerol-3-phosphate dehydrogenase and enantiomeric pure triols were prepared from D- and L-malic acid. Only D-butane-1,2,4-triol derived from D-malic acid was phosphorylated in the reaction catalyzed by glycerol kinase. We have not explicitly examined the absolute configuration of phosphorylated products listed in Table I but not listed in Table II. We assume, however, that all fall in the same stereochemical series.[47]

**Kinetics.** The mechanism of phosphorylation of glycerol catalyzed by glycerol kinase from rat liver is believed to follow an ordered bi–bi mechanism in which glycerol binds first to the enzyme, followed by ATP·Mg; *sn*-glycerol-3-phosphate leaves last.[12] The pattern of product inhibition observed in glycerol kinase from *E. coli* suggests a similar ordered mechanism.[12] Although straightforward in many details, the phosphorylation of glycerol has one peculiarity: previous examinations have generated biphasic double reciprocal plots, with the implication of different values of $K_m$ for ATP at low and high concentrations of ATP.[12,25] A related effect has recently been observed with glycerol itself with glycerol kinase from adipose tissues.[48] The origin of these kinetic peculiarities has not been established in detail, but they have been suggested to be due to two types of sites that differ in their affinity for ATP.[12] We have chosen conditions such that the ATP concentration used is higher than the highest reported value of $K_m$.[49] Michaelis–Menten parameters which are not limited by cofactor concentrations are more relevant for use in enzyme-catalyzed organic synthesis than those obtained using dilute reagents. Under these circumstances, we observed good linear behavior in Eadie–Hofstee plots (Figure 6). We have compared the kinetics observed for phosphorylation of glycerol and several of its analogues using glycerol kinase from four different microbial sources. We hoped in making this comparison that we might detect differences in kinetic behavior among these enzymes which could be used to advantage in organic synthetic procedures. In fact, the values observed for the different enzymes were very similar (Table III). We therefore conclude that the availability of enzymes from different microbial sources provides no advantage in this particular system, and that the most attractive enzyme for most synthetic applications is simply the least expensive.

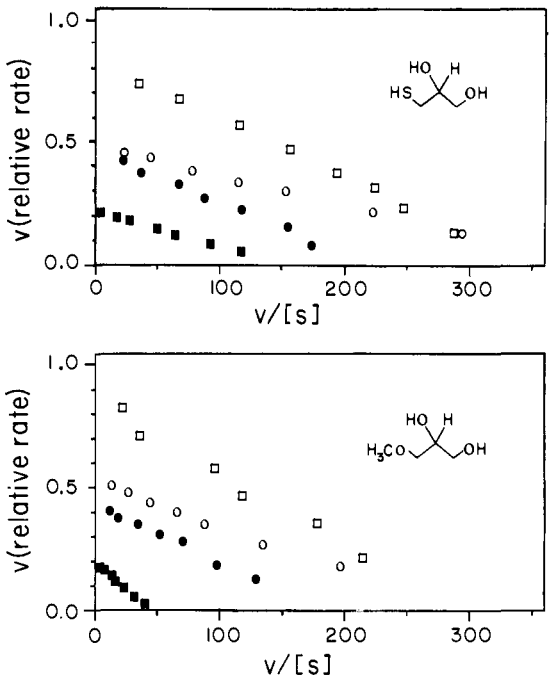



Figure 6. Eadie–Hofstee plots for phosphorylation of DL-3-mercaptopropane-1,2-diol (upper) and DL-3-methoxypropane-1,2-diol (lower) catalyzed by glycerol kinase. The sources of the glycerol kinase used in these experiments were from *C. mycoderma* (●), *S. cerevisiae* (■), *B. stearothermophilus* (●), and *E. coli* (□).

Several features of these kinetic data deserve brief comment. First, Cleland and co-workers have observed for glycerol kinase from *C. mycoderma* that $K_m$ for MgATP changed from 0.009 to 0.02 mM when glycerol was replaced by dihydroxyacetone.[21,50] We determined $K_m$ for ATP using glycerol kinase from *E. coli* and DL-3-chloropropane-1,2-diol as substrate ($K_m$ = 0.5 mM). The similarity between this value and that observed for glycerol ($K_m$

(47) Prelog, U. *Pure Appl. Chem.* **1964**, *9*, 119–30.
(48) Barrera, L. A.; Ho, R.-J. *Biochem. Biophys. Res. Commun.* **1979**, *86*, 145–52.
(49) The highest $K_m$ for ATP is reported for glycerol kinase from *E. coli*. The values are in the range 0.1–4 mM. See ref 12 and 25.
(50) The $K_m$ for ATP for the following substrates are: L-glyceraldehyde, 0.04 mM; D-glyceraldehyde, 0.007 mM; and D-propane-1,2-diol, 0.1 mM.

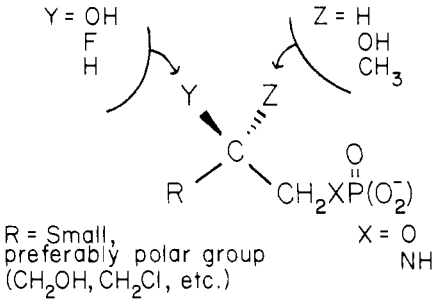



Figure 7. Structural characteristics of substrates for glycerol kinase.

= 0.3 mM) suggests that such effects are not likely to be large enough to influence our data.

The substrates included in Table III cover the range from the most active observed in Table I to those with relatively low activity (DL-butane-1,2,4-triol and ethylene glycol). In going from highly reactive substrates to inactive substrates, the values of $K_m$ increase from 0.04 to 100 mM, while the values of $V_{max}$ decrease by approximately a factor of 1000. The increase in $K_m$ is not a matter of great synthetic concern; the enzyme is still active in highly concentrated solutions of organic substrates, and the rates of conversion are only greatly reduced at concentrations considerably lower than $K_m$. The increase in $K_m$ can, however, become a problem for the few racemic substrates (DL-propane-1,2-diol and DL-3-fluoropropane-1,2-diol), where the enantiomer that is not a substrate inhibits the enzyme. The decrease in $V_{max}$ is of more importance for synthetic uses; this decrease corresponds to a real decrease in the fastest possible rate of reaction and necessitates larger quantities of enzyme to maintain useful reaction rates. It is, thus, ultimately the values of $V_{max}$ which limit the utility of this enzyme as a catalyst for practical-scale synthesis with weak substrates.

## Conclusions

The data summarized in Table I permit the hypothesis of a usefully detailed model summarizing the structural requirements for acceptability as a substrate with glycerol kinase (Figure 7). The position to which phosphate transfer occurs can be either a $CH_2OH$ group or a $CH_2NH_2$ group, since it appears that the enzyme is reluctant to accept groups other than hydrogen on the carbon attached to the phosphorylated nucleophile. No secondary hydroxyl group was phosphorylated. Compounds with multiple sites—if substrates—orient themselves such that the primary hydroxyl group is phosphorylated. The group Y should clearly be a hydroxyl group for highest activity. Substitution of hydroxyl by fluorine or hydrogen leads to poor substrates; substitution by $NH_2$ or $NH_3^+$ eliminates substrate activity. The range of variation accepted in the group Z is dependent on the enzyme source. Hydrogen is obviously acceptable; the observation that dihydroxyacetone phosphate reacts (probably in the hydrated form) suggests that OH is also acceptable for enzyme from all four sources. The activity of DL-2-methylpropane-1,2,3-triol and 2-(hydroxymethyl)butane-1,2-diol (2-ethylpropane-1,2,3-triol) with glycerol kinase from *C. mycoderma* suggests that small alkyl groups are acceptable for this enzyme, whereas even small alkyl groups are too large for glycerol kinase from both *E. coli* and *S. cerevisiae*. Other groups have not been systematically examined. The enzyme will accept a significant amount of variation in the group R, although small, preferably polar groups lead to highest rates. Variations are observed in substrate activities for different enzyme sources; the enzymes from *S. cerevisiae* and *C. mycoderma* accept larger substituents than the enzymes from *E. coli* and *B. stearothermophilus*.[51]

The range of rates summarized in Table I is approximately $10^4$ from reactivity $r$ = +++ to reactivity $r$ = +. Compounds showing reactivity $r$ = +++ provide the basis for enzymatic transformations sufficiently efficient to generate 100-g quantities of product; those classified as $r$ = ++ can be phosphorylated on 10-g scale without major difficulty, and those classified as reactivity $r$ = + are not practical for synthetic uses. Practical illustrations giving experimental details for procedures on the larger scales are summarized in the accompanying paper.[11] The examples in this paper are restricted to small scales (<5-g), but the rates given in Table I correlate with practical synthetic experience. The group classified $r$ = + comprises substrates from three groups. In the first group (e.g., DL-propane-1,2-diol and DL-3-fluoropropane-1,2-diol), no substrate activity is observed for a DL mixture because weak activity of the DL enantiomer was suppressed by inhibition due to the L enantiomer. In the second group (e.g., acetol and DL-glyceric acid), the substrate induces significant ATPase activity (>40% of total activity) in glycerol kinase concurrently with substrate phosphorylation. In the third group (e.g., DL-*N*-acetyl-3-aminopropane-1,2-diol and glycidol), the substrate and its hydrolysis product generate a mixture in which phosphorylation catalyzed by glycerol kinases produces nonspecific mixtures. Practical examples of syntheses are summarized in an accompanying paper.[11] The group classified $r$ = 0 includes the crude preparations of compounds described in the Experimental Section which contain impurities. It is, of course, possible that small quantities of adventitious impurities in the crude preparations tested for substrate activity act as inhibitors. In the case of DL-*N*-methylpropane-1,2-diol, based on the $^1H$ NMR spectrum, we estimate the amount of impurity to be less than 10%; for other compounds levels of impurities (unless otherwise indicated) were lower.

An interesting result that emerges from this work is the observation that highly selective phosphorylation on nitrogen can be achieved in DL-3-aminopropane-1,2-diol. This substrate activity was not duplicated by other structurally analogous amines although DL-3-amino-1-methoxypropane-2-ol induced ATPase activity.[52] The interest in this observation is less in the utility of the reaction itself than in the implication that an enzyme may have useful activity under nonphysiological conditions which is masked under physiological conditions by protonation of nucleophilic centers.

In summary, this study bears on two concerns. First, it establishes that the range of structures which are in substrates for glycerol kinase is wider than has been suggested in previous work. In particular, the substrate activity of DL-3-chloropropane-1,2-diol is relevant to the antifertility activity of this substance.[34,35] This work also suggests that glycerol kinase should be a useful catalyst in synthetic organic chemistry. It is possible in one step to prepare a number of phosphorylated, chiral analogues of glycerol from the racemic mixtures. These materials should find use in synthesis of phosphorylated organic substances (especially analogues of phospholipids[15]).

## Experimental Section

**Materials.** Glycerol kinase from *E. coli* was obtained as lyophilized powder from Sigma. Glycerol kinase from *C. mycoderma* was obtained as a crystalline suspension in ammonium sulfate from Sigma and from Boehringer Mannheim. No differences were observed in the kinetic parameters for the *C. mycoderma* enzyme from these two sources. Glycerol kinase from *B. stearothermophilus* was obtained as a solution in Tris buffer from Boehringer Mannheim. Glycerol kinase from *S. cerevisiae* was obtained from Genzyme. Other enzymes and biochemicals were obtained from Sigma. All enzymes were used as received; absolute purities were not established. Chemicals were reagent grade and used without further purification unless otherwise indicated. DL-3-Chloropropane-1,2-diol and DL-3-bromopropane-1,2-diol were distilled before use for kinetic measurements (distillation was not necessary for the $^{31}P$ NMR assays); aqueous solutions of DL-3-chloropropane-1,2-diol and

(51) DL-3-Thiomethylpropane-1,2-diol showed substrate activity with glycerol kinase from *C. mycoderma*, whereas no activity was observed for glycerol kinase from *B. stearothermophilus*.

(52) The activity of D-6-amino-6-deoxyglucose in phosphorylation catalyzed by hexokinase was examined by a modification of the assay described in the Experimental Section; glycerol kinase was substituted with hexokinase from yeast. After 24 h in a signal at 9 ppm was observed in the $^{31}P$ NMR spectrum. The reactivity was less than 20 times the ATPase activity of hexokinase from yeast (as determined by integration of the $^{31}P$ NMR peak heights of the phosphoramidate and the inorganic phosphate produced in the reaction). No isolations were carried out.

DL-3-bromopropane-1,2-diol were freshly prepared before use. Water was distilled twice, the second time from glass.

**Methods.** Spectrophotometric measurements were performed at 25 °C using a Perkin-Elmer Model 552 spectrophotometer equipped with a constant-temperature cell. Phosphorus NMR spectroscopy was routinely done on a 40.48-MHz (23-T) Varian instrument in 12-mm NMR tubes. Samples contained 20% deuterium oxide as internal NMR lock. Chemical shifts for phosphorus-containing compounds were reported as positive upfield relative to external 85% phosphoric acid; the temperature was 30 °C unless noted otherwise. Accumulation parameters used for assays were: pulse angle, 21°; pulse delay, 1.5 s; accumulation time, 1.02 s. Quantitative $^{31}P$ NMR measurements were carried out using a 90° pulse angle and a 2-min pulse delay, and an internal standard of known concentration (sodium phosphate). Carbon-13 NMR spectroscopy was routinely done on a 67.8-MHz (63 T) Jeol spectrometer in a 5-mm NMR tube. Samples (100–200 mg/mL) were dissolved in deuterium oxide and $^{13}C$ chemical shifts were reported relative to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The spectra were completely proton decoupled and the accumulation parameters used for the spectra were: pulse angle, 90°; pulse delay, 1.45 s; accumulation time, 0.55 s. Mass spectra were recorded on an AEI MS9 mass spectrometer at 50–100 °C.

**$^{31}P$ NMR Assay for Substrate Activity with Glycerol Kinase.** The $^{31}P$ NMR assay carried out in a 12-mm NMR tube could conveniently observe 1 to 10 μmol of phosphorus-containing compound. The buffer for the $^{31}P$ NMR assay at pH 7.5 was prepared as follows. Triethanolamine hydrochloride (5.57 g, 30 mmol), magnesium chloride (0.25 g, 1.25 mmol), PEP trisodium salt (2.34 g, 6.5 mmol), and ATP trisodium salt (0.39 g, 0.65 mmol) were dissolved in water (100 mL) and the pH was adjusted to 7.5 with 2 M NaOH. The $^{31}P$ NMR assay carried out at high pH (when substrates contain amine groups) used a modified buffer in which the triethanolamine hydrochloride was replaced by glycine (2.25 g, 30 mmol) and the pH was adjusted to 9.5.

A representative assay for DL-3-mercaptopropane-1,2-diol followed this course. Triethanolamine buffer (0.3 M, 3.2 mL) containing magnesium chloride (12.5 mM), PEP (65 mM), and ATP (6.5 mM) was added to deuterium oxide (0.7 mL). DL-3-Mercaptopropane-1,2-diol (0.1 mL, 0.8 mmol) was added to the buffer solution and generated a substrate concentration of 0.25 M. The assay was started by addition of pyruvate kinase (1 U) and glycerol kinase (5 U), and was left for 24 h at room temperature. The $^{31}P$ NMR chemical shifts of the assay solution without substrate are: ATP [−5 (d), −10 (d), −19 (t)], PEP [−1 (s)]. After 24 h the following chemical shifts were observed in the $^{31}P$ NMR spectrum of the DL-3-mercaptopropane-1,2-diol assay solution: ADP [−5 (d), −10 (d)], the organic phosphate produced by phosphorylation of DL-3-mercaptopropane-1,2-diol [3.5 (t, $^3J_{POCH}$ = 6.3 Hz)]. For reference, the $^{31}P$ chemical shift of inorganic phosphate was 2.5 ppm.

**Purity.** The enantiomeric purity of the isolated materials was determined by quantitative enzymatic analysis. The chemical purity of the organic phosphates were determined by quantitative $^{31}P$ NMR. The characterizations were carried out as follows.

**Enzymatic Analysis.** The procedure for quantitative determinations of *sn*-glycerol-3-phosphate was based on the glycerol-3-phosphate dehydrogenase catalyzed reduction of NAD. Since most of the *sn*-glycerol-3-phosphate analogues are substrates for glycerol-3-phosphate dehydrogenase, the procedure for assay of *sn*-glycerol-3-phosphate was expanded to include these analogues. The assay was carried out as described previously with minor modifications.[43,45] A representative assay for D-3-aminopropane-1,2-diol-3-phosphate follows. A buffer containing hydrazine and glycine (2.00 mL, 0.04 M hydrazine, 0.5 M glycine, pH 9.8) and NAD (0.1 mL, 31 mM) was placed in a 3-mL cuvette. The cuvette was equilibrated for 2–4 min at 25 °C, glycerol-3-phosphate dehydrogenase (20 μL, 20 U) was added, and the absorbance at 340 nm was recorded ($\epsilon_{NADH}$ = 6220 $M^{-1}$ $cm^{-1}$). When a steady absorbance level (baseline) had been reached, a small volume (0.1 mL) of the sample solution was added to the assay solution and the increase in absorbance was recorded. After 5 to 10 min no further increase in the absorbance was observed. The sample solution contained D-3-aminopropane-1,2-diol-3-phosphate barium salt (32 mg) dissolved in water (25 mL). The assays were run in duplicates (or triplicates if more than 3% variation was observed in the first two determinations). The absorbance at 340 nm was measured against a blank, although this control appeared not to be critical for the determinations. In the blank, water was substituted for the sample solution.

**Quantitative $^{31}P$ NMR.** The phosphorus-containing compounds were assayed quantitatively by integration of their $^{31}P$ NMR signals and comparison of the observed intensities with that of an internal standard of known concentration.[53] The $^{31}P$ NMR spectra were recorded in 20% deuterium oxide (pH 7–9) using a pulse angle of 90° and a pulse delay of 2 min.[54] The data were processed twice and the average was reported. A representative sample solution was prepared as follows: D-3-thiomethylpropane-1,2-diol-1-phosphate barium salt (124 mg) was dissolved in water (3 mL) by addition of excess sodium sulfate (150 mg). The precipitated barium sulfate was removed by centrifugation, and the precipitate was washed with deuterium oxide (0.8 mL). After the precipitate was removed from the deuterium oxide, the supernatant and the deuterium oxide wash were placed in a 12-mm NMR tube and a $^{31}P$ NMR spectrum was recorded. The data were processed twice (without sensitivity enhancement) and the average was reported. An internal standard, inorganic phosphate, was added (in the case of DL-3-thioethylpropane-1,2-diol-1-phosphate, 0.5 mL of 0.4 M monosodium phosphate solution) and another $^{31}P$ NMR spectrum was recorded. The intensities in the $^{31}P$ NMR spectrum were measured and the purity of the organic phosphate was calculated based on the inorganic phosphate used as internal standard. The inorganic phosphate used as internal standard had previously been calibrated against DL-glycerol-3-phosphate.[55] The sensitivity of the method was examined by adding increasing amounts of inorganic phosphate to a sample solution containing known amounts of DL-glycerol-3-phosphate disodium salt. This assay was able to detect inorganic phosphate at a concentration equal to 2 mol % of the major phosphate component (DL-glycerol-3-phosphate). Impurities were therefore detected at the level of 2 mol % but the overall accuracy of the quantitative determinations was not higher than 5%.

**Kinetic Determinations.** Initial rates of enzymatic reactions were all determined by UV spectroscopy at 25 °C. The phosphoryl group transfer from ATP to substrate was coupled to the oxidation of NADH using phosphoenolpyruvate, pyruvate kinase (E.C. 2.7.1.40), and L-lactic dehydrogenase (E.C. 1.1.1.27). The formation of NAD (or the disappearance of NADH) was monitored at 340 nm. Solutions contained triethanolamine hydrochloride (50 mM, pH 7.6), ATP disodium salt (5.0 mM), NADH (0.16 mM), PEP trisodium salt (3.04 mM), magnesium sulfate (15.6 mM), potassium chloride (1.04 mM), pyruvate kinase (20 U), L-lactic dehydrogenase (40 U), and 0.01–1 U of glycerol kinase.[23] The total assay volume was 2.5 mL. The measurements of rates of reaction for glycerol analogues were done at the following substrate concentrations: 0.5, 1, 2, 3, 5, 10, 15, and 20 mM. In two cases (propane-1,3-diol and glycerol), the $K_m$ values were significantly different from those of the other glycerol analogues. The substrate concentrations were varied from 10 to 500 mM for propane-1,3-diol and from 0.01 to 0.2 mM for glycerol. ATP and the magnesium ion were present in excess at all times. Reactions were started by addition of substrate. Control experiments were carried out to ensure that the rate-limiting step was that catalyzed by glycerol kinase. The glycerol analogues had no effect on the rates of reaction of pyruvate kinase and L-lactic dehydrogenase; rates of reaction did not change by the addition of glycerol analogues in assays in which glycerol and glycerol kinase had been substituted with glucose and hexokinase. A standard assay for glycerol kinase activity using 0.4 mM glycerol as substrate was performed before and after each series of experiments. One unit of glycerol kinase activity is defined as the amount of enzyme that is required to catalyze the transformation of 1 μmol of glycerol per min to *sn*-glycerol-3-phosphate at pH 9.8 and 37 °C. The commercially available glycerol kinase from *E. coli* was assayed according to the above procedure.[56] Assay of glycerol kinase from other sources followed this standard. The assay concentrations were as follows: hydrazine (0.88 M), glycine (0.18 M), magnesium chloride (0.25 mM), ATP (1.4 mM), NAD (0.25 mM), glycerol (1.5 mM), and glycerol-3-phosphate dehydrogenase (10 U).

**Glycerol Kinase Catalyzed Phosphorylation of Glycerol Analogues: General.** The syntheses of phosphorylated glycerol analogues were carried out on scales from 0.2 to 1 mmol using in situ ATP regeneration and soluble enzymes. PEP was used as the ultimate phosphoryl group donor. The glycerol kinase used for these enzyme-catalyzed syntheses was from *S. cerevisiae* or from *E. coli*. The syntheses were performed under similar conditions for all the *sn*-glycerol-3-phosphate analogues. Three representative syntheses (D-3-chloropropane-1,2-diol-1-phosphate, D-3-aminopropane-1,2-diol-1-phosphate, and ethylene glycol phosphate) illustrate the variations in the reactions. Table IV summarizes the data for other *sn*-glycerol-3-phosphate analogues.

**D-3-Chloropropane-1,2-diol-1-phosphate.** DL-3-Chloropropane-1,2-diol (reagent grade from Aldrich, 0.10 mL, 1.2 mmol), PEP monopotassium salt (83 mg, 0.40 mmol), and ATP disodium salt (25 mg, 0.040 mmol) were dissolved in a 0.1 M solution of triethanolamine (3 mL) containing magnesium chloride (10 mM) and deuterium oxide (0.7 mL). The pH

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(56) Glycerol kinase, Sigma, Product No. G-4509, Lot No. 101F-6805. Assay and product are described on a product chart.

**Table IV.** Summary of Isolated Yields and Experimental Conditions for Phosphorylation Catalyzed by Glycerol Kinase[a]

| product | | | | scale | RT[b] | GK[c]/PK | special | $^{13}$C NMR data[d] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| X | Y | Z | R | (mmol) | (days) | (U/U) | conditions | $^2J_{PXC}$ | $^3J_{PXCC}$ | yield[e] (%) |
| $^{-2}O_4P$ | OH | H | $CH_2OH$ | 0.22 | 1 | 20/10 | | 5.5 | 7.3 | 95 |
| | | | H | 0.47 | 6 | 150/20 | $P_i$[f] | 3.7 | 7.3 | 60 |
| | | | $CH_2Cl$ | 0.42 | 2 | 20/10 | | 3.7 | 7.3 | 96 |
| | | | $CH_2Br$ | 0.53 | 2 | 20/10 | | 4.4 | 6.7 | 95 |
| | | | $Ch_2SH$ | 0.61 | 2 | 100/20 | $P_i$[f] | 3.7 | 7.3 | 72 |
| | | | $CH_2OCH_3$ | 0.25 | 2 | 25/20 | $P_i$[f] | 5.5 | 7.3 | 73 |
| | | | $CH_2SCH_3$ | 0.35 | 4 | 100/20 | | | | 92 |
| | | | $CH_2OCH_2CH_3$ | 0.23 | 4 | 25/10 | | | | 88 |
| | | | $CH_2CH_3$ | 0.22 | 3 | 150/20 | $P_i$,[f] *S. cere.*[g] | 3.0 | 7.3 | 94 |
| | | | $CH_2CH_2OH$ | 0.48 | 2 | 200/100 | | 5.5 | 9.2 | 96 |
| | | | $CH_2CN$ | 0.10 | 3 | 100/20 | $P_i$[f] | | | 45 |
| | | | $CH(CH_3)OH$ | 0.20 | 3 | 150/100 | *E. coli*[g] | 3.7 | 5.5 | 87 |
| $^{-2}O_3PN$ | | | | 0.29 | 2 | 100/30 | pH 10.0[h] | 1.6 | 7.8 | 87 |

[a] The reaction was carried out as described for DL-3-chloropropane-1,2-diol unless otherwise indicated (special conditions). [b] The indicated reaction times (RT) are upper limits for completion of the reactions. [c] The reactions were carried out with glycerol kinase from *S. cerevisiae* unless otherwise indicated. Similar results are obtained with glycerol kinase from *E. coli*. [d] The product structure indicated was verified by observing the two- and three-bond coupling constants of the carbons $\alpha$ and $\beta$ to phosphorus in the $^{13}$C spectrum. [e] The isolated yields are calculated based on the total phosphoryl group donors added in the reaction. [f] During the reaction inorganic phosphate formed. The inorganic phosphate was removed by precipitation as barium phosphate before the organic phosphate was isolated (as described for ethylene glycol phosphate). [g] Only the indicated glycerol kinase catalyzed the formation of product. [h] The reaction was carried out at pH 10.0.

was adjusted to 7.5 and the reaction was started by addition of glycerol kinase (20 U) and pyruvate kinase (10 U). After 3 days the reaction was complete; PEP and ATP were no longer observed in the $^{31}$P NMR spectrum. The solution was passed through charcoal (0.1 g) to remove ADP and enzymes. Barium chloride (0.24 g, 1.0 mmol) and ethanol (25 mL, 95%) were added to the solution to precipitate the D-3-chloropropane-1,2-diol-1-phosphate barium salt. After the solid was removed by centrifugation and decanting the supernatant, the solid was washed once with ethanol (25 mL, 95%) and dried over $CaSO_4$ at 1 torr overnight. The white solid was 97% pure as determined by enzymatic assay and contained more than 95% organic phosphate as determined by quantitative $^{31}$P NMR (no other phosphate-containing compound was observed). The yield based on combined phosphoryl group donors was 96%: $^1$H NMR ($D_2O$, pH ~1) $\delta$ (DSS) 3.95–4.15 (m, 1 H), 3.60–3.85 (m, 4 H); $^{13}$C NMR ($D_2O$, pH ~1) $\delta$ (DSS) 75.3 (d, $^3J$ = 7.3 Hz), 70.4 (d, $^2J$ = 3.7 Hz), 49.9 (s); $^{31}$P NMR ($D_2O$, pH 9.2) $\delta$ 4.2 (t, $^3J$ = 6.5 Hz).

**D-3-Aminopropane-1,2-diol-3-phosphate.** DL-3-Aminopropane-1,2-diol (0.10 mL, 1.3 mmol), PEP monopotassium salt (80 mg, 0.39 mmol), and ATP disodium salt (25 mg, 0.040 mmol) were dissolved in 0.1 M glycine (4 mL) containing magnesium chloride (10 mM) and deuterium oxide (0.7 mL). The pH was adjusted to 10.3 and the reaction was started by addition of glycerol kinase (100 U) and pyruvate kinase (30 U). The reaction was complete after 2 days; the $^{31}$P NMR spectrum showed no remaining PEP or ATP. The solution was passed through charcoal (0.1 g) to remove ADP and enzymes. Barium chloride (0.12 g, 0.50 mmol) and ethanol (25 mL, 95%) were added to precipitate the organic phosphate. The suspension was stirred at 0 °C for 30 min and the solid was separated by centrifugation. The supernatant was discarded and the solid was washed once with ice-cold ethanol (50 mL, 95%). The suspended precipitate was isolated by centrifugation and the supernatant discarded. The solid was dried over $CaSO_4$ at 1 torr overnight. The resulting barium D-3-aminopropane-1,2-diol-1-phosphate (20 mg, 0.38 mmol) was 98% pure as determined by enzymatic assay and contained more than 95% organic phosphate as determined by quantitative $^{31}$P NMR (no other phosphate-containing compound was observed). The yield based on combined phosphoryl group donors was 87%: $^1$H NMR ($D_2O$, pH 11) $\delta$ (DSS) 3.70–3.80 (m, 1 H), 3.50–3.65 (m, 2 H), 2.75–2.90 (m, 2 H); $^{13}$C NMR ($D_2O$, pH 11) $\delta$ (DSS) 76.8 (d, $^3J$ = 7.3 Hz), 68.1 (s), 49.0 (d, $^2J$ = 1.6 Hz); $^{31}$P NMR ($D_2O$, pH 11) $\delta$ 9.4 (t, $^3J$ = 8.9 Hz).

**Ethylene Glycol Phosphate.** Ethylene glycol (0.25 mL, 0.45 mmol), PEP monopotassium salt (150 mg, 0.70 mmol), and ATP (60 mg, 0.090 mmol) were dissolved in 0.1 M triethanolamine (6 mL) containing magnesium chloride (10 mM) and deuterium oxide (1 mL). The pH was adjusted to 7.8 and soluble glycerol kinase (150 U) and pyruvate kinase (20 U) were added to the solution. After 2 days excess ethylene glycol (0.25 mL, 0.45 mmol) was added to the reaction mixture, and after 4 more days the reaction was complete; $^{31}$P NMR spectrum showed no remaining ATP or PEP. The solution was passed through charcoal (0.1 g) to remove ADP and enzymes. Barium chloride (24 mg, 0.10 mmol) was added to the reaction mixture and most of the inorganic phosphate precipitated as barium phosphate. The precipitate was removed by centrifugation, and then barium chloride (240 mg, 1.0 mmol) and 5 volumes of ice-cold acetone were added to the supernatant. The precipitate was allowed to settle for 30 min at 0 °C and centrifuged. The supernatant was decanted and the solid was washed once with acetone (20 mL). The resulting barium ethylene glycol phosphate (160 mg) was dried over $CaSO_4$ at 1 torr overnight. As determined by quantitative $^{31}$P NMR, the purity was 81% and corresponded to a yield of 60% based on combined phosphoryl group donors. The major impurity, as determined by $^{31}$P NMR, was inorganic phosphate (15%): $^1$H NMR ($D_2O$, pH 7.5) $\delta$ (DSS) 3.75–3.85 (m, 2 H), 2.65–3.75 (m, 2 H); $^{13}$C NMR ($D_2O$, pH 7.5) $\delta$ (DSS) 67.9 ($^2J_{POC}$ = 3.7 Hz), 64.7 ($^3J_{POCC}$ = 7.3 Hz); $^{31}$P NMR ($D_2O$, pH 7.5) $\delta$ 4.2 (t, $^3J_{POCH}$ = 5.8 Hz).

**DL-3-Chloropropane-1,2-diol-1-phosphate** was prepared from epichlorohydrin and phosphoric acid according to the procedure of Zetzsche and Aeschlimann:[37] $^{13}$C NMR ($D_2O$, pH ~1) $\delta$ (DSS) 74.2 (d, $^3J$ = 7.3 Hz), 70.6 (d, $^2J$ = 3.7 Hz), 49.9 (s); $^{31}$P NMR ($D_2O$, pH 9.8) $\delta$ 4.4 (t, $^3J$ = 6.8 Hz).

**DL-3-Aminopropane-1,2-diol-3-phosphate.** Phosphorus oxychloride (1.9 mL, 20 mmol) and DL-3-aminopropane-1,2-diol (1.9 g, 20 mmol) were dissolved in acetonitrile (25 mL) and cooled in a salt water–ice bath. Triethylamine (2.0 g, 20 mmol) was added dropwise to the solution over a period of 30 min. After being stirred for 1 h at 0 °C the solution was slowly added to an aqueous solution of NaOH (25 mL, 2 M) at 0 °C. The reaction mixture was stirred for 30 min at 0 °C and for an additional 2 h at ambient temperature. Barium chloride (2.4 g, 10 mmol) was added to precipitate the inorganic phosphate. The precipitate was removed by filtration. Additional barium chloride (2.4 g, 10 mmol) and ethanol (75 mL, 95%) were added to the filtrate to precipitate the barium DL-3-aminopropane-1,2-diol-3-phosphate. The suspension was allowed to settle for 3 h at 0 °C before the solid was separated by filtration and dried over KOH at 1 torr overnight. The isolated solid (3.2 g) was 85% pure barium DL-3-aminopropane-1,2-diol-3-phosphate (8.8 mmol, 44% reaction yield) as determined by quantitative $^{31}$P NMR, and contained 45% D-3-aminopropane-1,2-diol-3-phosphate as determined by enzymatic assay: $^1$H NMR ($D_2O$, pH ~12) $\delta$ (DSS) 3.7–3.8 (m, 1 H), 3.50–3.65 (m, 2 H), 2.75–2.95 (m, 2 H); $^{13}$C NMR ($D_2O$, pH ~12) $\delta$ (DSS) 76.6 (d, $^3J$ = 7.8 Hz), 68.1 (s), 49.1 (d, $^2J$ = 1.9 Hz); $^{31}$P NMR ($D_2O$, pH ~12) $\delta$ 9.1 (t, $^3J$ = 8.7 Hz).

**D-3-Aminopropane-1,2-diol-1-phosphate.** D-3-Chloropropane-1,2-diol-1-phosphate barium salt (600 mg, 1.8 mmol) was added to a water solution (5 mL), and cooled in an ice bath. Dowex 50W-X8 was added until the organic phosphate dissolved (pH <2). The Dowex 50W-X8 beads and the faint yellow color of the solution were removed by passing the solution through charcoal. The Dowex 50W-X8 resin was washed by passing an additional 20 mL of water through the charcoal. The D-3-chloropropane-1,2-diol-1-phosphate solution was added dropwise to a stirred solution of ammonium hydroxide (25 mL) placed in an ice bath. After the end of this addition the solution was stirred for 30 min at 0 °C, the ice bath was removed, and the reaction solution was stirred for 1 h at ambient temperature. The excess ammonia and water were removed by evaporation at 40–50 °C and the resulting oil was dissolved in 2 mL of water. Barium chloride (0.44 g, 1.8 mmol) and ethanol (20 mL, 95%) were added to precipitate the barium D-3-chloropropane-1,2-diol-1-phosphate. The suspension was stirred for 15 min at 0 °C, and the solid was separated by centrifugation. The supernatant was discarded. The solid was washed with ethanol (10 mL, 95%), and after centrifugation

the solid was dried over $CaSO_4$ at 1 torr overnight. The resulting barium D-3-aminopropane-1,2-diol-1-phosphate (0.50 g, 94%) was more than 95% pure as determined by quantitative $^{31}P$ NMR (no other phosphate-containing compound was observed): $^{13}C$ NMR ($D_2O$, pH 13) $\delta$ (DSS) 76.8 (d, $^3J$ = 5.5 Hz), 70.5 (d, $^2J$ = 3.7 Hz), 47.6 (s); $^{31}P$ NMR ($D_2O$, pH 20) $\delta$ 4.5 (t, $^3J$ = 6.3 Hz).

A similar preparation was carried out for DL-3-chloropropane-1,2-diol-1-phosphate on a 1-mmol scale. The isolated DL-3-aminopropane-1,2-diol-1-phosphate barium salt (0.28 g) was more than 95% pure (no other phosphate-containing compound was observed) as determined by quantitative $^{31}P$ NMR (88% reaction yield).

**DL-*S*-Acetyl-3-mercaptopropane-1,2-diol.** DL-3-Mercaptopropane-1,2-diol (1.1 g, 10 mmol) was dissolved in water (25 mL) and cooled in an ice bath. The solution was stirred for 30 min at 0 °C and acetic anhydride (2.8 mL, 30 mmol) was slowly added to the solution. The mixture was stirred for 2 h at 0 °C before the water, acetic acid, and residual acetic anhydride were removed by evaporation at 1 torr. (The temperature of the solution must not exceed 20 °C during these manipulations since acetyl migration occurs very readily.) The colorless liquid (0.65 g, 43% reaction yield) was used without further purifications: IR (neat) 1690 (S—C=O); $^1H$ NMR (acetone-$d_6$) $\delta$ 3.6–3.7 (m, 1 H), 3.4–3.5 (m, 2 H), 2.85–3.1 (m, 2 H), 2.3 (s, 3 H); $^{13}C$ NMR (acetone-$d_6$) 196.1 (s), 70.4 (s), 64.3 (s), 31.5 (s), 29.9 (s). The $^1H$ NMR and IR data correspond to those of D-*S*-acetyl-3-mercaptopropane-1,2-diol prepared from D-1,2-isopropylidene-3-*S*-acetyl-3-mercaptopropane-1,2-diol.[57] On storage at 4 °C the acetyl group migrates from sulfur to oxygen over the course of 2 months. No thioester bond was observed at the end of this time.

**DL-*N*-Methyl-3-aminopropane-1,2-diol Hydrobromide.** The DL-methyl-3-aminopropane-1,2-diol hydrobromide was prepared by a modification of the procedure of Knorr and Knorr.[58] DL-3-Bromopropane-1,2-diol (0.45 g, 3.2 mmol) was dissolved in ethanol (5 mL, 95%). The ethanol mixture was slowly added to a vigorously stirred solution of aqueous methylamine (40%, 5.0 g) which had been placed in an ice bath. After 15 min of stirring at 0 °C, the solution was heated for 5 h at 40–50 °C. The solvent and excess methylamine were removed by rotary evaporation. Residual volatiles were removed at 1 torr over KOH resulting *in* 0.35 g of colorless oil. The DL-*N*-methyl-3-aminopropane-1,2-diol hydrobromide was used without further purification: $^1H$ NMR ($D_2O$) $\delta$ (DSS) 3.9–4.2 (m, 1 H), 3.6–3.7 (m, 2 H), 3.1–2.3 (m, 2 H), 2.72 (s, 3 H).

**DL-*N*,*N*-Dimethyl-3-aminopropane-1,2-diol hydrobromide** was prepared by a modification of the procedure of Knorr and Knorr.[58] DL-3-Bromopropane-1,2-diol (0.59 g, 3.8 mmol) was dissolved in ethanol (5 mL). The ethanol solution was added to a stirred solution of aqueous dimethylamine (5.0 g). The mixture was heated for 6 h at 40–50 °C. The solvent and excess dimethylamine were removed by rotary evaporation, and residual volatiles were removed at 1 torr over KOH overnight. The crude DL-*N*,*N*-dimethyl-3-aminopropane-1,2-diol hydrobromide (yellow oil, 0.44 g) was used without further purification: $^1H$ NMR ($D_2O$) $\delta$ (DSS) 3.7–31.9 (m, 1 H), 3.3–3.4 (m, 2 H), 2.8–2.9 (m, 2 H), 2.55 (s, 6 H). The spectral data are in accord with those previously published.[59]

**DL-*N*-Acetyl-3-aminopropane-1,2-diol.** DL-3-Aminopropane-1,2-diol (9.1 g, 100 mmol) was dissolved in acetic anhydride (25 mL) and stirred overnight at ambient temperature. The acetic anhydride and acetic acid were removed by rotary evaporation, and the resulting oil was dissolved in methanol. Solid potassium carbonate was added to the methanol solution and the suspension was stirred for 2 days. After the solvent was removed by rotary evaporation, the liquid contained DL-*N*-acetyl-3-aminopropane-1,2-diol, residual sodium acetate, and methanol (total 9.5 g). This mixture containing DL-*N*-acetyl-3-aminopropane-1,2-diol was used without further purification: $^1H$ NMR ($D_2O$) $\delta$ (DSS) 3.63–3.73 (m, 1 H), 3.35–3.5 (m, 2 H), 3.05–3.25 (m, 2 H), 1.9 (s, 3 H); $^{13}C$ NMR ($D_2O$) $\delta$ (DSS) 178.6 (s), 74.6 (s), 67.7 (s), 46.2 (s), 26.3 (s).

**DL-Glyceraldehyde Dimethyl Acetal** was prepared by osmium tetroxide catalyzed oxidation of acrolein dimethyl acetal.[60] *N*-Methylmorpholine *N*-oxide (14.8 g, 107 mmol) and osmium tetroxide (70 mg) were dissolved in a mixture of water (40 mL) and acetone (20 mL). The reaction mixture was placed in an ice bath. Acrolein dimethyl acetal (11.8 mL, 100 mmol) was added to the solution, and the mixture was slowly equilibrated to ambient temperature (4–6 h). The solution was stirred overnight. Sodium hydrosulfite (0.5 g) and magnesium silicate (Woelm, Eschwege, 6 g) were added to the black solution, and the suspension was stirred for 20 min before the solids were removed by filtration. This step was repeated until all osmium esters were removed and the filtrate was red brown. The solvent and *N*-methylmorpholine were removed by evaporation. The residual volatiles were removed overnight over $CaSO_4$ at 1 torr; a colorless liquid (9.6 g) resulted. The liquid contained *N*-methylmorpholine (~25%), but was used without further purification to test substrate activity with glycerol kinase: $^1H$ NMR ($D_2O$) $\delta$ (DSS) 4.3 (d, $J$ = 6.0 Hz), 3.65–3.70 (m, 3 H), 3.61 (s, 3 H), 3.62 (s, 3 H); $^{13}C$ NMR ($D_2O$) $\delta$ (DSS) 109.3 (s), 75.8 (s), 66.4 (s), 60.2 (s), 59.7 (s) (*N*-methylmorpholine; $^{13}C$ NMR ($D_2O$) $\delta$ (DSS) 69.1 (s), 57.9 (s), 48.2 (s)); mass spectrum, $m/z$ 136 (M), 135 (M – 1), 104 (M – $CH_2OH$-($OCH_3$)), 105 (M – 1 – $CH_2OH(OCH_3)$), 75 (M – $CH_2OHCHOH$), 31 ($CH_2OH$). The $^1H$ NMR spectrum is in accord with a partial spectrum published previously.[61]

**DL-3-Amino-1-chloropropan-2-ol Hydrochloride.** DL-3-Amino-1-chloropropan-2-ol was prepared by acidic hydrolysis of 1-benzalimino-3-chloropropan-2-ol according to the procedure of Paul et al.:[62] mp 101–102 °C [lit.[62] 101–102 °C]; $^1H$ NMR ($D_2O$, pH 7) $\delta$ (DSS) 3.9–4.2 (m, 1 H), 3.50–3.65 (m, 2 H)8 3.0–3.2 (m, 2 H); $^{13}C$ NMR ($D_2O$, pH 7) $\delta$ (DSS) 71.7 (s), 50–55 (s), 46.6 (s).

**DL-3,4-Dihydroxybutanonitrile** was prepared from DL-3-chloropropane-1,2-diol by nucleophilic substitution of the chloride with sodium cyanide.[63] DL-3-Chloropropane-1,2-diol (2.2 g, 20 mmol) was dissolved in water (5 mL) and cooled in an ice bath. Sodium cyanide (1.0 g, 21 mmol) was added to the solution and stirred for 3 h at 0 °C. The reaction mixture was passed through an ion-exchange column (AG 501W-X8, 60 g of resin) and washed with ice-cold methanol (100 mL). The combined fractions were evaporated to dryness. The resulting oil (1.4 g) was used without further purification: IR (neat) 2260 (C N); $^1H$ NMR ($D_2O$) $\delta$ (DSS) 3.2–3.6 (m, 3 H), 2.5–2.7 (m, 2 H); $^{13}C$ NMR ($D_2O$) (DSS) 123.9 (s), 71.9 (s), 68.7 (s), 26.4 (s); mass spectrum, $m/z$ 102 (M + 1), 101 (M), 84 (M + 1 – $H_2O$), 70 (72) (M + 1 – $CH_2OH$), 61 (M – $CH_2CN$), 41 (M + 1 – $CHOHCH_2OH$), 31 ($CH_2OH$). The spectral data correspond to those reported by Jung and Shaw.[64]

**DL-3-Amino-1-methoxypropan-2-ol Hydrochloride.** DL-3-Amino-1-methoxypropan-2-ol was prepared in two steps. First, acid-catalyzed methanolysis of epichlorohydrin formed DL-3-chloro-1-methoxypropan-2-ol.[65] Second, nucleophilic displacement of the chloride by ammonia produced DL-3-amino-1-methoxypropan-2-ol. DL-Epichlorohydrin (0.93 g, 10 mmol) was dissolved in dry methanol (10 mL), and cation-exchange resin (Dowex 50W-X8, 100 mg) was added to the mixture. The solution was heated at 50 °C for 15 h. The solution was filtered to remove the cation-exchange resin, and the solvent was removed by evaporation. The resulting oil was dissolved in water (5 mL) and this mixture was added dropwise to an ice-cold stirred aqueous solution of ammonium hydroxide (20 mL, 14 M) which had been placed in an ice bath. The reaction was stirred at 0 °C for 2 h. After evaporation of the solvent and washing of the solid with ether, 0.6 g (as a colorless oil) of crude DL-3-amino-1-methoxypropan-2-ol hydrochloride resulted. The compound was used without further purification: $^1H$ NMR ($D_2O$, pH 7) $\delta$ (DSS) 4.0–4.2 (m, 1 H), 3.5–3.6 (m, 2 H), 3.4 (s, 3 H), 3.1–3.3 (m, 2 H); $^{13}C$ NMR ($D_2O$, pH 7) $\delta$ (DSS) 78.3 (s), 70.5 (s), 63.3 (s), 46.5 (s); mass spectrum, $m/z$ 106 (M + 1), 105 (M), 88 (M + 1 – $H_2O$), 87 (M – $H_2O$).

**2-Methylpropane-1,2,3-triol** was prepared according to the procedure of Eisenthal et al.:[24] $^1H$ NMR ($D_2O$) $\delta$ (DSS) 3.3 (s, 4 H), 0.7 (s, 3 H).

**2-(Hydroxymethyl)butane-1,2-diol (2-Ethylpropane-1,2,3-triol)** was prepared according to the procedure of Eisenthal et al.:[24] $^1H$ NMR ($D_2O$) $\delta$ (DSS) 3.4 (s, 4 H), 1.3 (q, 2 H), 1.1 (t, 3 H); mass spectrum, $m/z$ 117 (M – 3), 103 (M – OH), 91 (M – $CH_2CH_3$), 89 (M – $CH_2OH$), 43 (M – OH – $CH_2OH$ – $CH_2CH_3$), 31 ($CH_2OH$).

**Supplementary Material Available:** Tables of $V_{max}$ and $K_m$ for a number of substrates for glycerol kinase for *C. mycoderma* and *B. stearothermophilus* (2 pages). Ordering information is given on any current masthead page.

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