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ENZYMATIC SYNTHESIS OF PHOSPHORIC MONOESTERS WITH ALKALINE PHOSPHATASE IN REVERSE HYDROLYSIS CONDITIONS

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Abstract :

Title compounds were synthesized on a preparative scale using alkaline phosphatase, orthophosphoric monoester phosphohydrolase E.C. 3.1.3.1, in reverse hydrolysis conditions. Optimization for one of the 25 phosphoryl acceptors investigated (glycerol) shows that up to 55% synthesis yield can be obtained using a large excess of substrate, conditions in which the enzymatic activity remains high. From the results obtained with different phosphoryl group donors, phosphate, pyrophosphate and polyphosphates and with enzymes of different sources, it comes up that the best results are obtained with pyrophosphate and with the weakly purified calf intestine alkaline phosphatase. The extent of enzymatic hydrolysis of the donor can be reduced owing to the existence of two different pH optima for the two reactions, phosphorylation and hydrolysis. The synthesis can be also performed using inert co-solvents which allow to reduce the amount of acceptor used, as long as Zn^{++} is added to the reaction medium. The results are discussed in terms of the catalytic mechanism of alkaline phosphatase.

INTRODUCTION

Among the different ways that organic chemists investigate to synthesize new compounds, enzymes occupy a key position owing to the regio and stereospecificity that they generally allow [1,2,3]. Our interest in chiral phosphoric esters both as biologically active molecules [4] and also for mechanistic purposes [5] has led us to consider enzymes to be used for synthetic routes, particularly kinases which are the phosphorylating enzymes [6]. However, kinases are generally specific for their substrates and also require the expensive ATP as coenzyme. Even with the improvements achieved with ATP recycling [7], the use of such enzymes suffers strong limitations [8, 9]. A way for an alternative may be offered by enzymes using inorganic pyrophosphate in place of ATP ; pyruvate kinase from some strains of bacteria [10] operates in such conditions. Pyrophosphate can indeed be considered as a high energy molecule with a $\Delta'G_o$ = -5.27 Kcal per mole compared to the value of -7.60 for ATP in the same conditions [11]. To the best of our knowledge, such an alternative has not yet been considered.

Bearing this in mind we investigated the possibilities opened by the use of hydrolytic enzymes because they generally are of low specificity towards acceptor molecules in reverse hydrolysis reactions. In particular, the readily available enzymes of the phosphatase group catalyze the hydrolysis of phosphoric monoesters [12] according to the following equation :

$$RO\text{-}P(O)(O^-)\text{-}O^- + H_2O \underset{(b)}{\overset{(a)}{\rightleftharpoons}} ROH + HO\text{-}P(O)(O^-)\text{-}O^-$$

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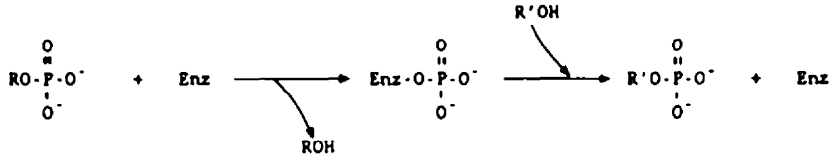
In aqueous dilute standard conditions, the equilibrium is shifted towards hydrolysis with a ΔG_o ~ -5 kcal mole^{-1} [13]; but if the equilibrium can be notably shifted to the left, a synthetic method for phosphoric esters becomes available. Such an attempt was made as early as 1928 [14], but further developments [15,16,17] have led to limited yields of phosphoric esters. The best value of 22% was obtained for glycerolphosphate with an immobilized phosphatase in a biphasic system [18]. Another route was to develop a transphosphorylation reaction, the phosphoryl group of a phosphoester being first transferred to the phosphatase, then in a second step to an acceptor, according to :



Different types of donors such as phosphoric esters [19, 20], phosphocreatine [19], phosphoenolpyruvate [19], phosphoroamidates [21], S-substituted monoesters of phosphorothioic acids [22], were used, the acceptors being alcohols [21], aminoalcohols [21, 23], Tris and polyols such as glycerol [20, 21] and also sugars [19]. Even though this type of reaction has led to a better understanding of the mechanism of the enzymatic reaction, the preparative interest remains low, since the synthesis of an ester with a yield of 30% in the best cases requires the consumption of a larger amount of an other ester.

We therefore considered the reaction again in the phosphorylation direction (b), with the idea that a more favourable equilibrium displacement might be obtained by using pyrophosphate instead of phosphate. We were encouraged in this approach by the fact that a pyrophosphotransferase activity has been demonstrated with alkaline phosphatase [24-27].

On this basis, we investigated the synthetic possibilities of this enzymatic reaction, with sodium pyrophosphate as a donor, and different acceptors such as alcohols, polyols, amino-alcohols used in large excess to shift the equilibrium on the ester side. In each case the reaction progress was followed by ^{31}P NMR spectroscopy and the products were isolated in the most significant cases. The first part of the work was devoted to setting down, for a reference reaction the phosphorylation of glycerol, the conditions leading to the best yields in phosphoric ester and to the lowest enzymatic hydrolysis of the inorganic pyrophosphate.

RESULTS

I - OPTIMIZATION OF GLYCEROL PHOSPHORYLATION

a) Standard assay. ^{31}P NMR spectra of a standard assay performed with sodium pyrophosphate as donor, glycerol as acceptor (55% v/v, in water) and calf intestine alkaline phosphatase, are given in Figure 1. From high field to low field are successively observed the peak of the decreasing pyrophosphate, then the phosphate formed and glycerophosphate. Each spectrum was recorded every 45 minutes. As the transfer of a phosphoryl group from the pyrophosphate to the acceptor liberates a phosphate group, 100% yield of phosphorylation corresponds to two equivalent peaks of phosphate (δ = 2.5 ppm) and glycerophosphate (δ = 4.2 ppm both at pH : 7.9). Actually, the peak for phosphate is higher than the peak for the glycerophosphate owing to a competitive enzymatic hydrolysis of the pyrophosphate to phosphate. Phosphorylation yields are calculated on this basis. For reference assays, particularly those concerning the determination of stereoselectivity (see below), the glycerolphosphate ester has been isolated (see experimental section). Concerning the glycerophosphate peaks, the major one corresponds to the α isomer as indicated by the triplet (the spectrum is recorded with coupling for protons) corresponding to the coupling of the phosphorus atom with the CH_2 group, the minor to the β isomer (Figure 2). As indicated below, the ratio of the two isomers depends upon the reaction conditions but selective formation of the α isomer (≥90%) is obtained in most cases.

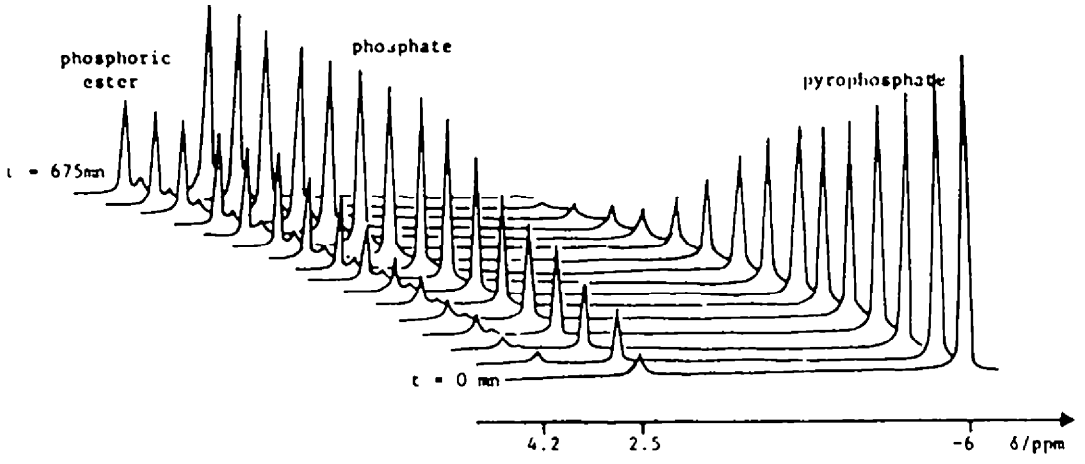


Figure 1 : NMR ^{31}P spectra (32.44 MHz) of glycerol phosphorylation kinetics in water (55%, v/v) by calf intestinal alkaline phosphatase (100 U/ml), pH : 7.9 ; sodium pyrophosphate 150 mM ; temperature : 37°C ; the NMR spectra are recorded every 45 mn.

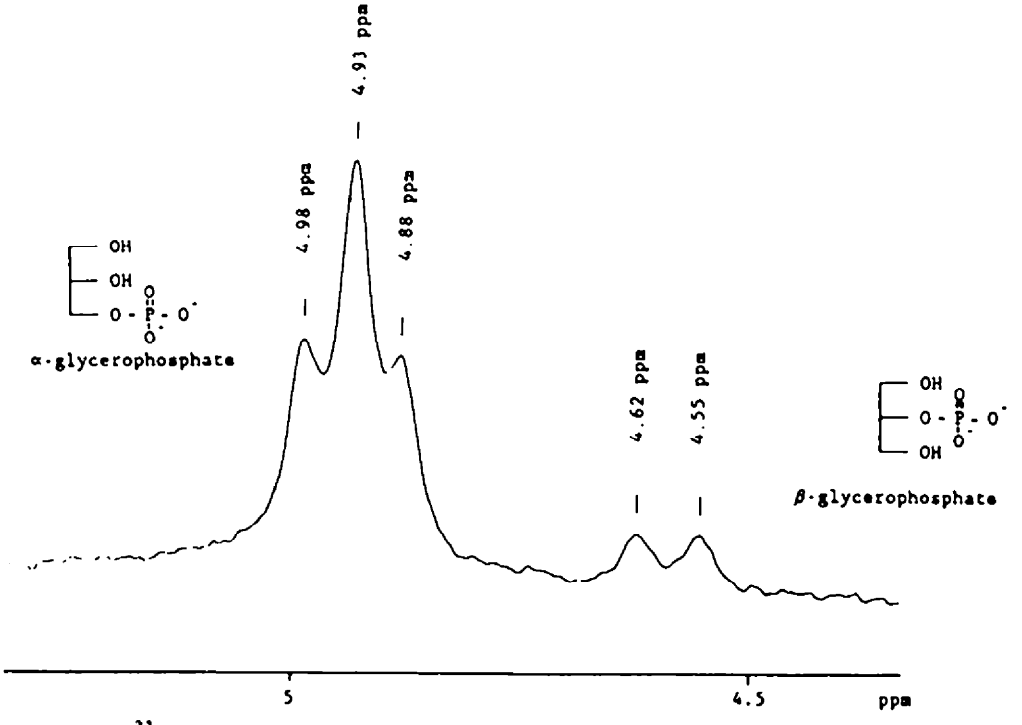


Figure 2 : NMR ^{31}P spectrum (121.49 MHz) of the mixture of the two isomers α and β-glycerophosphate obtained in the last run in figure 1 (t = 675 mn). The spectrum was recorded coupled for proton. The coupling constant for the triplet (δ = 4.9 ppm) corresponding to the α isomer is 6.5 Hz. The doublet for the β isomer gives δ = 4.6 ppm and $^3J_{H.P}$ = 8.2 Hz.

b - Influence of pH. One of the possibilities for increasing the yield in ester and decreasing the hydrolysis of the pyrophosphate, was a possible difference in pH sensitivity for the two activities, since it is known for hydrolytic enzymes that pH optima for hydrolysis and synthesis reactions are different [28] Figure 3 shows the results obtained in this respect : the two maxima for activity are distinct : pH : 5.8 for hydrolysis reaction of the pyrophosphate and pH : 8.5 for phosphorylation reaction. It comes out also that ester yield is appreciable since 55% is obtained : this value is significantly higher compared to that previously obtained [18].

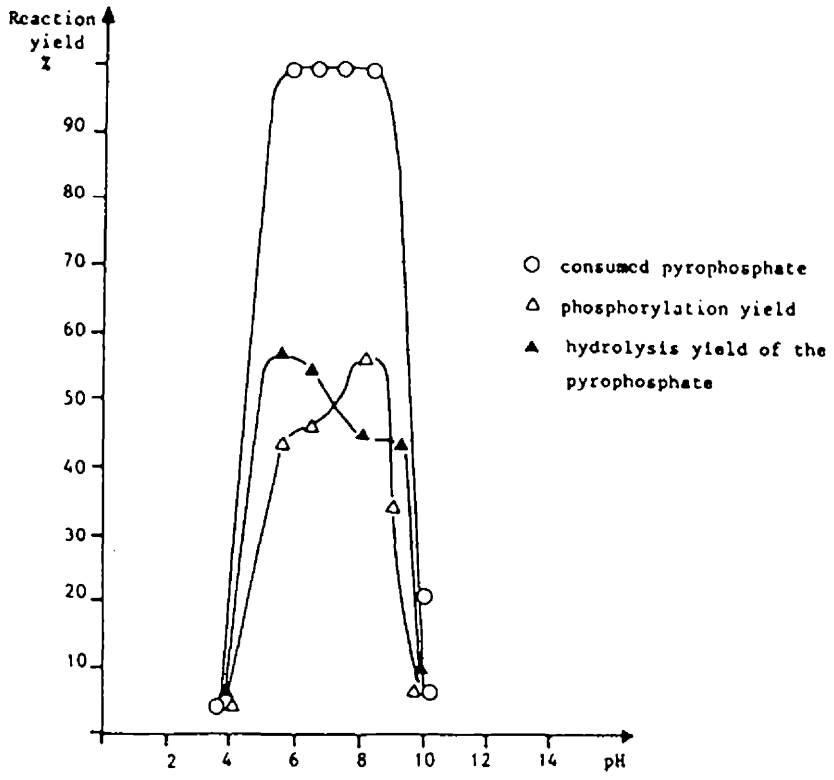


Figure 3 : Influence of the pH on glycerol phosphorylation, by alkaline phosphatase, using sodium pyrophosphate as donor, 48h incubation time. Experimental conditions were the same as Fig. 1.

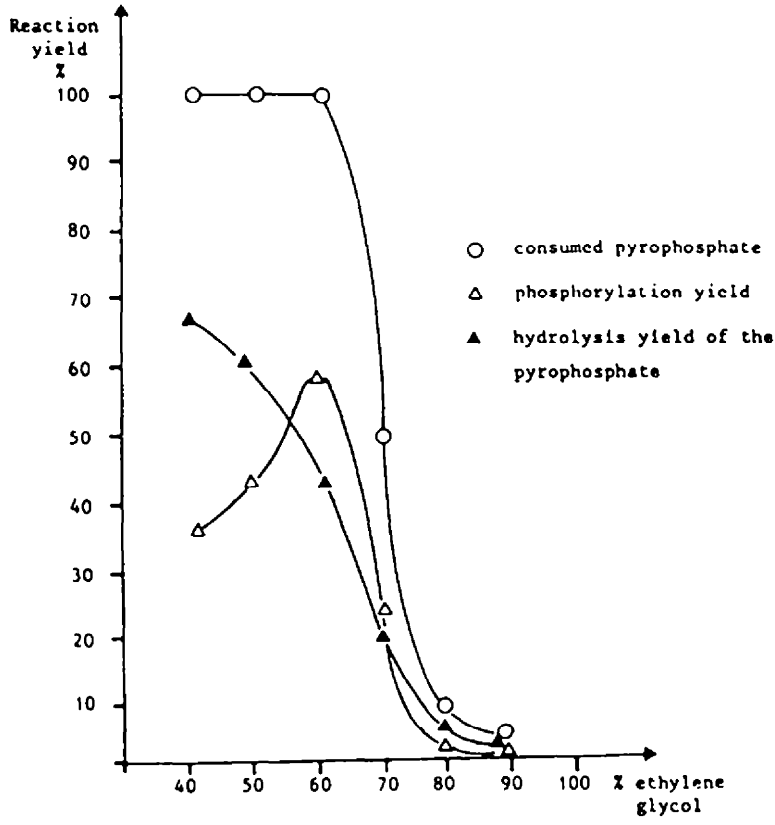


Figure 4 : Phosphorylation of ethyleneglycol using pyrophosphate as donor by alkaline phosphatase (calf intestine, 100 U/ml). Influence of the concentration of the acceptor (ethyleneglycol) on the yield (%, y axis) of phosphorylation, hydrolysis and pyrophosphate consumption, pH : 7.9 ; temperature : 37 °C, 48h incubation time.

c - Influence of the acceptor concentration. A second approach to shift the equilibrium more towards phosphoric ester synthesis, was to use a large excess of acceptor, with possibly a subsequent effect on decreasing the extent of hydrolysis ;but this effect of using an excess of substrate must remain compatible with a significant activity of the enzyme. Figure 4 indicates the results corresponding to the phosphorylation of ethylene glycol. It is first noticeable that the enzymatic activity remains high, up to 70% of ethylene glycol in terms of consumed pyrophosphate (a blank shows that the donor does not hydrolyze in these conditions). Moreover whereas the hydrolytic activity is decreasing, the phosphorylation goes through a maximum (at 60% v/v). At this maximum, phosphorylation yield is higher than the hydrolysis yield. For other acceptors described in part II, this 60% concentration of the acceptor was used in all experiments.

d - Influence of the chain length of the phosphoryl donor. We tested various chain lengths for polyphosphates to determine if they were recognized as substrates and what would be the effect of the chain on the yield. Table I indicates the results obtained with different polyphosphates, the values being compared with those for pyrophosphate (first row). In all cases, it comes out first that phosphorylation yields are higher than hydrolysis yields and second that polyphosphates with more than five phosphate groups are poor substrates for both hydrolysis and phosphorylation reactions. Also by comparing results obtained after 42 and 91 hours respectively, for pyrophosphate (assays 1 and 8) the increase in ester yield (33%) is higher than what would be expected from the remaining pyrophosphate after 48 h (9%). This indicated that the phosphate formed in the reaction with pyrophosphate (from both hydrolysis and phosphorylation reactions) is in turn transferred to the acceptor at a slower rate. This point was checked as indicated below.

TABLE I

Influence of the chain length of the phosphoryl donor

assay nr.	Donor	consumed polyphosphate %	hydrolysis yield %	phosphorylation yield %
1	n = 0	90.6	48.4	42.2
2	n = 1	51.0	16.0	35.0
3	n = 3	38.8	9.8	29.0
4	n = 13	10.5	4.0	6.5
5	n = 23	6.8	3.4	3.4
6	n = 43	4.8	1.1	3.7
7	n = 73	2.8	0.6	2.2
8	n = 0	100.0	24.8	75.2
9	n = 1	88.0	24.0	64.0
10	n = 3	63.7	20.4	43.3
11	n = 13	16.0	6.0	10.0
12	n = 23	6.6	1.9	4.7
13	n = 43	6.4	1.9	4.5
14	n = 73	2.6	0.4	2.2

Calf intestine alkaline phosphatase 100 U/ml, pH : 7.9. Glycerol concentration : 60% v/v; temperature : 37 °C ; concentration polyphosphate 20 g/l. n : number of internal phosphoryl groups for polyphosphates.

Experiments 1 to 7 : 48h incubation time, experiments 8 to 14 : 91h incubation time

e - Comparison of glycerol phosphorylation rates with pyrophosphate and phosphate as donors. The reaction conditions are as follows : pH : 7.9 (diethanolamine 0.4 M as buffer), the concentration in glycerol being kept at 60% v/v. Results shown in figure 5 indicate that not only the yield of phosphorylation but also the rates are higher with pyrophosphate than with phosphate (by a factor of 3.5 for initial velocities). Besides the fact that pyrophosphate is a better group from a preparative aspect, it appears that pyrophosphate is a better substrate (initial velocities) than the normal phosphate substrate for the phosphatase. Moreover these plots allow a direct comparison in the phosphoric ester synthe-

sis of the relative efficiencies of phosphate and pyrophosphate. These two donors behave differently and phosphoryl transfer with pyrophosphate proceeds directly and not with preliminary hydrolysis to phosphate followed by transfer. We also noticed a difference in the shape of the two plots (curvature and initial rate), which may be due to the inhibition effect by the phosphate [29].

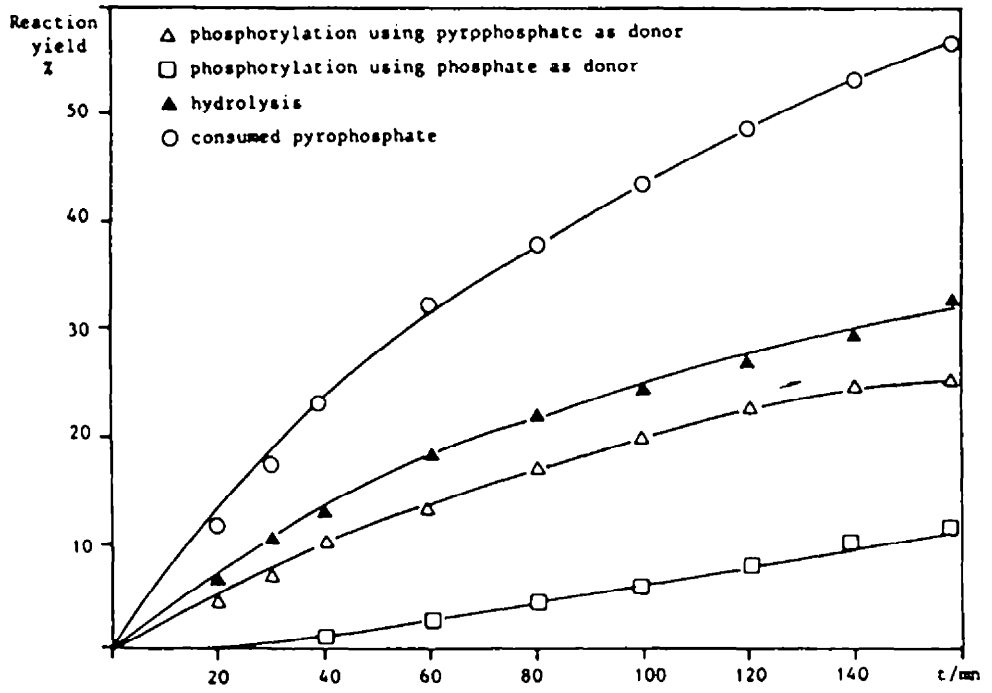


Figure 5 : Comparison between the rate of glycerol phosphorylation with pyrophosphate and phosphate by calf intestinal phosphatase (100 U/ml) in glycerol/water (60% v/v) ; pH :7.9 ; sodium phosphate and sodium pyrophosphate : 150 mM ; temperature : 37°C. The NMR spectra are recordad every 20 mn.

f - Effect of the origin and purity of the phosphatase. Three phosphatase preparations from *E. Coli*, chicken and calf intestine, were successively tested in the same conditions with glycerol as substrate (60%, v/v in water) and pyrophosphate as a donor. Results expressed in standardized activities were plotted as indicated in figure 6 and summarized in table II. Initial velocities, yields of phosphorylation and hydrolysis determined after the same time (675 mn) are successively indicated. It comes out that both in terms of initial velocities and yield of reaction, the calf intestine enzyme is the most efficient and therefore should be used for preparative purposes. Also we tested the effect of the purity of the enzyme using two samples of 12 U/mg lyophilisate and 80 U/mg protein respectively. Results are given in table II. They indicate that the purity of the enzyme exerts a weak effect, the best results being however obtained with the less purified enzyme, both in terms of initial velocities and yields of phosphorylation. These results are in agreement with the general observation that the stability of enzymes decreases when increasing their purity. This effect might be important in our conditions, as the denaturating effect of large concentrations of substrate is to be considered. From a preparative point of view, it is noteworthy that the best results are obtained with the crude enzyme.

g - To summarize, optimum conditions for synthesis are as follows : i-competitive enzymatic hydrolysis of the donor pyrophosphate can be limited by making profit of the difference in the two pH optima for synthesis and hydrolysis (see b) ; ii-large concentrations of acceptor can be used since the enzymatic activity remains high up to 60 % (v/v) concentration, at least with glycerol and ethyleneglycol (see c) ; iii-pyrophosphate and short chain polyphosphates behave as good donors, particularly the former which gives faster rate than phosphate (see d and e) ; iiii-among the different sources and purities of enzymes investigated, crude calf intestine alkaline phosphatase gives the best results (see f).

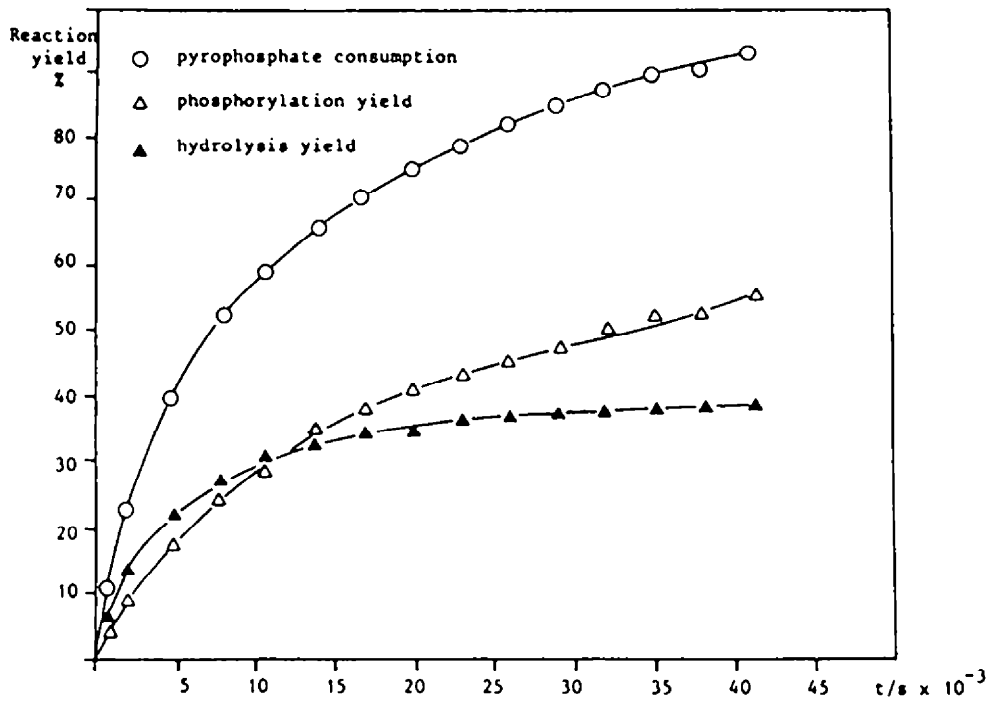


Figure 6 : Example of influence of the enzyme origine on the glycerol phosphorylation using pyrophosphate as donor in glycerol/water (60% v/v) ; pH : 7.9 ; sodium pyrophosphate 150 mM ;temperature : 37 °C ; enzyme : weakly purified calf intestine alcaline phosphatase (10 U/mg lyophilisate) ; pH : 7.9 ; sodium pyrophosphate : 150 mM ; temperature : 37°C ; the NMR spectra are recorded every 45 mn.

TABLE II

Influence of the origin of the enzyme

Enzyme	i.v.(pp.)	i.v.(hyd)	i.v.(tr)	R_1(pp)	R_2(hyd)	R_3(tr)	R_3/R_2
E_1	1.35	0.78	0.56	92.8	39.4	53.4	1.35
E_2	0.49	0.34	0.15	57.1	28.4	28.7	0.99
E_3	0.20	0.13	0.06	6.9	3.4	3.5	0.97
E_4	1.13	0.65	0.47	83.3	34.3	49.0	1.42

Enzymes were assayed with paranitrophenylphosphate and used at 117 U/ml

E_1 : calf intestine alkaline phosphatase (12 U/mg lyophilisate)
E_2 : *E. Coli* alkaline phosphatase (217 U/mg protein)
E_3 : chicken intestine alkaline phosphatase (15 U/mg protein)
E_4 : calf intestine alkaline phosphatase (80 U/mg protein)
i.v.(pp.) : initial velocity of pyrophosphate consumption : U/ml
i.v.(hyd) : initial velocity of pyrophosphate hydrolysis : U/ml
i.v.(tr) : initial velocity of phosphorylation : U/ml
R_1 : yield of reaction for pyrophosphate consumption : %
R_2 : yield of reaction for pyrophosphate hydrolysis : %
R_3 : yield of reaction for phosphoryl transfer : %

R_1, R_2, R_3 were determined after 44h incubation time.

II - PHOSPHORIC MONOESTERS SYNTHESIS IN THE OPTIMIZED CONDITIONS

a) Preparative assays. Table III gives the results which were obtained with different types of substrates including alcohols, sugars and polyols. The yields are expressed in terms of consumed pyrophosphate, yields of phosphorylation and hydrolysis of the pyrophosphate. Concentrations used were those settled for glycerol.

TABLE III

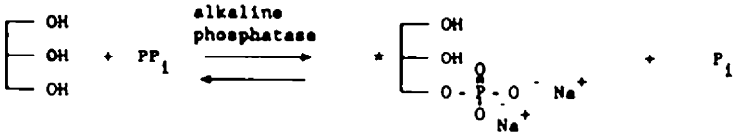
Phosphorylation by calf intestine alkaline phosphatase of different substrates.

Acceptor	experiment nr.	Consumed phosphate %	Yield phosphorylation %	Yield hydrolysis %
Sugars				
glucose	1	82.0	15.2	66.8
ribose	2	66.0	9.4	56.6
Alcohols				
CH_3-CHOH-C≡CH	3	83.5	12.1	71.4
CH_2=CH-CH_2OH	4	53.5	9.8	43.7
Diols and polyols				
$HOCH_2$-CHOH-CH_2OH	5	100.0	51.4	48.6
$HOCH_2$-CH_2OH	6	56.4	19.5	36.9
$HOCH_2$-CHOH-CH_3	7	61.2	15.6	45.6
$HOCH_2$-CH_2-CH_2OH	8	62.2	20.2	42.0
$HOCH_2$-CH_2-O(CH_2-CH_2)$_2$-OCH_2-CH_2OH	9	66.9	11.4	55.5
$HOCH_2$-CH_2-OCH_2-CH_2OH	10	53.2	16.6	36.6
CH_3-CHOH-CHOH-CH_3	11	71.0	12.2	58.8
HO(CH_2-CH_2-O)$_2$-CH_2-CH_2OH	12	60.5	11.4	49.1
$HOCH_2$-CHOH-CH_2-CH_2OH	13	100.0	52.1	47.9
$HOCH_2$-CH_2-CH_2-CH_2OH	14	58.0	18.7	39.3
$HOCH_2$-CH_2-CH_2-CH_2-CH_2OH	15	64.5	29.5	35.2
$HOCH_2$-CH=CH-CH_2OH	16	78.8	26.0	52.8
$HOCH_2$-CH_2-S-CH_2-CH_2-OH	17	86.0	31.3	54.7
$HOCH_2$-CHOH-CHOH-CHOH-CHOH-CH_2OH	18	91.3	21.1	70.2
$HOCH_2CH_2NH_2$	19	2.4	0	2.4
HO(CH_2)$_3$$NH_2$	20	0	0	0
HO-CH_2CH_2-N⟨◇⟩O	21	35.8	3	32.8
HO(CH_2)$_2$NH-(CH_2)$_2$$NH_2$	22	1.8	0	1.8
HO-CH_2CH_2SH	23	6.9	0	6.9
SH-CH_2CH_2SH	24	14.8	0	14.8
SH(CH_2)$_3$SH	25	100	0	100

Enzyme : 100 U/ml ; pH : 7.9 ; temperature : 37 °C ; sodium pyrophosphate : 150 mM ; 48h incubation time.

- First, concerning alcohols, a large set have been tested, including propanol, isopropanol, butanol-1, butanol-2 and cyclohexanol ; in each case, there is no phosphorylation and only quantitative hydrolysis of the pyrophosphate ; phosphorylation only occurs if another functionality is present (ethylenic or acetylenic group) as in experiments 3 and 4. The yield of phosphorylation is in the range of 10%.
- On the other hand, diols and polyols are good substrates and only monophosphorylation occurs. This was indicated from the single peak obtained for the phosphoric ester formed particularly in representative cases (experiments nr. 7, 13 and 18) . The yields of phosphorylation (based on consumed pyrophosphate) ranges from 10 to 50%, the best results being obtained with triols and terminal diol structures (experiments 5,13 and 6,8,15,16,17 respectively).
- With substrates bearing an heteroatom such as nitrogen or sulfur, different situations occur : with aminoalcohols (used in large excess), phosphorylation is not observed. Also the enzymatic activity is reduced, since the hydrolysis reaction of the pyrophosphate proceeds to a small extent. With substrates bearing a sulfur atom, the enzymatic activity is maintained, but phosphorylation is not observed.

b - Test for a stereoselectivity of the phosphorylation. As the phosphorylation of compounds such as glycerol leads to a product bearing a chiral center, we tested possible enantiomeric excess formation in the corresponding reaction, first by using optical rotation measurements.



But owing to the weak specific optical rotation of α-glycerol phosphate ($[\alpha]_D^{20}$ ~ 1.5°), the recorded activity, either in the solution after elimination of the enzyme or on the isolated product, is questionable, since possibly also due to impurities of the enzyme. We therefore proceeded to an enzymatic assay using L-α glycerophosphate dehydrogenase, specific for this enantiomer [30]. This assay was associated with a colorimetric titration of the total phosphate liberated by the hydrolysis after treatement with phosphatase of the same α-glycerophosphate sample, this hydrolysis being claimed as non specific [16]. We also checked this point by proceeding to the enzymatic hydrolysis of the L-α-glycerophosphate and the racemic mixture, we found the same value (1.4 ± 0.1 micromole of ester hydrolyzed per minute and mg of enzyme, see experimental section). From these determinations comes the conclusion that the enzymatic phosphorylation is leading to a racemic mixture. The reaction is therefore only regioselective and not stereoselective.

c - Change in regioselectivity induced by use of different cosolvents. In order to shift the equilibrium towards synthesis, experiments were performed using inert water miscible cosolvent such as glymes where enzymes keep their activity [31] and which can even be used for enzymatic synthyesis [32]. Glyme/water mixtures containing up to 50% of the cosolvent can be realized in which sugars and polyols (such as glycerol) are soluble, whereas the water activity is significaltly decreased [33]. Experiments were performed with keeping the amount of water constant (40 % v/v) ; the amount of glycerol acceptor was progressively reduced by replacing it by the inert cosolvent. Such binary mixtures water/glyme might also be of great interest for substrates for which high concentrations in water cannot be obtained. Table IV gives the corresponding results obtained with glycerol the concentration of which varies from 60 to 10 % v/v, the complement to 60% being introduced by monoglyme or triglyme. Results are compared to the reference reaction (60% glycerol, experiment nr. 1, table IV) where 49 % phosphorylation and 38 % hydrolysis are observed. The results indicate that the enzymatic activity is reduced : for instance with 10% of added cosolvent only 46.5 % of pyrophosphate is transformed versus 87% without this cosolvent, everything else being equal. Moreover the phosphoryl transfer reaction is more affected than hydrolysis since this second reaction becomes predominant ; and the same is observed for larger amounts of either glyme. A striking difference in regioselectivity was observed with these cosolvents : as indicated on table IV, the largely predominant α-isomer in the absence of glyme is reduced when glyme is added ; in such mixtures, by increasing the amount of glyme, the β-isomer becomes even predominant (see experiment nr. 15). But it is interesting to note -and commented later on - that both normal phosphoryl transfer reaction and selectivity are restored by adding 10^{-4} M of zinc chloride to the mixture (for pyrophosphate 150 mM and enzyme 100 U/ml, see experiments nr. 5,6,12 and 13 in table IV).

TABLE IV

Effect of co-solvent addition on enzymatic activity

experiment nr.	glycerol	glyme %	consumed pyrophosphate %	hydrolysis %	phosphorylation %	selectivity (a) α/β
Cosolvent triglyme						
1	60	0	87.0	49.0	38.0	95/5
2	50	10	46.5	26.5	20.0	77/23
3	40	20	46.9	38.4	18.5	74/26
4	30	30	45.4	29.2	16.2	68/32
5 10^{-4} M Zn^{++}	30	30	89.2	52.4	37.8	72/28
6 5.10^{-4}M Zn^{++}	30	30	93.0	55.8	37.2	72/28
7	20	40	42.2	27.0	15.2	61/39
8	10	50	33.1	22.3	10.8	52/48
Cosolvent monoglyme						
9	50	10	45.9	26.8	19.1	79/21
10	40	20	44.3	27.9	16.4	75/25
11	30	30	42.4	28.8	13.6	69/31
12 10^{-4} M Zn^{++}	30	30	88.4	56.6	31.8	69/31
13 5.10^{-4}M Zn^{++}	30	30	95.0	56.5	38.5	74/26
14	20	40	43.6	31.0	12.6	56/44
15	10	50	40.8	31.0	9.8	40/60

Enzyme : 100 U/ml ; sodium pyrophosphate 150 mM ; pH : 7.9 ; water is kept constant 40% v/v ; temperature : 37 °C ; 44h incubation time. in run 5, 6, 12, 13 : $ZnCl_2$ added.
(a) α/β is the concentrations ratio of the two phosphoglycerol isomers given in figure 2.

DISCUSSION

First, comments concerning the preparative interest of these reactions are the following : high regioselectivity and appreciable yields in phosphoric esters of polyols are obtained in simple conditions, with a hydrolytic enzyme. Using a large excess of acceptor, pyrophosphate as donor and a little purified enzyme allows the synthesis of up to 55% yield of ester of glycerol. The optimized conditions, settled for this substrate have then been extended to other acceptors. The reaction with phosphatase largely competes for preparative purposes with other enzymes particularly glycerol kinase which proves to be too specific [8,9] and also competes with chemical phosphorylation which leads for polyols to a mixture of products [34,35]. The success of such an enzymatic synthesis relies first upon the difference in the two pH optima corresponding to the phosphoryl transfer and hydrolysis reactions, and also on the fact that the enzymatic activity remains high even when using large excess of substrate, required to shift the equilibrium toward synthesis of phosphoric ester. Concerning then the results obtained with different types of structures, we first note that with ribose and glucose, yields of phosphorylation are weak owing to the low concentration of substrate allowed for solubility reasons. For the following substrates (assays 3 to 18 in table III), we note that phosphorylation occurs only when an other functionality than the hydroxyl group being phosphorylated is present, for instance with an unsaturated group (assays 3 and 4), whereas phosphorylation does not occur for simple alcohols. Concerning polyols we note that terminal diol structures (assays 4,6,8,10,13,15,16, table III) give better results than internal diols (assays 7 and 11). Although it has been shown that for hydrolysis steric effects are weak on the k_{cat}/K_M parameter [36], transfer yield seem to be more dependent on such effects. We also note that longer diol structures such as in runs 9 and 12 even with terminal diols give again poor results. It revealed from these experiments that for phosphorylation to occur, two requirements have to be met : high concentration of acceptor, and presence on this acceptor of two electron-rich centers at a proper distance.

However, these features seem to be restricted to polyols since amino alcohols, hydroxythiols and dithiols are not phosphorylated (assays 19-25 table III). In fact with aminoalcohols, these results are the consequence of a too excessive increase in pH which cannot be balanced by the buffer ; for such a high pH value, the enzymatic activity is too low. We checked that at lower concentrations in aminoalcohol where the enzymatic activity is restored, phosphorylation occurs as previously indicated by other authors [13,14,20] (table III). However there is no longer preparative interest at such a weak concentration. Hydroxythiol and dithiols do not lead to a phosphorylated product, even when enzymatic activity remains high (assay 25).

Concerning the phosphoryl group donor, results indicate that valuable yields are obtained with pyrophosphate instead of more expensive phosphoric esters ; this supplements the synthetic interest of this enzymatic phosphorylation. Also comparative experiments between pyrophosphate and phosphate (Fig. 5) indicate that the former is the actual phosphorylating agent and not the phosphate resulting from a preliminary hydrolysis of the pyrophosphate. This point had not yet been observed in the synthesis direction. However the phosphate resulting from both hydrolysis and transfer reaction with the pyrophosphate reacts then, but at a slower rate. The use of polyphosphates might be also of some preparative interest. But our results indicate that only the first terms up to five phosphoryl groups have some interest, since longer chains make them poor substrates.

These results and also those concerning the selectivity and the use of cosolvents have to be put together of with the available data concerning the active site of alkaline phosphatase. In the hydrolytic direction it has been shown that at low phosphoric ester concentration and pH : 8, the rate limiting step is the formation of a phosphoenzyme at serine 102, whereas in saturating conditions, the reaction of the water molecule on the intermediate phosphoserine becomes rate determining [36,37]. Bearing this in mind, we can consider that, in the conditions used (large excess of substrate), the relative yields of transfer versus hydrolysis will depend on the relative reactivities of the acceptor and the water molecules. It is indeed known that the water molecule firmly binds to the zinc cation present in the active site [38] rendering it a better nucleophile, the pK being shifted from 15 to 7.2 [38,39]. To compete with the water molecule, the acceptor first has to be recognized by the enzyme and be bound to the cation. Results obtained using glymes (reduced enzymatic activity restored by adding zinc) show that the acceptor actually binds to the zinc cation, since it can be displaced by the glyme which is a good ligand for such cations [40]. Therefore the best transfer reactions will be obtained for acceptors able to bind as bidentate, the result being an increase in their nucleophilicity towards phosphoserine. As for the change in selectivity induced by glymes, this result may be interpreted by a modification in conformation of phosphatase as this has been observed for other enzymes [41].

To summarize, this study exemplifies the possibilities extended by the use of enzymes of the phosphatase type, for the synthesis of phosphoric esters. Work is now in progress to add other improvements and develop at a larger scale such enzymatic synthesis.

Experimental section

Chemicals : Tetrasodium pyrophosphate, magnesium chloride and chemicals used as buffers were purchased from Merck, 4-nitrophenyl phosphate from Sigma, and the different polyphosphates from Benckiser-Knapsack GMBH.

Enzymes : alkaline phosphatase EC 3.1.3.1, calf intestine specific activity 10 U/mg lyophilisate was purchased from Sigma, calf intestine phosphatase with a specific activity of 80 U/mg protein from Boehringer ; alkaline phosphatase *E. Coli* from Sigma (217 U/mg protein) and chicken intestine alkaline phosphatase from Sigma (15 U/mg).

Assays : The enzymes were assayed by the hydrolysis of 4-nitrophenyl phosphate at pH 9.8 (tris buffer) and 37 °C ; the U is defined as the number of micromoles of 4-nitrophenyl phosphate hydrolysed per minute and per mg of lyophilized enzyme or per mg of proteine.

^{31}P NMR spectroscopy : The ^{31}P NMR spectra were recorded on Brucker AC 80 operating at 32.44 MHz and on Brucker AM 300 WB operating at 121.497 MHz, both equipped with Fourier Transform ; the reference was phosphoric acid 85% and lock on deuterium was used.

The progress of the enzymatic reactions was followed by sampling every Δt the reaction mixture ; the sample was frozen to -18 °C to stop the reaction. After rewarming up, 500 microliters of each sample were added to 200 microliters of D_2O and the resulting solution analyzed.

Determination of phosphorylation yields : phosphorylation yields were determined by integration in ^{31}P NMR of the signals corresponding to the pyrophosphate, the phosphate and the phosphoric ester formed I_1 . I_2 and I_3 respectively (see fig. 1) (we checked that intensities were proportional to concentrations). In a transfer reaction for an equivalent of phosphoric ester, an equivalent of phosphate is also formed. Therefore a yield of 100% corresponds to two signals I_2 and I_3 of equal intensities. As phosphorylation but also partial hydrolysis of the donor pyrophosphate is also occuring, I_2 is greater than I_3. The pyrophosphate consumed for phosphorylation is I_3, the part transformed by hydrolysis being $I_2 - I_3 / 2$. Therefore the total consumed pyrophosphate is defined as CP, the yield of phosphorylation as RP and the yield of hydrolysis as RH :

$$CP = \frac{I_2 - I_3}{2} + I_3 \quad ; \quad RP = \frac{I_3}{CP} \quad ; \quad RH = \frac{I_2 - I_3}{2 \times CP}$$

Quantitative assay of phosphate by colorimetry :

Was performed using three solutions A,B,C

A : sodium acetate, (4.5g), cupper sulfate (0.25g) in 100 ml of acetic acid 2N.

B : Ammonium molybdate 5% in water

C : Methyl amino-4 phenol sulfate 2% and sodium sulfite 5% in water.

For the assay, to 0.5 ml of solution (0 to 1 mM in phosphate) with 1 ml of water, were added 2.5 ml of solution A, 0.5 ml of solution B and 0.5 ml of solution C successively. The optical density at 700 nm was read after 5 minutes of incubation. Concentrations were determined from a reference plot.

Enzymatic reactions :

a) To determine the influence of the pH (Fig. 3), the reference solution was prepared as follows : buffer ethanolamine 0.4 M (pH ajusted by adding HCl) magnesium chloride 2 mM, sodium pyrophosphate 150 mM, amount of enzyme : 10 mg of lyophilized powder corresponding to 100 U ;glycerol 55% v/v with water ; temperature : 37 °C ± 0.5 the total volume being 5 ml. The progress of the reaction was followed for 48h.

b) The same conditions were used to investigate the influence of the concentration of ethyleneglycol in water varying from 40 to 90% v/v (Fig. 4).

c) For experiments with polyphosphates, conditions described in a) were used, with pH : 7.9, except that sodium pyrophosphate was replaced in each case by 0.1 g of polyphosphate for 5 ml of reaction solution ; progress was followed for 91 hours (Table I).

d) For comparison between pyrophosphate and phosphate as donor, conditions were as in c) the concentration in phosphate or pyrophosphate being 150 mM. Progress was followed for 160 minutes with sampling for ^{31}P NMR analysis every 20 minutes (Fig. 5).

e) For reactions with acceptors other than glycerol and ethyleneglycol, conditions were the following : pH : 7.9, donor sodium pyrophosphate 150 mM, enzyme 100 U/ml, temperature : 37 °C, total volume 5 ml (Table III).

For liquid acceptors, we used a 60% v/v concentration. For solids, they were dissolved at saturation in the buffer and triglyme (triethylene glycol dimethyl ether) and monoglyme (dimethoxy-1,2 ethane) 60% v/v were successively used as co-solvent. Products analysis and their quantitative determination were made after 48h reaction at 37°C.

Influence of the origin of the enzymes : Each enzyme was assayed as described above. The amount of enzyme used was standardized to 350 U/3 ml of reaction medium ; pH : 7.9 (diethanolamine buffer), 2 mM in magnesium chloride, 150 mM in pyrophosphate and concentration in glycerol 60% v/v ; temperature : 37°, reaction rates were followed for 675 mn, with NMR sampling every 45 minutes.

Enzymatic assay of L-α-glycerophosphate by glycerol-3 phosphate dehydrogenase : The following reaction was used for the assay :

$$\text{glycerol-3P} + NAD^+ + \text{hydrazine} \xrightarrow{GDH} \text{glycerol-3P-hydrazone} + NADH + H_2O + H^+$$

NADH concentration was determined by spectrophotometry at 340 nm.

The corresponding solutions were prepared : for 50 ml solution in water, 2.6 g of hydrazine sulfate 3.75 g of glycin 0.1 g of $EDTAH_2Na_2$, and 25.5 ml of sodium hydroxide 2M.

The solution of NAD^+ was 40mg in 1 ml water (60 mM) ; GDH (Boehringer) was 1700 U/ml.For each assay, were mixed 1 ml of buffer solution (pH : 9.5), 0.1 ml of NAD^+, 0.05 ml of solution to be analysed, 0.95 ml of water and 0.005 ml of GDH solution (total volume 2.105 ml). The optical density at 340 nm was read after 5 minutes at 20°C.

Hydrolysis of glycerophosphate by alkaline phosphatase : was performed using the calf intestine phosphatase (10 U/mg lyophilisate). The reaction solution was 1 ml of 10.25 mM in L α glycerophosphate 0.2 ml of phosphatase solution (10 U) and 0.5 ml of diethanolamine buffer 50 mM, pH : 9.8, temperature : 37 °C ; incubation time : 22 hours.

Comparison of initial velocities of L and D,L-α-glycerophosphate hydrolysis by alkaline phosphatase : was performed using the same enzyme as above and same buffer and pH. Concentrations of L-α-glycerophosphate and D.L-α-glycerophosphate were 20 mM, the concentration in enzyme 0.5 mg/ml. The reaction progress was followed by colorimetric titration of the formed phosphate.

Preparation and Purification of glycerophosphate : In a 100 ml flask containing 30 ml of glycerol, 20 ml of water and 1.66 g of sodium pyrophosphate (150 mM), pH adjusted at 7.9 adding HCl, 0.5 g of the lyophylised enzyme was added and temperature fixed at 40 °C. After 48 hours of incubation, the reaction mixture (55 % yield for glycerophosphate) was first ultrafiltrated, then diluted twice with water. A stoechiometric amount of magnesium chloride was then added and the pH increased at 10 adding sodium hydroxide ; the phosphate quantitatively precipitated. The phosphoric ester was then separated from the excess of glycerol by ion exchange chromatography (IRA 35 Amberlit, elution of the ester with sodium hydroxide 0.4 N).The pH of the solution was then adjusted to 6.5 with sulfuric acid, and the formed sodium sulfate was precipitated by adding three volumes of ethanol. The remaining solution was then lyophilized. The yield after purification for glycerophosphate was 45 % (0.35 g)(yield for purification 81 %), the purity was checked as for other esters by thin layer chromatography (polygram Cel 400 ; elution with mixture isopropanol 140 ml, water 60 ml, trichloroacetic acid 10g and ammonia 0.4 ml ; detection with spray of a solution containing ammonium molybdate 1g, hydrochloric acid 1N : 10 ml, perchloric acid 5 ml, water 25 ml ; after spray,the plate was heated 80°C for ten minutes. The purity was also checked by ^1H and ^{31}P NMR spectroscopy and spectra were identical to those of authentic samples.

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