# Regioselective Phosphorylation of Carbohydrates and Various Alcohols by Bacterial Acid Phosphatases; Probing the Substrate Specificity of the Enzyme from *Shigella flexneri*

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**Abstract:** Bacterial non-specific acid phosphatases normally catalyze the dephosphorylation of a variety of substrates. As shown previously the enzymes from *Shigella flexneri* and *Salmonella enterica* are also able to catalyze the phosphorylation of inosine to inosine monophosphate and D-glucose to D-glucose 6-phosphate (D-G6P) using cheap pyrophosphate as the phosphate donor. After optimization high yields (95%) are achieved in the latter reaction and we show here that it is possible to use these enzymes in a preparative manner. This prompted us to investigate by using $^{31}$P NMR and HPLC also the phosphorylation of a broad range of carbohydrates and alcohols. Many cyclic carbohydrates are phosphorylated in a regioselective manner. Non-cyclic carbohydrates are phosphorylated as well. Phosphorylation of linear alcohols, cyclic and aromatic alcohols is also possible. In all cases the acid phosphatase from *Shigella* prefers a primary alcohol function above a secondary one. We conclude that these enzymes are an attractive alternative to existing chemical and enzymatic methods in the phosphorylation of a broad range of compounds.

**Keywords:** enzymatic phosphorylation; glucose 6-phosphate; monophosphate; non-specific acid phosphatase; primary alcohol; regioselectivity.

## Introduction

Phosphate esters play an important role in a wide variety of structurally diverse natural and biologically active compounds such as glycolipids, nucleic acids, nucleotides, proteins, coenzymes, steroids and in particular carbohydrates.[1,2] Given the importance of this functional group it is not surprising that many methods have been developed for the phosphorylation of alcohol functions. Among the more widely used methods are those using phosphoramidites and chlorophosphates as phosphate donors.[1–4] Although these are methods with moderate to high efficiency, they all require elaborate manipulation of protecting groups to achieve regioselectivity with, for example, carbohydrate substrates. Potential disadvantages of these chemical methods[4–6] are the low overall yields of phosphorylated product, formation of oligophosphate esters as undesired side products arising from overphosphorylation, the need for multi-step procedures (e.g., phosphitylation, ligand exchange, oxidation and deprotection), and the use of toxic reagents and organic solvents.

Employing enzymes for the regioselective formation of phosphate esters will eliminate many of these disadvantages thus making these syntheses more efficient. Enzymatic phosphorylation ensures mild reaction conditions and, furthermore, protection of functional groups is not necessary. The usefulness of existing enzymatic methods, usually kinases, however, is limited by their narrow substrate specificity. These kinases also require ATP as phosphate donor. Due to its high cost ATP cannot be employed in stoichiometric amounts and thus ATP needs to be regenerated. It is clear that enzymes with broader substrate specificity, which can use other phosphate donors than ATP, are desired. The synthesis of organic phosphates by enzymic transfer of the phosphate group from organic 'donor' compounds to a suitable alcohol function was first demonstrated by Axelrod[7] and Appleyard.[8] Phosphatases hydrolyzing monophosphate esters are expected to yield equal amounts of inorganic phosphate ($P_i$) and an alcohol. However, Axelrod found that some phosphatase preparations (e.g., orange juice phosphatase) acting on nitrophenyl phosphate in the presence of certain alcohols gave lower amounts of $P_i$ compared to the nitrophenol formed.[7] It was then proposed that the preparation contained both a phosphatase and a transphosphatase, which was also the conclusion of Appleyard. Later it

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was found that phosphate transferase activity is a property of the phosphatase itself.[9]

The term non-specific acid phosphatases (NSAPs) refers to a group of secreted bacterial enzymes which do not exhibit a marked substrate specificity, and show hydrolytic activity towards several different and structurally unrelated organic phosphate monoesters such as nucleotides, sugar phosphates, phosphorylated proteins and lipids, phytic acid, etc.[10,11] These enzymes are believed to function essentially in scavenging organic phosphoesters that cannot cross the cytoplasmic membrane. Inorganic phosphate ($P_i$) and organic products are released, that can be transported across the membrane, thus providing the cell with essential nutrients. Some phosphohydrolases have evolved specialized functions in microbial virulence, signal transduction as well as in several metabolic pathways. On the basis of amino acid sequences, NSAPs are categorized into three classes, designated as class A, B and C.[10] The active site of class A acid phosphatases [$KX_6RP$-($X_{12-54}$)-PSGH-($X_{31-54}$)-$SRX_5HX_3D$[10,11]] is also conserved in mammalian glucose 6-phosphatases (G6Pase), lipid phosphatases and vanadium-containing haloperoxidases.[12,13] The active site residues participate in the binding of phosphate, compensate the negative charges of the oxygen atoms, act as nucleophiles, stabilize the pentacoordinated transition state and play a role in leaving group protonation.[14,15] G6Pase is a key enzyme in glucose homeostasis, catalyzing the hydrolysis of G6P to glucose and phosphate; the terminal steps in gluconeogenesis and glycogenolysis. G6Pase catalyses not only the hydrolysis of G6P but it also has phosphotransferase activity, being able to synthesize G6P from glucose and a wide variety of phosphate donors including pyrophosphate ($PP_i$).[16,17]

Recently, Asano et al. reported a new enzymatic method of phosphorylation of inosine to produce inosine 5′-monophosphate (5′IMP) by the recombinant PhoC from *Morganella morganii*, a class A1 non-specific acid phosphatase, using $PP_i$ as a phosphate donor. They investigated the phosphotransferase activity of a number of enterobacteria, and using $PP_i$ as the phosphate donor they showed that especially class A1 acid phosphatases, exhibit high regioselective transphosphorylation activity.[18–20] As we have previously shown,[21] acid phosphatases are able to phosphorylate and dephosphorylate glucose and G6P and based on a steady-state kinetic analysis an overall mechanism of phosphorylation and dephosphorylation was derived. The key intermediate in the mechanism is an activated phosphorylated enzyme intermediate that may react with a phosphate acceptor to yield a phosphorylated product. The intermediate may also react with water, resulting in hydrolysis and formation of free phosphate.[21] This prompted us to see if we can use the acid phosphatases from *Shigella flexneri* (PhoN-Sf, a class A1 enzyme) and *Salmonella enterica* (PhoN-Se, a class A2 enzyme) not only in preparative phosphorylation of glucose and related compounds, but also in the phosphorylation of structurally different alcoholic substrates using $PP_i$ as a cheap phosphate donor [Equation (1)].

$$R\text{–}OH + PP_i \xrightarrow{PhoN} R\text{–}OPO_3H_2 + P_i \quad (1)$$

## Results and Discussion

### Efficient Phosphorylation of Glucose

As published previously, PhoN-Sf mediates the phosphorylation of D-glucose (D-**1**) to D-glucose 6-phosphate (D-G6P) using pyrophosphate ($PP_i$) as the phosphate donor.[21] A maximum concentration of D-G6P of 35–40 mM at pH 4 after 3 hours was reported. Our $^{31}P$ NMR experiments reveal a maximal concentration of 65 mM at pH 4 after 200 minutes (Figure 1), which was confirmed by HPLC measurements.

The yield of D-G6P could be further increased by increasing the concentration of D-**1** in the reaction mixture to 400 mM and 1 M. As a result maximal D-G6P concentrations were found of 80 and 95 mM, respectively, formed from 100 mM $PP_i$. Thus we are dealing with a very efficient transphosphorylation process. Apparently hydrolysis of the activated phosphorylated enzyme (or indirectly the hydrolysis of $PP_i$) by competing water is limited and transphosphorylation of a phosphoryl group to D-**1** is favored over transphosphorylation to water (hydrolysis). As previously described,[21] D-**1** inhibits the D-G6P dephosphorylation. The mechanism of inhibition is complex and indicates a mixed type. Competitive inhibition with a $K_{ic}$ of 50 μM and uncompetitive inhibition with a $K_{iu}$ of 150 μM were observed. Effectively

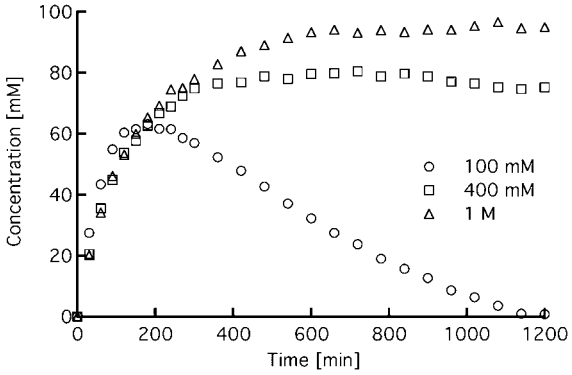



**Figure 1.** Time course of the synthesis of G6P from glucose and $PP_i$ by PhoN-Sf at 30 °C as followed by $^{31}P$ NMR. The reaction mixture contains 1 μM of phosphatase, 100 mM $PP_i$, 100 mM sodium acetate buffer (pH 4.0) and different concentrations of D-glucose.

as outlined[21] high concentrations of D-**1** will drive the equilibrium in the direction of D-G6P. Thus the high D-**1** concentration present in our reaction mixture inhibits the hydrolysis of D-G6P. This explains the very slow decrease of D-G6P under these reaction conditions. The D-G6P concentration could also be increased by addition of more $PP_i$. When a reaction mixture of 100 mM $PP_i$ and 100 mM D-**1** in 100 mM sodium acetate (pH 4.0) containing 1 μM PhoN-Sf was allowed to react for 3 hours at 30 °C the addition of another aliquot of 100 mM $PP_i$ and subsequent incubation for 3 hours increased the D-G6P concentration from 65 to 90 mM. A third aliquot of $PP_i$ resulted in 95 mM of D-G6P. A further increase in D-G6P is hard to reach since the $K_m$ for D-**1** at pH 4.0 is 8.2 mM[21] and hydrolysis of the formed D-G6P ($K_m$ of 0.021 mM at pH 6.0) takes over when the concentration of D-**1** drops.

To assess the potential usefulness of the enzymatic method to produce D-G6P, a small-scale pilot experiment was carried out. A 5 mL reaction mixture containing 100 mM of $PP_i$, 1 M of D-glucose in 100 mM acetate buffer pH 4.0 was used for preparative phosphorylation by 1 μM PhoN-Sf. Based on $PP_i$, conversion reached 97.3% after 16 hours. $Ba(CH_3COO)_2$ was used to precipitate the D-G6P. This yielded 76.5% D-G6P in a >95% purity. This shows that the enzyme can indeed be used in preparative methods.

## Stereospecificity of the Phosphorylation

A clear difference was observed when D-**1** and L-**1** were phosphorylated by PhoN-Sf with $PP_i$ as phosphate donor (Figure 2, panel A). Whereas D-G6P formation continues for over 360 minutes to a concentration of 50 mM, the L-G6P concentration reaches only a maximal concentration of 15 mM. After 150 minutes the L-G6P formed has been hydrolyzed completely. Clearly D-**1** has a much higher affinity for the substrate site than L-**1**. The amount of inorganic phosphate ($P_i$) formed in the process was also measured. When 1 μM PhoN-Sf was added to 100 mM $PP_i$ in the absence of acceptor alcohol, $PP_i$ is hydrolyzed completely to 200 mM $P_i$ within 150 minutes. In the presence of L-**1** (Figure 2, panel B) also 200 mM of $P_i$ is formed within 150 minutes. In contrast when D-**1** is present as an acceptor about 60 mM of $P_i$ is formed after 150 minutes. These observations confirm that D-**1** is a much better substrate for the enzyme than L-**1**

When the phosphorylation of D-**1** is studied in more detail (Figure 3) it becomes clear that the phosphorylation is efficient. At 100 minutes of incubation, 60 mM of D-G6P and 80 mM of $P_i$ are produced from 100 mM of $PP_i$. The 20 mM difference between the two values reflects $P_i$ that is enzymatically formed from either $PP_i$ or D-G6P with water as acceptor. Thus during phosphorylation of D-**1** by $PP_i$ the ratio of transphosphorylation

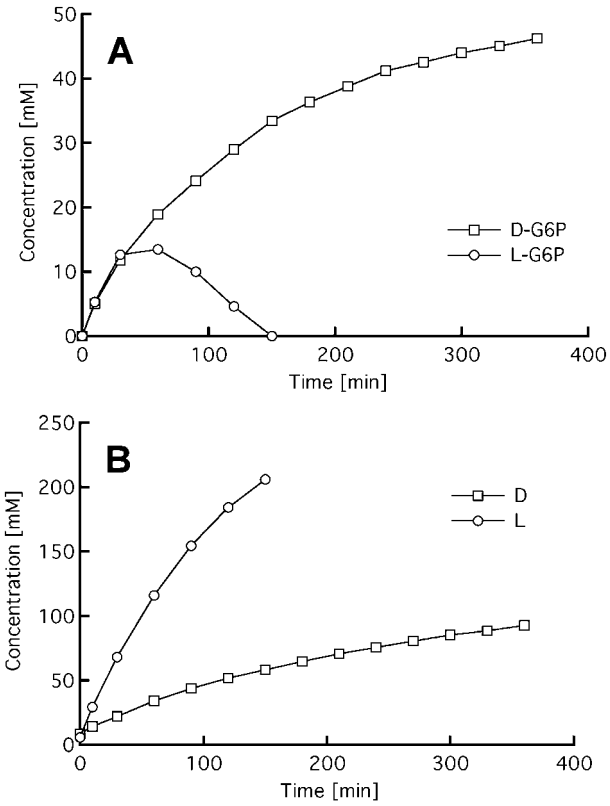



**Figure 2.** Time course of D- and L-G6P synthesis and $P_i$ formation by PhoN-Sf. The reaction mixture contains 1 μM of PhoN-Sf, 100 mM glucose, 100 mM $PP_i$, 100 mM sodium acetate buffer (pH 5.3) at 30 °C. The reactions were monitored by HPLC. Panel **A:** G6P synthesis. Panel **B:** $P_i$ formation during G6P production.

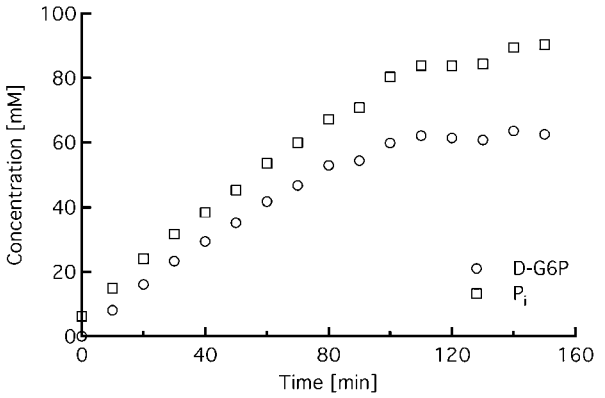



**Figure 3.** Time course of G6P and inorganic phosphate formation from D-glucose and $PP_i$ by PhoN-Sf as determined by HPLC. The reaction mixture contains 100 mM D-glucose, 100 mM $PP_i$, 100 mM sodium acetate buffer (pH 4.0), 1 μM PhoN-Sf at 30 °C.

to hydrolysis is 6:1 and the transphosphorylation catalyzed by the acid phosphatase is indeed a very efficient process. The known enzymatic method for D-G6P production with yeast hexokinase needs an ATP regenera-

tion system.[5] This method using either phosphoenol pyruvate/pyruvate kinase or acetylphosphate/acetate kinase has major disadvantages, e.g., the kinases are strongly inhibited by the phosphate donors. Thus the phosphorylation system described here is an attractive alternative to produce D-G6P.

### Phosphorylation of Other Carbohydrates.

Because of these findings, a variety of polyhydroxy compounds were investigated to evaluate the synthetic applicability of the phosphatase. Figure 5 shows the phosphorylation of D-glucose (D-**1**) and its derivatives D-mannose (**2**), D-galactose (**3**), and D-allose (**4**) at a starting concentration of 400 mM. These compounds were all phosphorylated, but compared to D-**1** the dephosphorylation is much faster. Dephosphorylation of D-G6P is not observed before 15 hours of incubation (Figure 1). However, as seen in Figure 5, dephosphorylation of phosphorylated **2**, **3** and **4** is observed after 60 to 120 minutes. The rapid hydrolysis was also seen with phosphorylated L-**1** (Figure 2). Hexose **3** is phosphorylated to a lesser extent compared to the other sugars. This may be related to the fact that the orientation of the hydroxy group closest to the phosphorylation site differs

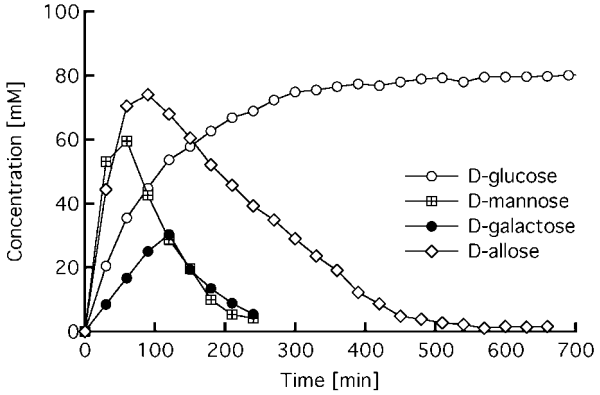



**Figure 5.** Time course synthesis of different D-hexose 6-phosphates from the corresponding D-hexoses by PhoN-Sf as determined by $^{31}P$ NMR. The reaction mixtures contain 1 μM of phosphatase, 400 mM D-hexose, 100 mM $PP_i$, and 100 mM sodium acetate buffer (pH 4.0), 30 °C.

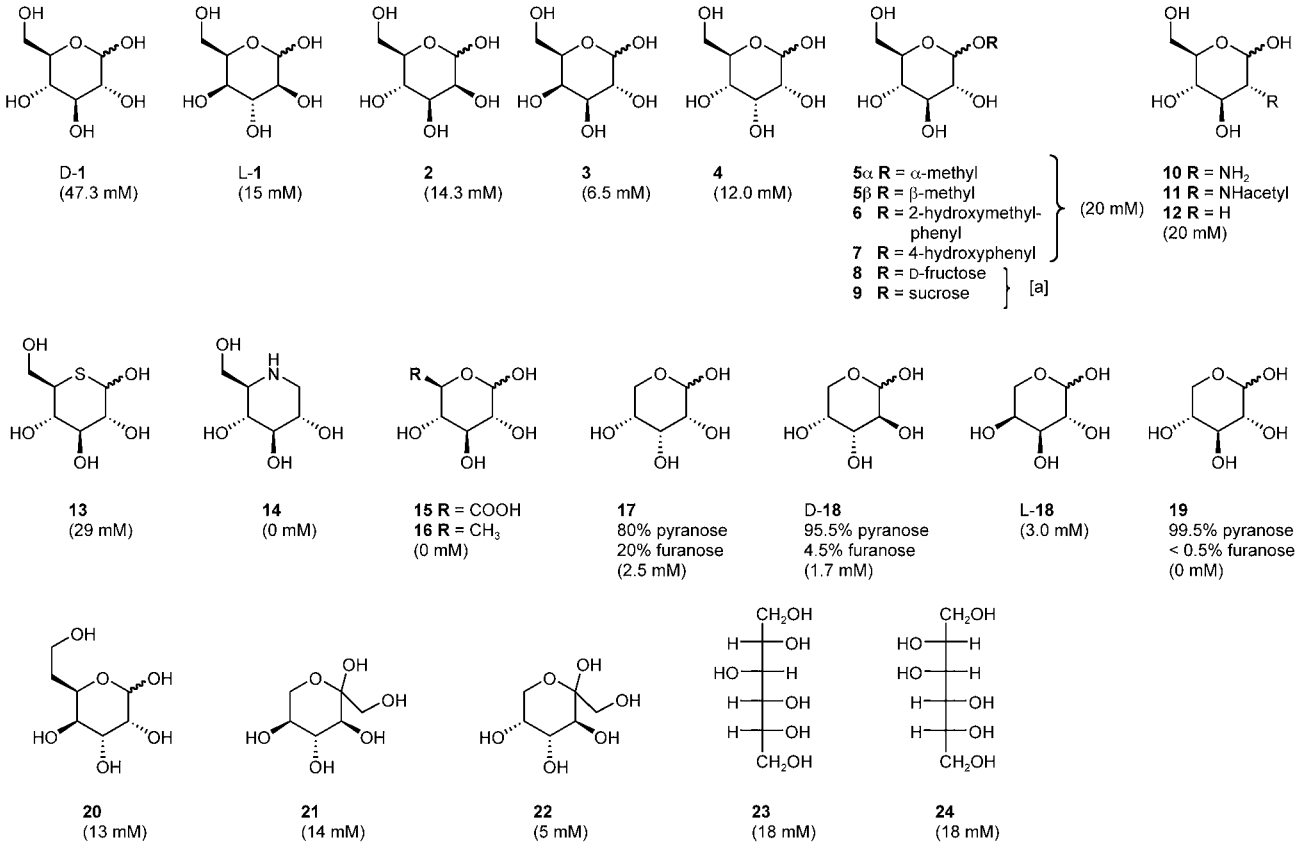



**Figure 4.** Carbohydrate substrates phosphorylated by PhoN-Sf. The values between brackets represent maximal concentrations of phosphate ester reached as determined by time course HPLC experiments (1–16 h). The reaction mixtures contain 1 μM of phosphatase, 100 mM of alcohol, 100 mM $PP_i$, and 100 mM sodium acetate buffer (pH 5.3) at 30 °C.
[a] Not quantified.

from that in D-**1**. Dephosphorylation of **4** is slower compared to **2** and **3**. So, subtle structural differences in the acceptor compounds give rise to a different behavior in the phosphorylation and dephosphorylation by PhoN-Sf, but the phosphorylation activity is not abolished. Phosphorylation of these sugars by yeast hexokinase has also been performed.[5] The same order of phosphorylation activity as in the present study was found, namely: D-**1** > **4** > **2** > **3**. The fact that hexokinase is not able to produce D-galactose 6-phosphate while it is a substrate for PhoN-Sf is striking. Thus, hexokinase is tolerant of modifications at the C-2 and C-3 positions, but is intolerant of structural changes at C-4, while PhoN-Sf accepts a more broad substrate range.

As reported before,[21] the phosphorylation of D-**1** is pH dependent. Table 1 shows that this is also the case for the D-glucose epimers. The yield of phosphomonoesters at pH 5.3 is lower compared to the yield from reactions carried out at pH 4.0. At higher substrate concentrations (0.4 M *vs.* 0.1 M) the equilibrium is driven in the direction of the phosphorylated product and hydrolysis is inhibited. At 1 M of D-glucose 96.6 mM of D-G6P is found (Figure 1).

Substituted sugars are substrates for PhoN-Sf as well. Glycosides, where an aglycone is bonded to the anomeric carbon atom (in these cases by an oxygen atom) are efficiently phosphorylated. When the anomeric hydroxy group is replaced by other substituents like a methyl group as in α- and β-methyl D-glucopyranoside (α-**5** and β-**5**), a 2-(hydroxymethyl)-phenyl group as in salicin (**6**) or 4-hydroxyphenyl as in arbutin (**7**), 20 mM of phosphorylated product are formed at pH 5.3. The amounts of α-**5** and β-**5** phosphorylated are equal. This means that the phosphatase does not recognize the difference between the two anomeric forms. Not only monosaccharides, but also disaccharides [D-sucrose (**8**)] and trisaccharides [D-raffinose (**9**)] are substrates for PhoN-Sf. Variation at the C-2 position is also allowed. Phosphorylation of D-glucosamine (**10**), *N*-acetyl-D-glucosamine (**11**) and 2-deoxy-D-glucose (**12**) resulted also in 20 mM of phosphorylated product (at pH 5.3). However, D-glucosamine 6-phosphate is not stable in solution[22] and breakdown products were seen. 5-Thio-D-glucose (**13**), which differs from D-**1** in substitution of the ring oxygen by sulfur is phosphorylated as well, while 1-deoxynojirimycin (**14**) that differs from D-**1** by substitution of the ring oxygen by nitrogen and absence of the anomeric hydroxy group, is not phosphorylated. Apparently, the enzyme does not accept NH on this position. Variation at position C-6, the phosphorylation site, is not allowed as expected. D-Glucuronic acid (**15**) and 6-deoxy-D-glucose (**16**) were not phosphorylated at all, showing that the enzyme is selective for the 6-position. Interestingly, **15** and **16** inhibit the hydrolysis of $PP_i$ by PhoN-Sf (not shown).

**Table 1.** Phosphorylation of different D-hexoses with PPi by PhoN-Sf.[a]

| | | pH 5.3 (0.1 M) | pH 4.0 (0.1 M) | pH 4.0 (0.4 M) |
|---|---|---|---|---|
| Glucose | (**1**) | 47.3 | 52.8 | 80.4 |
| Mannose | (**2**) | 14.3 | 20.9 | 63.9 |
| Galactose | (**3**) | 6.5 | 8.5 | 30.4 |
| Allose | (**4**) | 12.0 | 43.3 | 69.0 |

[a] The concentrations of the D-hexose 6-phosphates given are the maximal values observed by HPLC and $^{31}P$ NMR in time course studies. The reaction mixtures contain 1 μM of PhoN-Sf, D-hexose (0.1 or 0.4 M), 100 mM $PP_i$, and 100 mM sodium acetate buffer (pH 5.3 or pH 4.0) at 30 °C.

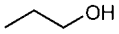
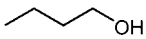
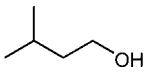
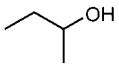
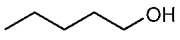
Because the phosphatase phosphorylates also inosine to inosine monophosphate[21] in which the D-ribose ring acts as an acceptor instead of D-glucose, a few riboses were investigated. In our hands a maximal concentration of 6.8 mM IMP at pH 5.0 is formed from 40 mM inosine and 100 mM $PP_i$. D-Ribose (**17**), the sugar part of IMP, is also phosphorylated, although the conversion is low and only 2.5 mM of product is formed starting from 100 mM **17** and 100 mM $PP_i$. The difference in yield between phosphorylation of the ribose structure in inosine and the molecule as such can be explained by the fact that ribose in inosine exists as a ribofuranose, in which a primary hydroxy group is present. In solution **17** itself exists mostly in the pyranose form and no primary hydroxy group is present.[23] Both L-arabinose (L-**18**) and D-arabinose (D-**18**) are phosphorylated, however, D-xylose (**19**) is not phosphorylated and inhibits the hydrolysis of $PP_i$ (not shown). When **19** is in its pyranose form (>99.5% in solution),[23] the geometry of the hydroxy substituents is the same as in D-**1** and, as shown already, D-**1** inhibits also the hydrolysis of $PP_i$. D-Glycero-D-*gulo*-heptose (**20**), a seven-carbon sugar, is converted to 13 mM of phosphomonoester.

L-Sorbose (**21**), a ketose, existing mainly as a cyclic hemiacetal, was phosphorylated to 14 mM of product. D-Fructose (**22**), a ketose as well, is phosphorylated to a considerably lesser extent. The difference in phosphorylation between **21** and **22** probably relates to the spatial projection of the hydroxy groups in the ring with respect to the primary hydroxy functions. In **21** these groups have the same orientation as in D-**1**. This is not the case in **22** and this might explain why **21** is phosphorylated almost three times better than **22**. Yeast hexokinase fails to react with **21**.[5]

For phosphorylation it is not necessary for the substrates to be in a cyclic hemiacetal form, as is shown for D-sorbitol (**23**) and D-mannitol (**24**). These compounds lack the carbonyl group that is necessary to form a hemiacetal and hence are not cyclic. Both compounds contain two primary alcohols as potential phosphorylation sites. Phosphorylation of either one of the terminal hydroxy groups in **24** results in the same product, whereas phosphorylation of **23** may result in two

**Table 2.** Phosphorylation of some simple alcohols by PhoN-Sf.[a]

| Compound | | Maximal concentration (mM) |
|---|---|---|
|  | (**25**) | 14.6 |
|  | (**26**) | 8.9 |
|  | (**27**) | 11.0 |
|  | (**28**) | 3.5 |
|  | (**29**) | 16.6 |
|  | (**30**) | 16.7 |
|  | *R*-(**31**)<br>*S*-(**31**) | 1.8 (13.2[b])<br>3.4 (13.3[b]) |
|  | (**32**) | 20.3 |

[a] The concentrations of the phosphate esters given are the maximal values observed by HPLC and $^{31}$P NMR in time course studies. The reaction mixtures contain 1 μM of PhoN-Sf, 100 mM of alcohol, 100 mM $PP_i$, and 100 mM sodium acetate buffer (pH 5.3) at 30 °C.
[b] PhoN-Se was used.

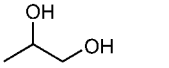
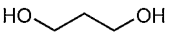
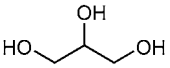
products. By $^{31}$P NMR it was confirmed that phosphorylation of **24** produced only one product peak, whereas phosphorylation of **23** resulted in two peaks with a ratio of 15:3 mM.

From these findings we conclude that PhoN-Sf is capable of phosphorylating a broad variety of carbohydrates. A range of hexoses [D-glucose epimers, C-1 derivatives (glycosides) and C-2 derivatives], pentoses, heptoses, ketoses and non-cyclic carbohydrates are allowed as substrates. Higher yields can be obtained by variation of pH and addition of excess $PP_i$. In addition to being regio-selective, PhoN-Sf is also capable of recognizing the difference between D- and L-sugars, therefore the enzyme is also stereoselective.

## Phosphorylation of Non-Sugar (Poly)hydroxy Compounds

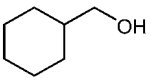
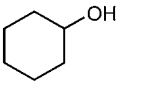
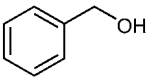
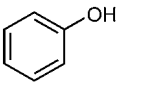
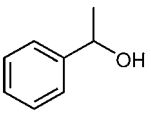
The observation that non-cyclic sugars **23** and **24** are phosphorylated by the phosphatase, suggests that other simple linear alcohols may also serve as substrates. As Table 2 shows, indeed simple alcohols are phosphorylated and increasing chain length resulted in increased yields of phosphorylated products. From ethanol (**26**) *via* 1-propanol (**27**), 1-butanol (**29**) to 1-pentanol (**32**), the yield increases from 8.9 to 20.3 mM starting from both 100 mM substrate and $PP_i$. Only methanol (**25**) does not fit in this series. Branched carbon chains are allowed as is seen with 3-methyl-1-butanol (**30**), which gives 16.7 mM phosphorylated product, compared to 16.6 mM for **29**. Secondary alcohols like 2-butanol (**31**) and 2-propanol (**28**) are hardly accepted by PhoN-Sf since only about 3.4 mM of phosphorylated product is formed. This is in agreement with our previous findings[21] that PhoN-Sf only produces 5′-IMP (primary alcohol function) and not 3′-IMP (secondary alcohol function) upon phosphorylation of inosine. When PhoN-Se was used in the phosphorylation of **31**, the yield is increased to 13.3 mM. This is in line with the observation that PhoN-Se is able to phosphorylate a secondary alcohol function in inosine to produce 3′-IMP.[21] No signifi-

**Table 3.** Phosphorylation of polyalcohols by PhoN-Sf.[a]

| Compound | | Maximal concentration (mM) |
|---|---|---|
|  | (**33**) | 7.3 |
|  | (**34**) | 10.7 |
|  | (**35**) | 9.1 (11.8[b]) |

[a] The concentrations of the phosphate esters given are the maximal values observed by HPLC and $^{31}$P NMR in time course studies. The reaction mixtures contain 1 μM of PhoN-Sf, 100 mM of alcohol, 100 mM $PP_i$, and 100 mM sodium acetate buffer (pH 5.3) at 30 °C.
[b] PhoN-Se was used.

**Table 4.** Phosphorylation of some cyclic and aromatic alcohols by PhoN-Sf.[a]

| Compound | | Maximal concentration (mM) |
|---|---|---|
|  | (**36**) | 39.0 |
|  | (**37**) | 3.1 |
|  | (**38**) | 13.4 |
|  | (**39**) | 3.7 |
|  | (**40**) | 0.2 (9.1[b]) |

[a] The concentrations of the phosphate esters given are the maximal values observed by HPLC and $^{31}$P NMR in time course studies. The reaction mixtures contain 1 μM of PhoN-Sf, 100 mM of alcohol, 100 mM $PP_i$, and 100 mM sodium acetate buffer (pH 5.3) at 30 °C.
[b] PhoN-Se was used.

cant difference is observed between phosphorylation of *R*-**31** and *S*-**31** by PhoN-Sf or by PhoN-Se.

Table 3 shows the phosphorylation of polyalcohols. 1,3-Propanediol (**34**) is a better substrate compared to 1,2-propanediol (**33**). This is in line with the observation that the secondary alcohol functions in **28** and **31** are hardly phosphorylated as shown in Table 2 and that **34** has 2 primary alcohol functions. Glycerol (**35**) is phosphorylated to almost the same extent as **34** and there is hardly any difference between the two acid phosphatases.

Not only linear alcohols are substrate for acid phosphatases, cyclic and aromatic alcohols are phosphorylated as well as is illustrated in Table 4. Again, as we have seen before, a primary alcohol function is phosphorylated much easier by PhoN-Sf compared to secondary ones. Furthermore, it is obvious that **38** is a less suitable substrate compared to its non-aromatic counterpart **36**. An explanation for this observation might be that **36** adopts a chair conformation as does glucose and this apparently results in a more efficient transphosphorylation to the corresponding monophosphate ester. The last substrate, 1-phenylethanol (**40**) was chosen because of its chirality. PhoN-Sf only phosphorylates 0.2 mM of the substrate whereas PhoN-Se produces 9.1 mM of phosphorylated product. For this reason, PhoN-Se was chosen to carry out further experiments. Unfortunately, the enzyme was not able to recognize the difference between *R*-**40** and *S*-**40**.

In conclusion the bacterial non-specific acid phosphatases from *Shigella flexneri* (PhoN-Sf) and *Salmonella enterica* (PhoN-Se) are convenient and selective tools in the preparation of a variety of phosphorylated products. These enzymes are able to phosphorylate a broad array of structurally different alcoholic compounds in a regiospecific manner. In addition, the NSAPs use (cheap) $PP_i$ as phosphoryl donor. This is in contrast to kinases which use ATP and for which regeneration systems have to be used. Furthermore, kinases exhibit a more narrow substrate specificity meaning that for each class of substrates another kinase has to be used. Employing NSAPs eliminates many of the disadvantages associated with classical chemical methods requiring protection and deprotection of groups under harsh reaction conditions. The preparative phosphorylation of D-glucose, resulting in D-G6P is also reported. The enzymatic method is easy and efficient and thus it may become a useful alternative to the existing chemical and enzymatic methods of producing phosphorylated compounds. It probably also allows the phosphorylation of more labile molecules of which the synthesis up to now has not been possible.

To explain the difference in reactivity towards the different substrates, docking of the substrates into the active site of the enzymes would be an option. However, crystal structures of PhoN-Sf and PhoN-Se are not (yet) available. The crystal structure of the enzyme from *Escherichia blattae* (EB-NSAP), a class A1 enzyme, has been reported,[24] but the similarity in sequence is only 84% with PhoN-Sf and 62% with PhoN-Se, respectively. Although the similarity between EB-NSAP and PhoN-Sf is quite high, we chose not to perform the modelling since small differences can cause major differences in the 3D structure near or at the active site.

## Experimental Section

Expression and purification of *Salmonella enterica* PhoN (PhoN-Se) and *Shigella flexneri* PhoN (PhoN-Sf) were as described elsewhere.[21,25] All chemicals and other enzymes were purchased from commercial suppliers and used without purification.

### Assay for Phosphotransferase Activity.

A standard reaction mixture contained 100 mM of substrate, 100 mM disodium pyrophosphate ($PP_i$), 100 mM acetic acid buffer (final pH 5.3 unless otherwise mentioned) and 1 μM of PhoN-Sf or PhoN-Se in a total volume of 0.5 mL at 30 °C. The amounts of phosphorylated products were determined by HPLC on a OA-column. The enantiomeric excess (ee) of the phosphorylation of 1-phenylethanol was determined as follows: a 50-μL sample of a standard reaction mixture was extracted with 200 μL *n*-octane and 20 μL of the extract were used for HPLC analysis on a Chiralcel OD column. For HPLC experiments, the decrease in substrate peak area was taken as a measure for conversion.

### $^{31}$P NMR

Phosphorylated products, $PP_i$ and $P_i$ were quantified by phosphorus nuclear magnetic resonance ($^{31}$P NMR). Spectra were determined on a Varian Unity Inova at 202 MHz using a 10-mm $^{15}$N-$^{31}$P probe. Chemical shifts (δ) are expressed in ppm relative to 85% phosphoric acid. At zero time a spectrum was taken from the reaction mixture containing 100 mM $PP_i$, 100 mM substrate (or 400 mM and 1 M), 100 mM sodium acetate buffer (final pH 5.3 or 4.0) in a 10-mm NMR tube. The reaction was initiated by addition of 1 μM PhoN-Sf or PhoN-Se, and was carried out in a 10-mm NMR tube at 30 °C. Concentrations of product and reactants were determined using 50 mM dimethyl methanephosphonate in deuterated water as an external standard, which was coaxially inserted in the NMR tube.

### Preparative Synthesis of G6P

A 5-mL reaction mixture containing 100 mM of $PP_i$, 1 M of D-glucose (**1**) in 100 mM acetate buffer pH 4.0 was stirred and kept at 30 °C. The reaction was started by the addition of 1 μM PhoN-Sf. Conversion reached 97.3% based on $PP_i$ after 16 hours. Isolation of G6P was based on the method described by Pollak et al.[26] and Wong et al.[27] The pH was adjusted to 9.8 with NaOH and $P_i$ was precipitated by the addition of 2 mL of 0.5 M $Ba(CH_3COO)_2$. The precipitate was washed with 2 times

with 2 mL of water. Cold ethanol (final concentration 75%) was added to the filtrate to precipitate the barium salt of G6P, which was washed with 2 mL of 75% ethanol after filtration. Drying over KOH resulted in 0.237 g of a white solid (>95% pure, determined by HPLC). The precipitate was dissolved using ion exchange resin (Dowex 50x8) in $H^+$ form. This isolation resulted in 78.6% recovery of the formed D-G6P (coupled enzymatic assay based on the method of Noltman et al.[28]). This quantity corresponds to a 76.5% yield based on $PP_i$. $^1H$ NMR (500 MHz, $D_2O$): $\delta = 5.22$ (d, $J = 3.8$ Hz, H-1α), 4.63 (d, $J = 8.0$ Hz, H-1β), 4.06–3.84 (m), 3.74–3.44 (m), 3.27 (t, $J = 8.9$ Hz). This is in agreement with reported values.[5] To determine the amount of G6P, 50 μL of a 10 or 20 × diluted fraction were added to 2 mL of a G6P assay mixture. This assay mixture contains 0.01 mg $mL^{-1}$ glucose 6-phosphate dehydrogenase, 1 mM of $NADP^+$ and 10 mM $MgCl_2$ in 100 mM Tris/acetate (pH 7.5). The formed NADPH can be monitored at 340 nm (extinction coefficient 6.22 $mM^{-1}\,cm^{-1}$).

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