Short Communication

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PHOSPHORYLATION OF ACYCLONUCLEOSIDES BY NUCLEOSIDE PHOSPHOTRANSFERASE FROM HIGHER PLANTS AND BACTERIA*

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New pyrimidine acyclonucleosides, prepared by us, were phosphorylated by nucleoside phosphotransferase from wheat shoots as well as from Enterobacter agglomerans. Conditions and parameters of enzymic phosphorylation were optimized and the results obtained with the two phosphotransferases were compared.

Phosphate esters of an antiherpetic agent acyclovir (acycloguanosine) were found to be more potent inhibitors of several enzymes than acyclovir itself [1]. Acyclonucleotides are also potential antiviral and anticancer compounds. This prompted us to study the synthesis of phosphate esters of acyclonucleosides. In this paper we describe enzymatic phosphorylation of a group of eight pyrimidine acyclonucleosides (listed in Scheme 1) which

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we synthesized in our laboratory [2]. We have used nucleoside phosphotransferase from two different sources [3]. For identification of acyclonucleotides obtained by the enzymatic method we also prepared these compounds using the chemical method according to Yoshikawa et al. [4], as modified by us [5].

Nucleoside phosphotransferase (EC 2.7.1.77) is widely distributed in nature; for practical use, however, it was isolated from carrot [6, 7], barley shoots [8] or wheat shoots [9, 10]. For our study we have used the last source, namely direct phosphorylation by wheat shoot homogenate as the enzymic preparation.

Another source of nucleoside phosphotransferase are bacteria. For phosphorylation, the enzyme from strains of Corynebacterium sp. AJ 1562 [11], Serratia marcescens IFM F420 [12] and Enterobacter agglomerans ATCC 14537 (previous name Erwinia herbicola) [13 - 15] has been used. Because of convenience of preparation and the good results described, we decided to use for our study the E. agglomerans nucleoside phosphotransferase. Moreover, it is convenient that an E. agglomerans suspension can be used in the phosphorylation procedure without homogenisation and enzyme isolation.

MATERIALS AND METHODS

Aicyclonucleosides. We selected for phosphorylation eight compounds (Scheme 1) from the collection of new pyrimidine acyclonucleosides [2]. As the standard we decided to use acycloguanosine (acyclovir, ACV in Scheme 1).

Plant material. Wheat grains were cultivated for 5 days and the shoots cut off. After two successive days, a second crop of shoots was collected.

Preparation of wheat shoot homogenate. The minced shoots were homogenized with the same quantity (w/w) of 0.02 M acetate buffer, pH 4.0, at 0°C. The crude homogenate was centrifuged at 1 000 g for 10 min. The clear supernatant was collected and frozen overnight. Next day the homogenate was defrozen and the upper, colourless, phase was discarded. The lower, pink phase was used for further determinations. The solution was frozen and stored at −20°C. Protein was determined colorimetrically by the use of Coomassie Brilliant Blue G-250 according to Spector [16].
Scheme 1. The phosphorylated acyclonucleosides. Codes employed:
C, 1-N-[(2'-Hydroxy/ethoxymethyl]-5,6-tetramethyleneuracil;
C-5, 1-N-[1',3'-Dihydroxy-2'-propoxymethyl]-5,6-tetramethyleneuracil;
D, 1-N-[(2'-Hydroxy/ethoxymethyl]-5,6-dimethyluracil;
Th-5, 1-N-[1',3'-Dihydroxy-2'-propoxymethyl]-5-methyluracil;
U, 1-N-[(2'-Hydroxy/ethoxymethyl]uracil;
U-5, 1-N-[1',3'-Dihydroxy-2'-propoxymethyl]uracil;
1-PrOH-CMU, 1-N-3'-Hydroxypropyl]-5,6-tetramethyleneuracil;
1-G-CMU, 1-N-2',3'-Dihydroxypropyl]-5,6-tetramethyleneuracil;
ACV, acycloguanosine = acyclovir, 9-N-[(2'-Hydroxy/ethoxymethyl]guanine

'The nucleoside phosphotransferase activity was assayed according to Brunngraber & Chargaff [6].

Bacterial material. Enterobacter agglomerans was obtained from a strain collection of the Department of Medical Microbiology, the Military
Medical Academy, Łódź. The suspension of cells was transferred from agar slant to the liquid medium described by Popow et al. [14]. Cultures of *E. agglomerans* were centrifuged and the biomass sediment was collected; a small sample (about 10 mg) was taken for further assays and the main portion was frozen and stored at −20°C. The withdrawn sample was weighed in a watch glass, kept at 37°C for 4 h, again weighed and the relation of mass between the moist and the dry sample was calculated.

**Phosphorylation of acyclonucleosides by the wheat shoot homogenate.** The acyclonucleoside was dissolved in 0.02 M acetate buffer (pH 4.0) to obtain a 0.04 M solution. A 15-fold molar excess of 4-nitrophenylphosphate was added and dissolved. The solution was brought to pH 4.0 by glacial acetic acid. An equal volume of defrozen homogenate (for an acyclonucleoside with two hydroxyl groups – a double volume) was added to this solution. The reaction mixture was incubated for 7.5 h at 37°C.

**Phosphorylation of acyclonucleosides by the *E. agglomerans* biomass.** The reaction mixture contained an acyclonucleoside, 3.8 μmol, 4-nitrophenylphosphate, 46 μmol, dry biomass, 20 mg (calculated from moist mass; for an acyclonucleoside with two hydroxyl groups – a double quantity), 1 M ZnSO₄ sol. 10 μl, 0.2 M acetate buffer (pH 4.5) up to 1.0 ml. The mixture was incubated for 4.5 h at 37°C. Then the reaction mixture was centrifuged at 2 500 g for 10 min and the clear supernatant was collected.

![Scheme 2](image)

Scheme 2. Scheme of an enzymic phosphorylation. 1, Initial compound; 2, donor of PO₄³⁻ group (4-nitrophenylphosphate); 3, product of phosphorylation; 4, 4-nitrophenol liberated (side-product)

**Further procedure.** The reaction mixtures containing the enzyme from either source were deproteinised using the perchloric acid/potassium
hydroxide method. The collected supernatants were loaded on silica gel plates. The 4-nitrophenol, released during the reactions (Scheme 2), was removed by a preliminary run with solvent system CHCl₃:methanol (4:1, by vol.). The products were isolated by chromatography in solvent system 1 (n-propanol:28% NH₄OH:H₂O = 22:17:1, by vol.). The spots correspond-

Table 1
Phosphorylation of acyclonucleosides

<table>
<thead>
<tr>
<th>Code of acyclonucleoside</th>
<th>Nucleoside phosphotransferase:</th>
<th>Chemical phosphorylation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>from wheat shoots</td>
<td>from Enterobacter agglomerans</td>
</tr>
<tr>
<td>C</td>
<td>57</td>
<td>47</td>
</tr>
<tr>
<td>C-5</td>
<td>54</td>
<td>44</td>
</tr>
<tr>
<td>D</td>
<td>53</td>
<td>42.5</td>
</tr>
<tr>
<td>Th-5</td>
<td>56</td>
<td>55</td>
</tr>
<tr>
<td>U</td>
<td>45</td>
<td>41.5</td>
</tr>
<tr>
<td>U-5</td>
<td>52</td>
<td>37.5</td>
</tr>
<tr>
<td>1-PrOH-CMU</td>
<td>25</td>
<td>46</td>
</tr>
<tr>
<td>1-G-CMU</td>
<td>27</td>
<td>41.5</td>
</tr>
<tr>
<td>ACV</td>
<td>20</td>
<td>37</td>
</tr>
</tbody>
</table>

* By the Yoshikawa method according to [5]
**Yields of monophosphates of corresponding acyclonucleosides (yields of the other products of phosphorylation, cyclic- and bis-monophosphates, are not included)

ing to standard, obtained by chemical phosphorylation [5] were detected under UV (254 nm). The spots were scrapped off and eluted with distilled water. The absorbance of the eluate was measured and the yield calculated (Table 1). The eluate was concentrated to a small volume in vacuo and analysed by t.l.c. on silica gel and cellulose plates (Merck) using solvent system 1. The eluates containing phosphorylation products of dihydroxyl acyclonucleosides were subjected to two-dimensional t.l.c. on cellulose plates, with alkaline solvent system 1 (described above) and acidic solvent
system 2 (n-butanol : acetic acid : water = 4:1:1, by vol.); the spots of products were homogeneous. In all the described experiments the presence of acyclonucleotides was confirmed by the use of the molybdate reagent

Table 2

**Thin-layer chromatography of starting compounds and their phosphorylated derivatives**

Abbreviations used: S, silica gel plates; C, cellulose plates. The Roman numeral I refers to the nonphosphorylated compounds and II to the phosphorylated derivatives. The arabic numerals 1 and 2 refer to the solvent systems described in the text

<table>
<thead>
<tr>
<th>Code of compounds</th>
<th>( R_f ) values</th>
<th>( R_f ) values</th>
<th>( R_f ) values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S(1) I I II I II I II</td>
<td>(1) I I II I II I II</td>
<td>(2) I I II I II</td>
</tr>
<tr>
<td>C</td>
<td>0.75 0.36 0.96 0.40</td>
<td>–     –     –     –</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>C-5</td>
<td>0.69 0.35 0.93 0.44</td>
<td>0.72 0.18</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>D</td>
<td>0.71 0.33 0.90 0.41</td>
<td>–     –     –     –</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>Th-5</td>
<td>0.60 0.32 0.80 0.36</td>
<td>0.65 0.12</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>U</td>
<td>0.55 0.24 0.74 0.30</td>
<td>–     –     –     –</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>U-5</td>
<td>0.56 0.24 0.72 0.29</td>
<td>0.38 0.05</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>1-PrOH-CMU</td>
<td>0.79 0.37 0.96 0.42</td>
<td>–     –     –     –</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>1-G-CMU</td>
<td>0.69 0.40 0.90 0.45</td>
<td>0.64 0.19</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>ACV</td>
<td>0.67 0.23 0.85 0.27</td>
<td>–     –     –     –</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>Urd</td>
<td>0.48 0.18 0.70 0.25</td>
<td>–     –     –     –</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>Thd</td>
<td>0.65 0.21 0.88 0.30</td>
<td>–     –     –     –</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>NPP*</td>
<td>– 0.60</td>
<td>– 0.75</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>NP**</td>
<td>0.84 – 0.95</td>
<td>–     –     –     –</td>
<td>–     –     –     –</td>
</tr>
<tr>
<td>P1***</td>
<td>– 0.05</td>
<td>– 0.15</td>
<td>–     –     –     –</td>
</tr>
</tbody>
</table>

*4-Nitrophenylphosphate  
**4-Nitrophenol  
***Inorganic phosphates (visualised by the molybdate reagent)  

[17] which also visualises inorganic phosphate. The \( R_f \) values are presented in Table 2.
RESULTS AND DISCUSSION

Phosphorylation by wheat shoots homogenate

We have introduced some changes in growing conditions of wheat shoots and homogenisation procedure (see Methods) in comparison to the procedure described by Giziewicz & Shugar [10].

The homogenate prepared by us contained 1.15 μg/ml protein and showed enzymatic activity of 46.7 μmol Urd/mg protein.

The optimalisation studies have shown that the maximum yield of phosphorylation was reached after 7.5 h incubation for all acyclonucleosides tested. In the case of dihydroxyl acyclonucleosides we have found that a double amount of homogenate is optimal for the yield, whereas an increase of the amount of 4-nitrophenyl phosphate does not improve the yield of the product.

In the preparative runs (1 millimole scale) after deproteination of the reaction mixture the supernatant was loaded on 2 mm thick t.l.c. silica gel plates. We have omitted the removal of 4-nitrophenol by ether extraction, as it was recommended in the original procedure [10]. Instead of extraction we have prerun the loaded plates in CHCl₃-methanol solvent in which 4-nitrophenol moves with the front of the solvent whereas other components of the reaction mixture stay at the origin. The subsequent run in propanol-ammonia solvent resulted in separation of the remaining components.

Phosphorylation by the biomass of E. agglomerans cells

Some changes and modifications were introduced in the use of E. agglomerans cells for phosphorylation of acyclonucleosides in comparison to the original procedure [15]. We have obtained the best yield of biomass by inoculating 100 ml of the cultivation medium by three drops of bacterial suspension (10⁹ cells/ml). The optimum time of phosphorylation at 37°C was found to be 4.5 h (the authors [15] incubated reaction mixtures at 50°C). The ratio of 5 mg of dry biomass to 1 μmol of acyclonucleoside (or
10 mg for dihydroxyl acyclonucleosides) resulted in the best phosphorylation yield.

**Comparison of the phosphorylation methods**

From various higher plant sources of nucleoside phosphotransferases, wheat shoots are that the most convenient and useful [10]. Direct use of the crude homogenate of shoots, without separation and purification of enzyme, is the main advantage of this method. However, the necessity of protein determination, and unstable enzymic activity cause some inconveniences.

*E. agglomerans* biomass is also used directly in reaction mixtures. The growth of *E. agglomerans* [15] is faster than that of wheat shoots, and preparation of the biomass is much simpler. The protein content and enzymatic activity is very stable, and it is not necessary to determine it in every run. Phosphorylation time is shorter. For these reasons we suggest the *E. agglomerans* method as preferable to the wheat shoots method. Yields of monophosphates obtained in both enzymatic methods are similar. The acyclonucleosides with two \(-\text{CH}_2\text{OH}\) groups possess a prochiral carbon atom, however none of studied enzymes gives stereospecific reactions (data not shown).

Acyclonucleosides with one OH group were phosphorylated chemically as well as enzymatically with a reasonably good yield. In laboratory practice, the chemical method is faster and less troublesome as it does not requires time for growing shoots or bacteria, and for preparation of the biological material. However, better results were obtained in enzymatic phosphorylation of acyclonucleosides with two OH groups, especially when the groups were chemically equal, because in the latter method we did not encounter any problems with formation of side products as, *e.g.*, cyclic phosphates and bismonophosphates.

We are indebted to M.Sc. Ewa Korczak for her assistance.

**REFERENCES**