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Enzymatic synthesis of nucleoside 5'-mono and -triphosphates

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The transformation of fluorodeoxy-nucleosides into 5'-monophosphates with the use of whole bacterial cells of *Erwinia herbicola* as a biocatalyst and PNP as a phosphate donor and next into 5'-triphosphates by using an extract of the *Saccharomyces cerevisiae* cells has been demonstrated.

Nucleoside 5'-monophosphate; Nucleoside phosphotransferase; Nucleoside 5'-triphosphate; Nucleotide kinase; (*Saccharomyces cerevisiae*, *Erwinia herbicola*)

1. INTRODUCTION

Recently we have reported on the chemical synthesis of dNTP(3'F) and NTP(3'F) and their use as terminators of the biosynthesis of DNA and RNA, respectively [1-4]. During the course of the chemical synthesis of fluorodeoxyribo- and 2'-deoxyribo-NMP and NTP, we have pointed out that the isolated yields of pure compounds are varied within the broad limits depending on the structure of a heterocyclic base and/or a carbohydrate moiety (cf. [5]), and from assay to assay as well. On the other hand, it has been illustrated that various enzymatic methods are more effective for the nucleosides transformation to NMP as compared to chemical ones (detailed discussion see [6]). Of particular interest is the use of enzymatic methods for the transformation of NMP to NTP (see e.g. the data in [7-9]).

The effectiveness of the enzymatic methods of phosphorylation depends on the specificity of pertinent enzymes that is of particular importance in the case of modified nucleosides and NMP [6,7]. The present paper is concerned with the investigation of the possibility of using the whole cells of *Erw. herbicola* [10] for conversion of natural and fluorodeoxy-nucleosides into NMP and the latter into NTP by using the kinase system of yeast *Sacch. cerevisiae* [8].

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Abbreviations: NTP(3'F), 3'-fluoro-3'-deoxyribunocleoside 5'-triphosphates with the bases adenine [ATP(3'F)], guanine [GTP(3'F)], uracil [UTP(3'F)] and cytosine [CTP(3'F)]; NMP(3'F), 3'-fluoro-3'-deoxyribonucleoside 5'-monophosphates with the natural bases; Ado(3'F), Guo(3'F), Urd(3'F), and Cyt(3'F), 3'-fluoro-3'-deoxyribonucleosides; dNTP(3'F) and dNMP(3'F), 3'-fluoro-2',3'-dideoxyribonucleoside 5'-tri- and -monophosphates, respectively; dAdo (3'F), dGuo(3'F), dThd(3'F) and dCyd(3'F), 3'-fluoro-2',3'-dideoxyribonucleosides; PNP, *p*-nitrophenylphosphate; NMP and NTP, nucleoside 5'-mono-and -triphosphates, respectively

2. MATERIALS AND METHODS

The biomass of *Erw. herbicola* 47/3 cells was prepared as described in [10]. The reaction mixture for 5'-monophosphorylation of nucleosides (1 ml) contained 15 mM nucleoside, 90 mM PNP, 0.2 M sodium acetate buffer (pH 4.5) and intact cells (1% dry wt.). Aliquots of the reaction medium were analysed by the TLC on Silufol UV-254 plates (Kavalier, CSSR; isopropanol/25% aq. ammonia/water, 7:2:1, w/w). The starting nucleoside and NMP were extracted with 0.05 M potassium phosphate buffer (pH 7.0) and quantified spectrophotometrically.

In the case of preparative (10-ml scale) NMP(3'F) synthesis, after the reaction was completed, the reaction medium was centrifuged (5000 × *g*, 10 min), the supernatant was diluted with water to 50 ml and extracted by ether (3 × 10 ml) to remove *p*-nitrophenol. The aqueous solution was applied on a DEAE-Toyopearl 650 M (Toyo Soda, Japan) column (300 × 20 mm; HCO_3^--form). The column was washed with 0.5 liters of water to recover the unreacted nucleoside. The eluate was evaporated to dryness, the residue was crystallized from an appropriate solvent, and the product was identified (TLC, UV spectroscopy) with the starting nucleoside and its amount was taken into consideration when determining the NMP(3'F) yield. NMP(3'F) was eluted using a linear gradient of triethylammonium bicarbonate (0.0-0.4 M). The fractions containing NMP(3'F) were collected and evaporated. To remove the traces of triethylammonium bicarbonate, the residue was coevaporated with ethanol (5 × 20 ml).

Strain *Sacch. cerevisiae* BMTP-91 was prepared from commercial baker's yeast. To obtain a phosphorylating extract, the cells grown on the beer's wort were dried at 28°C for 24 h and treated with 0.1 M solution of potassium phosphate buffer (pH 7.0; 10 ml/g of dry yeast) containing 1% glucose and 3 mM $MgCl_2$. The suspension was kept for 3 h at 28°C and then centrifuged (8000 × *g*, 5 min). The supernatant was used as the source of enzymes.

For the NTP synthesis 0.25 ml of the phosphorylating extract were added to 0.25 ml of 0.1 M potassium phosphate buffer containing 15 mM NMP, 1% glucose and 3 mM $MgCl_2$. The mixture was incubated at 22-23°C. The aliquots were analysed by the TLC (Silufol UV-254;

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dioxane/isopropanol/water/25% aq. ammonia, 4:2:4:1), the initial NMP and NTP formed were extracted with 10 mM potassium phosphate buffer (pH 7.0) and their quantity was determined spectrophotometrically. In the case of preparative (6.5-ml scale) synthesis of ATP(3'F) and GTP(3'F), after the reaction was completed the mixture was centrifuged (5000 × g, 15 min), the supernatant was diluted with water to 50 ml and applied on a DEAE-Sephadex A-25 column (400 × 20 mm; HCO_3^-). The column was eluted using a linear gradient triethylammonium bicarbonate (0.0–0.8 M). The NMP(3'F) and NTP(3'F) containing fractions were collected, evaporated, and coevaporated with ethanol (5 × 20 ml).

NMP(3'F) and NTP(3'F) Na^+-salts were obtained as amorphous powders according to [11]. The purity of all isolated compounds was checked by HPLC and UV spectroscopy.

3. RESULTS AND DISCUSSION

The transformation of nucleosides to NMP was studied by using the selected bacterial strain *Erw. herbicola* 47/3 the intact cells of which contain high-active nucleoside phosphotransferase (EC 2.7.1.77) and can promote the adenosine transformation to AMP by employing PNP as a phosphate group donor [10].

The whole cells of *Erw. herbicola* 47/3 exhibit the phosphatase activity alongside the phosphotransferase one. In particular, it has been found that at equimolar concentrations of substrates, the phosphorylation of adenosine and AMP transformation to adenosine proceed at the same rate (12 $mmol \cdot min^{-1} \cdot mg^{-1}$ of cells) at the initial stages of the reaction. An analogous ratio of the nucleoside phosphotransferase and phosphatase activities was revealed for the cells of other bacterial strains used as biocatalysts for nucleosides phosphorylation [12,13]. On the contrary, in the case of enzymatic preparations from plants, the phosphatase activity exceeds that of nucleoside phosphotransferase 10–40 times [14–16] which necessitates one to use a phosphate donor when preparing NMP at least in 15-fold molar excess relative to nucleoside. Next, it was found that the whole cells of *Erw. herbicola* 47/3 under the study conditions did not exhibit the adenosine, cytidine and guanosine deaminase activity in relation to natural and fluorodeoxy-nucleosides. Thus, the effectiveness of NMP synthesis is a result of the action of nucleoside phosphotransferase and phosphatase.

The results of NMP and NTP synthesis are listed in table 1. To evaluate the efffectiveness of NMP and NTP synthesis we have chosen a maximum degree of the conversion of initial substrates into the desired compounds and the time required to attain the maximum conversion.

It should be noted that the nucleoside transformation to NMP does not depend on the structure of a heterocyclic base and on the presence of hydroxyl groups at C-2' and C-3' atoms of a furanose ring though in the case of deoxynucleosides the prolonged time is required to attain the maximum conversion to NMP.

In accordance with the data of [8], the extract of the used *Sacch. cerevisiae* cells had the ability to transform adenosine to ATP. However, the other natural ribo- and 2'-deoxyribonucleosides as well as fluorodeoxy-nucleosides did not display any substrate activity in relation to the kinase system of the yeast. Moreover, we also did not observe the formation of NMP – primary products in a chain of nucleosides transformations to NTP. Apparently, the kinases of pyrimidine nucleosides and guanosine are absent in the cells of the

Table 1

The effectivity of NMP and NTP synthesis by the whole cells of *Erw. herbicola* and by the kinase system of the extract of the *Sacch. cerevisiae* cells, respectively

Initial compound	Max. degree of conversion to NMP (%)	Time of attaining max. conversion (h)	Max. degree of NMP conversion to NTP (%)	Time of attaining max. conversion (h)
Ado	73	3	69	6
2'dAdo	65	3.5	67	9
Ado(3'F)	76 (63)*	4	90 (85)*	5
2'dAdo(3'F)	50	4	66	24
Guo	80	1	50	4
2'dGuo	73	1	84	10
Guo(3'F)	83 (54)*	2	97 (93)*	5
2'dGuo(3'F)	56	4	76	24
Urd	78	1	75	4
2'dUrd	78	2	+	
Urd(3'F)	80 (79)*	3.5	0	
rThd	79	1.5	+	
2'dThd	82	3	26	10
2'dThd(3'F)	81	4	0	
Cyd	84	2	70	4
2'dCyd	78	3	56	10
Cyd(3'F)	74 (72)*	4	0	
2'dCyd(3'F)	+ +		0	

+ = conversion has not been studied; + + = the compound was obtained chemically
* isolated yield of the corresponding NMP(3'F) or NTP(3'F)

given strain of microorganisms while adenosine kinase exhibits the extremely pronounced specificity.

For the synthesis of natural and modified in carbohydrate moiety NTP, proceeding from the corresponding NMP, Imazawa and Eckstein have used the kinase system of calf thymus cells [7]. The method has turned out to be effective for the synthesis of pyrimidine NTP (with the exception of 2'dTMP and 5-methyl-2'dCMP which have not displayed the substrate activity at all) and natural purine NTP.

Simultaneously, essential limitations have been revealed for the use of modified purine NMP as substrates of enzymatic reactions. On the contrary, the kinase system of the yeast studied has transformed effectively all the purine and natural pyrimidine NMP to NTP. Also, some peculiar features of the NMP transformation to NTP should be noted. Firstly, in the case of purine NMP, with transition from NMP to 2'-deoxy derivatives the time required for attaining maximum conversion essentially increases. This effect manifests itself more strongly for the corresponding pairs of NMP(3'F) and dNMP(3'F). Secondly, in the case of pyrimidine NMP, the presence of 3'-hydroxyl group turns out to be a critical factor for the transformation of NMP to the corresponding NTP. Thirdly, it should be pointed out that 2'dTMP is the substrate of the studied kinase system of yeast.

It is evident from the present study that the whole *Erw. herbicola* 47/3 might be used for preparative synthesis of different NMP from nucleosides. Furthermore, we demonstrate for the first time that the kinase system of the extract of *Sacch. cerevisiae* cells is an advantageous alternative to the chemical synthesis of some modified purine NTP from NMP.

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