PURIFICATION AND PROPERTIES OF A PYRIMIDINE DEOXYRIBOSIDE PHOSPHORYLASE FROM *ESCHERICHIA COLI**

W. E. RAZZELL AND H. G. KHORANA
British Columbia Research Council, Vancouver (Canada)

INTRODUCTION

Since the first demonstration by KALCKAR[1] of a phosphorolytic pathway in the enzymic cleavage and synthesis of purine ribonucleosides a number of reports have appeared describing ribo- and deoxyribonucleoside phosphorylases from various sources[2]. While the phosphorolytic enzymes acting on purine nucleosides have been clearly distinguished from those acting on the pyrimidine analogues, information concerning their specificities with respect to the carbohydrate moiety has been less conclusive. The recent development of the syntheses of α-D-ribofuranose-1-phosphate[3], a natural substrate for the ribonucleoside phosphorylases, and related sugar phosphates[4,5] led us to examine further the question of substrate specificities of the enzymes of this group. In the present communication we report on the purification of a "pyrimidine deoxyribonucleoside phosphorylase" from *Escherichia coli*, which, while tolerating a variety of substitution in the pyrimidine ring, has been found to be absolutely specific for deoxyribose-1-phosphate. Previously, MANSON AND LAMPEN[6] demonstrated the existence of purine and pyrimidine nucleoside phosphorylases in *E. coli*. They implicated only one enzyme in the phosphorolysis of both the ribonucleosides and deoxyribonucleosides. PAEGE AND SCHLENK[7] have independently reported on the isolation and purification of a different enzyme from the same source which they termed "uridine phosphorylase".

MATERIALS AND METHODS

Cultivation of cells

Escherichia coli, strain B, was grown aerobically in an inorganic salts–glucose medium and the cells harvested by centrifugation. The cell paste obtained was stored frozen for use as required**.

Materials

Pyrimidines and nucleosides used were commercial preparations.

Deoxyribose-1-phosphate samples were the gifts of Dr. M. FRIEDKIN, Washington University School of Medicine, St. Louis, and Dr. H. L. A. TARR, Pacific Fisheries Experimental Station, Vancouver.

D-ribopyranose-1-phosphate, β-D-ribofuranose-1-phosphate, D-arabinofuranose-1-phosphate, L-arabinofuranose-1-phosphate, D-arabinopyranose-1-phosphate and α-D-ribofuranose-1-phosphate were synthesized in this Laboratory[3–5].

* This work was supported by a grant from the Canadian Cancer Society.
** We are indebted to Dr. ARTHUR KORNBERG, Washington University School of Medicine, St. Louis, for the cells used in the later stages of this work.

References p. 566.

We are grateful to Dr. J. J. Fox of the Sloan-Kettering Institute for a gift of 1-β-D-xylofuranosylthymine.

Assays

Arsenolysis of thymidine, according to the procedure of FRIEDKIN AND ROBERTS[8], was employed to follow the purification of the pyrimidine deoxyriboside phosphorylase. To 0.2 ml of 0.1 *M* succinate – 0.1 *M* potassium arsenate buffer, pH 6, containing 32 μmoles thymidine per ml was added water and enzyme to a final volume of 0.4 ml. Samples of 0.05 ml were removed immediately after the addition of enzyme and at subsequent intervals (usually 2, 5 and 10 min.) and were transferred directly to 0.95 ml of 0.3 *N* NaOH in silica cuvettes. The rate of thymine release was calculated from the increase in absorbance observed at 300 mμ ($\Delta E_m = 3610$). The reaction in the direction of synthesis was followed by measuring the formation of P_i by a modification of the procedure of FRIEDKIN[9]. To 0.3 ml of a solution containing 1.5 μmoles of pyrimidine, 1.5 μmoles of pentose phosphate and 50 μmoles of tris (hydroxymethylaminomethane) buffer, pH 7.4, was added water and enzyme to 0.4 ml. Aliquots of 0.05 ml were transferred to 0.05 ml of 0.5 *M* $MgCl_2$–5.0 *M* NH_4Cl, mixed, stored overnight at 4°, and centrifuged. The precipitates were resuspended in 0.1 ml of 0.25 *M* $MgCl_2$–2.5 *M* NH_4Cl, stood at 4° for 4 h, and centrifuged. The final precipitates were dissolved in 6% $HClO_4$ and P_i determined by GOMORI's[10] procedure.

Uridine phosphorylase activity was measured similarly by replacing thymidine by uridine and observing the increase in absorbance at 290 mμ ($\Delta E_m = 5410$) due to uracil formation.

All incubations were at 37°.

All manipulations were carried out in an ice bath or a cold room (2°).

EXPERIMENTAL AND RESULTS

Enzyme purification

Extracts were prepared by sonic oscillation of cells resuspended in 0.05 *M* potassium phosphate buffer, pH 7.3, containing 0.02 *M* cysteine (50 ml/g cell paste)*, and the debris sedimented by centrifugation (7000 × *g*, 20 min).

Ammonium sulfate fractionations both before and after protamine treatment (to remove nucleic acid) yielded a preparation containing both uridine and pyrimidine deoxyriboside phosphorylases, which could only be separated by adsorption and elution from alumina Cγ gel; but if the original cell-free extract was acidified to pH 4.8 with 2 *N* acetic acid in the cold, a large precipitate formed, carrying down all the uridine phosphorylase and leaving the pyrimidine deoxyribonucleoside phosphorylase in solution. The sequence of fractionation procedures finally employed was as follows. (For results see Table I.)

TABLE I

PURIFICATION OF PYRIMIDINE DEOXYRIBONUCLEOSIDE PHOSPHORYLASE

Preparation	*Volume ml*	*Protein mg/ml*	*Specific activity μmoles/h/mg protein*	*Total units μmoles/h*
1. Supernate, 7000 × *g*, 20 min.	150	14.0	9.3	19,500
2. pH 4.8, precipitate, redissolved	100	17	1.7	3,000
3. pH 4.8, supernate	100	5.0	32	16,000
4. protamine supernate of No. 3	120	3.6	36	15,600
5. $(NH_2)_2SO_4$, 0.4–0.7 satd. ppt. of No. 4	40	5.0	70	14,100
6. Dowex-1 column peak from 10 ml of No. 5	5.0	.28	1520§	—

§ Uridine phosphorylase < 7.

* It was found to be important to maintain a high concentration of cysteine in all solutions of the enzyme.

References p. 566.

To the supernate obtained after centrifuging the sonicated cell suspension, 2 *N* acetic acid was added dropwise, with stirring, until the pH fell to 4.8. Stirring was continued about 5 min or until all clumps of precipitated material were evenly dispersed. Centrifugation (10,000 × *g*, 10 min.) yielded a clear amber supernate containing 25 % of the protein and 82 % of the pyrimidine deoxyribonucleoside phosphorylase, and a grey precipitate. The supernate was adjusted to pH 6 with *N* KOH and to every 100 ml was added 10 ml of protamine sulfate, pH 5 (20 mg/ml)*. The mixture was stirred for 10 min and centrifuged (7,000 × *g*, 5 min). Some protein and 90 % of the nucleic acid was removed in this way. The supernate was fractionated with solid ammonium sulfate, the precipitate obtained between 0.4 and 0.7 saturation being dissolved in 30 ml of 0.02 *M* sodium acetate –0.02 *M* cysteine buffer pH 6.8 and dialyzed against the same buffer for 10 h. One quarter of the resulting solution was added to the top of a column of Dowex-1, 2 % cross-linked, exchange resin (15 cm × 2 cm diameter) equilibrated with 0.02 *M* acetate –0.01 *M* cysteine, pH 6.5. About 20 ml of water and then 50 ml of 0.02 *M* sodium acetate –0.01 *M* cysteine, pH 6.5, were run into the column, followed by 0.1 *M* sodium acetate –0.01 *M* cysteine, pH 5.5. Fractions of about 5 ml were collected and both the protein concentration and pyrimidine deoxyribonucleoside phosphorylase activity were determined for each fraction. The results are shown in Fig. 1. Unless the uridine phosphorylase was removed by means of the acid precipitation step, both enzymes were eluted together from the column.

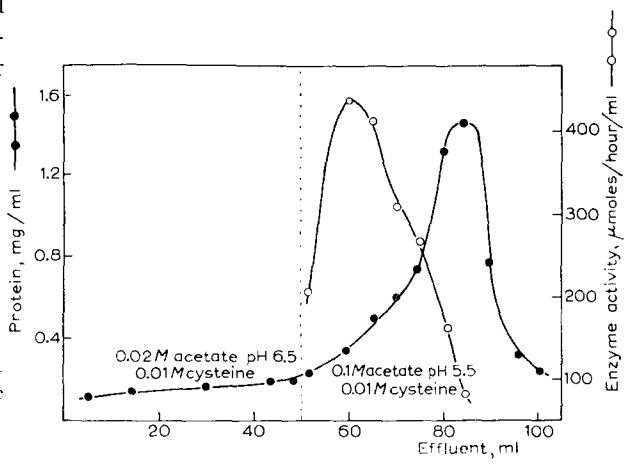

Fig. 1. Anion exchange chromatogram of pyrimidine deoxyriboside phosphorylase.

Substrate specificity

In Table II are the results of testing various pyrimidines with deoxyribose-1-phosphate and ribose-1-phosphate. The lower specific activity observed in the direction of synthesis appears to result from the approach to equilibrium, since the rate of P_i formation decreased rapidly. It is evident that no measurable reaction occurs with ribose-1-phosphate. Furthermore, no P_i was formed in reaction mixtures containing thymine, enzyme (85 μmoles/h/mg protein) and D-arabinofuranose-1-phosphate, L-arabinofuranose-1-phosphate, D-arabinopyranose-1-phosphate, D-ribopyranose-1-phosphate or β-D-ribofuranose-1-phosphate. Since xylose-1-phosphate was not available, evidence was sought for the phosphorolysis of xylofuranosylthymine under assay conditions identical for thymidine, but no reaction was observed.

* The protamine was the generous gift of the Eli Lilly Co.

References p. 566.

TABLE II

NUCLEOSIDE SYNTHESIS WITH VARIOUS PYRIMIDINES

Pyrimidine	*Pentose-1-P*	*Enzyme concn. μg/ml*	*Rate μmoles/h/mg protein*
Thymine	D-1-P*	9.4	360
Uracil	D-1-P	9.4	350
2-Thiouracil	D-1-P	9.4	268
5-Aminouracil	D-1-P	9.4	413
5-Bromouracil	D-1-P	9.4	114
2-Thiothymine	D-1-P	9.4	247
Thymine	R-1-P**	94.0	< 5
Uracil	R-1-P	94.0	< 7
2-Thiouracil	R-1-P	108.0	8 ± 1

In 0.4 ml: 1.5 μmoles pyrimidine, 1.5 μmoles pentose-1-P (cyclohexylammonium salt), 50 μmoles trishydroxymethylaminomethane·HCl, pH 7.4, enzyme (specific activity 1210 μmoles/h/mg). Aliquots were transferred to an equal volume of 0.5 *M* $MgCl_2$–5.0 *M* NH_4Cl, mixed, stored overnight at 4°, and centrifuged. The precipitates were resuspended in 0.25 *M* $MgCl_2$–2.5 *M* NH_4Cl and stood at 4° for 4 h, then centrifuged. The final precipitates were dissolved in 6% $HClO_4$ and P_i determined by GOMORI's[10] procedure.

* Deoxyribose-1-phosphate.
** Ribose-1-phosphate.

No phosphorolysis of deoxyguanosine, deoxyadenosine, the corresponding ribosides, inosine, or deoxycytidine, was found with the purified enzyme.

Confirmation of the reaction, thymidine + $P_i \rightleftharpoons$ thymine + deoxyribose-1-phosphate, was obtained by phosphorolysing thymidine (assay conditions, except arsenate replaced by P_i), and isolating deoxyribose-1-phosphate as the cyclohexylammonium salt according to the procedure of FRIEDKIN AND ROBERTS[8].

Uridine phosphorylase

A number of experiments were performed with the precipitate obtained at pH 4.8, which contains some pyrimidine deoxyriboside phosphorylase (1.7 μmoles/h/mg) as well as the uridine phosphorylase (0.96 μmoles/h/mg). Since PAEGE AND SCHLENK[7] showed that the uridine phosphorylase was inactive towards thymidine and cytidine, but did not examine the reaction between thymine and ribose-1-phosphate, we have incubated thymine and ribose-1-phosphate with the fraction precipitated at pH 4.8. No reaction was observed (less than 0.02 μmoles/h/mg), indicating that the uridine phosphorylase possesses greater specificity towards pyrimidines than does the pyrimidine deoxyriboside phosphorylase.

DISCUSSION

The results reported above demonstrate that this purified *E. coli* preparation is specific for deoxyribose-1-phosphate, but will tolerate a variety of substitution in the pyrimidine base. The name "pyrimidine deoxyribonucleoside phosphorylase" is suggested for the enzyme.

This enzyme obviously differs from the "uridine phosphorylase" purified by PAEGE AND SCHLENK[7], but it was not clear whether their preparation was correspondingly specific for ribose-1-phosphate. Since our experiments with the fraction rich in uridine phosphorylase showed that thymine does not replace uracil in a reaction

References p. 566.

with ribose-1-phosphate, the lack of thymidine phosphorolysis may be due to specificity for the base alone. In this connection the formation of thymine riboside from thymine, inosine and traces of inorganic phosphate, reported by LAMPEN[11], is difficult to explain. If a phosphorolytic mechanism is involved in the synthesis of this riboside, it is not due to either uridine or pyrimidine deoxyribonucleoside phosphorylase.

Although present evidence indicates the existence in bacteria of specific enzymes for pyrimidine ribosides and deoxyribosides, the situation is less clear with phosphorylases of animal tissues. The "thymidine phosphorylase" preparation obtained from horse liver by FRIEDKIN AND ROBERTS[8] is not absolutely specific for deoxyribose-1-phosphate[8,12]. The "uridine phosphorylase" preparation obtained from rat liver by CANELLAKIS[15] has not yet been tested for its specificity toward sugar phosphates.

Information is also lacking on the specificity of purine nucleoside phosphorylases toward sugar phosphates. The only evidence available is from the data obtained by FRIEDKIN AND KALCKAR[14] who used a partially purified liver enzyme. They showed that the Michaelis-Menten constants for hypoxanthine riboside and deoxyriboside were the same and that a system saturated with one substrate did not release purine bases any more rapidly upon the addition of the other. They were thus led to conclude that only one enzyme was functioning. It is possible that further studies on the purine nucleoside phosphorylases of *E. coli* would reveal more than one enzyme for the two series of purine nucleosides.

SUMMARY

1. By methods including chromatography on an anion exchange resin, an enzyme which catalyzes a reversible phosphorolytic cleavage of pyrimidine deoxyribosides has been purified 164-fold from sonic extracts of *Escherichia coli*. The rate of thymidine arsenolysis is 1520 μmoles/h/mg protein.

2. The enzyme is absolutely specific for deoxyribose-1-phosphate. Thus no reaction is observed with ribose-1-phosphate or other pentose-1-phosphates. A number of pyrimidines other than thymine serve as acceptors of deoxyribose.

This enzyme is distinct from the uridine phosphorylase previously reported in *E. coli*.

REFERENCES

1 H. M. KALCKAR, *J. Biol. Chem.*, 167 (1947) 477.
2 For a recent review see A. KORNBERG, *Advances in Enzymol.*, 18 (1957) 191.
3 G. M. TENER, R. S. WRIGHT AND H. G. KHORANA, *J. Am. Chem. Soc.*, 79 (1957) 441.
4 R. S. WRIGHT AND H. G. KHORANA, *J. Am. Chem. Soc.*, in the press, (1958).
5 R. S. WRIGHT AND H. G. KHORANA, *J. Am. Chem. Soc.*, 78 (1956) 811.
6 L. A. MANSON AND J. O. LAMPEN, *J. Biol. Chem.*, 193 (1951) 539.
7 L. M. PAEGE AND F. SCHLENK, *Arch. Biochem. Biophys.*, 40 (1952) 42.
8 M. FRIEDKIN AND D. ROBERTS, *J. Biol. Chem.*, 207 (1954) 205.
9 M. FRIEDKIN, *J. Am. Chem. Soc.*, 74 (1952) 112.
10 G. GOMORI, *J. Lab. Clin. Med.*, 27 (1942) 955.
11 J. O. LAMPEN, in W. D. MCELROY AND B. GLASS, *Phosphorus Metabolism*, Vol. II, Johns Hopkins Press, Baltimore (1952), p. 368.
12 D. B. STROMINGER AND M. FRIEDKIN, *J. Biol. Chem.*, 208 (1954) 663.
13 E. S. CANELLAKIS, *J. Biol. Chem.*, 227 (1957) 329.
14 M. FRIEDKIN AND H. M. KALCKAR, *J. Biol. Chem.*, 184 (1950) 437.

Received November 12th, 1957