Biochemical Pharmacology, Vol. 31, No. 6, pp. 1097–1102, 1982
Printed in Great Britain

0006-2952/82/061097-06 $03.00/0

PYRIMIDINE RIBONUCLEOSIDE PHOSPHORYLASE ACTIVITY VS 5- AND/OR 6-SUBSTITUTED URACIL AND URIDINE ANALOGUES, INCLUDING CONFORMATIONAL ASPECTS

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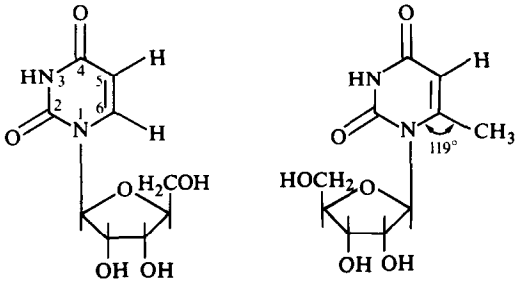
(*Received* 22 *July* 1981; *accepted* 8 *October* 1981)

Abstract—The pyrimidine ribonucleoside phosphorylase from *Salmonella typhimurium* phosphorylyses 6-methyluridine, a uridine analogue sterically constrained to the *syn* conformation about the glycosylic bond, as effectively as uridine itself. In conjunction with the observation that 3-methyluridine is a very poor substrate compared to 5-methyluridine and 5,6-dimethyluridine, it follows that the phosphorolysis reaction involves the initial conversion of uridine, and other 5-substituted uridines (including 5-fluorouridine), to the *syn* conformation during interaction with the enzyme. Furthermore, and consistent with the foregoing, the enzyme recognizes as substrates, to varying degrees, the N(3)-ribosides of xanthine and uric acid, and will also catalyze the formation of these ribosides from the corresponding purines, which may be considered formally as 5,6-disubstituted uracils. Similar observations are reported for the synthetic 5,6-trimethyleneuridine. The enzyme does not, however, recognize 6-methyluracil and 5,6-tetramethyleneuridine in the reverse, synthetic, reaction. The conformational aspects of these reactions are discussed. Since it was previously shown that 6-methyluridine is an equally effective substrate for the pyrimidine phosphorylase of primary rabbit kidney cells, at least some of these conformational requirements apply to the enzyme from mammalian sources, and are consequently of relevance in the design of chemotherapeutic agents, for which some examples are cited.

Enzymes involved in the metabolism of pyrimidines and pyrimidine nucleosides tolerate considerable modification at C(5) of the pyrimidine ring, a fact profited from in the design of nucleoside analogues with antimetabolic activity [1]. Relatively little, however, is known about susceptibility of analogues with substituents at C(6), the nucleosides of which are known to be predominantly, or exclusively, in the *syn* conformation about the glycosidic bond [2]. Our interest in this problem was originally stimulated by the observation that 6-methyluridine, known to be in the *syn* conformation [3] (see Scheme 1) is as good a substrate as uridine for uridine phosphorylase [4, 5].



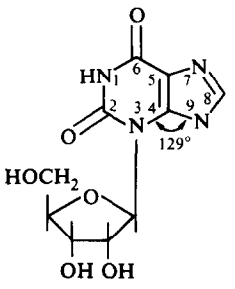
Uridine 6-Methyluridine

Scheme 1

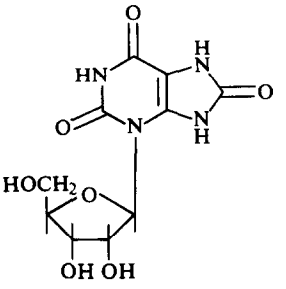
The foregoing, furthermore, directed our attention to purine N(3)-ribosides, which may be considered as formal analogues of 5,6-disubstituted pyrimidine nucleosides (see Scheme 2). The N(3)-riboside of uric acid is found in several animal organisms [6–10]; and the analogous N(7)-riboside of oxypurinol (Scheme 2) in the urine of allopurinol-treated patients [11] and in tissues of rats to whom this drug is administered [12]. The N(3)-riboside of xanthine is not found *in vivo*, probably because of the rapid oxidation of xanthine to uric acid. But the N(3)-deoxyriboside of xanthine [13], the N(3)-riboside of xanthine [14] and the N(7)-riboside of oxypurinol [11] have been synthesized *in vitro* with the aid of pyrimidine nucleoside phosphorylases. Attempts to obtain in this way the N(3)-riboside of uric acid were unsuccessful [15], although phosphorolysis of this nucleoside by uridine phosphorylase from different sources has been reported [11, 15].

Following the demonstration of the *in vitro* synthesis of the 5′-phosphates of the N(3)-ribosides of the foregoing purines by beef erythrocyte pyrimidine ribonucleotide pyrophosphorylase [16a, b, 17], it was assumed that the *in vivo* occurrence of N(3)-ribosyluric acid is due to enzymatic dephosphorylation of the nucleotide. We report here the direct synthesis, by a cell-free extract of *Salmonella typhimurium*, of the N(3)-ribosides of uric acid and related purine analogues, and additional pertinent observations.

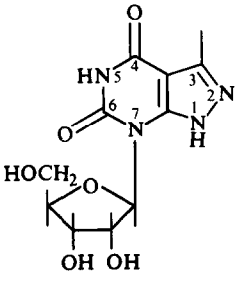
The overall results also lead to some conclusions regarding the conformation about the glycosylic bond of substrates of uridine phosphorylase, of potential significance in the chemotherapeutic activity of some nucleoside analogues.



3-β-D-Riboside of xanthine



3-β-D-Riboside of uric acid



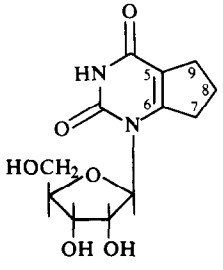
7-β-D-Riboside of oxypurinol

Scheme 2

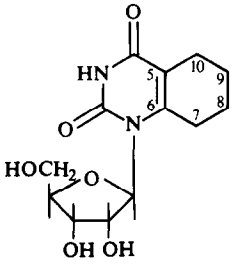
MATERIALS AND METHODS

Most of the purine and pyrimidine bases, and nucleosides, were grade A compounds from Sigma Chemical Co. (St. Louis, MO), CalBiochem (Zurich, Switzerland) and Merck (Darmstadt, F.R.G.). All of these were checked for purity by spectral and chromatographic methods.

5,6-Dimethyluracil, 5,6-trimethyleneuracil and 5,6-tetramethyleneuracil and their nucleosides (Scheme 3) were synthesized as elsewhere described



5,6-Trimethyleneuridine



5,6-Tetramethyleneuridine

Scheme 3

[18] and were gifts of Dr. M. Draminski of Lodz. We are indebted to Dr. A. Holy of Prague for a sample of 6-methyluridine. The dicyclohexylammonium salt of α-D-ribose-1-phosphate was a product of Sigma.

A crude preparation of N(3)-ribosyluric acid was a gift of Dr. H. Witzel of Münster (F.R.G.). This was purified by ascending chromatography on Whatman 3 MM paper with the solvent system methanol–H_2O–conc. NH_4OH (6:1:1, v/v), R_f = 0.57, and the major band eluted with water. Attempts at crystallization were unsuccessful, but the amorphous white powder did not melt below 360° (upper limit of our instrument), cf. > 350° reported by Forrest *et al.* [7]. It was homogeneous on TLC in several solvent systems (Table 1), and its u.v. absorption spectrum at different pH values was in good agreement with that reported by others [15, 16a].

Thin-layer chromatography. TLC made use of Merck F_{254} cellulose sheets. The major solvent systems employed, and the R_f values of the various compounds and products of enzymatic reactions, are listed in Table 1.

Ultraviolet absorption spectrophotometry. This was carried out with a Unicam SP-500 manual instrument and a Zeiss (Jena, G.D.R.) recording spectrophotometer, using 10-mm path length cuvettes.

Enzyme extracts. The source of enzymes was the wild type strain LT2 of *Salmonella typhimurium*, grown in the presence of 2–4 mM uridine or cytidine, the natural inducers of nucleoside catabolizing enzymes in this organism. The procedure for growth of the cells, and the preparation of cell-free extracts, is described elsewhere [4, 5]. The level of induction of uridine phosphorylase was such that the specific activity of the extracts was 30-fold higher than with cells grown in the absence of the inducer (see Table 2 for specific activities). These extracts also contained purine nucleoside phosphorylase, the presence of which did not interfere with experiments involving uridine phosphorylase (see below).

Enzymatic reactions. Unless otherwise indicated, these were run at 37° in buffered medium pH 7.4. Two controls accompanied each experiment, one without enzyme, the other without substrate.

Uridine phosphorylase (uridine:orthophosphate ribosyltransferase, EC 2.4.2.3) activity, involving conversion of bases to nucleosides, and vice versa,

Table 1. Mobilities of nucleosides and their bases on thin-layer cellulose plates*

Base or nucleoside	R_f in solvent system A	B	C	D	E	F
Uracil	0.74	0.73	0.66	0.71	0.42	0.32
Uridine	0.85	0.82	0.63	0.87	0.26	0.12
3-Methyluracil	0.75	0.78	0.85	0.78	0.70	0.60
3-Methyluridine	0.89	0.81	0.87	0.82	0.50	0.35
5-Fluorouracil	0.78	0.74	0.63	0.72	0.50	0.40
5-Fluorouridine	0.88	0.84	0.62	0.80	0.35	0.30
5-Methyluracil	0.79	0.73	0.78	0.74	0.56	0.48
5-Methyluridine	0.83	0.85	0.74	0.86	0.38	0.25
5-Ethyluracil	0.77	0.75	0.88	0.73	0.77	0.68
5-Ethyluridine	0.86	0.84	0.81	0.85	0.57	0.36
6-Azauracil	0.58	0.76	0.56	0.70	—	—
6-Azauridine	0.67	—	0.53	0.78	—	—
6-Carboxyuracil	0.66	0.75	0.54	0.75	0.05	0.04
6-Carboxyuridine	0.86	0.85	0.47	0.93	0.24	0.16
6-Methyluracil	0.74	0.76	0.71	0.73	0.53	0.46
6-Methyluridine	0.86	0.89	0.65	0.89	0.30	0.21
5,6-Dimethyluracil	0.73	0.71	0.80	0.70	0.60	0.57
5,6-Dimethyluridine	0.87	0.87	0.76	0.88	0.38	0.32
5,6-Trimethyleneuracil	0.64	0.66	0.79	0.60	0.67	0.56
5,6-Trimethyleneuridine	0.84	0.86	0.76	0.82	0.46	0.30
5,6-Tetramethyleneuracil	0.64	0.62	0.89	0.58	0.80	0.70
5,6-Tetramethyleneuridine	0.83	0.82	0.85	0.78	0.60	0.50
Xanthine	0.38	0.36	—	—	0.24	0.12
N(3)-Riboside	0.61	0.48	—	—	0.21	0.06
N(9)-Riboside	0.62	0.49	—	—	0.12	0.03
Uric acid	0.26	0.28	0.18	0.28	0.11	0.06
N(3)-Riboside	0.54	0.50	0.40	0.58	0.14	0.05

* Solvent systems: (A) 1 M ammonium acetate–methanol (9:1, v/v); (B) 4% sodium citrate; (C) n-propanol–1% conc. NH_4OH (3:2); (D) 16% NH_4HCO_3; (E) water-saturated n-butanol; (F) n-butanol saturated with 5% borate.

was monitored in two ways: (a) spectrophotometrically, as previously described [4, 5]; (b) by TLC with appropriate standards as controls (Table 1), using plates prewashed with the solvent system, and spotting of aliquots of the incubation mixture directly on the plates. With the spectral procedure, the extent of the reaction was followed by the change in absorbance at alkaline pH resulting from conversion of nucleoside (or base) to the base (or nucleoside). For conversion of 3-methyluridine to the corresponding base, this was $\Delta E_{290} = 8.2 \times 10^3$ [19, 20]; for conversion of 6-methyluridine to 6-methyluracil, $\Delta E_{290} = 5.4 \times 10^3$ [21], for conversion of 5,6-dimethyluridine, 5,6-trimethyleneuridine and 5,6-tetramethyleneuridine to the corresponding bases, $\Delta E_{300} = 4.1$, 6.4 and 3.3×10^3, respectively [18].

Conversion of xanthine and uric acid to their N(3)-ribosides, and phosphorolysis of the latter, was also followed spectrophotometrically at pH 8, at 265 nm and 305 nm, respectively, the corresponding absorbance changes being $\Delta E_{265} = 4.25 \times 10^3$ and $\Delta E_{305} = 11.6 \times 10^3$ [16a].

Purine nucleoside phosphorylase (purine nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1) activity was followed by TLC.

Kinetic constants. These were determined graphically from double reciprocal plots of substrate concentration vs initial velocity.

Protein. Protein was estimated according to Lowry *et al.* [22], with bovine serum albumin as standard.

The standard reaction mixture for following the phosphorolysis reaction was essentially as described elsewhere [4, 5], slightly modified as follows: reactions were terminated by addition of ice-cold 0.2 N NaOH, or, in the case of xanthine and uric acid, of ice-cold 0.2 M Tris–HCl buffer pH 8, followed by readings of absorbancy at the appropriate wavelength. The medium for monitoring of nucleoside synthesis consisted of Tris–HCl buffer pH 7.4 (50 mM), α-D-ribose-1-phosphate (5 mM), the base being tested (2 mM, and less for uric acid, because of its lower solubility), and enzyme. Ribosyl transfer reactions, monitored by TLC, were performed with incubation media containing Tris–HCl buffer pH 7.4 (50 mM), uridine (ribosyl donor, 2 mM), the desired base (ribosyl acceptor, 2 mM), phosphate buffer pH 7.4 (1 mM), and enzyme.

For nucleoside synthesis on a milligram scale, incubation times varied from 4 to 6 hr, and ribose-1-phosphate was added in two portions, one at the start of the reaction, the second after 2–3 hr incubation. The reaction was terminated by heating to 100° for 2 min, followed by centrifugation. The nucleoside was then isolated from the supernatant by ascending chromatography on Whatman 3mm paper with methanol–H_2O–conc. NH_4OH (6:1:1, v/v), and eluted with water.

Identity of reaction products was determined by chromatography, and by comparison of ultraviolet absorption spectra of eluates at various pH values with those of authentic controls. In the absence of authentic N(3)-ribosylxanthine, the ultraviolet

absorption spectrum of our enzymatically synthesized product was compared with data reported for N(3)-ribosylxanthylic acid [16a, b], and the corresponding N(3)-methylxanthine [23].

RESULTS

Table 2 lists the relative rates of synthesis (from α-D-ribose-1-phosphate and the appropriate base) and phosphorolysis of various uracil/uridine analogues by the pyrimidine nucleoside phosphorylase of *S. typhimurium*. The measured K_m and V_{max} values for the phosphorolysis reaction are presented in Table 3.

The susceptibility of 5-substituted uridines to pyrimidine nucleoside phosphorylases is well known. It is, nonetheless, of interest to note that this susceptibility decreases appreciably with an increase in the bulk of the 5-substitutent from H, through CH_3, to CH_2CH_3. Although the susceptibility of 5-fluoridine to phosphorolysis also decreases relative to uridine, but to a lesser extent, it will be seen that 5-fluorouracil is almost as good a substrate as uracil in the reverse reaction. This may be related to the fact that the pK_a for dissociation of 5-fluorouracil analogues is in the physiological pH range [24], so that they consist of a mixture of the neutral and ionic forms.

It is of some interest that 3-methyluracil is inactive as a substrate in the synthetic reaction. The rather low susceptibility of 3-methyluridine to phosphorolysis is equally striking, and reminiscent of the resistance to pancreatic ribonuclease of poly(3-methylU) and 3-methyluridine-2′,3′-cyclic phosphate [25]. We shall revert to this point below.

In contrast to 6-azauracil, which is a feeble substrate in the synthetic reaction, and 6-azauridine, which is fully resistant to phosphorolysis, both 6-methyluracil and 5,6-dimethyluracil are inactive in the synthetic reaction, whereas 6-methyluridine and 5,6-dimethyluridine are readily phosphorolysed. In fact, 6-methyluridine is almost as good a substrate as uridine, in confirmation of previous observations on the phosphorolysis of this analogue by both a bacterial [4] and a mammalian [5] enzyme.

Table 2. Relative rates of synthesis (from α-D-ribose-1-phosphate and appropriate base) and phosphorolysis (of 5(6)-substituted uridines or their analogues) by *S. typhimurium* pyrimidine nucleoside phosphorylase*

Nucleoside (or base)	Relative activity (%) Synthesis	Phosphorolysis
Uridine	100	100
3-Methyl-	0	<5
5-Methyl-	61	35
5-Ethyl	52	24
5-Fluoro-	94	70
6-Aza-	5†	0
6-Methyl-	0	56
6-Carboxy-(orotidine)	0	0
5,6-Dimethyl-	0	50
5,6-Trimethylene-‡	12	26
5,6-Tetramethylene-‡	0	25
N(3)-Ribosylxanthine	18	29
N(3)-Ribosyluric acid‡	7	23

* Reactions monitored spectrophotometrically (see Materials and Methods). The specific activities of the *S. typhimurium* extracts for synthesis and phosphorolysis of uridine were, respectively, 0.87 and 0.94 μmole/mg protein/min at 37°.

† Estimated from TLC.

‡ This is a formal analogue of a 5,6-disubstituted uracil or uracil nucleoside.

The somewhat unexpected behaviour of 6-methyluridine, and 5,6-dimethyluridine, directed our attention to the N(3)-ribosides of xanthine and uric acid, both of which may be considered formally as 5,6-disubstituted uridine analogues. As will be noted from Tables 2 and 3, both of these do, in fact, undergo phosphorolysis and, furthermore, at rates comparable to those for 6-methyl uridine and 5,6-dimethyluridine. Such substrate activity cannot be ascribed to the purine nucleoside phosphorylase present in our extract, since it had previously been shown that purified preparations of this bacterial

Table 3. Apparent kinetic constants for phosphorolysis of 5- and/or 6-substituted uridine analogues*

Nucleoside	K_m (mM)	V_{max} (μmoles/mg protein/min)
Uridine	0.72	1.40
3-Methyl-	3.72	0.09
5-Methyl-	1.60	0.64
5-Ethyl-	1.90	0.42
5-Fluoro-	1.64	1.20
6-Methyl-	1.04	0.90
5,6-Dimethyl-	2.03	0.53
5,6-Trimethylene-	1.0	0.40
5,6-Tetramethylene-	3.44	0.43
N(3)-Ribosylxanthine	1.85	0.50
N(3)-Ribosyluric acid	4.1	0.60

* Kinetic parameters determined spectrophotometrically (see Materials and Methods). The corresponding parameters for inorganic phosphate (P_i), with uridine as the non-variable substrate, were $K_m = 3.6$ mM and $V_{max} = 1.4$ μmoles/mg protein/min at 37°.

enzyme [26] are totally inactive against xanthosine and xanthine.

Furthermore, both of the corresponding bases, xanthine and urate, readily undergo the reverse synthetic reaction, i.e., conversion to the N(3)-ribosides. The spectral properties of these products unequivocally eliminated the possible formation of N(9)-ribosides.

The substrate properties of xanthine and urate, and their N(3)-ribosides, prompted us to examine those of 5,6-trimethyleneuracil, 5,6-tetramethyleneuracil, and the ribonucleosides of both of these (see Scheme 3). As will be seen from Tables 2 and 3, both nucleosides are good substrates in the phosphorolysis reaction, with rates comparable to those for the N(3)-ribosides of xanthine and urate. However, only the trimethylene derivative of uracil is a substrate in the reverse, synthetic, reaction.

Ribosyl exchange reaction. In the absence of exogenous inorganic phosphate, there was no detectable ribosyl transfer between uridine (as ribosyl donor) and the various modified bases; nor uridine formation from uracil (as ribosyl acceptor) and the modified nucleosides as ribosyl donors.

Addition of P_i to the incubation medium led to ribosyl exchange between nucelosides and free bases, with results listed *qualitatively* in Table 4. It will be noted that, of the bases examined, only those were active in the ribosyl exchange reactions which were also active acceptors of the ribosyl moiety from α-D-ribose-1-phosphate (Table 2). Similarly, only those nucleosides which were ribosyl donors to uracil were also substrates in the phosphorolysis reaction (Table 2). It follows that the ribosyl exchange reaction catalyzed by the *S. typhimurium* enzyme occurs via ribose-1-phosphate, and that it is mediated via nucleoside phosphorolysis and synthesis.

DISCUSSION

In considerations on the substrate properties of a 6-substituted (or 5,6-disubstituted) uracil or uracil nucleoside, it should be recalled that 6-methyluridine, as well as other 6-methylpyrimidine nucleosides, are constrained by steric hindrance of the 6-methyl substituent to the *syn* conformation about the glycosylic bond, both in the solid state [27] and in solution [3]. Uridine itself, as well as other non-substituted uracil and cytosine nucleosides, exist in solution as an equilibrium mixture, *syn* ⇌ *anti*, with the *anti* conformation predominating [2], and a relatively low energy barrier for interconversion between the two forms [2]. The presence of a 5-substituent, such as halogeno or alkyl, does not affect this situation.

The facility with which such an interconversion of the *syn* and *anti* conformations occurs is well illustrated in the case of 4-thiouridine which, when crystallized from water, is in the *syn* conformation; but, when crystallized from butyric acid, is in the conformation *anti* [28].

Since 6-methyluridine, exclusively in the *syn* conformation, exhibits a K_m for phosphorolysis of 1.04×10^{-3} M, as compared to 0.72×10^{-3} M for uridine, it may be concluded that interaction of the latter with the enzyme is accompanied by its conversion to the *syn* form, followed by phosphorolysis. It might be argued that the enzyme does not distinguish between the *anti* and *syn* conformations of the uracil moiety about the glycosylic bond. That this is not so is shown by the fact that 3-methyluridine is a very poor substrate, while 5-methyluridine (and 5,6-dimethyluridine) is a good one (see Tables 2 and 3).

Orotidine (6-carboxyuridine), which is exclusively in the *syn* conformation, is not a substrate; nor is 6-azauridine, which prefers the conformation *anti*. This suggests that it is not the conformational properties which account for this behaviour, but the electronegative N(6) in 6-azauridine, and the ionized carboxyl group in orotidine, which lead to electrostatic repulsion of the inorganic phosphate involved in the reaction. Such an effect may occur when the phosphate approaches C(1′) in the direction *cis*oidal to the electronegative substituent, which is in fact the case when the base is oriented in the conformation *syn* about the glycosidic bond.

In the reverse, synthetic, reaction, however, the enzyme does not accept 6-methyluracil (or 5,6-dimethyluracil) as a substrate. It follows that the

Table 4. Effect of modification of uracil ring on ribosyl exchange reaction in presence of 1 mM orthophosphate*

Uracil ring modification	Ribosyl acceptor from uridine	Ribosyl donor for uracil
3-Methyl-	−	n.t.†
5-Methyl-	+	+
5-Ethyl-	+	+
5-Fluoro-	+	+
6-Aza-	+	n.t.
6-Methyl-	−	+
5,6-Dimethyl-	−	+
5,6-Trimethylene-	+	+
5,6-Tetramethylene-	−	+
Xanthine	+	+‡
Uric acid	+	+‡

* Reactions were followed by TLC.

† n.t., not tested; +, ribosyl transfer occurs; −, no detectable ribosyl transfer.

‡ N(3)-Ribosyl product.

ribose-1-phosphate and pyrimidine ring in this case are mutually oriented such that the approach of the pyrimidine to the ribose C(1′) is sterically hindered by interference between the pyrimidine 6-methyl and the plane of the sugar ring, i.e., the orientation of the base relative to the sugar is diametrically different in the synthetic reaction (as compared to that for phosphorolysis), being formally *anti* if we consider both components as a single entity.

The substrate properties of 5,6-trimethylene uracil, which does participate in the synthetic reaction, points to the existence of some tolerance in the geometry of the substituent at C(6). The ability of this analogue to overcome steric interference with the furanose ring is probably due to the fact that the 5,6-trimethylene ring is puckered, so that the planar angle N(1)–C(6)–C(7) is larger than in the case of 6-methyluracil or 5,6-tetramethyleneuracil.

The behaviour of 5,6-trimethyleneuracil is reflected also by xanthine and uric acid, both considered as formal analogues of 5,6-disubstituted uracils, and which are converted to the corresponding N(3)-ribosides. The planar angle N(3)–C(4)–N(9) of the xanthine ring is 129.2° [29], hence appreciably larger than the planar angle N(1)–C(6)–C(H_3) in 6-methyluracil, 119° [27] (see Schemes 1 and 2). And in uric acid the corresponding angle is probably also larger than in 6-methyluracil, since the saturated imidazole ring is puckered as in 5,6-trimethyleneuracil.

In the light of the foregoing results, suggesting that phosphorolysis of uridine, and 5-substituted uridine analogues, by pyrimidine nucleoside phosphorylase, proceeds via the initial conversion of the nucleoside to the conformation *syn* about the glycosidic bond, it will be of obvious interest to examine the conformational requirements in the reactions catalyzed by pyrimidine nucleotide pyrophosphorylase. Bearing in mind that this enzyme catalyzes the formation of the 5′-phosphates of the N(3)-ribosides of xanthine and uric acid [16a,b, 17], it appears most likely that in this instance as well the conformation about the glycosidic bond is also of importance. Furthermore, the conformational requirements of the uridine phosphorylase reaction may be of relevance in relation to the activity of some chemotherapeutic agents, e.g., 5-fluoro-2′-deoxyuridine, which is no more effective than 5-fluorouracil because of its rapid phosphorolysis *in vivo* by pyrimidine nucleoside phosphorylase, whereas 5-fluorouracil is converted eventually to the active 5-fluoro-2′-deoxyuridine-5′-phosphate.

The present results may also be relevant to the known potent inhibitor properties of 5-fluoro-2′-deoxyuridine-5′-phosphate with respect to thymidylate synthetase. Although a wide variety of 5-substituted 2′-deoxyuridine-5′-phosphate analogues have been examined as inhibitors of this important enzyme, with a view to their use as anti-tumour agents [30], no attention has hitherto been devoted to the possible role of the conformation of these analogues about the glycosidic bond.

It should be recalled that, although the present results were obtained with the use of an enzyme of bacterial origin, it had been previously noted that 6-methyluridine is effectively phosphorylysed by the analogous enzyme from a mammalian source, viz. primary rabbit kidney cells [5]. Hence, at least some of the conclusions derived from this study are equally applicable to the enzyme(s) from mammalian sources.

Acknowledgements—We are very much indebted to Dr. M. Draminski, Dr. A. Holy, and Dr. H. Witzel, for gifts of compounds, and to Mrs. M. Dutkowska for technical assistance. This investigation profited from the support of the Polish National Cancer Research Program (PR-6).

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