Acta Biochimica Polonica

Vol. 43 No. 4/1996

733–742

QUARTERLY

Uridine phosphorylase from *Hymenolepis diminuta* (Cestoda): Kinetics and inhibition by pyrimidine nucleoside analogs

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Received: 4 October, 1996

Keywords: uridine phosphorylase, kinetics, inhibitors, *Hymenolepis diminuta*

A single pyrimidine nucleoside phosphorylase was found in the cytoplasmic extract from *Hymenolepis diminuta*. This enzyme preferentially cleaves uridine and, to a much lesser extent, thymidine. Its presence directly indicates the existence of pyrimidine nucleoside salvage pathway in this parasite.

Detailed kinetic studies in the phosphorolytic and synthetic direction pointed to the sequential mechanism of these reactions. For phosphorolysis, K_{urd} = 33 μM and K_P = 806 μM. For synthesis of uridine, K_{ura} = 204 μM and $K_{1\text{-P-rib.}}$ = 50 μM. Over six times higher K_m for uracil than for uridine indicates that phosphorolysis is the favoured reaction in this tapeworm.

Well known inhibitors of mammalian uridine phosphorylase: 2,2'-anhydro-5--ethyluridine and 1-(1,3-dihydroxy-2-propoxymethyl)-5-benzyluracil (DHPBU), both with K_i = 0.07 μM were potent competitive inhibitors of the enzyme from *H. diminuta*. The newly synthesized 2,3'-anhydro-5-ethyluridine (K. Felczak, unpublished) showed only moderate inhibitory activity (K_i = 14 μM) similarly as 1-(1,3-dihydroxy-2--propoxy-methyl)-5-benzyluracil. The same order of K_i values obtained for the investigated inhibitors *vs* uridine phosphorylase, irrespective whether the enzyme was isolated from rat intestinal mucosa (Drabikowska *et al.*, 1987, *Biochem. Pharmacol.* 36, 4125–4128) or *H. diminuta* may point to a great similarity between binding sites on the parasite and the host enzyme.

Uridine phosphorylase (UrdPase; EC 2.4.2.3) plays a key role in pyrimidine metabolism because of its participation in nucleoside degradation and in the salvage pathway. In most mammalian, especially neoplastic cells, and in some bacteria, its function seems to be catabolic (Krajewska *et al.*, 1978; Leyva *et al.*, 1982; Monks *et al.*, 1983; Neuhard, 1983; Pérignon *et al.*, 1987) but, under special conditions, the enzyme can utilize uracil and 1-P-ribose for nucleoside synthesis. Thus, UrdPase is believed to be able to modulate the salvage pathway by either anabolizing or catabolizing nucleosides.

Free pyrimidine bases and pyrimidine nucleosides, arising from digestion of nucleic acids in the host's ingestia, are available to intestinal parasites (Wilson & Wilson, 1958). These compounds may also be provided effectively by surface phosphohydrolases present on the worm's tegument (Dike & Read, 1971; Pappas, 1980). Exogenous pyrimidine compounds are actively transported across the *H. diminuta* plasma membrane (Page & MacInnis, 1975; Uglem *et al.*, 1983), and probably can be incorporated into nucleic acids by the salvage pathway. As shown by Campbell (1960), pyrimidines can also be degraded in *H. diminuta* to

products serving as sources of carbon, energy and nitrogen.

In extracts of *H. diminuta* five of six enzymes of the biosynthetic pathway have been identified, providing evidence that these parasites are capable of *de novo* synthesis of UMP (Hill *et al.*, 1981). However, the high rate of tapeworm reproduction suggests that this biosynthetic route, due to low activities of the participating enzymes, may not be sufficient to fulfill the pyrimidine requirement for the necessarily high rate of nucleic acid synthesis. Thus it would be reasonable if these parasites took advantage of the pyrimidine salvage pathway.

Presently, there is little information regarding this pathway, but elucidation of pyrimidine metabolism in cestoda and characterization of the enzymes involved in this process will, certainly, direct attention to the potential targets for the use of pyrimidine analogs as antiparasitic agents.

The purpose of this study was to examine an isolated, partially purified *H. diminuta* uridine phosphorylase, the enzyme of the pyrimidine salvage pathway. It represents potential site for chemotherapy with antipyrimidine agents. The kinetic studies, as well as inhibitory effects of some pyrimidine nucleoside analogs on this enzyme, are also presented.

MATERIALS AND METHODS

Chemicals. [U-^{14}C]Uridine (485 mCi/mmol) and [methyl^{14}C]thymidine (50–62 mCi/mmol) were obtained from Amersham International; [2-^{14}C]uracil (12.5 mCi/mmol) was from Institute for Research and Application of Radioisotopes (Czechoslovakia). DEAE-Sephacel, dithiothreitol (DTT) and D-ribose 1-phosphate, cyclohexylammonium salt, uracil, uridine, thymine, thymidine and EPPS (*N*-2-hydroxyethylpiperazine-*N'*-3-propane sulfonic acid) were from Sigma Chemical Co. (St. Louis, U.S.A.) and silica gel 60F_{254} aluminium sheets from Merck (Darmstadt, Germany). All other chemicals were of analytical grade from POCh (Gliwice, Poland).

Source of worms and preparation of extract. Wistar inbred male rats (bred at the Nencki Institute of Experimental Biology, Warsaw, Poland), 10–12 weeks old, were infected with *Hymenolepis diminuta* cysticercoids. The animals were killed 11 days after infection.

The tapeworms were recovered from small intestines, the extract was prepared and further procedures of thymidylate synthase purification (Jastreboff *et al.*, 1982) were performed by Cieśla *et al.* (1987). The outflow remaining after thymidylate synthase isolation by affinity chromatography was stored at –20°C. Since this fraction contained pyrimidine nucleoside phosphorylase activity, it was used for further purification of the enzyme.

The above-mentioned enzymatic fraction was thawed and dialysed thoroughly against several changes of 20 mM phosphate buffer, pH 8.0, containing 1 mM DTT and 1mM EDTA, for 10 h.

Isolation of uridine phosphorylase. A DEAE-Sephacel column (75 mm × 23 mm) was equilibrated with the buffer used for dialysis. About 60 mg protein of the enzymatic fraction was applied on this column and eluted first with the same buffer (64 ml) and then with a linear gradient of NaCl (0–1.0 M). Fractions of 4 ml were collected at 4°C and assayed for uridine or thymidine cleavage activity.

Enzyme assays. The phosphorolysis of nucleosides was measured radioisotopically. The standard incubation medium contained 50 mM phosphate buffer, pH 7.5, 1 mM DTT, 1 mM EDTA, [U-^{14}C]uridine or [methyl-^{14}C]thymidine in the total volume of 100 µl. Following preincubation for 5 min, the reaction was initiated by addition of enzyme. Incubation was carried out for 5–15 min at 37°C and terminated by immersion of the sample tubes in boiling water for 2 min. The tubes were next transferred to an ice bath for about 10 min and then 100 µl of ice-cold methanol added. The resulting precipitate was removed by centrifugation, and 30–40 µl of a clear sample spotted on silica-gel thin-layer chromatography plates along with a cold reference sample of uridine + uracil, or thymidine + thymine, followed by development with methanol/chloroform (1:9, v/v) for thymidine and thymine, and supplemented with 0.1% H_3BO_3 for uridine and uracil. The spots corresponding to uracil and uridine, or thymine and thymidine, were localized with a UV lamp, cut out, transferred to scintillation vials and radioactivity counted in a toluene scintillator with an LKB 1209 RACKBETA instrument.

R_F values obtained were: uridine, 0.00; uracil, 0.30; thymidine, 0.16; thymine, 0.35.

Kinetic studies. The enzyme used in all kinetic studies was that purified on a DEAE-Sephacel column, desalted and concentrated with the use of Sephadex G-25 gel. For synthesis of uridine from uracil and 1-P-ribose, phosphate was eliminated from the preparation by thorough dialysis against 20 mM EPPS, 1 mM DTT, 1 mM EDTA buffer, pH 8.0.

Detailed conditions of initial velocity studies with uridine and phosphate, or uracil and 1-P-ribose as substrates, are described in legends to the Figures.

Apparent K_i values were determined from Dixon plot $1/v$ *versus* [I] in the presence of different fixed concentrations of uridine (32–80 μM). Apparent K_m was determined from a Lineweaver-Burk plot. The values were calculated by the Cricket-Graph computer program or Linear Regression Program for K_i and K_m, respectively.

Protein determination. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Properties of uridine phosphorylase

The partially purified cytosolic fraction of *H. diminuta* remaining after thymidylate synthase isolation (further referred to as the enzymatic fraction) showed uridine cleavage activity. This activity was pH-dependent, with an optimum at pH 7.5–8.0, as for uridine phosphorylases isolated from other sources (Pontis *et al.*, 1961; Leer *et al.*, 1977; Yamada, 1968). The enzymatic fraction, in addition to uridine, also cleaved thymidine, albeit to a much lesser extent. To verify whether these activities were due to two distinct enzymes or to a single nonspecific uridine phosphorylase, the enzymatic fraction was subjected to DEAE-Sephacel chromatography, which has been shown to separate efficiently uridine and thymidine phosphorylases from different organisms and tissues (Krenitsky *et al.*, 1964; Niedzwicki *et al.*, 1981; el Kouni *et al.*, 1988a; el Kouni *et al.*, 1993).

The elution profile obtained from DEAE-Sephacel (Fig. 1) indicated that only uridine, but not thymidine phosphorylase was present in the enzymatic fraction from *H. diminuta* since thymidine phosphorylase activity did not occur in any fraction prior to application of the NaCl gradient. Thus the *H. diminuta* enzymes resembles the enzymes from mouse intestinal epithelium, mouse liver and Ehrlich ascites tumor (Krenitsky *et al.*, 1964) and suggests that the enzyme, unlike the enzymes from human liver and placenta (el Kouni *et al.*, 1993) carries a negative charge at pH 8.0 or has a significantly lower hydrophobicity.

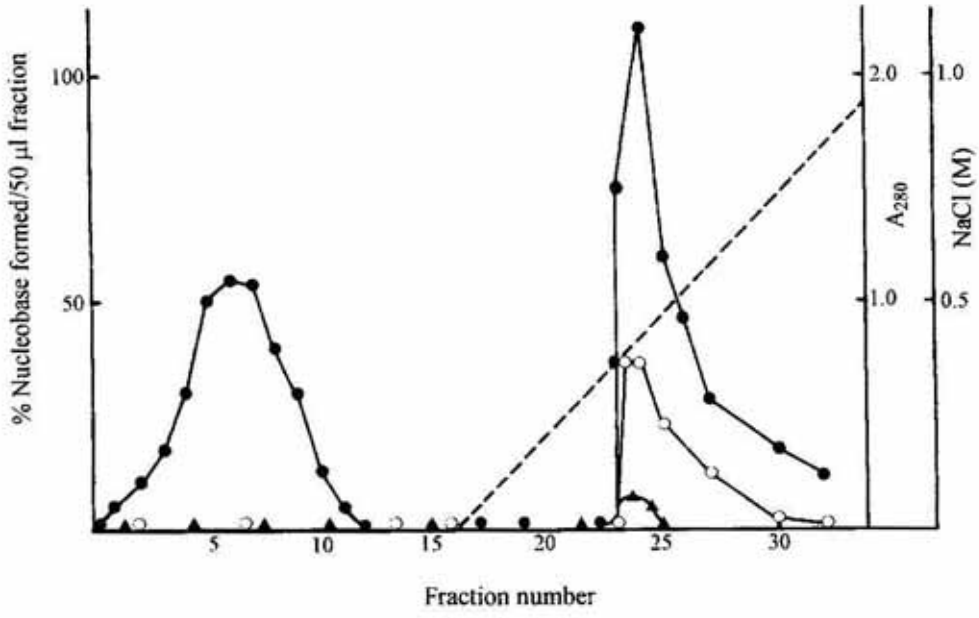

Fig. 1. Elution profile of the pyrimidine nucleoside phosphorylase from a DEAE-Sephacel column as outlined in Materials and Methods.
The column was eluted first with the buffer and then with an increasing gradient of NaCl (– – –). Protein was measured spectrophotometrically at 280 nm (●). Fractions before and after applying the NaCl gradient were assayed for activity *vs* uridine (○) and thymidine (▲) as substrates.

Table 1

Uridine and thymidine phosphorolysis catalyzed by uridine phosphorylase from Hymenolepis diminuta after DEAE-Sephacel column chromatography.

Standard reaction mixture was used as described in Materials and Methods. Uridine (112 μM) and thymidine (96 μM) as substrates, and 2,2'-anhydro-5-ethyluridine (1 μM) a specific inhibitor of uridine phosphorylase were used, in the presence (+) or absence (–) of phosphate. In the experiments without phosphate the reaction was carried out in 50 mM EPPS buffer, pH 8.0, supplemented with 1 mM EDTA and 1 mM DTT. The results represent data from two separate experiments. The ratio of activity *vs* thymidine to uridine was 10–12%.

Substrate	P_i	Cleavage activity (nmol/min × mg protein)	Inhibition (%)
Uridine	+	5.4	
Uridine + inhibitor	+	0.0	100
Uridine	–	0.0	
Thymidine	+	0.5	
Thymidine + inhibitor	+	0.0	100

The results presented in Fig. 1 and Table 1 indicate that non specific uridine phosphorylase from *H. diminuta* cleaves phosphorolytically both uridine and thymidine to their nucleobases. The activity *vs* uridine was about 10-fold higher than with thymidine as a substrate, in both the enzymatic preparation before (not shown) and after DEAE-Sephacel purification.

The enzyme preparation was very unstable and lost part of its activity on each cycle of freezing and thawing but the ratio of the activity *vs* uridine to thymidine remained constant.

To further check that the phosphorolytic activity *vs* thymidine was not due to thymidine phosphorylase that may still have been present in the enzymatic preparation, 2,2'-anhydro-5-ethyluridine, a potent and specific inhibitor of uridine, but not of thymidine, phosphorylase (Veres *et al.*, 1985), was used. In the presence of this inhibitor the activity against both substrates was completely abolished. This indicates that the pyrimidine nucleoside cleavage activity can be ascribed to the presence of only uridine phosphorylase and that uridine phosphorylase is the only pyrimidine nucleoside cleaving enzyme present in *H. diminuta*. A single pyrimidine nucleoside phosphorylase was also shown to be present in *Schistosoma mansoni* (el Kouni *et al.*, 1988a), in the intestinal mucosa of the rat (Veres *et al.*, 1985), the host organism of this parasite as well as in several neoplastic cells (Krenitsky *et al.*, 1964; Niedzwicki *et al.*, 1981).

Moreover, in the absence of phosphate, uridine was not cleaved hydrolytically, excluding the existence of any hydrolase in this preparation.

Kinetic studies

Experimental data for uridine phosphorolysis by *H. diminuta* uridine phosphorylase, plotted as a double-reciprocal plot of initial velocities *vs* inorganic phosphate concentration at several constant concentrations of uridine gave a series of lines intersecting at a point to the left of the ordinate above the abscissa (Fig. 2).

A similar pattern (not shown) was obtained when uridine was the variable substrate at several constant concentrations of phosphate.

In the reaction of nucleoside synthesis (Fig. 3), with either 1-P-ribose or uracil as variable substrates and 1-P ribose or uracil as nonvariable substrates, the same pattern of kinetics was observed. For both the phosphorolytic and synthetic directions of these reactions, the point of intersection of the lines suggests that the sequential mechanism (Segel, 1975) is obeyed by uridine phosphorylase from *H. diminuta*. The values of the kinetic constants for *H. diminuta* uridine phosphorylase are presented in Table 2.

These results together with those of others (Krenitsky, 1976; Bose & Yamada, 1974; Vita & Magni, 1983; el Kouni *et al.*,1988a; Avraham *et al.*, 1990) suggest that this reaction mechanism may be common to all uridine phosphorylases, irrespective of the source.

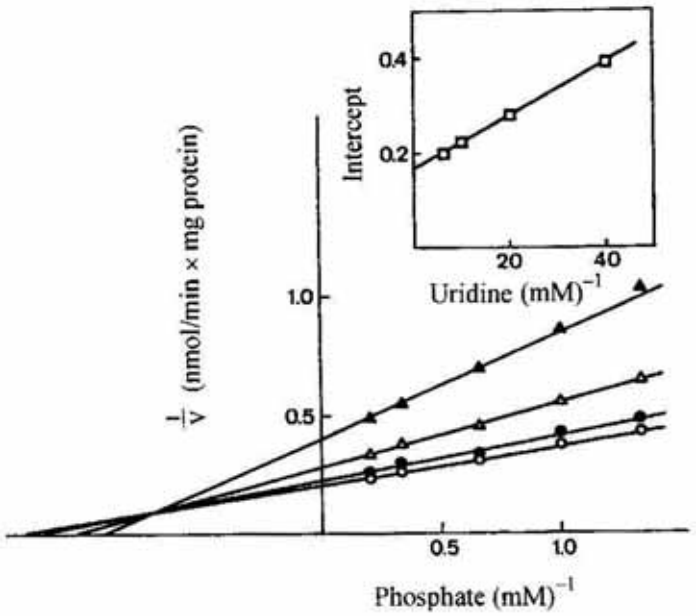

Fig. 2. Initial velocity studies of uridine phosphorolysis.
Plots of $1/v$ *vs* $1/P$ as variable substrate at various constant millimolar concentrations of uridine: 0.025 (▲), 0.05 (Δ), 0.10 (●), 0.15 (○). Assay conditions as described in Materials and Methods. K_{ia} for phosphate was determined from the intersection point of the lines at the coordinate 1/P. Insert: replot of y intercepts *vs* reciprocals of uridine concentration for K_b determination. For kinetic data see Table 2.

A six-fold higher K_m value of uracil (204 ± 19 μM) in the reaction of uridine synthesis than in uridine phosphorolysis (33 ± 2.5 μM) indicates that the last mentioned reaction is favoured. At this stage of purification, the specific activity for uridine cleavage was about 6 nmol/min × mg protein.

Inhibition of *H. diminuta* phosphorylase by pyrimidine nucleoside analogs

Several inhibitors effective against uridine phosphorylase from rat intestinal mucosa (Veres *et al.*, 1985; Drabikowska *et al.*, 1987b), *E. coli* (Park *et al.*, 1986; Drabikowska *et al.*, 1987a) and Sarcoma-180 cells (Niedzwicki *et al.*, 1981; Lin & Liu, 1985) were tested with the *H. diminuta* enzyme.

Table 3 presents apparent K_i data for inhibition of uridine phosphorolysis by *H. diminuta* uridine phosphorylase. Of the two anhydrouridines tested, 2,2'-anhydro-5-ethyluridine (I), was a more potent inhibitor than 2,3'-anhydro-5-ethyluridine (II). A similar K_i value for compound II was obtained with the rat intestinal mucose uridine phosphorylase (14 μM, unpublished).

The significant difference in the inhibitory potency between compound I (K_i = 0.07 μM) and II (K_i = 14 μM) may be simply explained by the lack of a free 3'-OH in the latter. This group

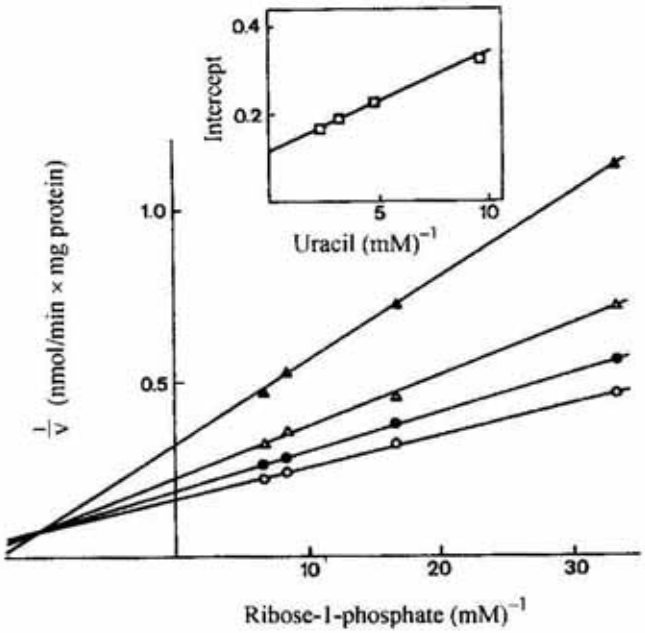

Fig. 3. Initial velocity studies of uridine synthesis.
Plots of $1/v$ *vs* 1/ribose-1-P as variable substrate at following constant millimolar concentrations of uracil: 0.103 (▲), 0.206 (Δ), 0.309 (●), 0.412 (○) mM. Assay conditions as outlined in Materials and Methods. The kinetic data were determined as described in the legend to Fig. 2 and are presented in Table 2.

Table 2

Kinetic constants for uridine phosphorylase from Hymenolepis diminuta.
Experimental conditions as described under Materials and Methods. K_{ia} and K_{ib} were estimated from the intersection points, K_a and K_b from the replots as shown, for example, in Figs. 2 and 3. K_{ia} designates a dissociation constant for the EA complex with substrate A, K_{ib} designates a dissociation constant for the EB complex with substrate B. K_a and K_b are the K_m for A or B, respectively, at a saturating concentration of the second substrate. Each value is the mean (±SD) of at least three independent experiments.

Reaction	Substrate	Kinetic constant (μM)	
Phosphorolysis	phosphate (A)	K_{ia}	1484 ± 56
		K_a	806 ± 75
	uridine (B)	K_{ib}	57 ± 6.8
		K_b	33 ± 2.5
Nucleoside synthesis	1-P-ribose (A)	K_{ia}	92 ± 12
		K_a	50 ± 8
	uracil (B)	K_{ib}	357 ± 34
		K_b	204 ± 19

was postulated to be essential for forming a hydrogen bond with mammalian uridine phosphorylase both with acyclonucleosides and substrates (Etzold *et al.*, 1968; Niedzwicki *et al.*, 1981; el Kouni *et al.*,1988b). At variance with this assumption are suggestions that a 3'-OH is essential only for nucleosides which undergo enzymatic catalysis, but is not a prerequisite for binding to the enzyme (Veres *et al.*, 1991).

One may assume that the shift of the anhydro bond from $O^2O^{2'}$ to $O^2O^{3'}$ causes a substantial change of the glycosidic torsion angle χ, thus decreasing binding affinity to the enzyme. Indeed, ^{14}C and ^{1}H NMR studies and computer-aided molecular modelling demonstrated a substantial change of χ from 109° for 2,2'-anhydro-5-ethyluridine to 78° for 2,3'-anhydro-2'-deoxy-5-ethyluridine (Veres *et al.*, 1988). A similar change of χ could then explain the differences between K_i values of inhibitor I and II. Undoubtedly, not only the conformation of the inhibitor, but also its electron distribution density (Delbaere *et al.*, 1973), are decisive in formation of an enzyme-inhibitor complex.

Table 3

Apparent kinetic constants: K_i for inhibition of phosphorolytic activity of uridine phosphorylase from Hymenolepis diminuta by acyclo- and anhydro-uridine analogs and K_m for uridine.
Assay conditions as outlined in Materials and Methods.

Inhibitors	App. K_i (μM)
2,2'-Anhydro-5-ethyluridine (I)	0.07 ± 0.01
2,3'-Anhydro-5-ethyluridine (II)	14.10 ± 1.1
DHPBU* (III)	0.07 ± 0.01
Tetramethylene acyclouridine (IV)	8.80 ± 0.9
Substrate	App. K_m (μM)
Uridine	47.0 ± 1.0

SD was calculated from at least three independent experiments. *DHPBU is 1-(1,3-dihydroxy-2-propoxymethyl)-5-benzyluracil (III); tetramethylene acyclouridine is 1-(1,3-dihydro xy-2-propoxymethyl)-5,6-tetramethyleneuracil (IV).

Recently, several anhydrouridines with various modifications to the uracil and sugar moieties and other pyrimidine nucleoside analogs, have been tested with respect to their potency to bind to uridine phosphorylase from *Toxoplasma gondii* (el Kouni *et al.*, 1996). These authors concluded that the capacity to adopt the *high syn* or *syn* conformation are critical structural and conformational requirements for nucleoside ligand to bind to *T. gondii* enzyme. They also observed that only those nucleoside ligands with the N3 of pyrimidine ring being a hydrogen acceptor can participate in hydrogen bonding with the parasitic but not host enzyme active site.

However, the identical inhibitory efficacy of anhydrouridine and acyclouridine (see Table 3, compound I and III, respectively) with the *H. diminuta* uridine phosphorylase cannot be ex-

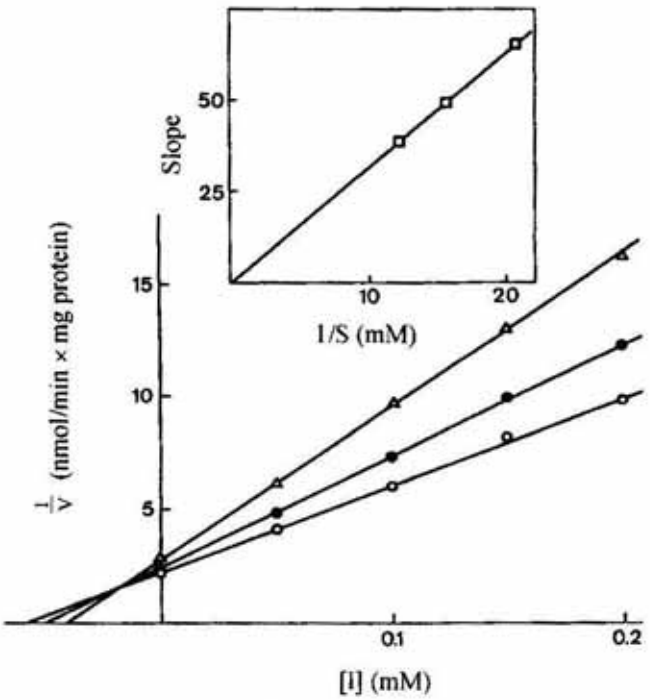

Fig. 4. Dixon plot for inhibition of H. diminuta uridine phosphorylase by 2,3'-anhydro-5-ethyluridine. Uridine concentrations (μM) were: 48 (Δ), 64 (●), 80 (○). The reaction was followed as described in Materials and Methods.

plained, in the case of both ligands, by participation of N3 of the pyrimidine ring in hydrogen bonding to the enzyme. Unlike the 2,2'-anhydrouridines, the N3 of the pyrimidine ring of the acyclouridines can be a hydrogen donor, but not an acceptor. Since N3 of the acyclouridine (III) cannot participate in the hydrogen bonding but can rotate around N-glycosyl bond to the favored *high syn* conformation (Birnbaum *et al.*, 1986) as that in the 2,2'-anhydrouridines, it seems likely that this orientation of the pyrimidine ring is a prerequisite for compound I and III to be bound tightly to the *H. diminuta* uridine phosphorylase.

Only two acyclouridines analogs: 1-(1,3-dihydroxy-2-propoxymethyl)-5-benzyluracil (DHPBU, III) and 1-(1,3-dihydroxy-2-propoxymethyl)-5,6-tetramethyleneuracil (IV) have been tested as inhibitors of the *H. diminuta* uridine phosphorylase. The effectiveness of inhibition by compound III was similar *vs* UrdPase isolated from Sarcoma cells (Lin & Liu, 1985; Siegel & Lin, 1985) and uridine phosphorylase from rat intestinal mucosa, but much less effective *vs* the *E. coli* enzyme (Drabikowska *et al.*, 1987b). Compound IV exhibits moderate inhibitory properties towards *H. diminuta* but K_i is only two times lower than with rat intestinal mucosa enzyme (Drabikowska *et al.*, 1987b).

All the pyrimidine nucleoside analogs investigated proved to be competitive inhibitors. Figure 4 presents as an example, the Dixon plot for 2,3'-anhydro-5-ethyluridine.

The inhibitory properties of only few available pyrimidine nucleoside analogs towards uridine phosphorylase used in the present investigation, together with those described previously (Drabikowska *et al.*, 1987b) emphasize the differences in their inhibitory *potency* towards bacterial, mammalian and tapeworm enzymes.

Only slight differences were found between the response of the host and parasite enzymes to the pyrimidine analogs. However too low number of analogs tested does not allow to draw definite conclusions. Thus, more analogs should be examined in an attempt to find the proper inhibitor which could serve as an antyhelminthic drug.

The author is grateful to co-workers of Professor W. Rode for providing the enzymatic preparation from *Hymenolepis diminuta*. I thank Professor M. Draminski for the gift of 1-(1,3-dihydroxy-2-propoxymethyl)-5,6-tetramethyleneuracil and Dr Z. Veres for 2,2'-anhydro-5-ethyluridine. The synthesis of 2,3'-anhydro-5-ethyluridine by Dr K. Felczak is gratefully acknowledged.

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