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Deoxyribosyl Transfer

III. CATALYSIS OF 3-PENTOSYLPURINE FORMATION BY PYRIMIDINE NUCLEOSIDE PHOSPHORYLASES*

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SUMMARY

The pyrimidine nucleoside phosphorylases, thymidine phosphorylase and uridine phosphorylase, catalyze the formation of 3-deoxyribosyl- and 3-ribosylpurines, respectively, from xanthine or some of its derivatives and the corresponding pentose phosphate. Although the rates of enzymatic cleavage of the deoxyribo- and ribonucleosides are approximately equal, the rate of synthesis of the deoxyribonucleoside is considerably greater than that of the ribonucleoside under comparable conditions.

In a previous report (1) it was shown that a deoxyribose-containing nucleoside was formed, in the presence of thymidine phosphorylase, from xanthine and deoxyribose 1-phosphate and that this nucleoside had properties consistent with the 3-deoxyribosylxanthine structure assigned to it. In this paper the purine substrate specificity of this pyrimidine deoxyriboside phosphorylase is examined, and in addition the catalysis of 3-ribosylpurine formation by uridine phosphorylase from *Escherichia coli* is reported.

EXPERIMENTAL PROCEDURE

Materials—Xanthine, 8-chloroxanthine, 6-thioxanthine, deoxyribose 1-phosphate, ribose 1-phosphate, 8-aza-2-thioxanthine, uric acid, and xanthine-8-^{14}C were purchased from Calbiochem; 8-azaxanthine was obtained from the Cancer Chemotherapy National Service Center, National Institutes of Health; 6,8-dihydroxypurine from Aldrich; 3-methylxanthine from Cyclo Chemical Corporation; and all other purines were obtained through the kindness of Dr. M. E. Balis of the Sloan-Kettering Institute, Rye, New York.

Enzyme Sources—Highly purified human spleen thymidine phosphorylase was prepared as described previously (2). This preparation is free of detectable purine nucleoside phosphorylase (EC 2.4.2.1) and uridine phosphorylase (EC 2.4.2.3).

E. coli strain B was chosen as the source of uridine phosphorylase, since it could be readily purified to a point where no other nucleoside phosphorylase activities could be detected. The procedure used was essentially that of Razzell and Khorana (3). All operations were carried out at 3°. A 10% sonic extract prepared in 0.05 M phosphate buffer, pH 7.0, from frozen, stored *E. coli* cells was centrifuged at 30,000 × *g* (1 hour) to remove debris. The supernatant fraction was adjusted to pH 4.8 (glass electrode), the precipitate was collected by centrifugation and then redissolved in 0.05 phosphate buffer, pH 7.0, containing 0.001 M EDTA and 0.01 M mercaptoethanol. After reprecipitating at pH 4.8 and redissolving the precipitate, the pH was adjusted to 6.5 and protamine sulfate solution was added in the amount described (3). At this point thymidine phosphorylase activity was absent, but some purine nucleoside phosphorylase activity was still detectable. The latter activity could be removed by heating at 50° for 8 to 10 min with little loss in uridine phosphorylase activity. The enzyme preparation was dialyzed against 0.02 M Tris-HCl buffer, pH 7.0, containing EDTA and mercaptoethanol, to remove phosphate. This enzyme preparation can be stored frozen (−20°) for 4 to 6 weeks. The preparative procedure is summarized in Table I. Xanthine oxidase was purchased from Worthington.

Enzyme Assays—Thymidine phosphorylase activity was determined spectrophotometrically by measuring the formation of thymine from 10 mM thymidine in 0.1 M phosphate buffer, pH 7.0, by using the change in optical density at 300 mμ in alkaline solution (4). Uridine phosphorylase, with 10 mM uridine as the substrate, was determined similarly by measuring the change in ultraviolet absorption at 290 mμ (5).

Preparation of Nucleosides—The preparation of the 3-deoxyribosyl- and 3-ribosyl purines was carried out with a 1 mM base and 2.5 mM pentose 1-phosphate in 0.05 M Tris-HCl buffer, pH 7.2, with thymidine phosphorylase and uridine phosphorylase, respectively. The incubation times were between 6 and 8 hours for maximum yields. The nucleosides were isolated from the reaction mixture as previously described (1), except that the base and nucleoside could be separated with either water or 4% aqueous sodium citrate as the developing solvent.

Comparisons of the rates of synthesis of xanthine and uracil nucleosides were performed in a similar manner with ^{14}C-labeled bases. Xanthine and its nucleosides were separated on paper strips with 4% aqueous sodium citrate as the developing solvent. Uracil and its nucleosides were separated on paper with the use of

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ethyl acetate-formic acid-H_2O (12:1:7, upper phase) (6). The amount of base converted to nucleoside was determined by an integrating radiochromatogram scanner.

Phosphorolysis of the xanthine nucleosides by the pyrimidine nucleoside phosphorylases was determined in the presence of xanthine oxidase. The change in absorbance at 290 mμ caused by formation of uric acid was followed with a recording spectrophotometer.

TABLE I

Preparation of E. coli uridine phosphorylase

Fraction	Volume	Uridine phosphorylase	Thymidine phosphorylase	Purine nucleoside phosphorylase
	ml	*units/ml*		
Extract	18	110	84	39
pH 4.8 precipitate	20	49	ND[a]	2.6
Protamine supernatant	22	40	ND	0.8
Heated enzyme	22	38	ND	ND

[a] ND, not detectable.

TABLE II

Deoxyriboside formation from purines and deoxyribose 1-phosphate in presence of thymidine phosphorylase

The incubation mixture contained 1 mM base, 2 mM deoxyribose 1-phosphate, 2.5 units per ml (micromoles of substrate reacting per hour) of human spleen thymidine phosphorylase in 0.02 M Tris-HCl buffer, pH 7.2. Incubation was at 37° for 5½ hours. The following purines did not react to form a nucleoside in this system: adenine, guanine, hypoxanthine, 2-thioxanthine, 2,6-dithiopurine, 6,8-dithiopurine, 2-hydroxypurine, 2,8-dihydroxypurine, 2-fluoropurine, 8-azaxanthine, 8-aza-2-thioxanthine, and uric acid.

Base	Nucleoside formation	R_F[a]	
		Base	Nucleoside
Xanthine	+	0.37	0.55
8-Chloroxanthine	+	0.23	0.44
6-Thioxanthine	+	0.39	0.53
6,8-Dihydroxypurine	+	0.50	0.69
2,8-Dichloro-6-hydroxypurine	Low	0.38	0.54

[a] R_F with 4% sodium citrate (aqueous) on cellulose thin layers.

RESULTS

Formation of 3-Deoxyribosylpurines—In an earlier communication (1), evidence that highly purified human spleen thymidine phosphorylase catalyzed the biosynthesis of 3-deoxyribosylxanthine from xanthine and deoxyribose 1-phosphate was presented. Other purines were examined for their ability to form deoxyribosides in the presence of thymidine phosphorylase. Initial determination of nucleoside formation was made by submitting the reaction mixtures incubated with and without deoxyribose 1-phosphate to thin layer chromatography on cellulose with 4% sodium citrate as developing solvent. Observation of the plates for ultraviolet-absorbing material was performed with the use of a short wave ultraviolet lamp. The results are shown in Table II. The deoxyribose 1-phosphate-dependent synthesis of a new ultraviolet-absorbing material occurred only with xanthine, 8-chloroxanthine, 6-thioxanthine, 6,8-dihydroxypurine, and, to a small extent, 2,8-dichloro-6-hydroxypurine. The first four bases were available in quantities sufficient for larger scale preparation of their nucleosides.

The deoxyribosides were prepared as described under "Experimental Procedure." After isolation, each nucleoside was checked for homogeneity by thin layer cellulose chromatography. No other ultraviolet-absorbing materials were present. The 3-deoxyribonucleoside of 6-thioxanthine could be isolated by

TABLE III

Ultraviolet spectra of 3-deoxyribosylpurines

Purine	HCl (0.1 N)		Phosphate buffer, (0.1 M), pH 7.0		NaOH (0.1 N)	
	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum
	mμ		*mμ*		*mμ*	
Xanthine	267	239	269	240	284	257
3-Deoxyribosylxanthine	267	240	269	241	277	250
6-Thioxanthine	340	287	340	288	338	292
3-Deoxyribosyl-6-thioxanthine	341	291	348	293	343	299
8-Chloroxanthine	272	244	277	250	287	250
3-Deoxyribosyl-8-chloroxanthine	272	244	277	249	280	250
6,8-Dihydroxypurine	256	224	256	229	270	241
3-Deoxyribosyl-6,8-dioxopurine	256	228	256	228	269	237

TABLE IV

Relative rates of reaction of xanthine, uracil, and their nucleosides with pyrimidine nucleoside phosphorylases

For the synthetic reaction 1 mM ^{14}C-labeled base and 8 mM pentose 1-phosphate were incubated with appropriate amounts of uridine or thymidine phosphorylase in Tris buffer, pH 7.2. The cleavage was determined in Tris buffer containing 0.01 M phosphate, the nucleoside concentration being 0.5 mM. A unit of enzyme activity was determined at the same pH by measuring the phosphorolysis of 10 mM nucleoside.

Base and pentose phosphate	Enzyme	Nucleoside formation	Nucleoside	Enzyme	Nucleoside cleavage
		μmoles/hr/unit			*μmole/hr/unit*
Xanthine					
Ribose-1-P	Uridine phosphorylase	0.01	3-Ribosylxanthine	Uridine phosphorylase	0.069
Deoxyribose-1-P	Thymidine phosphorylase	0.15	3-Deoxyribosylxanthine	Thymidine phosphorylase	0.072
Uracil					
Ribose-1-P	Uridine phosphorylase	0.23	Uridine	Uridine phosphorylase	0.95
Deoxyribose-1-P	Thymidine phosphorylase	1.0	Deoxyuridine	Thymidine phosphorylase	0.50

chromatography, and ultraviolet spectra determined, but the nucleoside could not be stored for any length of time since it degraded spontaneously. The characteristic yellow fluorescence under a short wave ultraviolet lamp and the absorption at 340 mμ disappeared, and chromatography showed the appearance of a number of new ultraviolet-absorbing and blue fluorescent bands. These were not studied further. The ultraviolet absorption maxima and minima for the bases and the nucleosides are given in Table III. Under these conditions the extent of formation of the four nucleosides does not differ by more than a factor of 2, falling in the order: xanthine = 8-chloroxanthine > 6-thioxanthine > 6,8-dihydroxypurine. Because of the long incubation times, no attempt was made to determine rates of formation of the various nucleosides from the bases.

As was the case with 3-deoxyribosylxanthine (1), the 8-chloroxanthine and 6-thioxanthine deoxyribonucleosides were also cleaved in acid and were shown to contain equimolar amounts of base and deoxyribose, determining the base by ultraviolet absorption and the sugar by the diphenylamine reaction. The 6,8-dihydroxypurine deoxyribonucleoside is considerably more resistant to cleavage in acid, and no accurate determination could be made of the deoxyribose content since prolonged acid hydrolysis required for complete conversion to the free base destroyed the sugar. In addition, this deoxyribonucleoside is not cleaved at a measurable rate by thymidine phosphorylase.

Formation of 3-Ribosylpurines with Use of Uridine Phosphorylase—The four purine bases listed in Table III were examined for their ability to serve as substrates for uridine phosphorylase. The reaction was studied first with ^{14}C-labeled xanthine and ribose 1-phosphate. Formation of a new, labeled material, dependent on the presence of ribose 1-phosphate, was shown with paper chromatography and a radiochromatogram scanner. The formation of 3-ribosylpurines was then examined under the same conditions as described previously for the deoxyribonucleosides. New ultraviolet-adsorbing materials were observed with all but 6,8-dihydroxypurine. The nucleosides were isolated after chromatography on thin layers (500 μ) of cellulose with 4% aqueous sodium citrate as the developing solvent. The ultraviolet absorption spectra at pH 1 and 13 were identical with those of the corresponding deoxyribonucleosides, as shown in Table III. The 3-ribosylpurines are much more stable to acid hydrolysis, and the quantitative determination of the pentose content of the nucleoside with the orcinol reagent was not possible. However, it was shown that 3-ribosylxanthine was phosphorolytically cleaved by uridine phosphorylase, as determined by formation of uric acid in the presence of xanthine oxidase (see below).

Relative Substrate Efficiencies of Xanthine and Uracil with Pyrimidine Nucleoside Phosphorylases—The relative rates of formation of uracil and xanthine nucleosides from ^{14}C-labeled bases with the two pyrimidine nucleoside phosphorylases are shown in Table IV. The rates of cleavage of these nucleosides are also shown. The rates are expressed in terms of micromoles of substrate reacting per unit of phosphorolytic activity determined at 10 mM pyrimidine nucleoside concentration. This concentration gives rates approaching maximum with deoxyuridine, although maximum rates begin at 1 mM with uridine as the substrate. In the case of the cleavage of the xanthine derivatives, the concentration (0.5 M) was chosen to allow direct spectrophotometric reading of the rate of cleavage. The two pentosylxanthines are cleaved by the respective phosphorylases at essentially the same rate at equal enzyme concentrations; however, the formation of the deoxyribosylxanthine is considerably more rapid than that of the ribosyl derivative.

Biological Activity of 3-Deoxyribosylpurines—The three stable 3-deoxyribosylpurines were examined to determine whether they were cytotoxic to the human tumor cell line, KB, in culture. No inhibition of growth was observed except in the case of the 8-chloroxanthine derivative in which inhibition occurred at relatively high concentrations, 50% inhibition of growth occurring at 100 μg per ml. This activity is probably caused by slow cleavage to the free base, which is 20 times as toxic on a weight basis as the nucleoside. None of the nucleosides listed in Table III had antibacterial activity at a concentration of 1 mg per ml against the following organisms: *Proteus vulgaris*, *Klebsiella pneumoniae*, *Salmonella schottmulleri*, *Pseudomonas aeruginosa*, *Staphylooccus aureus*, *Diplococcus pneumoniae*, *Escherichia coli*, and *Bacillus subtilis*.

DISCUSSION

The utilization of certain purines as substrates for the pyrimidine nucleoside phosphorylases to form atypical purine nucleosides is consistent with the observation that a pyrimidine ribonucleotide pyrophosphorylase can also accept purines as substrates (7).

The specificity of these enzymes for certain purine structures is of interest. Substitution of a sulfur for an oxygen in position 2 of xanthine renders the purine inactive as a substrate, whereas a similar transposition at position 6 has no effect. Similarly, 2,8-dihydroxypurine does not appear to be a substrate, whereas a nucleoside is formed with 6,8-dihydroxypurine. The latter, which is monosubstituted in the six-membered ring, is a substrate, but the 2- and 6-hydroxypurines are not. Substitution of a chlorine at position 8 does not seriously affect the ability of the substituted xanthine to form a nucleoside, but replacement of carbon 8 with a nitrogen, as in azaxanthine, or substitution on the ring with an oxygen, as in uric acid, prevents significant nucleoside formation. Since substituents in the five-membered ring of the conjugated bicyclic ring structure affect the electronic configuration of the six-membered ring, such substitution could have an effect both on binding of the purine to the enzyme and on the ease of substitution on N_3 of the six-membered ring.

Uridine phosphorylase has a much lower capacity to form nucleosides from purines and in addition differs from thymidine phosphorylase in that it does not convert 6,8-dihydroxypurine to a nucleoside. However, the rate of cleavage of the xanthine nucleosides by the two pyrimidine nucleoside phosphorylases is approximately equal.

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