6-Oxa Isosteres of Uracil and Thymine[1a]

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Received June 17, 1968

6-Oxadihydrouracil has been demonstrated to inhibit growth in several microbial systems, and its toxicity is competitively reversed by uracil and uridine, whereas 6-oxadihydrothymine is not significantly inhibitory to growth of any of the organisms studied. In contrast, growth of mammalian tissue culture cells is essentially unaffected by the presence of either oxygen isosteric analog. In general, the oxygen isosteres are slightly more toxic than the corresponding nitrogen analogs for *Escherichia coli* strain; however, the reverse is true for lactic acid bacteria.

Isosteric replacement of a methylene group in naturally occurring metabolites by –O– or –NH– has often resulted in the production of effective metabolite antagonists.[2a] For example, 6-azauracil and its conjugate derivatives have been widely studied in several laboratories and in a variety of biological assay systems and have proved to be functional metabolite antagonists of the corresponding uracil derivative.[2b] Thus, both 6-azauracil[3] and 6-azathymine[4] have been reported to be competitive inhibitors of the corresponding pyrimidine metabolites in microbial systems. The corresponding isosteric oxygen analogs, 6-oxadihydrouracil and 6-oxadihydrothymine, have recently been reported in conjunction with some organic synthesis studies on hydroxyurea condensation products;[5,6] however, no biological studies were described on these potential metabolite antagonists. Accordingly, these derivatives were prepared, and their biological properties were examined in several microbial and mammalian cell culture systems. The 6-oxadihydrouracil derivative proved to be a highly effective competitive antagonist of uracil for bacteria; however, the 6-oxadihydrothymine analog was relatively nontoxic in the assay systems studied; neither compound appeared to affect appreciably mammalian cell growth.

Experimental Section

Microbiological Assay Procedures.—For the lactic acid bacteria a previously described amino acid medium[7] was employed with the exclusion of uracil in the purine–pyrimidine supplement, the addition of calcium pantothenate at 0.2 μg/ml, and with additional modifications as noted: for *Streptococcus faecalis* 8043, 20 μg/ml of L-glutamine was added without heating to each assay tube; for *Leuconostoc dextranicum* 8086, 0.02 μg/ml of pantotheine was added and the phosphate concentration was increased fourfold. All of the lactic bacterial assays were incubated at 30°, *Lactobacillus arabinosus* 17-5 and *S. faecalis* were read at 15–18 hr, whereas *L. dextranicum* usually required 24 hr for optimum growth. Assays with *Escherichia coli* 9723 and Texas strain were carried out in a previously described inorganic salts–glucose medium,[8] whereas an alternate basal medium was utilized for studies with *E. coli* W.[9] All strains of *E. coli* were incubated at 37° for 15–18 hr.

The amount of growth was determined using a Bausch and Lomb Model 20 spectrophotometer set at 600 mμ. Since in these studies the interpretation of the data is based on essentially complete inhibition of growth, the intermediate spectrophotometric readings are not required; however, in order to establish growth rates, control curves have been determined in which dry weight of cells is plotted *vs.* per cent transmission and thus serve as a direct measure of bacterial growth (Table I).

TABLE I
DRY WEIGHT OF BACTERIAL CELLS AS A FUNCTION OF PER CENT TRANSMISSION AS DETERMINED ON A SPECTRONIC 20 SPECTROPHOTOMETER AT 600 mμ

	Bacteria, mg/ml dry weight of cells			
% transmission	*E. coli*	*L. arabinosus*	*S. faecalis*	*L. dextranicum*
90	0.01	0.01	0.07	0.06
80	0.03	0.06	0.13	0.10
70	0.06	0.11	0.21	0.20
60	0.09	0.16	0.32	0.28
50	0.12	0.23	0.45	0.46
40	0.18	0.32	0.62	0.60
30	0.24	0.43	0.83	0.80
20	0.34	0.62	...	0.99

Tissue Culture Assay Procedures.—Procedures for testing metabolite structural analogs in mammalian cell cultures have been described in detail previously.[10] Briefly, stock monolayer-type cultures of HEp-2 human carcinoma, Jensen rat sarcoma, and WI-38 human diploid fibroblast cells were subcultured to replicate T-25 flasks so that initial inocula ranged from 0.2 to 3.3 × 10^6 cells/flask. After 1–2 days of incubation, test compounds were added at 0.25, 2.5, 5.0, 25, or 50 μg/ml final concentrations. Each culture contained 3 ml of medium[11] and fresh medium changes were made at 48, 72, or 96 hr, at which times three replicate control and test cultures at each level were terminated for determination of cell numbers in a hemocytometer.

Organic Syntheses.[12] **6-Oxadihydrouracil and 6-Oxadihydro-**

(1) (a) Supported in part by the National Cancer Institute, National Institutes of Health (CA 08102) and by North Texas State University Faculty Research Fund. (b) To whom reprint request should be addressed.

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(11) No exogenous pyrimidines were added to this media; however, it does contain 10% serum. An analysis of serum for the presence of pyrimidine compounds was carried out using *E. coli* ATCC 12632 which responds to uracil, uridine, cytosine, and cytidine, but not to their metabolic precursors; it was demonstrated that the tissue culture medium contained less than 0.6 μg/ml of these metabolites.

(12) All melting points were determined in a liquid bath, and the elemental analyses were carried out by Mr. Ed Hoff on campus at North Texas State University.

thymine.—A previous report has described the synthesis of these compounds through the sequence: aminoxyacetic acid → ethyl aminoxyacetate → ethyl ureidooxyacetate → 6-oxadihydropyrimidine;[5,6] however, in the present study an alternate route was utilized which is somewhat shorter.

Ethyl ureidooxyacetate was synthesized by treating 20 g of hydroxyurea[13] in 150 ml of EtOH with a solution of 5.85 g of Na in 100 ml of EtOH. The resulting solution was stirred for about 30 min at room temperature, and then 42.5 g of ethyl bromoacetate was added dropwise over a period of about 2 hr; finally, the reaction mixture was stirred for an additional 48 hr at room temperature. The solvent was removed *in vacuo*, the semisolid residue was repeatedly extracted (hot EtOAc), and, upon removal of the solvent, there was recovered 12.2 g of product which was recrystallized (EtOAc), mp 122–124° (lit.[5] mp 125°). Cyclization of ethyl ureidooxyacetate to **6-oxadihydrouracil** was effected by dissolving 5 g of ester in EtOH (50 ml) containing 1 equiv of NaOEt and allowing the reaction mixture to stand at room temperature for 4 hr to produce a gelatinous mixture. The solvent was removed *in vacuo*, and the resulting residue was dissolved in a minimal quantity of H_2O; the solution was adjusted to about pH 7 with concentrated HCl and the resulting precipitate was recrystallized (H_2O) to yield 2.95 g of product, mp 180–181° (lit.[5] mp 182°).

6-Oxadihydrothymine was prepared in a comparable two-step synthesis by condensing 3 g of hydroxyurea with 9 g of ethyl α-iodopropionate in the presence of EtOH (200 ml) containing 2.2 g of KOH, and heating the reaction mixture under reflux for about 2 hr. The solvent was removed *in vacuo*, and the resulting oily residue was taken up in EtOH (100 ml) containing 1 equiv of NaOEt and allowed to stand at room temperature for 2 hr. After removal of the solvent, the resulting solid residue was dissolved in H_2O and adjusted to pH 6 with concentrated HCl and the precipitated material was recrystallized from H_2O to yield 1.9 g of product, mp 152–153° (lit.[5] mp 153°).

TABLE II

RELATIVE TOXICITIES OF 6-OXA AND 6-AZA ISOSTERES OF URACIL AND THYMINE

Microorganism	Concn for complete growth inhib, μg/ml			
	6-Oxa-dihydro-uracil	6-Aza-uracil	6-Oxa-dihydro-thymine	6-Aza-thymine
E. coli 9723	2	6	600	>2000
E. coli W	6	6	2000	2000
E. coli Texas	2	6	>2000	>2000
L. dextranicum 8086	0.2	0.6	>2000	>2000
L. arabinosus 17-5	0.2	0.06	200	>2000
S. faecalis 8043	0.2	0.06	60	0.6

TABLE III

REVERSAL OF 6-OXADIHYDROURACIL GROWTH INHIBITION IN *L. arabinosus* BY URACIL AND DERIVATIVES

6-Oxa-dihydro-uracil, μg/ml	Growth response, % transmission[a]									
	Uracil, μg/ml					Uridine, μg/ml				Uridylic acid, 20 μg/ml
	None	0.02	0.06	0.2	0.6	0.02	0.06	0.2	0.6	
0	28	23	23	25	19	21	24	23	22	25
0.06	64	27				29	24			29
0.2	93	34	33			97	44	31		83
0.6		94	87	55			99	64	29	
2			94	88	61			97	71	
6				89	78				84	
20					83					

[a] Determined using a Spectronic 20 spectrophotometer at 600 mμ.

Results and Discussion

6-Oxadihydrouracil is significantly inhibitory to growth of a number of microorganisms as indicated in Table II. Two representative systems were chosen for more extensive study, *L. arabinosus* and *E. coli*, and the reversal of toxicity by uracil and/or its conjugate derivatives is presented in Tables III and IV, respectively. In *L. arabinosus*, 6-oxadihydrouracil is reversed in a competitivelike manner by uracil over a 30-fold range of concentrations with an inhibition index (ratio of analog to metabolite required to inhibit growth completely) of about 10 at the upper levels (Table III). Uridine also reverses the toxicity of 6-oxadihydrouracil in a comparable fashion with an inhibition index of about 10 over a 30-fold range of increasing substrate concentration. Uridylic acid even at high concentrations is relatively inactive in reversing the toxicity of 6-oxadihydrouracil, presumably due to the inability of such phosphate derivatives to penetrate cell walls.

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Precursors of uracil biosynthesis and related compounds were essentially ineffective in reversing the inhibitory effect of 6-oxadihydrouracil,[14] and comparable results have also been reported in the case of 6-azauracil.[3] In this respect it should be noted that the aza analog possesses potential pyrimidinelike nuclear structure, whereas the corresponding oxa derivative cannot tautomerize to such an aromaticlike ring system because of the divalent character of the oxygen atom. Thus, the oxa analog is in reality an isostere of dihydrouracil. Since both of these analogs appear to function as metabolite antagonists of uracil in these microbiological systems, and neither are reversed appreciably by dihydrouracil, orotic acid, or dihydroorotic acid, the enzymic site of inhibition would suggest a metabolic block after the formation of orotic acid in

TABLE IV

REVERSAL OF 6-OXADIHYDROURACIL GROWTH INHIBITION IN *E. coli* TEXAS BY URACIL

6-Oxa-dihydro-uracil, μg/ml	Growth response, % transmission[a]				
	Uracil, μg/ml				
	None	0.06	0.2	0.6	2.0
0	19	19	18	19	19
0.6	19				
2	94	19			
6		83	24		
20			90	34	
60				83	43
200					73

[a] Determined using a Spectronic 20 spectrophotometer set at 600 mμ.

(14) The following metabolites did not produce any significant reversal of growth inhibition induced by 2 μg/ml of 6-oxadihydrouracil at their limit of solubility in the assay medium: β-alanine, β-ureidopropionic acid, N-carbamoylaspartic acid, orotic acid, dihydroorotic acid, and dihydrouracil.

the biosynthesis of pyrimidines. *E. coli* cultures inhibited by azauracil have been observed to accumulate orotic acid and orotidylic acid[15] suggesting a block in the synthesis of the dihydroxypyrimidine nucleus rather than at sites of utilization of uracil, and, more specifically, at the decarboxylative conversion of orotidylic acid to uridylic acid.[16]

6-Oxadihydrouracil also inhibits the growth of *E. coli* Texas and its toxicity is competitively reversed by uracil over a 30-fold range of concentrations with an inhibition index of about 100. Further, uracil precursors and related compounds also did not reverse the inhibitory action of the analog in *E. coli*.

Neither 6-oxadihydrouracil nor 6-oxadihydrothymine were appreciably inhibitory to mammalian cells grown in tissue culture as indicated in Table V. Studies were carried out using HEp-2 human carcinoma, Jensen rat sarcoma, and WI-38 diploid human embryonic lung cells. Using HEp-2 cells, the per cent of control growth in the presence of 0.25, 2.5, and 5.0 μg/ml of the oxauracil derivative was 103, 88, and 101, respectively; the control growth was tenfold that of the initial inoculum. Subsequent assays at levels of inhibitor up to 50 μg/ml did not affect proliferation *in vitro* of these human cancer cells appreciably. In the same system, 6-oxadihydrothymine at levels of 5, 25, and 50 μg/ml gave values of 100, 114, and 88% that of control growth, respectively (with a sevenfold increase of cell growth over that of the inoculum). No striking differences in results were obtained with either of these analogs at these concentration levels using Jensen rat sarcoma and WI-38 lung cells in comparable tissue culture assays. In summary, neither of the oxa analogs proved to be appreciably inhibitory to growth of mammalian cell cultures even though they exhibited a relatively high toxicity to microbial growth.

TABLE V

EFFECT OF OXA ISOSTERES ON GROWTH OF MAMMALIAN CELLS *in Vitro*

Isostere, μg/ml	% of control proliferation[a]		
	HEp-2	Jensen	WI-38
6-Oxadihydrouracil			
0.25	103	90	...
2.5	88	87	...
5.0	101	115	86
25	...	...	86
50	104	62	85
6-Oxadihydrothymine			
5	100	89	75
25	114	86	82
50	88	73	65

[a] Calculated by dividing the number of new cells produced in test compound cultures by those produced in nonsupplemented Medium 7a control cultures (× 100). Test compounds were introduced in the log phase of proliferation; for culture conditions see text. The HEp-2 and WI-38 cells are derived from human carcinoma and normal embryonic lung tissue, respectively; the Jensen cells were obtained from freshly excised Jensen sarcomas carried in Holtzman rats.

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Synthesis of Carbonate Analogs of Dinucleosides. 3′-Thymidinyl 5′-Thymidinyl Carbonate, 3′-Thymidinyl 5′-(5-Fluoro-2′-deoxyuridinyl) Carbonate, and 3′-(5-Fluoro-2′-deoxyuridinyl) 5′-Thymidinyl Carbonate[1]

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Received July 1, 1968

The synthesis of (3′→5′) carbonate analogs of dinucleosides is described. 3′-Thymidinyl 5′-thymidinyl carbonate (**10**), 3′-thymidinyl 5′-(5-fluoro-2′-deoxyuridinyl) carbonate (**15**), and 3′-(5-fluoro-2′-deoxyuridinyl) 5′-thymidinyl carbonate (**18**) have been synthesized. Thymidine was converted to 5′-O-tritylthymidine and treated with phosgene to give 3′-(5′-O-tritylthymidinyl) chloroformate (**6**). Subsequent treatment with thymidine and removal of the protective group afforded **10**. Compounds **15** and **18** were prepared by the same method. 3′-(5′-Phosphorylthymidinyl) 5′-thymidinyl carbonate (**14**) was prepared from **10** by reaction with diphenyl phosphorochloridate followed by hydrogenolysis of the protective groups. Compounds **10**, **14**, and **15** did not show significant inhibition of *Escherichia coli* growth or thymidylate synthetase.

In vitro inhibition of nucleic acid formation by nucleotides and their derivatives has been demonstrated for 5-fluoro-2′-deoxyuridine 5′-monophosphate (FUDRP) and 5-trifluoromethyl-2′-deoxyuridine 5′-monophosphate (F_3 TDRP).[2]

A potential site of inhibiting nucleic acid synthesis is the enzyme deoxyribonucleotidyltransferase (DNA polymerase).[3,4] Studies on the inhibitory action of nucleosides and nucleotides have demonstrated that the latter do not pass through cell membranes.[5] Recently Bloch and coworkers[6] have synthesized dinucleoside phosphates containing 5-fluorouracil; cellular permeability also limits the uptake of these compounds and the observed biological activity appears to be derived

(1) This work was supported by a Public Health Service predoctoral fellowship 5-F1-GM-29, 336 to E. A. C. and by Grants CA-5639 and 1K3-CA-10,739 from the National Cancer Institutes, National Institutes of Health. Presented at the XXIst IUPAC Meeting, Prague, Sept 1967.
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