Biological Studies of Analogs of Orotic Acid: 6-Uracil-sulfonic Acid, 6-Uracilsulfonamide, and 6-Uracil Methyl Sulfone*

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Investigations of the metabolism of orotic acid have shown that this compound is utilized by certain bacteria (20) and by the mammal for the synthesis of pentosenucleic acid (1, 5). Although it has not been definitely established that orotic acid (or a metabolic derivative of it) is an obligatory intermediate in the biosynthesis of nucleic acids, there is considerable evidence in support of this possibility (15, 16).

An approach to the elucidation of the biological significance of orotic acid is afforded by the design, synthesis, and biological investigation of potential antimetabolites of this compound. Conceivably, active compounds of this class could have chemotherapeutic utility by virtue of an interference with the utilization of orotic acid or its derivatives, if qualitative or quantitative differences in their action on different types of cells were to be encountered. The well established antimetabolic activity of sulfonic acids, sulfonamides, and substituted sulfones analogous to certain naturally occurring carboxylic acids suggested an investigation of similar analogs of orotic acid. The syntheses and chemistry of compounds of this new series have been presented elsewhere (2, 3).

In the present study it has been shown that the parent compound, 6-uracilsulfonic acid, is relatively ineffective as a growth inhibitor of Lactobacillus bulgaricus 09. On the other hand, 6-uracil-sulfonamide and 6-uracil methyl sulfone inhibited the growth of this organism noncompetitively, as well as that of another orotic acid-utilizing strain, namely, Lactobacillus bulgaricus (Hanson). These compounds found to have little if any effect on the growth of Streptococcus faecalis 8043 or of Leuconostoc citrotorum (Pediococcus cerevisiae).

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MATERIALS AND METHODS

1-Dihydroorotic acid and N-carbamylaspartic acid (N-ureidosuccinic acid) were prepared by the methods of Miller (13) and Nyc and Mitchell (14), respectively. Unless otherwise stated, all other materials used were commercial preparations.

Lactobacillus bulgaricus 09 was grown in the basal medium described by Wright et al (19), except that uracil was omitted. The culture used was derived from a single colony selected from organisms grown on a solid purified medium containing orotic acid (15 μg/ml) as the only pyrimidine. This strain was transferred weekly on agar slants composed of the same purified medium and also daily in purified liquid media containing orotic acid. Standardized inocula were prepared by washing 24-hour cultures twice with a solution of NaCl (0.9 per cent) and resuspending the cells to give a turbidity reading of about 80; 1 drop of this suspension was added to each assay tube (containing 4 ml of medium).

Lactobacillus bulgaricus 09-X, a uracil-utilizing variant obtained during these studies (Chart 1), was carried in the same manner as L. bulgaricus 09, except that the medium contained uracil (15 μg/ml) as the only pyrimidine.

Lactobacillus bulgaricus (Hanson) was maintained by biweekly transfer in milk-broth medium. Inocula were prepared by transferring a loopful of the milk-broth to purified media containing erotic acid (15 μg/ml), incubating at 37° C. for 24 hours and then following the procedure outlined for L. bulgaricus 09.

Streptococcus faecalis 8043 and Leuconostoc citrotorum (Pedicoccus cerevisiae) were grown according to published procedures (10 and 18, respectively).

Solutions of the orotic acid analogs in distilled water were freshly prepared for each experiment; the pH was adjusted to that of the medium with NaOH. These solutions were sterile-filtered and added aseptically to autoclaved tubes containing the media.

Unless otherwise stated, the amount of microbial growth was determined turbidimetrically after incubation for at least 48 hours at 37° C. The 50 per cent molar inhibition index, frequently mentioned, is the ratio of inhibitor to metabolite which is required to suppress growth to 50 per cent of the maximum obtained in the absence of the inhibitor.

1 Turbidity measurements were made with the Klett-Summerson colorimeter (610 με filter).

2 Obtained from Dr. S. M. Hauge, Department of Biochemistry, Purdue University.
RESULTS

The effect of 6-uracilsulfonic acid on the growth of *L. bulgaricus* 09.—When tested over a considerable range (50–2500 µg/ml of culture medium), 6-uracilsulfonic acid was inactive as an inhibitor of the growth of *L. bulgaricus* 09 in a pyrimidine-deficient medium containing limiting amounts of orotic acid. The compound also was inactive as an inhibitor of orotic acid in the uracil-containing medium described by Wright et al. (19). The analog could not be substituted for orotic acid as a growth factor for *L. bulgaricus* 09. In a medium containing uracil (5 µg/ml) and limiting amounts of orotic acid, high concentrations of the sulfonic acid (1.2–2.5 mg/ml of medium) stimulated the growth of the organism (Chart 1). However, in contrast to the organisms of the parent culture, those which developed in the presence of the sulfonic acid and uracil had the ability to grow rapidly on uracil as well as on orotic acid (Table 1). The development of this strain, which was designated *L. bulgaricus* 09-X may be attributed to the selection of a spontaneously occurring variant. During fifteen serial passages in a uracil-free medium (containing orotic acid), the ability of the strain to grow rapidly when uracil was substituted for orotic acid was retained.

The effect of 6-uracilsulfonamide and 6-uracil methyl sulfone on the growth of *L. bulgaricus*.—The growth of *L. bulgaricus* 09, in a medium supplemented with orotic acid (or L-dihydroorotic acid or DL-carbamylaspartic acid), was markedly inhibited by either 6-uracilsulfonamide or 6-uracil methyl sulfone (Chart 2). Also, these compounds inhibited the response of *L. bulgaricus* 09-X and *L. bulgaricus* (Hanson) to orotic acid. The 50 per cent molar inhibition indices, shown in Table 2, indicate that the inhibition of growth of *L. bulgaricus* 09 by either compound is noncompetitive with respect to orotic acid, and that the methyl sulfone is approximately twice as active as the sulfonamide as an inhibitor of growth. An indication of the rela-
tion between molecular structure and biological activity is afforded by the finding that 2,4-di- 
methoxy-uracil-6-methyl sulfone was inactive as an inhibitor of the growth of *L. bulgaricus* 09 (in 
concentrations as high as 1000 µg/ml of medium).

Recent studies, in which orotic acid was shown to be converted by soluble pigeon liver enzymes 
(8, 19) to uridine-5'-phosphate (U-5'-P) and in which U-5'-P and its derivatives were shown to be 
the first demonstrable products of orotic acid in a rat liver homogenate (4), suggest that U-5'-P is a 
direct intermediate in the anabolic conversion of orotic acid to the pyrimidines of nucleic acids.

**TABLE 2**

<table>
<thead>
<tr>
<th>INHIBITION OF THE GROWTH OF <em>L. bulgaricus</em> 09 BY 6-Uracil Sulfonamide and 6-Uracil Methyl Sulfone</th>
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<tbody>
<tr>
<td>GROWTH SUPPLEMENT µg/ml</td>
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<tr>
<td>-------------------------</td>
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<tr>
<td><strong>STRAIN</strong></td>
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<tr>
<td>09 Orotic acid</td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td>L-Dihydroorotate</td>
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<td></td>
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<tr>
<td>DL-Carboxymethyl-</td>
</tr>
<tr>
<td>aspartate</td>
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<tr>
<td></td>
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<tr>
<td>09-X Uric acid</td>
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<tr>
<td>Hanson Orotic acid</td>
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</table>

That U-5'-P will support the growth of *L. bulgaricus* 09, in molar concentrations equivalent to 
those of orotic acid, had been shown by Rose and Carter (17). Accordingly, U-5'-P might be 
expected to overcome the inhibitory effect of the analogs; it was found, however, that 6-uracil 
methyl sulfone markedly inhibited the growth of *L. bulgaricus* 09 in a medium supplemented with 
U-5'-P and that this inhibition of growth was noncompetitive (Table 2). Even more notable was the 
finding (Table 2) that this substance also inhibited noncompetitively the growth of *L. bulgaricus* 
09-X in a medium supplemented with uracil.

Since, in the experiments described, the antagonists were added to the culture medium prior to 
inoculation, the effect of these compounds on cultures in the log phase of their response to the 
metabolite was also determined. Even under these conditions (Chart 3) each compound caused a 
marked inhibition of the growth of the organism; in this respect also the methyl sulfone was about 
twice as active as the sulfonamide. Although a derivative of orotic acid, the methyl ester of 2,4-
dichloro-6-carboxy-pyrimidine, when added to an orotic acid-supplemented medium prior to inoculation, 
also inhibited the growth of *L. bulgaricus* 09 noncompetitively, it had little if any effect on the 
growth of the organism when added to the culture medium during the log phase of growth (20 hours 
after inoculation). It may be suggested that, under the latter conditions, this compound was rapidly 
inactivated.

The effect of 6-uracilsulfonamide and 6-uracil methyl sulfone on the growth of other organisms.— 
Certain micro-organisms which do not utilize orotic acid for growth were not inhibited markedly 
by these compounds (except *S. faecalis* by a very high concentration of the methyl sulfone) (Table 
3). For purposes of comparison, the response of *L. bulgaricus* 09 to the analogs also is shown in 

![Chart 3](chart3.png)

*CHART 3.*—The effect of 6-uracilsulfonamide and 6-uracil methyl sulfone on the growth of *L. bulgaricus* 09, when the antagonists were added to the culture media 19 hours after inoculation (as indicated by the arrows).

Table 3. *S. faecalis* was grown in the presence of thymine, as well as in the presence of pteroyl-glutamic acid, and in the presence and absence of uracil. The analogs, particularly the methyl sulfone, at very high concentrations (500–2000 µg/ml) had a somewhat greater effect on the growth of *S. faecalis* in the presence of pteroyl-glutamic acid; the degree of inhibition of growth, however, was quite small when compared with the effect of these compounds on the growth of *L. bul-

1 Kindly supplied by the Squibb Institute for Medical Research, New Brunswick, N.J.
garicus 09. Leuconostoc citrovorum, grown in the presence of thymidine or synthetic citrovorum factor (CF), was only slightly inhibited at very high levels of either compound (2000 µg/ml). The results with these organisms have the virtue of indicating that the antagonists do not affect the—The finding that this compound noncompetitively inhibited the growth of L. bulgaricus 09 in a medium supplemented with either orotic acid or U-5'-P suggested that the methyl sulfone may interfere with nucleic acid metabolism at more than one point or perhaps at a specific point be-

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>METABOLITE</th>
<th>Percentage growth in the presence of varying amounts of analog</th>
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<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>6-Uracilsulfonamide (µg/ml)</td>
</tr>
<tr>
<td>S. faecalis 8043</td>
<td>Thymine</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Thymine (Uracil-free medium)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>PGA*</td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>PGA* (Uracil-free medium)</td>
<td>0.0008</td>
</tr>
<tr>
<td>L. citrovorum 8081</td>
<td>Synthetic CF†</td>
<td>0.0008</td>
</tr>
<tr>
<td></td>
<td>Thymidine</td>
<td>0.3</td>
</tr>
<tr>
<td>L. bulgaricus 09</td>
<td>Orotic acid</td>
<td>15.0</td>
</tr>
</tbody>
</table>

* PGA = pteroylglutamic acid.
† CF = citrovorum factor.

BU0003131.png

Chart 4.—Reversal by partially purified ribonucleic acid (1 mg/ml) of the inhibition by 6-uracil methyl sulfone (250 µg/ml) of the growth of L. bulgaricus 09 in the presence of orotic acid (15 µg/ml). The methyl sulfone and the RNA were added aseptically 19 hours after inoculation.

growth of bacteria nonspecifically and make the premise more reasonable that they interfere with the metabolism of compounds derived from orotic acid.

Attempts to reverse the inhibition of the growth of L. bulgaricus 09 induced by 6-uracil methyl sulfone.

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4 We are indebted to Dr. C. E. Carter of this department for kindly supplying this dinucleotide and the highly purified preparations of liver and yeast ribonucleic acid.
complete alkaline or enzymatic hydrolysates of these compounds, affected the growth of the organisms inhibited by the methyl sulfone. However, a commercial preparation of yeast RNA, at a level of 2 mg/ml, overcame completely or nearly completely the effect of the methyl sulfone; the results of a typical experiment are shown in Chart 4. After further purification this material still retained the ability to overcome the inhibition of growth of L. bulgaricus 09 by the methyl sulfone. Also, this property was retained after hydrolysis with acid (or alkali [KOH, N/1, 37° C., 18 hr.]). Other lots of the commercial RNA had the same effect on the growth of organisms inhibited by the methyl sulfone. The inactivity of the compounds of known composition and the fact that further purification, as well as acid and alkaline degradation, did not destroy the activity of the commercial RNA suggest that the active material is probably not a known low molecular weight polynucleotide or derivative thereof.

DISCUSSION

6-Uracil sulfonic acid, in the absence of orotic acid, had no activity as a growth factor for L. bulgaricus 09; also, it did not inhibit growth of the organisms on orotic acid. However, in a medium containing uracil and suboptimal amounts of orotic acid, but not in a uracil-free medium, the analog significantly stimulated bacterial growth. The resultant bacterial population, in contrast to the parent strain, utilized uracil efficiently for growth. It may be suggested, therefore, that 6-uracil sulfonic acid interferes sufficiently with the metabolism of the orotic acid-requiring organism to permit the preferential growth of a variant which is able to utilize uracil.

Either 6-uracil sulfonamide or 6-uracil methyl sulfone noncompetitively inhibited the growth of L. bulgaricus 09 in a medium supplemented with orotic acid, whether the antagonist was added prior to inoculation or during the log phase of growth; in this respect the methyl sulfone was about twice as active as the sulfonamide. The compounds also inhibited the growth of this organism in media supplemented with DL-carbamyl aspartate (ureidosuccinate) or L-dihydroorotate, and the orotic acid-induced growth of another strain (Hanson) of L. bulgaricus. In contrast to the marked effect of these analogs on the growth of those micro-organisms which can utilize an exogenous source of orotic acid for growth, they had but little effect on the growth of Leuconostoc citrovorum (Pediciococcus cerevisiae) grown on either citrovorum factor or thymidine, and of Streptococcus faecalis in a medium supplemented with purines and thymine. The growth of S. faecalis, when pteroylglutamic acid was provided as the essential growth factor, was inhibited only by very high concentrations of these compounds. It is suggested, therefore, that these analogs exert their toxic effect on certain micro-organisms by interfering with one or more stages in the utilization of exogenous orotic acid (and certain precursors of it). Several possible explanations of the failure of the antagonists to inhibit S. faecalis and L. citrovorum could be considered. Thus, the continually recurring question of penetration of the bacterial cells by the analogs must be raised. That orotic acid, per se, may not be an endogenous metabolite could be regarded as another possibility; however, it is unlikely that uridine-5'-phosphate is not a normal intermediate, even in the insensitive organisms, and it will be recalled that growth of L. bulgaricus 09 on this metabolite is antagonized noncompetitively by the antagonists. Obviously, more work must be done to explain these bacterial findings. Accordingly, it may be particularly pertinent to mention at this time that, in preliminary collaborative experiments with Dr. L. W. Law of the National Cancer Institute and Dr. Maire Hakala of this department, 6-uracil methyl sulfone inhibited the growth of three strains of lymphoma (L1810, L4946, L5178) and of Sarcoma 180 in mice.

Since either 6-uracil sulfonamide or 6-uracil methyl sulfone inhibits the rate of decarboxylation of C14-carboxyl-labeled orotic acid by a crude soluble pigeon liver enzyme and also by a combined pigeon liver-yeast enzyme system (described by Kornberg et al., 8, 12), a possible explanation for the findings in animals may be afforded. During the early phase of the enzymatic reactions the inhibition by the analogs is competitive, but as the reaction progresses the kinetics become more complex, and, as in L. bulgaricus 09, the inhibition becomes noncompetitive in character. Accordingly,
it is possible that one of the actions of the analog is to compete with orotic acid for the enzyme which, with the acid of 5-phosphoribosyl-pyrophosphate (9), converts orotic acid to orotidine-5'-phosphate, a precursor of uridine-5'-phosphate (8, 11, 12), and experiments designed to test this possibility are now in progress.

If the action of the antagonists could be explained entirely in this manner, it would be expected that the addition to the bacterial cultures of one of the products of the inhibited reaction or reactions might overcome the effect of the analog. However, as mentioned previously, the growth of L. bulgaricus 09 in a medium supplemented with uridine-5'-phosphate was inhibited noncompetitively by either the sulfonamide or the methyl sulfone. Furthermore, the uracil-supported growth of a strain of L. bulgaricus (09-X), which responds to uracil as well as to orotic acid, also was inhibited noncompetitively by these compounds. These observations suggest that the analogs (or their metabolic derivatives) may exert their effects at more than one point in the anabolic pathways of pyrimidine metabolism and that one site of action lies beyond the mononucleotide stage. A clue concerning a possible second site of inhibitory activity by the analogs or their metabolites was afforded by the finding that a substance of as yet unknown composition, which is closely associated with certain commercially prepared batches of ribonucleic acid but which does not appear to be a known derivative of nucleic acid, overcomes the effect of the antagonists on the growth of L. bulgaricus 09. Additional work must be done to determine the chemical nature of the substance or substances responsible for this antagonism.

SUMMARY

6-Uracilsulfonic acid was neither a growth factor nor an antagonist of the activity of orotic acid as a growth factor for Lactobacillus bulgaricus 09. However, in the presence of the sulfonic acid and uracil the strain which evolved had the capacity to utilize uracil in place of orotic acid.

Either 6-uracilsulfonamide or 6-uracil methyl sulfone noncompetitively inhibited the growth of L. bulgaricus 09 in a medium supplemented with orotic acid, whether the antagonist was added prior to inoculation of the medium or during the logarithmic phase of growth. The growth of the organism in the presence of n-carbamylaspartate (ureidosuccinate), d-dihydroorotate, or uridine-5'-phosphate also was noncompetitively inhibited by the same antagonists, as was the orotic acid-supported growth of another strain (Hanson) of L. bulgaricus, and the growth of a variant of L. bulgaricus 09 (termed 09-X), which utilized uracil in lieu of orotic acid.

These antagonists inhibited the growth of Leuconostoc citrovorum (Pedioceucus crenescens) (8081) and of Streptococcus faecalis (8048) only slightly at very high concentrations, but the response of the latter organism was more marked in the presence of pteroylglutamic acid than when it was grown on purines and thymine. Reversal of the effect of the antagonists on L. bulgaricus 09 was obtained with a material associated with samples of crude or partially purified ribonucleic acid (RNA) of yeast, and with alkaline or acid hydrolysates of the material, but not with highly purified samples of RNA of yeast or liver. The significance of the observations with the antagonists, with respect to their possible mechanisms of action, has been discussed.

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Biological Studies of Analogs of Orotic Acid: 6-Uracilsulfonic Acid, 6-Uracilsulfonamide, and 6-Uracil Methyl Sulfone

William L. Holmes and Arnold D. Welch