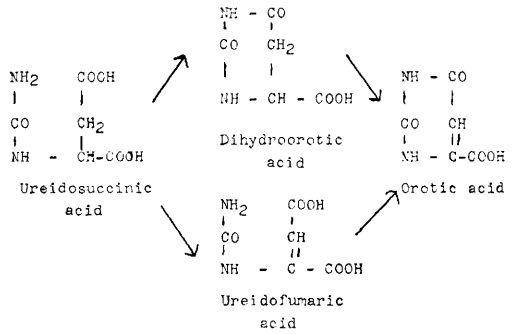
Dihydroorotic Acid in Nutrition of Lactic Acid Bacteria. (20763)

LEMUEL D. WRIGHT, CHARLOTTE A. DRISCOLL, CHARLES S. MILLER, AND HELEN R. SKEGGS.

From the Departments of Microbiological Chemistry and Organic Chemistry, Sharp and Dohme Division, Merck and Co., West Point, Pa.

Lactobacillus bulgaricus 09 fails to grow on a semi-synthetic medium that supports excellent growth of related species(1,2). Good growth is obtained, however, in the presence of added orotic acid. Ureidosuccinic acid in considerably larger amounts may be substituted for orotic acid. Both are utilized for pyrimidine nucleotide and presumably, therefore, nucleic acid synthesis(3). If ureidosuccinic acid is an acylic precursor of the pyrimidine ring it can be visualized to yield orotic acid by one of two alternate pathways (a) cyclization to dihydroorotic acid followed by dehydrogenation to orotic acid, or, (b) dehydrogenation to ureidofumaric acid followed by cyclization to orotic acid.



In an attempt at elucidating the mechanism involved, "dihydroorotic acid" was synthesized by the method of Bachstez and Cavallini(4) and tested for growth-promoting activity with *Lactobacillus bulgaricus* 09(5). The compound proved to be inactive in promoting growth. This finding was taken as evidence for the participation of ureidofumaric acid rather than dihydroorotic acid in the biosynthesis of orotic acid from ureidosuccinic acid. The hypothesis could not be tested, however, because of the unavailability of ureidofumaric acid. Lieberman and Kornberg recently have shown the existence in a cell-free preparation from an anaerobic bacterium of an enzyme that, in the presence of reduced diphosphopyridine nucleotide, reduces orotic acid. The product of the reduction was isolated and characterized by elementary composition and equivalent weight as dihydroorotic acid(6). Enzymatically derived dihydroorotic acid is different in a number of ways from the "dihydroorotic acid" of Bachstez and Cavallini and promotes growth of *Lactobacillus bulgaricus* 09(7). Dihydroorotic acid is asymetric about position 4 and consequently both D- and L- forms are possible theoretically. Both enantiomorphs of dihydroorotic acid as well as the racemate have now been synthesized in these laboratories by a new procedure and were made available for microbiological testing(8).

It is the purpose of this paper to show that (a) L-Dihydroorotic acid is utilized by some but not by all of a number of "pyrimidineless" lactobacilli (b) D-dihydroorotic acid in large amounts is an antimetabolite of L-dihydroorotic acid in pyrimidine utilization, and (c) differential microbiological procedures appear to be available for the qualitative and quantitative estimation of uracil, orotic acid, and dihydroorotic acid in a mixture of the three or in natural material free of interfering compounds.

Methods. The D-, L-, DL-dihydroorotic acids employed in this study were prepared as described by Miller *et al.*(8). The strains of lactobacilli and the media on which they were grown are summarized as follows: For *Lactobacillus arabinosus* ATCC 8014 the basal medium was composed of: 1 g norit-treated, acid-hydrolyzed, vitamin-free casein, 20 mg L-cystine, 40 mg DL-tryptophane, 1 mg each of adenine, guanine, and xanthine, 2 g sodium acetate, 4 g glucose, 1 ml each of salts A and B, 200 μg each of thiamin chloride, riboflavin, nicotinic acid, calcium pantothenate, 400 μg pyridoxine, 100 μg p-aminobenzoic acid, 1 μg biotin to a value of 100 ml of double-strength medium. The pH was 6.8. For

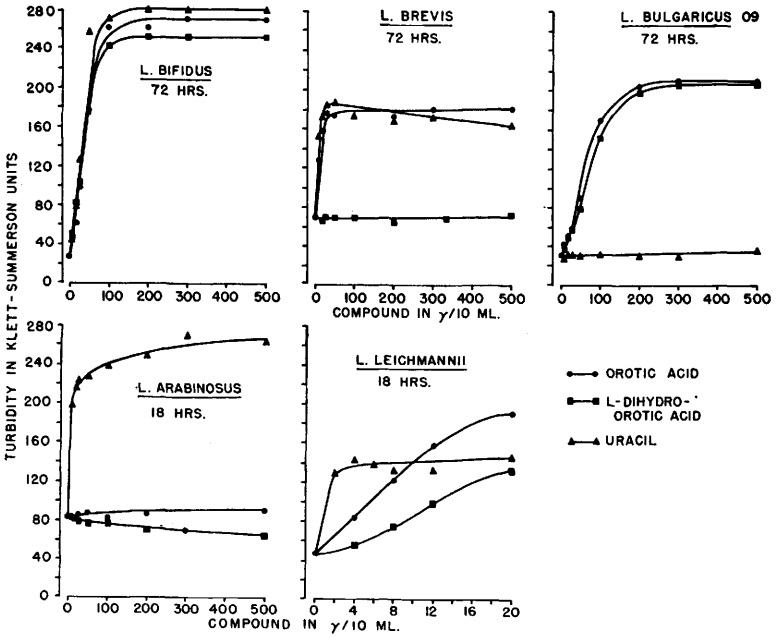


FIG. 1. Response of lactic acid bacteria to dihydroorotic acid and related compounds.

Lactobacillus leichmannii ATCC 4797 the basal medium was further supplemented with 100 mg DL-alanine, 20 mg guanylic acid, 0.2 ml tween 80, 100 mg thiomalic acid, 50 μg pyridoxal, 50 μg folic acid, and 0.002 μg vit. B_{12}. The pH was 6.8. For *Lactobacillus bulgaricus* 09 the medium was supplemented with 1 g norit-treated, trypsin-digested, vitamin-free casein, 0.2 ml tween 80, 50 μg pyridoxal, 50 μg folic acid, and 0.4 μg vit. B_{12}. The pH was 5.8. For *Lactobacillus brevis* ATCC 8287 the basal medium was further supplemented with 1 g norit-treated, trypsin-digested, vitamin-free casein, 0.2 ml tween 80, 50 μg pyridoxal, and 50 μg folic acid. The pH was 6.8. For *Lactobacillus helveticus* ATCC 335 the basal medium was supplemented with 1 g norit-treated, trypsin-digested, vitamin-free casein, 20 mg L-cysteine, 100 mg DL-alanine, 20 mg L-asparagine, 0.2 ml tween 80, 50 μg pyridoxal, 50 μg folic acid, and 0.04 μg vit. B_{12}. The pH was 6.5. For *Lactobacillus bulgaricus* Sarles and *Lactobacillus bulgaricus* ATCC 8001 the basal medium was further supplemented with 1 g norit-treated, trypsin-digested, vitamin-free casein, 20 mg L-cysteine, 100 mg DL-alanine, 20 mg L-asparagine, 0.2 ml tween 80, 50 μg pyridoxal, 50 μg folic acid, 0.04 μg vit. B_{12}, and 1650 units of pantetheine. The pH was 6.5. For *Lactobacillus bifidus* ATCC 4963 the basal medium was further supplemented with 500 mg norit-treated, trypsin-digested, vitamin-free casein, 0.2 ml tween 80, 50 μg pyridoxal, 50 μg folic acid and 2 μg vit. B_{12}. The pH was 6.8.

The lactobacilli studied vary with respect to the completeness of their pyrimidine requirement. For *L. bulgaricus* 09, *L. brevis*, *L. bulgaricus* 8001, *L. bulgaricus* Sarles, and *L. bifidus* a utilizable source of pyrimidine is *essential* for growth. For these species a prolonged incubation period of 72 hours was possible. Visual inspection of the tests showed that for these strains utilization of the various pyrimidine sources described in this paper is not influenced by the period of incubation up to 72 hours. For *L. arabinosus*, *L. leichmannii*, and *L. helveticus* a utilizable source of pyrimidine is only *stimulatory* for growth. With these species it was necessary to determine the amount of growth after a relatively short incubation period, usually of 18 hours. The effect of D-dihydroorotic acid on the utilization of orotic acid or L-dihydroorotic acid by *L. bulgaricus* 09 was studied in tests that

TABLE I. Effect of D-Dihydroorotic Acid on Growth of *Lactobacillus bulgaricus* 09.

Pyrimidine/tube		Turbidity* 24 hr	Turbidity* 48 hr
0 γ orotic acid		0	0
10		22	20
20		39	34
30		54	52
40		60	69
60		70	102
100		81	150
20	+ 500 γ D-dihydroorotic acid	36	38
20	1000	19	42
20	1500	9	50
20	2500	10	41
50	+ 500	54	88
50	1000	28	102
50	1500	10	108
50	2500	7	51
0 γ L-dihydroorotic acid		0	0
10		24	16
20		38	31
30		49	50
40		57	65
60		51	93
100		62	143
20	+ 500 γ D-dihydroorotic acid	24	37
20	1000	9	40
20	1500	8	48
20	2500	4	32
50	+ 500	39	86
50	1000	19	96
50	1500	13	104
50	2500	6	31

* Corrected for color of medium.

were incubated for 24 hours and 48 hours. A Klett-Summerson photoelectric colorimeter was used to determine the extent of bacterial growth which is given in terms of arbitrary scale readings. Other non-specified procedures were as described previously(1,2) or according to customary technics with lactic acid bacteria.

Results. A summary of the data demonstrating the extent to which L-dihydroorotic acid can satisfy the pyrimidine requirement of certain lactobacilli is summarized in Fig. 1. For *L. bifidus, L. bulgaricus* Sarles and *L. bulgaricus* 8001 uracil, orotic acid, and L-dihydroorotic acid are equally effective. Data obtained with *L. bifidus* are given as an example. For *L. brevis* uracil and orotic acid are utilized and L-dihydroorotic acid is inactive. For *L. bulgaricus* 09 orotic acid or L-dihydroorotic acid satisfies the pyrimidine requirement but, as pointed out previously(1,2,9), uracil is inactive. For *L. arabinosus* and *L. helveticus* uracil alone is stimulatory with orotic acid and L- dihydroorotic acid essentially inactive. Data obtained with *L. arabinosus* are given as an example. The results with *L. leichmannii* are more difficult to interpret. At low levels of compound, uracil is more active followed by orotic acid and dihydroorotic acid in that order. At high levels orotic acid is more active than uracil.

D-dihydroorotic acid alone at a level up to 500 γ per tube (10 ml) is completely inactive in satisfying the pyrimidine requirement of *L. bulgaricus* 09. DL-Dihydroorotic acid is 50% as active as L-dihydroorotic acid or orotic acid. With an incubation period of 18-24 hours D-dihydroorotic acid in large amount is an antimetabolite of L-dihydroorotic acid and orotic acid (Table I). After 48 hours of incubation a large excess of D-dihydroorotic acid in the presence of L- dihydroorotic acid is utilized so that inhibition that was present during early growth dis-

appears and growth occurs to an extent greater than that attributable only to the L-dihydroorotic acid present.

The data of Fig. 1 form the basis for possible microbiological methods of determining uracil, orotic acid, and dihydroorotic acid in a mixture of the three compounds. The uracil content would be given by the difference between the results obtained by *L. bifidus* assay and that obtained by *L. bulgaricus* 09 assay. The orotic acid content would be given by the difference between the result obtained by *L. brevis* assay and the value previously found for uracil. The dihydroorotic acid content would be given by the difference between the result obtained by *L. bifidus* and that obtained by *L. brevis*. Obviously other interfering compounds such as, for example, cytosine, thymine, and ureidosuccinic acid must be absent.

Summary. L-Dihydroorotic acid is utilized by a number of "pyrimidineless" lactobacilli to promote growth. D-Dihydroorotic acid in comparable amount is not utilized and under suitable conditions is an antimetabolite of L-dihydroorotic acid. Methods are outlined by which the microbiological determination of uracil, orotic acid or dihydroorotic acid in a mixture of the three is possible by differential assay.

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Received November 20, 1953. P.S.E.B.M., 1953, v84.

Isolation of West Nile Virus from Hooded Crow and Rock Pigeon in the Nile Delta.* (20764)

TELFORD H. WORK,† HERBERT S. HURLBUT,‡ AND R. M. TAYLOR.§
(Introduced by J. Casals.)
From the U. S. Naval Medical Research Unit No. 3, Cairo, Egypt.

West Nile virus has repeatedly been isolated from the blood of febrile children(1-4) and from *Culex* mosquitoes(5) collected in an endemic area in the Nile Delta of Egypt. Because of the high incidence of immunity found in the human population during these studies, investigation was extended to the com-

* The study was conducted under the auspices of the Ministry of Public Health of the Egyptian Government, the U. S. Naval Medical Research Unit No. 3, and the Division of Medicine and Public Health of The Rockefeller Foundation.

The opinions or assertions contained herein are the private ones of the authors, and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

† Staff member of the Division of Medicine and Public Health, The Rockefeller Foundation, New York, assigned to the Department of Virology, U. S. Naval Medical Research Unit No. 3, Cairo, Egypt.

‡ CDR MSC USN, Department of Entomology.

§ Consultant to the Division of Medicine and Public Health, The Rockefeller Foundation, New York, assigned to the Department of Virology, U. S. Naval Medical Research Unit No. 3, Cairo, Egypt.