and that some at least of the spheres may arise by segmentation of the long forms. Studies are being continued to learn more about the proliferation of this virus and to make more accurate observations on the obvious differences in the way strains of influenza attack and destroy the tissues they infect.

Summary. Particles having the known dimensions of the influenza virus are observed in electron micrographs of thin sections cut from infected chorioallantoic membranes and mouse lungs. These particles are in groups and clusters apparently developing from the borders of membrane cells and from the walls of the alveoli.

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Orotic Acid and Related Compounds in the Nutrition of *Lactobacillus bulgaricus* 09. (18175)

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Certain strains of *Lactobacillus bulgaricus* grow readily on a synthetic medium containing yeast extract as the source of an unknown nutritive essential (LBF)(1). Wright *et al.* reported(2) that one strain of *Lactobacillus bulgaricus,* identified as *Lactobacillus bulgaricus* 09, is incapable of growth on a basal medium supplemented with a tryptic digest of casein and yeast extract as crude components and requires much larger amounts of natural material such as milk products, liver or yeast to furnish another growth factor(s).

During the course of studies directed toward elucidating the nutritive requirements of *Lactobacillus bulgaricus* 09 it was found that orotic acid will replace the requirement for large amounts of natural material. When it became possible to grow *Lactobacillus bulgaricus* 09 with orotic acid in the basal medium described, studies were undertaken to determine the extent to which the norit-treated tryptic digest of casein and the yeast extract contributed to the growth factor requirements of the organism. It has been found that the yeast extract is a dispensable component of the medium provided adequate amounts of the norit-treated tryptic digest of casein are present. The growth factor contributed by the norit-treated tryptic digest of casein has been identified with "strepogenin"(3). With the availability of a basal medium that promotes good growth of *Lactobacillus bulgaricus* 09 when supplemented with orotic acid, data have been obtained with respect to (a) the extent to which certain compounds can substitute for orotic acid and (b) the distribution of orotic acid. As a consequence of these findings it has been possible to make certain deductions concerning possible pathways of pyrimidine synthesis in nature and the possible role of orotic acid in nucleic acid metabolism.

Experimental. Lactobacillus bulgaricus 09 was obtained from Dr. I. C. Gunsalus to whom we are indebted. The organism was maintained in stock culture by weekly transfer in Difco skim milk to which 1% Difco tryptose was added. For the preparation of inocula day to day transfer of the organism in the milk-tryptose medium with return to stock culture at weekly intervals was carried out. For seeding microbiological assays 0.1 ml of a 24-hour culture in milk-tryptose medium was dispersed with shaking into 10

1. Williams, W. L., Hoff-Jorgensen, E., and Snell, E. E., *J. Biol. Chem.*, 1949, v177, 933.

2. Wright, L. D., Huff, J. W., Skeggs, H. R., Valentik, K. A., and Bosshardt, D. K., *J. Am. Chem. Soc.*, 1950, v72, 2312.

3. Wright, L. D., Fruton, J. S., Valentik, K. A., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 687.

TABLE I. Basal Medium.*

Norit-treated acid-hydrolyzed casein	1.0	g
Norit-treated tryptic digest of casein	0.5	
Sodium acetate	1.2	
Glucose	4.0	
L-Tryptophane	20	mg
L-Cystine	20	
Adenine	1	
Guanine	1	
Xanthine	1	
Uracil	1	
Salts A	1	ml
Salts B	1	
Thiamine chloride	200	γ
Riboflavin	200	
Calcium pantothenate	200	
Nicotinic acid	200	
Pyridoxine	400	
Pyridoxal	50	
PABA	100	
Folic acid	50	
Biotin	1	
Vit. B_{12}	0.04	
Tween 80	0.2	ml
pH to 5.7 and dilute to	100	

* Concentrations given are for double strength medium.

ml of sterile saline. One drop per tube of this saline suspension was the inoculum used. 37°C was the incubation temperature employed. The basal medium used in these studies had the composition given in Table I.

Turbidimetric measurement of bacterial density with the Klett-Summerson photoelectric colorimeter or titration of the lactic acid produced against 0.1 N NaOH with bromthymol blue as indicator were used as alternative methods for measuring the extent of bacterial growth. Seventy-two hours was the usual incubation time employed. Other microbiological procedures were in accordance with standard methods(4). The orotic acid used in these studies was synthetic material(5,6) (Fig. 2). The orotic acid and intermediates studied had ultraviolet absorption spectra identical with those published by Mitchell and Nyc(5,6). The miscellaneous compounds tested for possible orotic acid activity were, except as noted, commercial or personal research samples.

4. Snell, E. E., Vitamin Methods, 1950, v1, Academic Press, Inc., New York, New York.

5. Mitchell, H. K., and Nyc, J. F., *J. Am. Chem. Soc.*, 1947, v69, 674.

6. Nyc, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, 1947, v69, 1382.

Results. Microbiological activity of orotic acid and related compounds. The response of *Lactobacillus bulgaricus* 09 to orotic acid is shown in Fig. 1. The same response is obtained in the absence of uracil from the basal medium. Also included in Fig. 1 are the data obtained on the microbiological activity of ureidosuccinic acid, 5-(acetic acid)-hydantoin, and 5-(carboxy-methylidine)-hydantoin. These compounds are obtained as intermediates in the synthesis of orotic acid by two accepted methods for the synthesis (Fig. 2) of this compound starting with diethyloxaloacetate and urea(5) or aspartic acid(6). 5-(Carboxy-methylidine)-hydantoin (V) has 30-40% of the activity of orotic acid, ureidosuccinic acid (II) is 10-20% as active, while 5-acetic acid hydantoin (III) is essentially inactive. To obviate the possibility that 5-(carboxy-methylidine)-hydantoin was converted partially to orotic acid during autoclaving, the compound also was tested following sterile filtration and aseptic addition to previously autoclaved media. The same results were obtained by the two methods of assay. 5-(Acetic acid)-hydantoin at levels up to 5000 γ/tube does not inhibit utilization of either 5-(carboxy-methylidine)-hydantoin or ureidosuccinic acid.

Compounds* that have been found to be inactive at levels up to at least 100 γ/tube include: adenosine, adenosine-3-phosphoric acid, adenosine-5-phosphoric acid,[1] β-alanine, allantoin, alloxan, γ-amino-butyric acid,[2] 2-

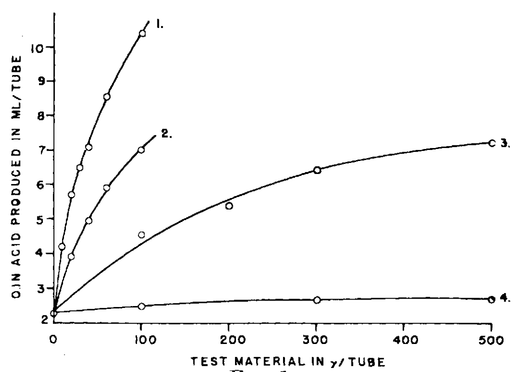

FIG. 1

The response of *Lactobacillus bulgaricus* 09 to orotic acid and related compounds. (1) orotic acid (VI), (2) 5-(carboxy-methylidine)-hydantoin (V), (3) ureidosuccinic acid (II), and (4) 5-(acetic acid)-hydantoin (III).

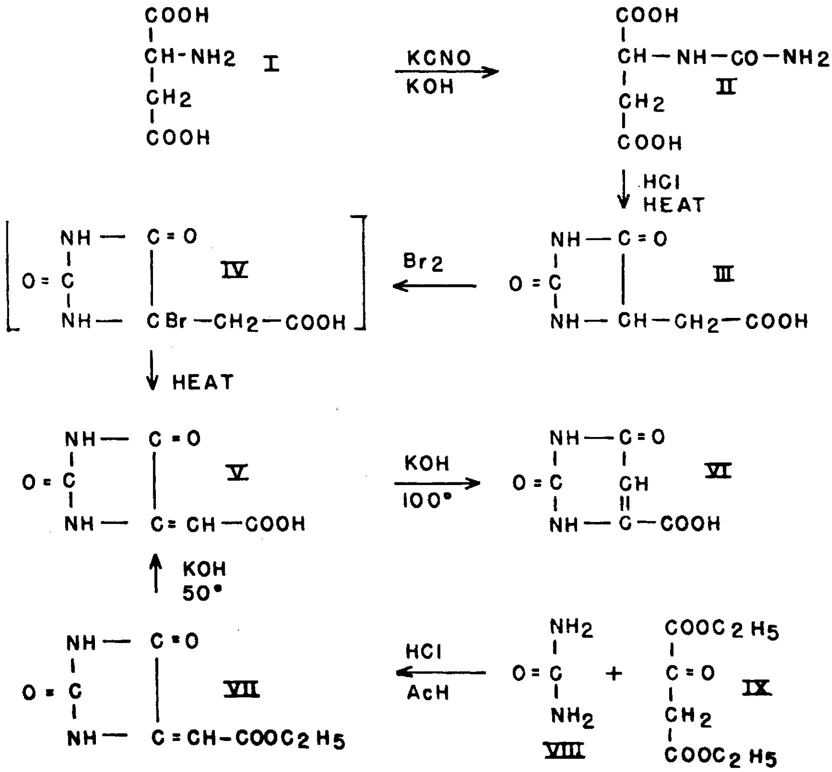

FIG. 2
Structures of orotic acid and intermediates involved in the synthesis of orotic acid by 2 methods(5,6). I aspartic acid, II ureidosuccinic acid, III 5-(acetic acid)-hydantoin, IV 5-bromo-5-(acetic acid)-hydantoin, V 5-(carboxy-methylidine)-hydantoin, VI orotic acid, VII 5-(carboxy-methylidine)-hydantoin, VIII urea, IX diethyl oxaloacetate.

amino-4-methyl-6-oxypyrimidine,[3] asparagine, aspartic acid, benzimidazole, caffeine, 5-carboxy-uracil,[4] choline, citrulline, creatine, creatinine, cytidine, cytidylic acid, cytosine, 2, 4-diamino-6-oxypyrimidine, desoxyribonucleic acid, fumaric acid, fumaric acid plus urea, guanidine, guanosine, guanylic acid, hypoxanthine, indole-3-acetic acid, inositol, lactose, maleic acid, maleic acid plus urea, malic acid, malic acid plus urea, N-methyl-3-carboxylamide-2-pyridone, N-methyl-3-carboxylamide-6-pyridone, N-methyl-3-carboxy-6-pyridone, 1, 7-dimethyl-5-oxy-(1,5-dihydro-1,6-napthyridine), 2-methyl-5-ethoxymethyl-6-aminopyrimidine, 4-methyl-uracil,[3] 5-methylthiouracil, nicotinamide, ornithine, oxaloacetic acid, o-phenylene urea,[5] pteroic acid,[6] 4-pyridoxic acid, 2-pyrolidone-5-carboxylic acid, pyruvic acid, ribonucleic acid, succinic acid, theobromine, theophylline, thymidine,[7] thymine, trigonelline, uracil, urea, uric acid, uridine,[8] uridylic acid, xanthopterin,[3] xanthurenic acid.[3]

Thymidine, uridylic acid, and cytidylic acid alone or in combination have no activity when tested in the presence of an amount of orotic acid permitting partial growth of *Lactobacillus bulgaricus* 09.

A concentrate of *Lactobacillus bulgaricus*

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* We are indebted to the following individuals for the indicated miscellaneous compounds:

1 Dr. H. A. Lardy, University of Wisconsin.

2 Dr. Karl Folkers, Merck & Company, Inc.

3 Dr. J. M. Sprague and coworkers, Sharp & Dohme, Inc.

4. Dr. H. K. Mitchell, California Institute of Technology.

5 Dr. J. O. Lampen, formerly of American Cyanamid Co.

6 Dr. E. L. R. Stokstad, Lederle Laboratories, Inc.

7 Dr. E. E. Snell, University of Wisconsin.

8 Dr. H. S. Loring, Stanford University.

TABLE II. Apparent Orotic Acid Content of Natural Materials.

Sample	Orotic acid, γ/g or ml
Delactosed whey	2600
Skim milk powder	580
Whey nutrient I	600
" " II	600
Corn steep solids	345
Yeast extract	2670
Liver fraction "S"	1600
" " "L"	2000
Dried distillers' solubles	1040
Dried mold mycelium	280
Urine I	94
" II	110
" III	84
" IV	76

factor (LBF)(1) from Dr. E. E. Snell was found to be inactive in lieu of orotic acid for *Lactobacillus bulgaricus* 09.

The Occurrence of Orotic Acid in Natural Materials. A variety of natural materials have been assayed for orotic acid following solution or dispersion in water. The orotic acid content of various natural materials, subject to the limitations mentioned in the Discussion, is summarized in Table II.

Discussion. The ability of ureidosuccinic acid and 5-(carboxy-methylidine)-hydantoin to replace orotic acid in the nutrition of *Lactobacillus bulgaricus* 09 is unexpected. The existence of an equilibrium between 5-(carboxy-methylidine)-hydantoin and orotic acid such that 5-(carboxy-methylidine)-hydantoin would appear to have 30-40% of the activity of orotic acid is quite unlikely under the conditions of microbiological assay employed. Acid conditions would appear to influence any such equilibrium in favor of the hydantoin rather than the pyrimidine since an hydantoin instead of a pyrimidine is obtained when ureidosuccinic acid is treated with strong acid. The microbiological activity of ureidosuccinic acid is difficult to explain on the basis of any equilibrium phenomenon. It is suggested that 5-(carboxy-methylidine)-hydantoin is utilized after hydrolysis to ureidofumaric acid. The apparent microbiological activity of ureidosuccinic acid and 5-(carboxy-methylidine)-hydantoin may indicate that the synthesis of the pyrimidine ring in nature involves cyclization of an aliphatic ureido dicarboxylic acid such as ureidosuccinic acid or ureidofumaric acid.

It should be appreciated that any data on the distribution of a component of natural material is subject to the specificity of the method of assay employed. It would appear, however, from the data of Table II that orotic acid or a microbiologically active related compound has an ubiquitous occurrence in nature. In the case of milk products the orotic acid content has been confirmed by actual isolation of the compound in essentially quantitative yield (see accompanying paper)(7). Other natural materials with high orotic acid activity by microbiological assay are, at present, under study.

The fate of orotic acid in the nutrition of *Lactobacillus bulgaricus* 09 is at present unknown but it may be assumed that it is involved in nucleic acid synthesis. Since uridine, cytidine, uridylic acid and cytidylic acid as well as uracil and cytosine are inactive in lieu of orotic acid with *Lactobacillus bulgaricus* 09, it is conceivable that the synthesis of nucleic acid, at least in this organism, does not involve the participation of uracil or the uracil nucleosides and nucleotides but that orotic acid becomes incorporated into nucleic acid before decarboxylation. Such an hypothesis is not incompatible with recent data, obtained from isotopic studies, indicating that orotic acid but not uracil or uridine is a precursor of nucleic acid pyrimidine in the rat(8-11).

Summary. The orotic acid requirement of *Lactobacillus bulgaricus* 09 can be met by ureidosuccinic acid (10-20% as active as orotic acid) or by 5-(carboxy-methylidine)-hydantoin (30-40% as active as orotic acid) but not by any one of a large group of re-

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8. Arvidson, H., Eliasson, N. A., Hammarsten, E., Reichard, P., and Ubisch, H. V., *J. Biol. Chem.*, 1949, v179, 169.

9. Hammarsten, E., Reichard, P., and Saluste, E., *J. Biol. Chem.*, 1950, v183, 105.

10. Reichard, P., *Acta chem. Scand.*, 1949, v3, 422.

11. Hammarsten, E., Reichard, P., and Saluste, E., *Acta chem. Scand.*, 1949, v3, 433.

lated or unrelated compounds studied. The data are taken as microbiological evidence that ureidosuccinic acid and possibly ureidofumaric acid (derived from 5-(carboxy-methylidine)-hydantoin) are precursors of the pyrimidine ring.

Data on the distribution of orotic acid in nature are presented.

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A Growth-Promoting Substance for *L. bulgaricus* 09 in Whey: Isolation and Identification as Orotic Acid. (18176)

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In 1949 Williams *et al.*(1) reported the presence of a factor in yeast extract required by certain strains of *Lactobacillus bulgaricus* for growth in a synthetic medium. During investigations in our laboratories on the growth requirements of several strains of the same species, it was found that one strain identified as *Lactobacillus bulgaricus* 09 was incapable of growth when the medium was supplemented with yeast extract but required a factor present in liver, milk products or other natural materials(2). It was found in a survey of known compounds that orotic acid (4-carboxy uracil) was capable of replacing the requirement of the organism for the natural material(2). Of the natural materials tested, whey was found to be a very good source of the growth-promoting factor. This source was selected for isolation studies to investigate the possible identity of the microbiological activity of whey with orotic acid. The isolation of the microbiological activity from this natural source and its characterization as orotic acid are presented in this communication.

Experimental. Assays for the factor were carried out microbiologically using *Lactobacillus bulgaricus* 09 as described in the accompanying paper(3). Orotic acid was used as a standard in the assay and the activities of the various fractions obtained are expressed in terms of this standard. 1.25 kg of powdered whey* were stirred for one hour with 25 liters of water and the mixture filtered with the aid of 1.2 kg of Hyflo filter aid. 21.3 liters of a yellow aqueous extract were obtained. This solution contained by microbiological assay 92 γ/ml of the factor expressed as orotic acid or a total of 1.94 g of activity. The aqueous extract was stirred for ½ hour at pH 3 with 426 g of Norit A; filtered using suction on a 12″ Buchner funnel and washed with approximately 3 liters of water. 0.18 g out of 1.94 g of activity remained in the charcoal filtrate. An elution of the substance was affected by allowing 1 N sodium hydroxide solution to pass slowly through the charcoal bed employing suction. Twelve 300-ml fractions were collected. A total of 1.7 g of activity were eluted as indicated by microbiological assay of the fractions. Fractions No. 5 through 10 which contained 1.01 g of activity were pooled, neutralized to pH 7 with concentrated hydrochloric acid and concentrated under vacuum to 300 ml. The dark yellow concentrate which contained at this point about 23% sodium chloride was allowed to stand overnight in the refrigerator. After filtering and washing with cold water and with alcohol there were obtained 1.2 g of grayish white crystalline material subsequently identified

1. Williams, W. L., Hoff-Jorgensen, E., and Snell, E. E., *J. Biol. Chem.*, 1949, v177, 933.

2. Wright, L. D., Huff, J. W., Skeggs, H. R., Valentik, K. A., and Bosshardt, D. K., *J. Am. Chem. Soc.*, 1950, v72, 2312.

3. Wright, L. D., Valentik, K. A., Spicer, D. S., Huff, J. W., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., in press.

* "Edible Quality" spray dried delactosed whey was purchased from the National Dairy Laboratories, Oakdale, Long Island, N. Y.