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 $\gamma$/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.

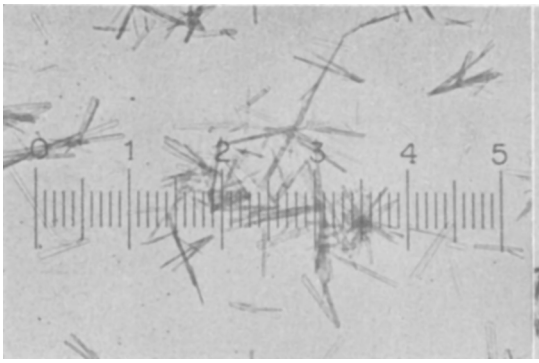



FIG. 1a

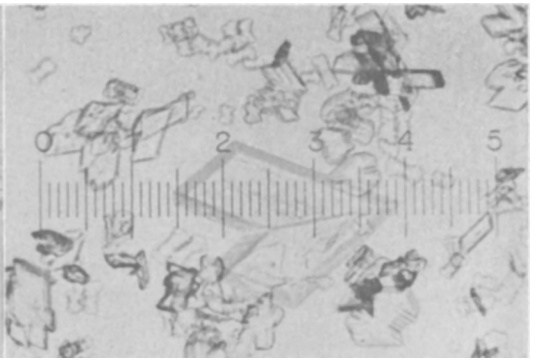



FIG. 1b

(a) Crystals of the sodium salt of orotic acid. (b) Crystals of orotic acid. Scale: 0 to 1 = 113 microns.

as a sodium salt. This material contained about 90% orotic acid as indicated by microbiological assay. About 9.6 mg of activity remained behind in the filtrate. In the presence of high concentrations of sodium chloride the sodium salt of orotic acid will crystallize readily as rather well defined needles even from solutions containing large amounts of impurities. The sodium salt also is obtained from strong acid solutions (pH 1) which contain high concentrations of salt.

The 1.2 g of the crude crystalline sodium salt were boiled for ½ hour with 125 ml of water and filtered while hot to remove 160 mg of an extremely water-insoluble microbiologically inactive crystalline compound. About 200 mg of Darco G-60 were added to the slightly yellow filtrate and after stirring for a few minutes the hot mixture was filtered and the colorless solution placed in the refrigerator overnight. After filtration, the crystalline mass was washed with water and alcohol and dried over $P_2O_5$. 670 mg of snow-white crystalline material were obtained. The crystals were well defined long needles as shown in Fig. 1a. An additional 230 mg of the crystalline salt were obtained by concentration and cooling of the filtrate. Thus of the 1.01 g of orotic acid activity indicated by microbiological assay to be present in the pooled charcoal eluates selected for purification, 900 mg actually were obtained in crystalline form. This material does not contain water of crystallization but is, however, slightly hygroscopic. 300 mg of the crystalline sodium salt were dissolved in 35 ml of boiling water and 0.4 ml of concentrated HCl added. On cooling overnight in the refrigerator 220 mg of the free acid were obtained as white crystalline plates as shown in Fig. 1b. The crystalline acid contains 1 molecule of very tightly bound water of crystallization.

A comparison of the sodium salt and the free acid of the isolated material was made with synthetic orotic acid† and its sodium salt and the results are summarized in Table I.

A micropotentiometric titration of 10 mg of the isolated acid with 1.39 N sodium hydroxide confirmed a molecular weight of 174 and indicated the presence of 2 acidic groups having pK values of 2.46 and 9.40 respectively as shown in Fig. 2. Similar data were obtained for synthetic orotic acid.

Ultraviolet absorption curves on the sodium salts dissolved in water at a concentration of 20 γ/ml were made on the Beckman quartz spectrophotometer and showed an absorption maximum at 278 mμ and a minimum of 240 mμ. The corresponding molecular extinction coefficients are shown in Table I.

The isolated material and synthetic orotic acid were equivalent in their ability to promote growth of *Lactobacillus bulgaricus* 09 when assayed under the conditions described in the accompanying paper(3).

*Discussion.* The data presented in Table I indicate that the growth promoting factor for *Lactobacillus bulgaricus* 09 present in

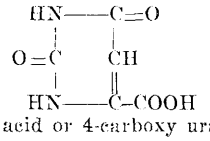
† Orotic acid was prepared from oxalacetic ester and urea according to the method described by Mitchell and Nyc(4).

TABLE I. Comparison of Properties of Orotic Acid and Growth-promoting Factor for *Lactobacillus bulgaricus* Isolated from Whey.

| Compound | M.W. by titration | $pK_1$ | $pK_2$ | $\epsilon 278\ m\mu$ | $\epsilon 240\ m\mu$ | $\epsilon 278/\epsilon 240$ | % N* |
|---|---|---|---|---|---|---|---|
| Isolated compound | 177 | 2.46 | 9.40 | 7130 | 1730 | 4.11 | 15.73 |
| Orotic acid | 180 | 2.51 | 9.40 | 7180 | 1710 | 4.19 | — |
| Theoretical | 174 | — | — | — | — | — | 15.73 |

* This analysis was carried out on the sodium salt of the isolated compound.

whey is identical with orotic acid. This compound has the structure shown in the accompanying diagram.



(Orotic acid or 4-carboxy uracil)

It is believed that practically all of the growth-promoting activity of the water extract of whey is due to the presence of orotic acid in view of the almost quantitative recovery of the activity as the crystalline compound. In the survey of other compounds ureidosuccinic acid and 5-(carboxy-methylidine)-hydantoin were found to replace orotic acid in the nutrition of *Lactobacillus bulgaricus* 09. These two substances are discussed in the accompanying paper(3).

Orotic acid was discovered and isolated from cow's milk in 1905 by Biscaro and Belloni(6). This substance was found by Loring and Pierce to replace cytosine and uracil in satisfying the growth requirements of 2 pyrimidine-deficient mutants of *Neurospora*(7). Mitchell *et al.* have reported that orotic acid is produced in large amounts by certain mutant strains of *Neurospora* and suggest that possibly this substance arises from a side reaction in the biosynthesis of nucleic acid(5). Chattaway reported that orotic acid can replace the folic acid requirement of certain microorganisms, indicating a possible role of this compound in the biosynthesis of nucleic acid(8). Recent isotopic studies with rats suggest that orotic acid rather than uracil is a precursor of nucleic acid pyrimidine(9-12).

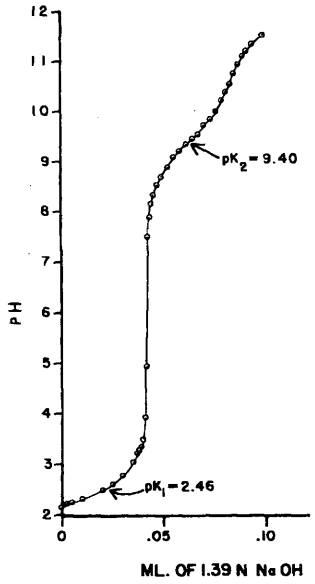



FIG. 2.
Titration curves of 10 mg (0.0574 mm) of crystalline orotic acid isolated from whey.

*Summary.* Various natural substances especially whey were found to contain a substance required for growth of *Lactobacillus bulgaricus* 09. In the survey of known compounds orotic acid was found to satisfy the requirement of this organism for natural material. The microbiologically active material was isolated from one of the natural sources, whey, and identified with orotic acid.

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

5. Mitchell, H. K., Houlahan, M. B., and Nyc, J. F., *J. Biol. Chem.*, 1948, v172, 525.

6. Biscaro, G., and Belloni, E., *Ann. Soc. Chim. Milano*, 1905, v11, Nos. 1 and 2; *Chem. Zent.*, 1905, v2, 64.

7. Loring, H. S., and Pierce, J. G., *J. Biol. Chem.*, 1944, v153, 61.

8. Chattaway, F. W., *Nature*, 1944, v153, 250.

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

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

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

12. Hammarsten, E., Reichard, P., and Saluste, E., *Acta chem. Scand.*, 1949, v3, 432.

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