INVESTIGATIONS ON THE BIOSYNTHESIS OF CITROVORUM FACTOR BY LACTIC ACID BACTERIA

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Sauberlich and Baumann (1948) have reported that Leuconostoc citrovorum, strain 8081, failed to grow on a synthetic medium unless the medium was supplemented with an unidentified growth factor isolated from liver extracts, designated citrovorum factor. More recent reports in the literature (Sauberlich, 1949; Nichol and Welch, 1950a) point to the conversion of pteroylglutamic acid to citrovorum factor, and to the implication of the latter compound as the physiologically-active agent in vivo (Bond et al., 1949). Since earlier papers have reported the biosynthesis of a folic acid-like factor by the bacterial cell from various precursors (Stokes and Larsen, 1945; Sarett, 1947; and Nimmo-Smith et al., 1948), it was of interest to investigate the nature of such biosyntheses and to evaluate the suggestion of Woods (1950) that the "folic acid" normally active for microorganisms may differ from pteroyl glutamic acid and may in fact belong to the folic acid group (Bond et al., 1949).

In an attempt to study the biosynthetic pathway of citrovorum factor, four organisms representative of variations in folic acid metabolism were chosen for study. These were: (1) Lactobacillus arabinosus, which does not require pteroyl glutamic acid for growth and is reported to synthesize it from p-aminobenzoic acid (Sarett, 1947); (2) Streptococcus faecalis, which has a requirement for pteroyl glutamic but responds even more efficiently to rhizopentin (Stokes et al., 1944); (3) Lactobacillus casei, which requires the intact pteroyl glutamic acid molecule (Stokes et al., 1944; Krueger and Peterson, 1944); and (4) Leuconostoc citrovorum, which responds to citrovorum factor and only partially to high levels of pteroyl glutamic acid (Sauberlich and Baumann, 1948).

This paper describes observations made on the biosynthesis of citrovorum factor by these microorganisms and on the factors which affect such synthesis.

METHODS

L. casei and S. faecalis cells were grown in the amino acid medium of Stokes and Gunness (1945) supplemented with 0.001 μg per ml pteroyl glutamic acid. Trypsinized casein (3 mg per ml) was added to the L. casei medium as a source of strepogenin (Stokes et al., 1949) to eliminate the lag in early growth. L. arabinosus cells were grown in the amino acid medium supplemented with 0.1 mg per ml p-aminobenzoic acid. L. citrovorum was grown in the medium of Sauberlich and Baumann (1948) with either citrovorum factor or pteroyl glutamic acid added for optimal growth. All organisms were incubated at 37°C for 17 to 20 hours in one or two liter batches under deep layer conditions to enhance growth.

The cells were harvested by centrifugation, washed twice with 0.15 phosphate buffer at pH 8.0, and resuspended to an appropriate volume in the buffer. The technique of Stokes and Larsen (1945) was employed in the resting cell studies with the following modifications: (a) larger concentrations of cells were used (4.0 to 6.0 mg per ml of suspension medium), (b) no nitrogen stream was used during the incubation period which was considerably longer (17 hours), and (c) the above buffer supplemented with 0.8 per cent glucose was used as the suspension medium. The pH of the buffer and glucose concentration were those found optimal by Stokes and Larsen (1945) for the synthesis of "folic acid" from rhizopentin. After overnight incubation at 37°C the suspensions were steamed for 10 minutes to destroy enzyme activity. To liberate citrovorum factor activity 1 mg of dried chicken pancreas per ml of suspension was added and

1 We are indebted to Mr. E. L. Rickes, Organic and Biochemical Research Department, Merck & Co., Inc., for generous supplies of dried chicken pancreas throughout this study.
the resulting mixture incubated overnight at 37 C under a layer of toluene. This incubation likewise was followed by the above steaming procedure. Aliquots of cellular suspensions were diluted then and assayed for L. citrovorum activity by a modification of the method of Sauberlich and Baumann (1948). Since pancreas preparations varied in citrovorum factor activity, each batch was assayed and appropriate corrections made. The amount of citrovorum factor synthesized was reported in units per mg dry cell weight, which was determined by overnight drying at 100 C of an aliquot of the bacterial suspension. Turbidimetric growth determinations of the suspensions before and after incubation revealed no appreciable change in cell mass.

RESULTS

Synergism between pteroylglutamic acid and citrovorum factor. Bardos et al. (1949) have described an apparent synergism between thymidine and citrovorum factor. They observed that in medium supplemented with levels of thymidine insufficient to elicit a growth response, the response to citrovorum factor was increased tenfold. Since in our experiments relatively high levels of pteroylglutamic acid were used, it was of interest to note whether any synergism existed between citrovorum factor and pteroylglutamic acid or related compounds. Under our conditions of assay we observed a three to fourfold increase in citrovorum factor activity when media were supplemented with subminimal levels of pteroylglutamic acid or formylpteroylglutamic acid (figure 1). For this reason care was exercised in assaying samples so that the pteroylglutamic acid levels were far below those which would affect the citrovorum factor assay.

For qualitative differentiation between pteroylglutamic acid and citrovorum factor activity when the concentration of the former was not sufficiently low so that its interference in the L. citrovorum assay was not eliminated, overnight incubation at room temperature after acidification to pH 2 with HCl was used (Keress-tesy and Silverman, 1950). Under these conditions approximately 90 per cent destruction of citrovorum factor occurred, whereas pteroylglutamic acid similarly treated showed no significant loss of activity as measured by the L. casei assay.

Citrovorum factor synthesis during growth. All of the organisms tested synthesized citrovorum factor when grown on synthetic media supplemented with appropriate precursor (table 1). Considerable variation was encountered in the amount of citrovorum factor synthesized, but in all cases except L. casei the concentration was found to be in excess of that which is required for maximal growth. It should be noted also that the major part of the citrovorum factor formed is in a bound form which is not utilized readily by the test organism, L. citrovorum, and becomes available only after enzymatic digestion.

Citrovorum factor synthesis in cellular suspensions. When washed cells were incubated under the conditions described in the presence of pteroylglutamic acid, synthesis of citrovorum factor could be demonstrated with suspensions of the test organisms (tables 2 and 3). S. faecalis grown in pteroylglutamic acid supplemented...
medium, and to a lesser extent *L. casei* and *L. arabinosus*, synthesized relatively high levels of citrovorum factor. It is interesting to note that while *L. citrovorum* cells grown on pteroylglutamic acid appear to store more citrovorum factor during the growth phase, they do not synthesize nearly as much in cellular suspensions as the other organisms (table 3).

Investigation of some of the factors which affect biosynthesis by "resting cells" was undertaken.

**TABLE 1**

The biosynthesis of citrovorum factor by lactic acid bacteria

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>CITROVORUM FACTOR ACTIVITY, UNITS PER ML</th>
<th>Whole broth</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A*</td>
<td>B*</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td></td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td></td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td><em>Lactobacillus arabinosus</em></td>
<td></td>
<td>5</td>
<td>46</td>
</tr>
<tr>
<td><em>Leuconostoc citrovorum</em></td>
<td></td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td><em>Leuconostoc citrovorum</em> (40 hr)</td>
<td></td>
<td>11</td>
<td>55</td>
</tr>
</tbody>
</table>

* A = sample assayed as is; B = sample treated with chicken pancreas preparation prior to assay. Samples were assayed after 17 to 20 hours' incubation.

*Leuconostoc citrovorum* grown in medium supplemented with 2 μg per ml pteroyl glutamic acid.

**Enzyme liberation of citrovorum factor.** Recent investigations by Hill and Scott (1952a,b) have demonstrated a citrovorum factor liberating enzyme in both hog kidney and chick liver. Using a chicken pancreas preparation as an enzyme source, we found that with every test organism overnight incubation was necessary to liberate citrovorum factor from the cells. Incubation of the pancreas preparation alone or in combination with pteroylglutamic acid showed no increase in citrovorum factor activity. The increase in citrovorum factor potency of suspensions after pancreas treatment was not uniform, however. Thus, it was observed that in cellular suspensions to which no metabolite had been added the citrovorum factor activity was present largely in the bound form. The addition of pteroylglutamic acid to suspensions not only increased the over-all synthesis of citrovorum

### TABLE 2

Effect of pteroylglutamic acid and p-aminobenzoic acid antagonists on synthesis of citrovorum factor by bacterial suspensions

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CITROVORUM FACTOR ACTIVITY, UNITS PER MG CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Streptococcus faecalis</em></td>
</tr>
<tr>
<td>Pteroylglutamic acid 10 μg/ml</td>
<td>1630</td>
</tr>
<tr>
<td>Pteroylglutamic acid 1 μg/ml</td>
<td>116</td>
</tr>
<tr>
<td>Pteroylglutamic acid 1 μg/ml</td>
<td>18</td>
</tr>
</tbody>
</table>

Cells without added precursor contained 6 to 10 citrovorum factor units per mg.

Samples were sufficiently diluted to rule out aminopterin effect in the assay.

* 10 μg per ml aminopterin used in this experiment instead of 1 μg.

### TABLE 3

Effect of pteroylglutamic acid and aminopterin on synthesis of citrovorum factor by cells of *Leuconostoc citrovorum*

<table>
<thead>
<tr>
<th>TREATMENT*</th>
<th>CITROVORUM FACTOR ACTIVITY—UNITS PER MG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium A†</td>
</tr>
<tr>
<td>None</td>
<td>1.1</td>
</tr>
<tr>
<td>1 μg per ml pteroylglutamic acid</td>
<td>5.2</td>
</tr>
<tr>
<td>10 μg per ml pteroylglutamic acid</td>
<td>51.0</td>
</tr>
<tr>
<td>1 μg per ml pteroylglutamic acid + 1 μg per ml aminopterin</td>
<td>0.26</td>
</tr>
</tbody>
</table>

* All treatments include 2 mg per ml ascorbic acid.
† Medium A = 4 units per ml citrovorum factor
Medium B = 2 μg per ml pteroylglutamic acid
Medium C = 0.6 units per ml citrovorum factor + 1 μg per ml pteroylglutamic acid.

factor but markedly increased the percentage of the free form.

Storage of samples in the refrigerator for a
week or longer also resulted in considerable increase in the "free" citrovorum factor activity of cells. Cell filtrates (relatively free of cells) also contain bound citrovorum factor (table 1), which cannot be liberated by heat. This lends support to the hypothesis that the bound citrovorum factor is not merely due to adsorption on the bacterial surface, but rather to the presence of a conjugate. These data also substantiate the conclusion that citrovorum factor produced by the growing or resting cell exists largely in a bound form which is not utilized readily by L. citrovorum.

Time of incubation. Under our experimental conditions it was found that approximately ten of more hours of incubation were required for maximal biosynthesis of citrovorum factor by resting cells. In actual practice an overnight incubation period (16 to 17 hours) was used because of its greater convenience.

Pteroylglutamic acid concentration. The conversion of pteroylglutamic acid to citrovorum factor was found to be proportional to the concentration of pteroylglutamic acid added (figure 2). Rhizopterin and N-10 formylpteroylglutamic acid could be utilized also by S. faecalis in the biosynthesis of citrovorum factor but not as efficiently as pteroylglutamic acid. With these compounds as well, citrovorum factor synthesis was proportional to concentration.

Ascorbic acid and dextrose. Since dextrose and anaerobiosis were found to increase "folic acid" formation from rhizopterin (Stokes and Larsen, 1945) and since chemical synthesis of citrovorum factor appears to involve reductive processes (Flynn et al., 1951), the effect of a reducing agent, ascorbic acid, on citrovorum factor biosynthesis was studied. Several experiments with suspensions of S. faecalis and L. casei indicated that the addition of ascorbic acid appreciably increases the amount of citrovorum factor produced (figure 2). These results are in substantial agreement with those of Nichol and Welch (1950a) and do not support the findings of Hill and Scott (1952b), who reported that the effect of ascorbic acid is apparently on the citrovorum factor liberating system. Our data, however, indicate that ascorbic acid also plays a role in the formation of citrovorum factor since, prior to enzyme treatment, ascorbic acid markedly increases the conversion of pteroylglutamic acid to citrovorum factor. Results with dextrose pteroylglutamic acid, the addition of dextrose markedly increased such synthesis.

Vitamin B₁₂. In experiments with S. faecalis and a B₁₂ requiring organism, L. lactis, we were unable to demonstrate any effect of vitamin B₁₂ on the conversion of pteroylglutamic acid to citrovorum factor, even when high levels of vitamin B₁₂ were used (0.1 µg per ml). This is interesting in light of the report of Dietrich, Monson, and Elvehjem (1951) that, in chicks, vitamin B₁₂ enhanced the conversion of pteroylglutamic acid to citrovorum factor. In the case of S. faecalis, which produces vitamin B₁₂ during growth, it could be argued that the bacterial cells might have retained sufficient stores of the vitamin so that its effect on the conversion could not be detected. However, the lack of such effect in the case of L. lactis (grown in medium containing ascorbate but no vitamin B₁₂) coupled with the absence of any sparing action of citro-
vorum factor on B₁₂ requirement of microorganisms strongly suggests that vitamin B₁₂ is not involved in the biosynthesis of citrovorum factor by microorganisms.

Effect of pteroylglutamic acid antagonists. In growth experiments it had been noted that citrovorum factor effectively overcomes growth inhibition of L. casei, S. faecalis, and L. citrovorum by several so-called folic acid antagonists, including aminopterin and x-methyl folic acid. It was found also that although citrovorum factor was more effective than pteroylglutamic acid in neutralizing aminopterin toxicity in L. citrovorum, the inhibition indices for pteroylglutamic acid compared favorably with citrovorum factor when L. casei and S. faecalis were used as test organisms. It was possible, therefore, that such inhibition did not necessarily involve a block in the pathway pteroylglutamic acid → citrovorum factor, but might act through interference at the enzyme level in which citrovorum factor might play the role of coenzyme. On the other hand, Nichol and Welch (1950b) have reported that aminopterin inhibits the formation of citrovorum factor from pteroylglutamic acid by rat liver slices. In order to elucidate the mode of action of aminopterin, studies on its effect on biosynthesis of citrovorum factor by resting cells were undertaken. With suspensions of all the test organisms it was possible to demonstrate a marked and consistent inhibition of citrovorum factor synthesis (tables 2 and 3). This would seem to indicate that aminopterin does block the biosynthetic pathway pteroylglutamic acid → citrovorum factor.

In a similar manner, attempts were made to study the role of p-aminobenzoic acid and pteroylglutamic acid in the metabolism of L. arabinosus. In the presence of p-aminobenzoic acid or pteroylglutamic acid, L. arabinosus synthesized a citrovorum factor-active substance, and such synthesis, as with the other lactobacilli, was inhibited almost completely by aminopterin. Sulfanilamide, however, while completely blocking the conversion of p-aminobenzoic acid to citrovorum factor, only partially (about 40 per cent) inhibited the formation of citrovorum factor from pteroylglutamic acid. The synthesis by L. arabinosus of an acid labile, L. citrovorum active compound and the inhibition of this synthesis by aminopterin demonstrate that this organism is capable of converting either p-aminobenzoic acid or pteroylglutamic acid to citrovorum factor. The incomplete inhibition by sulfanilamide of citrovorum factor synthesis from pteroylglutamic acid and the total inhibition of such synthesis from p-aminobenzoic acid lend further support to the step-wise conversion, p-aminobenzoic acid → pteroylglutamic acid → citrovorum factor or citrovorum factor-like compound. Such interpretation of the L. arabinosus data is not incompatible with the recent findings of Koft et al. (1950) and Sarett (1951). These investigators indicate that pteroylglutamic acid and citrovorum are not true stimulants for L. arabinosus. Activity of pteroylglutamic acid and citrovorum factor is ascribed to their degradation to p-aminobenzoic acid. It is conceivable that p-aminobenzoic acid may play more than one role in the nutrition of lactic acid bacteria. Not only can it serve as a direct precursor of pteroylglutamic acid, but it may act also as a catalyst in several enzyme systems. Such a hypothesis would explain not only the bioconversion of citrovorum factor from p-aminobenzoic acid and pteroylglutamic acid by L. arabinosus, but also the relatively low order of activity of pteroylglutamic acid and citrovorum factor for the growth of this microorganism.

SUMMARY

Both growing and resting cells of several lactobacilli were found to synthesize citrovorum factor from pteroylglutamic acid. This citrovorum factor was largely in a bound form which was available to Leuconostoc citrovorum only after enzyme digestion.

The conversion of pteroylglutamic acid to citrovorum factor by resting cells was proportional to the concentration of pteroylglutamic acid added and was enhanced by the addition of ascorbic acid. Lactobacillus arabinosus synthesized citrovorum factor from both p-aminobenzoic acid and pteroylglutamic acid.

Aminopterin inhibited the conversion of pteroylglutamic acid to citrovorum factor in all the test organisms. In the case of L. arabinosus sulfanilamide totally inhibited the conversion of p-aminobenzoic acid to citrovorum factor but only partially blocked the formation of citrovorum factor from pteroylglutamic acid. These data have been interpreted as indicating
the formation of citrovorum factor through the biosynthetic pathway p-aminobenzoic acid \( \rightarrow \) pteroylglutamic acid \( \rightarrow \) citrovorum factor.

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