CONVERSION OF PTEROYLGLUTAMIC ACID TO THE CITROVORUM FACTOR BY PREPARATIONS FROM LACTOBACILLUS CASEI

CHARLES R. HEISLER and B. S. SCHWEIGERT

American Meat Institute Foundation, and Department of Biochemistry, the University of Chicago, Chicago, Illinois

Received for publication December 22, 1958

The conversion of folic acid to citrovorum factor (N³-formyl tetrahydrofolic acid) had been demonstrated for preparations of Streptococcus faecalis (Broquist et al., 1953), Lactobacillus arabinosus (Hendlin et al., 1953), Lactobacillus casei (Bond, 1953), and a number of animal tissues (Wisten and Eigen, 1950; Nichol and Welch, 1950; Nichol, 1952). It has been shown that ascorbic acid, ATP, Mg²⁺, DPN, and a source of a one-carbon unit (Nichol, 1954) are required for optimal conversion of folic acid to citrovorum factor or to an active formylating form of this vitamin. Pyridoxal phosphate, Mn²⁺, and TPN (Jaenicke, 1956) have been reported as needed for a one-carbon donor system involving serine. We wish to report the requirement of another cofactor for the conversion of folic acid to citrovorum factor by a preparation of L. casei, which is found in the boiled extract of crude enzyme preparations.

MATERIALS AND METHODS

Two grams (wet weight) of washed cells of L. casei ATCC 7469 were suspended in 20 ml of water at 0 C and disrupted by a Raytheon 9-ke Magnetostriiction Oscillator for 2 hr. The sus-

1 Data in this paper are taken from a dissertation submitted by Charles R. Heisler to the Faculty of the Division of the Biological Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry at the University of Chicago. Preliminary results on this work have been reported earlier (Heisler and Schweigert, 1955; 1956). The study was supported, in part, by a grant from the National Institutes of Health. Journal Paper No. 157 of the American Meat Institute Foundation.

2 Present address: Department of Agricultural Chemistry, Oregon State College, Corvallis, Oregon.

3 The following abbreviations are used: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

pension was centrifuged at 3000 × G for 30 min to remove the heavy cell debris, then at 20,000 × G for 30 min. The straw colored liquid resulting from this was used as the enzyme source. The boiled supplement was produced by placing a sample of this enzyme preparation in boiling water for 3 min and centrifuging off the coagulated protein.

The majority of the enzymatic activity after ammonium sulfate treatment (pH 5.0) was found in two fractions which precipitated at 50 to 60 and 60 to 75 per cent of saturation.

After incubation of folic acid with the enzyme preparation, the folic acid and citrovorum factor activities were assayed microbiologically using L. casei and Pediococcus cerevisiae ATCC 8081 (Leuconostoc citrovorum), respectively, as the test organisms. The chemically defined medium used in the assay was a modification of that described by Flynn et al. (1951). All determinations were done in duplicate at three different levels of the sample. Turbidimetric readings were taken at 24 to 48 hr at 660 mµ.

Paper chromatography was used to check the purity of the folic acid samples, to identify the products of folic acid conversion, and to partially purify components in the boiled supplement. Paper chromatography was carried out by the ascending technique at 25 C on strips of Whatman no. 1 filter paper with 3 different solvent systems: (a) phosphate (0.1 m, pH 7.0): chloroform (2 volumes), (b) water saturated with secondary butanol, and (c) 12 per cent Na₂HPO₄ with 0.2 per cent ethylenediaminetetraacetate. The chromatograms were examined under ultraviolet light and the strips were further analyzed by bioautographic procedures using L. casei and P. cerevisiae as test organisms for the folic acid and citrovorum factor activity, respectively. A finer resolution of the less well defined bioautographic spots was obtained using tripheynltetrazolium chloride as indicator.
RESULTS

Examination of the activity of the enzyme preparation and the cellular debris after centrifugation at 20,000 × G and 3 C showed that 95 per cent of the total enzymatic activity was in the supernatant liquid. The cell-free enzyme lost formation and the amount of enzyme added was not observed, as seen in figure 1. Chromatographic examination of folic acid by the method of Zakrzewski and Nichol (1953) did not reveal the presence of an inhibitor which might affect the reactions at low concentrations of enzyme.

Figure 1. Effect of the addition of boiled supplement on citrovorum factor (CF) production with the enzyme concentration varied. Each vessel contained the following additions: folic acid (FA), 0.23 μmoles; ATP, 4.0 μmoles; DPNH, 1.35 μmoles; TPN, 1.35 μmoles; Mg++, 5.0 μmoles; serine, 0.19 mmole; ascorbic acid, 0.115 mmol; S, untreated enzyme preparation (1.5 to 2.5 mg nitrogen per ml), amounts as indicated; and bS, boiled supplement, amounts as indicated. The phosphate buffer concentration was 0.06 M, pH 6.3. Final volume, 10 ml. Incubation, 3 hr at 26 C.

However, additions of a boiled supplement, in amounts such that the total additions of heated and unheated enzyme were constant, yielded an approximate straight line relationship. Apparently, an additional heat stable cofactor found in the enzyme preparation was needed for the enzyme reaction to proceed at optimal rates particularly at lower concentrations of enzyme.

Further evidence for the stimulatory effect of...
the boiled supplement was obtained with enzyme fractions prepared by ammonium sulfate treatment. Fraction A (the precipitate resulting from 50 to 60 per cent of saturation of the enzyme preparation) carried out the conversions, although it was less active than the original preparations (designated S), as shown in figure 2. Fraction B (material precipitable by the addition of ammonium sulfate to 60 to 75 per cent of saturation) showed almost no activity alone, but in combination with fraction A approximately doubled the capacity of fraction A to convert folic acid to citrovorum factor. The addition of the boiled supplement to all of the enzyme preparations (particularly fraction A) brought about an increase in the conversion.

The stimulatory effect of the boiled supplement could not be replaced by any of the cofactors known to be required although the addition of ascorbic acid, serine, and TPN was found to be needed for optimal conversion of folic acid to citrovorum factor in confirmation of studies with other systems (Nichol and Welch, 1950; Nichol, 1954; and Jaenicke, 1956). Homocysteine, reported by Doctor et al. (1954) to stimulate the conversion in rat liver homogenate, was markedly inhibitory for the L. casei preparations.

Nature of factor found in boiled supplement. The stimulatory factor present in the boiled supernatant preparation was heat-stable in the absence of ascorbate and was dialyzable, but was not found in the ash of the whole cells or boiled supplement. The factor was found to be quite stable to autoclaving at 120°C under 15 pounds pressure for 15 min at pH 5 and above, but was labile below pH 5.

Table 1 shows the results of two typical experiments in which the boiled supplement (bS) is added to various enzyme preparations. Fractions A and B have been dialyzed and although they do not show an absolute requirement for the boiled supplement, this addition causes an increase in the production of citrovorum factor.

TABLE 1
Activity of enzyme preparations with and without additions of boiled supplement*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme Fraction Tested</th>
<th>Citrovorum Factor Produced with Graded Additions of Boiled Supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>1 ml S</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>2 ml S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 ml S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 ml S</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 ml S</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1 ml bS</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>1 ml S</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>1 ml A</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1 ml A + 1 ml B</td>
<td></td>
</tr>
</tbody>
</table>

* For descriptions of preparations designated S, A, B, and bS see Results in text and legend for figure 2.
from folic acid up to 35 times that produced by the enzyme alone (table 1). In tables 1 and 3, S, A, B, and bS are equal in volume (1 ml) unless indicated otherwise, and were derived from S which contained 1.5 to 2.5 mg nitrogen per ml. These enzyme preparations were incubated for 3 hr with the system outlined in the legend of figure 1. The activity of the boiled supplement is not attributable to the presence of citrovorum factor compounds active for the test organism. Table 2 shows that only a very small amount of material which is active for P. cerevisiae is present in the boiled supplement even when the supplement was incubated with a conjugase enzyme preparation. The boiled supplement was concentrated to one tenth its initial volume and chromatographed on Whatman no. 1 filter paper, using two solvent systems. Four fluorescing bands were observed with only the fastest moving band displaying cofactor activity. This band was rechromatographed and gave a blue fluorescent band with an Rf of 0.90 in water saturated with secondary butanol and an Rf of 0.97 in 12 per cent NaHPO4 and 0.2 per cent ethylenediaminetetraacetate. The strip was analyzed by means of a bioautograph using P. cerevisiae as the test organism and similar Rf values were observed. The Rf values for folic acid and citrovorum factor in water saturated with secondary butanol were 0.10 and 0.57, respectively.

The boiled supplement (1 ml) was incubated for 17 hr at 37 C under toluene with and without 5 mg of an alkaline folic acid conjugase (a preparation from chick pancreas which cleaves polyglutamate derivatives of folic acid to monoglutamates). The folic acid and citrovorum factor activity was increased by this treatment as measured by L. casei and P. cerevisiae (table 2).

These data suggested that most of the folic acid and citrovorum factor activity of the boiled supplement was present in the polyglutamyl form. It should be pointed out that polyglutamate derivatives containing more than three glutamic acid moieties are inactive, or essentially inactive, for the test organisms (Andrews and Schweigert, 1953). Conjugase activity in the unheated enzyme preparations could not account for the increase in citrovorum factor produced with the L. casei enzyme system after addition of the boiled supplement because, (a) the cofactor effect was considerably larger than the citrovorum factor found in the boiled supplement after treatment with conjugase, and (b) the action of unheated enzyme on the boiled supplement produces only a slight increase in citrovorum factor content.

It was of importance to determine if the boiled supplement treated with the conjugase preparation lost its cofactor activity (table 3). On the basis of these data and those obtained in the previous experiments, the cofactor activity of the boiled supernatant appears to be attributable to the presence of small quantities of a polyglutamate form of citrovorum factor acting catalytically in the conversion of folic acid to citrovorum factor.

**DISCUSSION**

Wright (1955, 1956) reported that catalytic levels of a new factor present in boiled extracts, termed Co C, were necessary for the synthesis of serine in an enzyme system from Clostridium cylindrosporum. Triglutamyl derivatives of folic acid would substitute for the boiled extracts; however, the active substances isolated from the

---

### TABLE 2

<table>
<thead>
<tr>
<th>Substances Tested</th>
<th>Folic Acid Activity</th>
<th>Citrovorum Factor Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiled supplement</td>
<td>0.015</td>
<td>0.050</td>
</tr>
<tr>
<td>Conjugase (5 mg.)</td>
<td>0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>Boiled supplement + conjugase</td>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>Boiled supplement + untreated enzyme*</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

* In the absence of folic acid.

### TABLE 3

<table>
<thead>
<tr>
<th>Substance Tested</th>
<th>Citrovorum Factor Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiled supplement (bS)</td>
<td>0.06</td>
</tr>
<tr>
<td>Untreated enzyme (S)</td>
<td>4.0</td>
</tr>
<tr>
<td>Untreated enzyme + boiled supplement</td>
<td>6.5</td>
</tr>
<tr>
<td>Untreated enzyme + boiled supplement, treated with conjugase</td>
<td>4.5</td>
</tr>
</tbody>
</table>
extracts were not identical with known folic acid derivatives.

Hakala and Welch (1957) identified three blue fluorescent spots present on chromatograms of teropterin as mono-, di-, and triglutamyl derivatives of folic acid. Similar blue fluorescent bands of teropterin, obtained by chromatography, were completely inactive in the present system. Hakala and Welch have also isolated a form of citrovorum factor from *Bacillus subtilis* which they have tentatively identified as the triglutamyl derivative. This compound has Rf values similar to that observed for the active material from the boiled supernatant obtained from *L. casei* and showed activity for *P. cerevisiae*. Wright (1955) has described a number of polyglutamyl derivatives of folic acid which were catalytically effective in her system, one of which was identified as the triglutamyl form of citrovorum factor.

A need for a similar cofactor has been established in the present work with an *L. casei* enzyme system in the conversion of folic acid to citrovorum factor. Further evidence is needed, however, to show conclusively that the active form of the vitamin occurring in the boiled extracts is indeed a specific polyglutamyl derivative. The ability of known folic acid derivatives to replace the cofactor activity for *C. cylindrosporum* and the destruction of the cofactor activity in boiled preparations of *L. casei* by conjugase treatment indicate that such a derivative is present in the active materials tested. It is likely, from the present work with *L. casei*, that a polyglutamyl derivative of citrovorum factor is active as the cofactor. The characteristics of the cofactor that support this latter conclusion and tend to eliminate other derivatives of folic acid and citrovorum factor are as follows:

1. The cofactor derived from *L. casei* is a fast-moving light blue fluorescing spot whose Rf is considerably greater than folic acid, citrovorum factor, N^9^formyl tetrahydrofolic acid, or teropterin. In addition, these folic acid derivatives do not possess cofactor activity. Polyglutamyl derivatives of citrovorum factor also are known to have higher Rf values in these particular solvent systems.

2. The fast-moving component of the boiled supplement has *P. cerevisiae* activity, as well as *L. casei* activity, indicating that it is a derivative of citrovorum factor.

3. When treated with alkaline conjugase, the folic acid activity of the purified material obtained from the boiled supplements increases for *L. casei* and for *P. cerevisiae*. This is interpreted to mean that the polyglutamyl derivative of citrovorum factor has been hydrolyzed to citrovorum factor. There is a possibility that this fast-moving component is contaminated with other polyglutamyl derivatives of folic acid which may account for the greater increase in *L. casei* activity. The destruction of the activity in this partially purified boiled supplement by the alkaline conjugase is an indication that a polyglutamyl form is required for activity.

The probable role of a polyglutamyl form of citrovorum factor in the reactions in which the monoglutamyl derivatives are inactive is not clear. It is possible that in all enzyme systems a polyglutamyl derivative(s) is the true cofactor form of this vitamin and that in the crude systems, where the monoglutamyl derivatives are active, a mechanism is available for the synthesis of the derivatives with more than one glutamic acid present.

ACKNOWLEDGMENTS

We are indebted to Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for the synthetic citrovorum factor (leucovorin), purified pteroylglutamic acid, and the teropterin used in this work.

SUMMARY

The characteristics of an enzyme system obtained from extracts of *Lactobacillus casei*, which converts folic acid to citrovorum factor, were investigated. Most of the enzyme activity was found in the protein precipitated by ammonium sulfate at 50 to 60 per cent saturation. A 2- to 3-fold purification was obtained as determined by specific activity measurements. The enzyme system lost more than one-half of its activity during storage in the frozen state and rapidly lost activity in the unfrozen state particularly at an alkaline pH.

Optimal conversion of folic acid to citrovorum factor occurred in 0.06 M phosphate at pH 6.3 at temperature of 25 to 26 C.

Serine, ascorbic acid, and triphosphopyridine nucleotide are required for optimal conversion in this enzyme system.

The need for an additional cofactor in the conversion of folic acid to the citrovorum factor was demonstrated. This cofactor, found in boiled soluble preparations from cells of *L. casei*, was...
dialyzable, stable to heat and alkali, but was unstable when stored at a pH of 5 or lower. The chromatographic behavior, activity for *Pediococcus cerevisiae*, and susceptibility to conjugase, suggested the factor is a polyglutamyl derivative of the citrovorum factor. The similarity of this cofactor with those described for the metabolism of serine by *Clostridium cylindrosporum* was discussed.

REFERENCES


Bond, T. J. 1953 Production of folinic acid from folic acid by *Lactobacillus casei*. Science, 117, 563–564.


Nichol, C. A. 1952 Enzymatic conversion of folic acid to citrovorum factor. Federation Proc., 11, 452.


