STUDIES ON THE IDENTIFICATION OF A FOLATE COMPOUND OF HUMAN SERUM*

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Human serum contains folate (pteroylglutamate) compounds that support the growth of Lactobacillus casei but not Streptococcus faecalis or Leuconostoc citrovorum (Pediococcus cerevisiae) (1). It has been generally accepted that any compound having folic acid activity for L. casei but not for S. faecalis must be a triglutamate (or polyglutamate) (1–4). On the basis of this similarity, serum folate compounds were considered to be pteroylpolyglutamates. Larrabee, Buchanan, Rosenthal and Cathou (5, 6) recently have isolated a monoglutamate (N\(^3\)-methyl tetrahydrofolate) as an intermediate of methionine biosynthesis, and Keresztesy and Donaldson (7, 8) have isolated a compound with similar properties (prenolic A) from horse liver. Both compounds exhibit the same response in growth to the three organisms as that shown by the serum folate derivative. This communication reports evidence that the L. casei-active material of serum is the monoglutamate, N\(^3\)-methyl tetrahydrofolate.

MATERIALS AND METHODS

Normal human serum was obtained from healthy young adult males as previously described (1).

Hog kidney conjugate (9, 10) was prepared as described to us by Wood and Hitchings (11) with several minor modifications. Fresh hog kidneys (285 g) were defatted and minced in 200 ml of distilled water in a Waring Blender for 45 seconds. This suspension was incubated under toluene at 37°C for 4 hours and then at room temperature overnight. Thirty ml of distilled water was added, and the coarse aggregates of fat were removed by filtration through gauze. The material was then centrifuged for 5 minutes in an International clinical centrifuge. The supernatant fluid was decanted and stirred with 4.5 g of Supercel for 30 minutes. After centrifugation the supernatant solution was decanted into 25 ml of a suspension of calcium phosphate gel (0.31 g per ml), stirred for 30 minutes, and centrifuged. The supernatant fluid was treated with 2 g of Darco G 60 activated charcoal\(^1\) per 100 ml of solution, mixed for 30 minutes, and centrifuged at 3,000 rpm as described before. The supernatant solution was then centrifuged at 30,000 rpm for 30 minutes in a Spinco model L centrifuge. The clear, red, supernatant solution was stored frozen overnight at −17°C, thawed the next day and centrifuged at 10,000 rpm for 10 minutes. The supernatant solution was the hog kidney conjugate used in this study. The final centrifugation proved necessary to remove a contaminant containing “bound” folic acid activity.

Chicken pancreas conjugase (10, 12, 13) was prepared by dissolving 2 mg of desiccated chicken pancreas (Difco Laboratories) per ml of water (14). The water solution of desiccated pancreas was centrifuged before use. The supernatant solution was the preparation of chicken pancreas conjugase used in this study.

N\(^3\)-methyl tetrahydrofolate is an intermediate of methionine biosynthesis isolated by Larrabee and Buchanan (5, 6) and was prepared by their procedure (6). It did not replace folic acid and N\(^3\)-formyl tetrahydrofolate as a growth factor for S. faecalis and L. citrovorum, respectively (6). Since on hydrolysis with 6 N HCl at 110°C this compound yielded 1 mole glutamate per mole of N-methyl group, it is a monoglutamate.

Teropeterin (pteroylglutamic acid)\(^2\) had been stored as a dry powder since 1950 in an amber bottle at 4°C; 5 mg was dissolved in a mixture of 40 ml of 0.01 N NaOH and 10 ml of absolute ethanol. Appropriate dilutions were made with deionized water.

N\(^3\)-methyl tetrahydropteroylglutamate was prepared from pteroylglutamic acid (Teropeterin) by a modification of the procedure of Keresztesy and Donaldson (7) for the synthesis of N\(^3\)-methyl tetrahydropteroyl monoglutamate from folate.

† Predoctoral Fellow of the National Science Foundation (1958–1960) and of the National Institutes of Health (1961–2).

\(^1\) Generously provided by Darco Department, Atlas Powder Co., New York, N. Y.

\(^2\) Kindly provided by Dr. T. H. Jukes and Dr. E. L. R. Stokstad, Lederle Laboratories, Pearl River, N. Y.
Microbiologic assays with *L. casei*, *S. faecalis*, and *L. citrovorum* have been described (1). Activity for the first two organisms was measured against a folic acid standard; activity for *L. citrovorum* was measured against the calcium salt of synthetic N⁵-formyl tetrahydrofolate.² Incubation of substrates with conjugase was performed at the optimal pH for each conjugase (hog kidney, pH 7.2; chicken pancreas, pH 4.5); all solutions, however, were adjusted to pH 6.1 before microbiological assay.

The *folic acid activity of serum* was concentrated by a method similar to that applied by Usdin (2) for the concentration of blood folic acid activity. To 500-ml aliquots of fresh normal human plasma was added 5 g of Darco G 60 activated charcoal and 5 g of sodium ascorbate. This mixture was refrigerated at 6°C for 1 hour with gentle shaking, and then was passed through Whatman no. 2 filter paper on a Buchner funnel. Folic acid activity was eluted from the charcoal by treatment of the latter with a solution containing 100 ml of concentrated NH₄OH, 400 ml of deionized water, and 500 ml of ethanol. The charcoal was removed by filtration through Whatman no. 2 filter paper and the filtrate was evaporated to dryness in a rotary flash evaporator. Folic acid activity was extracted from the dried residue with three 2-ml aliquots of 0.001 M ammonium carbonate. The insoluble material was removed by centrifugation for 5 minutes in a clinical centrifuge and discarded.

Since the high salt concentration of the dark amber-colored concentrate interfered with paper chromatography, filtration with dextran gel (Sephadex G-25 medium)³ was employed to separate most of the salts from the folate activity. Three ml of the viscous concentrate was diluted with an equal volume of water and placed on a column of Sephadex (2 × 26 cm). The column was prepared as described by Flodin (15) and eluted with 0.001 M Na₂HPO₄·7H₂O. Fractions of 2 ml were collected; compounds active for *L. casei* were concentrated in fractions 36 through 48. Fraction 44, which had maximal activity, was concentrated tenfold in vacuo, and used for chromatographic studies.

*Paper chromatography* was done on Whatman no. 1 paper (18 × 30 cm). The sheets were spotted with 0.50-μl aliquots of 7 to 10 × 10⁶ M solutions of synthetic sodium folate, N⁵-formyl tetrahydrofolate, chemically prepared N⁵-methyl tetrahydropteroylglutamate, enzymatically prepared N⁵-methyl tetrahydrofolate, and with 2-μl aliquots of serum concentrate. Four developing solvent systems were used: 1) saturated Na₂HPO₄·7H₂O; 2) one-third saturated Na₂HPO₄·7H₂O; benzyl alcohol (2:1, vol/vol; aqueous phase used); 3) 5 per cent citric acid brought to pH 9 with concentrated NH₄OH; isooamyl alcohol (2:1, vol/vol; both phases used); 4) 0.10 M sodium bicarbonate buffer, pH 9.3, containing 6 mg of ascorbic acid per ml. Similar solvent systems have been used by others (2, 16, 17). After approximately 3 hours of development by the ascending method, the paper chromatograms were allowed to dry in a hood.

### TABLE I

<table>
<thead>
<tr>
<th>Experimental preparations</th>
<th><em>L. casei</em></th>
<th><em>S. faecalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10⁻² g. Teropterin</td>
<td>34</td>
<td>4.3</td>
</tr>
<tr>
<td>2. Hog kidney conjugase</td>
<td>4</td>
<td>2.7</td>
</tr>
<tr>
<td>3. Chicken pancreas conj.</td>
<td>8.5</td>
<td>16.4</td>
</tr>
<tr>
<td>4. No. 1 + no. 2</td>
<td>51</td>
<td>16.4</td>
</tr>
<tr>
<td>5. No. 1 + no. 3</td>
<td>47</td>
<td>68</td>
</tr>
</tbody>
</table>

*All samples were preincubated for 18 hours at 37°C under 1 ml toluene with 350 mg per 100 ml ascorbic acid prior to assay.
| The hog kidney conjugase was diluted to 30 per cent of original concentration with deionized water; 1 ml was used in the assays. |

**Bioautography** was done as follows: a fresh 7- to 10-hour culture of *L. casei* was grown in 10 ml of basal medium to which had been added 10⁻⁷ g of folic acid as the sodium salt. The bacteria were separated by centrifugation for 5 minutes in a clinical centrifuge. The supernatant solution was discarded, and the organisms were resuspended in 8 ml of 0.9 per cent NaCl. A mixture of 100 ml of basal medium, 100 ml of water, 1 g of sodium ascorbate, and 4 g of Bacto-Difco agar ⁵ was autoclaved and immediately placed in a water bath at 45°C. The resuspended *L. casei* culture (8 ml) was added after 10 minutes. This inoculated solution was poured into a sterile glass tray (20 × 36 cm) and allowed to solidify. The tray was covered with a lid made from aluminum sheet. A developed paper chromatogram was placed on the surface of the moist agar for 2 minutes and then removed and dried. The tray was incubated at 30°C for 12 to 18 hours. At this time the zones of dense *L. casei* growth were clearly visible in dim light and were traced on the dried paper chromatogram.

**RESULTS**

**Test of conjugase activity.** Teropterin was chosen as a substrate to test the activity of the prepared conjugases. Marked degradation of our Teropterin sample apparently had occurred during its storage for 11 years (a preparation of Teropterin is not commercially available at the present time). Table I demonstrates that this sample of Teropterin has only one-third of its original activity. Furthermore, only one-third of this remaining activity was made available to *S. faecalis* by hog kidney conjugase at the concentration of enzyme tested, although all of this remaining activity was made available by chicken pancreas conjugase under our experimental conditions.

³ Purchased from Pharmacia, Uppsala, Sweden.

⁴ *L. casei* folic acid assay medium, purchased from Baltimore Biological Laboratory, Baltimore, Md. (1).

⁵ Purchased from Difco Laboratories, Detroit, Mich.
TABLE II
Failure of hog kidney or chicken pancreas conjugase to make folic acid activity in human serum available to S. faecalis or L. citrovorum *

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Folic (or folinic) acid activity</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>L. casei</td>
<td>S. faecalis</td>
<td>L. citrovorum</td>
</tr>
<tr>
<td>A</td>
<td>m(\mu)g/ml</td>
<td>m(\mu)g/ml</td>
<td>m(\mu)g/ml</td>
</tr>
<tr>
<td>1. HS (human serum)</td>
<td>14.5</td>
<td>3.3</td>
<td>1</td>
</tr>
<tr>
<td>2. Chicken pancreas conjugase†</td>
<td>7</td>
<td>4.1</td>
<td>1</td>
</tr>
<tr>
<td>3. No. 1 + No. 2 ‡</td>
<td>20</td>
<td>5.8</td>
<td>1</td>
</tr>
<tr>
<td>4. No. 3 — no. 2</td>
<td>13</td>
<td>1.7</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>m(\mu)g/ml</td>
<td>m(\mu)g/ml</td>
<td>m(\mu)g/ml</td>
</tr>
<tr>
<td>1. HS</td>
<td>5.5</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2. Hog kidney conjugase‡</td>
<td>7.4</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>3. No. 1 + no. 2 ‡</td>
<td>13</td>
<td>6.9</td>
<td>5.4</td>
</tr>
<tr>
<td>4. No. 3 — no. 2</td>
<td>5.6</td>
<td>0.9</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* All samples were preincubated for 18 hours at 37°C under 2 ml toluene with 350 mg per 100 ml ascorbic buffer (1) prior to assay.
† 0.5 ml.
‡ 1 ml.

Level of folate compounds in serum and effect of conjugase preparations. As shown in Table II, an analysis has been made for the levels of folate compounds present in normal human serum. The results of these experiments demonstrate that the folate compounds of serum support the growth of L. casei to a much greater extent than they do the growth of S. faecalis or L. citrovorum. Other investigators have also reported the existence of classes of folate compounds in blood and other tissues with these same characteristics as growth factors for the three organisms (2, 7, 8). Since some polyglutamates of folinic acid also support the growth of L. casei but not that of S. faecalis or L. citrovorum, we have incubated serum with crude preparations of chicken pancreas and hog kidney conjugase. If the unknown folate compounds of serum were polyglutamates, it might be expected that conversion to the monoglutamates by the action of conjugase would make them available to S. faecalis or L. citrovorum. The results of experiments in which normal human serum has been treated with preparations of conjugase are included in Table II. Incubation of normal human serum with either conjugase preparation does not result in the appearance of folate compounds that serve as growth factors for any of the three organisms beyond that level found in the untreated serum. These experiments therefore eliminate, as components of human serum, polyglutamates of folinic acid or its derivatives known to be attacked by these conjugases.

The data therefore leave the possibilities that 1) the folate compound of human serum is a monoglutamate such as N\(^2\)-methyl tetrahydrofolate which, as shown in Table III, serves as a growth factor for L. casei but not for the other two organisms; or 2) the folate compound of serum is a polyglutamate which is not attacked by conjugase or which is converted into a product with the same growth characteristics as the initial substrate. In order to distinguish between these possibilities, both N\(^2\)-methyl tetrahydropteroylglutamate and N\(^8\)-methyl tetrahydropteroylglutamate were prepared and compared with the folate compound of human serum by chromatographic analysis.

Tentative identification of a folate component of serum. The folate activity of serum was adsorbed on Norite, eluted, and concentrated as described in Materials and Methods. The concentrated serum extract was tested for microbiological activity; 5 \(\mu\)l of the concentrate contained 78 \(\mu\)g of “folate activity” for L. casei and 4.7 \(\mu\)g for S. faecalis. In Table IV a comparison has been

<table>
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<th>TABLE III</th>
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<tr>
<td>Approximate quan-</td>
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<tr>
<td>tity of N(^2)-methyl tetrahydrofolate</td>
</tr>
<tr>
<td>(\mu)g</td>
</tr>
<tr>
<td>13</td>
</tr>
<tr>
<td>39</td>
</tr>
</tbody>
</table>
made of the migration of this product with that of other known folate compounds on Whatman no. 1 paper with a variety of developing solvents. The unknown folate activity migrates as a single component in all four buffer systems and with an Rf value similar to that of N⁵-methyl tetrahydrofolate. When the serum concentrate is mixed with a solution of N⁵-methyl tetrahydrofolate and then spotted on paper, only one spot is found after development of the chromatogram with any one of the four solvent systems. The Rf value of the materials of the mixed solution is the same as the Rf of the N⁵-methyl tetrahydrofolate tested separately.

Although the mono- and triglutamates of N⁵-methyl tetrahydrofolic acid cannot be distinguished from each other on the basis of their growth-promoting properties for the three microorganisms, they may be identified by paper chromatographic analysis. With solvents 1 and 2 (Table IV) the triglutamate migrates more rapidly than does the monoglutamate or the folate compound of human serum. These chromatographic comparisons thus eliminate the triglutamate as the folate component of human serum and provide strong evidence that the folate component is the monoglutamate, N⁵-methyl tetrahydrofolate. This identification, however, must be held as tentative until sufficient material has been isolated from serum for determination of its spectral characteristics and its ability to serve as a substrate for methionine biosynthesis.

**DISCUSSION**

These studies as well as those of others (7) have led to the correction of a previous erroneous assumption that the only folate compounds that support the growth of *L. casei* but not *S. faecalis* or *L. citrovorum* are di- or triglutamates of pteroic acid. The fact that the N⁵-methyl tetrahydrofolate supports the growth preferably of *L. casei* is an obvious exception to the previous assumption.

Liver also contains N⁵-methyl tetrahydrofolate as an important folate constituent. Keresztesy and Donaldson (7, 8, 18) have isolated from horse liver a monoglutamate compound (prefolic A) which supports growth of *L. casei* but not of *S. faecalis* or *L. citrovorum*. Silverman, Law and Kaufman (19) have shown that prefolic A is the major folate constituent of mouse liver. Moreover, extracts, of human liver possess marked growth activity for *L. casei* but little for *S. faecalis* or *L. citrovorum* (20). A recent comparison of the chemical and physical properties of prefolic A and N⁵-methyl tetrahydrofolate shows that they are undoubtedly the same (6). The experiments of these several laboratories thus provide convincing evidence that N⁵-methyl tetrahydrofolate is a major constituent of at least two tissues, namely liver and serum, and has an important metabolic function in the synthesis of methyl-containing compounds such as methionine and, indirectly, choline (21).

**SUMMARY**

1. Folic acid activity in human serum for *Lactobacillus casei* is not made available to *Streptococcus faecalis* or *Leuconostoc citrovorum* by the action of hog kidney or chicken pancreas conjugase.

2. A new intermediate of methionine biosynthesis, N⁵-methyl tetrahydrofolic acid, has marked folic acid activity for *L. casei* but relatively little for *S. faecalis* or *L. citrovorum*. The fact that this material is a monoglutamate indicates the existence of a previously unknown class of folic acid-active materials: monoglutamates microbiologically active for *L. casei*, but relatively inactive for *S. faecalis* or *L. citrovorum*.

3. The possibility that folic acid activity in human serum may be due largely to such a monoglutamate form is supported by the demonstration that a concentrate of serum folic acid activity and N⁵-methyl tetrahydrofolate migrates identically in four different solvent systems.
ACKNOWLEDGMENT

The authors are indebted to Mrs. Rebecca Fisher Dunn, Mrs. Barbara Bean Mummey, Miss Brenda Conti, and Miss Nancy Cunneen for technical assistance and to Dr. Jane Desforges (Boston City Hospital) and Dr. Sherwin Kevy (Children's Hospital Medical Center) for human blood plasma.

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11. Wood, R. C., and Hitchings, G. H. Personal communication.