Distribution of Folic Acid Derivatives in Natural Material

I. CHICKEN LIVER FOLATES

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The occurrence of polyglutamyl folic acids has been demonstrated in a variety of materials including algae, yeast extract, chicken liver, bacterial cells, and human blood (1). However, due to the limitations of the extraction, fractionation, and assay conditions, only the stable N^5-formyl-reduced folate conjugates have been adequately characterized. Furthermore, the recently identified N^2-methylfolic acid-H_4 which is involved in methionine biosynthesis (2, 3) had eluded characterization. Because of its growth stimulation for Lactobacillus casei alone (2, 4) it is possible that previous investigators considered it to be a polyglutamyl form.

In the present paper we have attempted to characterize the forms of folic acid occurring in chicken liver by means of a chromatographic fractionation procedure with the use of N,N'-DEAE-cellulose columns (5). Microbiological growth responses to the eluted fractions before and after digestion with two types of γ-glutamyl conjugase preparations were used to detect the polyglutamyl forms and to characterize the nature of the 1-carbon fragments found to be associated with the native forms.

EXPERIMENTAL PROCEDURE

Preparation of Chicken Liver Extract—An acetone powder was prepared from 225 g of frozen commercial liver. The dried powder, 57 g, was extracted by stirring for 30 minutes at 70° in 1 liter of an aqueous 1% ascorbate solution, pH 6. The extract was filtered through a bed of Hyflo Super-Cel and the filter cake was washed with 100 ml of the ascorbate solution. The filtrate and wash were combined.

Chromatographic Fractionation of Folate Derivatives—The combined extract was fractionated on a preparative scale employing a DEAE-cellulose column (5), the elution being effected by a gradient phosphate buffer, pH 7.0 (see legend, Fig. 1). The peak fractions were used for further characterization studies. They were first lyophilized and desalted (see legend, Fig. 2) before chromatography on standardized analytical DEAE-cellulose column (6). Fig. 3 shows an analytical chromatogram of the end products of digestion by the Physalia conjugase preparation for each polyglutamyl peak fraction.

Conjugase Preparation—Detection of the polyglutamyl derivatives in the eluted fractions by the conventional microbiological growth responses requires prior conjugase digestion. The autolysed chick pancreas conjugase preparation of Mims and Laskowski (7) was employed without further purification. The folic values obtained were corrected for the small folic activity of the conjugase preparation. The end product of digestion with this conjugase preparation is the corresponding diglutamyl derivative (8).

In order to determine the nature of the 1-carbon moiety associated with the folate conjugates the polyglutamyl peak fractions were subjected to analytical DEAE-cellulose chromatography directly and also after treatment with a conjugase preparation obtained from the gas glands of Physalia physalis L (gift of Dr. J. B. Wittenberg). The preparation used was obtained by extracting 20 mg of an acetone powder prepared from the gas glands in 2.0 ml of 0.05 M phosphate buffer, pH 6. This extract was incubated at 37° for 1 hour, then clarified by centrifugation at 0° (total volume, 1.8 ml). The supernatant solution was treated with 300 mg of Dowex-1(Cl⁻) for 15 minutes at 0° with occasional stirring. The clear extract was used directly after centrifugation (1.4 mg of protein per ml). No correction for endogenous folic activity was found necessary. The product of digestion of the polyglutamates with this preparation appears to be the corresponding monoglutamate without any significant alteration of the 1-carbon moiety associated with the naturally occurring polyglutamyl forms (Fig. 3). Scarcity of the source material prevented the exclusive use of this conjugase preparation in the entire study reported here.

Determination of Folic Acid Activity—After suitable conjugase treatments and dilution with the 1% ascorbate solution, pH 6, the samples were analyzed for folic acid growth activity with L. casei, Pseudomonas aeruginosa (801), and Streptococcus faecalis R with Lencovorin (Lederle) (dl-N^5-formyl folic acid-H_4) as standard. Assay conditions which prevented oxidative alteration of the l-carbon moiety associated with the naturally occurring polyglutamyl forms (Fig. 3). Scarcity of the source material prevented the exclusive use of this conjugase preparation in the entire study reported here.

RESULTS

Fig. 1 represents the L. casei growth activity for fractions resolved on the preparative column. The L. casei and the S. faecalis R activities after pancreatic conjugase digestion of the eluted fractions are also indicated.

The monoglutamyl derivatives appear in the earlier fractions. They were unaffected by pancreatic conjugase digestion and correspond to the N^1-formyl (A, Tube 5) and the N^3-formyl (B, Tube 7) derivatives of pteroylglutamic acid-H_6, both of which are active for all three test organisms. Fraction C (Tube 8) represents the N^5 methyl derivative which is inactive for L. casei alone (2, 4). The conclusions are based on the chromatographic and microbiological properties of these compounds as described in previous work (6, 9).

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Some conclusions as to the probable structure of some of the folate compounds detected may be arrived at after evaluating the data in Table I, Fig. 1, and Fig. 2 for each fraction.

The activity of Fraction I for L. casei alone resembles that of a N³-methyl derivative. This growth stimulation was unaltered by conjugase digestion, characteristic of a mono- or diglutamyl derivative of pteronic acid-H₄ (10). Because of its location (Fig 2, Tube 16) on the analytical column and since it gives rise to a monoglutamyl derivative which behaves similarly to N³-methyl folic acid-H₄ only after Physalia conjugase treatment (Fig. 3), it is probably a N³-methyl derivative of diglutamyl pteronic acid-H₄.

Fraction II behaves as a N⁴-formyl derivative with more than 2 glutamyl residues since its growth stimulation for all three organisms is enhanced after conjugase digestion. Its chromatographic and microbiological properties are identical with those of a chemically synthesized sample of N⁴-formyltriglutamyl-pteronic acid-H₄ which has been described in an earlier study (9).

Fraction III has the characteristic growth response of a formylpolyglutamyl derivative of pteronic acid-H₄, probably containing more than 3 glutamyl residues. Fraction IV represents the major component of chicken liver folates and, similar to Fractions

Fig. 2. Chromatograms of the individual polyglutamyl peak fractions after analytical chromatography on DEAE-cellulose. Separate portions (5 to 15 ml) of the peak folate fractions (Fig. 1) were lyophilized and the concentrates extracted with three 5-ml portions of an ethanol-water (3:1) mixture containing 1 mg per ml of ascorbate, pH 6. The combined extracts for each fraction, representing partially desalted preparations, were concentrated under reduced pressure to 0.5 ml and then made to volume (2.5 ml) adjusted to contain 1% ascorbate, pH 6. From 1 to 2 ml of these preparations were employed for analytical chromatography. The chromatograms represent L. casei activity before and after pancreatic conjugase digestion (see legend, Fig. 1) of the fractions eluted from the analytical columns (5) and are expressed as µg of folic acid per ml of the individual 100-ml peak fractions collected initially from the preparative column (Fig. 1).
V and VI, is active for *L.* casei alone after conjugase treatment. Thus, these fractions (IV, V, and VI) have properties of N^4- methyl derivatives of increasing complexity. Further characterization of these polyglutamyl folates was not attempted.

Fig. 3 presents further evidence for the nature of the 1-carbon moiety associated with the polyglutamyl derivatives. These peak fractions (I through VI) were digested with the *Physalia* gas gland conjugase preparation. This preparation degrades conjugated forms stepwise, resulting in accumulation of the corresponding monogluta- nyl derivative as the major end product of reaction. Traces of the intermediate polyglutamyl forms appeared as minor components when the mixture was chromatographed before completion of the reaction.

Samples of N^5-formylpteroylglutamate-H_4 and N^5-methyl pteroylglutamate-H_4, presumably the final end products of *Physalia* conjugase digestion, can be located in Tubes 12 and 13, respectively, after chromatography on freshly packed analytical columns (6). However, with repeated use of a column these components may both appear in Tube 12 and can then be differentiated only by the difference in microbiological growth responses. Accordingly, the components in Fractions I, IV, V, and VI giving rise stepwise to a monogluta- nyl end product with growth activity for *L.* casei alone would correspond to N^5-methyl-reduced folate derivatives (2, 4). Fractions II and III with end products active for all three test organisms in the digests of Tubes 12 and 13, respectively, after chromatography on freshly packed analytical columns (6). However, with repeated use of a column these components may both appear in Tube 12 and can then be differentiated only by the difference in microbiological growth responses. Accordingly, the components in Fractions I, IV, V, and VI giving rise stepwise to a monogluta- nyl end product with growth activity for *L.* casei alone would correspond to N^5-methyl-reduced folate derivatives (2, 4). Fractions II and III with end products active for all three test organisms would correspond to N^5-formyl-reduced folate derivatives.

Although the major component after *Physalia* conjugase digestion is the monogluta- nyl derivative, N^5-formylpteroylglutamate-H_4 (Tube 12), some N^10-formyl-pteroylglutamate-H_4 (Tube 8), and the oxidized form N^10-formylpteroylglutamate (Tube 10, inactive for *P.* cerevisiae) also appear (Fig. 3) in the digests of Tubes 12 and 13. These may represent artifacts of isomerization and oxidation reactions resulting from treatment with the crude conjugase preparation. The 1-carbon units attached to the polyglutamyl forms remained essentially unaltered. The results of the analytical procedures are summarized in Table II.

**Table I**

Effect of pancreatic conjugase on release of folic acid activity

<table>
<thead>
<tr>
<th>Peak fraction tube No.</th>
<th>Preparative column</th>
<th>Test organism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>After treatment</td>
</tr>
<tr>
<td></td>
<td><em>L.</em> casei</td>
<td><em>S.</em> faecalis</td>
</tr>
<tr>
<td>10 (I)</td>
<td>795</td>
<td>3.5</td>
</tr>
<tr>
<td>12 (II)</td>
<td>456</td>
<td>7.5</td>
</tr>
<tr>
<td>13 (III)</td>
<td>130</td>
<td>5.0</td>
</tr>
<tr>
<td>15 (IV)</td>
<td>200</td>
<td>5.0</td>
</tr>
<tr>
<td>17 (V)</td>
<td>15</td>
<td>5.0</td>
</tr>
<tr>
<td>27 (VI)</td>
<td>1.7</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Large amounts of polyglutamyl-reduced folates of varying complexity with attached 1-carbon fragments were found in an extract of chicken liver. Their significance is still obscure, but in other reports on bacterial (11-13) and liver (14, 15) systems, polyglutamates have been found to be as or more effective than monogluta- nyls as cofactors in enzymatic reactions. In the present study with chicken liver the predominant formyl-containing compounds are the N^5-formyl derivatives. Similar analytical procedures in previous studies with rat liver (16), leukemic cell lines of the mouse (6), and the gas glands of *Physalia physalis* L (9) revealed that the N^4, rather than the N^5-formyl derivatives were predominant. The folic acid active material in human blood erythrocytes characterized by high *L.* casei activity which could be released by the plasma enzymes or after hog kidney-chick pancreas conjugase digestions (17) possibly also contains methylpolyglutamyl derivatives. The occurrence of polyglutamates cannot be excluded in studies using *L.* casei to detect folates without prior exogenous conjugase treatment (6).

The present findings, employing the combined use of analytical DEAE-cellulose chromatography, conjugase digestion,
Folate conjugates detected:

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SUMMARY

Some of the multiple forms of folates occurring in chicken liver have been characterized. An initial preparative scale fractionation on DEAE-cellulose columns of chicken liver extract was followed by analytical chromatography of the crude fractions on standardized DEAE-cellulose columns.

Prior digestion with a chick pancreas conjugase preparation of the peak fractions was found necessary in order to detect the polyglutamyl forms. Similar studies with the corresponding monoglutamate end products obtained after digestion with a conjugase preparation from the gas glands of Physalia have been employed to characterize the nature of the l-carbon units associated with the native polyglutamyl forms.

The monogluta mates identified include the N5-formyl-, N5-formy1-, and the N5-methyl- derivatives of tetrahydrofolic acid. Of the two N5-formylpolyglutamate derivaties shown to occur, one appears to be identical to N5-formyltriglutamylpteroc acid H4. There is evidence for a N5-methylglutamate derivative of pteroc acid H4, together with at least three as yet uncharacterized N5-methylpolyglutamyl derivatives.

and differential microbiological growth response studies, provide the first evidence for the natural occurrence of at least four N5-methylpolyglutamyl-reduced folates.

REFERENCES

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