Enzymatic deconjugation of erythrocyte polyglutamyl folates during preparation for folate assay: investigation with reversed-phase liquid chromatography

CHRISTINE M. PFEIFFER and JESSE F. GREGORY III*

Erythrocyte (RBC) folates occur mainly as 5-methyltetrahydrofolate polyglutamates. Determination of RBC folate concentration requires an initial deconjugation of these polyglutamates. In this study, existing HPLC methods were adapted to investigate the rate and extent of this deconjugation process. The action of endogenous plasma pteroyl-polyglutamate hydrolase activity was strongly affected by the conditions of sample preparation, with pH of the incubation mixture more critical to effective deconjugation than incubation time. Dilution of whole blood with 10 g/L ascorbic acid yielded fast hydrolysis of long-chain polyglutamates, and total conversion to 5-methyltetrahydrofolate monoglutamate occurred after 90 min of incubation at 37 °C. In contrast, dilution of whole blood with 10 g/L sodium ascorbate, with up to 90 min of incubation at 37 °C, yielded a mixture of polyglutamates of 5-methyltetrahydrofolate (glu₃, 1–8). As documented by direct HPLC analysis and in concurrent assays with Lactobacillus casei, acidification provided by ascorbic acid can have dramatic effects on the measurement of RBC folates.

INDEXING TERMS: ascorbic acid • sample preparation

The value of serum and erythrocyte (RBC) folate assays in diagnosis of folate status is well established.° RBCs contain higher concentrations of folate than plasma, and practically all the RBC folates are 5-methyltetrahydrofolate polyglutamates [1]. Because RBC and liver folate concentrations correlate significantly [2], RBC folate concentrations are regarded as indicative of body stores, and the estimation of RBC folate is often of greater clinical value than that of serum folate [3]. Yet, there exists no reference method for the preparation of whole-blood samples for use in determination of RBC folate.

Variability in the ability to assay RBC folate with commercial competitive-binding kits and microbiological assays involving Lactobacillus casei has been reported repeatedly [4–7]. Explanations offered for the different results and the imprecision have primarily focused on assay conditions, e.g., pH, extraction temperature, the condition and type of binders, and the standards used [7]. Shane et al. determined that the microbiological assay was unsuitable for the measurement of folate polyglutamates but gave accurate results for their assessment of folates provided they are converted to the monoglutamate forms before assay; commercial competitive-binding assays gave variable responses to polyglutamates compared with monoglutamates [8]. This emphasizes the importance of sample preparation and of verification of the extent of deconjugation of the 5-methyltetrahydrofolate polyglutamates in the whole-blood sample applied to the folate assay.

Neither the microbiological assay nor competitive binding assays offer the possibility to determine simultaneously the composition of the different folate vitamers in the tested sample. However, chromatographic methods such as ion exchange or reversed-phase HPLC can ensure the separation and determination of various folates [9]. Different HPLC methods for the separation of folate polyglutamates have been developed, but most of them involve gradient separation and have a run time of ~1 h, and none has been applied for the separation of RBC polyglutamates [10–17]. The objective of this study was to develop a rapid, sensitive, and specific chromatographic assay that allows simultaneous determination of RBC folates and verification of the deconjugation of RBC folate polyglutamates depending on varying conditions of sample preparation. This communication describes a modification and application of the reversed-phase fluorometric HPLC procedure of Gregory et al. [18, 19]. A 3-cm-long HPLC column packed with 3-μm octadecylsilsilica was used in conjunction with isocratic mobile phase at pH 2.3, which allowed rapid and efficient separation of

Food Science and Human Nutrition Department, University of Florida, P.O. Box 110370, Gainesville, FL 32611.

*Author for correspondence. Fax 352-392-9467; e-mail jfg@ufl.edu.

°Nonstandard abbreviations: RBC, erythrocytes; PteGlu, pteroylglutamic acid; FBP, folate binding protein; DHFR, dihydrofolate reductase; and UV, ultraviolet.

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5-methyltetrahydrofolate polyglutamates, eluting with increasing chain length (HPLC method I). An adaptation of the gradient HPLC method for the separation of folate polyglutamates (elution order with decreasing chain length) developed by Cashmore et al. [11] was used to evaluate the identity and purity of folic acid heptaglutamate preparation, and of synthesized 5-methyltetrahydrofolate heptaglutamate (HPLC method II). These methods are based on the observations of Bush et al. regarding the pH dependence of reversed-phase HPLC behavior in separation of folate polyglutamates [20]. A secondary objective of this study was to determine optimal conditions for preparation and handling of blood before folate assay.

**Materials and Methods**

**CHEMICALS**

All chemicals used were reagent grade or the highest available purity, and the solutions were prepared with water obtained from a MilliQ water purification system (Millipore, Bedford, MA). Pteroylheptaglutamic acid (PteGlu7) was obtained from ARC (St. Louis, MO) and pteroyltetrahydroglutamic acid (PteGlu4) from Schirch's Labs (Jona, Switzerland). The PteGlu prepert used for the synthesis of 5-methyltetrahydrofolate heptaglutamate contained ~25% PteGlu and trace amounts of PteGlu4, PteGlu5, and PteGlu6, as analyzed by the presented HPLC method II. Pteroylglutamic acid (PteGlu4), 5-methyltetrahydrofolate (disodium salt), 2-mercaptoethanol, l-ascorbic acid, l-ascorbic acid sodium salt, and potassium phosphate tribasic were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium phosphate monobasic and dibasic were obtained from Fisher Scientific (Fair Lawn, NJ). For the HPLC analysis, HPLC-grade methanol, acetonitrile, and phosphoric acid were used (Fisher Scientific). An affinity chromatography sorbent with immobilized folate binding protein (FBP) was prepared by a minor modification of the method of Selhub et al. [21] as described by Gregory and Toth [18].

**SYNTHESIS OF 5-METHYLTETRAHYDROFOLATE POLYGLUTAMATE**

Synthesis of 5-methyltetrahydrofolate heptaglutamate was performed by a minor modification of the method described by Horne et al. [22, 23]. This method with enzymatic reduction of folic acid to tetrahydrofolate was chosen because it was developed for small quantities (0.2 mmol starting amount), it is mild, very fast, it yields the natural stereoisomer at C-6 position, and only one purification step has to be performed at the end of the synthesis. Dihydrofolate reductase (DHFR; EC 1.5.1.3) from L. casei has the advantage that it can use folic acid as a substrate, whereas DHFR from other sources greatly favors dihydrofolates. We chose PteGlu as starting material because RBC folates occur mainly as long-chain 5-methyltetrahydrofolate polyglutamates [24, 25].

The concentration of the folic acid heptaglutamate preparation (PteGlu7) used for the synthesis was determined spectrophotometrically from molar absorptivity values reported by Blakley [26]. The identity and purity of the PteGlu7 preparation and of the resulting 5-methyltetrahydrofolate heptaglutamate were determined with the aid of the developed gradient HPLC method II, with ultraviolet (UV)-diode array detection for spectral peak identification (see below).

**BLOOD COLLECTION AND PREPARATION**

Whenever needed, blood was drawn from healthy, fasting donors into 7-mL EDTA Vacutainer Tubes (Becton Dickinson, Rutherford, NJ). These blood collections were a component of a protocol that had been reviewed and approved by the University of Florida Institutional Review Board. Whole-blood samples were diluted 1:10 with 50 mmol/L sodium ascorbate or 57 mmol/L ascorbic acid solution (10 g/L), sonicated for 30 s, and then either analyzed immediately for endogenous folates (no incubation) or incubated for various times at ambient temperature or at 37 °C for deconjugation of polyglutamyl folates by the action of plasma pteroylglutamyl hydrolase activity. To freshly diluted whole-blood samples, 5-methyltetrahydrofolate (43 nmol/L whole-blood dilution) or 5-methyltetrahydrofolate heptaglutamate (50 nmol/L whole-blood dilution) was added immediately after sonication to further assess the identity of some of the HPLC peaks and to evaluate the activity of the endogenous plasma pteroylglutamyl hydrolase.

**FOLATE EXTRACTION AND PURIFICATION BY AFFINITY CHROMATOGRAPHY**

All preparative and analytical procedures were performed under gold fluorescent lights (General Electric #F40G0) to minimize photochemical degradation of 5-methyltetrahydrofolate. To a 500-μL portion of sample (whole-blood 1:10 dilution), five volumes (2.5 mL) of 0.1 mol/L potassium phosphate buffer (pH 7.0) containing 50 mmol/L sodium ascorbate and 10 mmol/L 2-mercaptoethanol were added. Nitrogen gas was bubbled through the sample for 10 s, followed by immediate capping. Thermal extraction was performed by incubation in a boiling water bath for 10 min. After cooling on ice, centrifugation followed at 10,000 g for 10 min at 5 °C. The supernatant was poured off, bubbled with nitrogen gas for 10 s, and loaded on a FBP-Affigel 10 column (0.5-mL bed volume, ~2 μg binding capacity) that had been previously equilibrated with 0.1 mol/L potassium phosphate buffer, pH 7.0. All sample application, wash, and elution steps were conducted at ambient temperature at a flow rate of 0.3 mL/min as controlled by a peristaltic pump. The columns were washed with two 1-mL portions of 0.025 mol/L potassium phosphate, pH 7.0, containing 1.0 mol/L NaCl, followed by two 1-mL washes with 0.025 mol/L potassium phosphate, pH 7.0. Folate was eluted with 0.1 mol/L HCl. The first 0.7-mL portion of 0.1 mol/L HCl contained no folate and was discarded. The following 1.2-mL portion of 0.1 mol/L HCl contained all of the eluted folate. This fraction was collected in a 1.5-mL microcentrifuge tube containing 50 μL of a 500 mmol/L sodium ascorbate solution (100 g/L) to stabilize the folate for HPLC analysis (final volume of the eluted sample was 1.25 mL with a final concentration of 20 mmol/L sodium ascorbate). Samples were flushed with nitrogen gas for 10 s, sealed, and kept in the refrigerator until used for the HPLC analysis with method I (typically within an hour).
HPLC PROCEDURES

HPLC method I. The concentrations of 5-methyltetrahydrofolate of different polyglutamate chain length in the affinity column eluates were determined by a minor modification of a reversed-phase HPLC procedure previously described [18, 19]. Analysis was performed with a PE H53-C18 cartridge (3.3-cm length, 4.6-mm i.d., 3-μm particle size; Perkin-Elmer, Norwalk, CT), a mobile phase of 33 mmol/L phosphoric acid (pH 2.3), 40 mL/L acetonitrile at a flow rate of 1.0 mL/min, and a 500-μL injection volume. The native fluorescence of 5-methyltetrahydrofolate and its polyglutamate forms was monitored with a SpectroVision FD-300 (Chelmsford, MA) detector at 295 nm excitation and 356 nm emission wavelengths.

HPLC method II. The separation of folic acid polyglutamates as well as the determination of the identity and purity of the PteGlu, preparation and of the synthesized 5-methyltetrahydrofolate heptaglutamate was achieved by a modification of two reversed-phase HPLC procedures previously described [11, 16]. Analysis was performed with the above-mentioned PE H53-C18 cartridge, and gradient elution with slightly increasing acetonitrile concentration at a flow rate of 1.0 mL/min. Both solvents (A and B) were 0.1 mol/L potassium acetate buffers, pH 5.0; the latter contained 75 mL/L acetonitrile. Table 1 shows the composition of the gradient. UV-diode array detection (LC-235, Perkin-Elmer) was used for peak identification by comparing the obtained spectrum with the spectrum of a reference compound.

MICROBIOLOGICAL ASSAY

The total folate content of whole-blood samples subjected to different sample preparations was also determined by microbiological assay with L. casei [27] with folic acid casei medium (Difco Labs., Detroit, MI), run at pH 6.8. At this pH, 5-methyltetrahydrofolate may be underestimated with a folic acid calibrant [28].

Results

Figure 1 shows a typical chromatogram for the separation of folate polyglutamates of different chain lengths obtained for a mixture of PteGlu, PteGlu, PteGlu, and 5-methyltetrahydrofolate (~0.3 mmol/L, each) with the presented gradient HPLC method II.

A chromatogram of 5-methyltetrahydrofolate heptaglutamate is shown in Fig. 2. The synthesized preparation contained mainly 5-methyltetrahydrofolate heptaglutamate, with ~25% 5-methyltetrahydrofolate hexaglutamate, and trace amounts of PteGlu, and PteGlu. No shorter chain lengths of either 5-methyltetrahydrofolate or folic acid were found.

Freshly drawn blood was subjected to various sample preparation methods to study the deconjugation of RBC folate polyglutamates. We found that the pH of the incubation mixture is a critical factor for effective deconjugation. Table 2 shows the percentage distribution of 5-methyltetrahydrofolate polyglutamates relative to the total folate content for the various sample preparations. Total folate contents were calculated on the basis of equal molar response for each 5-methyltetrahydrofolate polyglutamate. Blood diluted with 10 g/L sodium ascorbate (final pH ~7) showed even after 90 min of incubation at 37 °C the whole array of 5-methyltetrahydrofolate polyglutamates, with only a small shift to polyglutamates of shorter chain length, compared with a sample without incubation (Table 2 and Fig. 3). Results obtained by supplementing whole blood (diluted with 10 g/L sodium ascorbate, no incubation) with 5-methyltetrahydrofolate monoglutarate and with 5-methyltetrahydrofolate heptaglutamate for reference are shown in Fig. 4 and contribute to the identification of the various 5-methyltetrahydrofolate polyglutamates.

Dilution of whole blood with 10 g/L ascorbic acid (final pH ~4) provided a fast deconjugation. Incubations of diluted whole blood for 0, 15, 30, 60, and 90 min at ambient temperature and at 37 °C were studied (Table 2). Although the chromatogram of a sample without incubation after ascorbic acid addition showed a mixture of all the different chain lengths (Fig. 5), after 15 min of incubation at ambient temperature, only 5-methyltetrahydrofolates with chain lengths of up to four glutamate residues could be found. After 30 min at 37 °C, only 5-methyltetrahydrofolate monoglu, diglutamate, and triglutamates were detected (Fig. 5); after 60 min at 37 °C, only monoglu- and diglutamate was present; and after 90 min at 37 °C, 5-methyltetrahydrofolate monoglutarate was the only derivative found (Fig. 5).

The activity of endogenous plasma pteroylpolyglutamate hydrolase was tested at different pH values by incubation of whole blood diluted with 10 g/L sodium ascorbate, pH adjusted to 4.5, 5.0, or 6.0 (with 0.1 mol/L HCl), for 90 min at 37 °C. Incubations at pH 4.5 and 5.0 resulted in mainly 5-methyltetrahydrofolate monoglutarate (75%), with 25% residual diglutamate, whereas samples incubated at pH 6.0 showed an incomplete deconjugation (50% monoglu-, 32% diglu-, 18% pentaglu- and hexaglutamate).

The stability of synthetic 5-methyltetrahydrofolate diluted with 10 g/L sodium ascorbate was tested at different pH values (4.5, 5.0, and 6.0) during incubation for 90 min at 37 °C. The stability of 5-methyltetrahydrofolate calibrator diluted with 10 g/L ascorbic acid also was tested during incubation for 30, 60, and 90 min at 37 °C. All of these treatments showed 100% stability of the 5-methyltetrahydrofolate calibrator under the conditions of blood handling. To verify that the observed hydrolysis was due to enzymatic action and not caused by a pH effect, 5-methyltetrahydrofolate heptaglutamate calibrator was

| Table 1. Composition of the gradient in HPLC method II (solvent A, 0.1 mol/L potassium acetate, pH 5.0; solvent B, 0.1 mol/L potassium acetate, pH 5.0, containing 75 mL/L acetonitrile). |
|---|---|---|
| Time, min | mL/L B | mL/L acetonitrile |
| 0 | 30 | 2.25 |
| 5 | 100 | 7.50 |
| 8 | 150 | 11.25 |
| 11 | 200 | 15.00 |
| 15 | 300 | 22.50 |
| 20 | 450 | 33.75 |
| 25 | 600 | 45.00 |
Fig. 1. Typical chromatogram for separation of folate polyglutamates of different chain lengths obtained for a mixture of PteGlu7, PteGlu3, PteGlu1, and 5-methyltetrahydrofolate (~0.3 mmol/L, each) with gradient HPLC method II (PG₆ is an impurity of PG₇) and respective UV absorption spectra.

Fig. 2. Chromatogram of 5-methyltetrahydrofolate heptaglutamate obtained with gradient HPLC method II and respective UV absorption spectra.
incubated in 10 g/L ascorbic acid (pH ~4) for 90 min at 37 °C, and then analyzed by HPLC. No hydrolysis of the polyglutamate chain length was found under these conditions.

A microbiological assay involving L. casei was performed for blood sample preparations from five different subjects to test our findings concerning the deconjugation of RBC polyglutamates. Table 3 summarizes the results. No difference was found in the response of the microorganism between no incubation and incubation for 90 min at 37 °C if blood was diluted with 10 g/L sodium ascorbate. Dilution of blood with 10 g/L ascorbic acid, however, yielded ~2 times higher values after only 20 min of incubation at 37 °C. There was no further increase noted if the sample was incubated for up to 90 min at 37 °C.

**Discussion**

Deconjugation of folate polyglutamates in RBCs was affected by the conditions of sample preparation, with the pH of the incubation mixture strongly influencing the activity of endogenous plasma pteroylpolyglutamate hydrolase. Dilution of whole blood with 10 g/L ascorbic acid caused rapid hydrolysis of long-chain folate polyglutamates, and yielded 5-methyltetrahydrofolate monoglutamate after 90 min of incubation at 37 °C. Dilution of whole blood with 10 g/L sodium ascorbate, however, showed even after 90 min at 37 °C a mixture of all different polyglutamates of 5-methyltetrahydrofolate. Findings of Krungkrai suggest that human serum pteroylpolyglutamate hydrolase (conjugase) functions as an exopeptidase at pH 4.5 [17].

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**Table 2. Distribution of endogenous 5-methyltetrahydrofolate polyglutamates of processed whole blood diluted with 10 g/L sodium ascorbate or ascorbic acid.**

<table>
<thead>
<tr>
<th>Chain length</th>
<th>no incub.</th>
<th>90 min, 37 °C</th>
<th>no incub.</th>
<th>15 min, AT</th>
<th>30 min, AT</th>
<th>60 min, AT</th>
<th>90 min, AT</th>
<th>15 min, 37 °C</th>
<th>30 min, 37 °C</th>
<th>60 min, 37 °C</th>
<th>90 min, 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.6</td>
<td>11.3</td>
<td>9.9</td>
<td>34.2</td>
<td>47.9</td>
<td>67.1</td>
<td>73.5</td>
<td>46.0</td>
<td>60.7</td>
<td>73.9</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>trace</td>
<td>7.1</td>
<td>6.7</td>
<td>26.4</td>
<td>28.9</td>
<td>28.4</td>
<td>26.5</td>
<td>29.9</td>
<td>29.4</td>
<td>26.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>trace</td>
<td>9.0</td>
<td>11.7</td>
<td>32.5</td>
<td>23.2</td>
<td>4.5</td>
<td>trace</td>
<td>24.2</td>
<td>9.9</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>4</td>
<td>8.8</td>
<td>17.5</td>
<td>16.2</td>
<td>6.9</td>
<td>trace</td>
<td>n.d.</td>
<td>n.d.</td>
<td>trace</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Total folate content: nmol/L whole blood 5-methyltetrahydrofolate equivalents

1–8 359 343 335 349 296 333 323 329 298 303 305

* Values reported represent a single determination.

* AT, ambient temperature.

* n.d., not detected.

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**Fig. 3. Typical chromatogram of processed whole blood diluted with 10 g/L sodium ascorbate obtained with isocratic HPLC method I (fluorometric detection).**

The number below each peak indicates its polyglutamyl chain length.
Our results from the incubation of whole blood diluted with 10 g/L ascorbic acid for up to 90 min support those findings. The long-chain folate polyglutamates are hydrolyzed to intermediate chain lengths in the first 15 to 30 min, and in the next 60 min tri- and diglutamates are hydrolyzed to monoglutamates.

Because there is no effective deconjugation of 5-methyltetrahydrofolate polyglutamates at neutral pH, the blood sample diluted with 10 g/L sodium ascorbate and no incubation represents most likely the endogenous RBC folate polyglutamate distribution (Fig. 3). Under these conditions ~95% of the 5-methyltetrahydrofolate content occurs as tetra- (8.8%), penta- (47.2%), hexa- (30.1%), and heptaglutamate (9.4%), while the rest occurs as monoglutamate (4.6%) and trace amounts of di-, tri-, and octaglutamate. This corresponds closely to reports of Shin et al. [29], who found that ~95% of total folates in RBCs are 5-methyl derivatives of reduced pteroylpolyglutamates, mainly as pentaglutamate (60%) and hexaglutamate (35%). Studies of Erbe [24] and Steinberg [25] showed that in RBCs, folates occur in the polyglutamate form, mainly as tetra-, penta-, and hexaglutamates.

The chromatographic separations of folate polyglutamates used here were based on observations of Bush et al. [20], who reported that in reversed-phase chromatography the selectivity of separation for polyionogenic compounds can be drastically modulated by changing the pH of the eluent. The elution of folate polyglutamates in reversed-phase chromatography, when the pH of the eluent is sufficiently high for the carboxylic acid groups to be predominantly ionized, should be in the order of decreasing number of glutamyl residues in the molecule (HPLC method II). If, on the other hand, the pH of the eluent is low
enough to suppress the ionization of glutamyl carboxyl groups, the elution order will be with increasing chain length (HPLC method I).

The adapted gradient HPLC method II provides a good separation for different folate polyglutamates within 20 min. Depending on the mixture to be separated, the gradient can be adjusted appropriately. This method has been applied successfully to the separation of RBC folate polyglutamates (data not shown), but the isocratic HPLC method I reported here seems to be more favorable for this task, since it is faster (no reequilibration time), and the short-chain 5-methyltetrahydrofolate polyglutamates elute first and show a better peak shape. With this HPLC method, RBC 5-methyltetrahydrofolate polyglutamates can be separated within 20 min.

Our findings concerning the importance of the pH and of the sample preparation for the deconjugation of RBC folate polyglutamates are consistent with the pH optimum of plasma Pteroylpolyglutamate hydrolase of 4.5 [30]. In spite of the documented pH profile of this enzyme, there seems to be confusion in the field of folate analysis regarding methods of sample preparation for whole blood. Some laboratories use ascorbic acid solution to dilute the blood [31–33], others use sodium ascorbate solution [34, 35], and still others use sodium ascorbate solution, but adjust the pH to 4.5 [36] or 6.1 [6, 37]. Depending on the method used for the whole-blood folate assay, the use of sodium ascorbate without a pH adjustment might result in an underestimation of the folate content and might lead to false diagnosis of deficiency.

Scott et al. studied the necessity of allowing whole blood to incubate with ascorbic acid before adding the blood–ascorbate mixture to the microbiological assay medium [31]. They found an increase in the microbiological response for up to 15 min of incubation time. If whole blood is analyzed with the microbiological assay, such a short incubation time might be sufficient, since long-chain polyglutamates can be hydrolyzed to triglutamates or shorter, and L. casei responds equally to folic acid mono-, di-, and triglutamates [38]. If other methods such as competitive binding assays or HPLC are used, longer incubation times are advisable to ensure complete deconjugation of folate polyglutamates. Quantification by HPLC is greatly simplified if polyglutamyl folates are fully deconjugated.

The results of the present study strongly emphasize the importance of sample preparation and the need for a reference method. Regardless of the method selected, one should always test the folate composition of the sample with a sensitive and specific method. Chromatographic methods with either UV-diode array detection or fluorometric detection provide these advantages, in contrast to rather nonspecific microbiological assays and competitive binding methods. The two HPLC methods are powerful tools for the separation of different folate polyglutamates.

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References

7. Life Sciences Research Office, Federation of American Societies for Experimental Biology. Assessment of folate methodology used

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Table 3. Endogenous whole-blood folate concentrations (nmol/L whole blood) determined by microbiological assay with L. casei.

<table>
<thead>
<tr>
<th>Subject</th>
<th>No Incubation</th>
<th>90 min, 37 °C</th>
<th>20 min, 37 °C</th>
<th>30 min, 37 °C</th>
<th>60 min, 37 °C</th>
<th>90 min, 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>191 ± 11.5</td>
<td>209 ± 22.8</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>207 ± 25.8</td>
<td>220 ± 34.5</td>
<td>231 ± 35.2</td>
<td>242 ± 35.2</td>
<td>253 ± 35.2</td>
<td>264 ± 35.2</td>
</tr>
<tr>
<td>3</td>
<td>218 ± 13.5</td>
<td>230 ± 24.8</td>
<td>241 ± 25.8</td>
<td>252 ± 26.8</td>
<td>263 ± 27.8</td>
<td>274 ± 28.8</td>
</tr>
<tr>
<td>4</td>
<td>229 ± 14.5</td>
<td>241 ± 25.8</td>
<td>252 ± 26.8</td>
<td>263 ± 27.8</td>
<td>274 ± 28.8</td>
<td>285 ± 29.8</td>
</tr>
<tr>
<td>5</td>
<td>240 ± 15.5</td>
<td>252 ± 27.8</td>
<td>263 ± 28.8</td>
<td>274 ± 29.8</td>
<td>285 ± 30.8</td>
<td>296 ± 31.8</td>
</tr>
</tbody>
</table>

Values reported are mean ± SD for n = 4 replicates.


