Erythrocyte Folate Analysis: Saponin Added During Lysis of Whole Blood Can Increase Apparent Folate Concentrations, Depending on Hemolysate pH

Anthony J. A. Wright,* Paul M. Finglas, and Susan Southon

Background: The analysis of red cell folate (RCF) depends on complete hemolysis of erythrocytes, and it is assumed that complete hemolysis is achieved by 10-fold dilution of whole blood with hypotonic solutions of 10 g/L ascorbic acid/ascorbate. This report challenges this assumption.

Methods: The conventional method of erythrocyte lysis was modified to include saponin, a known effective hemolyzing agent. The influence of saponin was determined at various lysate pHs, using the microbiological (Lactobacillus rhamnosus) folate assay. The effect of saponin during lysate preparation was subsequently compared with either the effect of 30 s of sonication or a single 1-h freeze-thaw cycle.

Results: Saponin addition was found to increase assayable RCF up to ninefold, depending on lysate pH. Sonication of lysates had no effect, and freezing-thawing lysates once did not always guarantee complete hemolysis. Lysates created with 10 g/L ascorbic acid (a historically widely used diluent) without pH adjustment produced assayable folate concentrations significantly lower than optimal.

Conclusions: A lysing agent should be incorporated into RCF assays to guarantee complete hemolysis. Tenfold dilution of blood with 10 g/L ascorbic acid, without pH adjustment, produces lysates with pHs (pH 4.0) below the point (pH 4.7) at which hemoglobin can denature irreversibly. The optimum pH for hemolysates is ~5.0.

© 2000 American Association for Clinical Chemistry

Some of the major causes of perinatal, infant, and childhood mortality and morbidity are spina bifida and the related neural tube defects (NTDs),1 anencephaly and encephalocele. Although the introduction of screening (maternal serum a-fetoprotein estimation or fetal ultrasound examination) has led to a reduction in the number of children born with NTDs, through termination of pregnancy (1, 2), it cannot prevent the development of these defects or the associated parental distress.

There now is firm evidence that an adequate intake of periconceptional folate (a B-group vitamin found especially in leafy green vegetables, some other vegetables and fruits, and whole grain cereals) can prevent a significant proportion of NTDs. Numerous studies (case-control and cohort studies and nonrandomized and randomized controlled trials) have shown that increased intake of either dietary folates or supplemental folic acid reduces the risk of NTDs (3). It is not surprising, therefore, that there is renewed interest in the analysis of red cell folate (RCF) as an indicator of folate deficiency risk in women of childbearing age. Moreover, inverse associations between RCF concentration and the risk of cardiovascular disease (4) and various cancers (5, 6) have generated additional interest in the analysis of RCF as an indicator of risk of folate deficiency in the general population.

Interlaboratory comparison studies of analytical methods for folate (7–9) not only demonstrated large interassay variation in whole-blood folate analysis, but also large intraassay variation. There are discussions in the literature about method comparisons (8, 10, 11) and possible sources of variability (9, 12–16). Recently, we suggested that the assumptions underpinning RCF analysis are flawed (17). The first assumption in erythrocyte folate analysis is that dilution of whole blood with hypotonic

1 Nonstandard abbreviations: NTD, neural tube defect; RCF, red cell folate; AA, ascorbic acid; and Hb, hemoglobin.
diluents produces complete hemolysis and, hence, the release of all folate; historically, 10-fold dilution of whole blood with ascorbic acid (AA) has been widely used to create lysates (7, 10, 18–20). However, this assumption has not been tested within the range of conditions currently used in RCF analysis, although it is known that elements within the RCF assay could influence the degree of hemolysis. It is also known that erythrocyte folate is electrostatically associated with deoxyhemoglobin, within the hollow central cavity of the quaternary structure of the hemoglobin (Hb) molecule (21–24). After hypotonic lysis of erythrocytes, transitory holes appear in the erythrocyte membrane that are large enough to allow the escape of Hb. However, these holes can reseal without complete release of Hb (25). To maximize the release of erythrocyte folate, and thus ensure appropriate measurement of folate status, it may be necessary to use a hemolyzing agent within the RCF assay to ensure complete release of Hb. Because pH is a major determinant of the rate of the resealing process (26, 27), characterization of the interaction of lysate pH with the selected hemolyzing agent is essential.

Saponin was chosen as the hemolyzing agent in the current studies not just because of its historical use for the hemolysis of erythrocytes, but because it was anticipated that the thick peptidoglycan outer cell wall of the gram-positive bacterium Lactobacillus rhamnosus (previously known as L. casei), used in the classical microbiologically assay of folate, would prevent the saponin molecule from reaching the outer bacterial membrane.

Saponins (sapogenin glycosides) consist of an aglycone unit (sapogenin; a sterol or triterpene) linked to a sugar unit (glycone; one or more carbohydrate chains); they possess surface-active or detergent properties because the carbohydrate portion of the molecule is water-soluble whereas the sapogenin is fat-soluble (28). It is thought that the release of Hb from erythrocytes is the direct result of membranolytic saponins combining irreversibly with membrane cholesterol to form permeable micelle-like aggregates, with a central pore ~80 Å (8 nm) in diameter, within the plane of the membrane (29). Transmission electron microscopy has revealed irreversible destruction of the erythrocyte lipid bilayer by saponin (30). Thus, saponin-lysed erythrocytes do not reseal.

Preexperimental work indicated that the microbiological growth response of L. rhamnosus to a folic acid calibration series was not perturbed even at a saponin concentration fivefold higher than would be encountered in diluted hemolysates in the current studies. Furthermore, under pH conditions where the use of saponin generated significantly higher RCF values, preexperimental work also indicated that the use of saponin at either twice or one-half the concentration actually used in the current studies gave RCF values that were not significantly different. Therefore, the saponin concentration used in the experiments described here neither bordered on being too low nor affected the growth of L. rhamnosus.

This report describes two studies designed to determine (a) the effect of the addition of a hemolyzing agent (saponin) on the RCF assay at a range of lysate pHs, and (b) the effectiveness of freezing-thawing or sonication of erythrocytes in comparison with the addition of saponin. Results obtained are discussed in relation to conditions currently used in RCF analysis and possible modification of the assay.

Materials and Methods

BLOOD COLLECTION

Nonfasting venous blood, from a female regular blood donor, was transferred immediately to sterile lithium heparin-coated blood chemistry tubes and mixed gently.

STOCK SAPONIN SOLUTION

Saponin (11.111 g, containing 90 mg/g sapogenins, “white suitable for hemolysis”; cat. no. 43650-2L; BDH-MERCK) was diluted to 100 mL with H2O to create a stock solution containing 10 g/L saponin.

BLOOD DILUENTS

Freshly prepared 10 g/L (57 mmol/L) solutions of AA were adjusted, as appropriate, with 1 mol/L NaOH through a range of pHs from 4.0 to 6.0. In addition, if appropriate, 1 mL of stock saponin solution was added to 99 mL of diluent (producing a final working saponin concentration of 100 mg/L) before the pH was adjusted. This working saponin concentration has recently been shown to produce 100% hemolysis, 50% hemolysis being achieved at a concentration of ~10 mg/L (30).

STUDY I

Study I was divided into two parts:

Study I(a). Study I(a) involved an investigation of assayable RCF concentration, comparing the use of AA at pH 2.7 (the historically used diluent) with pH-adjusted AA or water, and concomitant determination of the effect of added saponin as a hemolyzing agent. Fresh anticoagulated whole blood was diluted 10-fold with 10 g/L AA (pH 2.7); 10 g/L AA adjusted with 1 mol/L NaOH to pH 4.0, 4.25, 4.5, 4.75, 5.0, and 6.0; or water (analytical reagent grade), each with or without added saponin; the pHs of the lysates were 4.0, 4.7, 5.2, 5.5, 6.0, 6.3, 7.0, and 7.4, respectively. Six independent lysates were made using each diluent.

Study I(b). Study I(b) investigated, in the absence of added saponin, whether incomplete hemolysis (seen with increasing diluent/lysate pH) is a function of pH per se or whether it is attributable to the decreased hypotonicity of diluents to which NaOH was added during pH adjustment. Fresh anticoagulated whole blood was diluted 10-fold with 10 g/L AA adjusted with 1 mol/L NaOH to pH 4.5, 4.75, and 5.0, and with 10 g/L AA first adjusted with 1 mol/L NaOH to pH 6.0 and then readjusted...
immediately with 1 mol/L HCl back to pH 4.5 (each diluent without added saponin). Four independent lysates were made using each diluent.

STUDY II
Study II was an investigation into the effects of freezing-thawing and saponin addition on lysis with water, and the effect of freezing-thawing, sonication, and saponin addition on lysis with AA, pH 5.0 (lysat, pH 6.3). Fresh anticoagulated whole blood was diluted 10-fold with water (analytical reagent grade), (a) without saponin, (b) without saponin again, and (c) with saponin. Eighteen separate lysates were prepared using each condition. The second set of 18 lysates without saponin was frozen immediately in solid CO₂ for 1 h and then thawed. Additionally, fresh anticoagulated whole blood was also diluted 10-fold with 10 g/L AA, pH 5.0, (a) without saponin, (b) without saponin again, (c) without saponin for a third time, and (d) with saponin. Eighteen separate lysates were prepared using each condition. The second set of 18 lysates without saponin was frozen immediately in solid CO₂ for 1 h and then thawed. The third set of 18 lysates was sonicated for 30 s in an ultrasonic water bath.

_CREATION OF LYSATES_
After gentle mixing, 100 µL of anticoagulated whole blood was reverse-pipetted into 900 µL of the appropriate diluent in 2-mL plastic screw-cap microcentrifuge tubes and mixed gently by multiple inversions.

_FOLATE POLYGLUTAMATE DECONJUGATION_
All lysates (including those that may have undergone preliminary freezing-thawing) were incubated for 2 h at 37 °C so that any released red cell folyl-polyglutamates could undergo deconjugation, with naturally occurring plasma folate conjugase (γ-Glu-X carboxypeptidase; EC 3.4.19.9), to the monoglutamate form, which is the appropriate form for assay. The pHs of lysates/hemolysates, once cooled, were monitored with the use of a micro pH probe (pH Boy KS501; CAMLAB Limited) that had been calibrated immediately before use.

_POSTDECONJUGATION TREATMENT_
Deconjugated samples were remixed gently by multiple inversions and diluted 20-fold by reverse-pipetting 100 µL into a 2-mL screw-cap microcentrifuge tube containing 1.9 mL of 0.1 mol/L sodium phosphate buffer, pH 6.2 (so that samples could be added to the microbiological assay at the same pH as the assay medium). The tubes were then capped, and the samples were remixed gently by multiple inversions and centrifuged in a microcentrifuge at 16 000g for 1 min to pellet partially hemolyzed or nonhemolyzed erythrocytes. Four 50-µL volumes of each supernatant were then transferred aseptically, by reverse-pipetting, to 96-well Cell Culture Cluster plates (cat. no. 3596; Corning-Costar®) for the microbiological assay.

PREPARATION OF GROWTH MEDIA
Difco Lactobacilli Agar AOAC (cat. no. 0900-15; Becton Dickinson UK) was used to prepare agar for the maintenance of the microorganism as “stab” cultures. Hot liquid agar (3 mL) was dispensed into 5-mL screw-cap bottles, which were subsequently securely capped, autoclaved for 15 min at 121 °C (103 × 10³ Pa), and stored at 2–8 °C.

Medium for the revitalization of either lyophilized or stab cultures was prepared with Difco Lactobacilli Broth AOAC (cat. no. 0901-15; Becton Dickinson UK). Aliquots (10 mL) were dispensed into thick-walled glass test tubes, capped with Clark Fincaps (Clark Scientific), autoclaved for 15 min at 121 °C (103 × 10³ Pa), and stored at 2–8 °C.

Medium for the preparation of cultures in log-phase growth and for the main folate assay was prepared with Difco Folic Acid Casei Medium (cat. no. 0822-15; Becton Dickinson UK). Dehydrated medium (47 g) was gently brought to the boil for 1–2 min in 900 mL of H₂O and then cooled to room temperature. At pH >6.2, there can be a divergence in growth response to folic acid (assay calibrant) and 5-methyltetrahydrofolic acid from deconjugated erythrocyte folate samples (I2) because of a peculiarity of _L. rhamnosus_’s metabolism of the monoglutamate form of 5-methyltetrahydrofolate not shared with mammalian metabolism (I3). For this reason, 1.0 g of l-FA was then added to the cooled medium, and if necessary, the pH was further adjusted with either 1 mol/L acetic acid or 1 mol/L NaOH to pH 6.2 before the addition of H₂O₂ to a final volume of 1 L. Aliquots (200 mL) were distributed into four or five 250-mL wide-mouthed screw-cap bottles, autoclaved at 121 °C (103 × 10³ Pa) for 5 min (to minimize caramelize of the medium), and stored at 2–8 °C. One bottle was enough for nine 96-well assay plates. Initially, and thereafter occasionally, 200 mL of medium was distributed in 10-mL aliquots into thick-walled glass test tubes, capped with Clark Fincaps (Clark Scientific), autoclaved for 5 min at 121 °C (103 × 10³ Pa), and stored at 2–8 °C. On the day of assay, 1 ng of folic acid would be added to one of these tubes to create an “inoculum” tube.

MICROORGANISM
Lyophilized cultures of _L. rhamnosus_ NCIMB 6375 (also known as ATCC 7469) were obtained from the National Collection of Industrial and Marine Bacteria. _L. rhamnosus_ was previously known as _L. casei_, subsp. _rhamnosus_.

PREPARATION OF CULTURE
Cultures were initiated by transfer of freeze-dried cells to 10 mL of Lactobacilli Broth AOAC and overnight incubation at 37 °C. A subsample (100 µL) of this broth was then recultured in fresh Lactobacilli Broth AOAC overnight. After loop transfer, six stabs (four “weekly” stabs, a “monthly maintenance” stab, and a “spare” stab) were incubated for 2 days at 37 °C and stored at 2–8 °C. _L. rhamnosus_ was maintained long-term as stab cultures and refreshed every 4th week from the monthly maintenance stab.
PREPARATION OF FOLIC ACID STOCK SOLUTION

Precisely 20 mg of folic acid (cat. no. F 7876; Sigma-Aldrich) was dissolved with 200 μL of 1 mol/L NaOH. Approximately 50 mL of H₂O was added, and the solution was neutralized to pH 7.0 with 1 mol/L HCl. After the addition of 20 mL of ethanol, the solution was finally made up to 100 mL with H₂O, transferred to an aluminum foil-covered brown glass volumetric flask, and stored at 2–8 °C. This folic acid stock solution had a nominal concentration of 200 mg/L.

ESTIMATION OF TRUE CONCENTRATION OF FOLIC ACID STOCK SOLUTION

It is critically important to note that not only do commercial supplies of folic acid contain water, but that opened supplies also pick up water rapidly. Hence, it should be considered mandatory that the absolute concentrations of folic acid stock solutions be determined using the specific absorptivity for a 10 g/L solution in 0.1 mol/L alkali at 365 nm, the absorption maximum (ε₃₆₅nm = 195) (31). In the present studies, the real concentration of the stock solution was actually 155.9 mg/L. In practice, it was easier to use the nominal folic acid concentrations (200 mg/L) of the stock solution and any subsequent dilutions throughout all experimental calculations and to correct final tabulated answers only; in our case, we corrected by multiplying by 155.9/200.

PREPARATION OF FOLIC ACID CALIBRATION SOLUTIONS (OF NOMINAL VALUES)

On the day of assay, the stock solution was diluted. For solution A (200 μg/L), 100 μL of stock solution was diluted to 100 mL with water. For solution B (4 μg/L), 2.0 mL of solution A was diluted to 100 mL with water (this solution was used to prepare the inoculum tube). For solution C (4 μg/L), 2.0 mL of solution A was diluted to 100 mL with 0.1 mol/L sodium phosphate buffer, pH 6.2 (this solution was used to prepare the folic acid calibration series). Solution D was 0.1 mol/L sodium phosphate buffer, pH 6.2. This solution was used to dilute deconjugated samples before plating for assay, and to dilute the folic acid calibration solution (solution C).

PREPARATION OF CALIBRATION SERIES

For the calibration series, 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1.0 mL of folic acid solution C (4 μg/L) were added to 2-mL plastic screw-cap microcentrifuge tubes already containing 2.0, 1.9, 1.8, 1.7, 1.6, 1.5, 1.4, 1.3, 1.2, and 1.0 mL, respectively, of solution D (0.1 mol/L sodium phosphate buffer, pH 6.2) to form a 10-point calibration series of 0–2000 ng/L such that a 50-μL aliquot from each solution contained 0, 20, 30, 40, 50, 60, 70, 80, and 100 pg, respectively. Aliquots (50 μL) of each calibrator were added aseptically (sterile laminar-flow cabinet) to four wells of every sterile, flat-bottomed 96-well Cell Culture Cluster plate that was to be used.

PREPARATION OF INOCULUM AND COMBINED INOCULUM/FOLIC ACID CASEI MEDIUM, AND TIMETABLE OF ASSAY

On the day before the assay, bacteria were transferred from one of the stab cultures to a tube of Lactobacilli broth and incubated overnight at 37 °C to reinitiate active growth. Early on the day of the assay, folic acid solutions A, B, and C (see above) were constructed from the stock folic acid solution. Solution B (250 μL, containing 1 ng of folic acid in H₂O) was added to a tube containing 10 mL of Folic Acid Casei Medium, together with 100 μL of bacterium from the Lactobacilli broth grown overnight, to create an inoculum tube, which was incubated 6 h at 37 °C to generate organisms in the logarithmic phase of growth. During this period of time, folic acid calibration samples were prepared together with blood samples for the assay.

Using sterile pipette tips, we added four 50-μL aliquots of the calibration samples (every plate) and the experimental treated/diluted blood samples to sterile 96-well plates in a sterile microbiological laminar-flow cabinet. After the inoculum had grown for 6 h, 2 mL was transferred to one bottle containing 200 mL of Folic Acid Casei Medium and mixed; 200 μL of the combined growth medium/inoculum was then added, by reverse-pipetting, to all plate wells with an eight-lane multitip pipette. Plates were subsequently placed in a sealable plastic box lined with wetted absorbent paper; assay plates were stacked on top of two unused plates to keep them well above the wetted absorbent paper lining the box bottom. Before the box was sealed with a lid and incubated at 37 °C, a small container of water was also placed inside to ensure that humidity was maintained and that there was no volume change in the wells of the assay plates during incubation.

After overnight (~18 h) incubation, we measured the bacterial growth in the sample wells by reading the absorbance at 630 nm with an automatic microplate reader. Before the absorbance in the sample wells was measured, the absorbance for the four 0 pg folic acid calibration wells in each plate was reset to an approximate zero absorbance reading.

CALCULATION OF RESULTS

For each separate plate, the mean absorbance for each group of four calibration or sample replicates was calculated, and any residual mean absorbance for the 0 pg/well calibrant was subtracted (thus ensuring a zero absorbance for the 0 pg/well calibrant and allowing the absolute growth on different plates, both within and between assays, to be compared). A spreadsheet (Microsoft EXCEL®) was used to plot (pg/well on the y axis, and absorbance on the x axis) the calibration for each plate and to calculate a polynomial (third degree) equation: y = C + ax + bx² + cx³. The resulting equation for each plate was used to convert the absorbances of experimental samples into pg of folate/well. Folate concentrations (in ng of folic acid/mL of whole blood = μg/L) were generated by multiplying pg of folate/well (50 μL of
lysate) by an overall factor of 4 (i.e., $\times 20$ for conversion to pg/mL diluted lysate; $\times 20$ for dilution of lysate; $\times 10$ for original dilution of whole blood; divided by 1000 to convert from pg to ng).

**Statistical Analysis**

**Study I(a).** The effect of added saponin at any one diluent pH was analyzed by the Student unpaired $t$-test. The effect of pH, separately for with or without added saponin, on assayable RCF compared with 10 g/L AA, pH 2.7 (lysate, pH 4.0), was assessed by parametric one-way ANOVA and the least significant difference test.

**Study I(b).** The effect of pH on assayable RCF was assessed by parametric one-way ANOVA and the least significant difference test.

**Study II.** With water as the diluent, the effect on assayable RCF (a) without saponin, (b) without saponin + freezing-thawing, and (c) with saponin was assessed by parametric one-way ANOVA and the least significant difference test. Because of nonhomogeneous variances with 10 g/L AA, pH 5.0, as the diluent, the effect on assayable RCF (a) without saponin, (b) without saponin + freezing-thawing, (c) without saponin + sonication, and (d) with saponin was assessed by nonparametric Kruskal-Wallis one-way ANOVA by ranks and the Mann-Whitney $U$-test.

**Results**

The results for study I(a) are given in Table 1. The addition of saponin to the 10 g/L AA diluent, pH 2.7, 4.0, and 4.25 (whole blood lysates, pH 4.0, 4.7, and 5.2, respectively), did not produce significantly different assayable RCF concentrations. In contrast, addition of saponin to the 10 g/L AA diluent, pH 4.5, 4.75, 5.0, and 6.0, and water (lysates, pH 5.5, 6.0, 6.3, 7.0, and 7.4, respectively) produced significantly higher ($P < 0.05$) assayable RCF concentrations: a 27% increase in the case of water (lysate, pH 7.4), and a ninefold increase in the case of 10 g/L AA diluent, pH 6.0 (lysate, pH 7.0). Even with added saponin, the use of 10 g/L AA diluents more neutral than the historically widely used diluent (10 g/L AA, pH 2.7) produced significantly higher (~14%) assayable RCF concentrations.

The results ($\mu$g/L folate) for study I(b) are given in Table 2. On the basis of results from study I(a), it would be expected that assayable RCF for a diluent of 10 g/L AA, pH 6.0, would be very poor and even lower than that for 10 g/L AA, pH 5.0. However, results from this study indicated that the immediate readjustment of 10 g/L AA, pH 6.0, diluent back to pH 4.5 produced assayable RCF concentrations considerably higher than expected: 85% of the values observed when 10 g/L AA adjusted directly to pH 4.5 was used as diluent.

The results for study II are given in Table 3. When water was used as the diluent (lysate, pH 7.4), the assayable RCF concentration when saponin was not added was 81.7% of the value measured when saponin was added. This value rose to 90.1% with one cycle of freezing-thawing before RCF deconjugation. The failure of freezing-thawing to completely hemolyze water lysates was confirmed by visual observation. After deconjugation and subsequent lysate dilution before microbiological assay, centrifugation of samples revealed the presence of small red pellets in the freeze-thawed samples that were not seen in saponin-treated samples. When 10 g/L AA, pH 4.5 was used as diluent.

**Table 1. Results for study I(a).**

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Lysate pH</th>
<th>Without saponin</th>
<th>With saponin</th>
<th>Ratio Saponin/AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA-2.7</td>
<td>4.0</td>
<td>236.5</td>
<td>19.1</td>
<td>123.5</td>
</tr>
<tr>
<td>AA-4.0</td>
<td>4.7</td>
<td>275.2</td>
<td>20.8</td>
<td>270.2</td>
</tr>
<tr>
<td>AA-4.25</td>
<td>5.2</td>
<td>261.41</td>
<td>19.9</td>
<td>267.0</td>
</tr>
<tr>
<td>AA-4.5</td>
<td>5.5</td>
<td>227.2</td>
<td>15.8</td>
<td>263.4</td>
</tr>
<tr>
<td>AA-4.75</td>
<td>6.0</td>
<td>83.41</td>
<td>23.4</td>
<td>266.4</td>
</tr>
<tr>
<td>AA-5.0</td>
<td>6.3</td>
<td>29.61</td>
<td>10.1</td>
<td>240.5</td>
</tr>
<tr>
<td>AA-6.0</td>
<td>7.0</td>
<td>12.61</td>
<td>3.5</td>
<td>114.5</td>
</tr>
</tbody>
</table>

**Table 2. Results for study I(b).**

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Lysate pH</th>
<th>Folate, $\mu$g/L</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA, pH 4.5</td>
<td>5.5</td>
<td>254.6</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>AA, pH 4.75</td>
<td>6.0</td>
<td>99.8</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>AA, pH 5.0</td>
<td>6.3</td>
<td>24.2</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>AA, pH 6.0</td>
<td>5.5</td>
<td>215.9</td>
<td>5.3</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Results for study II. a

<table>
<thead>
<tr>
<th>Lysate pH</th>
<th>Water (pH 7.4)b</th>
<th>AA-5.0 (pH 6.3)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Without saponin (a)</td>
<td>149.0</td>
<td>15.5</td>
</tr>
<tr>
<td>Without saponin; frozen 1 h in solid CO2 (b)</td>
<td>164.4</td>
<td>20.7</td>
</tr>
<tr>
<td>Without saponin; sonicated 30 s (c)</td>
<td>Not assayed</td>
<td>34.2f</td>
</tr>
<tr>
<td>With saponin (d)</td>
<td>182.4</td>
<td>19.8</td>
</tr>
</tbody>
</table>

One-way ANOVA. h

Without saponin; frozen 1 h in solid CO2 (b)

Mean SD Mean SD

a/d (−sap/+sap) b 81.7% 15.1%
b/d (−sap + frozen/+sap) 90.1% 98.9%
c/d (−sap + sonicated/+sap) Not assayed 15.7%

a Folate concentrations (µg/L) of whole blood diluted (n = 18 independent dilutions) 10-fold with water or 10 g/L AA preadjusted with 1 mol/L NaOH to pH 5.0, with or without added saponin (but in this case, with or without additional freeze-thaw or sonication), were assessed after 2-h incubation at 37 °C (to allow for erythrocyte folate polyglutamate deconjugation) by microbiological assay with L. rhamnosus. b Lysate pH.

Table 3. Results for study II. a

<table>
<thead>
<tr>
<th>Lysate pH</th>
<th>Water (pH 7.4)b</th>
<th>AA-5.0 (pH 6.3)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Without saponin (a)</td>
<td>149.0</td>
<td>15.5</td>
</tr>
<tr>
<td>Without saponin; frozen 1 h in solid CO2 (b)</td>
<td>164.4</td>
<td>20.7</td>
</tr>
<tr>
<td>Without saponin; sonicated 30 s (c)</td>
<td>Not assayed</td>
<td>34.2f</td>
</tr>
<tr>
<td>With saponin (d)</td>
<td>182.4</td>
<td>19.8</td>
</tr>
</tbody>
</table>

One-way ANOVA. h

Without saponin; frozen 1 h in solid CO2 (b)

Mean SD Mean SD

a/d (−sap/+sap) b 81.7% 15.1%
b/d (−sap + frozen/+sap) 90.1% 98.9%
c/d (−sap + sonicated/+sap) Not assayed 15.7%

a Folate concentrations (µg/L) of whole blood diluted (n = 18 independent dilutions) 10-fold with water or 10 g/L AA preadjusted with 1 mol/L NaOH to pH 5.0, with or without added saponin (but in this case, with or without additional freeze-thaw or sonication), were assessed after 2-h incubation at 37 °C (to allow for erythrocyte folate polyglutamate deconjugation) by microbiological assay with L. rhamnosus. b Lysate pH.

Discussion

An international interlaboratory study of folate methods, estimating erythrocyte folate concentrations (7), demonstrated both large intraassay (CV = 35.7%) and interassay variation (up to ninefold). After the interlaboratory study (7), it was suggested that the inaccuracy and incomparability of erythrocyte folate measurements would hinder evaluation of the effects of national folic acid supplementation programs and the emerging understanding of the relationships between erythrocyte folate concentrations (folate status) and the risk of NTDs, vascular disease, and cancer (32).

Recently, we suggested that several assumptions underpinning RCF analysis are deeply flawed (17). The studies presented here addressed the first of these assumptions, that the dilution of whole blood with hypotonic diluents always produces red cell lysis and, hence, the release of all erythrocyte folate. Most lysates are prepared initially by diluting whole blood 10- to 21-fold with AA solutions (10 to 0.5 g/L), although it has been reported (14, 20) that some laboratories use sodium ascorbate solutions or sodium ascorbate adjusted to pH 4.5 or 6.1. These variations in diluent type (AA vs ascorbate), concentration, and dilution factor will produce lysates with different pHs. The studies presented here assessed the effect of lysate pH, using a historically widely used diluent (10 g/L AA) and dilution factor (10-fold).

Results from study I(a) demonstrated that RCF concentrations can be up to ninefold higher in the presence of added saponin, depending on diluent/lysate pH. We consider these results indicative of incomplete hemolysis. Unexpectedly, when lysis was induced with plain water, the ultimate hypotonic diluent, the presence of saponin increased the RCF concentration by 27%, which also was indicative of incomplete hemolysis. Study I(a) showed that an ascorbate diluent at pH 6.0 produced very poor hemolysis and, hence, low assayable RCF concentrations. It is arguable that this was a direct consequence of the decrease in diluent hypotonicity produced by the ions added when NaOH was used to adjust the 10 g/L AA diluent to pH 6.0. However, study I(b) showed that if diluent adjusted to pH 6.0 was immediately readjusted back to pH 4.5 with 1 mol/L HCl before use (the added ions decreasing its hypotonicity even further), assayable RCF essentially reverted to the much higher concentration seen when 10 g/L AA was adjusted directly to pH 4.5. This result implies that the substantially lower assayable RCF concentration obtained with increasing diluent pH is mainly a function of lysate pH per se and is not attributable to an inherent decrease in the hypotonicity of diluents caused by the addition of increasing amounts of NaOH as a pH modifier.

Study I(a) also demonstrated, surprisingly, that 10-fold dilution of whole blood with unadjusted 10 g/L AA (pH 2.7), historically a widely used diluent and dilution factor that produces a lysate pH of 4.0, yields a RCF concentration significantly lower than that achieved at the higher lysate pHs of 4.7, 5.2, or 5.5. This result was unexpected and raises a question concerning hemolysis conditions that have been accepted as a “gold standard” for many decades. An empirical observation made during the course of the current studies was that hemolysates generated with 10 g/L AA changed from a dark red/purple to a dark brown color within minutes, which suggested changes in the structure of Hb. It previously has been shown that Hb denatures irreversibly in solution at pH <4.7, with the degree of denaturation being directly
related to increasing acidity and time (33). Because RCF associates with Hb (21–24), it is perhaps not surprising that the use of 10 g/L AA, pH 2.7 (lysate, pH 4.0), leads to lower RCF concentrations. Under such conditions, RCF would be expected to coprecipitate with denatured Hb. This may explain problems encountered in a field study investigating the potential for screening blood folate concentrations by the collection of small finger-prick samples stored on filter paper as dried blood spots, where a generally decreased stability of blood folate concentrations in lysates or dried blood spots in the presence of AA was reported (34).

Results from study II, which used a diluent of 10 g/L AA, pH 5.0 (lysate, pH 6.3), demonstrated that although sonication for 30 s in an ultrasonic water bath had no significant impact on RCF concentration and, by implication, the degree of hemolysis, subjecting samples to one freeze-thaw cycle before RCF deconjugation increased the assayable folate concentration from 15% to ~100% of the comparative values seen in lysates with added saponin. Although there have been reports that freezing and thawing lysates once (14, 35), but not repeatedly (35), increases assayable folate concentrations, we have yet to find a currently used commercial RCF assay with blood preparation procedures that suggests that freezing and thawing may induce higher assayable values or that requires a single freeze-thaw cycle of lysates before RCF deconjugation. However, in contrast to the effect seen when 10 g/L AA, pH 5.0, was used as the diluent, results from study II demonstrated that subjecting samples to one freeze-thaw cycle before RCF deconjugation of whole blood previously diluted 10-fold with water still did not produce complete hemolysis, a failure confirmed by visual observation. Thus, freezing and thawing lysates once may not always guarantee complete hemolysis.

The results from studies I and II demonstrate that it should not be assumed that 10-fold dilution of whole blood with hypotonic solutions containing 10 g/L AA/ascorbate will always produce complete hemolysis. The results for water diluent suggest that the assumption of complete hemolysis should not be made unless lysing diluents are accompanied by appropriate addition of a hemolyzing agent. Of course, any hemolyzing agent that interferes with terminal folate analysis, microbiological assays, or any of the various commercially available competitive folate binding assays should not be used.

Once complete hemolysis has been achieved (in the absence of Hb denaturation), the next important step before assay is the complete enzymatic deconjugation of erythrocyte polyglutamyl folates through the judicious choice of pH, time, and temperature. The most obvious optimum pH is ~5.0 (4.7–5.2), an acidic pH close to the optimum for folate deconjugases, whose maximum activity is at pH 4.5 (36), but not low enough to induce Hb denaturation. Although deconjugase activity at pH 6.0 should be ~40% of the maximum exhibited at pH 4.5 (36), incomplete deconjugation has been reported in pH 6.0 lysates incubated for as long as 90 min at 37 °C (20).

However, it may be important to reevaluate this report because the authors initially diluted blood 10-fold with 10 g/L sodium ascorbate before adjusting the resulting lysate to pH 6.0. We have observed (unreported observations) that a diluent containing 10 g/L sodium ascorbate (pH 8.0) produces a lysate with a pH of 7.1, incomplete hemolysis, and a very low assayable RCF concentration that is indistinguishable from the concentration obtained when 10 g/L AA, pH 6.0, is used as the diluent (lysate, pH 7.0). In any future work (on completely hemolyzed solutions) into the efficiency of deconjugation at any chosen hemolsate pH and temperature, it may be informative to attempt to distinguish between incomplete deconjugation attributable to low deconjugase activity per se and that attributable to binding of RCF to Hb. This could be accomplished by assessing both the distribution of folate polyglutamate forms in subsamples of hemolysates by HPLC (20) and the distribution of folate polyglutamate forms in the filtrate of subsamples centrifuged through a molecular size-exclusion membrane that would prevent the passage of Hb. Samples showing incomplete folate deconjugation in hemolysates, but complete deconjugation in filtrates, would indicate that potentially effective deconjugase activity is being hindered by the binding of erythrocyte folate to Hb, perhaps in a manner (“oxyhemoglobin folate-trap hypothesis”) that we have previously proposed (17). Dimerization of Hb would negate any potential physical “trapping” of folate within the hollow central cavity of the quaternary structure of the Hb molecule. The choice of an acidic hemolsate pH that maximizes folate deconjugase activity, short of inducing denaturation of Hb (i.e., pH ~5.0) will also tend to maximize Hb tetramer-dimer dissociation, which has been shown to occur extensively at pH <6.0 (37).

The degree of hemolysis and/or the completeness of deconjugation will obviously influence the estimation of erythrocyte folate concentrations, but may affect the accuracy and stability of the classical biological method (microbiological assay) and the now widely used competitive folate-binding protein assays (radioassays and immunosassays) differently. The microbiological assay is more tolerant of the presence of polyglutamate RCF forms and will tend to underestimate total folate where hemolysis is complete but deconjugation is not. In contrast, under similar conditions, folate-binding protein assays may bind polyglutamate forms with a greater affinity than monoglutamate and thus overestimate total folate (38).

In conclusion, the inaccuracy and incomparability of erythrocyte folate measurements are hindering evaluation of the effects of national folic acid supplementation programs and our emerging understanding of the relationships between erythrocyte folate concentrations (folate status) and the risk of NTDs, vascular disease, and cancer. Erythrocyte folate assays vary in the strength of the
AA/ascorbate diluent and the dilution factor used for preparing lysates. This produces different lyse pHs, some of which may not fully induce either complete hemolysis and/or complete RCF deconjugation, and some which may irreversibly denature Hb, thus potentially coprecipitating any RCF electrostatically held within its central cavity (17, 21–24). This report concludes that:

- Lysing diluents should contain a chemical agent that guarantees complete hemolysis in the absence of interference with terminal folate assays. In this respect, it is not known whether saponin would interfere with commercial competitive folate-binding assays, or whether the use of other membrane-disrupting compounds, such as sodium dodecyl sulfate (sodium lauryl sulfate) or Triton X-100, would substitute for, or confer advantages over, the crude saponin preparation used in this report.
- The use of 10 g/L AA without pH adjustment, historically a widely used diluent, is not recommended because the acidity of the resulting hemolysate (pH 4.0) is well below the threshold (pH 4.7) for inducing irreversible Hb denaturation.
- The optimum pH for hemolysates should be ~5.0, an acidic pH that does not induce Hb denaturation but would maximize both plasma folate deconjugase activity and Hb dimerization.
- Before a fixed time is set for deconjugation at any chosen temperature (e.g., 2 h at 37°C), the time-dependent distribution of RCF mono- and polyglutamate forms should be monitored by HPLC such that complete RCF deconjugation can be guaranteed well within the allotted time.

The authors have no commercial interests. The Institute of Food Research is a company limited by guarantee, with charitable status (Reg. Charity No. 1058499), grant-aided by the Biotechnology and Biological Sciences Research Council (BBSRC). We thank Vivienne Davidson, Research Nurse and Manager of the Institute of Food Research’s Human Nutrition Unit, for volunteer recruitment and for general facilities, and Dr. Siân Astley for obtaining blood samples.

References