Assay of serum and whole blood folate by a modified aseptic addition technique¹,²,³


There are wide discrepancies in studies published by different laboratories between results of assays of serum and red cell folate activity (SFA and RCF) in normal subjects, but many of these differences are likely to be the result of variations of technique, rather than of the population being studied. There are basically three techniques in the assay of SFA using Lactobacillus casei as the test organism. The serum may be diluted 1 in 10 with phosphate buffer before extracting the folate activity by autoclaving (1, 2). Chanarin and Berry (3) modified this method by diluting serum 1 in 70 or more before heat extraction. The third method is by aseptic addition of serum to the assay medium without previous heat extraction (4).

Most whole blood folate (WBF) assay procedures have involved heat extraction (5), but an automated method utilized the aseptic addition technique (6).

It was found that a SFA method slightly modified from that of Chanarin and Berry (3) gave higher results that were more closely reproducible than those obtained from the 1 in 10 dilution heat extraction method (7). Further work showed that when the extract was added to assay medium to give final serum dilutions of 1 in 100 and 1 in 200, discrepant results were obtained. The present work describes an investigation into every stage of the assay protocol, with special reference to specimen dilution and to a comparison of the heat extraction with the aseptic addition technique. These have culminated in a much simplified and accurate aseptic addition SFA technique. Similar experiments with WBF have resulted in the successful application of the aseptic addition technique to hemolysate.

Methods

Reagents

It was suspected that some distilled water contained an inhibitory substance that was removed by resin, so deionized water was used in preparing all reagents, in every stage of the assay, and for the final rinsing of glassware; growth of organisms (8), usually algae, in reservoirs and resins was avoided by running only distilled water through the Elgastat deionizer.

Media. Bacto microassay culture agar (Difco Laboratories), Bacto B₃ inoculum broth, and folic acid (PGA) assay medium (Baltimore Biological Laboratory) were used according to the manufacturers' instructions.

Test organism. Lactobacillus casei (NCIB 6375) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aber

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² Supported by the Arnold Yeldham and Mary Raine Medical Research Foundation, the National Health and Medical Research Council of Australia, and Smith, Kline & French Laboratories (Australia) Ltd.
³ Address reprint requests to: J. D. Martin, FRCOG, Department of Obstetrics, King Edward Memorial Hospital, Subiaco, Western Australia, 6008.
⁴ Present address: Department of Pathology, Ahmadu Bello University, Zaria, Nigeria.
⁵ It is important that the assay medium be kept cool while being shipped from factory to laboratory, and that it is stored at 4°C over a desiccant. The medium should be a fine dry powder, and lumpy or discolored medium must be rejected.
dean, Scotland. The lyophilized preparation was subcultured to inoculum broth, incubated overnight, and subcultured to agar stabs, which were incubated overnight and stored at 4°C for 1 month. The strain was maintained by subculture from agar to broth and to agar again once per month.

**Standard solution.** Pteroylglutamic acid (PGA) (British Drug House Ltd.), 10 mg, was dissolved in 100 ml phosphate buffer pH 7, 0.1 M. The stock standard was divided into aliquots of approximately 1 ml in disposable plastic vials, and stored at −20°C for up to 6 months. One aliquot was thawed on the day of the assay, used once, and discarded.

Standard solutions were stored in this manner for 6 months and compared with freshly prepared solutions microbiologically and fluorometrically, using an Amino-Bowman spectrophotofluorometer, excitation wavelength 360 μm and emission 450 μm; the two solutions gave nearly identical results by both methods, whereas a PGA solution which had been stored in an amber bottle at 4°C for 6 months had approximately 60% of the original microbiological activity.

**Control serum and hemolysate.** Plasma from discarded unhemolyzed stored blood from the blood transfusion service was used. Ascorbic acid (Nutritional Biochemicals Corporation) 0.5 g was added to 100 ml plasma, which was divided into aliquots of approximately 1 ml and stored at −20°C for up to 6 months. One aliquot was thawed on the day of the assay, used once, and discarded.

Five milliliters of whole blood from a normal individual was diluted into 95 ml ascorbic acid solution, 1 g/100 ml; this ensured complete rupture of the red cells in a solution with pH value optimal for the activity of plasma conjugase, which makes RCF available for L. casei (5, 9). The hemolysate was divided into approximately 1-ml aliquots, stored, and used in the same manner as the control serum.

**Apparatus and glassware**

Seventy-five by 12 mm (3 by 0.5 inch) tubes were held in a stainless steel rack with holes grouped and numbered to identify 10 triplicate standards and 50 sets of 6 tubes for each test specimen; the whole was covered by a close-fitting lid. The rack was filled with tubes the night before the assay and autoclaved at 15 lb/square inch (psi), the autoclave being evacuated to dryness at the end of 15 min. The pipettes were soaked overnight in Pyroge (Diversay) solution after use, rinsed automatically with tap water for 6 hr, rinsed finally in deionized water, and sterilized in a hot air oven. All glassware was soaked in Pyrogen overnight, rinsed at least three times in tap water and once in deionized water before being dried and sterilized in a hot air oven.

The 75 by 12 mm tubes were used once and discarded, but may be treated as other glassware and reused. A preliminary experiment showed that there was no significant difference in PGA standard and serum assay readings when 125 by 15 mm (5 by 0.625 inch) tubes were used, but the assay medium had an appreciably darker color.

**Serum storage vials.** A Pasteur pipette was broken off short and weighed on an accurate balance. The tip was pressed into ascorbic acid, and the amount adjusted until the pipette contained 5 mg. The top of the solid column of ascorbic acid was marked and the pipette used to distribute ascorbic acid to a stock of plastic vials with push-in caps, which were stored over silica gel at 4°C until used for serum specimens. This pipette delivered a mean of 5.30 mg (SD ± 0.61 mg) on 20 weighings.

**Aseptic addition technique**

**Specimen.** Blood was collected by venipuncture after at least 2 hr fasting and divided into one clotted specimen (at least 2 ml) and one ethylenediaminetetraacetate (EDTA) anticoagulated specimen (approximately 2 ml). Serum was separated and 1 ml was pipetted into a storage vial containing ascorbic acid. Whole blood (0.1 ml) was added to 1.9 ml freshly prepared ascorbic acid solution (1 g/100 ml) in a plastic vial. The serum and hemolysate were stored at −20°C until assayed. The remaining EDTA specimen was used for routine hematology, which included the hematocrit (packed cell volume, PCV) reading. The techniques of assay of serum and hemolysate were identical from this point, except that the hemolysates were centrifuged for 2 min at 3,000 rpm after thawing in order to dispose of the red cell stroma. Preincubation of specimens or incubation with chicken pancreas was found not to alter results when allowance had been made for the folate content of the pancreatic preparation (5).

**Assay procedure.** An aqueous solution of 200 mg/100 ml ascorbic acid was prepared and autoclaved at 10 psi for 10 min on the day of the assay. A fresh solution of assay medium was sterilized at the same time and all solutions and glassware were allowed to cool before the assay was commenced to avoid precipitation of proteins.

The actual concentration of ascorbic acid before and after autoclaving was determined on one occasion by titration with 2,6-dichlorophenolindophenol (10); titration values were 194 mg/100 ml and 186 mg/100 ml, respectively. Preliminary experiments showed no significant differences of growth in standard or specimen tubes when 150, 200, 250 mg/100 ml ascorbic acid solution was used, nor did the use of phosphate buffer of pH 6.1, 0.1 M as diluent affect results.

An aliquot of stock PGA standard was diluted 1 in 500,000 with sterile ascorbic acid solution. Multiple standards were set up in triplicate in the tubes in the metal rack containing 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, 2.0 ml of the dilute standard, and the volume made up to 2 ml with ascorbic acid solution.

The specimens assayed (serum or hemolysate) were pipetted into two sets of triplicate tubes, 0.04
ml and 0.02 ml. The control serum or hemolysate, or both, was included in each assay. Sterile ascorbic acid solution (2 ml) was added to all tubes by means of a semiautomatic pipetting unit (F. Froud and Sons). Two milliliters of assay medium was dispensed to all standard and assay tubes after the ascorbic acid solution, as a precipitate sometimes formed if the reverse order was followed; one drop of inoculum (see below) was added except to the first triplicate blank standard and the first of each triplicate assay tube. The close-fitting lid was placed onto the rack, and the whole incubated at 37 C for 48 hr.

**Inoculum.** On the day before the assay, a subculture of *L. casei* was made from agar to broth, and incubated overnight. The overnight growth was subcultured to broth on the morning of the assay. Four 30-ml screw-cap bottles containing 10 ml assay medium and 10 ml ascorbic acid solution were autoclaved at the same time as the bulk of the assay medium and ascorbic acid solution. The culture was centrifuged after 6 hr incubation, and the supernatant broth discarded. The organisms were resuspended and washed three times in the sterile 20-ml aliquots of single strength assay medium; the final resuspension was the inoculum.

**Readings.** All tubes were shaken to disperse the growth before reading turbidity on an Electro Engineering Laboratories (EEL) nephelometer using a red filter. The average reading of the triplicate uninoculated standard tubes was subtracted from the average of the triplicate standard readings, and the appropriate blanks were subtracted from the mean of the duplicate assay readings.

The nephelometer was preferred to a spectrophotometer, as it was found that readings using a Spectronic 20 (Bausch & Lomb Inc.) at 520 mµ gave consistently higher readings with hemolysate assays, the difference being greater with 0.04-ml tubes, although serum results were identical; this was interpreted as being due to light absorbance by blood pigments.

**Calculation.** A curve was constructed relating turbidity to PGA content of the standard tubes. From this was read the folate content of the assay tubes. If the first serum tubes (0.04 ml) contained "x" nanograms and the second tubes (0.02 ml) contained "y" nanograms, then the SFA was 25x ng/ml and 50y ng/ml. If the first hemolysate tubes contained "a" nanograms, and the second tubes contained "b" nanograms, then the WBF activity was 500a ng/ml and 1,000b ng/ml. Red cell folate activity was calculated from the equation:

$$\text{RCF} = \frac{\text{WBF} - \text{SFA}(1 - [\text{PCV}/100])}{(\text{PCV}/100)} \text{ ng/ml}$$

SFA and RCF results were transformed to logarithmic values before statistical analysis, so that the mean results of the control subjects were expressed as the antilog of $\Sigma \log x/n$ and the 95% confidence limits as the antilogs of 2 sd from the log mean.

**Effect of lactic acid on turbidity**

The possibility of precipitation of protein from serum and hemolysate by lactic acid production in the aseptic addition technique was investigated. Uninoculated assay tubes were prepared in the usual manner and one to five drops of concentrated lactic acid added from a Pasteur pipette; the tubes were incubated for 48 hr, turbimetric readings taken, and pH measured at the same time as an assay of folate in serum and hemolysate.

**Comparison of heat extraction and aseptic addition methods**

Preliminary experiments had shown that there was a complete precipitation by heat of protein from serum and hemolysate containing ascorbic acid, when diluted in a phosphate buffer of pH 6.1, 0.1M, with 200 mg/100 ml additional ascorbic acid. Precipitation was incomplete at greater acidity and growth was diminished at pH 6.5.

Serum or hemolysate (0.4 ml) was added to 19.6 ml phosphate buffer-ascorbic acid solution and autoclaved 10 psi for 10 min; the supernatant obtained after centrifugation was added 2 ml and 1 ml to triplicate tubes. The volume of the second dilution tubes was made up to 2 ml with autoclaved phosphate buffer-ascorbic acid solution and 2 ml assay medium was added to all tubes. Standard methods involve autoclaving the assay for sterility before inoculation with *L. casei* (1–3), but second autoclaving was avoided by using sterile glassware and the assay medium was sterilized at the time of the extraction process.

**Results**

**Precipitation of protein by lactic acid**

Protein was not precipitated from either serum or hemolysate by lactic acid over a pH range 6.2 to 4.3 in conditions similar to those of an actual assay, although the acidity was increased beyond that of the supernatant from the top PGA standard (pH 5.1) and assay tubes with high folate content (pH 4.9).

**Comparison of heat extraction and aseptic addition methods**

The estimated folate content of 57 sera and hemolysates was consistently lower when calculated from the heat extraction assay containing 0.02 ml of a specimen than in an assay containing 0.04 ml (Table 1, compare variables 5 with 6, 15 with 16). Results from the two dilutions were closer using the aseptic addition technique, but the 0.02-ml assay tended to give the higher result, both with
TABLE 1
Bioassay of 57 sera and whole blood folates

<table>
<thead>
<tr>
<th>Variables</th>
<th>Specimen</th>
<th>Technique</th>
<th>Specimen volume, ml</th>
<th>Mean</th>
<th>( \bar{x}_1 - \bar{x}_2 )</th>
<th>S²</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2</td>
<td>Serum</td>
<td>Aseptic</td>
<td>0.04/0.02</td>
<td>5.24</td>
<td>-0.29</td>
<td>0.08</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>5 and 6</td>
<td>Serum</td>
<td>Heat</td>
<td>0.04/0.02</td>
<td>5.60</td>
<td>0.45</td>
<td>0.07</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>1 and 5</td>
<td>Serum</td>
<td>Aseptic/Heat</td>
<td>0.04</td>
<td>5.46</td>
<td>-0.73</td>
<td>0.28</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>2 and 6</td>
<td>Serum</td>
<td>Aseptic/Heat</td>
<td>0.02</td>
<td>5.38</td>
<td>0.02</td>
<td>0.24</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>11 and 12</td>
<td>Hemolysate</td>
<td>Aseptic</td>
<td>0.04/0.02</td>
<td>86.12</td>
<td>-5.43</td>
<td>45.12</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>15 and 16</td>
<td>Hemolysate</td>
<td>Heat</td>
<td>0.04/0.02</td>
<td>87.43</td>
<td>19.70</td>
<td>32.66</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>11 and 15</td>
<td>Hemolysate</td>
<td>Aseptic/Heat</td>
<td>0.04</td>
<td>90.34</td>
<td>-13.87</td>
<td>68.78</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>12 and 16</td>
<td>Hemolysate</td>
<td>Aseptic/Heat</td>
<td>0.02</td>
<td>83.21</td>
<td>11.26</td>
<td>102.28</td>
<td>( P &lt; 0.001 )</td>
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</tbody>
</table>

* Comparison of results from the aseptic addition and heat extraction methods, including 0.04 ml and 0.02 ml specimen/4 ml assay volume.

TABLE 2
Bioassay of 57 sera and whole blood folates

<table>
<thead>
<tr>
<th>Variables</th>
<th>Specimen</th>
<th>Technique</th>
<th>Specimen volume, ml</th>
<th>Mean</th>
<th>( \bar{x}_1 - \bar{x}_2 )</th>
<th>S²</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 3</td>
<td>Serum</td>
<td>Aseptic</td>
<td>0.04</td>
<td>5.07</td>
<td>0.05</td>
<td>0.16</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>2 and 4</td>
<td>Serum</td>
<td>Aseptic</td>
<td>0.02</td>
<td>5.70</td>
<td>0.09</td>
<td>0.20</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>5 and 7</td>
<td>Serum</td>
<td>Heat</td>
<td>0.04</td>
<td>5.35</td>
<td>0.25</td>
<td>0.27</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>6 and 8</td>
<td>Serum</td>
<td>Heat</td>
<td>0.02</td>
<td>5.16</td>
<td>0.44</td>
<td>0.20</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>11 and 13</td>
<td>Hemolysate</td>
<td>Aseptic</td>
<td>0.04</td>
<td>85.34</td>
<td>-3.86</td>
<td>28.14</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>12 and 14</td>
<td>Hemolysate</td>
<td>Aseptic</td>
<td>0.02</td>
<td>96.25</td>
<td>-1.28</td>
<td>77.33</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>15 and 17</td>
<td>Hemolysate</td>
<td>Heat</td>
<td>0.04</td>
<td>89.48</td>
<td>2.07</td>
<td>123.32</td>
<td>( P &gt; 0.05 )</td>
</tr>
<tr>
<td>16 and 18</td>
<td>Hemolysate</td>
<td>Heat</td>
<td>0.02</td>
<td>78.49</td>
<td>-1.82</td>
<td>115.52</td>
<td>( P &gt; 0.05 )</td>
</tr>
</tbody>
</table>

* Reproducibility of results in duplicate assays by the aseptic addition and heat extraction methods, including 0.04 ml or 0.02 ml specimen/4 ml assay volume.

serum and hemolysate (compare variables 1 with 2, 11 with 12).

The aseptic addition technique using 0.04 ml serum gave a mean result slightly but significantly lower than the same dilution in the heat extraction technique (variables 1 and 5), but there was no significant difference between the 1 in 200 dilution results (variables 2 and 6). Aseptic addition of hemolysate gave a lower mean result in the 0.04-ml assay (variables 11 and 15) and a higher result in the 0.02-ml assay (variables 12 and 16) when compared with the heat extraction results at the same dilutions.

All assays were repeated (Table 2). There was no significant difference between the means of results of serum assays performed by the aseptic addition technique at either dilution (variables 1 and 3, 2 and 4), but SFA results from heat extraction assay were significantly lower when repeated, and the variance was greater (variables 5 and 7, 6 and 8). Results were consistently higher from the second aseptic addition of 0.04 ml of hemolysate (variables 11 and 13), but the variance was small, suggesting that there was one factor common to all results accounting for the difference of means. There was no significant difference in the means of repeat assays using aseptic addition at 0.02 ml hemolysate (variables 12 and 14), or in either dilution of heat extraction assay (variables 15 and 17, 16 and 18), but the variation between results was greater with heat extraction.

**Effect of specimen dilution in the aseptic method**

The mean SFA and WBF results were significantly higher with 0.02 ml than with
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0.04 ml of specimen, using the aseptic addition technique (Table 1, variables 1 and 2, 11 and 12), but the difference was not uniform over the whole range of results. The 0.04-ml serum result tended to give lower results in the upper range of serum folate activity (Fig. 1). This was more noticeable and the slope of the regression was steeper with hemolysate assays, in which the 0.02-ml assays gave low results in the lower range of WBF (Fig. 2).

**Serum and red cell folate in nulliparous woman**

Ninety-five nulliparous, apparently well-nourished female nurses and laboratory technologists aged 18 to 41 years were studied (Table 3). Three had hemoglobin (Hb) between 11.2 to 11.9 g/100 ml, and these were among the 15 women who showed evidence of iron deficiency on the peripheral blood film (microcytosis, hypochromia, and poikilocytosis). Two subjects had 4% or more lobed neutrophil polymorphs (Table 4); of these, no. 298 was classified as being iron deficient and her folate assay results were normal, but no. 1847 had SFA and

![Graph](image-url)

**Fig. 1. Serum folate assay: effect of dilution in aseptic addition technique.**

![Graph](image-url)

**Fig. 2. Whole blood folate assay: effect of dilution in aseptic addition of hemolysate technique.**

<table>
<thead>
<tr>
<th>TABLE 3</th>
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<tbody>
<tr>
<td>Age, hematological values, and folate results in 95 nulliparous female nurses and laboratory technologists</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Age, years</td>
</tr>
<tr>
<td>Hb, g/100 ml</td>
</tr>
<tr>
<td>PCV, %</td>
</tr>
<tr>
<td>MCHC, %</td>
</tr>
<tr>
<td>5-lobed neutrophil polymorphs, %</td>
</tr>
<tr>
<td>SFA, ng/ml</td>
</tr>
<tr>
<td>RCF, ng/ml</td>
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</table>

<table>
<thead>
<tr>
<th>TABLE 4</th>
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<tbody>
<tr>
<td>Hematological details of six subjects with hyperlobulated neutrophil polymorphs, low SFA, or low RCF</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Hb g/100 ml</th>
<th>PCV, %</th>
<th>MCHC, %</th>
<th>Anemia</th>
<th>Hypochromia</th>
<th>Microcytosis</th>
<th>SFA ng/ml</th>
<th>RCF ng/ml</th>
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<tbody>
<tr>
<td>298</td>
<td>12.9</td>
<td>39</td>
<td>33</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4.8</td>
<td>269</td>
</tr>
<tr>
<td>1847</td>
<td>14.6</td>
<td>44</td>
<td>33</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6</td>
<td>124</td>
</tr>
<tr>
<td>1781</td>
<td>13.0</td>
<td>40</td>
<td>33</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>2.3</td>
</tr>
<tr>
<td>1851</td>
<td>13.6</td>
<td>42</td>
<td>32</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>2.1</td>
</tr>
<tr>
<td>1763</td>
<td>13.8</td>
<td>41</td>
<td>34</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>1843</td>
<td>12.6</td>
<td>39</td>
<td>32</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>2.5</td>
</tr>
</tbody>
</table>
RCF values near the lower limits of 95% confidence limits and was probably folate deficient. Two subjects had SFA below the 95% confidence limits, and were likely to be in negative folate balance, if not deficient; one, no. 1781, was iron deficient also. Two subjects had RCF below the 95% confidence limits, and both had SFA at the lower limit of 95% confidence. There was a significant correlation between the SFA and RCF ($r = 0.5834$, $P < 0.001$, Log SFA = 0.7119, Log RCF = 0.9584).

In summary, five control subjects were marginally deficient in folate; one of these was iron deficient, but otherwise there was no correlation between hypochromia and microcytosis and measures of folate deficiency.

Discussion

It would not be feasible to test all the variations of folate assay procedure in every possible combination, but the present work examines each stage to some extent. This has resulted in a method of great simplicity and acceptable accuracy, which is applicable to both serum and whole blood. Each tube contained only specimen or standard solution, sterile ascorbic acid solution and assay medium, the last two being added by a semi-automated pipetting unit. One technologist was able to prepare easily an assay containing 100 specimens in a working day. The method could be readily automated, following the principles of Davis and associates (6).

The work of washing glassware, the preparation of reagents, the maintenance of the organism, and the preparation of the PGA standard was reduced to a minimum (8, 11). The dilution of whole blood into an aqueous solution of ascorbic acid was modified from the procedure of Hoffbrand and associates (5), and ensured complete rupture of the red cells in a solution with pH value optimal for the activity of plasma conjugase, which may not be achieved with dilution in buffer. The inclusion of standard serum and hemolysate specimens in each assay ensured the stability of the method, and would seem a simple essential of the protocol. The major advantage claimed for this over other protocols is the successful application of the aseptic addition technique to WBF assay. A close-fitting lid to a rack with numbered holes avoided time-consuming adjustment of caps and labeling individual tubes; contaminating organisms affecting the assay were rare. Better mixing resulted from pipetting the specimen first. The BBL assay medium contained sufficient buffering, and additional phosphate buffer made no difference in growth in either standard or specimen tubes. Baltimore Biological Laboratory (BBL) folic acid (PGA) assay medium was superior to any prepared by ourselves or by another laboratory which we tested, and to one other commercial product; it had negligible growth in inoculated blank tubes and a wide differentiation of growth over the required range. The washing of the inoculum may not have been strictly necessary (11), but this step was retained because of a reluctance to add a single drop of folate-rich medium.

There may be physical or other differences between the specimen and standard tubes. *L. casei* is able to utilize the reduced folate in serum more rapidly than the PGA standard, and a true comparison can be made only after growth has exhausted completely all folate available; this was found to be after 46 hr incubation in a similar assay protocol (12). More than one dilution of the specimen must be included to make certain that comparisons are valid. Cooperman (8) proposed as many serum as standard dilutions, but this is impracticable. Two dilutions will reveal most sources of error. Results from two dilutions were closer with aseptic addition than with the heat extraction technique (Table 1) and differences were negligible over the middle range of the standard curve (Figs. 1 and 2), which covers the range of the majority of results from control subjects. Discrepancies were more noticeable in the assay of whole blood than serum folate, and were more pronounced when readings were beyond the penultimate points of the standard curve. The lower results at the upper range were possibly the result of greater clumping of organisms in the test than the standards, whereas the poor growth in the lowest range was the reverse of the observa-
tions by Streeter and O’Neill (12) and was unlikely to be the result of too short an incubation time. Assays involving these low or high turbidity readings should be repeated at dilutions calculated to give growth nearer the center of the standard curve.

Greater turbidity from the larger volume of specimen is another possible cause of error, especially if there is precipitation of protein by acid produced during bacterial growth (8). Ascorbic acid (150 to 200 mg/100 ml) in the diluent is known to prevent precipitation of serum proteins in the aseptic addition assay (11), and the same is true of whole blood, as no precipitation or increase of turbidity was demonstrated by addition of lactic acid to simulated assays.

Hemolysates gave apparently falsely high results in the higher concentration assay when turbidity was measured by light absorbance, not light scatter. The greater optical density (OD) was probably due to absorbance by blood pigments, and readings of scatter of red light by a nephelometer avoided this error.

Repeat assays showed that the aseptic addition technique had better reproducibility of results than the heat extraction method (Table 2). One apparent exception was the higher results on second assay of whole blood, but this was associated with a constant factor, not with greater scatter of duplicate readings, and a small error in pipetting the standard could have been responsible.

The preparation and storage of stock standard PGA solutions were found to be serious sources of error, and it is recommended that solutions are made up from dry PGA of a well-known manufacturer and divided into aliquots, which are frozen, thawed on the day of the test, used once, and discarded. Chanarin (9) recommends checking the purity of a PGA by reading its absorption in 0.1 N NaOH at 365 mm, the E1% value being 206. It is a deplorable habit to keep a large volume of standard in the laboratory refrigerator and to sample it each assay until it is exhausted.

The SFA results in the nulliparous Australian women were low compared with other control groups (2, 7, 13). Similar low results were obtained by Davis and Kelly working also in Western Australia (14) and by recent workers in other countries with high standards of living (15, 16). Stricter insistence on fasting, care in avoiding hemolysis, and accurate PGA reference solutions could account for the lower range of normal results, but women with high incomes may be deficient in folate because of destruction by cooking, weight reducing diets, or the use of oral contraceptives (17–19). One woman in this series had hypersegmentation of the neutrophil polymorphs and SFA and RCF near the lower extreme of the 95% confidence limits and four others were also marginally deficient of folate (Table 4). Fifteen out of 95 control subjects showed evidence of iron deficiency and three of these had Hb below 12 g/100 ml.

The aseptic addition technique of bioassay of SFA and RCF described in this paper has been used in an investigation into the incidence, complications, and prevention of folate deficiency in pregnancy: results to be published show that there is a close correlation between low SFA and RCF with other indices of folate deficiency.

Summary

An aseptic addition method for the measurement of serum and red cell folate is described and compared with a technique involving heat extraction. Results are similar, but the aseptic addition method is simpler, gives better reproducibility on duplicate assays, and better agreement between results obtained from two dilutions of the specimens.

Several important sources of error were discovered, including incorrect preparation and storage of reference solutions, and interference of blood pigments giving falsely high readings when turbidity was measured by light absorbance.

The mean serum folate in 95 nulliparous female nurses and laboratory technologists was 5.1 ng/ml (95% confidence limits 2.39 to 10.72 ng/ml); the mean red cell folate was 216 ng/ml (95% confidence limits 116.8 to 400.2 ng/ml). Five of the subjects were considered to be marginally folate de-
cient by the criteria of hyperlobulated neutrophil polymorphs, low serum or red cell folate. Fifteen subjects had evidence of iron deficiency, but their folate levels did not differ significantly from the rest of the control group.

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