

## Results with Commercial Radioassay Kits Compared with Microbiological Assay of Folate in Serum and Whole-Blood

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We compared results with three commercial folate radioassay kits [Bio-Rad, New England Nuclear (NEN), and RIA Products] with those by microbiological assay for more than 200 samples of human serum and whole blood. All but one kit (NEN) compared favorably with the microbiological assay for serum samples, although there were notable diagnostic discrepancies. Two kits (NEN and Bio-Rad) were tested on whole-blood samples; both yielded values significantly higher than those by microbiological assay. The frequency distributions of erythrocyte folate data differed strikingly between the two kits; the NEN method yielded a much narrower range of normal values than did either the Bio-Rad or the microbiological assay. Radioassay kits appear to be suitable diagnostic agents for serum folate, if the behavior of a particular kit is investigated thoroughly before its routine use. However, the diagnostic value of radioassays of erythrocyte folate needs to be validated.

Assay of serum folic acid is the most commonly used procedure to detect deficiency of the vitamin. Although it offers a sensitive test, serum folate is subject to short-term fluctuations resulting from dietary changes. Erythrocytes do not become depleted as quickly as serum and therefore they may better indicate the degree of folic acid deficiency. Erythrocyte folate correlates well with other indexes of folate deficiency such as bone-marrow cell morphology and hematological changes (1-3).

In the past, most assays of serum and erythrocyte folate have been done by microbiological techniques. Serum folate, which exists primarily as methyltetrahydrofolic acid (Me-THF) is readily utilized by test organisms. In contrast, erythrocyte folate exists as polyglutamate derivatives that are unavailable to microorganisms unless exposed to plasma conjugases (autolysis) or treated with external conjugases. Correctly and carefully done, the microbiological assay remains the most sensitive and reliable method available. The organism commonly used for folate assay (*Lactobacillus casei*) responds similarly to all physiological derivatives of the pteridine moiety of the vitamin, a feature not true of radioassays.

Ligand-binding radioassays for folate have been developed recently, and several commercial kits are now available, in most of which a binding protein from milk is used. These procedures offer the advantage of speed, technical ease, and the fact that they are less affected by antibiotics or other

growth-inhibiting substances in serum. In addition, there have been reports (4, 5) that the affinity of the milk binding-protein for folate is unaffected by the number of glutamate residues on the molecule. Thus, it should be possible to assay hemolysates directly, without prior autolysis (6, 7). The binding of pteroylglutamic acid (PGA) appears to be identical to that of Me-THF if the pH is 9.3 (8). Therefore, in many radioassay procedures the pH is adjusted and PGA standards are used rather than the unstable Me-THF derivative.

A possible drawback to folate radioassays is the fact that many metabolic forms of the coenzyme exist: numerous combinations of (a) three oxidation states of the pteridine ring, (b) at least five different substitutions on the *N*-5 or *N*-10 position, and (c) glutamate chain varying from one to seven units. The first two variables are known to affect the affinity between the folate substrate and the milk binding-protein (6). However, this has not been considered a serious obstacle because most circulating folate is Me-THF.

Several folate radioassays have been published, some including comparisons with the microbiological assay (5, 7, 9-15). In general, serum radioassay values have agreed reasonably well with the *L. casei* microbiological assay values, and reports are accompanied by claims ranging from "essentially identical" to "separate [the] samples into similar diagnostic groups." However, there are some disagreements. One group concluded that when discrepancies were observed the microbiological assay gave the more accurate indication of folate stores (10). In contrast to results for serum, comparisons between the *L. casei* microbiological assay and radioassays for folate in whole blood have agreed less consistently (5, 13-15). Nevertheless, most commercial suppliers claim that their kits will yield valid data for erythrocyte folate.

The purpose of the present study was to compare rigorously several commercial radioassay kits with the *L. casei* microbiological assay, for both serum and whole-blood folate. A major motivation was the paucity of studies comparing microbiological and radioassays of erythrocyte folate and the generally poor correlations found in the few data reported. Our ultimate goal was to determine whether or not a radioassay procedure could be used to detect as reliably those individuals in the population with low folate concentrations in serum and erythrocytes as would be the case by microbiological assay.

### Methods

Serum folate assays were performed with a chloramphenicol-resistant strain of *L. casei* (ATCC 27773) according to the procedure of Scott et al. (16). Whole-blood folate assays were done by the *L. casei* (ATCC 7469) method of Cooperman (17).

Preparation of folate standards used for these assays was based on the millimolar absorptivities: 25.8 at 256 nm and 25.1

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Received July 28, 1978; accepted Sept. 19, 1978.

**Table 1. Sallent Features of Folate Radioassay Kits Compared**

Kit	Type of assay	Std.	Carrier for std.	Zero std. or serum blank provided	Tracer	Binder	Normal range <sup>a</sup> folate, µg/liter of serum
Bio-Rad	competitive	PGA	protein	Yes	<sup>125</sup> I-labeled folate	β-lactoglobulin	1.9–14.0
New England Nuclear	competitive	PGA	protein	No	<sup>125</sup> I-labeled folate	β-lactoglobulin	2.3–15.2
RIA Products	noncompetitive	Me-THFA	serum	Yes	<sup>125</sup> I-labeled folate	β-lactoglobulin	3–16
Amersham/Searle	competitive	Me-THFA	goat serum	No	<sup>75</sup> Se-labeled folate	porcine serum protein	>4

<sup>a</sup> As stated in kit instructions.

at 283 nm, both at pH 13.0 (18). The purity of the crystalline PGS (Sigma Chemical Co., St. Louis, Mo. 63178) was calculated to be 92%. A final stock standard was diluted to 200 µg/liter in 10 mmol/liter NaOH/ethanol (80/20 by vol) and stored in the dark at 0–4 °C.

Samples for this study were collected during two surveys of nutritional status of military personnel. Group 1 (n = 216) was composed of men stationed at the Alameda Naval Air Station (August 1976); group 2 contained 312 men and 42 women stationed at Twentynine Palms Marine Corps Base (March 1977). Blood samples were taken in the morning, after the individuals had fasted overnight. For serum analysis, blood was drawn in Vacutainer Tubes, allowed to clot at room temperature, centrifuged, and the serum removed. For erythrocyte analysis, blood was drawn in Vacutainer Tubes containing heparin as anticoagulant and diluted 10-fold with an ascorbic acid solution (10 g/liter). All sample manipulations were performed in subdued light and the samples were kept frozen at –20 °C until they were analyzed.

Serum and whole-blood composites, obtained by pooling samples from several donors, were treated in the same manner as the other samples. Composites were included in duplicate with each batch of analyses. In addition, Control Serum II (Ortho Diagnostics Inc., Raritan, NJ 08869) was run in duplicate with each batch of radioassays except those with RIA Products kits. Erythrocyte folate was in all cases calculated by dividing the whole-blood folate by the hematocrit. In using this method, one assumes that the serum folate concentration is insignificant as compared to its concentrations in whole blood.

The following kits were purchased between October 1976 and May 1977: <sup>125</sup>I-Folate Kit, RIA Products, Inc., P.O. Box 914, Waltham, MA 02154; Folate (<sup>125</sup>I) Radioassay Kit, New England Nuclear, North Billerica, MA 01862; Quanta-Count Folate, Bio-Rad Laboratories, 32nd. and Griffin Ave., Richmond, CA 94804. A sample kit was obtained late in the study from Amersham/Searle Corp., Arlington Heights, IL 60005. In this kit a different tracer and binder are used than the other three kits. A group of 22 serum samples was run with this kit. All assays were performed according to the instructions included with the respective kit. All kits were tested on group 1 samples; only the Bio-Rad and microbiological assay were done on group 2 samples.

Table 1 summarizes major features of the kits. Each buffer contained borate, but other components varied. The procedures for all kits included a heat treatment of samples to release folate from endogenous folate binder. In all kits, dextran-coated charcoal was used to remove unbound folate after incubation with the tracer and binder. There were differences among the kits as to the sequence of heat treatment and additions of binder and tracer. The Bio-Rad kit was the only one that provided a protein-based diluent for use with whole-blood samples, to compensate for differences in protein content of standards and diluted whole blood.

Statistical analysis of data was performed by use of two packaged computer programs. Scatter plots, linear regression equations, and correlation coefficients were obtained with programs in the Statistical Package for Social Sciences (19). Analyses of variance, frequency distributions, and normality plots were obtained with the BMDP Biomedical Computer Programs (20).

## Results and Discussion

Table 2 summarizes precision data for the kits and microbiological assay. Figure 1 shows scatter plots of serum folate values obtained by the *L. casei* microbiological method and each brand of radioassay kit. Radioassay values tended to decline with increasing folate concentrations, thus the regression lines (except that of the Amersham/Searle kit) had slopes of less than 1.0, and positive intercepts. Many of the values that accounted for the low correlation coefficient of the NEN plot fell within one run. However, these data were not thrown out because the standard curve and results for quality-control samples with that run were reasonable. Those few

**Table 2. Between-Run Precision of Folate Analyses by Microbiological and Radioassay Methods**

	n <sup>a</sup>	Range ± SD, µg/liter	CV, %
<i>Serum composite</i>			
Microbiological	7	10.0 ± 0.8	8.0
Bio-Rad	10	8.9 ± 0.7	7.8
RIA Products	3	11.4 ± 1.2	10.5
<i>Whole-blood composite</i>			
Microbiological	7	187 ± 26.1	14.0
Bio-Rad	7	244 ± 28.4	11.6
New England Nuclear	7	196 ± 24.4	12.2
<i>Ortho control serum</i>			
Bio-Rad	8	8.9 ± 1.1	11.2
New England Nuclear	10	9.0 ± 2.1	23.3
RIA Products	3	20.0 ± 2.2	11.0
<i>NEN control serum</i>			
New England Nuclear	13	3.7 ± 0.3	8.6
<i>Whole-blood composite, % recovery<sup>b</sup></i>			
Microbiological assay	6	91.8 ± 16.5	—

<sup>a</sup> All quality-control samples were assayed in duplicate with each run. The average of the two was taken as the value for a given run.

<sup>b</sup> Added pteroylglutamate standard. "Recovery" values were collected routinely with each group of microbiological assays. However, they may not represent true recoveries, because pteroylglutamate is more stable than those forms occurring naturally in blood and plasma.

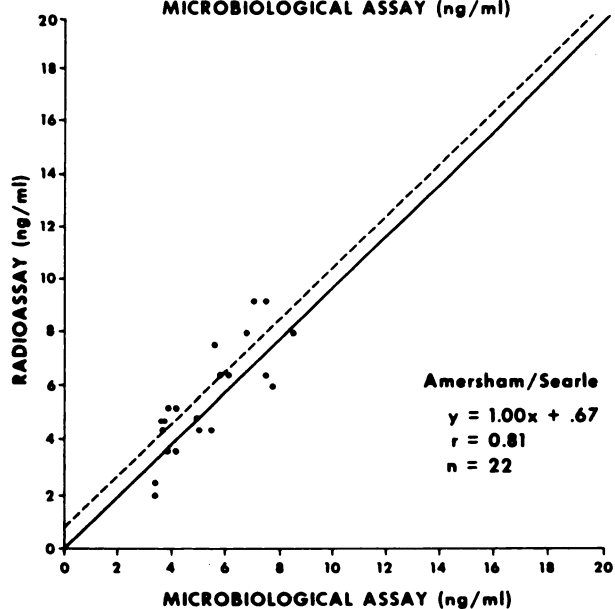
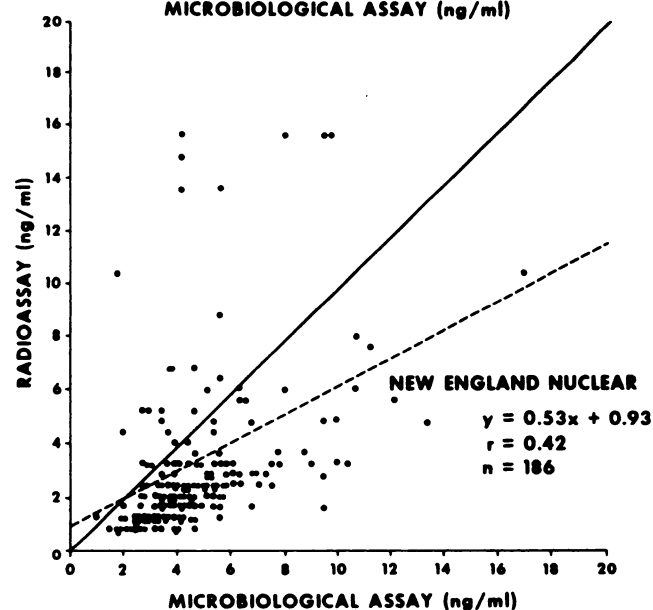
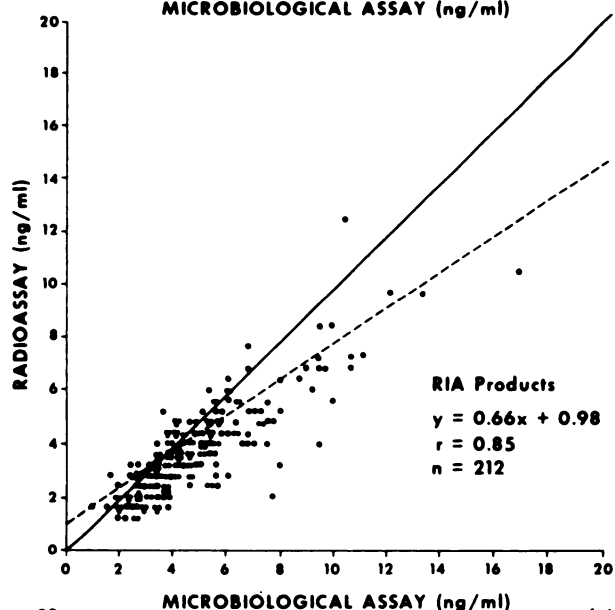
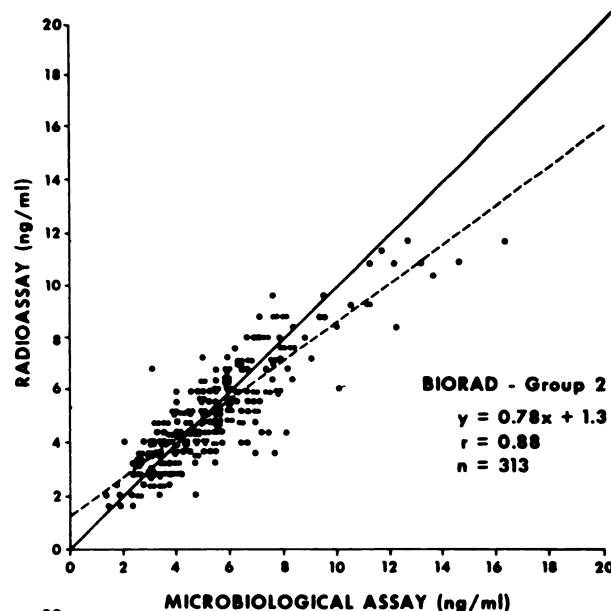
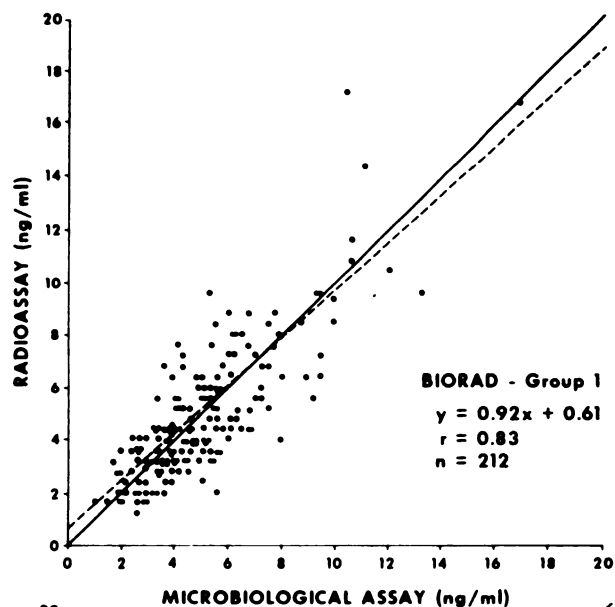


Fig. 1. Scatter plots of serum folate values by radioassay kits vs. microbiological assay method

The solid line in each is the line of identity ( $y = x$ ); the dotted line represents the plot of the linear regression equation. Occasional samples were lost and therefore n values varied for individual scatter plots

samples assayed by use of the Amersham/Searle kit gave values that agreed well with the microbiological assay values.

Values for folate in whole blood are compared in Figure 2. The slope of the regression line for the NEN kit was again less than 1.0 and the intercept was rather large. In contrast, values for erythrocytes with the Bio-Rad kit were higher than microbiological assay values throughout the folate concentration range. Because the RIA Products kit supplied standards in a serum base and made no provisions for diluting whole blood in a suitable protein-containing medium, we did not test this kit on whole-blood samples.

Table 3 summarizes the overall means and 95% ranges. The data were not distributed normally but did fit into reasonable log-normal distributions. Therefore, medians have been included and the normal ranges were estimated according to log distributions. Although all radioassays of serum differed statistically from the microbiological assay ( $p < 0.01$ ), the means were similar for all but one of the assay methods. The NEN kit yielded values about 30% lower than the other kits and the microbiological assay.

Both kits tested on whole-blood samples gave significantly higher means than the microbiological assay and different from each other ( $p < 0.01$ ). In addition, there were distinct differences in frequency distribution curves (Figure 3). Al-

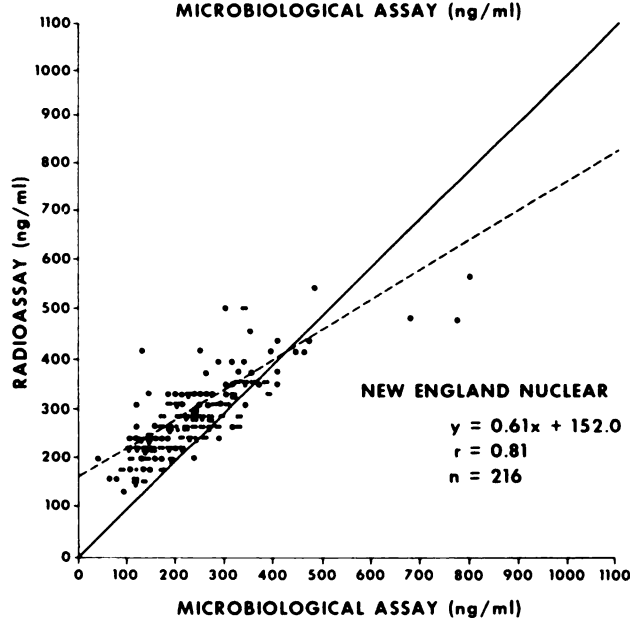
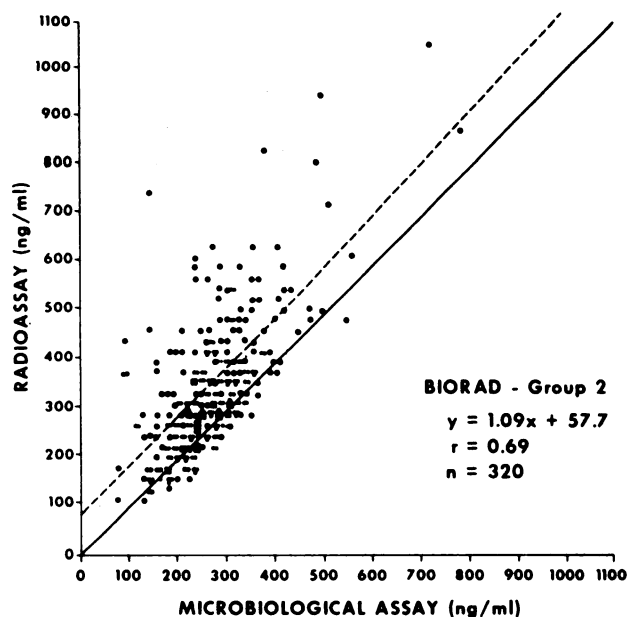
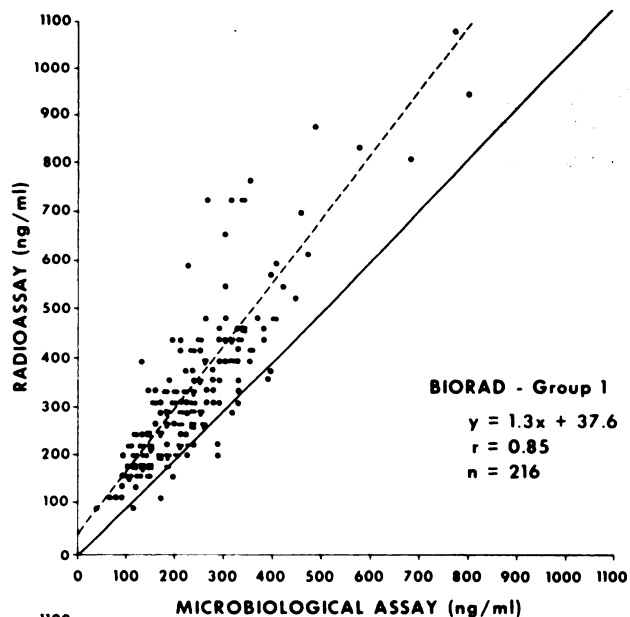


Fig. 2. Scatter plots of erythrocyte folate values by radioassay kits vs. microbiological assay method  
The solid line in each is the line of identity ( $y = x$ ); the dotted line represents the plot of the linear regression equation

Table 3. Serum and Erythrocyte Folate Concentrations in Military Personnel as Ascertained by Different Assay Methods

	Median	Mean	Estimated normal range <sup>a</sup>
Serum folate, $\mu\text{g/liter}$			
<i>Group 1 (n = 212)</i>			
Microbiological	4.2	4.7	1.8–10.4
Bio-Rad	4.3	5.0 <sup>b</sup>	1.8–11.0
New England Nuclear	2.5	3.4 <sup>b</sup>	0.8–9.4
RIA Products	3.9	4.1 <sup>b</sup>	1.7–8.5
<i>Group 2 (n = 313)</i>			
Microbiological	5.0	5.3	2.2–10.9
Bio-Rad	5.1	5.4 <sup>b</sup>	2.4–10.5
Erythrocyte folate, $\mu\text{g/liter}$			
<i>Group 1 (n = 216)</i>			
Microbiological	214	230	85–508
Bio-Rad	299	331 <sup>b</sup>	120–741
New England Nuclear	282	291 <sup>b</sup>	164–481
<i>Group 2 (n = 320)</i>			
Microbiological	257	264	129–484
Bio-Rad	312	345 <sup>b</sup>	153–674

<sup>a</sup> Estimation based on log distribution: geometric mean  $\pm$  2 SD.

<sup>b</sup> Significantly ( $p < 0.01$ ) different from the microbiological assay.

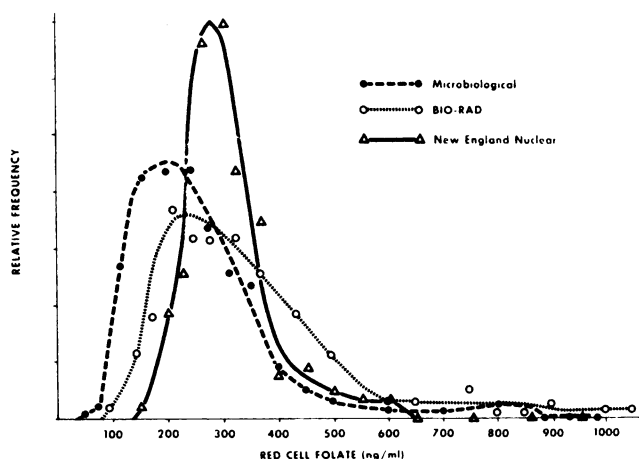


Fig. 3. Frequency distribution curves of erythrocyte folate data obtained by radioassay and microbiological assay methods  
 $n = 216$

though the Bio-Rad curve is displaced to the right of the microbiological, they both show distinct positive skewness. In contrast, NEN data were clustered in a sharp peak, with little skewness. Thus, the estimated normal range for erythrocyte folate was much narrower according to the NEN kit than with either the Bio-Rad or the microbiological assay. The reasons for these fundamental differences in response to whole-blood folate are not known.

While this study was in progress, Hill and Dawson (14) reported comparisons of the Bio-Rad kit and *L. casei* microbiological assay of serum and whole blood samples. Their results are similar to those of the present study, although their microbiological assay values were slightly higher for serum samples than their radioassay values. Their whole-blood

**Table 4. Summary of Agreement between Radioassays and Microbiological Assay of Serum and Erythrocyte Folate at Low Folate Concentrations<sup>a</sup>**

	Agreement of radioassay with microbiological assay <sup>b</sup>	No. falsely low by radioassay
<i>Serum folate</i>		
<i>Group 1 (n = 212)</i>		
Bio-Rad	23/38	7
RIA Products	33/38	15
New England Nuclear	33/38	80
<i>Group 2 (n = 313)</i>		
Bio-Rad	12/25	6
<i>Erythrocyte folate</i>		
<i>Group 1 (n = 216)</i>		
Bio-Rad	11/43	1
New England Nuclear	2/43	0
<i>Group 2 (n = 320)</i>		
Bio-Rad	2/12	0

<sup>a</sup> Serum criterion, 3.0 µg of folate per liter; erythrocyte criterion, 140 µg of folate per liter.  
<sup>b</sup> Number low by both assays/number low by microbiological assay.

values appeared to be greater by radioassay, particularly in high-folate samples.

A primary objective of this study was to estimate the diagnostic potential of the kits, as compared with that of the microbiological assay. Thus, we paid particular attention to discrepancies at low folate concentrations. For purposes of comparison, limits of 3 µg of folate per liter of serum and 140 µg of folate per liter of erythrocytes were used, because these values have been recommended as diagnostic for folate deficiency when one uses the *L. casei* microbiological assay (3). Borderline values were not considered as discrepancies if they were within reasonable experimental error (10% CV). Table 4 summarizes the number of samples that were found to be below the cutoff lines by the microbiological assay and by each radioassay. The first column represents the positive agreement between each kit and the microbiological assay; e.g., of the 38 serum samples judged to be low by the microbiological assay, 23 to 33 were also found to be low by the kit methods. The second column summarizes the number which were judged low by radioassay, but not confirmed by microbiological assay. Because of the large number of falsely low values by the NEN method, the 3.0 µg/liter value for serum obviously is not appropriate for this kit. Agreement between the radioassays and microbiological assay was much poorer for erythrocyte folate than for the serum analyses—a finding not unexpected because of the different population means and distributions of data. Some attempt was made to redefine new criteria for each kit other than those just mentioned, on the basis of difference in means, or by use of the regression lines. Although apparent agreement could be improved somewhat, such arbitrary adjustments cannot be assumed to be valid clinically. Such mathematical exercises are particularly risky for erythrocyte folate values, which do not have qualitatively similar frequency distributions.

The discrepancies between radioassays and the microbiological method can be attributed partly to variations in concentration of the standards. For example, microbiological assay of Bio-Rad standards indicated them to be about 90% of the stated values. RIA Products' "zero standard" (char-

coal-treated serum) assayed 1.0 µg of folate per liter by the microbiological method, which suggests that not all the folate had been removed. These facts could account for the average difference between each of these kits and *L. casei* microbiological assay. The reasons for the low NEN values are not known.

Erythrocyte folate values differed too greatly to be explained by the standards. One explanation for the higher radioassay values could be that forms of folate are present that bind more tightly to the binding protein than to the assay standards, thereby giving false high values. Gutcho (21) has offered support recently for this possibility when he noted that at pH 9.3 pteroylpolyglutamates were three- to fivefold more reactive in binding to the milk protein than are monoglutamates. Previous reports of equivalent binding of folate mono- and polyglutamates were based on systems with a pH of 8.0 or less (4, 5). Thus, autolysis or conjugase treatment of hemolysates may be necessary for radioassay procedures as well as for the microbiological assay. In the present study, autolysis of hemolysates according to the procedure for microbiological assay caused a consistent drop in radioassayable erythrocyte folate, but the values still remained higher than microbiological assay values (unpublished observations, E.L.M. and C.M.L.).

A disconcerting problem encountered in this study was that major changes were made in two of the kits after the preliminary tests were completed. In the early phases, NEN included ascorbate in the buffer, but later omitted it and supplied ascorbic acid tablets to be added to each sample. Before this change (and during our initial recovery and reproducibility studies) addition of ascorbate to the samples made little difference; after the buffer was altered, the amount of ascorbate added was critical. In our case, the tablets were unsuitable because they were intended for too large a sample (1.0 ml of serum or 5 ml of whole blood). The other major change was the increase of the buffer pH of the RIA Products kit from 8.6 to 9.3. The reason for this change was apparently to equalize binding of Me-THF with that of PGA. Thus, "reasonable" values would be obtained for various commercial control sera that contain added PGA rather than Me-THF. However, this change seems to defeat the purpose of having "natural" Me-THF standards in this kit.

Radioassay kits appear promising as diagnostic agents for serum folate. However, anyone contemplating routine use of a particular kit is cautioned to investigate thoroughly the behavior of the kit in advance. In particular, it is essential to determine the performance of the kit and establish the normal range before a decision is made as to criteria for distinguishing deficient and borderline individuals in a population. Because of the considerable differences between radioassays and the microbiological assay in terms of erythrocyte folate values and distribution of data, the diagnostic capability of the kits cannot be assumed. The reasons for the discrepancies must be understood first. Further studies are needed with subjects with known folate status and controlled intake to compare adequately the reliability and diagnostic value of radioassay methods with that of the microbiological assay.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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