The Determination of Erythrocyte Folate Concentration Using a Two-Phase Ligand-binding Radioassay

By Sheldon P. Rothenberg, Maria da Costa, John Lawson, and Zoltan Rosenberg

The concentration of folate in erythrocytes was determined using a two-phase ligand-binding radioassay procedure described previously for measuring serum folate. The mean (± SD) folate concentration in erythrocytes of 20 normal subjects was 210 ± 57 ng/ml. In 12 patients clinically folate deficient who had normal serum B_{12} concentration, the mean (± SD) erythrocyte folate was 71 ± 39 ng/ml. Incubation of the lysed erythrocytes for 2 hr prior to boiling increased the radioassayable folate. The radioassayable folate decreased rapidly if the whole blood was stored at 4° C without ascorbate. Extracts of blood prepared with ascorbate could be stored at −20° C for several days. The radioassayable concentration of erythrocyte folate was similar to the values obtained using *Lactobacillus casei* when the concentration was 200 ng/ml or less. With values higher by *L. casei*, the radioassayable folate was significantly lower even though the normal and folate-deficient groups were distinctly separated. This radioassay provides a rapid and reliable method of measuring erythrocyte folate, a parameter which reflects folate stores more reliably than serum folate concentration.

THE DETERMINATION OF serum folate can be helpful in the assessment of nutritional status and for the etiologic evaluation of megaloblastic anemia. Not infrequently, however, the serum folate concentration may be subject to perturbation as a result of short-term dietary changes and, in such instances, the value may not truly reflect the tissue stores of folate.[1] For this reason, erythrocyte folate levels have been recommended as a more reliable index of folate stores since this "tissue folate" is not subject to the more dynamic changes observed with serum folate concentrations.[2]

The measurement of either serum or erythrocyte folate concentration has required microbiologic assay techniques because physicochemical methods have generally lacked the sensitivity to measure the low physiologic concentration of this vitamin. Recently, however, competitive ligand-binding radioassays for folate have been described[3–5] with sufficient sensitivity to measure physiologic concentrations of folic acid as well as N^5-methyltetrahydrofolate, the natural folate. In a previous report from this laboratory, we described a ligand-binding radioassay to measure

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serum folate using a partially purified folate binder from milk and tritiated folic acid (^{3}H-PGA or tracer PGA) in a sequential incubation system.[4] We now wish to report the application of this same radiometric system for the determination of red cell folate concentration.

MATERIALS AND METHODS

Tritiated folic acid (^{3}H-PGA or tracer folate) was purchased commercially (Amersham/Searle). ^{5}N-methyltetrahydrofolate (methyl-FH_4) was purchased from Sigma Chemical. The folate binder was prepared from unpasteurized fresh milk as previously described.[4*] Serum and anticoagulated whole blood was obtained from normal subjects and patients with megaloblastic and/or macrocytic anemias. Serum B_{12} was determined using a radioassay procedure.[6] Microbiologic folate was determined using *Lactobacillus casei* by the method of Cooperman.[7]

Preparation of Whole Blood Extracts

The standardized method was to collect 5 ml whole blood into test tubes containing 60 U of heparin (Becton, Dickenson & Co.). One volume of the anticoagulated blood was diluted with 24 volumes of distilled water to lyse the erythrocytes and then with an additional 25 volumes of Ringer's solution containing 500 mg per 100 ml ascorbic acid. The pH of this Ringer's-ascorbate solution was adjusted to different values to determine the optimum hydrogen-ion concentration for the extraction. In some experiments, the diluted blood was extracted immediately by boiling for 5 min, and, in others, the hemolyzed blood was first incubated for 2 hr at 37°C before boiling for 5 min. After the boiling procedure, the precipitated proteins were removed by centrifugation, and an aliquot of the supernatant solution (usually 0.1 ml) was assayed for folate by the radioassay procedure and microbiologically.

The details of the radioassay procedure have been previously reported.[4] Briefly, the folate binder which is partially purified from whole milk is diluted in 0.05 *M* borate buffer containing 20% charcoal-treated normal human serum such that a standard volume (0.1 ml) binds 60%–70% of the ^{3}H-PGA tracer used in the radioassay. The procedure is a two-phase sequential incubation system where diluted binder is first incubated with the methyl-FH_4 standards or extract to be assayed in 0.05 *M* borate-Ringer's buffer pH 8.0 containing 200 mg/100 ml ascorbate. The reaction is incubated for 30 min at room temperature and then for any convenient period of time from 2 to 24 hr at 4°C. The tracer PGA is then added and the reaction mixture incubated an additional 30 min at 4°C. The unbound ^{3}H-PGA is removed by the addition of cold 5% charcoal in 0.5% dextran, and the bound ^{3}H-PGA remaining is determined by counting the radioactivity in an aliquot of the supernate using a liquid scintillation system. The standard curve is constructed by plotting the ratio of bound to free ^{3}H-PGA (B/F) against the methyl-FH_4. The folate concentration in the red cell extracts is determined by referring the B/F ratio obtained for these samples to the standard curve. It is not necessary to run controls without binder reagent because the deproteinated red cell extracts do not bind the ^{3}H-PGA. The folate concentration per ml of red cells is obtained by dividing the whole blood folate by the corrected hematocrit. In this study we corrected for the serum folate concentration, but this need not be done routinely because of the low concentration of serum folate relative to red cell folate.

RESULTS

Figure 1 illustrates a typical standard curve and a sample calculation used to obtain the serum and whole blood concentrations. The sensitivity of the curve is apparent from the sharp decrease in the B/F ratio of the tracer PGA as the methyl-FH_4 concentration of the standards increased. Usually the curve approaches the abscissa when the standard contains 300 pg methyl-FH_4. Samples with a B/F value corresponding to greater than 200 pg of methyl-FH_4 were reassayed at a greater dilution.

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*In the original report, a sentence was omitted from the part describing the preparation of the binder. This was corrected in a later issue of that journal (N Engl J Med 287:208, 1972).

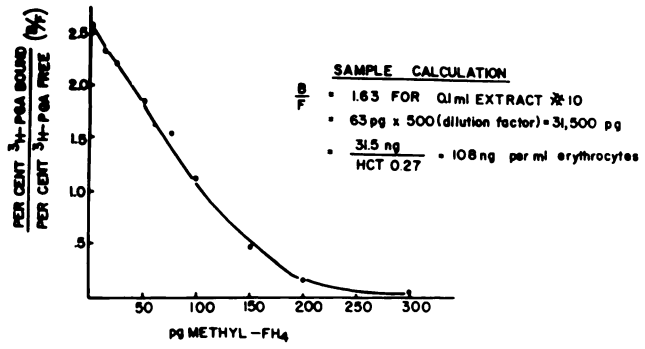

Fig. 1. Typical curve obtained using 5-methyltetrahydrofolate as the standard folate. An example of determining the folate concentration per ml of erythrocytes is shown.

The effect of incubation of lysed red cells at 37° C for 2 hr before boiling on the folate concentration is shown in Table 1. In eight of the ten samples studied, the erythrocyte folate was considerably higher if the lysed red cells were incubated before boiling. In samples 1, 2, 3, 5, and 9, the values without incubation might have been considered low or low normal, whereas following incubation, they fell more definitely within the normal range.

The folate concentration of heparinized blood incubated and extracted with Ringer's–ascorbate containing different hydrogen ion concentrations is shown in Table 2. There was no significant difference in the values obtained at the different pH levels. We have, therefore, standardized the procedure using heparinized whole blood, Ringer's–ascorbate buffer, pH 4.9, and incubation of the diluted whole blood for 2 hours at 37° C before boiling.

The results of replicate assays of whole blood kept at 4° C without and with ascorbate is summarized in Table 3. Without ascorbate, whole blood kept at 4° C rapidly loses its radioassayable folate. Stability of the folate is clearly preserved when the blood contains 5 mg ascorbic acid per ml.

Freshly prepared extracts were assayed and then frozen to determine the stability of the folate under these conditions. The data summarized in Table 4 indicates that the folate remains fairly stable when extracted and frozen with ascorbate.

Table 1. Effect of Incubation of Lysed Red Cells at 37°C for 2 hr in pH 4.9 Ringer's-Ascorbate Buffer on Erythrocyte Folate Concentration

	ng Folate per ml Erythrocytes	
Sample	Nonincubated	Incubated
1	148	172
2	143	189
3	156	210
4	126	121
5	142	200
6	146	137
7	17	71
8	32	103
9	70	181
10	172	321

Heparinized whole blood was diluted as described in text using water and Ringer's-ascorbate, pH 4.9, and incubated for 2 hr at 37° C before boiling for 5 min.

Table 2. Concentration of Folate in Erythrocytes Extracted With Ringer's-Ascorbate of Varying Hydrogen-ion Concentration With 2 hr Incubation at 37°C

	Folate Concentration ng/ml Erythrocytes pH of Ringer's-Ascorbate		
Sample	4.5*	4.9†	6.0‡
1	209	222	229
2	232	250	245
3	175	193	199
4	211	223	223
5	185	191	185

*pH of extract after 2 hr incubation at 37°C was 4.9 ± 0.2.
†pH of extract after 2 hr incubation at 37°C was 5.8 ± 0.2.
‡pH of extract after 2 hr incubation at 37°C was 6.5 ± 0.2.

Table 3. Erythrocyte Folate Concentration of Whole Blood Stored at 4°C Without and With Ascorbic Acid

	Folate Concentration ng/ml Erythrocytes Days between Assays								
Sample	0	1	2	3	4	5	6	7	8
Whole blood*									
1	244	74							
2	256	173	161	130					
3	100	81		67					
4	99						0		
5	378							236	
6	505								291
7	35								0
Whole blood† + ascorbate									
1	47	63	32						
2	187	209	187					183	
3	215	215	215					168	
4	128	139	117					104	

*Heparinized whole blood was extracted immediately and then kept at 4°C until extracted again on the indicated day.

†Heparinized whole blood containing 5 mg ascorbic acid per ml was extracted immediately and kept at 4°C until extracted again on the indicated day.

Table 4. Stability of Folate in Extracts of Whole Blood Stored at -20°C

	Folate Concentration ng/ml Erythrocytes Days Extract Frozen		
Sample	0	3	5
1	260	334	267
2	103	132	132
3	189	183	246
4	155	195	195

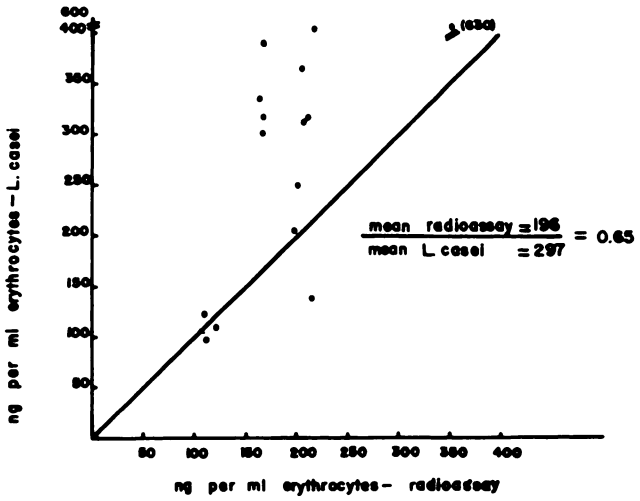

Fig. 2. Folate concentration of erythrocytes determined by radioassay compared to the values obtained for the same extracted samples by microbiological assay using *Lactobacillus casei.*

The comparison of erythrocyte folate concentration assayed by the standardized extraction of heparinized whole blood and microbiologically using *L. casei* is shown in Fig. 2. The agreement between the assays was good when the folate concentration by *L. casei* was 200 ng/ml or less. With folate concentrations greater than this value by *L. casei,* the radioassay results were considerably lower. The ratio of the mean of the radioassay concentrations to the mean of the *L. casei* concentrations for these samples was 0.65.

The erythrocyte folate concentration of heparinized whole blood extracted by the standardized incubation with Ringer's–ascorbate, pH 4.9, for normal subjects and patients with clinical folate deficiency and pernicious anemia is illustrated in Fig. 3. The values obtained for randomly selected hospital personnel ranged from

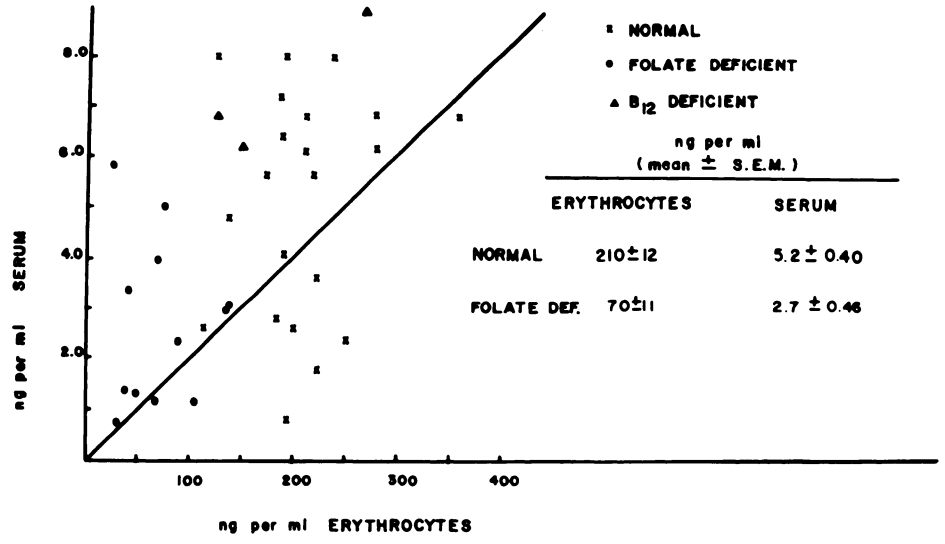

Fig. 3. Correlation of serum and erythrocyte folate concentrations determined by radioassay in normal subjects and patients with clinical folate deficiency.

119 to 358 ng/ml with a mean ± SD of 210 ± 57 ng/ml. The range of values of erythrocyte folate in 12 patients with clinical folate deficiency and normal serum B_{12} was 25 to 137 with a mean ± SD of 71 ± 39 ng/ml. The significance of the difference of these mean values was $p < 0.001$.

All patients clinically folate deficient had erythrocyte folate values below 137 ng/ml. Although most of these patients had serum folates below normal, three of this group had distinctly normal values. Of three patients with pernicious anemia tested, one had low, one had low normal, and one had a normal erythrocyte folate concentration.

We selected heparin as the anticoagulant because of the concern that citrate, oxalate, or EDTA might affect the properties of binder reagent. However, we have assayed some samples of whole blood containing these anticoagulants and found no significant difference from the values obtained for heparinized samples. Heparin solution (1000 U/ml) had a radioassayable folate concentration of 1 ng/ml.

DISCUSSION

The results of this study demonstrate that the radioassay for folate using a partially purified binding ligand prepared from milk, which had been previously used to measure serum folate,[4] can also be applied to measure erythrocyte folate. The values for the erythrocyte folate obtained by radioassay are similar in range to those obtained using *L. casei* and can be used to facilitate the diagnosis of folate deficiency where the serum folate is in the indeterminate range or even normal. The low erythrocyte folate concentrations observed in two of the patients with pernicious anemia are consistent with previous reports of this observation using microbiological folate assays[8,9] and is probably secondary to impaired uptake of folate by red cells in B_{12} deficiency.[10]

It is of interest that our previous studies demonstrated that the radioassay values for serum folate were only slightly more than 50% of the microbiological values, suggesting that the folate binding ligand from milk may not have binding determinants for all the folates in serum. This same observation for erythrocyte folate, when the *L. casei* results are greater than 200 ng/ml, suggests that there may also be a microbiologically active folate in red cells which does not react with milk binder.

Incubation of the lysed red cells at 37° C for 2 hr before extraction by boiling in most instances gave higher folate concentrations than the corresponding blood extracted without incubation. Toennies and co-workers reported this observation using the microbiological assay[11] and attributed it to the conjugase action of plasma on the microbiologically inactive polyglutamate form of folate in red cells yielding microbiologically active oligoglutamates. A similar phenomenon was not expected using the radioassay procedure because unpublished observations in this laboratory had demonstrated that folate heptaglutamate was as competitive as PGA for the binding determinant of the milk binder. The higher radioassayable erythrocyte folate concentration obtained after incubation may be due to release of a form(s) of the vitamin from macromolecular binding moieties within the erythrocyte. Herbert (personal communication) has also suggested that polyglutamate folates greater than heptaglutamate which were not tested in this radioassay may be present in the erythrocyte and, upon incubation, be broken down to the assayable oligoglutamates.

The radioassay for red cell folate can be completed in a single day or following an overnight incubation of the first phase of the procedure. Unlike microbiologic assays, the radioassay is not affected by antibiotics or drugs such as methotrexate which will inhibit bacterial growth. One cautionary note, however, is the necessity to maintain the whole blood with ascorbate if it is not going to be extracted on the day of collection.

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