Measurement of Folates in Human Plasma and Erythrocytes by a Radiometric Microbiologic Method

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A radiometric microbiologic assay for the determination of folic acid in human plasma and red blood cells is described. The assay is based upon the measurement of $^{14}CO_2$ produced from the metabolism of [1-^{14}C] gluconate by* Lactobacillus casei. *The $^{14}CO_2$ evolved was shown to be proportional to the amount of added DL-N-5-methyltetrahydrofolate (DL-N-5-methyl FH_4).

A total of 26 normal plasma and 57 blood hemolysates were assayed in parallel by this radiometric and the standard (turbidimetric) microbiologic assay. The correlation coefficients for the two assays were r = 0.96 for plasma and r = 0.98 for red-cell folate.

Lyophilization of* L. casei *was found to simplify this radiometric assay by eliminating routine maintenance and culture of this microorganism.

J Nucl Med 19: 906–912, 1978

The measurement of the folate content in serum and red cells by the microbiologic method using *Lactobacillus casei* has been well validated (*1–9*). The microbiologic assay is both sensitive and specific, but is tedious and time-consuming.

In recent years, several competitive and noncompetitive protein-binding radioassays for measuring folates have been described (*10–19*). The competitive protein-binding radioassays that have been compared with the *L. casei* assay gave comparable values in the assay of human sera. The noncompetitive protein-binding radioassay gave half the serum folate values when compared with the *L. casei* assay (*17*). Neither of these radioassay techniques gave folate values for red blood cells in agreement with the microbiologic assay (*14,19*). It has been shown that the red-cell folate value is more indicative of tissue folate stores than is the serum folate value, since the latter fluctuates according to dietary intake (*3,20–24*).

We recently developed a radiometric microbiologic assay for measuring vitamin B_{12} in human serum (*25*). In the present study, we describe a radiometric microbiologic assay for the measurement of folate content in both plasma and red blood cells. The folate values obtained by this radioassay correlated well with those obtained by the standard microbiologic assay.

MATERIALS AND METHODS

Preparation of blood samples. Heparinized venous blood was taken from normal subjects at least three hours after breakfast using either disposable syringes and needles or greentop vacutainer tubes. Informed consent was obtained from each subject. The whole blood was mixed well and the hematocrit was determined with a microhematocrit.

For the determination of red-cell folate, 1 ml of whole blood was diluted with 9 ml of deionized distilled water, vortexed to ensure complete hemolysis of red cells, and then further diluted with 10 ml of 0.05 *M* phosphate buffer (pH 6.1) containing 200 mg% of freshly added ascorbic acid. The resulting hemolysate was incubated at 37°C for 20 min and then stored in aliquots in folate-free vials* at

Received Nov. 7, 1977; revision accepted Feb. 11, 1978.

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—70°C. On the day of assay, the hemolysate was thawed and further diluted 1:5 with 0.05 *M* phosphate buffer containing 200 mg% of freshly added ascorbic acid. For the radiometric microbiologic assay, 0.25 ml and 0.5 ml of the diluted hemolysate were added to duplicate vials. The remaining hemolysate was autoclaved for 2–3 min at 15 psi and the coagulated proteins removed by centrifugation (400 g for 10 min). For the turbidimetric microbiologic assay, 0.25 ml and 0.5 ml of this protein-free hemolysate supernatant were added to triplicate tubes. Results were expressed as folate per milliliter of packed red blood cells, based on the prior determination of the sample hematocrit.

For the determination of plasma folate, the remaining blood was centrifuged (400 g) for 10 min. The plasma was stored at —70°C in aliquots with 5 mg ascorbic acid added per milliliter of plasma. On the day of assay, the plasma was thawed and diluted 1:50 with the phosphate-ascorbate buffer and 0.5 ml, 1 ml, and 2 ml were added to duplicate vials for the radiometric microbiologic assay. The remaining diluted plasma was autoclaved in the same way as the diluted hemolysate and the coagulated proteins removed by centrifugation. For the turbidimetric microbiologic assay 0.5 ml, 1 ml, and 2 ml of this protein-free plasma supernate were added to triplicate tubes.

Glassware. For cleaning glasswares, the same procedure was followed as in the radiometric microbiologic assay for vitamin B_{12} (*20*), but the serum vials used were 20 ml instead of 50 ml.

Maintenance of stock cultures and preparation of inoculum. *Lactobacillus casei* (ATCC 7469a) was obtained commercially†. Stock cultures were prepared monthly by stab inoculation of assay culture agar. Tubes were incubated at 37°C for 24 hr, then stored in the refrigerator (2–4°C). The day before the assay, bacteria were transferred from the agar to 10 ml of Bacto Micro Inoculum Broth‡. The broth culture was incubated for 16–18 at 37°C. On the morning of the assay, 0.5 ml of the broth culture were transferred to another 10 ml of Bacto Micro Inoculum Broth and incubated for 6–7 hr. The bacteria were centrifuged, washed three times with 10 ml of sterile Folic Acid Casei Medium and resuspended in 10 ml of this medium. This suspension was further diluted 1:100, and one drop of the final dilution was delivered to each turbidimetric assay tube using a sterile Pasteur pipette. One-tenth ml of the same suspension delivered from a sterile 1-ml tuberculin syringe was used to inoculate each radiometric assay vial.

Lyophilization of L. casei. A 6-hr growth culture of *L. casei* was washed three times with sterile single-strength assay medium. The final bacterial pellet was resuspended in 1 ml of the same medium. Aliquots (0.1 ml) of the bacterial suspension were lyophilized in 1-ml ampoules in an automatic freeze-dryer for 3–4 hr under a high vacuum (5–10 μ Hg). The ampoules were flame-sealed and stored at 4°C. On the day of assay, the lyophilized culture was resuspended in 1 ml of the assay medium and 0.2 ml were added to 10 ml of the assay medium. Each radiometric assay vial was then inoculated with 0.1 ml of this dilution.

Standards. Twenty milligrams of dried pteroylmonoglutamic acid (PGA)|| were suspended in 100 ml solution containing 10 ml ethanol and 90 ml distilled water. The pH was adjusted to 10.0 with 0.1 *N* sodium hydroxide to dissolve the folic acid, and then brought to pH 7.0 with 0.05 *N* hydrochloric acid. The stock solution was stored frozen in aliquots at —70°C. The working standard, pteroylmonoglutamic acid, prepared on the day of assay, was diluted in 0.05 *M* phosphate buffer to contain 1 ng PGA/ml.

DL-N-5-methyl FH_4 (barium salt) was purchased in 10-mg ampoules, of the same lot number. The contents were dissolved in 100 ml of 0.05 *M* phosphate buffer, pH 6.1, containing 200 mg% of as-

TABLE 1. PRODUCTION OF $^{14}CO_2$ BY *L. CASEI* IN THE PRESENCE AND ABSENCE OF PGA

	Radioactivity added (μCi)	$^{14}CO_2$ Production (metabolic index units)*		Net $^{14}CO_2$ production (metabolic index units)
		0 ng PGA	1 ng PGA	
L-[U-^{14}C] arginine	1	2	2	0
L-[guanido-^{14}C] arginine	1	0	0	0
[L-^{14}C] glycine	1	0	2	2
L-[U-^{14}C] histidine	1	2	4	2
DL-[carboxyl-^{14}C] histidine	1	0	0	0
L-[U-^{14}C] valine	1	2	2	0
[2-^{14}C] propionate	1	0	1	1
L-[U-^{14}C] glutamic acid	1	0	0	0
L-[U-^{14}C] isoleucine	1	0	0	0
L-[U-^{14}C] aspartic acid	1	0	0	0
L-[U-^{14}C] malic acid	1	16	30	14
[1,5-^{14}C] citric acid	1	10	15	5
[U-^{14}C] glycerol	1	0	0	0
D-[1-^{14}C] glucose	2	0	47	47
D-[U-^{14}C] gluconate	1	8	60	52
D-[1-^{14}C] gluconate	1	0	234	234

* 100 metabolic index units are equivalent to 31 nCi of $^{14}CO_2$.

corbic acid, and aliquots were stored at −70°C. The exact concentration of N-5-methyl FH_4 was determined spectrophotometrically using the extinction coefficient of $30.8 \times 10^3\ M^{-1}\ cm^{-1}$ at 290 nm, according to Gupta and Huennekens (*26*). A working standard, corrected for the measured concentration and the DL racemic mixture, was prepared on each day of assay and diluted in 0.05 *M* phosphate buffer to contain 1 ng/ml of L-N-5-methyl FH_4.

For each assay, six to seven different volumes of the working standard containing from 0 to 1.2 ng of folic acid, each in duplicate tubes or vials, were prepared. Five milliliters of assay medium containing 1 mg ascorbic acid/ml were added to each tube or vial, and the volume was made up with the 0.05 *M* phosphate buffer.

Radioactive compounds. The following C-14-labeled compounds were purchased commercially: L-[U-^{14}C] arginine monohydrochloride, 270 mCi/millimol; L-[guanido-^{14}C] arginine monohydrochloride, 55.3 mCi/millimol; [1-^{14}C] glycine, 55 mCi/millimol; L-[U-^{14}C] histidine, 330 mCi/millimol; DL-[carboxyl-^{14}C] histidine, 20 miC/millimol; L-[U-^{14}C] valine, 240 mCi/millimol; [2-^{14}C] propionic acid sodium salt, 31.4 mCi/millimol; L-[U-^{14}C] glutamic acid, 270 mCi/millimol; L-[U-^{14}C] isoleucine, 290 mCi/millimol; L-[U-^{14}C] aspartic acid, 232 mCi/millimol; L-[U-^{14}] malic acid, 50 mCi/millimol; [1,5-^{14}C] citric acid monohydrate, 27 mCi/millimol; D-[U-^{14}C] gluconic acid sodium salt, 3.9 mCi/millimol; D-[1-^{14}C] gluconic acid sodium salt, 56.6 mCi/millimol; [U-^{14}C] glycerol, 46 mCi/millimol; D-[1-^{14}C] glucose, 60 mCi/millimol. All the C-14-labeled compounds were prepared using sterile techniques with deionized water to contain 10 μCi/ml. D-[1-^{14}C] gluconate was prepared to contain 5 μCi/ml.

Turbidimetric microbiologic assay. Triplicate tubes were prepared for each plasma or hemolysate sample, one of which was kept as a plasma or hemolysate blank (no bacteria added). These tubes contained 5 ml of Folic Acid Casei Medium, and the volume was made up to 10 ml with 0.05 *M* phosphate buffer. The tubes were autoclaved (2½–3 min at 10 psi) and, after cooling, 0.5 ml, 1 ml, and 2 ml of plasma supernate, and 0.25 ml and 0.5 ml of hemolysate supernate, were added aseptically. The bacteria were added last and the tubes were incubated at 37°C for 20 hr. Bacterial growth was measured using absorbance at 640 nm. With the proper blanks subtracted, the standard curve was plotted, and the amount of folate activity in the sample was calculated.

Radiometric microbiologic assay. Duplicate 20-ml vials were prepared for each plasma or blood hemolysate. The diluted plasma or hemolysate sample was autoclaved directly after adding to the assay medium and buffer. It was not necessary to remove the coagulated proteins. After cooling, 1 μCi (0.2 ml) of [1-^{14}C] gluconic acid and 0.1 ml of the bacterial inoculum were added. The vials were incubated at 37°C for 16–17 hr. Bacterial growth was then measured by quantifying the amount of $^{14}CO_2$ released, using an ionization chamber§ as previously described (*25*).

RESULTS

Substrate selection and standard curve. All C-14-labeled compounds were evaluated using PGA. This standard was used initially because it is stable and available in a pure form. As shown in Table 1, D-[1-^{14}C] gluconate was the substrate best metabolized by *L. casei* in the presence of added folate, with significant release of $^{14}CO_2$ after 16–18 hr of incubation at 37°C. D-[U-^{14}C] gluconate resulted in the production of approximately one-fourth of the amount of $^{14}CO_2$ that D-[1-^{14}C] gluconate produced when 1 μCi of each was used.

A representative standard curve using N-5-methyl FH_4 and 2 μCi of D-[1-^{14}C] gluconate is shown in Fig. 1. A linear relationship was observed from 0 to 0.8 ng of N-5-methyl FH_4.

Originally we used 2 μCi of D-[1-^{14}C] gluconate in the radiometric assay system, but comparable values were obtained using either 2 μCi or 1 μCi. Thereafter 1 μCi was used for economy.

With a constant amount of folic acid (1 ng) and

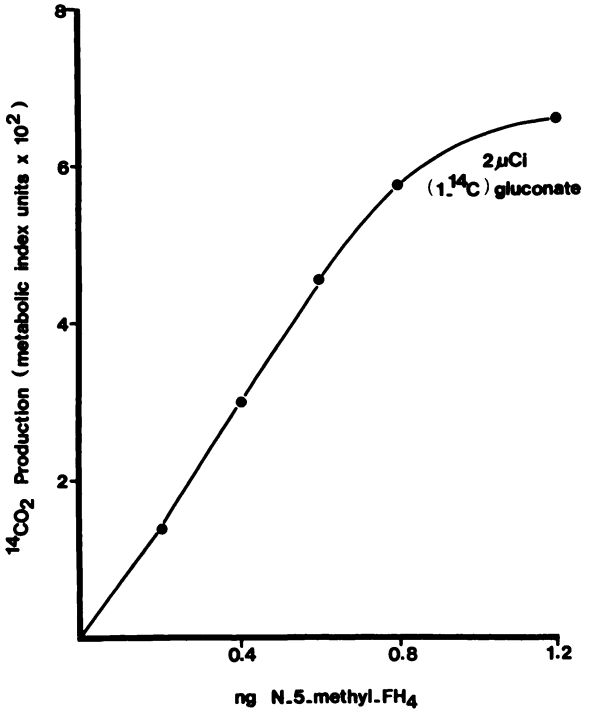

FIG. 1. Metabolism of 2 μCi of D-[1-^{14}C] gluconate by *L. casei* with increasing concentrations of N-5-methyl FH_4. Incubation time 16–17 hr. Each point represents mean of triplicate determinations.

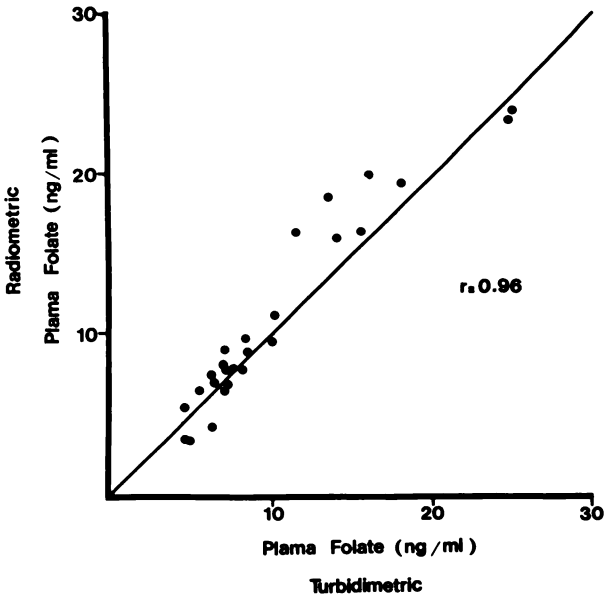

FIG. 2. Comparison of 26 plasma folate levels assayed by radioactive and turbidimetric procedures using *L. casei*. Correlation coefficient, $r = 0.96$. Each point represents mean of duplicate determinations. Line of identity is drawn in.

varying amounts of ascorbic acid (from 0–20 mg), we found that a minimum of 1 mg of ascorbic acid per tube (turbidimetric assay) and 3–4 mg of ascorbic acid per vial (radiometric assay) were required to protect folic acid during the autoclaving step. No differences in growth of *L. casei* were observed, however, in the range of 1–20 mg of ascorbic acid per turbidimetric assay tube or 3–20 mg of ascorbic acid per radiometric vial. Accordingly, we settled on 5 mg of ascorbic acid per assay tube or vial.

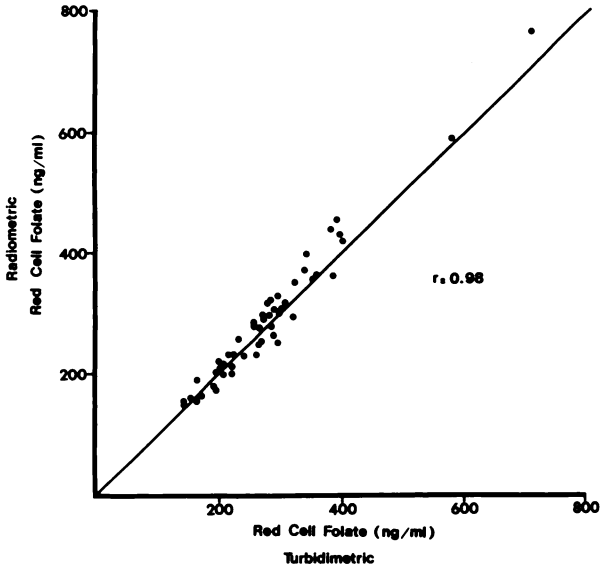

FIG. 3. Comparison of 57 hemolysate samples assayed by radiometric and turbidimetric procedures using *L. casei*. Correlation coefficient, $r = 0.98$. Each point represents mean of duplicate determinations. Line of identity is drawn in.

Plasma folate. Plasma samples from 26 normal volunteers were assayed in parallel by the radiometric and turbidimetric microbiologic methods. Excellent correlation was obtained ($r = 0.96$, Fig. 2). Levels ranged from 3.3 to 24.3 ng/ml plasma (mean 10.9) for the radiometric assay, and 4.5 to 24.7 ng/ml plasma (mean 10.2) for the turbidimetric assay.

Red-cell folate. Blood hemolysates from 57 normal volunteers were assayed by radiometric and turbidimetric microbiologic methods in parallel. As shown in Fig. 3, the values obtained by the two methods correlated well ($r = 0.98$). The mean red-cell folate value obtained by the radiometric assay was 270.0 ng/ml with a range of 149.4–769.2 ng/ml red cells. By turbidimetric assay, the mean red-cell folate value was 281.7 ng/ml with a range of 142.7–707.1 ng/ml.

Accuracy of the radiometric microbiologic method. Both the reproducibility and sensitivity to the addition of folate of the radiometric method were evaluated. Four hemolysates were assayed 8–10 times over a period of 6 mo, and the coefficients of variation were 8.6%, 12.9%, 11.5%, and 8.5% (Table 2).

Recovery studies were performed by adding 5 ng of N-5-methyl FH_4 to seven plasma samples, and 50 ng and 100 ng of N-5-methyl FH_4 to seven hemolysates (Table 3). The mean recovery percentage for the seven plasma samples was 102.7 ± 8.7% (s.d.). The mean recovery percentage for the hemolysates was 107.5 ± 13.5% (s.d.) when 50 ng was added, and 106.0 ± 4.5% (s.d.) when 100 ng was added.

Lyophilization of L. casei. Twenty-six hemolysates were compared by the radiometric assay using lyophilized and standard cultures (Fig. 4). A correlation coefficient of 0.94 was obtained when samples were assayed in parallel using previously lyophilized and stored *L. casei* and using conventional serial cultures of this organism.

DISCUSSION

The development of this radiometric microbiologic assay for plasma and red-cell folate levels was based on the measurement of $^{14}CO_2$ produced by *L. casei* in the presence of human plasma or whole-blood hemolysates (or folate standard) and D-[1-^{14}C] gluconate. The $^{14}CO_2$ produced was proportional to the amount of folate present.

Buyze et al. (27) showed that *L. casei* possesses the enzyme 6-phosphogluconate dehydrogenase. When this microorganism was grown with gluconate

TABLE 2. RADIOMETRIC MICROBIOLOGIC ASSAY: REPRODUCIBILITY OF ERYTHROCYTIC FOLATE LEVELS

Assay	Folate (ng/ml)			
	Sample 1	Sample 2	Sample 3	Sample 4
1	217.7	76.9	226.1	200.0
2	232.9	92.3	261.1	181.7
3	201.5	67.1	263.1	170.4
4	234.8	72.1	223.5	195.4
5	227.5	70.4	223.5	188.1
6	206.1	62.3	189.6	172.1
7	194.9	71.0	210.6	180.6
8	185.4	83.8	193.4	188.8
9	—	—	218.4	223.3
10	—	—	—	171.5
Mean ± s.d.	219.6 ± 18.4	74.5 ± 9.6	223.3 ± 25.6	187.2 ± 16.2
C.V. (%)	8.6%	12.9%	11.5%	8.5%

TABLE 3. RECOVERY OF N-5-METHYL-FH_4 ADDED TO PLASMA AND HEMOLYSATES

Sample No.	Plasma	Hemolysate	
	5 ng	50 ng	100 ng
1	102.5	92.1	112.6
2	110.8	103.0	110.2
3	110.8	116.3	102.8
4	86.2	119.7	108.2
5	99.2	126.7	104.6
6	109.0	92.4	100.1
7	100.5	102.8	103.3
Mean ± 1 s.d.	102.7 ± 8.7	107.5 ± 13.5	106.0 ± 4.5

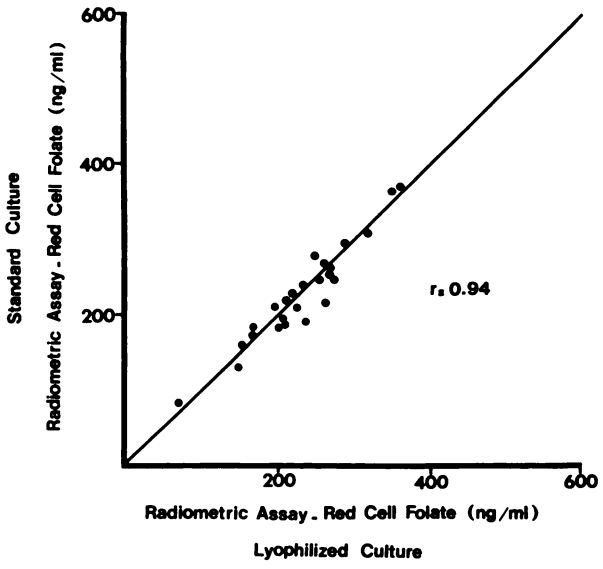

FIG. 4. Comparison of 26 hemolysate samples assayed by the radiotracer procedure using standard and lyophilized cultures of *L. casei*. Correlation coefficient $r = 0.94$. Each point represents mean of duplicate determinations. Line of identity is drawn in.

as the sole carbon source, the compound was metabolized through the hexosemonophosphate shunt with release of CO_2.

$$\text{gluconate} \xrightarrow[\text{glucokinase}]{+\text{ ATP}} 6\text{ phosphogluconate}$$

$$\xrightarrow[\text{dehydrogenase}]{6\text{ PG}} \text{ribulose-5-PO}_4 + \text{CO}_2$$

This pathway has been shown to exist also in *B. subtilis* (*28*), *E. coli* (*29*), *Acetobacter* (*30*), and many other microorganisms. Production of $^{14}CO_2$ by *L. casei* in the presence of folate was greater from D-[1-^{14}C] gluconate than that from D-[U-^{14}C] gluconate because a) the 1-^{14}C-compound has a higher specific activity (× 14.5), and b) $^{14}CO_2$ is produced by this enzyme by cleavage of the carbon at the one position of gluconate.

It has been shown that folate in human serum is mainly N-5-methyl FH_4, while that in erythrocytes is mainly conjugated derivatives of N-5-methyl FH_4 (*31–33*). To test the metabolic activity of *L. casei* for D-[1-^{14}C] gluconate in the presence of different folates, we assayed both PGA and N-5-methyl FH_4 on a molar-equivalent basis by the radiometric method. *L. casei* had essentially identical metabolic activity with each of these folates (Fig. 5).

It has been pointed out that both ascorbic acid and phosphate can stimulate the growth of *L. casei* when the folate concentration is limiting (*34*). We therefore used phosphate buffer both to dilute all reference standards, plasma and hemolysate samples, and to make up the volumes for all assays. Each reaction tube or vial was similarly prepared to contain an excess (5 mg) of ascorbic acid.

Like Magnus (*35*) we elected to assay plasma folate rather than serum folate for several reasons. First, a single (3-ml) heparinized blood sample per-

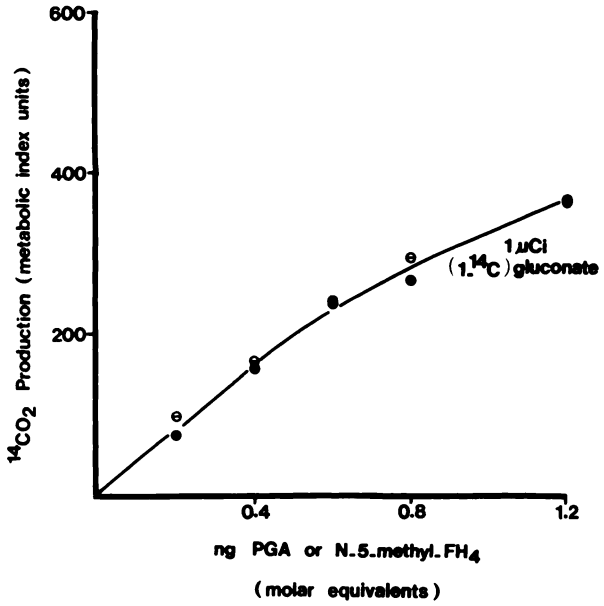

FIG. 5. Response of *L. casei* to PGA Θ and N-5-methyl FH_4 ● on a molar basis in presence of 1 μCi of D-[1-^{14}C] gluconate. Incubation time 16–17 hr. Each point represents mean of triplicate determinations.

mits determination of the hematocrit and the whole-blood and plasma folates. Second, with both plasma and RBC folate values determined, it is possible to differentiate transient dietary folate deprivation (low plasma with normal RBC folate values) from: a) true folate deficiency (low plasma with very low RBC folate values); b) transient spurious elevation of plasma folate by recent ingestion of folate or folate-rich foods; and c) vitamin-B_{12} deficiency (plasma values normal to high, with moderately low RBC folate values) (*3,7,36,37*).

A significant reduction of the time required for each assay can be achieved by lyophilization of *L. casei*. This eliminates the need for much of the routine maintenance of the organism (Table 4). Also, a more reproducible standard curve is obtained (*38*) using lyophilized cultures, presumably because it is possible to prepare a large supply (6 mo to 1 yr) of homogeneous cultures at one time.

The radiometric microbiologic assay has several advantages over the turbidimetric assay. A separate plasma or hemolysate blank for each blood sample at each dilution is not required, as it is for the turbidimetric microbiologic assay. Coagulated proteins in the plasma and whole-blood extracts do not interfere with the radiometric assay. Further, this assay method is adaptable to automation.

The detection of $^{14}CO_2$ evolved by *Lactobacillus* organisms has been shown, both in this radiometric assay for folates and the previously reported radiometric vitamin B_{12} assay (*25*), to be unaffected by turbid or colored solutions, unlike the conventional turbidimetric assays. Accordingly, these radiometric assays seem to offer the opportunity to measure the content of these essential nutrients in other biologic materials, such as tissue homogenates and foodstuffs.

TABLE 4. LYOPHILIZATION AS METHOD OF SHORTENING ASSAY TIME

Standard technique	Lyophilization technique
Stock cultures of *Lactobacillus casei* are kept as stab inoculum in micro assay culture agar. These are subcultured monthly in micro assay culture agar.	
↓	
Before day of assay, subculture into micro inoculum broth and grow for 16–18 hr at 37°C.	
↓	
On morning of assay, subculture from 16 hour growth to fresh micro inoculum broth. Grow for 6–7 hr at 37°C.	
↓	
Centrifuge bacteria. Wash three times with assay medium.	→ Lyophilize bacteria and seal the ampoules. Store at 4°C.
↓	↓
Resuspend, dilute and inoculate assay tubes or vials.	On day of assay, break open ampoule, resuspend, dilute and immediately inoculate assay vials.

FOOTNOTES

* Wheaton, Millville, N.J.

† American Type Culture Collection, Rockville, Md.

‡ Difco Laboratories, Detroit, Mich.

|| Obtained from USP Reference Standards, USP-NF Reference Standards, Rockville, Md.

§ Bactec Model R301, Johnston Laboratories, Cockeysville, Md.

ACKNOWLEDGMENTS

This work was supported by U.S.P.H.S. Grant GM 10548.

We are grateful to Dr. Min-Fu Tsan for obtaining many of the blood samples used in this study.

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