Cryoprotected *Lactobacillus casei*: An Approach to Standardization of Microbiological Assay of Folic Acid in Serum

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Folate-depleted, cryoprotected preparations of *Lactobacillus casei* are stable for at least eight months at −18 °C, capable of reproducible growth and suitable as a ready source of inoculum for measurement of folates in physiological fluids. Cryoprotected microorganisms can be a commercially available laboratory reagent, thus simplifying and further standardizing the microbiological assay of various nutriments. Standard folate growth curves of cryoprotected *L. casei*, prepared at intervals over eight months, are superimposable, have a low blank, and should eliminate the variations encountered with the continuous passages of microorganisms required for the classic microbiological assays. Serum folate values obtained by use of the cryoprotected *L. casei* fall into the same diagnostic groups as determined by the classic microbiological assay.

Microbiological assays of vitamins, purines, pyrimidines, and amino acids in serum and (or) whole blood, although sensitive and specific, are increasingly being replaced by radioassay. This trend is mainly ascribable to frequent variations of the bacterial inocula used in the assay and the requirements for microbiological expertise and facilities for the maintenance of bacterial cultures, requirements not easily met in the usual clinical-nutrition laboratory. We have eliminated these obstacles by using, for the purposes of obtaining a low blank and sensitive assay, folate-depleted cryoprotected preparations of *Lactobacillus casei* that are capable of reproducible growth and are stable for at least eight months at −18 °C.

Preserving bacteria by freezing was first described by Howard (1) and further exploited by Tanguet et al. (2, 3) in the assay of various nutriments. In contrast, cryoprotected microorganisms are stored in glycerol/water (40/60 by vol) and consequently are not frozen or lyophilized and are ready for use without thawing (4). We show here that cryoprotected microorganisms can be used in the clinical setting as a substitute for the usual serially maintained bacteria. This diminishes the need for microbiological expertise on a technical level and ensures reproducible growth curves with every assay. Here we have used the assay for serum folate to illustrate the principle of cryoprotected microorganisms. Except for the preparation of the cryoprotected inoculum, the assay is based on the similar principles of microbiological assays of folate in serum (5–7) and involves the aseptic addition method of Herbert (8).

Materials and Methods

**Materials**: *Lactobacillus casei* (ATCC no. 7469) was from the American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852, as an agar-slant culture. De-ionized water was used in preparing all media and solutions. *L. casei* maintenance broth and *L. casei* assay medium were products of Difco Laboratories, Detroit, MI 48232. L-Ascorbic acid was from Sigma Chemical Co., St. Louis, MO 63178, and glycerol from Fisher Scientific Co., Pittsburgh, PA 15219. Pteroylglutamic acid (folic acid) was from Lederle Laboratories, Pearl River, NY 10965, under the trademark FOLVITE. Solutions of FOLVITE were standardized by use of the molar absorbptivity value 27 000 at 282 nm. We used 18 x 150 mm glass culture tubes with plastic closures, and “Nephele” flasks of the appropriate sizes from Belco Glass, Vineland, NJ 08360. A Coleman Junior Spectrophotometer was used in determining absorbance.

**Preparation of the cryoprotected microorganisms**: Maintenance medium was prepared according to the package insert (38 g/L); 5 mL was apportioned per tube, sterilized by autoclaving at a pressure of 103 KPa (15 lb/in.²) for exactly 15 min at “slow” setting, removed from the autoclave immediately after the pressure dropped, to prevent caramelization of the glucose, and stored at 4 °C. The initial “log cultures” are prepared as follows. (a) Between 4 and 5 p.m. on the day preceding the experiment, inoculate a tube of medium with the most recently made culture and incubate it overnight at 37 °C. (b) At about 10 a.m. the next morning, transfer between 0.1 and 0.3 mL to a second tube of medium and reincubate for 5–7 h. This is the log culture.

Prepare the folate-depleted inoculum as follows.

1. Prepare the *L. casei* assay medium at a concentration of 47 g/L and add to this 250 mg of L-ascorbic acid. Warm to facilitate solution, then distribute to Nephele flasks as follows: (a) 150 mL in flask one, which is supplemented with folic acid, 30 ng/L final concentration, and (b) 300 mL in flask two, with no additional folic acid. Autoclave as previously described. The total amount of medium needed depends on the quantity of bacteria in each batch but is kept at a 3:1 ratio of a to b. Because the *L. casei* assay medium is folate free, the supplementation with folic acid is necessary for growth.

2. Incubate an amount of the log culture, determined by experience and usually in the range of 0.1–0.3 mL, in the folate-supplemented flask overnight.

3. The following morning, record the absorbance at 650 nm. Repeat at hourly intervals and, when growth is seen to slow, pour the culture into the flask that contains no additional folic acid (this will deplete intracellular folate) and record the absorbance. Continue the incubation, taking absorbance readings hourly. When growth slows down, centrifuge at 6500 X g for 10 min at 4 °C. Wash once with assay medium to remove any remaining folate from the cells, pool the pellets, and reconstitute with 10 mL of assay medium.

4. Dilute the mixture 100-fold with assay medium and record the absorbance. Re-adjust the original sample so that...
the 100-fold dilution will give an absorbance of 0.2, then dilute with an equal volume of glycerol/water (80/20 by vol).

(5) Store at -18 °C, in 0.5-mL aliquots.

(6) On the day of assay, reconstitute with 2 mL of assay medium. This is the undiluted cryoprotected inoculum.

Preparation of the standard curve: Prepare L. casei assay medium as previously described, and autoclave 5-mL portions in 18 x 150 mm glass tubes with plastic closures. Prepare the standard curve by adding 0–1000 pg of folic acid per tube, in duplicate. After adding one drop of the cryoprotected inoculum, incubate the tubes at 37 °C for 24–72 h. Record the absorbance at 650 nm vs an uninoculated control and plot it vs folic acid concentration, on linear graph paper.

Note: These curves should be run with cryoprotected inoculum also diluted 10-, 50-, and 100-fold. The dilution that gives the best curve should be used for all assays performed with that particular preparation. The standard curves were superimposable when repeated at intervals over an eight-month period. Thus one batch of cryoprotected L. casei, prepared as described, can be used for several thousand assays if stored in appropriate portions at -18 °C.

Assay of serum folate: Venous blood was drawn from fasting subjects (disposable plastic syringes and Vacutainer Tubes, Becton-Dickinson no. 3200) to ensure freedom from contamination by traces of folate. The blood was allowed to clot at room temperature, centrifuged at 1000 x g, and the supernatant serum was stored frozen. On the day of assay, the serum was thawed and 0.05 and 0.1 mL was added to tubes containing 5 mL of the assay medium. The tubes were inoculated and incubated at 37 °C. Results were determined by direct reference to the standard curve after correction for the dilution factor.

Results

We compared the curves for folic acid dependent growth from the same preparation of cryoprotected L. casei throughout a period of eight months (Figure 1A). The growth curves exhibit folate dependency, sensitivity, and remarkable stability and reproducibility. The growth rates are slightly slower but not as variable as for the two strains of L. casei maintained and grown simultaneously in the classic manner at the same institution (Figure 1B). While we initially thought that a more rapid folate-dependent growth would be obtained when we used a more concentrated inoculum, the results show that dilution produces more consistent growth, which closely approximates that of the classic noncryoprotected L. casei (Figure 2). As can be seen in Figure 3, serum folate values by the classic and the cryoprotected assay are comparable.

Discussion

Microbiological techniques for assay of vitamins have enjoyed wide popularity. Direct low-temperature storage of test organisms in an appropriate suspension medium makes it possible to keep the cultures on hand for prompt use. While techniques for preparing frozen microorganisms have been in existence since 1956 (1), until now no one has used cryoprotected microorganisms for the purpose of clinical nutritional status surveys. Because the cryoprotected microorganisms are stable for many months at -18 °C, commercially available preparations would standardize the assay methodology in nutritional laboratories world-wide. Although the methodology described here is for microbiological assay of folate, cryoprotected test organisms can be prepared for the assay of other vitamins, with resulting improvement in the standardization and a simplification of the microbiological assay. The technology is neither complex nor time consuming. In 8 or 9 h, one technician can prepare a sufficient quantity of inoculum to last a busy laboratory for at least six months. Most important is the finding that the standard curves made from a single batch of cryoprotected inoculum are superimposable over an eight-month period and that serum assayed with these preparations fall into the previously established diagnostic categories. The methodology used for the actual microbiological assay is basically a modification of the aseptic addition method (7, 8).
There is a basic difference between the cryoprotected inoculum and that used in the classic assay, which may account for their different growth characteristics. The former is folate-depleted, while the latter is folate-rich because it is carried in folate-replete maintenance medium to prevent the outgrowth of folate-independent mutants. The folate-rich inoculum must be diluted to prevent "carry over" of folates, which are taken up avidly by L. casei and may account for the growth variation in individual strains (9, 10).

Initially we thought that a more rapid folate-dependent growth would be obtained when using a heavy inoculum of cryoprotected folate-depleted microorganism, but we found that dilution of the cryoprotected inoculum results in more rapid growth, which approaches the growth rate of the classical non-cryoprotected L. casei (Figure 2). The reason for this unexpected observation is still unclear, but it could result from over-aeration and (or) the production of toxic substances (e.g., peroxides) formed during the preparation of the folate-depleted inoculum that are toxic to anaerobic organisms, or L. casei may contain an extremely low activity of a folate splitting enzyme (11), which may inhibit growth when a heavy inoculum is used.

Supported by NIH Grant AM-16690-06 and the Chemotherapy Foundation of New York. We thank Dr. Edward Bottone (Department of Microbiology) for determining the viability and purity of the cryoprotected L. casei preparations.

References