Bacteria as Specific Binding Agents for an Isotopic Assay of Serum Folate (34830)

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The current technique for the determination of the levels of serum or tissue folate depends upon the ability of the test material to support the growth of a given microorganism. The microbiologic assay requires the determination of the replication or growth rate of the test organism, and the methods are tedious, time-consuming, and not easily adapted to a routine laboratory.

This communication details a "radiomicrobiologic" assay for serum folate which utilizes the technique of saturation analysis, wherein the addition of a known quantity of a labeled compound competitively inhibits the binding of its unlabeled counterpart. In this assay, bacteria (*Lactobacillus casei*) are employed as specific binders for folate just as antibodies have been used to assay polypeptide hormones (1) or as naturally occurring proteins have been used to measure thyroxine, steroids and B_{12} (2, 3).

Pure cultures of *L. casei* (ATCC No. 7469) were transferred to maintenance media (Difco Laboratories) and incubated overnight at 37°. After washing three times in pteroylglutamic acid (PGA) media (BBL), the packed-cell volume of the organisms (microhematocrit method) was adjusted to 35%, then diluted 1 to 20 in PGA media and placed in an ice bath for subsequent use.

Ascorbic acid (500 mg/100 ml) was added to the human sera collected for study and stored at −20°. Attempts to assay whole sera by this technique were unsuccessful; therefore, serum extracts were prepared by autoclaving 4 ml of patient's serum with 12 ml of phosphate buffer, 0.05 *M*, pH 6.2, containing 200 mg/100 ml ascorbic acid. After autoclaving for 15 min at 15 psi, the samples were cooled and centrifuged. Duplicate 4-ml aliquots from each extract were assayed as follows: 0.2 ml PGA media (×10 normal concentration), 0.1 ml PGA media, and 0.1 ml PGA media containing 0.5 ng tritiated folic acid (specific activity 30–40 Ci/mmole) were combined in a polypropylene tube and chilled in an ice bath. Then 0.1 ml of the previously prepared *L. casei* was added. The tubes were incubated 3 hr at 37° and placed in a refrigerated centrifuge (0°) for 10 min at 8,000–10,000 rpm. One milliliter of the supernatant fluid was removed and counted in 10 ml of a standard PPO–POPOP–toluene mixture containing 13% "Bio-Solv" (formula BBS-3, Beckman Instruments). Total counts were obtained by counting 1 ml of the 4.5 ml mixture from which only the bacteria had been deleted. Quench correction was done by internal standardization. Since the same aliquots and total volumes were used throughout, the fraction of the supernatant fluid counts represented the percentage of free folic acid, although the reciprocal of these values, the percentage bound to the bacteria, was plotted as shown in Fig. 1.

In preparing suitable standard curves, selection of a substitute for serum extract posed a unique problem. Proportional dilution studies clearly indicated that phosphate buffer and/or PGA media mixtures altered the binding kinetics when compared to serum extracts. Ultimately, this problem was overcome by substituting a pool of serum extract previously prepared in bulk and stored at −20°. This was prepared by adding one part of pooled human serum to three parts of phosphate buffer. In order to completely oxidize the endogenous folate in this serum, the

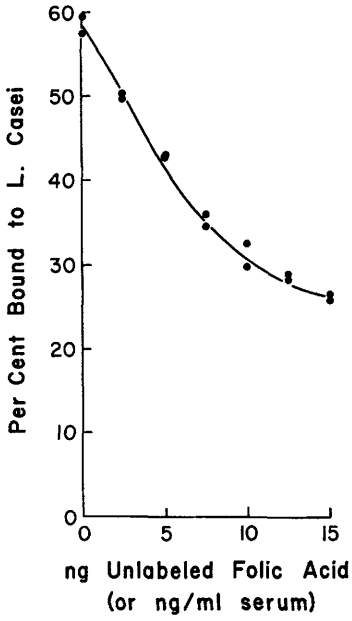

FIG. 1. A representative standard curve. The *L. casei* were incubated with 0.5 ng of tritiated folic acid and 0–15 ng of unlabeled folic acid for 3 hr. Since 4 ml of extract of a 1:4 dilution of serum was assayed, the unknown concentration (in ng/ml serum) can be read directly from the curve.

ascorbate was deleted from the buffer, and the mixture was adjusted to a pH of 2.0 with 6 *N* HCl and autoclaved for 30 min (4). After adjusting again to 6.4, the pH of the sample mixture, with 6 *N* NaOH, the ascorbate was then added (11 mg/ml) and reautoclaved for 15 min. The mixture was cooled and centrifuged, and the supernatant fluid was frozen in aliquots for subsequent use.

Serum samples were assayed by this "radiomicrobiologic" technique and compared with the classic microbiologic method which uses growth rates of the same test organism. These microbiological assays were performed at two separate independent laboratories (Bio-Science, Van Nuys, California and Bio-Assay, Dallas, Texas). The comparison of the two methods is recorded in Fig. 2. Good correlation of the two methods can be seen especially in the range consistent with significant folic acid depletion (3 ng/ml, or less). Of interest are the two samples denoted by circles in Fig. 2. Neither of these would have been considered folic acid-deficient by either method and, indeed, clinical and laboratory investigation corroborated this impression. Both of these samples came from patients who were extremely ill and on several drugs. The relationship of these agents to binding is now under further study. Proportional dilution studies were performed by diluting serum with water prior to extraction and assay. The concentration was proportional to the volume, suggesting the absence of interfering substances in normal serum. Furthermore, since the line intersected the 0 point, it can be assumed that all of the folate was removed from the "standard" serum.

The recovery of exogenous folic acid (pteroylglutamic acid) added to serum is demonstrated in Fig. 3. Excellent recovery of the added folate is achieved.

The present study demonstrates that the competitive binding of folic acid to *L. casei,* during the lag phase of growth of the organism, permits a radiomicrobiologic determination of the amount of endogenous folic acid present in serum. The radiomicrobiologic method in our preliminary studies appears to correlate well with the more tedious and time-consuming, classical microbiologic growth-rate assay, especially in the clinically criti-

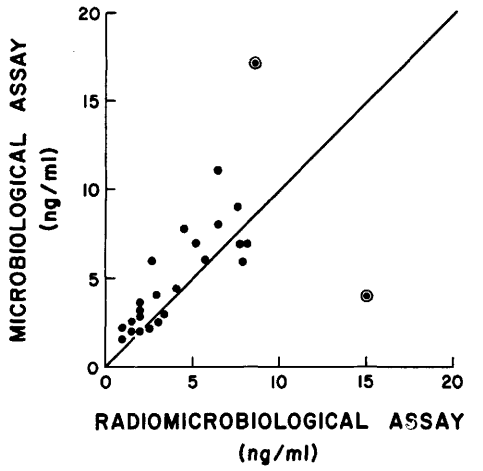

FIG. 2. Comparison of the classical microbiological assay for folate and the radiomicrobiologic method. The normal range for the microbiological assay is 5–20 ng/ml, while values from 3–5 ng/ml are considered of equivocal significance. (The diagonal line represents a theoretical perfect correlation between the two methods.)

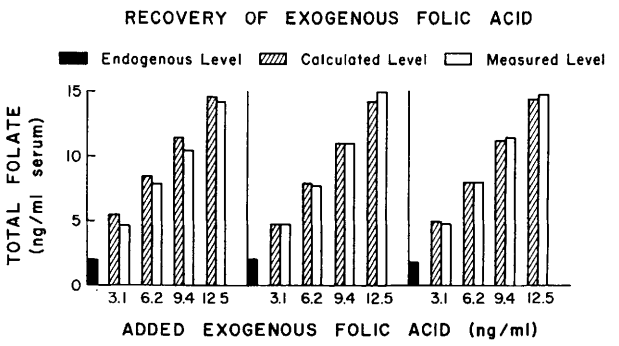

FIG. 3. The recovery of exogenous folic acid (pteroylglutamic acid) added to serum. Three separate specimens were assayed before and after the addition of amounts of folic acid as noted on the abscissa.

cal range of folic acid depletion. Furthermore, the evidence of proportional dilution suggests that interfering substances do not occur in normal serum. Extended studies are currently in progress to further evaluate the biologic characteristics of this reaction.

Summary. Saturation analysis or competitive inhibition utilizing bacteria (*L. casei*) as the specific binder permitted the measurement of serum folate. Unlike conventional microbiologic techniques, organism growth rate was not determined. This new "radiomicrobiologic" approach quantitated serum folate by competitive binding of tritiated folic acid during the early "lag" phase of bacterial growth.

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