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## Serum Folic Acid Activity by Improved *L. casei* Assay.*† (25900)

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Serum folic acid activity by microbiological assay with *Lactobacillus casei* has recently been proposed as a diagnostic test for folic acid deficiency(1,2,3). Levels less than 7.5 mμg/ml were reported indicative of the deficiency condition. Although folic acid activity of whole blood, plasma and serum had been extensively investigated by a variety of microbiological assays it had not previously been recommended as a means of assessing folic acid nutrition because activity levels in normals often were zero(4-14) and because the folic acid active compounds normally present in blood were presumably not folic acid or folinic acid(15). Nevertheless, because clinical folic acid deficiency is probably more common than generally appreciated and because laboratory means of assessing it are few(16), we undertook to evaluate the test proposed by Herbert *et al.*(1,3) and Baker *et al.*(2). In so doing, we found the assay they proposed lacked sensitivity at the lower levels which were of critical clinical importance. We wish to describe herein several improvements of the *L. casei* assay which greatly increase its sensitivity and accuracy and our experience with it in normal and folic acid deficient subjects. We found that levels in folic acid deficient subjects overlapped those of normals to such an extent that the assay had no clinical usefulness as a diagnostic test.

*Materials and methods. Maintenance of stock culture. Lactobacillus casei* ATCC 7469 was used in these studies, grown on the liver-tryptone agar of Nymon and Gortner(17) and maintained according to the scheme shown in Fig. 1. Three agar stabs, designated as monthly stabs, were prepared from the original culture. One monthly stab was used to prepare 4 weekly stabs, and the 2 remaining monthly stabs refrigerated until used. Each week 5 daily stabs were prepared from a weekly stab and the latter discarded. The remaining weekly stabs were refrigerated until used. A liquid broth inoculum was prepared each day from a daily stab, and the latter discarded. At end of month an unused monthly was employed to make 3 new monthly stabs and the scheme repeated. Spares were kept several months to be used in case of contamination in subcultures. *Inoculum preparation.* For liquid broth inoculum, 10 ml of skim milk medium previously described was used(18). A transfer from the daily agar stab was allowed to incubate at 37°C overnight (16-18 hours). 0.2 ml of uniformly suspended broth culture was transferred to a tube containing 6 ml of single strength assay medium to which 200 μμg folic acid had been added. This was allowed to incubate at 37°C for about 6 hours until O.D. of 0.1 to 0.12 was obtained in Coleman Jr. spectrophotometer at 660 mμ. The culture

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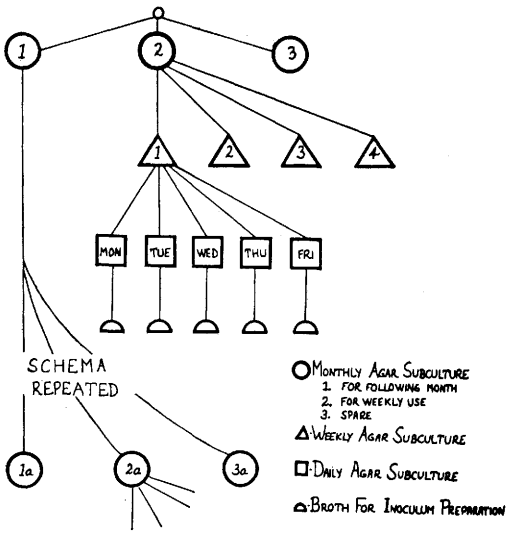


FIG. 1. Scheme for maintenance of *L. casei* on agar to insure culture stability.

was then centrifuged at 3000 rpm for 6 minutes, the supernatant discarded, and the bacteria resuspended in 6 ml sterile saline. A suitable aliquot was then diluted 10-fold with sterile saline. One drop of the latter was used to inoculate each assay tube. *Preparation of samples.* Venous blood was obtained from subjects in preprandial state and allowed to clot. Serum separated, was stored at –20°C until used. Serum was diluted 1:100 with phosphate-ascorbic acid buffer of Toennies *et al.*(19), incubated at 37°C for 90 minutes and autoclaved for 2½ minutes at 15 lb. pressure to precipitate protein. The clear supernatant when added to the assay tubes in amounts of 0.2, 0.5 and 0.8 ml provided dilutions of original serum of 1:500, 1:200 and 1:125 respectively. This compared with 1:20, 1:10 and 1:5 dilutions of original serum added to assay tubes by Baker *et al.*(2). *Assay medium and procedure.* The modified medium of Toennies *et al.*(19,20) was used. Assay was carried out in a total of 2 ml of liquid. This was found to increase its sensitivity. 0.2, 0.5, and 0.8 ml of serum sample prepared as above and sufficient distilled water to make 1 ml were added to duplicate 13 x 100 mm test tubes containing 1 ml of medium. For standard curve, folic acid was used at levels of 30, 50, 70, 100, 150, 200, 250, 300, 400 and 500 $\mu\mu$g per tube. Tubes were covered with aluminum caps, autoclaved at 15 lb. pressure for 2½ minutes, inoculated after cooling and allowed to incubate at 37°C for 16 hours. Turbidity was then read in a Coleman Jr. spectrophotometer at 660 m$\mu$. The standard curve was plotted on semilogarithmic paper. *Clinical material.* Subjects classified as normal included laboratory personnel and clinic patients who showed no signs of malnutrition and had normal hemograms. Subjects classified as folic acid deficient had macrocytic anemia, various degrees of megaloblastic erythroid and myeloid changes in their bone marrow, and made a classical reticulocyte, erythrocyte and hemoglobin response to less than 0.8 mg of folic acid per day(21,22). All folic acid deficient patients excreted significantly increased amounts of urinary formiminoglutamic acid after a 72 hour 15 g L-histidine monohydrochloride metabolic load (23).

*Results.* Comparison of standard curves obtained using the method described here with that of Toennies *et al.*(19,20) and that of Baker *et al.*(2) are shown in Fig. 2. The greatest sensitivity was obtained with our method. The straight line portion of the curve extends from 30 to 500 $\mu\mu$g per tube. The assay method of Baker *et al.*(2) is least sensitive because the usable portion of the curve extends from 300 to 3000 $\mu\mu$g per tube.

*Serum levels.* Serum folic acid activity determined by our method in normal adults,

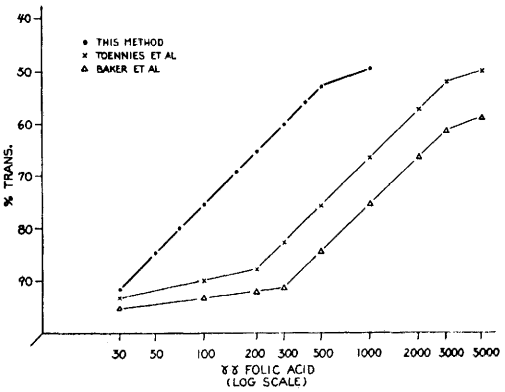


FIG. 2. Comparison of standard curves obtained by this method with that of Toennies *et al.* and Baker *et al.* Folic acid concentration expressed in $\mu\mu$g/assay tube; vol/tube our method, 2 ml; Toennies *et al.*, 6 ml; Baker *et al.*, 10 ml.

TABLE I. Serum Folic Acid Activity in Normal and Folic Acid Deficient Subjects Determined by Improved *L. casei* Assay.

| | No. of subjects | mμg/ml Mean and S.D.* | Range |
|---|---|---|---|
| Adults: | | | |
| Normal | 38 | 15.9 ± 9.5 | 4.0- 45.6 |
| Folic acid deficient | 30 | 9.9 ± 6.4 | 2.4- 31.5 |
| Pregnant women: | | | |
| Normal | 10 | 17.3 ± 6.5 | 9.5- 28.3 |
| Folic acid deficient | 10 | 10.2 ± 5.4 | 3.8- 22.8 |
| Normal infants and children (5 wk to 3 yr) | 40 | 27.5 ±33.8 | 2.7-201.2 |

* S.D. = stand. dev.

normal pregnant women in the last trimester of pregnancy, and normal infants from 5 weeks to 3 years of age is shown in Table I. Range of values found in each group was wide, for example, varying from 4.0 to 45.6 mμg/ml in normal adults. Mean level for normal pregnant women in the last trimester did not differ significantly from normal adults. The greatest variation occurred in normal infants, 5 weeks to 3 years of age, in whom levels from 2.7 to 201.2 mμg/ml were found. The mean level in this age group tended to be higher than in the other 2 groups.

In folic acid deficient adults and pregnant women mean levels of 9.9 and 10.2 mμg/ml respectively were moderately lower than mean value in corresponding normal subjects. However, the variation in serum folic acid activity in these deficient individuals was as great as in normals, 2.4 to 31.5 mμg/ml and 3.8 to 22.8 mμg/ml respectively.

*Discussion.* The improved assay method for serum folic acid activity described here has several advantages over previously described procedures. Maintenance of the culture in the prescribed manner on agar stabilizes growth characteristics of the organism so that consistently good standard curves are obtained. When the stock culture is maintained through broth transfer as recommended by Baker *et al.*(2), the organism eventually loses its folic acid requirement and considerable growth occurs in the growth control tubes containing no folic acid. The further modification of reducing the assay culture volume from 10(2) to 2 ml, permits extension of sensitivity from 300 μμg(2) to 30 μμg/tube. This makes possible more accurate assessment of serum folic acid activity in the low ranges. It is here that precise measurement is important to evaluate the possible clinical significance of this serum determination. The 10-fold increase in sensitivity also allows performance of an accurate assay on 0.1 ml of serum. This micromethod modification is of practical clinical advantage when larger amounts of serum are not obtainable.

Drs. Usdin and Toennies assayed serum of laboratory personnel at the Inst. of Cancer Research, Philadelphia, Pa., for folic acid activity with their *L. casei* method and have kindly allowed us to quote their unpublished results. These workers found serum folic activity to range from 2.65 to 10.6 mμg/ml with a mean of 5.49 and a standard deviation of ± 2.42 mμg/ml. These results are in agreement with our experience.

All the above results are contrary to those reported by Herbert *et al.*(1,3) and Baker *et al.*(2) in which a serum folic acid activity level below 7.5 mμg/ml by *L. casei* was *consistently* associated with clinical folic acid deficiency. Furthermore, the *wide overlap* of serum folic acid activity among normal subjects and folic acid deficient patients found in our studies makes this determination of little value as an index of folic acid deficiency.

Baker *et al.*(10,12,14) using a thermophile, *Bacillus coagulans,* assay, which presumably measures the same compounds as *L. casei*(2), found no correlation between serum folic acid activity and anemia in pregnant women. More recently, Baker *et al.*(24) reported serum folic acid activity by *L. casei* assay of *normal* pregnant women to range from 1 to 100 mμg/ml. Similarly the cord serum "activity" of *normal* newborns varied from 2.5 to 250 mμg/ml. These findings, more in conformity with our own results in normal adults, young infants and children, also cast doubt on the usefulness of this assay as a criterion of folic acid deficiency.

*Summary.* 1) An improved *L. casei* assay procedure for serum folic acid activity is described. Its range of usefulness over previ-

ous procedures is increased because of greater sensitivity, better accuracy in lower ranges, stability of test organism and micromethod applicability. 2) With this improved assay, the overlap of serum folic acid activity levels among normal subjects and folic acid deficient patients is so great that this determination has no value as an index of folic acid deficiency.

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## Effects of DDD on Steroid Response and Blood Flow Through the Adrenal After ACTH.* (25901)

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The effects of ACTH in increasing corticosteroid secretion of the adrenal cortex and blood flow through the gland have been reported. That these 2 responses are not directly related does not seem to be commonly known. This paper records the dissociation of these phenomena and sets forth some quantitative measurements on vascular reaction. Administration of DDD (2,2-bis(parachlorophenyl)-1,1-dichloroethane) causes a decreased secretion of 17-hydroxycorticosteroids and the adrenal becomes refractory to stimulus from ACTH. However, in doses sufficient to prevent steroid formation, DDD does not affect vascular responses of the gland. These findings are considered important because some isomers of DDD are being used currently in human therapy(1).

*Method.* Data were derived from 185 mongrel dogs of both sexes. The preparation of DDD used was purchased commercially, and reportedly contains approximately 90% of the p,p'-isomer and 5-7% of the o,p'-isomer(2).

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