Microbiologic Determination of Folic Acid Derivatives in Blood

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Folic acid (F.A.) deficiency in man may be due to a variety of factors. The most important ones are considered to be unusually poor diets, faulty absorption of the vitamin from the intestinal tract, derangement in the intestinal flora resulting in low synthesis of the vitamin and faulty metabolism of the vitamin in the body. Human blood contains several members of the F.A. family of compounds. Therefore, in order to obtain valid information about the F.A. activity in human blood or serum it seems desirable to measure as many forms of the vitamin as possible. Such approach may facilitate detection of the biochemical lesions responsible for, or connected with, the deficiency.

In the present paper, various methods modified or developed in our laboratory to achieve this end are described. Most of them have been in use in our laboratory for several years.

Materials and Methods

Assay Organisms

a) Lactobacillus casei ATCC No. 7469 is used to measure "total folic acid" (total F.A.) activity, as in addition to pteroylglutamic acid (P.G.A.) and folic acid it utilizes certain polyglutamates of P.G.A. as well. This designation is given despite the further increase in the activity for L. casei after incubation of human blood with chicken pancreas conjugase; this increase is presumably due to deconjugation of hepta- and other polyglutamates. The L. casei culture is maintained in yeast-peptone-agar stabs (see table 4 for composition; it will be referred to as stock-agar), transferred bimonthly, and stored at 4 °C. between transfers. The other two assay organisms (see below) are grown in the same medium and are similarly maintained.

b) Streptococcus faecalis No. 9. A strain isolated in this laboratory, is used for determination of "free F.A.," including several tetrahydrofolic acid derivatives. This strain is considerably more sensitive than the strain ATCC No. 8043 usually employed for determination of F.A. (see below, fig. 1).

c) Pediococcus cerevisiae (Leuconostoc citrovorum) ATCC No. 8081 is used for the determination of folic acid and other tetrahydro-derivatives of folic acid. Calcium leucomorin (Lederle) is employed as the standard. As the synthetic racemic product possesses about 50 per cent of the activity of the natural citrovorum factor, the assay figures are halved in order to arrive at correct values.

Assay Medium

All three assays are performed with the medium of Toennies et al. (See table 3 for composition of assay medium.) However, for the determination of folic acid (with P. cere-
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visiae), thymidine (1 μg./ml.) is added, as it enhances the growth in the presence of folinic acid.9,10 The medium is prepared in double strength and 2.5 ml. aliquots are dispensed into matched 18 x 150 mm. test tubes. After addition of the material to be assayed, the volume is made up to 5.0 ml. with distilled water and tubes plugged with cotton (or covered with aluminum caps) and autoclaved for 10 minutes at 15 psi. Twice distilled water or singly distilled and subsequently deionized water is used.

Standard Folic and Folinic Acid Solutions

Concentrated solutions (100 μg./ml.) of P.G.A. and calcium leucovorin are prepared by weighing out 10 mg. of these compounds, dissolving them with a few drops of 0.05N NaOH (to bring to pH 7.0) and filling the volume to 100 ml. with doubly distilled water; these solutions are stable for one month if kept frozen. Standard solutions are made up for each assay from the concentrated solutions and added to make final concentrations of 10, 20, 40, 60, 80, 120, 160 and 200 μg./ml. of either P.G.A. or folinic acid. Standard assay curves are run in triplicate. Extracts are assayed for their F.A. or folinic acid activity at three different dilutions, employing duplicate tubes per dilution. Results of the triplicates or duplicates are averaged; differences between duplicates are usually small.

P.G.A., folinic acid, pteroic acid and teropterin (sodium pteroyl glutamate) were obtained from Lederle Laboratories, Pearl River, N. Y. We thank Dr. L. Ellenbogen for providing us with these compounds.

Inoculum Preparation

A loopful from a 24-hour stock-agar-culture is transferred to 5 ml. of assay medium supplemented with 150 μg./ml. P.G.A. or folinic acid and incubated overnight at 37 C. Cultures of L. casei and S. faecalis are diluted 1:100 and those of P. cerevisiae 1:10. One drop of the diluted cultures is used to inoculate each tube.

Incubation

Assay tubes are incubated for 24 or 48 hours at 37 C., according to the organism used; the S. faecalis assay is read after 24 hours incubation, while the other two assays are read after 48 hours. Growth density is measured at 650 μ in a Coleman junior spectrophotometer or at 660 μ in a Klett-Summerson colorimeter with an adapter to hold 18-mm. wide tubes.

Release of Folic Acid Derivatives

a) Whole blood. In order to release the various F.A. derivatives and render them active towards the assay organisms, the procedure of Toennies et al.5,11 is modified as follows: 1 ml. of citrated blood is hemolyzed by diluting it with 17 volumes of distilled water. Ascorbic acid (30 mg., freshly prepared) and 2 ml. of 0.5 M phosphate buffer (pH 6.0) are added, the pH adjusted to 6.0 with 0.25 N NaOH and the volume made up to 20 ml. The hemolyzed samples are incubated for 2 hours at 37 C. and then autoclaved (20 minutes at 15 psi) to stabilize the labile forms of the vitamin11 and conjugate the serum proteins. Aliquots of the clear supernatant (obtained by centrifugation) are added to the double strength medium and assayed for their F.A. activity (F.A.A.). Samples thus treated will be referred to as activated hemolysates.

b) Serum. Samples are diluted 1:10 with 0.1 M phosphate buffer pH 6.0, and ascorbic acid added (10 mg./ml. of serum). The samples are autoclaved for 20 minutes at 15 psi (without any preincubation at 37 C.) and the supernatant is assayed for its F.A.A. Incubation prior to heating the serum sample in presence of ascorbate did not further increase activity and was, therefore, omitted. Similar results were obtained by Herbert.6

RESULTS

Comparative Growth Response to P.G.A. and Folinic Acid of S. faecalis ATCC No. 8043 and S. faecalis No. 9

Figure 1 shows the markedly higher sensitivity of the No. 9 strain of S. faecalis as compared to that of strain No. 8043. Whereas 200–250 μg./ml.
Calcium leucovorin, on a weight basis, is about 50 per cent as active as P.G.A., since it is a synthetic racemic form of folinic acid. The difference in sensitivity between the two strains is also maintained in respect to folinic acid (fig. 1).

_Teropentin and Pteronic Acid_

As with P.G.A. and folinic acid, the assay of pteroic acid and teropentin is more sensitive with the No. 9 strain; both strains show the same specificity towards the latter compounds.

_Effect of Thymidine on Growth of P. cerevisiae in Presence of Folinic Acid_

This is shown in figure 2. At low concentrations of folinic acid (50 μg./ml.), addition of thymidine causes a four-fold increase in growth, while at higher concentrations the stimulation is two-fold. The effective concentrations of thymidine are between 0.1 and 5 μg./ml. However, thymidine is ineffective in the absence of folinic acid even if added in concentrations as high as 50 μg./ml.

_Effect of Ascorbic Acid on F.A. Activity of Whole Blood and Activated Blood Hemolysates_

As some of the F.A. compounds—especially the tetrahydro derivatives—are very unstable in air, the effect of ascorbic acid was examined on the microbiological activity of blood during storage at 4 C. and 30 C., respectively. Two types of treatment were studied: 1) whole blood was hemolyzed (see Methods) and added to aliquots of ascorbic acid in increasing concentrations (from 7.5-50 mg. per ml. of blood). The samples were incubated for 2 hours.
at 37 C. and then autoclaved. The supernatants from the respective samples were divided into two parts: one (a) was tested immediately for F.A.A., while the other (b) was incubated for 4 days at 30 C. prior to the assay. 2) Samples of undiluted, citrated blood were incubated at 30 C. for 4 days with no addition of ascorbic acid. The samples were hemolyzed, a small amount of ascorbate added (7.5 mg. per ml. of blood), and activated by incubation for 2 hours at 37 C.; they were then autoclaved and the extracts obtained assayed immediately for F.A.A. The results, which are summarized in table 1, show almost no loss of F.A.A. of untreated blood (undiluted, citrated) on incubation at 30 C. for 4 days in the absence of added ascorbic acid. This holds particularly true for assays done with \textit{L. casei}, while a slight drop in activity is noticed when it is determined with \textit{S. faecalis} or \textit{P. cerevisiae}. On the other hand, a loss of as much as 65 per cent of the F.A.A. may occur in activated hemolysates.

Table 1.—Effect of Ascorbic Acid on Folic Acid Activity of Blood and Blood Hemolysates (mg./ml.) Stored at 4 C. and at 30 C.

<table>
<thead>
<tr>
<th>Ascorbic acid (mg./ml. of blood)</th>
<th>Unincubated Blood hemolysates (series a)</th>
<th>Incubated at 30 C. for 4 Days Blood hemolysates (series b)</th>
<th>Blood (series c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assayed Blood sample No. 1</td>
<td>7.5 20 33 50</td>
<td>7.5 20 33 50</td>
<td>7.5</td>
</tr>
<tr>
<td>with \textit{L. casei}</td>
<td>2 24 38 52</td>
<td>30.4 31.7 34.1 32.9</td>
<td>31</td>
</tr>
<tr>
<td>Assayed Blood sample No. 1</td>
<td>6.6 8 9.9 10.3</td>
<td>5.1 8.4 7.2</td>
<td>9.0</td>
</tr>
<tr>
<td>with \textit{S. faecalis}</td>
<td>2 2 2.1 2.6</td>
<td>1.4 2.1 1.9 2.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Assayed Blood sample No. 1</td>
<td>5.4 7.4 7.6 6.4</td>
<td>4.2 7.0 7.2 7.3</td>
<td>7.3</td>
</tr>
<tr>
<td>with \textit{P. cerevisiae}</td>
<td>2 1.7 1.5 2.0</td>
<td>1.0 1.1 1.5 1.7</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Whole blood was hemolyzed (see Methods) and to aliquots, ascorbic acid, in increasing amounts (as indicated in the table), was added. After incubation for 2 hours at 37 C., the samples were autoclaved. The supernatants of each sample were divided into two parts: one was tested immediately for F.A.A. (series a) while the other was incubated for 4 days at 30 C. prior to the assay (series b).

Whole blood (citrated, undiluted) was incubated at 30 C. for 4 days in absence of ascorbic acid. Thereafter, hemolysates were prepared, activated with ascorbate (7.5 mg./ml. of blood), autoclaved, and assayed for F.A.A. activity (series c).

The assays were carried out with three different organisms. Samples No. 1, 2 and 3 represent blood from different donors.
incubated at 30 C. for 4 days in the presence of low ascorbate (7.5 mg. per ml. of blood); addition of high concentrations of ascorbic acid (30 mg. or more per ml. of blood) affords 75 to 100 per cent protection of the activity.

**Folic Acid Activity in Healthy Subjects**

Table 2 presents results obtained with the procedure as outlined under Methods. In healthy subjects, more than 90 per cent of all F.A.A. is found in the blood, while serum contains only 5-10 per cent. Much higher activity is found in assays carried out with *L. casei* than in those employing either *S. faecalis* or *P. cerevisiae*. The F.A.A. in blood of healthy infants is about the same as in normal adults. The respective *L. casei* blood F.A.A. for infants and adults ranges between 35 and 160 μg./ml. (mean 96.4 μg./ml.) and 47-149 μg./ml. (mean 89.0).

**DISCUSSION**

The *S. faecalis* No. 9 strain used to assay folic acid (F.A.) derivatives is considerably more sensitive than the standard strain ATCC No. 8043 as shown by the response to P.G.A. and folinic acid (fig. 1), yet it exhibits the same specificity as the standard strain towards the substances examined. The *P. cerevisiae* (*L. citrovorum*) assay has also been improved; addition of thymidine results in increased sensitivity and specificity of the assays. Thymidine, however, should not be added to *L. casei* and *S. faecalis* assays as it replaces the F.A. requirement in these organisms. Therefore, presence in the samples to be assayed of microgram quantities of thymine or thymidine would interfere with *S. faecalis* and *L. casei* assays.

Preincubation of blood hemolysates with ascorbic acid and phosphate buffer at pH 6.0 greatly enhances the folic acid activity (F.A.A.) of the blood. The protective effect of ascorbic acid on F.A.A. in serum has recently been shown by Herbert, confirming previous findings of Baker et al. The experiments summarized in table 1 show that ascorbic acid protects the F.A.A. both during the short heating (autoclaving) of the samples and during the prolonged storage at 30 C. The amount of ascorbic acid added to the samples (a total of 30 mg. per ml. of blood and 10 mg. per ml. of serum) is ample in view of the storage experiments presented and is also in agreement with recent findings. Undiluted, citrated blood shows little, if any, loss of F.A.A. on storage at 30 C. in the absence of added ascorbate (when assayed with *L. casei*). Apparently the intact membrane of the red cell is responsible for the protection. Since the assay medium contains both phosphate and ascorbic
Table 3.—Composition of Double Strength Assay Medium*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% casein hydrolysate enzymatic (Nutritional Biochemicals Corp. Cleveland, Ohio)</td>
<td>125 ml.</td>
<td></td>
</tr>
<tr>
<td>Sodium acetate, anhydrous</td>
<td>20.0 Gm.</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>20.0 Gm.</td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>3.5 Gm.</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>3.5 Gm.</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>250 mg.</td>
<td>prepare a 2% solution; add 10 ml.</td>
</tr>
<tr>
<td>DL-alanine</td>
<td>200 mg.</td>
<td>prepare a 1% solution; add 10 ml.</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>100 mg.</td>
<td>prepare a 2% solution in 2N HCl; add 10 ml.</td>
</tr>
<tr>
<td>L-cystine</td>
<td>200 mg.</td>
<td>prepare a 1% solution; add 10 ml.</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>200 mg.</td>
<td>weigh out 2.0 Gm. dissolve in 100 ml.</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 mg.</td>
<td>weigh out 100 mg. of each ml of 1N HCl; add 10 ml.</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>10 mg.</td>
<td>weigh out 100 mg. of each ml of 1N HCl; add 10 ml.</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>10 mg.</td>
<td></td>
</tr>
<tr>
<td>Adenine sulfate</td>
<td>17 mg.</td>
<td>prepare a 0.17% solution; add 10 ml.</td>
</tr>
<tr>
<td>Guanine hydrochloride</td>
<td>12.5 mg.</td>
<td>prepare a 0.125% solution; add 10 ml.</td>
</tr>
<tr>
<td>Uracil</td>
<td>10 mg.</td>
<td>prepare a 0.1% solution; add 10 ml.</td>
</tr>
<tr>
<td>Xanthine</td>
<td>10 mg.</td>
<td>prepare a 0.1% solution; add 10 ml.</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5 mg.</td>
<td></td>
</tr>
<tr>
<td>Ca-pantothenate</td>
<td>0.5 mg.</td>
<td>weigh out 50 mg. of each ml of water; dissolve in 1000</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>0.5 mg.</td>
<td></td>
</tr>
<tr>
<td>Pyridoxamine hydrochloride</td>
<td>0.2 mg.</td>
<td>weigh out 20 mg. add 10 ml.</td>
</tr>
<tr>
<td>P-Aminobenzoic acid</td>
<td>0.1 mg.</td>
<td>weigh out 10 mg.</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.5 mg.</td>
<td>weigh out 10 mg. dissolve in 200 ml. water with 0.6 ml acetic acid; add 10 ml.</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.01 mg.</td>
<td>weigh out 10 mg. dissolve in 1000 ml.; add 1 ml.</td>
</tr>
</tbody>
</table>

The stock solutions are kept at 4 C. under toluene.
The pH of the medium is adjusted to 6.45 with 10 N NaOH;
The volume is made up to 500 ml.

*For assay with P. cerevisiae: add thymidine 1 µg./ml. final concentration. For assay with S. faecalis: add Tween 80 (Atlas Powder Co., Wilmington, Del.) 0.1 per cent (V/V) final concentration; Tween enhances growth of this organism in presence of folic acid.

acid, a nonspecific growth stimulation of the assay organism as shown to occur with L. casei is eliminated.

F.A.A. of whole blood of healthy individuals is about 10- to 20-fold higher than that of serum. The highest activity is found with L. casei and is assumed to be due to the ability of the organism to utilize polyglutamates of F.A. rather than to the presence in the blood of F.A. bypassing substances. Experiments not described in this paper have shown a two to three-fold increase in L. casei activity of blood hemolysates after incubation with chicken pancreas conjugase. However, it is plausible that L. casei also responds to as yet unidentified F.A. compounds not necessarily polyglutamates; methyltetrahydrofolic acid may be one of those compounds.

The ratio of F.A.A. in the blood of healthy individuals with the three assays used (P. cerevisiae, S. faecalis, L. casei) is about 1:2:15. Similar ratios of activity are found in sera of healthy individuals (1:25:25). It is, therefore, highly suggestive that both serum and blood cells contain the same assortment of F.A. active substances, the concentrations of which are much higher in the blood cells than in the serum. Thus, it seems advisable to determine F.A.A. in
blood rather than in serum. This view is strengthened by the more pronounced decrease of F.A.A. of whole blood than that of serum in patients suffering from megaloblastic anemia.\textsuperscript{19}

The simultaneous assay with the three organisms used allows one to distinguish between various forms of the F.A. derivatives in the body, and their relative abundancy. Thus, the activities of the tetrahydro-forms of F.A. (as measured with the \textit{P. cerevisiae} assay) are much higher in fetal than in maternal blood.\textsuperscript{20} The differential assay should, therefore, aid in discovering deficiency due to lack of specific F.A. derivatives.

SUMMARY

A microbiological method for the determination of folic acid activity (F.A.A.) in blood, and serum with three organisms (\textit{L. casei}, \textit{S. faecalis} and \textit{P. cerevisiae}), is described. The \textit{S. faecalis} No. 9 strain employed shows a considerably higher sensitivity than the standard ATCC No. 8043 strain, whilst the specificity towards the various F.A. derivatives tested is similar. The sensitivity of the \textit{P. cerevisiae} (\textit{L. citrovorum}) assay is also increased by adding thymidine, which spares the requirement for folinic acid. Finally, the procedure of extraction of the F.A.A. from blood has been modified to assure release and stabilization of the compounds assayed.

F.A.A. in blood and serum of healthy adults and infants are given.
REFERENCES


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