Clinical and Laboratory Observations on Serum Folate-binding Protein

By Edward R. Eichner, Charles J. Paine, Valerie L. Dickson, and Marion D. Hargrove, Jr.

We studied the effect of serum folate-binding protein (FBP) on folate radioassays and the relationship of the serum level of unsaturated FBP to the serum folate level in various clinical states. Our modification of a heat-extracted radioassay was compared to a whole serum radioassay and to the Lactobacillus casei assay. Our results confirmed the existence of elevated serum levels of unsaturated FBP in some normal subjects, in some women taking oral contraceptives, and in most patients with uremia. Elevated levels of unsaturated FBP will produce falsely low results in folate radioassay unless the FBP has been destroyed by heat, as was done in the modified radioassay here presented. In normal and uremic subjects, serum folate and unsaturated FBP levels tended to correlate, whereas in patients taking large doses of folic acid the level of unsaturated FBP fell as the level of serum folate rose.

The nature, specificity, and importance of serum folate-binding protein(s) are the subjects of increasing research activity. The advent of radioassays for serum folate has quickened interest in folate binders, especially in the endogenous serum folate-binding protein (FPB) that binds the tritiated folic acid used in the assays. Studies of serum FBP by Tisman and Herbert and by Waxman and colleagues have shown it to be a relatively large glycoprotein that retards, rather than enhances, delivery of folic acid to cells. It is similar to the intracellular and serum folate binder first reported and extensively studied by Rothenberg and colleagues in patients with chronic myelogenous leukemia and later in women pregnant or taking oral contraceptives. Elevated levels of serum FBP have also been reported in patients with uremia and with cirrhosis.

Waxman and Schreiber have shown that folate-deficient serum binds significantly greater amounts of exogenous tritiated folic acid than does normal serum. This inverse relationship between serum folate level and level of unsaturated FBP suggests that normally the serum folate binders are relatively saturated with endogenous folate but become unsaturated with folate deficiency.

The use of whole serum rather than FBP-free, heat-extracted serum for radioassay of folate has been questioned, although the possible adverse effect of FBP on the radioassay seems to vary among laboratories. Zettner and Duly, who found significant (> 1 ng/ml) levels of unsaturated FBP in about 25% of normal serums and in a larger percentage of serums from hospitalized patients,
argued that serum folate must be separated from its binder before competitive binding assays are used. They did not, however, actually compare any of the radioassays. The data in this report, comparing three assays for serum folate, show the advantage of a modified, heat-extracted radioassay in the presence of elevated serum levels of unsaturated FBP and suggest a direct, rather than inverse, relationship between serum folate level and unsaturated FBP level in a normal subject and in certain pathophysiologic states.

MATERIALS AND METHODS

The three assays for serum folate were the Lactobacillus casei microbiologic assay, the radioassay of Waxman and Schneiber, and the radioassay of Dunn and Foster. The L. casei assay method was essentially that of Herbert; our modifications are described elsewhere. The radioassay of Waxman and Schneiber, which introduced commercially available crystalline beta lactoglobulin, a minor contaminant of which acts as the triitated folic acid binder, was followed exactly. The per cent of unsaturated serum FBP (termed FABP in prior publications) in this assay was calculated by dividing the radioactivity in the serum binding control tube (after correction for radioactivity not adsorbable to charcoal in the absence of beta lactoglobulin) by the radioactivity in the corrected standard. All the measurements of per cent FBP to follow are of unsaturated FBP (UFBP), and 1% UFBP equates to the binding of 5 pg of exogenous triitated folic acid by 0.4 ml of serum.

The method of Dunn and Foster differs from that of Waxman and Schneiber mainly in that the folate binders in serum are destroyed by heating serum in alkaline lysine buffer in a boiling water-bath for 15 min to denature the proteins. We followed the method of Dunn and Foster with one major exception. In that assay, the source of protein in the N5 methyltetrahydrofolate (MTHF) standard curve was bovine albumin, which was added to the standard diluent buffer. In our hands, the slope of the standard curve obtained when albumin was used, when plotted via the method of Dunn and Foster, was consistently flatter than the slope of the curve obtained when MTHF was added to folate-free serum in recovery studies. This difference resulted in recoveries exceeding 100%. We modified the method of Dunn and Foster by preparing the standard diluent without albumin, and by adding 10% folate-free serum by volume to the standard curve tubes. To compensate for this additional volume, the same amount of albumin-free standard diluent was added to each test serum assay tube. This simple modification equaled the slopes of standard curve and serum tubes and yielded recoveries of MTHF averaging 100%. Folate-free serum was prepared by treating serum with 15 mg of neutral Norit A charcoal per ml as described by Rothenberg and colleagues.

To investigate the possibility of an exaggerated elevation of the serum folate level in the heat-extracted radioassay because of preferential binding to milk folate binder of oxidized folates which are potentially released from serum binding by heat extraction, we did L. casei assays on selected serums (from fasting normal subjects and hospitalized patients) before and after boiling as done in the heat-extracted radioassay. We also compared the performance in the heat-extracted radioassay of a standard curve containing folic acid, an oxidized folate, to that of the routine MTHF standard curve.

To investigate the possible saturation of serum FBP by the administration of folic acid, L. casei folate levels and unsaturated FBP levels were determined on serums obtained at 0 and 4 hr after the administration of 40 µg/kg folic acid orally to six fasting hospitalized patients as a test of intestinal absorption. The other serums assayed in this study were from the following sources: a.m. (fasting) and p.m. (before dinner) samples from a normal physician volunteer on a standard, normal diet for 11 days; a.m. fasting serums from 19 male and 19 female normal laboratory and hospital personnel; a.m. fasting serums from eight patients with proven, untreated pernicious anemia; midafternoon serums from 50 women taking oral contraceptives and 50 women of similar age and socioeconomic level not taking such agents; a.m. fasting serums from 27 nonuremic hospitalized patients; and a.m. fasting serums from 30 nondialyzed patients with renal disease and azotemia. All serums had 5 mg/ml ascorbic acid added and were stored at -20°C. Informed consent was obtained from all subjects investigated.
RESULTS

The comparison of the three assays for serum folate in 38 normal subjects is shown in Table 1. The overall correlation of each radioassay with the *L. casei* assay was almost identical (*r* = 0.88 for whole serum, *r* = 0.85 for heat-extracted), and the radioassays correlated well with each other (*r* = 0.76). Moreover, the mean values for the radioassays were almost identical at about 75% of the mean *L. casei* value. At low levels of unsaturated FBP (UFBP), the

<table>
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<th>L. casei Value (ng/ml)</th>
<th>Whole-serum Radio-assay Value (ng/ml)</th>
<th>Heat-extracted Radio-assay Value (ng/ml)</th>
<th>UFBP (%)</th>
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Mean 6.3 4.8 4.7
Per cent of L. casei 76% 75%
whole-serum radioassay value tended to be slightly greater than the heat-extracted value, probably because of slight loss of radioassayable folate in the heat extraction step. Thus, for the 26 serums with UFBC levels under 8%, the whole-serum value correlated slightly better with the L. casei value than did the heat-extracted value ($r = 0.96$ and $0.84$, respectively). As indicated in Table 1, high levels of UFBC were associated with false low whole-serum but not heat-extracted serum radioassay values.

The inverse relationship between level of UFBC and the whole-serum radioassay value could be demonstrated even with relatively low UFBC values. Figure 1A shows the comparison of a.m. (fasting) versus p.m. (before dinner) UFBC levels in a normal subject during 11 days on a standard, normal diet. On 10 of the 11 days, the p.m. value was greater than the a.m. value. These samples were coded and run in single assays on two occasions with similar results. Although all the UFBC values were within our normal range, and although our coefficient of variation for the repetitive measurement of UFBC in single samples of serum was 20%–25%, the mean p.m. value from this protocol was significantly greater than the mean a.m. value ($p < 0.01$). This rise during the day in UFBC level was associated with a significant rise during the day in serum L. casei folate level (Fig. 1B). Even this slight rise in UFBC was associated with lower whole-serum radioassay values relative to corresponding L. casei values. Figure 1C shows the p.m. values for each assay expressed as a percentage of the a.m. values. The mean p.m. rise in L. casei value was significantly greater than the mean p.m. rise in radioassay value, and there was a significant inverse correlation between the per cent rise in UFBC and the per
Fig. 2. The relationship between the level of unsaturated FBP (FABP) and the serum folate level by whole-serum radioassay expressed as a percentage of the L. casei level. Higher levels of unsaturated FBP are associated with a significant lowering of the radioassay value relative to the L. casei value in all three populations.

Figure 2 shows the same inverse relationship between the level of UFBP and the whole-serum radioassay value for normal subjects (Fig. 2A) compared to women taking oral contraceptives (Fig. 2B) and to nonuremic hospitalized patients with diverse diseases (Fig. 2C). There was no significant direct correlation between the level of UFBP and the level of L. casei serum folate in any of these three groups.

Figure 3 shows levels of UFBP in all 38 normals, in a pilot group of 31 women taking oral contraceptives, and in 30 patients with uremia. Elevated...
levels of UFBP were not common in the normal subjects. The high of 56% was in a normal man and remains unexplained. The other three high values in the normal group were in women taking oral contraceptives. Approximately one in three in the pilot group of 31 women taking oral contraceptives had elevated UFBP levels. We expanded this group to 50 women and compared them to 50 women not taking such agents. The mean UFBP level was greater in the women taking oral contraceptives (18.3% versus 15.2%), but the difference between the means was not significant ($p > 0.2$). The UFBP level in the 30 uremic patients was generally elevated, and the mean level shown in Fig. 3 is significantly greater than that in normals ($p < 0.01$).

Figure 4 includes data from 51 subjects (normals, women taking oral contraceptives, and some patients with uremia) chosen from the prior groups to represent a variety of UFBP levels. It shows that elevated levels of UFBP are associated with a significant lowering of the whole-serum radioassay value relative to the heat-extracted radioassay value.

The amount of *L. casei*-active folate released by the boiling of 25 serums is shown in Table 2. There was only a moderate correlation between the amount of folate released by boiling and the initial *L. casei* value ($r = 0.51$, $p < 0.01$). Some serums released appreciable amounts of folate upon boiling, although the mean rise was only 8.2% of the total level. This released folate, presumably mainly oxidized, would theoretically be magnified 1.6 times its actual level when assayed by the heat-extracted method, because comparison of the folic acid standard curve, as a prototype of an oxidized folate, to the routine MTHF standard curve in our heat-extracted assay showed that the folic acid bound 1.6 times more avidly to milk folate binder than did the MTHF. Serums with low UFBP which released relatively large amounts of folate upon boiling did not,
Table 2. Comparison of the Rise in Serum *L. casei* Folate Level After Heat Extraction to the Level of Serum Folate-binding Protein (UFBP)

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<tr>
<th>Initial <em>L. casei</em> Value (ng/ml)</th>
<th>Rise After Heat Extraction (ng/ml)*</th>
<th>Rise as Per Cent of Total Value</th>
<th>Serum UFBP (%)</th>
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*Three of the serums had a fall in value after boiling; these are indicated by a minus sign and were taken as zero in the calculations.

†Patient was taking folic acid pills.

‡Folate-free serum.

however, register significantly higher values by the heat-extracted as compared to the whole-serum assay. Further, the higher values by heat-extracted assay for serums with elevated UFBP could not be attributed to release of oxidized folate upon boiling, because there was a poor correlation between the level of UFBP and the amount of folate released upon boiling ($r = 0.39$).

Some of our data indicated a direct relationship between level of UFBP and level of serum folate. Thus, in the normal subject shown in Fig. 1, UFBP and *L. casei* folate rose in concert during the day. The UFBP levels of the 30 patients with uremia correlated with *L. casei* levels ($r = 0.45$, $p < 0.025$) and with heat-extracted radioassay levels ($r = 0.48$, $p < 0.01$); the direct correlation between UFBP levels and *L. casei* levels in the 100 women from the oral contraceptive study did not, however, reach significance. Figure 5A shows that there tended to be a relationship between high serum *L. casei* levels and high, rather than low, UFBP levels in eight patients with untreated pernicious anemia. However, when large oral doses of folic acid were given to six hospitalized patients, serum folate binders were fully saturated with endogenous folate, thus...
inhibiting binding of the exogenous tritiated folic acid and resulting in a strong inverse correlation between UFBP and \textit{L. casei} level in these patients (Fig. 5B).

**DISCUSSION**

The radioassays for serum folate\textsuperscript{1,8} have advanced the study of folate metabolism but have introduced problems of interpretation and standardization. A major problem is the presence in some serums of an endogenous folate-binding protein (FBP) which may interfere with the radioassay of serum folate.\textsuperscript{18,21} In this study we have compared a modified heat-extracted radioassay to the whole-serum radioassay of Waxman and Schreiber, using the \textit{L. casei} assay as a reference point. The data show that high levels of unsaturated FBP are associated with falsely low whole-serum radioassay values, but that the heat-extracted radioassay is apparently unaffected by high levels of unsaturated FBP such as reported before and confirmed here in some normal subjects, in some women taking oral contraceptives, and in most patients with uremia.\textsuperscript{14,15,21}

The mean unsaturated FBP level of 50 women taking oral contraceptives was greater than that of a similar group not taking such agents, but the difference between the means was not statistically significant. There is convincing evidence for a relationship between oral contraceptives and elevated leukocyte and serum FBP levels,\textsuperscript{14} and it seems likely that our data would support the same link if the study group were enlarged or if other variables could be better controlled. We cannot fully explain the reasons why the mean unsaturated FBP level for the 50 women not taking oral contraceptives was appreciably greater than the mean for the 38 normal subjects in our study, although the serums
from the former group were obtained in midafternoon, and this timing may have elevated unsaturated FBP levels as it did in the normal subject (Fig. 1). Also, half of the 38 normal subjects were men, and it is possible that expanded studies will show that men have lower FBP levels than do women.

There is an inverse relationship between serum folate level and unsaturated FBP level in folate-deficient patients\textsuperscript{14} and the suggestion of an inverse relationship between serum folate level and leukocyte folate binder in pregnant women.\textsuperscript{14} Our data from normal subjects in Table I do not support the proposition of an inverse relationship between serum \textit{L. casei} folate and level of unsaturated FBP, because none of the 14 serums with \textit{L. casei} levels less than 5 ng/ml, of which four were less than 3 ng/ml, had unsaturated FBP levels of 10\% or higher. Our data do suggest a direct relationship between serum folate level and unsaturated FBP level in certain situations. Thus, a direct correlation was found between serum folate level and unsaturated FBP level in patients with uremia, and high levels of serum folate tended to be linked with high, rather than low, levels of unsaturated FBP in patients with pernicious anemia. Serum folate level correlated with unsaturated FBP level in the normal subject on a standard diet (Fig. 1), and preliminary data from another volunteer show the same correlation.

This new evidence, suggesting a direct relationship between serum folate level and level of unsaturated FBP in certain situations, is not necessarily at variance with the earlier work reporting an inverse relationship. Decreased renal excretion of serum FBP in uremia\textsuperscript{17} could produce elevated serum levels of FBP with increased binding and perhaps increased release from stores of endogenous folate and thus higher serum levels of folate. Likewise, in the normal subject studied here, the direct relationship between serum folate level and unsaturated FBP level may reflect entrance into the blood during the day of dietary and/or storage folate complexed with partially unsaturated FBP which may be primarily an intracellular folate binder.\textsuperscript{11} In pernicious anemia, the high unsaturated FBP with high \textit{L. casei} serum folate is consistent with the methylenetetrahydrofolate trap hypothesis\textsuperscript{28} in that the MTHF would not saturate the FBP. The increase in FBP in pernicious anemia could be secondary to ineffective granulocytosis. The study of FBP is in its infancy, and a better understanding of the physiology and pathophysiology of FBP will undoubtedly lead to integration of diverse relationships between serum folate level and FBP.

We have shown that the whole-serum radioassay used in this study is not optimal when the serum level of unsaturated FBP is elevated. Unsaturated FBP binds the tracer tritiated folic acid as well as does the assay binder, which results in a shift of the experimental value to the low end of the dose-response standard curve. Our results support those of other workers who have reported that serum folate must be separated from its binder to yield accurate serum folate radioassays.\textsuperscript{3,7,21} Such a separation was accomplished by heat in the study reported here and by a serum folate-releasing factor in a new radioassay.\textsuperscript{29} For optimal results, folate released by heat extraction must be assayed in a system where oxidized and reduced folate behave similarly with respect to the binding ligand used in the radioassay. These conditions were approached at the alkaline pH of our assay and are apparently met in other radioassays.\textsuperscript{5,29}
ACKNOWLEDGMENT

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Clinical and laboratory observations on serum folate-binding protein

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