Total Folate Binding Capacity of Normal Human Plasma, and Variations in Uremia, Cirrhosis, and Pregnancy

By Neville Colman and Victor Herbert

The current study presents evidence that all human serum contains a class of high-affinity folate binders ($K_a = 2.8 \times 10^{10}$ liters/mole), which migrate as a single peak on gel filtration. Failure of previous studies to detect this characteristic in all but a minority of subjects is attributable to its variable, often total, saturation. Direct measurement of the total folate binding capacity (TFBC) has been made possible by dissociation of endogenous folate-binder complexes at acid pH, removal of free folate by coated charcoal, and radiofolate tagging. This procedure does not appear to significantly denature the binders, which release and rebind similar quantities of $^3$H-PGA. In 20 normal subjects, TFBC ranged from 100 to 325 pg/ml (mean $\pm$ SE = 174 $\pm$ 16), and was always at least 33% saturated. In three clinical conditions, all associated with elevated unsaturated folate binding capacity, three different patterns emerged when TFBC was also measured. Uremic subjects had significantly elevated mean TFBC with normal saturation. In cirrhotic subjects, mean TFBC approximated normal, but saturation was significantly decreased. In pregnancy, two groups were seen: one with increased TFBC and the other with a normal TFBC, some of whom had decreased saturation. Lactobacillus casei serum folate level was about 30 times greater than the TFBC; there was no correlation between the two measurements.

USEFUL NUTRITIONAL INFORMATION has been obtained from assays of serum folate, vitamin B$_{12}$, and iron, and, in the case of the latter two, considerable additional useful information has been obtained by measurement of the total binding capacity of serum for the corresponding hematin. Early studies suggested that human serum contained largely nonspecific binders for added folic acid, despite evidence that a fraction of serum folate eluted with high molecular weight complexes on gel filtration. Thus when folic acid was added to serum, the percentage bound remained constant over a wide concentration range, the binding was reversible, and the bound vitamin could easily be extracted with coated charcoal.

Specific binding of added radioactive folic acid by serum was first unequivocally reported by Rothenberg and da Costa in three patients with chronic granulocytic leukemia, and was subsequently observed in pregnancy, women taking oral contraceptives, folate deficiency, cirrhotic and uremic patients,

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Measurement of total folate binding capacity (TFBC) and its saturation. Based on the ob-

This paper presents evidence that all normal human serum contains a high-

affinity folate binder in variable states of saturation, and that prior failures to
detect this binder have been due to it frequently being largely saturated with
endogenous folate. An assay method for the binder is described, and three
different patterns of alteration are reported in three conditions in which the
binder has previously been reported.

MATERIALS AND METHODS

Samples. Serum was prepared from clotted blood, and plasma from blood drawn into 10-ml
vacuum tubes containing 10 mg disodium EDTA and 20 mg sodium fluoride (Vacutainers No.
3200XF92, reorder No. 4601, Becton-Dickinson & Co., Rutherford, N.J.). Samples were frozen
at -20°C until assayed.

Reagents. The buffer used was a mixture with buffering capacity in the pH range of 2.6-
11.8.12 The acid component was 0.0285 M for each of citric acid, monopotassium phosphate,
barbital, and boric acid, and the basic component was 0.2 N NaOH. Other reagents were a
2.5 g/100 ml suspension of Norit A charcoal (pharmaceutical grade neutral, Amend Drug, Hill-
side, N.J.) coated with 125 mg/100 ml hemoglobin (HbCC),13 and a commercial scintillant fluid
(Unogel; Schwarz/Mann, Orangeburg, N.Y.).

Folic acid. Tritiated pteroylglutamic acid (1H-PGA), nominally labeled in the 3', 5', and
9 positions, with specific activity 26 Ci/m mole (Amersham/Searle, Arlington Heights, Ill.), had a
radiochemical purity of 97% by paper chromatography before being shipped by the manufacturer.
After storage at -60°C as a 100 ng/ml solution in Tris-buffered Hanks' solution, it was diluted
to 5 ng/ml for assay. The working solution was chromatographed on Whatman DE-52 DEAE-
cellulose in a linear phosphate-buffered saline gradient. The isotope emerged in three peaks, with
3% eluting with the 0.02 M loading buffer (peak 1), 18% eluting at 0.19 M (peak 2), and 79%
eluting at 0.45 M (peak 3). It was confirmed that only peak 3 could be bound by folate binder,14
and peaks 1 (3%) and 2 (18%) were interpreted to represent the original radiochemical impurity
and folate degradation products, respectively. Since this interpretation corresponded well with
microbiologic assay indicators of folate degradation, it was concluded that the specific activity
of intact 1H-PGA was that stated by the supplier. Microbiologic assay for folate was done using
a chloramphenicol-resistant strain of L. casei by a modification15 of the aseptic addition tech-
nique.16

Measurement of total folate binding capacity (TFBC) and its saturation. Based on the ob-

Fig. 1. Assay method for unsaturated (1) and total (2) folate binding capacity.
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servations discussed under Results, the following method was arrived at and became the standard method used in this study (see Fig. 1):

Prior to each assay, the buffer was tested to ensure that two parts acid plus one part base formed a mixture of pH 7.4. For measurement of TFBC, 0.2 ml of sample plus 0.5 ml of the acidic buffer component were incubated for 15 min, and then decanted onto a 12.5-mg hemoglobin-coated charcoal pellet. Dextran-coated charcoal could not be used in this step because unacceptably high coated charcoal controls resulted. The sample was vigorously mixed (Vortex) and centrifuged at 1000 g for 10 min, and then decanted into 0.25 ml of the basic component of the buffer. While these tubes were centrifuging, preparation was made for simultaneous measurement of unsaturated folate binding capacity (UFBC) by addition of 0.2 ml of sample to 0.75 ml of buffer pH 7.4.

The rest of the assay for UFBC and TFBC was the same. After addition of 250 pg of $^3$H-PGA, and incubation for 30 min, 0.5 ml coated charcoal (0-4°C) was added, and the tubes were immediately mixed vigorously and centrifuged at 1000 g for 10 min. The supernatant was poured directly into 10 ml scintillation fluid, mixed, and counted in a Beckman LS-250 counter; quench correction was achieved using the external standard ratio and appropriate quench curves. Separate coated charcoal controls were run for the UFBC and TFBC assays, and all tubes were run in duplicate. The results were calculated as follows:

\[
\text{Binding capacity (UFBC and TFBC)(pg}$^{3}$H-PGA/ml sample) = \frac{\text{dpm sample} - \text{dpm charcoal control}}{\text{dpm standard} - \text{dpm background}} \times \frac{\text{pg}^3\text{H-PGA added}}{\text{ml sample}}.
\]

Percentage saturation = \( \frac{\text{TFBC} - \text{UFBC}}{\text{TFBC}} \times 100 \).

Gel filtration studies. The binder(s) responsible for UFBC and TFBC were compared by subjecting the serum to each procedure in triplicate. The triplicate supernatants were pooled and eluted in turn with 0.02 M phosphate buffer containing 0.15 M saline, pH 6.0, at a rate of 7 ml/hr from a 2.5 x 70 cm Sephadex G-200 column calibrated with blue dextran, chymotrypsin A, ovalbumin, and aldolase (Pharmacia, Inc.). The apparent molecular weight of the binder-$^3$H-PGA complex was estimated, as recommended by the supplier, by expressing the partition coefficient ($K_{av}$) as a function of the logarithm of the molecular weight.

Kinetic studies. The apparent association constants of the serum binders for $^3$H-PGA were studied by addition of increasing quantities of the ligand, purified by DEAE-cellulose chromatography, to constant amounts (0.2 ml) of the same normal serum, prepared and buffered by the acid-charcoal TFBC method. The binding data were analyzed by plotting $v/c$ against $v$ (where $v$ is the number of picomoles bound per 0.2 ml serum, and $c$ is the molar concentration of free folic acid). As indicated by Scatchard and co-workers\textsuperscript{17,18} if the protein under study contains only a single class of specific binding sites, this plot should be linear and is described by the

Fig. 2. Effect of pH on plasma binding of added $^3$H-PGA. The method used was as described in the text, using the conventional buffer\textsuperscript{12} and 1250 pg $^3$H-PGA/ml plasma.
function $\bar{v}/c = kn - k \bar{v}$ (where $k$ is the apparent association constant and $n$ is the number of binding sites with association constant $k$).

**RESULTS**

**Properties of the Serum Folate Binder**

The effect of pH on $^{3}$H-PGA binding by the plasma of a cirrhotic patient is shown in Fig. 2. Binding was optimal at pH 6.2, and was decreased by more than 95% at pH 4.2. In a second experiment, plasma which had been incubated at pH 4.2 for 30 min was brought to pH 7.2 prior to addition of $^{3}$H-PGA for binding; the binding of $^{3}$H-PGA by three samples with high unsaturated binder was 98%, 98%, and 103% of the corresponding result obtained without acid exposure.

The effect of coated charcoal adsorption of acidified plasma on $^{3}$H-PGA binding was tested by addition of the 0.7 ml serum–buffer mixture at pH < 4 to a 12.5 mg hemoglobin-coated charcoal pellet. After centrifugation, the supernatant was brought to pH 7.2 by addition of the basic buffer component, and $^{3}$H-PGA binding compared with that of the corresponding samples prior to acidification. The results, shown in Table 1, indicated that this technique substantially increased plasma folate binding in samples with and without elevated unsaturated folate binder. Since removal of previously bound folate at acid pH by charcoal adsorption was apparently responsible for this increased binding capacity, the additional binder uncovered by this technique was referred to as "saturated folate binder," and the binding capacity measured by this method was called total folate binding capacity (TFBC).

The hypothesis that the elevation of TFBC over UFBC was associated with removal of bound endogenous folate was tested by *L. casei* folate assay of the supernatants obtained by exposing five normal serum samples to both the UFBC and TFBC techniques, omitting the $^{3}$H-PGA addition step from each method. The mean serum folate of these samples was 6.0 ng/ml. After the UFBC steps, the mean ± SD was 1.7 ± 1.2 ng folate per milliliter of added serum. After the TFBC steps, folate concentration was too low to allow accurate assay, but was in all cases less than 0.25 ng/ml of added serum. This finding was a further indication that normal serum contained some folate bound sufficiently tight to resist charcoal adsorption, and that the TFBC method removed this. Parallel assay for UFBC and TFBC by including $^{3}$H-PGA addition gave means ± SD of 88 ± 70 pg/ml (UFBC) and 233 ± 38 pg/ml (TFBC). Thus the mean increase in binding capacity of serum after acid–charcoal exposure (0.145 ng $^{3}$H-PGA per ml) was roughly one-tenth as great as the amount of microbiologically active folate removed by this method (≥ 1.45 ng per ml).

| Table 1. Effect of Acid Exposure on Plasma Binding of $^{3}$H-PGA at pH 7.2 (pg/ml) |
|---------------------------------|-----------------|-----------------|
|                                | Basal           | Without HbCC    | With HbCC       |
| Plasma A (normal)              | 15              | 11              | 109             |
| Plasma B (normal)              | 25              | 19              | 207             |
| Plasma C (uremic)              | 491             | 507             | 696             |

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The ability of saturated folate binder to release and rebind PGA was tested in three plasma samples by measuring the TFBC, acidifying the sample to pH < 4 and exposing it to a coated charcoal pellet, and neutralizing again before readdition of labeled PGA for binder measurement. The results at each stage of the experiment are shown in Table 2. All samples released bound ³H-PGA at acid pH and bound approximately the same amount of folate again after neutralization.

The effect of duration of acid exposure before the charcoal adsorption step was tested by allowing exposure for 0.15, 1.5, 3, 5, 15, and 30 min. The mean TFBC of ten sera did not differ significantly between any two time periods, and thus dissociation of most of the binder–ligand complexes was apparently instantaneous. To avoid possible deterioration of folate binder by acid with time, the 15-min period was chosen. Less than a 15-min incubation was considered to be less practical for batch assays.

Gel filtration of both the UFBC and TFBC supernatants, carried out separately, resulted in elution of the major part of the radioactivity at exactly the

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**Table 2. Release and Rebinding of ³H-PGA by Plasma (pg bound/ml plasma)**

<table>
<thead>
<tr>
<th></th>
<th>Plasma X (Normal)</th>
<th>Plasma Y (Normal)</th>
<th>Plasma Z (Uremic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. UFBC</td>
<td>13</td>
<td>25</td>
<td>113</td>
</tr>
<tr>
<td>B. TFBC</td>
<td>163</td>
<td>213</td>
<td>225</td>
</tr>
<tr>
<td>C. Tube &quot;B&quot; acidified to pH &lt; 4 exposed to coated charcoal</td>
<td>9</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>D. Tube &quot;C&quot; alkalinized to pH 7.4, incubated with labeled PGA for 30 min, exposed to coated charcoal</td>
<td>188</td>
<td>244</td>
<td>213</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Sephadex G-200 gel filtration of the unsaturated (○—○) and total (———) serum folate binder. Both peaks eluted in the same fraction, with an apparent MW of 42,000, and are compared here to three reference proteins.
same position, corresponding to an apparent molecular weight of 42,000 (Fig. 3). The TFBC sample peak exceeded the UFBC sample peak by an amount which accounted for the entire increase in binding by the former sample over the latter. The remainder of the radioactivity eluted from both columns was alike in position corresponding to a molecular weight below 5000, and alike in amplitude, corresponding to the 2%-3% of added radioactivity not removed by coated charcoal and present in the charcoal control tubes.

**Kinetic data** obtained from study of native and acid-charcoal-treated sera are shown in Fig. 4. Native serum had a lower number of apparent binding sites and an apparently lower association constant for $^3$H-PGA than acid-charcoal-treated serum, reflecting the predictable effect of endogenous bound and free folate (see Discussion, below). The serum folate binder, studied after removal of endogenous folate by the TFBC method, was found to follow a linear Scatchard function ($r = 0.9921$), indicating a single class of binding sites with an apparent association constant of $2.77 \times 10^{10}$ liters/mole.

**Reversibility of binding.** Under the conditions of the current study, maximal
binding of added $^3$H-PGA occurred within 10 min. Incubation of TFBC supernatant at room temperature with 500-fold concentrations of nonradioactive PGA or methyltetrahydrofolate for 1–100 min resulted in less than 4% loss of the tracer from binding sites. Prolongation of the charcoal exposure step at its conventional temperature of 0–4°C for 1–100 min caused less than 1% decrease in $^3$H-PGA binding below that observed when samples were centrifuged immediately after addition of charcoal.

**TFBC and its Saturation**

The results of TFBC measurement in 144 subjects, comprising four main groups, are diagrammatically shown in Fig. 5. The mean UFBC, TFBC, and percentage saturation for each group are listed in Table 3.

**Normal subjects.** This group comprised 20 healthy hospital workers of both sexes with normal hematologic indices. All had greater than 33% saturation of the TFBC, which ranged from 100 to 325 pg/ml. Three of the 20 subjects had UFBC greater than 125 pg/ml, i.e., more than 10% of the added $^3$H-PGA was bound.

**Uremic subjects** were 20 males studied prior to dialysis. In eight subjects (40%), TFBC exceeded the observed range in normals, and the mean was significantly higher than that of the normal group ($p < 0.001$). The mean percentage saturation was similar to the mean of the normal group but, in accordance with the high TFBC, UFBC was greater than 10% of the added $^3$H-PGA in 12 subjects (60%).

**Cirrhotic subjects.** These were ten alcoholic males with histologically proven cirrhosis of the liver. Their mean TFBC was slightly but not significantly higher than that of the normal group ($p > 0.2$), and two subjects (20%) had TFBC levels marginally above the observed range in normals (350 and 354 pg/ml). The mean percentage saturation was significantly lower than that in normals ($p < 0.05$). Thus, despite the normal TFBC range, six subjects (60%) had UFBC greater than 10% of the added $^3$H-PGA. There was no correlation between percentage saturation and the serum folate level.

**Pregnant subjects.** These were 94 women studied prior to prophylactic folate therapy, 25 in the first trimester, 49 in the second trimester, and 20 in the third trimester. As seen in Fig. 5, they appeared to fall into two distinct groups with respect to TFBC. One group of subjects had normal TFBC, whereas another, comprising 27% of the sample, had TFBC levels twice or three times normal. This phenomenon was most notable in the second trimester, resulting in a significant elevation of the mean above that of the normal group ($p < 0.01$). Of the 24 pregnant subjects with elevated TFBC, all but three had less

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>UFBC (pg/ml)</th>
<th>TFBC (pg/ml)</th>
<th>Saturation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>20</td>
<td>61 ± 11</td>
<td>174 ± 16</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>Uremic</td>
<td>20</td>
<td>119 ± 18</td>
<td>344 ± 44</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>Cirrhotic</td>
<td>10</td>
<td>131 ± 32</td>
<td>236 ± 30</td>
<td>45 ± 9</td>
</tr>
<tr>
<td>First trimester</td>
<td>25</td>
<td>131 ± 40</td>
<td>248 ± 46</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>Second trimester</td>
<td>49</td>
<td>207 ± 33</td>
<td>299 ± 36</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>Third trimester</td>
<td>20</td>
<td>155 ± 52</td>
<td>257 ± 49</td>
<td>53 ± 7</td>
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</tbody>
</table>
than 33% saturation of the binder. In addition, among subjects in the second and third trimester, 10 of 48 samples had less than 33% saturation of a normal TFBC. The mean percentage saturation in the second trimester subjects was significantly lower than that in normal subjects ($p < 0.01$). There was no correlation between the percentage saturation of TFBC and either the serum or red cell *L. casei* folate level.

**DISCUSSION**

The current study describes in detail the first method for direct measurement of total folate binding capacity of serum and plasma. Using this method, a partially saturated binder capable of releasing and rebinding folate has been identified in all subjects tested.

The procedure is based on dissociation of endogenous folate-binder complexes at acid pH, allowing removal of free folate by coated charcoal. This process results in removal of all folate detectable by conventional *L. casei* assay, including that not removed by charcoal at neutral pH. The sample is then neutralized, making the binding sites available for radiofolate tagging. It is possible to compare TFBC and its saturation in different groups of subjects, despite the fact that only a small percentage of the serum folate is actually bound by this binder.

Following removal by this procedure of the specific ligand (i.e., endogenous folate) present in untreated serum, the kinetics of the binder were studied using added radiofolate. The binder was of high affinity ($k_a = 2.8 \times 10^{10}$ liter/mole) and the linear Scatchard plot ($r = 0.9921$) indicated a single class of binding sites. Since this preparation contained both sites that were previously unsaturated and those previously saturated with endogenous folate, the linear plot suggested that the unsaturated and saturated binders represented the same substance. This view gains support from the fact that both binders migrate at an identical rate on Sephadex G-200 gel filtration.

The apparently decreased association constant ($k_a$) for $^3$H-PGA in native serum compared with acid- and charcoal-treated serum reflects a well-described effect of competing nonradioactive ligand in Scatchard plot analysis. When the binder is exposed to the competing ligand and the radioactive tracer at the same time, the difference in apparent $k_a$'s for the tracer can be used to calculate the $k_a$ for the competing ligand. Such a calculation cannot be made under the conditions of the current study because endogenous folate was exposed to binder prior to, rather than concurrent with, exposure to $^3$H-PGA.

It has been noted that the folate binder in milk loses some activity after acidification for prolonged periods of time. This observation raises the question of whether a similar phenomenon occurs in the current experiments. However, as shown in the data in the Results section, the short acid exposure step used in the present study leaves unsaturated binder intact, and repetition of the procedure in the same sample does not decrease TFBC.

Microbiologic serum folate assay suggests that the bound endogenous folate fraction is about ten times greater than the TFBC for $^3$H-PGA, on a mole for mole basis. Explanation for this may lie in the reports that methyltetrahydro-
folate, which constitutes the vast majority of serum folate, must be present in higher concentrations than PGA in order to compete with it for serum binding sites. The report suggesting that serum folate radioassay is up to 3 ng/ml lower before protein extraction than after is difficult to interpret because it does not delineate how, or whether, results were controlled for serum binding of tracer \(^{3}\text{H}-\text{PGA}\). Previous workers had demonstrated that unsaturated binder in unextracted serum, by increasing the binding capacity of the radioassay system, causes artificially low folate radioassay results that can account for the difference compared with extracted serum. Accurate direct measurement of bound endogenous folate has not yet been achieved.

Results in normal subjects have been compared with those in uremia, cirrhosis, and pregnancy, three conditions in which the stigmata of folate deficiency are more common. In all three groups, there was an increase in the number of subjects in whom more than 10\% of the added \(^{3}\text{H}-\text{PGA}\) was bound by native serum, but this resulted from three different patterns. In the uremic group, there was an elevated mean TFBC with normal saturation. In the cirrhotic group, there was significantly decreased saturation of a normal mean TFBC. Among pregnant subjects, both phenomena were evident and did not necessarily occur simultaneously; i.e., some subjects had elevated TFBC, some had decreased saturation, and some had both.

It has been suggested that the rise in unsaturated folate binder in pregnant women might be due to hormonally induced synthesis of folate binder. The current studies indicate that there is a significant increase in the total folate binding capacity in pregnant subjects and thus support the hypothesis of daCosta and Rothenberg. In addition, however, there is a significant decrease in percentage saturation of TFBC in both pregnant and cirrhotic subjects which is not related to the serum folate level. The mechanisms that influence percentage saturation have not been elucidated in this study and are under investigation.

Kamen and Caston suggested that protein extraction released from all sera a variable amount of folate which was previously unavailable for radioassay but available to \(L.\text{ casei}\). In preliminary studies, they had obtained evidence that the folate radioassay level before protein extraction best reflected clinical status, and that megaloblastosis could be found in chronic renal and liver disease in the presence of normal \(L.\text{ casei}\) folate levels. Since available evidence has suggested that serum folate binder was a withholding rather than a delivery protein, the current observations in uremic subjects support the findings of Kamen and Caston.

Investigation of the role of the saturated binder in folate transport and metabolism is currently being undertaken in our laboratory, with a view to delineating the clinical significance of the observations reported here. It appears probable that the finding of altered binding patterns in conditions associated with altered folate metabolism is not purely fortuitous. On the technological level, our preliminary abstract, reporting the presence of an endogenous folate–binder complex and describing the method for dissociating it, has made it possible for subsequent workers to study the physical and chemical properties of saturated binder in cord serum, and to achieve the first reported success...
in the use of affinity chromatography to purify serum folate binder. Prior to our report, other workers\textsuperscript{27} had been unsuccessful in attempts to uncover saturated folate binders in serum by removing endogenous bound folate.

The method described in this study forms the basis of an additional preliminary report of TFBC patterns in folate deficiency and in pregnancy,\textsuperscript{28} and it has been used to identify animal models for the study of folate-binding proteins.\textsuperscript{29}

**ACKNOWLEDGMENT**

We wish to acknowledge the technical assistance of Jean Richardson and Eli Saleeby.

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