The Weak Binding Reaction Between Folate and Human Serum Proteins

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ABSTRACT

New evidence is presented that folic acid in serum binds with low affinity to major serum proteins. This low affinity binding is distinct from the high affinity binding by folate binding protein (FBP), a minor protein which is known to occur in serum with great quantitative variability. These conclusions are based on results obtained by equilibrium dialysis of serum containing only negligible amounts of FBP. At 4°, the equilibrium value of the ratio of bound to free folate was approximately 0.81 and remained the same even with up to 1,000 times greater than normal folic acid concentrations; however, with higher concentrations the ratio decreased progressively. These results are predicted by the laws of mass equilibria for a binding system with low ligand concentration vis-a-vis high binder concentration and low affinity between the reactants. A rough estimate of the mean affinity constant $K$ governing this weak folate interaction with serum proteins yielded a value of $1.12 \pm 0.13 \times 10^3 \text{ M}^{-1}$.

Introduction

Folate appears to be bound by human serum in two different ways: (1) firmly and specifically to special, minor serum proteins with high affinity and (2) weakly and perhaps nonspecifically to major serum proteins with low affinity. These two binding phenomena up to now have not been clearly distinguished from each other in the literature concerned with serum folate binding, even though that evidence for at least two types of binding has been accumulating for a number of years.

The presence of high affinity folate binding proteins (FBPs) in human serum is now firmly established, and their partial purification by various techniques has been reported. The complex of folate with FBPs is characterized by an affinity high enough to withstand such dissociating conditions as charcoal adsorption, chromatography, and dialysis. A survey of over 1,000 sera from patients and healthy volunteers showed that the serum content of unsaturated FBP is highly variable, the binding capacity ranging from less than 0.1 to 8.5 μg of folate per liter of serum. The physiologic functions or importance of serum FBP are not clear, and the elevation of unsaturated FBP in some sera may be incidental to tissue turnover. In most sera, however, the endogeneous folate
appears to exceed the binding capacity of the serum FBP considerably, so that the major fraction of the serum folates is not bound by FBP and therefore available for the weaker interactions with the other serum proteins.

The weak binding of folate by serum is demonstrable with such non-dissociating methods as equilibrium dialysis, Sephadex gel filtration and ultrafiltration. These weak complexes of folate and serum proteins dissociate readily under such treatment as DEAE-cellulose column chromatography, charcoal adsorption, electrophoresis or dialysis. Furthermore, this weak binding reaction presented the rather puzzling phenomenon of being seemingly nonsaturable because the ratio of free and bound ligand when using folic acid or methyltetrahydrofolic acid remained the same over three orders of magnitude of folate concentrations. The bound folate fraction was variously reported as 50 percent, 46 to 51 percent, 64 percent, 51 to 61 percent when the data of Alter et al are recalculated as the actual bound fraction, and 59 to 76 percent.

Some theoretical considerations and experimental data are presented to support the concept that serum folate, in addition to its interaction with FBP, normally forms weak complexes with major serum proteins. Furthermore, it is shown that the maintenance of a seemingly constant binding ratio over large concentration ranges of folate is the behavior predicted in principle by the laws of mass equilibria for a binding system of low affinity when the binder concentration exceeds that of its ligand by orders of magnitude. On the basis of these theoretical considerations, the present authors predicted and have shown experimentally that higher concentrations of folate than those tested by previous workers are needed to demonstrate a decrease of the binding ratios.

**Theoretical Considerations**

For the purpose of formulating a model approximating the weak folate-serum protein interaction, it shall suffice to make the following simplifying assumptions:

1. The major serum proteins capable of forming such weak complexes react identically with folates;
2. The concentration of these serum proteins is approximately \(7.0 \times 10^{-4}\) M (based on a protein content in serum of 70 g per l and an average mol wt of 100,000);
3. These proteins possess only one binding site for folates;
4. The concentration of folates in serum is approximately \(2 \times 10^{-8}\) M (based on a serum folate level of 9 \(\mu\)g per l and an average mol wt of the endogenous folates of 450); and
5. The affinity constant \(K\) for the weak binding reaction of folates by serum proteins is of the order of \(1.43 \times 10^4\) M\(^{-1}\). This estimate is derived from the basic relationship

\[ K = \frac{[PQ]}{[P][Q]} \quad \text{(formula \#1)} \]

where \([PQ], [P]\) and \([Q]\) are the molar concentrations of the complex, free folate and free protein, respectively. If it is assumed for the bound folate fraction, based on the reported findings (vide supra), an approximate value of 50 percent, the ratio of \([PQ]/[P]\) then becomes 1 and \(K = 1/[Q]\). Since the protein complexed with folate thus is only a minute fraction \(1/70,000\) of total protein, it can set \([Q]\) equal with total protein concentration and thus \(K = 1/7 \times 10^{-4}\) M = \(1.43 \times 10^4\) M\(^{-1}\) can be obtained.

Under these restricting assumptions, the equilibrium ratio of bound to free folate is then given by the quadratic equation

\[ R^2 + R(1 + Kp - Kq) - Kq = 0 \quad \text{(formula \#2)} \]

where:

\[ R = \text{bound folate}/\text{free folate}, \]
\[ K = \text{the affinity constant}, \]
p = the molar concentration of total folate, and
q = the molar concentration of protein.

Using equation 2, the R values for six folate concentrations have been calculated ranging from \(2 \times 10^{-8}\) to \(2 \times 10^{-3}\) M and a constant protein concentration of \(7.0 \times 10^{-4}\) M (table I). When \(p < q\) (folate concentrations from \(2 \times 10^{-8}\) to \(2 \times 10^{-5}\) M), changes of p result only in minute changes of R, differences obviously too small to be detected experimentally. Only when the concentration of p approaches \((2 \times 10^{-4}\) M\) or exceeds \((2 \times 10^{-3}\) M\) the order of magnitude of q, the predicted changes of R become sufficiently large to be discernable by measurement.

In the following are reported equilibrium dialysis experiments involving whole serum with the folate content raised in ten-fold increments to extend over the concentration range shown in table I. The results are in good agreement with the theoretical predictions.

Materials

**Phosphate-citrate buffer:** 0.05 M, pH 7.4.9 The osmolality of this buffer was raised to 290 milliosmols by the addition of NaCl (approximately 1.5 g per l) in order to obtain the same osmolality as was found in the serum pool.

\([35^2\text{H}]\text{folic acid}: ([\text{H}]	ext{PGA}) 22 \text{ Ci per millimol}.\) Working solutions were made in phosphate-citrate buffer to contain 500 \(\mu\text{g per l}\) of \([\text{H}]	ext{PGA}.\) The concentration of \([\text{H}]	ext{PGA}\) was determined through competitive binding assays using unlabeled folic acid as described elsewhere.31

**Folic acid:** (PGA) crystalline.\(\dagger\) Working solutions were made in phosphate-citrate buffer and stored at \(-20^\circ\). These were stable for several months.

**Serum pool:** Patients’ sera obtained routinely by the clinical chemistry labora-

\(\dagger\) Lot No: BR-2574 from Schwartz/Mann, Orangeburg, NY 10962.
\(\dagger\) Sigma Chemical Co., St. Louis, MO 63178.

\(\ddagger\) Obtained from Colle-Farmer Instruments Co., Chicago, IL 60648.
\(\ddagger\) Obtained from Scientific Products, Irvine, CA 92714.
\(\ddagger\) Obtained from Clay Adams, Parsippany, NJ 07054.
\(\ddagger\) Obtained from Hamilton Co., P. O. Box 10030, Reno, NV 89510.
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TABLE I

<table>
<thead>
<tr>
<th>Folate Concentration</th>
<th>Protein Concentration</th>
<th>Affinity Constant</th>
<th>( R = \text{Bound folate/ free folate} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 2 \times 10^{-8} ) M</td>
<td>( 7 \times 10^{-4} ) M</td>
<td>( 1.43 \times 10^{3} ) M(^{-1} )</td>
<td>0.9999857</td>
</tr>
<tr>
<td>( 2 \times 10^{-7} ) M</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.999857</td>
</tr>
<tr>
<td>( 2 \times 10^{-6} ) M</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.99857</td>
</tr>
<tr>
<td>( 2 \times 10^{-5} ) M</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.9858</td>
</tr>
<tr>
<td>( 2 \times 10^{-4} ) M</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.865</td>
</tr>
<tr>
<td>( 2 \times 10^{-3} ) M</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.315</td>
</tr>
</tbody>
</table>

Methods

*Equilibrium dialysis* was carried out in the following way: mixtures of 5 ml of serum, 0.1 ml of working solution of \(^{3}H\)PGA and 1.0 ml of solutions containing 0, 500, 5,000, 50,000, 500,000 and 5,000,000 \( \mu g \) per 1 of unlabeled PGA were placed into one side of the dialysis chambers and mixtures identical to these in \(^{3}H\)PGA and unlabeled PGA contents but substituting buffer for serum were placed into the other chamber side. In preparing the 5,000,000 \( \mu g \) per 1 solution of unlabeled PGA, the entire solids did not dissolve in the diluting buffer as some fine crystals remained after several hours of mixing. However, when 1.0 ml aliquots of these well-mixed suspensions were added to the 5 ml aliquots of serum or buffer, the crystals dissolved completely.

The chambers were protected from light with black paper, placed onto the shaker and rotated at 90 rpm for 52 hours. The experiments were carried out in a walk-in refrigerator at 4° since at room temperature or at 37° the \(^{3}H\)PGA did not remain sufficiently stable over the time required for equilibrium dialysis. After periods of 16, 20, 28, 44 and 52 hours of dialysis, aliquots of 100 \( \mu l \) from the serum and the buffer sides were aspirated with the Hamilton syringes and placed into 5 ml of scintillant for radioactive counting. The level of radioactivity in these aliquots ranged from approximately 20,000 to 50,000 cpms, and the coefficient of variation of the entire procedure calculated from replicate measurements was 1.4 percent. Equilibrium was reached between 18 and 20 hours.

The stability of the \(^{3}H\)PGA was monitored repeatedly throughout the period of dialysis by determining the bindability of the \(^{3}H\)PGA to milk folate binding protein\(^2\) in the samples without unlabeled PGA. No reduction in the bindability of \(^{3}H\)PGA was seen throughout the 52 hours of dialysis. The absorption of the radioactivity by polyvinylpyrrolidone-coated charcoal\(^3\) from the dialysis mixtures was checked throughout the dialysis period. The "blank" values remaining in the supernate after charcoal treatment did not rise above 1.5 percent of initial total radioactivity in the serum and above 1.0 percent in the buffer, a further indication of the good stability of the \(^{3}H\)PGA during dialysis.

After equilibrium was reached, the molar concentrations of folic acid in either side of the chambers were determined at 20, 28, 44 and 52 hours of dialysis from the distribution of the radioactivity minus the charcoal "blank" values between the two chamber sides and the total amount of folic acid (labeled and unlabeled) known to have been added at the beginning of dialysis. The "blank" values in the serum included the radioactivity bound by FBP. Thus, in effect, four replicate determinations were obtained of the equilibrium conditions spanning a total of 32 hours. The small amount of endogenous folate (0.6 \( \mu g \) per 1) remaining in the serum after seven days of aging at 4° was considered negligible and therefore not included in these estimations. The concentration of bound folate was calculated from the differences in the folate concentrations between the buffer and the serum dialysates.
Results and Discussion

The results are summarized in Table II. The equilibrium values shown are the means of the values found at 20, 28, 44 and 52 hours. As is readily apparent, during dialysis the folic acid concentrations in the serum increased and those in the buffer decreased. The value of R, the ratio of bound to free folic acid, remained constant with folic acid concentrations ranging from $2.03 \times 10^{-8}$ to $1.86 \times 10^{-5}$ M. With concentrations of $1.86 \times 10^{-4}$ M or higher, a decrease of the values of R is seen. The mean affinity constant K calculated from these data was $1.12 \pm 0.13 \times 10^3$ M$^{-1}$. This compares well with the results published by Soliman and Olesen who studied the binding of [$^3$H]PGA to pure human plasma albumin by Sephadex gel filtration and found a K value of $0.9 \times 10^3$ M$^{-1}$.

Our experimental data are in principal agreement with the theoretical values in Table I. The results of the interaction of folate and serum proteins in the equilibrium dialysis experiments are those expected of a binding system which is characterized by low ligand concentration and higher binder concentration exceeding that of the ligand by several orders of magnitude, and which is governed by a low affinity. When such a binding system comes to equilibrium, a large fraction of the total ligand will be free even though a huge excess of binder is present. This is reflected in the low R values (e.g. <1) which show little change unless the ligand concentration approaches that of the binder. Association and dissociation of these weak complexes must be rather rapid processes since it is possible with charcoal to adsorb folates, that are not bound to FBP, from serum within less than one minute, as rapidly and completely as from buffer solutions free of protein. Similarly, the complexing of PGA by cow's milk folate binder, requiring less than three minutes for completion at room temperature, is not recognizably slowed down in the presence of serum. Both these processes, the charcoal adsorption and the complexing with milk binder, depend on the dissociation of the folate-serum protein complex. It is also known that association and dissociation of the weak complexes between ligands and serum proteins in general have half-times of the order of a few milliseconds, although there are exceptions to this rule.

The results of our binding studies, as well as those of others, with folic acid were quantitatively quite similar to those with methyltetrahydro-

<table>
<thead>
<tr>
<th>TABLE II</th>
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<tbody>
<tr>
<td><strong>Equilibrium Dialysis Experiments</strong></td>
</tr>
<tr>
<td>Folate Concentrations Before and After Dialysis* and Resulting R Values</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration Factor</th>
<th>At start of dialysis in both sides of chambers</th>
<th>Serum Side</th>
<th>Buffer Side</th>
<th>Bound Folate</th>
<th>Percent Bound</th>
<th>R = Bound folate/ free folate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-8}$</td>
<td>2.03</td>
<td>2.615</td>
<td>1.445</td>
<td>1.170</td>
<td>44.8</td>
<td>0.810</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>2.07</td>
<td>2.665</td>
<td>1.475</td>
<td>1.190</td>
<td>44.7</td>
<td>0.807</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>1.88</td>
<td>2.427</td>
<td>1.333</td>
<td>1.094</td>
<td>45.1</td>
<td>0.821</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>1.86</td>
<td>2.395</td>
<td>1.325</td>
<td>1.070</td>
<td>44.7</td>
<td>0.806</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>1.86</td>
<td>2.366</td>
<td>1.354</td>
<td>1.012</td>
<td>42.8</td>
<td>0.747</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>1.86</td>
<td>2.066</td>
<td>1.654</td>
<td>0.412</td>
<td>19.9</td>
<td>0.249</td>
</tr>
</tbody>
</table>

*Folic acid concentrations are in μg per ml. Values are the means of four replicate determinations obtained after 20, 28, 44 and 52 hours of dialysis.
folic acid, \( \text{25} \) the major endogenous serum folate. Also, various temperatures of \( \text{4°} \) as used by us, Neal and Williams \( \text{18} \) and Spector et al\( \text{,25} \) \( \text{5°} \), \( \text{23°} \), \( \text{37°} \) \( \text{11} \) and \( \text{43°} \) \( \text{25} \) seemed to have little effect on the binding ratios obtained with either folate derivative. These observations imply that the endogenous folate in circulating plasma normally is partially bound in these weak complexes and that the \text{in vivo} \) binding ratios are similar to those demonstrated \text{in vitro}.

The weak binding of folate by serum proteins raises the question as to what effect such complex formation may have on the rate of folate delivery to the tissues, particularly since an interference with the uptake of folates by tumor cells has been demonstrated for the strong FBP complex. \( \text{29} \) The effect of weak folate-serum protein complexes on tissue delivery would be expected to be much smaller since, as the experimental data indicate, at physiologic concentrations approximately 50 percent of the total plasma folate is not bound by proteins and presumably readily available to the high affinity folate receptors\( \text{2,3,13,21,22} \) and dihydrofolate reductase\( \text{27} \) in the cells. Furthermore, any withdrawal of free folate from the circulating plasma by the tissues would be followed by rapid dissociation of the folate protein complexes until equilibrium is re-established.

Lastly, the effect that drugs interacting with serum proteins may have on folate binding should be considered.

In general, a drug may interfere with the binding of another substance (exogenous or endogenous) to serum proteins either by competition for the same binding sites or by lowering the binding affinity. \( \text{15,16} \) Both mechanisms result in a decrease of the bound fraction. The effect of a drug on the binding ratio of folates, when competing for binding sites with the same affinity, can be formulated by the general equation for competitive binding\( \text{5} \):

\[
R^2 + R (1 + K_{p_{\text{fol}}} + K_{p_{\text{drug}}} - K_q) - K_q = 0
\]

(Formula \#3)

where \( p_{\text{fol}} \) is the total molar concentration of folate and \( p_{\text{drug}} \) the total molar concentration of drug.

Two interesting aspects derive from this formulation: (1) that the effect of the drug on \( R \) is simply additive with that of folate and (2) a consequence of 1, that the molar concentration of the drug in serum would have to approximate that of the proteins in order to lower significantly the percentage of bound folate, analogous to the effect of raising folate concentrations (tables I and II). Alter et al\( \text{1} \) showed that folate binding by serum was decreased when acetylsalicylic acid was added to the dialysis mixtures. They suggested that the decrease of folate binding may have been due to the trans-acetylation of albumin by acetylsalicylic acid known to occur \text{in vitro} \( \text{8} \) as well as \text{in vivo} \( \text{8} \) and that this structural alteration changed the binding affinity for folates. However, the simple quantitative relationships of drug interferences as outlined suffice to explain this effect of acetylsalicylic acid on the basis of its competition with folates for binding sites on the albumin molecule.

The \text{in vitro} \) and \text{in vivo} \) concentrations of the drug producing the effect seen by Alter et al\( \text{1} \) were given as from 5 to 40 mg per dl, that is from 0.28 to \( 2.23 \times 10^{-3} \) M, concentrations ranging just below and above those of serum proteins. These are also the drug concentrations for which formula \#3 predicts significantly and measurably lower \( R \) values for folates. Since the free folate is in dynamic equilibrium not only with the bound folate in plasma but also with tissue folate, the free folate concentration would be expected to change only insignificantly when drugs displace the bound fraction. Clinically, lower serum folate levels should be observed without the signs of folate deficiency. This expectation is well
borne out by the in vivo observations of Alter et al.1

References


