Quantitative Aspects of the Binding of Folic Acid by Folate Antiserum

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SUMMARY

Rabbits and guinea pigs immunized with folic acid coupled to methylated bovine serum albumin produced antibodies which bound the haptenic vitamin. The antibodies were of the high-affinity type with equilibrium constants in the range of $10^8$ liters/mole and binding energies from -11 to -14 kcal/mole. The folate-binding capacity of the antiserum approximated $10^{-7}$ mole/liter, sufficient to bind all physiological folate with determinants for this antibody. Since the biological activity of antibody is a function of the product of the equilibrium constant and antibody concentration, these studies demonstrate the feasibility of inducing antibodies against folate which might interfere with the transport and metabolic function of this vitamin.

INTRODUCTION

The function of folate coenzymes in the transfer of carbon units for the synthesis of the purine ring and the methylation of uridylate to thymidylate is a fundamental requirement for cell replication. The mammalian cell cannot synthesize the basic pteroylglutamate structure and, consequently, growth and maturation of both normal and neoplastic cells require an exogenous source of this vitamin. For this reason, the folate coenzymes have been the target of intensive investigation, particularly as related to tumor growth. Indeed, the objective of cancer and leukemia chemotherapy with antagonists of folate acid is to interfere with the biological function of this vitamin and thereby inhibit neoplastic cell growth.

It has long been known that folate deficiency frequently accompanies the disordered growth of neoplasia. Morphological evidence of erythroid megaloblastosis is a fundamental requirement for cell replication. The mammalian cell cannot synthesize the basic pteroylglutamate structure and, consequently, growth and maturation of both normal and neoplastic cells require an exogenous source of this vitamin. For this reason, the folate coenzymes have been the target of intensive investigation, particularly as related to tumor growth. Indeed, the objective of cancer and leukemia chemotherapy with antagonists of folate acid is to interfere with the biological function of this vitamin and thereby inhibit neoplastic cell growth.

Of considerable interest in this regard is the observation that folate deficiency coexists with acute leukemia, the administration of folic acid will usually correct the megaloblastosis but at the same time may also increase the number of leukemic cells (4). This observation is supported by the report that AKR mice with transmitted leukemia, normally resistant to the 4 amino folate analogs, have significantly prolonged survival when a folate-free diet and oral Sulfasuxidine are used in addition to the administration of a folate antagonist (20). This close dependence of cancer cell growth on adequate folate metabolism has suggested the notion that interference with intercellular transport of folic acid might be a way of producing an intracellular deficiency of this coenzyme, thereby interfering with cell replication.

Immunization of rabbits (7, 13, 17, 19) and guinea pigs (17) with folic acid coupled to carrier macromolecules can induce antibodies which bind the haptenic vitamin. Antibody-bound folate cannot be reduced to tetrahydrofolate by hepatic folate reductase (17), and plasma clearance and tissue distribution of folic acid-$^3$H in immunized animals deviate from normal (18). Thus, one approach to the perturbation of folate transport could be by means of specific antibodies which would sequester the circulating vitamin. Moreover, a high concentration of antibody in the extracellular fluid bathing cells theoretically could block the uptake of folic acid. Because this biological effect of the hapten-antibody complex will be dependent, at least in part, on the concentration and binding properties of the antibody, we have investigated at this time the kinetic and quantitative aspects of folate binding by antifolate antiserum.

MATERIALS AND METHODS

Folic acid was coupled to methylated bovine albumin with the use of carbodiimide [1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate] by the method of Ricker and Stollar (13), slightly modified in this laboratory (17). White New Zealand rabbits and guinea pigs were then immunized as previously described (17).

Blood was obtained from the ear lobe artery of the rabbit and by cardiac puncture from the guinea pig. Following clot retraction, the supernatant serum was stored either at -20° or at 4°. At the latter temperature, kanamycin (10 mg/ml) or Merthiolate (final concentration, 1:10,000) was added to prevent bacterial growth.

The binding of the vitamin by antiserum was determined by a radioimmunoassay with folic acid-$^3$H (Amersham/Searle, Inc., Des Plaines, Ill.) as the labeled hapten (17). The reaction...
mixtures contained 75 to 100 pg of folic acid-3H, 0.1 ml of antiserum in a total volume of 0.5 ml of 0.02 M Tris-Ringer’s buffer, pH 7.4. This buffer was prepared by diluting 1 volume of 1 M Tris-HCl; pH 7.4, in 50 volumes of Ringer’s solution. Slight adjustments of pH to 7.4 were made with either 1 N NaOH or 1 N HCl. Increasing concentrations of total radioactivity were obtained by adding the indicated quantity of unlabeled folic acid in 10-μl volumes.

The reaction mixtures were incubated at 37° for 2 hr following preliminary rate studies with folate-3H alone which indicated that initial binding velocities ranged from 20 to 80%/min. Free and antibody-bound vitamin were separated by the addition of 0.3 ml of a 2.5% suspension of albumin-coated charcoal which adsorbs virtually all free folic acid. The charcoal suspension was prepared by mixing equal volumes of 5% charcoal and 1% bovine albumin, removing the supernatant albumin solution after centrifugation, and resuspending the coated charcoal in deionized water to a 2.5% suspension. To minimize dissociation of the antibody-folate complex following the addition of the charcoal suspension, the mixtures were quickly shaken for 1 or 2 min and then centrifuged at 4°. An aliquot of the supernatant solution was added to 15 ml of scintillation solution containing 5 g PPO and 100 ml of BBS-3 solubilizer (Beckman Instruments, Inc., Fullerton, Calif.) per liter of toluene, and the radioactivity was determined with an ambient temperature Nuclear-Chicago liquid scintillation counter. Sufficient counts were recorded for a counting error of 2% or less.

As a consequence of the high specific activity of the folic acid-3H, some radiolysis of the labeled compound occurred even when stored at −65°. In order to correct for this nonfolic acid radioactivity, which was primarily p-aminobenzylglutamate (16) and nonimmunoreactive (5), a sample of tracer folate used in the reaction mixtures was precipitated with stable folic acid and 5% ZnSO₄ as previously described (15). The nonprecipitable radioactivity was then subtracted from the total radioactivity of each reaction mixture to obtain the folate-3H radioactivity.

A small fraction of radioactive could not be absorbed by the charcoal from a mixture containing folic acid-3H and normal rabbit serum. Accordingly, this additional control was run with each series of experimental reactions and the radioactivity of this blank subtracted from the total to obtain the value for the immunologically bound radioactivity. There was little variability of this control value when different samples of normal rabbit serum were tested.

Bound and free hapten were also determined by equilibrium dialysis. A series of reaction mixtures containing aliquots of immune serum in 1 ml of 0.02 M Tris-Ringer’s buffer, pH 7.4, were pipetted into 6-mm-wide Visking cellulose dialysis bags and placed into test tubes containing 4 ml of buffer with a constant quantity of folic acid-3H acid and increasing quantities of unlabeled folic acid. The reactions were incubated for 20 to 24 hr at 37° with periodic shaking, since preliminary experiments indicated that this was sufficient time for tracer folate alone under similar conditions to reach an equilibrium distribution. For determination of the concentration of free hapten, an aliquot of the dialysate was counted in 15 ml of scintillation solution. The bound radioactivity was then determined by subtracting the total free folate-3H (distributed in a 5-ml volume) from the total radioactivity added initially. One control containing buffer without antiserum and a 2nd control containing normal rabbit serum were run simultaneously with each experiment. Replicate tests of different samples of normal rabbit serum demonstrated little variation in the binding of folate-3H. The small percentage of bound radioactivity due to adsorption to the dialysis bag or to nonimmune binding by serum was then subtracted from the calculated bound radioactivity in the experimental reactions to obtain the antibody-bound folic acid.

Calculations. The ratio of molar concentrations of bound to free hapten, determined from the experimentally obtained values of percentages of folic acid-3H bound and free, was plotted against the molar concentrations of bound unlabeled folic acid as described by Berson and Yalow (1) for the insulin-antinsulin models. Thus,

\[ \frac{B}{F} = K(\text{Ab}^a - B) \]

where \( B \) and \( F \) represent molar concentrations of bound and free folic acid, respectively; \( K \) is the equilibrium constant; and \( \text{Ab}^a \) is the total molar concentration of antibody-combining sites. A straight-line plot of \( B/F \) versus \( B \) indicates an antibody population with a single order of combining sites. The slope of this line is \(-K\) and the ordinate intercept is \( K[\text{Ab}^a] \). The total concentration of antibody-combining sites in the reaction mixture can then be determined from the \( y \)-intercept or from intercept with the \( x \)-axis, for when all the binding sites are occupied by unlabeled hapten, \( B/F = 0 \) and \( \text{Ab}^a = B \).

If the plot of \( B/F \) versus \( B \) is curvilinear with concavity upward, more than 1 order of antibody combining site is present. The complexity of the equation would depend on the number of antibody classes with different \( K \) values. The following equation applies when the curve is analyzed for 2 orders of combining site:

\[ \frac{B}{F} = K_a(\text{Ab}^a_a - B_a) + K_b(\text{Ab}^b_b - B_b) \]

where \( B_a \) and \( B_b \) are the molar concentrations of hapten bound to each order of combining site, \( B = B_a + B_b \), \( \text{Ab}^a_a \) and \( \text{Ab}^b_b \) are the molar concentrations of each order of combining site, and \( K_a \) and \( K_b \) are their respective equilibrium constants. The \( y \)-intercept of the \( B/F \) versus \( B \) plot is equal to \( K_a[\text{Ab}^a_a] + K_b[\text{Ab}^b_b] \), and the \( x \)-intercept is equal to the molar concentration of total binding sites \( \text{Ab}^a \) which is equal to \( [\text{Ab}^a_a] + [\text{Ab}^b_b] \). The \( K \) values were obtained by approximating tangents to the extremities of each curve and determining the slope of each line. The molar concentration of antibody-combining sites in serum was calculated from the dilution of antiserum in the reaction mixture and the value of \( [\text{Ab}^a] \).

The heterogeneity index, \( a \), was calculated according to the Sips distribution function in a plot suggested by Karush (8):

\[ \log \left[ \frac{r}{(n - r)} \right] = \log C + \log K_0 \]

5 The concentration of each order of binding sites can be determined by solving the simultaneous equations \( B/F = K_a[\text{Ab}^a_a] + K_b[\text{Ab}^b_b] \) and \( [\text{Ab}^a] = [\text{Ab}^a_a] + [\text{Ab}^b_b] \).
where \( r \) is the mole of hapten bound per mole of antibody, \( C \) is the free hapten concentration, \( n \) is the valency of the antibody, and \( K_0 \) is the equilibrium constant. For determination of \( r \), the molar concentration of antibody was obtained from the value of \([\text{Ab}^0]\) divided by 2, the valency of the antifolate antibody (19). A plot of \( \log \frac{r}{(n-r)} \) against \( \log C \) was analyzed by the method of least squares to obtain the slope \( a \), the index of heterogeneity. The average value for the equilibrium constant was also obtained from this plot since at half-saturation of antibody-combining sites \( \log \frac{r}{(n-r)} = 0 \), \( K_0 \) is equal to the reciprocal of \( C \).

RESULTS

Curvilinear and straight lines were obtained for different serums when the experimental data were graphically analyzed as \( B/F \) versus \( B \). Examples of each are shown in Chart 1. Sometimes a replicate study showed some deviation from linearity after initial studies were analyzed as linear. In such case, the linear analysis was used because such slight deviations indicate that \( K_a \) and \( K_b \) were of approximately similar magnitude. It should be appreciated that there are inaccuracies associated with extrapolating curvilinear plots. Berson and Yalow (1) estimated the accuracy of \( K_b \) as \pm 10 to 20% and of \( K_a \) as 2-fold reciprocally to the error of \([\text{Ab}^0]\). These variations were minimized by running replicate determinations. It was observed that repeat determinations on serum stored frozen varied to some extent. This was attributed to experimental error as well as to some effect on the antibody by freezing and thawing the antiserum.

The quantitative data for several different antiseraums are summarized in Table 1. A single \( K \) value indicates that the derivation was from a linear plot, whereas 2 values for \( K \) indicate that this plot was curvilinear. The range of \( K \) values was 1.13 to 65 \( \times 10^8 \) liter/mole, and serum-binding capacities for folic acid ranged from 0.32 to 3.12 \( \times 10^{-7} \) mole/liter. The free energy change calculated from the equilibrium isotherm reaction,

\[
\Delta F^o = -RT \ln k
\]

ranged from about \(-11\) to \(-14\) kcal/mole.

The equilibrium constant for the antibody in R-5 antiserum was similar whether determined by equilibrium dialysis or charcoal separation of bound and free hapten, but the total binding capacity of the antiserum was lower by the former technique. Since the dialysis reactions were incubated for 18 to 20 hr at 37°, some protein denaturation could have reduced the total binding capacity without altering the binding property of the remaining antibody molecules. The experimentally determined equilibrium constant is a mean value for an antibody population with varying affinities (8). The heterogeneity index, as determined by the Karush plot (8), assumes a continuous distribution of such affinities (although a discontinuous distribution is possible) and reflects the dispersion of equilibrium constants around the mean. A value of 1 determined from the plot of \( \log \frac{r}{(n-r)} \) versus \( \log C \) indicates a homogenous population (Chart 2). The values listed in Table 2 for the antiseraums studied range from 0.88 to 1.06 and indicate considerable homogeneity. The average \( K \) value determined from this analysis is within reasonable agreement to the mean of \( K \)'s determined by the \( B/F \) versus \( B \)

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**Table 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>( K_0 ) or ( K_a/K_b ) (liter/mole) ( \times 10^8 )</th>
<th>( \Delta F^o ) (kcal/mole)</th>
<th>Serum binding capacity (mole/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1</td>
<td>CC</td>
<td>2.5</td>
<td>-11.9</td>
<td>0.6 ( \times 10^{-7} )</td>
</tr>
<tr>
<td>R-5</td>
<td>CC</td>
<td>1.7b</td>
<td>-11.7</td>
<td>3.12 ( \times 10^{-9} )</td>
</tr>
<tr>
<td>R-5</td>
<td>ED</td>
<td>2.2c</td>
<td>-11.8</td>
<td>1.5 ( \times 10^{-7} )</td>
</tr>
<tr>
<td>R-19</td>
<td>CC</td>
<td>65/2.5d</td>
<td>-13.9/-11.9</td>
<td>2.0 ( \times 10^{-7} )</td>
</tr>
<tr>
<td>R-20</td>
<td>CC</td>
<td>39/15</td>
<td>-13.6/-13.0</td>
<td>0.32 ( \times 10^{-7} )</td>
</tr>
<tr>
<td>G. P.-12</td>
<td>CC</td>
<td>1.1</td>
<td>-11.4</td>
<td>0.85 ( \times 10^{-7} )</td>
</tr>
</tbody>
</table>

a The abbreviations used are: CC, coated charcoal separation; ED, equilibrium dialysis.

b Mean of replicate values of 2.9 and \( 0.6 \times 10^8 \) for serum stored frozen.

c Mean of replicate values of 3.6 and \( 0.9 \times 10^8 \) for serum stored frozen.

d These values were calculated from the graph illustrated in Chart 1. Replicate determinations on serum stored frozen were 17 and \( 0.5 \times 10^7 \) for \( K_a \) and \( K_b \), respectively.

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Chart 1. Graphic analysis of ratio of antibody-bound to free folic acid (\( B/F \)) plotted as a function of molar concentration of bound folic acid. *Left,* curvilinear result indicates an antibody population with more than 1 order of combining sites, *right,* straight-line plot indicates an antibody population with a single order of combining sites. \( K \), equilibrium constant; \([\text{Ab}^0]\), molar concentration of antibody-combining sites. (See text for mathematical analysis.)

Chart 2. Graphic analysis used for the determination of the heterogeneity index of the antibody population. The experimental points (*) were analyzed by the method of least squares to obtain the equations for each line (\( X = X \)).

The equilibrium constant for the antibody in R-5 antiserum was similar whether determined by equilibrium dialysis or charcoal separation of bound and free hapten, but the total binding capacity of the antiserum was lower by the former technique. Since the dialysis reactions were incubated for 18 to 20 hr at 37°, some protein denaturation could have reduced the total binding capacity without altering the binding property of the remaining antibody molecules.
can influence the property of the antibody since Rubenstein with correspondingly high binding energies. The method of because, translated into quantitative terms, the biological investigation has shown that the antibodies obtained by this laboratory has demonstrated that antibody-bound folate does not react with the antifolate antibody, a small fraction "immunoreactive," the binding capacity of each of the antiserums tested would have been sufficient to sequester most of the circulating vitamin. Toward this end, we are now studying the response of animals to immunization with a variety of folate analogs in order to obtain a wide-spectrum antiserum.

Although the major fraction of endogenous serum folate does not react with the antifolate antibody, a small fraction does (5). It is, nevertheless, theoretically significant that if all the serum folate were "immunoreactive," the binding capacity of each of the antiserums tested would have been sufficient to sequester most of the circulating vitamin. Toward this end, we are now studying the response of animals to immunization with a variety of folate analogs in order to obtain a wide-spectrum antiserum.

Binding alone, however, will not be the sole criterion to predict whether immunological sequestration will inhibit biological function of the hapten. Probably the most important consideration is the fate of the antibody-bound vitamin when it is cleared from the circulation. Is the folate sequestered as such or does it become attached to the antibody? This question is presently under investigation.

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