Kinetics of Binding of Carrier-Free Ga-67 to Human Transferrin

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A new method has been developed for calculation of a relative intrinsic association constant, K, for the binding of carrier-free gallium-67 to human transferrin. The approach is based on the method of Scatchard, and depends on the relatively large molar abundance of transferrin compared with the molar concentration of carrier-free Ga-67. Values obtained are in the range of K = 2.5 × 10⁵ liters/mol.

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Despite nearly 10 years of clinical research experience with gallium-67 citrate, the biochemical basis for the tumor affinity of gallium is not yet known. In order to understand the mechanism of this tumor affinity, we have been studying the concentration of Ga-67 by tumor cells growing in Waymouth's tissue-culture medium. We have considered the possibility that the biologic properties of carrier-free gallium are determined largely by the degree to which gallium is bound to transferrin, the iron-binding protein of plasma. Information regarding the quantitative interaction between gallium and transferrin is lacking. For this reason, we sought a method that would allow us to measure the binding of Ga-67 to transferrin in Waymouth's medium.

In 1949, Scatchard reported a method for the evaluation of the attractions of proteins for small molecules and ions (*1*). With this technique it is possible to calculate "how many" metal ions are bound, and "how tightly," to a protein molecule. The basic concept requires chemical side chains on the protein molecule to form a functional group (receptor site) that binds metal ions. There may be multiple metal-binding receptor sites on a single protein molecule. According to Scatchard,

"If the various groups on a protein molecule act independently, we can apply the law of mass action, as though each group were on a separate molecule, and the strength of binding can be expressed as a constant for each group" (*1*).

Based on the law of mass action, a mathematical formulation was developed that answered the question "how tightly" in terms of an intrinsic association constant (K), and "how many" in terms of the number of metal ions bound per molecule of protein.

The Scatchard formulation has been used extensively to study binding reactions of diverse sorts in biologic systems. For example, Berson and Yalow (*2–4*) applied this method of radioimmunoassay, to quantitate the binding interaction between small amounts of antigen and antibody. Also, similar kinetic considerations have been used to evaluate the binding of hormones by cellular receptors (*5*).

These same kinetic considerations may be applied to the binding of transferrin and gallium. Usually an experimental approach would be to vary the concentration of stable metal ion at a constant transferrin concentration; then, using a radioactive tracer, to determine the molar concentration of bound gallium at various total concentrations of gallium. There are technical problems with this, however, because of the relative insolubility of gallium with a variety of anions present in serum and various buffers; also, $GaNO_3$, the most suitable salt for such experiments, is extremely hydrophilic, and special methods must be used to make up solutions of accurately known concentration.

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We have developed an alternative approach, which we believe is suitable for evaluation of the binding interaction between carrier-free metal ions and a binding protein with known properties of molecular weight and binding sites. Although in the example below we specifically discuss gallium and transferrin, the description is a general one and depends on the relatively large molar abundance of the binding protein (e.g., transferrin), compared with carrier-free metal (e.g., gallium).

MATERIALS AND METHODS

Theoretical considerations. From the law of mass action:

$$[TF_f] + [Ga_f] \underset{k_2}{\overset{k_1}{\rightleftarrows}} [Ga\text{-}TF] \quad (1)$$

$$K = \frac{k_1}{k_2} = \frac{[Ga\text{-}TF]}{[TF_f]\,[Ga_f]}, \quad (2)$$

where $[TF_f]$ is the molar concentration of unoccupied transferrin binding sites; $[Ga_f]$ is the molar concentration of unbound ("free") gallium; [Ga-TF] is the molar concentration of bound gallium; K is the intrinsic association constant at equilibrium; and k_1 and k_2 are the forward and reverse rate constants, respectively.

The total concentration of transferrin binding sites, [TF], may be defined as follows:

$$[TF] = [TF_f] + [Ga\text{-}TF]. \quad (3)$$

Rearranging (2) above we get:

$$K([TF_f]) = \frac{[Ga\text{-}TF]}{[Ga_f]}, \quad (4)$$

since from (3) above:

$$[TF_f] = [TF] - [Ga\text{-}TF]. \quad (5)$$

Substituting into (4):

$$K([TF] - [Ga\text{-}TF]) = \frac{[Ga\text{-}TF]}{[Ga_f]}. \quad (6)$$

Let B = [Ga-TF] = the molar concentration of gallium bound; F = $[Ga_f]$ = the molar concentration of gallium free; Eq. (6) then becomes

$$\frac{B}{F} = K([TF] - B). \quad (7)$$

Eq. (7) is analogous in every respect to the Berson and Yalow formulation for radioimmunoassay systems (*3*).

There are several underlying assumptions. First, that a single molecule of gallium is bound by a single receptor site. This is analogous to the "univalency" that is assumed for most hormones in radioimmunoassay systems (*3*). In the case of the gallium interaction with transferrin, the "valency" of gallium has been settled. One molecule of Ga^{3+} is bound per binding site and there are two binding sites per transferrin molecule (*6*). Furthermore, the two metal-binding sites for gallium are the same ones that bind other metal ions, including iron. Another assumption is that the two sites are independent of each other—that is, binding to one site does not affect binding to the other site. A third assumption is that the two sites have about the same *K* values.

(Deviation from assumptions 2 and 3 would be expected to introduce nonlinearities into the graphical analysis, which we shall discuss later.)

Suppose we want to determine the binding of Ga-67 ("carrier-free") to transferrin in an experimental system. We add a constant amount of carrier-free Ga-67 to a vessel containing various amounts of transferrin, and we allow the system to reach equilibrium. Using a dialysis system, we separate transferrin-bound Ga-67 (*B*) from unbound Ga-67 (*F*). We can determine the ratio *B/F* from the following considerations. Let $[Ga_t]$ be the total molar concentration of "carrier-free" Ga-67 in the reaction mixture, and *b* equal the fraction of total (radioactive Ga-67 bound. The fraction of (radioactive) Ga-67 free is simply 1-b.

$$\frac{B}{F} = \frac{b\,[Ga_t]}{(1\text{-}b)\,[Ga_t]} = b/(1\text{-}b)$$

The ratio *b*/1-*b* is easy to measure, since *b* is the fraction of total radioactivity (counts) that is bound and 1-*b* is the fraction of total radioactivity (counts) that is free.

Equation (7) then becomes

$$B/F = b/(1\text{-}b) = K([TF] - b[Ga_t]. \quad (8)$$

In Eq. (8), the absolute magnitude of $[Ga_t]$ is not known with certainty, but the order of magnitude is known. For "carrier-free" Ga-67, the specific activity is 1.7×10^{-6} mg/mCi (*7*). In these experiments we use Ga-67 in the 0.5 μCi/ml range, so $[Ga_t] \simeq 10^{-11}$ mol/l. The fraction bound, b, will vary from 0 to 1. Thus, over the entire range of binding, the term $b[Ga_t] \leq 10^{-11}$ mol/l.

The molecular weight of transferrin is 77,000 (*8*), and the number of metal-binding sites per transferrin molecule is two (*6*). This permits us to calculate the molar concentration of gallium-binding sites on transferrin for varying concentrations of transferrin. We wish to study the binding of carrier-free Ga-67 to transferrin, over the range $\simeq 10^{-7}$ to $\simeq 10^{-4}$ mol/l. In this range $[TF] \geq 10{,}000\ b[Ga_t]$. From Eq. (8), we see that the term $b[Ga_t]$ may be neglected and (8) becomes:

$B/F = b/(1 - b) = K([TF] - b[Ga_t]) \simeq K[TF]$. Thus, if $b/(1 - b)$ is plotted against [TF], a linear relationship should be obtained with slope $= K$. The line should pass approximately through the origin.

Experimental studies. In order to evaluate the binding of carrier-free Ga-67 and human transferrin, the following protocol was used.

Carrier-free Ga-67 citrate was added to a total volume of 3 ml of a transferrin solution in Waymouth's tissue-culture medium. The final concentration of Ga-67 was 0.5 μCi/ml, and transferrin concentrations, ranging up to 2.5 mg/ml (6.5×10^{-5} mol/l), were run in duplicate: 0, 0.1, 0.2, 0.75, 1.0, 1.5, 2.0, and 2.5 mg/ml. This mixture was allowed to incubate for 24 hr at 37°C. Apotransferrin* was used.

An equilibrium dialysis cell† was used for these experiments. The membrane was No. 103 (MW cut-off 12–14,000 Daltons) and was pretreated by boiling for 5 min in a 5% sodium carbonate and 1.86% NaEDTA solution. The membrane was then washed twice in distilled water and then soaked for 1 hr in Waymouth's tissue-culture medium.

One-milliliter aliquots of the incubation mixtures were placed in the retentate chamber, using a 20-gauge needle coated with siliclad. To the dialysate chamber, 1 ml of Waymouth's medium was added. The cells were sealed with Dennison dots, and the system was allowed to rotate for 23 hr at room temperature. Sixteen samples were run simultaneously. At the end of the experiment the solution was withdrawn and aliquots of 100 μl were counted. The membrane was also counted.

For the calculation of the bound (B) over the (F) ratios, the radioactivity in the retentate chamber was considered to contain $B + F$ activity, whereas the activity in the dialysis chamber was F only. In this experiment, no correction was made for membrane activity. This varied from a maximum of 9% of the total activity with no added transferrin to 2% of total activity at 2.5 mg/ml.

RESULTS AND DISCUSSION

When B/F [$b/(1 - b)$] is plotted against varying concentrations of transferrin, expressed as molar concentration of transferrin binding sites, [TF], the data are well approximated by a straight line (Fig. 1). Using a least-squares method for linear regression, the relationship was $B/F = 2.5 \times 10^5$ l/mol [TF] − 0.44, with $r = 0.99$. K, the relative association constant at equilibrium, was 2.5×10^5 l/mol.

With this K value it is possible to calculate the fractional association of Ga-67 and TF over a wide range in [TF]. In other words, the K value permits us to calculate, at any given [TF], the fraction of Ga-67 that will be bound.

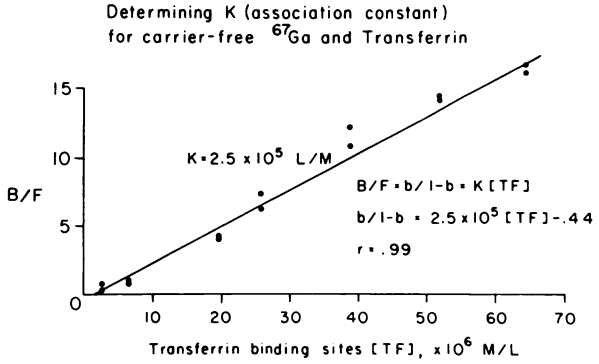

FIG. 1. A plot of B/F (the bound-to-free ratio) against [TF] (molar concentration of transferrin binding sites for Ga-67) can be used to determine K, the intrinsic association constant.

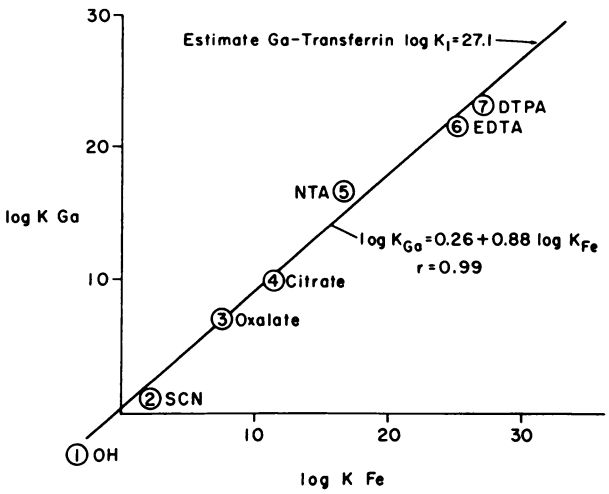

FIG. 2. K, the intrinsic association constant, may be predicted from a plot of log K_{Ga} (log of association constant for gallium) against log K_{Fe} (log of association constant for iron) for a variety of known association constants for the ligand-metal interaction. OH = hydroxide; SCN = thiocyanate; NTA = nitriloacetic acid; EDTA = ethylene diamine tetracetic acid; DTPA = diethylene triamine pentacetic acid.

The K value that we have obtained is considerably lower than what would be expected on purely theoretical grounds. If one follows the analogy of Welch and Welch (*9*) and plots log K_{Fe} against log K_{Ga} for various metal complexes, the K value for the interaction between Ga-67 and TF should approach 10^{25} to 10^{30}. Such a plot is shown in Fig. 2 and is based on known affinity constants for Ga and Fe binding by various ligands (*10,11*).

We believe the difference between the expected and our observed association constants is the result of two factors. First of all, our experiments were performed in Waymouth's tissue-culture medium because of our interest in evaluating the interaction of

carrier-free Ga-67, transferrin, and a tumor cell (EMT-6 sarcoma of BALB/c mice) in the tissue-culture system. Waymouth's medium is a complex solution, with several ligands capable of binding Ga-67. These ligands probably compete with transferrin for Ga-67, thereby lowering the amount of ^{67}Ga-TF complex formed. Thus, the K value that we have determined describes the relative affinity of transferrin for Ga-67 in this complex system. It is this "relative association constant" that has relevance in our complex system, whereas the true value for K, determined in a simple system without competing binders, is probably much higher.

A second factor that might affect our experimentally determined value of K is the tendency for Ga-67 citrate to form polymeric forms and gallium hydroxides in aqueous solutions. Harris and Martell (*10*) were unable to determine K accurately for gallium citrate due to the phenomenon of multinucleate polymeric forms of gallium citrate that are formed at pH 4–5. At physiologic pH, the polymeric forms of gallium citrate are dissociated and become more complex species of gallium citrate and hydroxide. These other forms of gallium could also have an influence on the value of K.

In nuclear medicine, one deals frequently with minute quantities of chemical substances. In the case of cyclotron-produced Ga-67, a zinc target is irradiated to produce Ga-67, and when the Ga-67 decays, Zn-66 is produced. Thus, the Ga-67 is truly carrier-free. Also, no carrier is added during processing of this compound, so the chemical quantity of Ga-67 present is determined by the specific activity of the radionuclide. In these studies, the specific activity of the Ga-67 was 1.7×10^{-6} mg/mCi.

Most studies of Ga-67 binding to transferrin have been qualitative in nature (*12–14*). An exception is the work of Clausen et al. (*15*), who found a K value, 1.772 l/mol, much lower than ours, and who predicted a much higher number of binding sites, 14, than is reported by Harris and Aisen (*6*). Clausen et al., however, worked under conditions different from ours: they used macroscopic quantities of gallium salts (added as $GaNO_3$) and added significant amounts of stable citrate to their system. Citrate is known to reduce the binding of Ga to serum proteins, but only when present in great excess (e.g. 100:1) (*9*). Nonetheless, perhaps differences in scale (macroscopic Ga and citrate), or qualitative differences in transferrin, account for the disagreement with our results.

Our method of calculating the intrinsic association constant is relatively simple and straightforward. By neglecting the term $b[Ga_t]$ in Eq. (8), an error of only 1 part in 10,000 is introduced in determining K. For most purposes, an order of magnitude error in K could probably be tolerated.

In conclusion, a new method has been developed for determination of the relative association constant, K, for the binding of a carrier-free metal ion to a specific metal-binding protein. This technique was used to determine a K value for the interaction of carrier-free Ga-67 and human transferrin in Waymouth's tissue-culture medium. The resulting K value was considerably lower than the value predicted on theoretical grounds for the interaction of gallium and transferrin in a simple solution. We conclude that secondary effects, such as competition with other ligands present in the tissue-culture medium, play a major role in determining the degree of transferrin binding of carrier-free Ga-67.

FOOTNOTES

* >90% iron free, Grade B, Sigma Chemical Co., St. Louis, Mo.

† EMD-101 8-place equilibrium microdialyzer, Hoefer Scientific Instruments, San Francisco, Cal.

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