In Vitro Transfer of Ga-67 from Transferrin to Ferritin

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Equilibrium dialysis was used to examine the binding of Ga-67 to horse spleen ferritin (HFE), and the ability of this protein to remove Ga-67 originally bound to human transferrin (TF). Seventy hours were required for the HFE to bind 70% of the activity. When HFE was placed in competition with preformed TF·Ga-67 complex, little nuclide was translocated to HFE. Upon the addition of compounds of low molecular weight that occur intracellularly, this transfer was dramatically enhanced. In the presence of 1 m*M* adenosine triphosphate (ATP), the most effective mediator examined, the final distribution was 17% bound to TF and 62% to HFE, with 16% not protein-bound. In the absence of any mediator, the same distribution was 84, 6, and 3%. Control experiments with ATP showed that little radionuclide was transferred from TF to albumin. These results add support to the previous suggestions of the potential role of ferritin in Ga-67 localization.

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Ferritin (FE), an important iron-storage protein found in most tissues (*1*) has received little attention with regard to its possible role in Ga-67 localization (*2*). This macromolecule, with a molecular weight near 450,000 daltons (*3*), binds up to 4300 atoms of iron per molecule (*4*). Hegge and coworkers (*5*) have shown that Ga-67 localized in rabbit hepatocytes is incorporated into the FE Fraction. Gallium-67 radioactivity has been associated with a FE-like fraction isolated from animal tumor cells (*6*), cultured tumor cells (*7*), and rabbit uterine tissue (*8*). These findings combined with the knowledge that other iron-binding proteins are likely involved in Ga-67 localization (*2*), suggested that FE may also play a role in localization. There is some evidence that transferrin acts as a delivery vehicle for Ga-67 (*7,9,10*). FE is found primarily intracellularly and therefore would probably function as a sequestering agent, binding Ga-67 brought into the cell. Whether or not these two proteins come in contact is not known. However, there is some evidence that transferrin enters the reticulocyte to deliver iron (*11,12*) and the tumor cell to deliver Ga-67 (*7*). Thus these two proteins may interact directly.

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It is known that when horse spleen ferritin (HFE) and human transferrin (TF) are incubated together, they exchange iron in both directions, although net flow is toward HFE. This exchange occurs whether or not a dialysis membrane separates the two proteins (*13,14*). Chelate molecules of small molecular weight dramatically enhanced the rate and amount of translocation. We have therefore attempted to characterize the transfer of Ga-67 from TF to HFE and the ability of various compounds to influence this transfer.

METHODS

The experiments with equilibrium dialysis were performed as previously described (*15*). The dialyzer consisted of five dialysis cells. In each cell, 1 ml of a 3–4 μM HFE* solution in buffer was added to the right-hand chamber, while 1 ml of buffer containing Ga-67 citrate was placed in the left. In later experiments, however, in an attempt to speed equilibrium, Ga-67 and HFE were added to the same chamber. Before its addition to the dialyzer, the HFE solution was dialyzed against buffer overnight at 4°C to remove any small molecules or peptides present (*1*). To vary the citrate concentration in the sample, an aliquot of a stock citrate solution was added before insertion of the sample into the dialyzer. The buffer was 50 m*M* hydroxyethyl-piperazine-N′-

2-ethane sulfonic acid (HEPES) containing 100 m*M* NaCl and 0.4 m*M* HCO_3^- (pH 7.4), and a small amount of benzyl alcohol as a bacterostatic. At specific times, 20 μl was withdrawn from each chamber using a piece of PE-50 polyethylene tubing attached to an automatic pipettor. The tubing was stretched to form a tip that fitted snugly into the chamber port. The radioactivity measured and the percentage of Ga-67 bound was calculated from:

$$\% \text{ Bound} = \frac{R - L}{R} \times 100$$

where R = activity in the right-hand chamber (HFE-Ga-67 and "free" Ga-67, not protein bound) and L = activity in the left chamber ("free" Ga-67).

In the competition experiments, 5 ml of a 25–30 μM TF* solution was incubated with 15–20 μCi of Ga-67 citrate (citrate <100 μM) for about 10–15 min at 37°C. It was then dialyzed overnight (4°C) with at least three changes of buffer. Routinely >95% of the activity was bound. To achieve a 1 m*M* final concentration of mediating ion, an aliquot (~500 μl) of a 10 m*M* stock solution was added to this sample and 5 ml of buffer. One milliliter of this sample was placed in each right-hand chamber of the five dialyzer cells, while 1 ml of buffer was placed in the left chamber. The TF was allowed to equilibrate with the mediating ion in the dialyzer for 1 hr. Then, 100 μl of a tenfold concentrated solution containing the competing protein—either HFE or bovine serum albumin (BSA)*—was added to the left-hand chambers of three cells to yield the appropriate final concentration, and 100 μl of buffer was added to the right. In the two other cells, only 100 μl of buffer was added. The time on the abscissa of the graphs represents time after the HFE addition. As above, the radioactivity in each chamber was measured as a function of time. Since protein was present in both chambers, the amount of protein-bound activity could not be directly ascertained. A difference percentage bound (D%B) was computed from the equation:

$$D\%B = R - L/R + L \times 100$$

[The parameter was previously designated "% bound" (*15*) but has been changed to avoid confusion]. Since the right-hand chamber contained TF-Ga-67 + "free" Ga-67, and the left contained HFE-Ga-67 + "free" Ga-67, this implies that

$$D\%B = \frac{\text{TF-Ga-67} - \text{HFE-Ga-67}}{\text{TF-Ga-67} + \text{FE-Ga-67} + 2\ (\text{"free" Ga-67})} \times 100$$

Thus, this parameter is a measure of the shift of radionuclide from TF to HFE.

The equilibrium time was estimated from the plateau of the D%B values. The experiments were carried out for at least 100 hr to ensure attainment of equilibrium. To ascertain the fractions of activity in the TF in competition with HFE, the HFE, and chelate-bound fractions after equilibrium dialysis, an aliquot of each was chromatographed on a column containing G-50 Sephadex gel. Control experiments showed that <1% of the Ga-67 activity chromatographed with BSA. The percentage values shown in Tables 1 and 2 were highly reproducible,

TABLE 1. EFFECT OF VARIOUS MEDIATORS ON TRANSFER OF Ga-67 FROM TF TO HFE

Mediating agents*	Ga-67 distribution at end of equilibrium dialysis experiment: TF %	HFE %	Non-protein %	Equilibrium time (hr)‡
None	98	NF†	2	90
	84	6	3	
ATP	72	NF	28	60
	17	62	16	
ATP Control	66	NF	34	no change
	73	4§	19	
Ascorbate	98	NF	2	54
	22	48	14	
Citrate	86	NF	14	25
	39	41	14	
Lactate	93	NF	7	11
	68	13	8	
EDTA	92	NF	8	11
	66	16	10	

* = mediator concentration 1 m*M*.
† = no HFE present.
‡ = estimated from plateau in curves of D%B vs. time.
§ = BSA used as competing protein.

TABLE 2. EFFECT OF VARIOUS PHOSPHATE-CONTAINING COMPOUNDS ON THE TRANSFER OF Ga-67 FROM TF TO HFE

Mediating agents*	Ga-67 distribution at end of equilibrium dialysis experiment: TF %	HFE %	Non-protein %	Equilibrium Time (hr)‡
None	98	NF†	2	90
	84	6	3	
ATP	72	NF	28	60
	17	62	16	
ADP	85	NF	15	25
	41	41	12	
AMP	95	NF	5	47
	54	16	16	
Adenosine	95	NF	5	74
	46	27	12	
Pyrophosphate	31	NF	69	no change
	28	0.7	57	
Orthophosphate	94	NF	6	7
	58	27	8	

* = mediator concentration 1 m*M*.
† = no HFE present.
‡ = estimated from plateau in curves of D%B vs. time.

and the estimated error was ~ ± 5%. The iron saturations of TF and HFE were determined to be 7.5 and 2.5%, respectively (*14*).

RESULTS

Figure 1 shows that a solution of HFE bound a large fraction of the Ga-67 after a prolonged incubation. The differences of citrate-ion concentration, 12 μM compared with 1 m*M*, had no significant influence on either the kinetics of binding or amount bound. This suggested a relatively tight binding of HFE to Ga-67, since the presence of a large excess of citrate ions did not compete at those concentrations. As a control, the ability of BSA to bind Ga-67 was examined over a similar length of time (data not shown). BSA bound little activity (2.2%) adding support to the specific nature of the Ga-67-HFE interaction.

To examine further the relative stability of the HFE-Ga-67 complex, and to ascertain the ability of HFE to remove and sequester Ga-67 brought to the cell by TF, the TF-Ga-67 complex was challenged by HFE. With a TF/HFE ratio of 10/1 and in the absence of any mediating ions, there was a small but significant transfer of Ga-67 from TF to HFE relative to the unchallenged TF-Ga-67 complex (Fig. 2). However, when the incubation mixture contained 1 m*M* adenosine triphosphate (ATP) as a mediating ion, there was a dramatic shift of activity from TF to HFE (Fig. 3A). The TF in competition with HFE lost Ga-67, whereas the D%B in the unchallenged TF remained constant. When BSA was used as the competing protein, there was no apparent transfer of radiogallium from TF to BSA in the presence of 1 m*M* ATP (Fig. 3, bottom). The challenged and unchallenged D%B values did not differ significantly.

When the samples from the challenge experiments were chromatographed to ascertain the actual % bound, ATP was the most effective mediator examined (Table 1). One-millimolar ATP added to the incubation mixture decreased the Ga-67 bound to TF from 72% to 17%, while increasing the HFE-bound Ga-67 to 62%. This

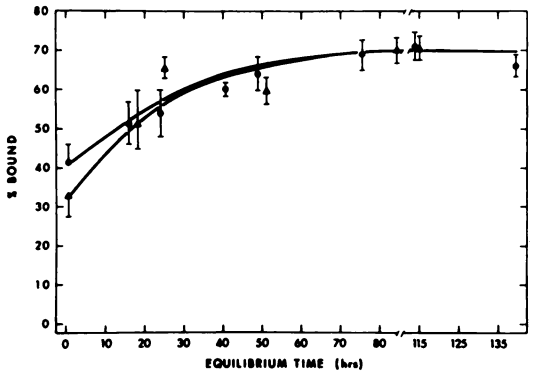


FIG. 1. Equilibrium dialysis of Ga-67 incubated with 3.8 μM horse spleen ferritin (HFE) in presence of 12 μM (▲) or 1 m*M* (●) sodium citrate. Incubation temperature was 37°C, buffer was 50 m*M* HEPES containing 100 m*M* NaCl and 0.4 m*M* HCO_3^- (pH 7.4), and Ga-67 concentration was 1.6 μCi/ml. Points and error bars represent means and s.e.m. (n = 5).

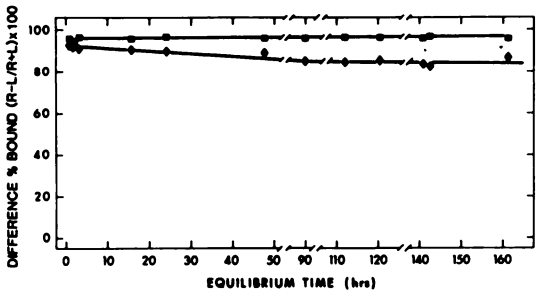
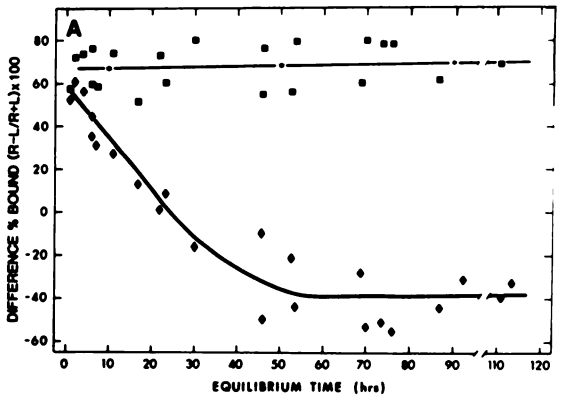


FIG. 2. Equilibrium dialysis of preformed 25 μM transferrin (TF) solution labeled with Ga-67, containing 2.6 μCi Ga-67/ml. This was challenged by buffer (■) and by 2.8 μM HFE (◆). R and L are radioactivity in dialysis chambers that contained TF + Ga-67 and HFE or buffer + Ga-67, respectively. Points represent means of at least duplicate samples. Other conditions described in Figure 1 legend.



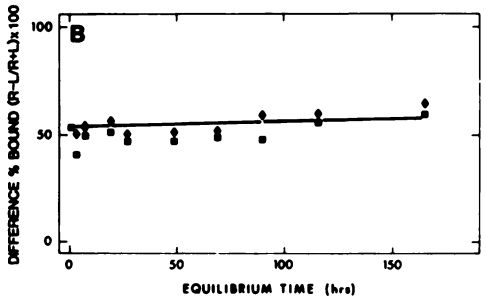


FIG. 3. Equilibrium dialysis of preformed TF-Ga-67 (27 μM) solution containing 2.3–3.1 μCi/ml. This was challenged in presence 1 mM ATP by: A. buffer (■) and 2.8 μM HFE (◆). B. buffer (■) and 45 μM bovine serum albumin (◆). R and L are defined in Fig. 2. Points represent means of at least duplicate samples. Other conditions described in Figure 1 legend.

process came to equilibrium at about 60 hr. It appears to be a specific effect due to the presence of HFE. In a control experiment, only a small amount of Ga-67 was translocated to BSA when the latter was substituted as the competing protein. The ability of ATP to stimulate radionuclide movement contrasts sharply with that in an experiment completed in the absence of any mediating ions. Only about 6% of the activity was translocated from TF to HFE, and most of the activity remained bound to TF (Table 1). Moreover, the time required to achieve equilibrium was considerably longer. Ascorbic acid was the most potent of the nonphosphate compounds, but was somewhat less efficient than ATP. Lactate, present in high concentrations in some tumor cells, was an ineffective mediator. In addition, EDTA, a compound with a high affinity for Ga-67, was also relatively ineffective.

The surprising effect of ATP led us to examine the influence of various phosphate-containing compounds on Ga-67 transfer. Comparing the nucleotides ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP), the amount of radiogallium transfer stimulated decreased concomitant with the phosphate content (Table 2). ATP was the most effective, whereas AMP was the least. Adenosine and orthophosphate, components of the nucleotides, were less effective than ADP or ATP. This suggested that the diphosphate structure was important in the mediation process. Lastly, pyrophosphate (PPi) at 1 mM concentration, removed a large fraction of Ga-67 from TF, but no significant activity was transferred to HFE (Table 2).

DISCUSSION

There is already evidence indicating that FE plays some role in Ga-67 localization. For example, a major component of a crude tissue extract derived from human lung cancer possessed electrophoretic mobility like that of HFE (*16*). This component also contained 33% of the Ga-67 activity. In animal tumor models, Ga-67 activity is significant in the protein fractions, with a molecular weight >250,000 daltons, isolated from the cell lysates. The protein in these fractions could have been FE (*6,7*). Hegge and co-workers (*5*) have shown that the Ga-67 is incorporated in significant amounts by a crude FE fraction prepared from rabbit hepatocytes. This fraction was obtained by $CdSO_4$ precipitation from a cytosolic protein solution. In contrast, recently Samezima et al. (*17*) have reported that only a small quantity of Ga-67 was bound to a crude FE fraction from rat liver. This fraction was isolated on a sucrose density gradient. Why little Ga-67 was incorporated into rat liver relative to rabbit liver is unclear, but it may be related to the crude nature of the preparations or the difference in the isolation procedures. Our results are the first to demonstrate in vitro the relatively high affinity of Ga-67 for highly purified HFE. These results suggest that the protein fraction of high molecular weight and crude rabbit FE (cited above) contained FE, although conclusive proof is still required. Our findings, taken together with the earlier data, provide additional support for the potential role of FE.

How would FE function in Ga-67 localization? Since FE is primarily intracellular, it is likely that Ga-67 would enter the cell, be bound to FE, and then the FE-Ga-67 complex would be incorporated into primary or secondary lysosomes (*18*). This scenario parallels the general mechanism in which iron is incorporated into normal cells (*19*). Exactly in what form the nuclide enters the tumor cell is in question. Evidence derived from experiments with tumor-cell cultures suggests the TF delivers the Ga-67 to the cell (*7,9,10,20*). In a rat-tumor model, by contrast, the saturation of the TF metal-binding sites with iron does not preclude Ga-67 incorporation into the tumor (*21*). Moreover, Sephton and Martin (*22*) have shown in a few patients that tumor masses can be imaged after Ga-67 injection even though a patient's unsaturated iron-binding capacity is significantly reduced. These data suggest Ga-67 not bound to TF is important for tumor localization. In either form, our data show that FE would be able to sequester the radionuclide delivered to the cell. Thus the concentration of FE could play a role in increased Ga-67 concentration in neoplastic and inflammatory lesions. FE concentration is considerably increased in the blood cells from patients with a variety of leukemias (*23*). Increased concentration of tissue FE has been demonstrated in patients with Hodgkin's Disease (*24–26*). The lesions in this disease are known to be gallium-positive (*27*).

Why FE would be increased or changed in neoplastic transformation is unclear. However, de Sousa and coworkers (*26,28*) have suggested that abnormal iron metabolism is the basic defect in lymphoreticular diseases. In agreement with this hypothesis, Broxmeyer and co-workers (*29,30*) have provided in vitro evidence that both FE and lactoferrin may participate as physiological regulators of granulocyte and monocyte proliferation. Thus, all iron-binding macromolecules—lactoferrin, FE, and TF—would play a role in these diseases and be involved in Ga-67 deposition and sequestration.

Our results clearly show that Ga-67 binds to HFE. Preliminary experiments suggest that HFE has greater affinity for gallium than EDTA (Weiner, unpublished results). Initially we were somewhat surprised by these findings. Iron must be in the ferrous state before it is eventually oxidized and rapidly incorporated into the ferric hydroxide micell in the protein's interior (*31*). One important distinction between iron and gallium is the unlikelihood that gallium could undergo such changes in oxidation state (*32*). However, there is evidence that small amounts of ferric iron (up to 200 atoms/molecule) can slowly bind to HFE (*33*). Therefore, if gallium and iron share similar binding characteristics (as we believe), our data appear confirmatory.

It was puzzling how gallium or iron could be rapidly translocated from TF to another protein, even one with a higher affinity, because of the tightness of the metal-to-TF bond (*34*). The presence of these small molecules appears to overcome, in some manner, the kinetic barrier to the release of the metal ion, and thus allow transfer. The translocation mechanism probably involves a number of factors. First is the ability of the mediator to bind the nuclide and transport it between the two proteins. The most effective transfer agent in this study, ATP, is known to form a low-affinity complex with gallium (*35,36*, and Weiner, unpublished results). If we examine the transfer ability of all the nucleotides, the amount of activity transferred decreases as the number of phosphate moieties is reduced. This transfer ability parallels the stability constants of these nucleotides for a wide variety of transition-metal ions, including iron (*37*). Also, since the metal affinity is not related to the base present, other nucleoside-triphosphates would probably be as effective as ATP.

Complex formation is not the only criterion, since EDTA does not fit this pattern. This molecule has a high affinity for gallium but transfers little radionuclide. EDTA is ineffective in transferring iron between TF and HFE (*13*). Part of the difficulty may be that, because of some steric barrier, EDTA cannot donate the metals to a site on the HFE molecule. Alternatively, the Ga-EDTA complex is resistant to isotopic exchange at neutral pH, and this kinetic barrier may prevent efficient transfer (*38*). The results observed with PP_i appear to be unique. Even though PP_i was more effective than EDTA in dissociating Ga-67 from TF, none ended up bound to HFE. At high concentrations (>1 m*M*) it is also comparatively poor as a transfer agent for iron (*31*). Thus, under our conditions (1 m*M* PP_i), it is likely that this chelator formed a Ga-67 complex that could not donate the metal to HFE.

Probably the most important aspect of the transfer mechanism is the ability of various compounds, particularly the organic phosphates, to destabilize the metal-ligand coordination on TF. Most phosphate compounds tested increased the concentration of chelate-bound Ga-67 (Table 2). Moreover, PP_i, ATP, and other nucleoside triphosphates are extremely effective in enhancing the release of iron from TF (*1,31,39*). This suggests the diphosphate structure is important in the process. Exactly how this destabilization is caused is unknown, but a number of factors have been considered (*1*). These include induced conformational changes, enhancement of the carbonate exchange rate (the carbonate oxygen is directly coordinated to the metal ion), and lastly, ligand exchange at the freely exchangeable water ligand. Each of these factors could weaken the metal-to-protein ligand bonds and provide for increased free metal ion to complex with a suitable acceptor.

The ability of the compounds studied to stimulate radionuclide translocation suggests that the presence of these compounds, concomitant with increased FE concentration, may play some role in Ga-67 accumulation. Increased levels of ascorbic acid have been reported in

malignant epithelial tumors when compared with the surrounding tissue (*40*). ATP and ADP are present in high concentrations (2-15 m*M*) in a variety of cells, including those of the Ehrlich ascites tumor (*41*). But their precise concentrations in other tumor cells, and how that relates to Ga-67 uptake, is unknown. A number of these compounds are present in most cells, and they could at least act additively and possibly in a synergistic manner.

FOOTNOTES

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