Radiogallium Localization in Tumors: Blood Binding and Transport and the Role of Transferrin

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As a crucial step toward the understanding of the tumor localization of gallium, we have re-investigated its binding and transport in blood. The studies were performed in vivo by injection of gallium-67 citrate in rabbits, and in vitro by incubation of gallium-67 citrate with individual plasma proteins. By ultrafiltration and gel filtration chromatography, rabbit plasma samples showed essentially complete protein binding, whereas dialysis indicated considerable nonprotein-bound gallium, the amount depending on the dialysis medium. According to electrophoresis, total binding was inversely proportional to electrophoresis time. Affinity chromatography showed all gallium to be bound to transferrin, whereas electrophoresis caused continuous dissociation of gallium from transferrin, with the resulting unbound radioactivity appearing in various other protein bands. Similarly, the binding of gallium to transferrin in the in vitro incubation studies was inversely proportional to electrophoresis time, whereas ultrafiltration and gel filtration chromatography showed all gallium to be transferrin-bound. No binding of gallium to other proteins, such as albumin, was observed. This study demonstrates that gallium at the tracer level in blood is exclusively bound to and transported by transferrin, and indicates that electrophoresis and dialysis of easily dissociable metal complexes are subject to significant artifacts. Accurate determination of protein binding of radiopharmaceuticals requires a combination of analytical techniques and cautious interpretation of the results.

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Gallium-67 citrate localizes in a wide variety of soft-tissue tumors and abscesses, and has been used as a diagnostic imaging agent in clinical nuclear medicine for a decade. Several studies have attempted to elucidate the in vivo behavior of gallium citrate, but the mechanism of its tumor localization remains uncertain. Investigations of even such fundamental properties as blood binding and transport of gallium have yielded divergent results, ranging from little or no interaction of gallium with plasma proteins to varying degrees of association of gallium with albumin, transferrin, and haptoglobin. Hartman and Hayes (*1*) first demonstrated that gallium in blood is bound to plasma proteins, and suggested (*2*) that alpha and beta globulins, particularly transferrin, are the active components. Gunasekera et al. (*3*) reported that gallium in blood remains mainly associated with serum proteins, with 70% apparently loosely associated with albumin, transferrin, and haptoglobin. In contrast, Hara (*4*) concluded that in blood gallium exists mostly in a nonprotein-bound form and is only partially associated with transferrin. Although the specific nature of gallium binding to any particular plasma protein was not established, Clausen et al. (*5*) showed that gallium in blood is firmly bound to transferrin, which has 14 binding sites for gallium. Larson (*6*), however, reported

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that transferrin has only two binding sites for gallium and that these two are the same ones that bind other metal ions. Many investigators have suggested that plasma-protein binding plays a major role in the transport of gallium to the target tissue. Harris and Sephton (*7*) have shown that the uptake of gallium by cultured tumor cells was greatly enhanced and also stimulated by serum transferrin. Larson (*8*, *9*) reported a transferrin-mediated uptake of gallium by EMT-6 sarcoma tumor cells, both in vitro and in vivo. In contrast, Anghileri et al. (*10*) suggested that competitive binding of gallium at calcium and magnesium binding sites, rather than a metabolic process, is involved in gallium localization.

In the light of this ambiguity and conflicting evidence, we have re-investigated the in vivo behavior of gallium citrate. The present study was aimed at characterizing the binding of gallium in the blood and the transport of gallium to the target tissue (*11*).

MATERIALS AND METHODS

Gallium-67 citrate, without added carrier, was injected intravenously into 25 male New Zealand white rabbits at 1 mCi/kg. The syringes were weighed before and after injection, and weighed aliquots of the injected dose were retained as counting standards. Serial blood samples were drawn into heparinized tubes at 15 min and at 3, 4, and 24 hr after injection. The samples were centrifuged at 2000 rpm for 10 min and weighed aliquots of the packed cells and supernatant plasma were taken. The samples and standard were counted in a scintillation well counter, with the results expressed as percent injected dose per gram sample. From these data and the plasma volumes of the animals the total radioactivity in the plasma at each time period was determined, as was the relative distribution of activity between the plasma and packed cells. The remaining plasma from each blood sample was subjected to a number of tests to determine total protein binding of gallium as well as relative distribution of gallium among the major protein fractions. Due to logistical limitations, not all tests were performed on each plasma sample. The 15-min time interval was examined more extensively than the others, since preliminary results indicated no significant differences at later times.

Total protein binding: ultrafiltration. A 0.2-ml sample of plasma was placed in a membrane ultrafiltration cone* having a 95% retention for molecules above 25,000 daltons. The cones were centrifuged at 2500–3000 rpm for 15 min and centrifuged again after addition of 0.2 ml water (pH adjusted to 7.5), following which the filtrate and the residue were counted. Total protein binding of gallium was calculated as percent of the radioactivity in the applied plasma sample that was retained in the cone. As a control, a similar procedure was followed for Ga-67 citrate alone and the percent gallium activity bound to the cone was determined.

Total protein binding: dialysis. A 1-ml sample of plasma was dialyzed at 4°C for 24 hour in normal saline and in 0.01 *M* sodium phosphate and 0.15 *M* sodium chloride buffer, both adjusted to pH 7.4, using a dialysis membrane that retained molecules above 12,000 daltons. Total protein binding of gallium was calculated as percent of the applied plasma radioactivity that was retained inside the membrane. As a control, Ga-67 citrate alone was dialyzed in saline and in the phosphate buffer, and the percent gallium retained in the bag was determined in both cases.

Total protein binding: gel filtration chromatography. A 1.5 × 30-cm Sephadex G-50 gel column was prepared and equilibrated with a 0.005 *M* sodium bicarbonate buffer, pH 7.5. A 1-mg sample of transferrin was passed through the column as a routine precaution to block any protein binding sites on the gel. A 0.1-ml sample of plasma was applied to the column and eluted with the bicarbonate buffer. The column eluate was consecutively monitored on-line by an ultraviolet spectrometer and a scintillation counter, the signals from the two detectors being displayed on a chart recorder. As a control, the elution pattern of Ga-67 citrate alone was determined. Total protein binding of gallium was calculated as percent of the applied radioactivity that appeared in the protein region at the void volume. The total radioactivity recovered from the column, expressed as a percent of the applied activity, was also determined.

Distribution among plasma proteins: agarose-film electrophoresis. Duplicate 1-μl plasma samples were applied to an agarose film plate. Electrophoresis was run at a constant current of 2 mA per sample at 4°C in 0.001 *M* barbital buffer, pH 8.6, for 15, 30, 45, and 60 min. As a control, the electrophoretic behavior of Ga-67 citrate alone was determined for each of the four time periods. At the end of each run, one of the duplicate electrophoretograms was stained in amido black, destained in 7% acetic acid, and aligned with the unstained electrophoretogram to mark the location of the six protein bands (albumin, and α_1, α_2, β_1, β_2, and γ globulins). The bands were individually cut out and counted, along with any radioactivity that had migrated beyond the albumin band. Relative distribution of gallium in the applied plasma sample was calculated as percent of the radioactivity that appeared in each electrophoretic fraction. The total radioactivity recovered on the agarose plate, expressed as a percent of the applied activity, was also determined. To test the effect of dialysis on the distribution of gallium among the protein fractions, plasma samples were first dialyzed in saline at 4°C for 24 hr and then electrophoresed under the same conditions.

Specific transferrin binding: affinity chromatography. The IgG fraction of goat anti-rabbit transferrin antiserum† (4.3 mg) was incubated with cyanogen-bromide-activated Sepharose 4B‡ (1 g) for 2 hr in a coupling

buffer of 0.1 *M* sodium bicarbonate and 0.5 *M* sodium chloride, pH 8.3. Unbound antibody was removed by washing with coupling buffer, after which the unoccupied binding sites on the Sepharose gel were inactivated by incubation overnight with 1 *M* glycine, pH 8.5. The gel was then washed with additional coupling buffer and finally with alternate portions of 0.1 *M* acetate buffer (pH 4.0) and 0.1 *M* borate buffer (pH 8.0). Columns with 2-ml bed volumes were prepared from disposable 3-ml plastic syringes. A 0.05-ml plasma sample was applied to the column and incubated with the gel for 15 min, followed by elution with normal saline (pH adjusted to 8.0) in 1-ml increments until no further radioactivity appeared in the eluate. As a control, Ga-67 citrate alone was applied to the column and eluted in the same fashion. Gallium binding to transferrin was calculated as percent of the applied radioactivity that was retained on the column. The efficiency of transferrin retention by the column was determined to be 98% using radioiodinated transferrin. To dissociate the antigen-antibody complex and recover the gallium activity retained by the gel, the column was thoroughly washed with 3 *M* guanidine hydrochloride (pH adjusted to 2.0).

In vitro binding to transferrin and albumin. Transferrin (0.2% w/v) and albumin (4% w/v) were prepared in a plasma-water medium (pH 7.4–7.6) obtained by ultrafiltration of normal rabbit plasma. Each solution was incubated separately with Ga-67 citrate (10 μCi/ml) for 3–4 hr. Aliquots of each incubation mixture were then electrophoresed as described previously. A sample of the gallium-transferrin mixture was also subjected to ultrafiltration and gel filtration chromatography under the conditions described earlier. To test the effect of the electrophoretic conditions on gallium binding, a 5-mA current was passed for 5 min between two platinum wires immersed in a portion of the gallium-transferrin incubation mixture. An aliquot was then electrophoresed in the usual way.

RESULTS

At all time intervals examined after injection of Ga-67 citrate in the rabbits, at least 99% of the whole-blood activity was present in the plasma. The total plasma activity fell from an initial (15 min) value of 57% of the injected dose to 7% by 24 hr. The results of ultrafiltration and gel filtration chromatography over this period indicated most of the gallium to be protein-bound, whereas dialysis results, especially in the phosphate buffer, suggested significant amounts of free gallium. In the control ultrafiltration experiments with Ga-67 citrate alone, the membrane cones retained 12% of the gallium activity. In the control dialysis in saline, only 50% of the gallium activity was dialyzable, while control dialysis in phosphate buffer resulted in essentially complete removal of gallium activity from the dialysis bag. On the gel-filtration column, gallium citrate alone showed a single distinct peak, well resolved from the protein peak at the void volume. The plasma-protein binding of gallium as determined by ultrafiltration and dialysis is summarized in Table 1, and the gel-filtration results are shown in Fig. 1.

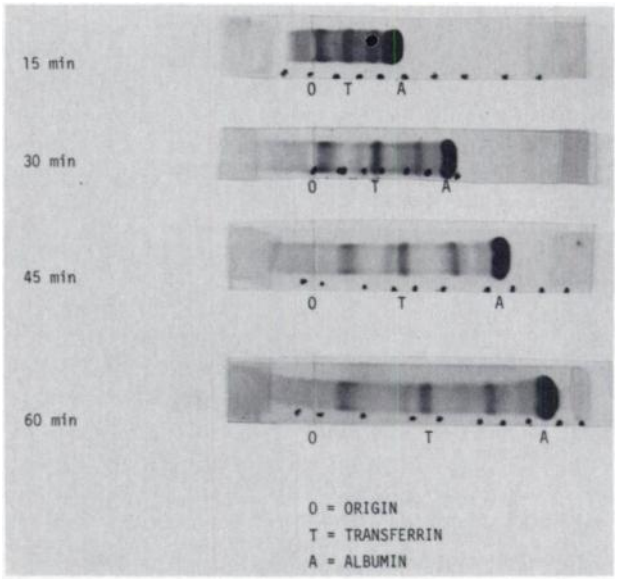


FIG. 1. Fractionation pattern of rabbit plasma obtained by agarose gel electrophoresis for various times.

TABLE 1. PLASMA-PROTEIN BINDING OF GALLIUM-67 CITRATE, BASED ON DIALYSIS AND ULTRAFILTRATION. PLASMA SAMPLES OBTAINED AFTER I.V. INJECTION OF GALLIUM-67 CITRATE INTO RABBITS

Time of blood sample	% injected dose in plasma*	% bound to plasma proteins as determined by		
		ultrafiltration†	24-hr dialysis* in 0.9% saline	24-hr dialysis* in 0.01 *M* phosphate buffer
15 min	57	98	79	62
3 hr	26	99	73	56
24 hr	7	99	87	65

* Five determinations.
†Ten determinations.

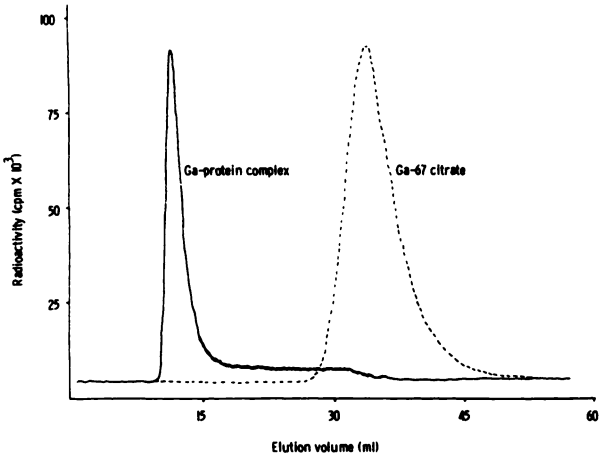
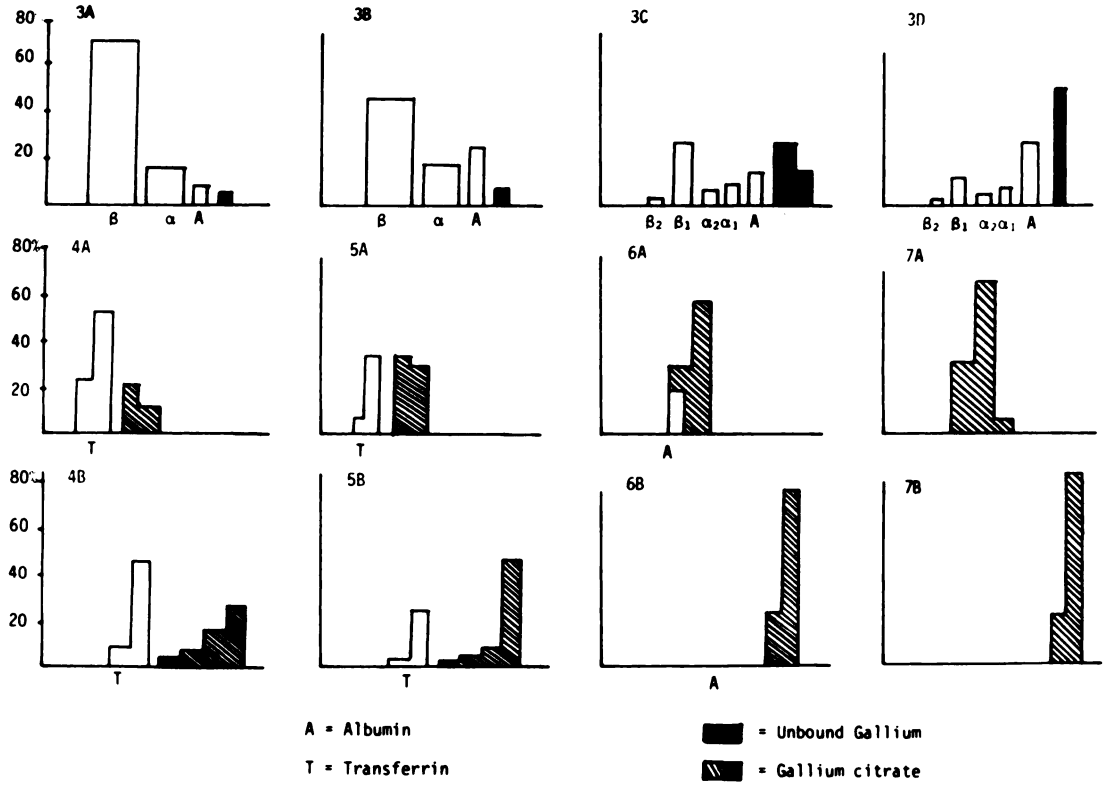


FIG. 2. Typical Sephadex G-50 gel filtration chromatogram (solid line) obtained both with in vivo gallium-plasma samples and with in vitro gallium-transferrin incubation mixture. Dotted line shows elution pattern of gallium citrate alone. Protein binding was calculated to be (86.34 ± 1.00)% for plasma sample and (90.49 ± 3.67)% for transferrin incubation mixture.

The characteristic plasma-protein fractionation patterns obtained in the electrophoretic studies at varying electrophoresis times are depicted in Fig. 2. Although higher resolution was obtainable at electrophoresis times of 45 or 60 min, four major fractions of protein could be discerned with as little as 15 min of electrophoresis. Radioactivity with greater mobility than albumin was considered unbound Ga-67. The apparent relative distribution of gallium among the various fractions was strongly dependent on electrophoresis time. Upon electrophoresis for 15 min, 71% of the gallium radioactivity was associated with the β fraction, whereas 14% appeared at the α band, 9% at the albumin band, and 6% beyond. After 30 min of electrophoresis, the analogous figures were 44, 19, 24, and 12%. After 45 min, the β_1 fraction (transferrin) retained only 26% of the gallium, and the unbound activity increased to 42%. After 60 min of electrophoresis, gallium activity associated with the β_1 band decreased further to 13%, and unbound activity increased further to 50%. These data are presented in summary form in Table 2 and depicted graphically in Figs. 3A-D.

The apparent total protein binding of gallium represented by the sum of the radioactivities in all protein fractions also varied considerably with electrophoresis time (Table 2). This total amounted to 94% of the applied activity after 15 min of electrophoresis but dropped to only 50% after 60 min. Dialysis of plasma samples before electrophoresis had little effect on the electrophoresis results, as summarized in Table 3. In all of these electrophoresis experiments, 100% of the radioactivity



FIGS. 3A-7B. Summary of electrophoresis results. Migration patterns and percent applied radioactivity appearing in fractions recovered from agarose gel electrophoretograms. 3A-3D: Rabbit plasma at 15, 30, 45, and 60 min, respectively. 4A-4B: Transferrin at 15 and 30 min. 5A-5B: Transferrin at 15 and 30 min following 5 min exposure to electric field. 6A-6B: Albumin at 15 and 30 min. 7A-7B: Gallium-67 citrate at 15 and 30 min.

TABLE 2. AGAROSE ELECTROPHORESIS* OF PLASMA OBTAINED AFTER I.V. INJECTION OF GALLIUM-67 CITRATE INTO RABBITS: GALLIUM DISTRIBUTION (% ± s.d.) AMONG VARIOUS PLASMA PROTEINS

Electrophoresis time	% Total protein binding	% Activity associated with each protein band					% Unbound activity
		γ β_2	β_1	α_2	α_1	Albumin	
15 min	93.7 ± 1.2	70.7 ± 3.3		13.7 ± 2.7		9.2 ± 1.9	6.2 ± 1.2
30 min	87.5 ± 3.0	44.2 ± 5.1		19.2 ± 3.3		24.0 ± 2.9	12.5 ± 3.0
45 min	58.2 ± 3.1	3.2 ± 0.6	25.7 ± 3.0	6.4 ± 1.3	8.5 ± 1.3	14.2 ± 1.7	41.7 ± 3.1
60 min	50.1 ± 5.7	2.1 ± 0.8	12.7 ± 2.5	4.5 ± 1.2	7.5 ± 1.3	23.2 ± 2.9	49.8 ± 5.7

* 12 determinations.

applied initially to the agarose plate was accounted for on the plate at the end of the run.

Similar electrophoretic behavior was observed in the in vitro incubation studies. A 15-min electrophoretogram of the gallium-transferrin incubation mixture showed 68% binding, which decreased to 48% after 30 min, 30% after 45 min, and 17% after 60 min (Figs. 4A and B). The results for 45- and 60-min electrophoresis are not shown in the figures, since for these times free gallium activity migrated off the anodic end of the agarose plate. Ultrafiltration and gel filtration chromatography of the same gallium-transferrin incubation mixture showed most of the gallium to be bound to transferrin. Passage of electric current through the mixture before electrophoresis decreased the binding to transferrin (Figs. 5A and B). Electrophoresis of the gallium-albumin incubation mixture showed no significant binding to albumin at any time period (Figs. 6A and B). The control runs with Ga-67 citrate alone, at electrophoresis times of 15 and 30 min, are shown in Figs. 7A and B; at 45 and 60 min the gallium activity migrated off the anodic end of the agarose plate.

In the affinity chromatography experiments, 96% of the applied plasma radioactivity was retained by the column and thus was bound to the transferrin. When Ga-67 citrate alone was applied to the affinity column, less than 2% of the radioactivity was retained. All radioactivity retained by the column from the gallium-plasma experiments was recovered by washing the column with 3 *M* guanidine hydrochloride. The affinity chromatography findings and a general summary of all the protein-binding results are presented in Table 4.

DISCUSSION

The apparent total protein binding of gallium, and its relative distribution among protein fractions, are both strongly dependent on the analytical techniques employed, some of which are subject to significant artifacts. Electrophoresis, especially, can cause serious problems of interpretation with an easily dissociated complex. When a plasma sample, obtained after Ga-67 citrate administration, was electrophoresed for increasing periods of time, the amount of bound gallium decreased. Ultrafiltration and gel filtration chromatography of the same sample, however, demonstrated almost complete protein binding of gallium. Increasing the electrophoresis time also caused different apparent distributions of gallium among the protein fractions, whereas affinity chromatography of the same plasma sample demonstrated binding only to transferrin. Evidently the electrophoretic conditions cause continuous dissociation of gallium from the initial transferrin-bound state. The resulting free gallium, the nature of which is uncertain, continuously migrates along the electrophoresis plate and appears in the various protein bands when they are cut off from the plate and counted.

The in vitro protein-binding patterns reflected similar behavior. In the gallium-transferrin incubation mixture, the amount of gallium bound to transferrin decreased with increasing electrophoresis time, whereas ultrafiltration and gel filtration chromatography showed nearly all gallium to be bound. Very little gallium was bound to albumin in vitro after electrophoresis for only 15 min, and complete dissociation occurred as electrophoresis time increased further. The decreased binding of gallium to transferrin after passage of an electric current through

TABLE 3. EFFECT OF DIALYSIS* ON PLASMA-PROTEIN BINDING OF GALLIUM-67 CITRATE

Plasma protein	% (± s.d.) associated with each protein band after 45-min electrophoresis	
	Before dialysis	After dialysis (in 0.9% saline)
γ-globulin		
β_2-globulin	4.2 ± 0.7	3 ± 0.8
β_1-globulin	25.5 ± 2.9	30 ± 2.8
α_2-globulin	9.3 ± 1.5	6.2 ± 1.1
α_1-globulin	10 ± 1.3	14 ± 1.9
Albumin	15 ± 2.1	19 ± 1.8
Unbound	36 ± 4.1	27.8 ± 2.6

* Six determinations.

TABLE 4. SUMMARY OF PLASMA-PROTEIN BINDING (% ± s.d.) OF GALLIUM-67 CITRATE

Method	In vivo: plasma sample obtained 15 min after i.v. injection of gallium citrate into rabbit		In vitro: gallium citrate incubated with transferrin
	% total protein binding	% binding to transferrin	% binding to transferrin
1. Electrophoresis*			
15 min	93.7 ± 1.2	70.7 ± 3.3	68 ± 4.5
30 min	87.5 ± 3.0	44.2 ± 5.1	48 ± 3.9
45 min	58.2 ± 3.1	25.7 ± 3.0	30 ± 4.1
60 min	50.1 ± 5.7	12.7 ± 2.5	17 ± 3.1
2. Ultrafiltration†	98.22 ± 0.89		98 ± 0.26
3. Gel filtration chromatography‡	86.34 ± 1.00		90.49 ± 3.67
4. Affinity chromatography‡		95.78 ± 1.86	

* 12 determinations.
† Ten determinations.
‡ Five determinations.

the incubation mixture suggests that current causes a major perturbation, leading to dissociation during electrophoresis. The nature and pH of the electrophoresis buffer may also influence dissociation.

If there were actually any nonprotein-bound gallium present in the plasma samples, it should have been removed by dialysis. Electrophoresis before and after dialysis gave nearly identical results, however, suggesting that the free gallium that appeared on the electrophoresis plate was formed during the electrophoresis itself. Dialysis, too, is subject to artifacts related to dissociation. Loss of an appreciable amount of the plasma radioactivity after 24 hr of dialysis in saline or in phosphate buffer most likely results from the prolonged interaction of the gallium-transferrin complex with the dialysis medium, resulting in competitive displacement of the gallium. Displacement of gallium by phosphate has been reported previously (*3*), and similar effects have been noted with several other common buffer systems (*12*). An additional problem, at least in saline, is the trapping of much of the free gallium inside the dialysis bag. Proper choice of dialysis medium, and minimal dialysis times, are critical.

The divergent conclusions in the literature regarding protein binding of gallium are probably due to similar artifacts and the difficulty of comparing results obtained by various investigators using different analytical methods. Most of the previous work on gallium binding was based largely on electrophoresis. As a result, radioactivity that was reported in those studies to be unbound or loosely associated with albumin and haptoglobin may actually have been gallium dissociated from the transferrin complex. Accurate determination of protein binding of radiopharmaceuticals in general, and of gallium in particular, requires judicious selection of the most appropriate technique and cautious interpretation of the results.

This study demonstrates that by 15 min after injection, gallium at the tracer radiopharmaceutical level in blood is exclusively bound to and transported by transferrin. The chemical structure of the transferrin complex—including nature and number of its gallium-binding sites, the presence or absence of citrate, and the stability constant—are not known definitively. The present results indicate, nevertheless, that this complex is responsible for delivery of gallium to the site of a tumor or abscess, and further strongly suggest that the complex is somehow intimately involved in the ultimate deposition of the gallium within the target tissue. Involvement of a gallium-transferrin complex at the tumor site has been implicated in the in vitro tissue-culture studies and in vivo work of Larson et al. (*8*, *9*), in which a specific transferrin receptor is proposed, and in the studies of Hoffer et al. (*13*), in which transfer of gallium from transferrin to lactoferrin in the tumor tissue is suggested. Clausen et al. have reported gallium association with intracellular ferritin (*5*), and Lawless et al. (*14*) have identified a gallium-binding glycoprotein present in significant levels in certain types of tumors. The exact role played by the transferrin complex at the tumor site remains unknown.

FOOTNOTES

* Amicon, Inc.
† Cappel Laboratories
‡ Pharmacia Fine Chemicals

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