# Mechanism of Ionophoric Transport of Indium-111 Cations Through a Lipid Bilayer Membrane

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The use of mobile ionophores to facilitate the transport of $^{111}$In through a lipid bilayer membrane has broad applications in liposome technology and cell labeling. However, the mechanism of such ionophore-mediated transport of $^{111}$In through a lipid bilayer membrane is not completely clear. The present report describes the correlations of the behaviors of ionophoric loading of $^{111}$In into liposomes with the lipophilicity and the indium-binding affinity of three ionophores, namely, 8-hydroxyquinoline, acetylacetone, and tropolone. Our results suggest that the mechanism of the ionophoric transport of $^{111}$In through a lipid bilayer membrane involves the rapid exchange of $^{111}$In cations among the ionophores in both the aqueous solution and the lipid bilayer. Furthermore, the effectiveness of an ionophore in facilitating the transport of $^{111}$In from the external aqueous compartment to the entrapped nitrilotriacetic acid depends not only on the lipophilicity of the [$^{111}$In]ionophore complex, but also on the lipophilicity of the free ionophore itself and the competiton of $^{111}$In between nitrilotriacetic acid inside the inner aqueous compartment of the liposome and the ionophore imbedded in the lipid bilayer membrane of the liposome.

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Liposomes encapsulating indium-111 ($^{111}$In) can be used to monitor the physical integrity and biodistribution of liposomes in vivo by the technique of gamma-ray perturbed angular correlation (PAC) (*1*) and scintigraphic imaging (*2*), respectively. In addition, cells such as platelets or leukocytes labeled with high levels of $^{111}$In are useful tools for detecting thrombi and inflammation in vivo (*3,4*). In past studies, mobile ionophores such as 8-hydroxyquinoline (*5*) and acetylacetone (*6*) have been employed to load high levels of $^{111}$In into the internal aqueous compartments of liposomes. The loaded $^{111}$In remains inside the liposome after forming a stable complex with the entrapped chelating agent, nitrilotriacetic acid (NTA). Although the efficiency of ionophore-facilitated loading of $^{111}$In can be as high as 90%, the concentrations of the above two ionophores that are required to achieve this maximal loading efficiency are markedly different. Similar observations of a marked difference in the concentration of ionophores that are required to achieve the highest efficiency of labeling cells with $^{111}$In have been reported previously (*7–9*).

To achieve the highest efficiency of labeling platelets with $^{111}$In in buffered saline, the optimal concentrations for tropolone, 8-hydroxyquinoline, and acetylacetone are 3–5 $\mu M$ (*7*), 34–70 $\mu M$ (*8*), and 19–37 m$M$ (*9*), respectively. Similarly, in loading $^{111}$In into liposomes, a concentration of 14–40 $\mu M$ of 8-hydroxyquinoline is needed to reach 90% loading efficiency of $^{111}$In (*5*), whereas an acetylacetone concentration of at least 30 m$M$ is needed to obtain 90% loading efficiency of $^{111}$In (*6*). The cause of the more than 1,000-fold difference in the effectiveness of the two ionophores in loading $^{111}$In into liposomes is not known. The detailed mechanism of the ionophoric transport of $^{111}$In across a lipid bilayer membrane and the factors that govern the behavior of loading $^{111}$In into liposomes and cells are not completely clear.

The present study investigates the effects of the lipophilicity and the indium-binding affinity of 8-hydroxyquinoline, acetylacetone, and tropolone on the transport of $^{111}$In through the membrane, using the lipid bilayer membrane of small unilamellar liposomes as a model. The results of the present study suggest that the mechanism of the ionophoric transport of $^{111}$In through

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a lipid bilayer membrane is through the process of a rapid exchange of $^{111}In$ cations among the binding pockets formed by several molecules of a given ionophore in both the aqueous solution and the lipid bilayer. Furthermore, the effectiveness of an ionophore in facilitating the transport of $^{111}In$ from the external aqueous compartment to the entrapped NTA depends not only on the lipophilicity of the ionophore-$^{111}In$ complex, but also on the lipophilicity of the free ionophore itself and the competition of NTA inside the inner aqueous compartment of the liposome with the ionophore imbedded in the lipid bilayer membrane of the liposome for $^{111}In$.

## MATERIALS AND METHODS

Bovine brain sphingomyelin, cholesterol, nitrilotriacetic acid, 8-hydroxyquinoline, acetylacetone, and tropolone were obtained commercially and were used as supplied. The radionuclidic purity of [$^{111}In$]chloride* at calibration time was at least 99% with <0.1% $^{114m}In$ and 0.1% $^{65}Zn$, and the specific activity of the $^{111}In$ sample was 2.41 ng (or 11 pmole) per mCi. Indium-111 chloride was further purified as described previously (*10*). Anion-exchange resin AG1-X8 and Sephadex G-50 were purchased commercially.

### Preparation of Liposomes

Bovine brain sphingomyeline(SM)/cholesterol(CH) (2M:1M) small unilamellar vesicles (SUV) were prepared by sonicating 20–40 mg of the dried thin film of lipids and 1 ml of 1 m*M* NTA in an appropriate isotonic buffered solution at the desired pH in a Branson 350 sonicator with a titanium microtip at the setting of 1.5 for 15 min as described previously (*1,5,6*). During the sonication, the microtip was immersed 1.4–1.5 cm into the buffered solution of liposomes, which was in a 3-ml conical glass vial. The glass vial was immersed in a 100-ml glycerol bath at room temperature. At the end of sonication, the temperature of the liposomes solution was ~47°C. The sonicated liposomes were centrifuged at 160,000 *g* for 1 hr or 10,000 *g* for 5 min to remove the titanium fragments and highly aggregated material. The supernatant was clear for SUV (SM/CH, 2*M*:1*M*).

The isotonic saline solutions containing 5 m*M* sodium acetate, pH 5.4; 7 m*M* Tris-HCl, pH 7.6; and 10 m*M* HEPES, pH 7.4 were used for preparing liposomes for subsequent steps of loading $^{111}In$ into liposomes by 8-hydroxyquinoline, acetylacetone, and tropolone, respectively. The nontrapped NTA was removed by passing the liposomes through a Sephadex G-50 column (0.8 × 35 cm) equilibrated with and eluted by one of the above isotonic buffered solutions. The average size of SUV (SM/CH, 2*M*:1*M*) purified by centrifuging at 160,000 *g* for 1 hr was estimated to be 187 ± 42 Å from negative-stain electron micrographs of the liposomes using potassium phosphotungstate as the stain. The purified liposomes were loaded with $^{111}In$ by an appropriate [$^{111}In$]ionophore loading solution as described below:

### Preparation of [$^{111}In$]8-Hydroxyquinoline Loading Solution (*1,5,6*)

Depending on the desired radioactivity, the loading solution was prepared by mixing 70–100 μl [$^{111}In$]chloride (0.1–10 pmol) in 3 m*M* HCl with an equal volume of 1.8% NaCl, 20 m*M* sodium acetate, pH 5.5, containing various concentrations of 8-hydroxyquinoline.

### Preparation of [$^{111}In$]Acetylacetone Loading Solution (1,5,6)

The loading solution of $^{111}In$ is prepared by mixing 10–20 μl [$^{111}In$]chloride (0.1–10 pmole) in 3 m*M* HCl with 100–200 μl of 10 m*M* Tris-buffered isotonic saline, pH 7.6, containing various concentrations of acetylacetone.

### Preparation of [$^{111}In$]Tropolone Loading Solution

The loading solution was prepared by dissolving an appropriate amount of the purified, dried [$^{111}In$]chloride (0.1–10 pmole) in 10–20 μl of 3 m*M* HCl and mixing with 100–200 μl of 10 m*M* HEPES, pH 7.4 buffered isotonic saline, containing various concentrations of tropolone.

### Loading Procedure

Within 15 min after the preparation of the loading solution, SUV (SM/CH; 2*M*:1*M*) were loaded with $^{111}In$ by adding 140–200 μl [$^{111}In$]ionophore loading solution to 1 ml liposomes (1–5 mg/ml) dropwise, while the suspension of liposomes was vortexed gently, and incubating at room temperature for 1 hr. The loaded liposomes were purified and isolated by passage over a small column (0.7 cm × 7 cm) of AG1-X8 equilibrated with and eluted by 0.106*M* sodium phosphate buffer, pH 7.4, as described previously (*1,5,6*). The resin adsorbed 99.9% of the [$^{111}In$]tropolone, [$^{111}In$]8-hydroxyquinoline, or [$^{111}In$]acetylacetone that was not associated with liposomes. The percentage of loading was estimated from the radioactivity of the $^{111}In$ associated with the purified, loaded liposomes and the radioactivity of the $^{111}In$ that was not internalized and was adsorbed by the AG1-X8 resin.

### Measurement of Lipophilicity

The partition coefficients of tropolone, 8-hydroxyquinoline, and acetylacetone were determined by measuring the equilibrium concentration of each of these ionophores in n-octonol and 0.106*M* sodium phosphate, pH 7.4 aqueous buffer at room temperature according to the procedure of Alhaider et al. (*11*). The concentrations of 8-hydroxyquinoline, acetylacetone, and tropolone were measured spectroscopically at 239 nm, 278 nm, and 239 nm, respectively. The octanol/buffer distribution ratio of the [$^{111}In$]ionophore complexes were determined from the radioactivity of each of the [$^{111}In$]ionophore complexes in n-octanol and in 9.6 m*M* sodium phosphate, pH 7.4 buffer, containing 1.8 m*M* sodium citrate at room temperature. The presence of a low concentration of citrate keeps the indium-ionophores from adsorbing to the test tube. The initial concentration of each ionophores in the aqueous phase was: 9.1 μ*M* tropolone or 8-hydroxyquinoline, or 90.9 μ*M* acetylacetone.

## RESULTS AND DISCUSSION

As depicted in Figure 1, the patterns of the dependence of the efficiency of loading $^{111}In$ into liposomes on the concentrations of tropolone and 8-hydroxyquinoline are quite similar. This indicates that the effectiveness of tropolone and that of 8-hydroxyquinoline for loading $^{111}In$ into liposomes are similar. Since the [$^{111}In$]

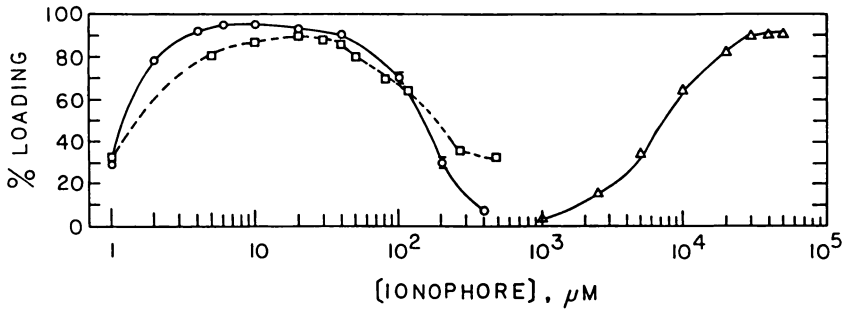


**FIGURE 1**
Efficiencies of loading $^{111}$In into small unilamellar liposomes as function of concentrations of ionophores. Small unilamellar sphingomyelin/cholesterol (2:1; mol/mol) liposomes were loaded with $^{111}$In by various concentrations of tropolone (○), 8-hydroxyquinoline (□), and acetylacetone (Δ), respectively, as described in the text. Each point is average of three measurements. Standard deviations are either shown or less than size of symbols.

ionophore complex is responsible for the transport of $^{111}$In across a lipid bilayer membrane, the higher lipophilicity of [$^{111}$In]tropolone and [$^{111}$In]8-hydroxyquinoline as compared with [$^{111}$In]acetylacetone (Table 1) is consistent with the observation of the same trend in their effectiveness in facilitating the transport of $^{111}$In from the external aqueous compartment to the entrapped NTA molecules (Fig. 1). In fact, the slightly higher lipophilic solubility of [$^{111}$In]tropolone as compared with 8-hydroxyquinoline-$^{111}$In is reflected in Figure 1, when the concentrations of the two ionophores are in the low region of the curve.

It is interesting to note that in contrast to 8-hydroxyquinoline and acetylacetone, the lipophilic solubility of [$^{111}$In]tropolone is higher than that of the parent ionophore (Table 1). This may be due to (a) the bearing of a partially negative charge in tropolone [pKa = 6.7 (*13*)] at pH 7.4 of the experiment; (b) the nonplanar structure of the seven member ring of tropolone; and (c) the resonance structures of tropolone. Thus, after forming a complex with $^{111}$In the ionic character of $^{111}$In cation is partially neutralized and the polar part of tropolone is well shielded at the same time. The high lipophilic solubility of [$^{111}$In]tropolone suggests that tropolone is more effective than 8-hydroxyquinoline or acetylacetone in loading $^{111}$In into liposomes or for labeling cells (*12*).

Previously, Rao and Dewanjee (*15*) showed that the partition coefficients (olive oil/buffer) of [$^{111}$In]8-hydroxyquinoline, [$^{111}$In]acetylacetone, and [$^{111}$In]tropolone were 3.54, 18.18, and 7.93, respectively. The discrepancy between their result and our result could be due to the following reasons. In their study, ACD + buffer solution (pH 6.5) was used to obtain the partition coefficients of [$^{111}$In]tropolone and [$^{111}$In]8-hydroxyquinoline, whereas HEPES buffer (pH 7.6) was used to measure the partition coefficient of [$^{111}$In]acetylacetone. It is difficult to make a meaningful comparison when their concentrations of ACD and buffer are not known. Furthermore, the distribution ratio (olive oil/buffer) of the radioactivity of an [$^{111}$In]chelate can be affected markedly by the presence of any potentially competing chelating agents in the aqueous phase. It is difficult to make an assessment of the relative lipophilicity of these three [$^{111}$In]chelates, using their data in which citrate was only present in the measurement of the partition coefficients of [$^{111}$In]8-hydroxyquinoline and [$^{111}$In] tropolone, while no citrate was present in the measurement of that of [$^{111}$In]acetylacetone.

It is important to point out that the concentration of $^{111}$In used in these studies was in the range of 0.1 to 10 n*M* and yet the concentrations of ionophores needed for an optimal loading of $^{111}$In into liposomes were in the range of μ*M* for 8-hydroxyquinoline and tropolone, and in the range of m*M* for acetylacetone. An interesting question is the function of the excess free ionophore. Presumably, a stable [$^{111}$In]ionophore complex can travel as a whole in the aqueous solution, reach a liposome, diffuse across the lipid bilayer membrane and deliver the $^{111}$In to the stronger chelator, NTA [$pK_1$ =

**TABLE 1**
Comparisons of Partition Coefficients of 8-Hydroxyquinoline, Tropolone, and Acetylacetone, Their Indium Chelates and Their Stability Constants*

| | 8-Hydroxyquinoline | Tropolone | Acetylacetone |
|---|---|---|---|
| Octanol/buffer partition coefficient | 86.70 ± 2.40 | 2.20 ± 0.04 | 1.91 ± 0.02 |
| Octanol/buffer distribution ratio of [$^{111}$In]ionophore | 3.19 ± 0.03 | 11.70 ± 1.18 | 0.006 ± 0.001 |
| p $\beta_3$, stability constant with $^{111}$In | 30.7 | — | 18.6 |

*Values of partition coefficients are average of three measurements ± s.d. Values of stability constants were obtained from Refs. *13* and *14*.

14.9 for NTA-$^{111}$In (*13*)], inside the liposome. However, if this were the only mechanism of transporting $^{111}$In across the lipid bilayer, an excess number of ionophores would not be needed. Thus, an alternative pathway must exist.

The most likely mechanism is that while the excess free ionophores are distributed in the aqueous and the lipid phases according to the partition coefficient of the ionophore, the $^{111}$In cation forms a complex with several molecules of a given ionophore in the aqueous solution initially (*12,15*). The $^{111}$In cations could rapidly dissociate from the original [$^{111}$In]ionophore complexes and become associated with a different set(s) of ionophores. In the course of this exchange process, $^{111}$In cations diffuse to the liposomes, become associated with the ionophores imbedded in the lipid bilayer membrane, diffuse across the lipid bilayer, and release the $^{111}$In to the stronger chelator, NTA, inside the liposomes.

Several lines of evidence suggest that the exchange of $^{111}$In cations among various chelating agents does happen rapidly. Firstly, from the PAC-study of the time course of loading liposomes with $^{111}$In by 8-hydroxyquinoline, the transfer of $^{111}$In cations from 8-hydroxyquinoline to NTA occurs and finishes in ~30 min (*16*). A similar observation of a rapid transfer of $^{111}$In cations from acetylacetone to NTA has also been documented (*6*). In addition, Rao and Dewanjee (*15*) reported that the efficiency of labeling red blood cells with $^{111}$In by 8-hydroxyquinoline, acetylacetone, and tropolone reached maximal values within 10–20 min. This suggests that the rates of dissociation of $^{111}$In cations from 8-hydroxyquinoline and acetylacetone must take place rapidly.

Second, as shown in Figure 2, tropolone at 44 $\mu M$ is able to induce an almost complete transfer of $^{111}$In from a small, entrapped volume of 1 m$M$ NTA inside the SUV to a large external volume of 1 m$M$ NTA outside the SUV within a period of 1 hr. This suggests that not only the rates of dissociation of $^{111}$In cations from tropolone and NTA take place rapidly, but also the rates of association of $^{111}$In cations to these two chelating agents take place rapidly as well.

Third, in using ionophore A23187 to load liposomes with $^{111}$In, the ionophore A23187 is imbedded in the lipid bilayer of liposomes before the loading process takes place (*17*). The loading process starts by incubating the A23187-containing liposomes, which has entrapped 1 m$M$ NTA, with a citrate solution of indium cations. The process of loading liposomes with $^{111}$In by ionophore A23187 finishes within ~40 min (*17*). This again suggests that the on-and-off rates of the exchange of $^{111}$In cations among the binding pockets formed by several molecules of given chelating molecules (NTA and citrate) or ionophore A23187 take place rapidly.

Based on the above kinetic evidences, one can conclude that the exchange of an $^{111}$In cation from the

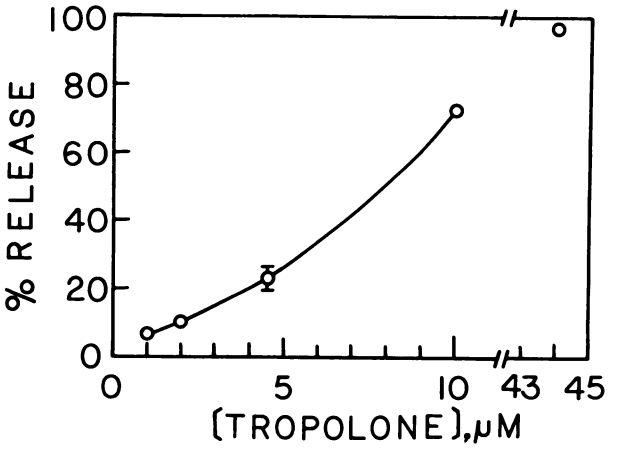


**FIGURE 2**
Tropolone-mediated release of liposome-entrapped $^{111}$In. Small unilamellar sphingomyelin/cholesterol (2:1; mol/mol) liposomes were loaded with $^{111}$In by 8-hydroxyquinoline and purified by an AG1-X8 column as described in Materials and Methods. Liposomes were incubated with 1 m$M$ NTA, 0.106$M$ sodium phosphate buffer, pH 7.4 in presence of various concentrations of tropolone at room temperature for 1 hr and passed through small column of AG1-X8. Percentage of release of liposome-entrapped $^{111}$In was calculated from ratio of radioactivity adsorbed by AG1-X8 resin to total radioactivity applied to column. Each point is average of three measurements. Standard deviations are either shown or less than size of symbols.

binding pocket formed by NTA molecules (or citrate molecules) to the binding pocket formed by the ionophore (8-hydroxyquinoline, acetylacetone, tropolone, or A23187) takes place rapidly. Furthermore, the rapid exchange of $^{111}$In cations between pockets formed by the chelating molecules (NTA or citrate) and by the ionophores indicates that the exchange of $^{111}$In cations

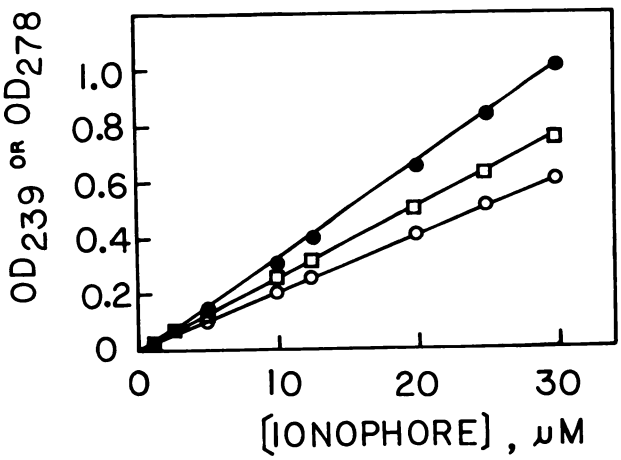


**FIGURE 3**
Calibration curves of ionophores. For simplicity, actual unit of concentration of acetylacetone is ten times of unit shown in figure. Each point is average of three measurements. Errors are less than symbols. (●) = HOQ; (□) = Tropolone; (○) = ACAC.

between the binding pockets formed by the molecules of 8-hydroxyquinoline (acetylacetone, tropolone, or A23187) itself can also take place rapidly. Moreover, it is quite possible that the mechanism of ionophoric transport of $^{111}$In cations through the liposomal membrane are operated by two parallel pathways. Some of the $^{111}$In cations are transported through the mechanism of the stable ionophore-$^{111}$In complex, and some are transported via the mechanism of the exchange of $^{111}$In cations among the chelating agents involved in the loading process.

Thus, there are dual functions for the excess free ionophores. First, excess ionophores maintain a sufficiently high concentration of free ionophores in the lipid bilayer to allow an efficient transport of $^{111}$In across the lipid bilayer. Second, the excess number of ionophores keeps $^{111}$In from forming colloids and/or binding to competing molecules, such as the water, buffer, and the phosphate head groups of phospholipids during the exchange of $^{111}$In.

While a critical level of free ionophores in the lipid bilayer is an important factor in the efficient transport of $^{111}$In across the lipid bilayer membrane, a large excess of the ionophore will hinder the transport of $^{111}$In to the entrapped NTA. This is indicated by the decrease in the efficiency of loading $^{111}$In into liposomes, as the concentrations of tropolone and 8-hydroxyquinoline exceed a critical concentration of 10 $\mu M$ and 20 $\mu M$, respectively (Fig. 1). The excess ionophores could interfere with the loading of $^{111}$In into liposomes in two possible ways.

The first possibility is that the excess ionophores in the aqueous phase outside the liposomes could diminish the gradient of chelating strength between the entrapped NTA molecules and the competing species in the external aqueous compartment of liposomes. The presence of such interference is supported by our previous finding that the efficiency of loading $^{111}$In into NTA [$pK_1$ = 14.9 for NTA-$^{111}$In (*13*)] entrapped in liposomes can be decreased by the presence of a weak chelator, citrate [$pK_1$ = 6.2 for [$^{111}$In]citrate (*13*)], in the aqueous medium (*5*). The second possible source of interference could be the competition of $^{111}$In between the ionophores imbedded in the lipid bilayer and the NTA in the internal aqueous phase. Figure 2 shows that in the presence of an appropriate acceptor of $^{111}$In, such as NTA, tropolone at high concentrations facilitates the release of the entrapped $^{111}$In from liposomes. Presumably, both the indium-binding affinity and the lipophilicity of the ionophore would determine when and how the efficiency of loading $^{111}$In into liposomes starts to decrease as the concentration of the ionophore increases.

In the cases of labeling platelets, leukocytes, or red blood cells with [$^{111}$In]ionophores, proteins situated in both the cytoplasm, granules, and membranes could play a role in binding $^{111}$In in the cell (*15,18*). The optimal concentrations of 34–70 $\mu M$ 8-hydroxyquinoline (*8*), 3–5 $\mu M$ tropolone (*7,12*) and 18–37 m$M$ acetylacetone (*9*) for labeling platelets with $^{111}$In are quite similar to the optimal concentrations of the respective ionophores in loading $^{111}$In into liposomes as shown in Figure 1. The phenomenon of a decrease in the efficiency of labeling platelets with $^{111}$In in the presence of an excess of ionophores has also been reported for these three ionophores (*7–9,12*). Thus, it is very likely that the mechanism proposed here for interpreting the behaviors of loading $^{111}$In into liposomes by ionophores will be applicable to the labeling of platelets, leukocytes, red blood cells, or other cells with $^{111}$In.

## NOTE

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