Mechanisms of Gallium-67 Accumulation by Tumors: Role of Cell Membrane Permeability

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The effects of citrate ion on in vitro and in vivo uptake of [^{67}Ga]citrate by tumor cells have been studied. Carrier-free [^{67}Ga]citrate seems to follow the physical diffusion of citrate ions into the cell, and the presence of carrier gallium inhibits that diffusion, reducing considerably its uptake. These results appear to support the hypothesis that increased permeability of tumor cells is the principal cause of [^{67}Ga]citrate accumulation by tumors.

J Nucl Med 29: 663–668, 1988

One of the most remarkable characteristics of malignant cells is the alteration of biologic systems located at the plasma membrane level. When compared with normal cells, marked amounts of intracellular material released from tumor cells into the surrounding fluid provided corroborative evidence of increased permeability of tumor cell membrane (*1*). This phenomenon was suggested due to different characteristics of the two cells surfaces (*2,3*). The permeability changes have been related to derangement on the structure of the plasma membrane lipid double layer and the corresponding proteins (*4*). More recently, the modifications of cell permeability during chemical carcinogenesis and virus transformation of normal cells have been extensively reviewed (*5,6*).

This study was undertaken to determine the role of plasma membrane permeability on gallium-67 (^{67}Ga) accumulation by tumors. With this purpose, we have investigated the effects of citrate ion on carrier-free (c.f.) [^{67}Ga]citrate uptake.

MATERIAL AND METHODS

The experiments were performed with Ehrlich ascites carcinoma cells, DS ascites sarcoma cells, and P388 ascites leukemia cells maintained in the ascitic form by weekly inoculation into Swiss mice, Sprague-Dawley rats, and B6D2F1 mice, respectively. Eight days after inoculation pools of cells from at least five mice and two rats were used for each experiment. Care was taken to minimize ascites contamination with blood.

Tumor cells (5×10^7 Ehrlich ascites or 8×10^7 DS sarcoma or 5×10^7 P388 leukemia) were suspended in Tyrode medium containing 0.25 μCi of c.f. [^{67}Ga]citrate (in 6.8 m*M* sodium citrate), and the amount of sodium citrate necessary to give the concentration to be assayed was added (0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 m*M* citrate. Series of four tubes were incubated at 37°C for 30 min (some experiments were also performed at 45°C).

To assay the effects of carrier gallium on [^{67}Ga]citrate uptake we have prepared citrate of gallium by precipitation of gallium hydroxide from gallium chloride solution with concentrated ammonia, followed by gallium hydroxide dissolution in citric acid solution. Series of three tubes were incubated at 37°C for 30 min and in the presence of various gallium citrate carrier concentrations (1, 5, 10, and 50 μg of gallium as citrate).

The study of in vitro uptake of citrate ions was performed using 1,5-^{14}C-sodium citrate (S.A. 34 mCi/mmol). This uptake was compared to the corresponding to c.f. [^{67}Ga]citrate (in the presence or absence of 10 μg of gallium carrier) using the same pool and number of Ehrlich ascites cells (9×10^7 cells/tube in series of four tubes).

In order to have cells presenting comparable physicochemical and permeability characteristics of the plasma membrane, in all experiments, the plasma membrane status of each cell pool was assessed before any treatment by the standard Trypan blue inclusion technique. Only pools having <5% inclusion of Trypan blue were used.

A preliminary series of experiments were performed to evaluate the amount of ^{67}Ga bound to the external part of the

Received June 23, 1987; revision accepted Dec. 1, 1987.

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TABLE 1
Radioactivity Released by EDTA- and Protease-Treatment from Tumor Cells Preincubated with [^{67}Ga] citrate (as Percentage of Total ^{67}Ga in the Cells and Mean Value ± s.d. of Three Tubes)

	Ehrlich ascites	DS sarcoma	P388 leukemia
EDTA	8.3 ± 2.1	7.9 ± 2.6	10.5 ± 3.4
Protease	10.2 ± 1.9	9.5 ± 2.3	11.6 ± 2.8

plasma membrane. After 30 min of incubation at 37°C with c.f. [^{67}Ga]citrate, the tumor cells were washed twice with pH 7.2 0.1*M* EDTA. Cells washed with normal saline were used as the control. In a parallel experiment, the cells were incubated for 15 min at 37°C with 5 mg of protease. After incubation, the cells were washed with saline and the radioactivity counted. The difference of radioactivity in control cells minus the radioactivity in EDTA-washed or protease-treated cells gives an estimation of the contribution of the cell coat (glycoproteins) to the binding of ^{67}Ga by the tumor cells.

In vivo [^{67}Ga]citrate uptake studies were performed on DS sarcoma-bearing rats having a 10-day-old subcutaneously transplanted tumor, 1–1.5 cm Ø). The radioactivity was injected intravenously or intraperitoneally. The animals were killed by decapitation, and the radioactivity was counted on samples of different tissues.

RESULTS

Preliminary experiments performed to assay the effects of EDTA on the ^{67}Ga already bound to tumor cells have shown that <10% of the total radioactivity is released during the treatment. Values of the same magnitude were observed after protease hydrolysis of the cell coat (Table 1).

All the three tumor cells showed a decreased ^{67}Ga uptake dependent on the citrate concentration in the incubation medium (Fig. 1). An experiment without the addition of sodium citrate, but diluting the original c.f. [^{67}Ga]citrate solution (containing 6.8 m*M* sodium citrate) presented a similar citrate concentration-dependence of ^{67}Ga-uptake (Fig. 2).

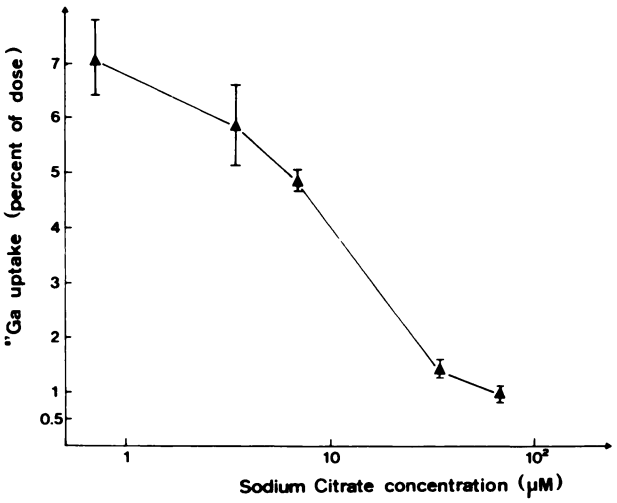

FIGURE 2
Effects of the dilution of c.f. [^{67}Ga]citrate solution on the uptake of ^{67}Ga by Ehrlich ascites cells (mean of 4 values ± s.d.).

In another series of incubations, using the same pool of Ehrlich ascites cells, the uptake of [^{67}Ga]citrate and [^{14}C]sodium citrate in the presence of increasing "cold" citrate concentrations exhibited two curves with an intersection point at about 0.5 m*M* sodium citrate (Fig. 3), and the increase of [^{14}C]citrate uptake was a direct function of citrate concentration in the medium. For c.f. [^{67}Ga]citrate, after a sharp citrate concentration-dependent decrease the slope is modified to reach an

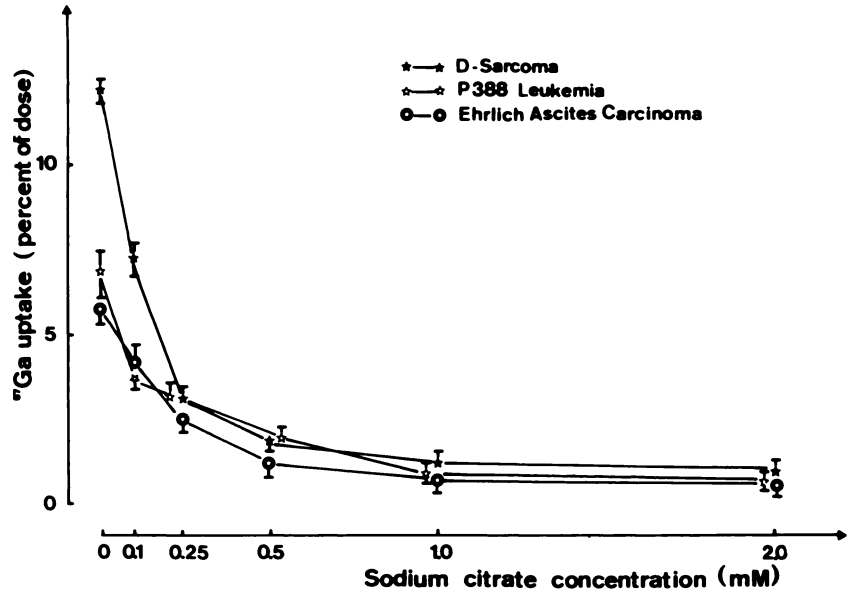

FIGURE 1
Effects of sodium citrate on the uptake of c.f. [^{67}Ga]citrate by tumor cells (mean of 4 values ± s.d.).

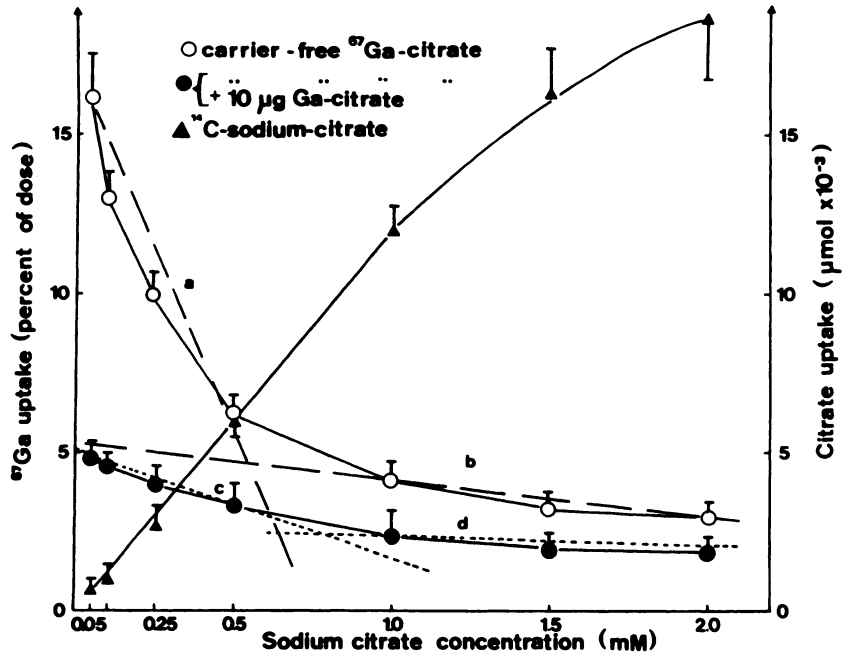

FIGURE 3
[⁶⁷Ga]citrate and [¹⁴C]citrate uptake by Ehrlich ascites cells as a function of sodium citrate concentration in the incubation medium (mean of 4 values ± s.d.).

almost steady value similar to the one observed in the presence of gallium carrier (Fig. 3).

A comparison of ^{67}Ga uptake at 37°C and 45°C showed a significantly higher uptake at 45°C, and the difference was specially greater at very low citrate ion concentrations (Fig. 4).

Sodium citrate modified the uptake of [^{67}Ga]citrate only if they were together in the incubation medium. No change in the ^{67}Ga taken up by the cells was observed when the incubation with sodium citrate was performed before or after ^{67}Ga uptake (Table 2).

Increasing concentrations of gallium carrier dramatically lower [^{67}Ga]citrate uptake, to give curves tending to a plateau unaffected by sodium citrate concentration (Fig. 5). For all these curves, the EDTA treatment dissolves a fairly similar percentage of total ^{67}Ga taken up (20–30%).

In vivo, sodium citrate concentration has shown inhibitory effects on the uptake of c.f. [^{67}Ga]citrate by DS sarcoma tumors. These effects are dependent on the route of injection, and the time elapsed after [^{67}Ga] citrate administration (Table 3).

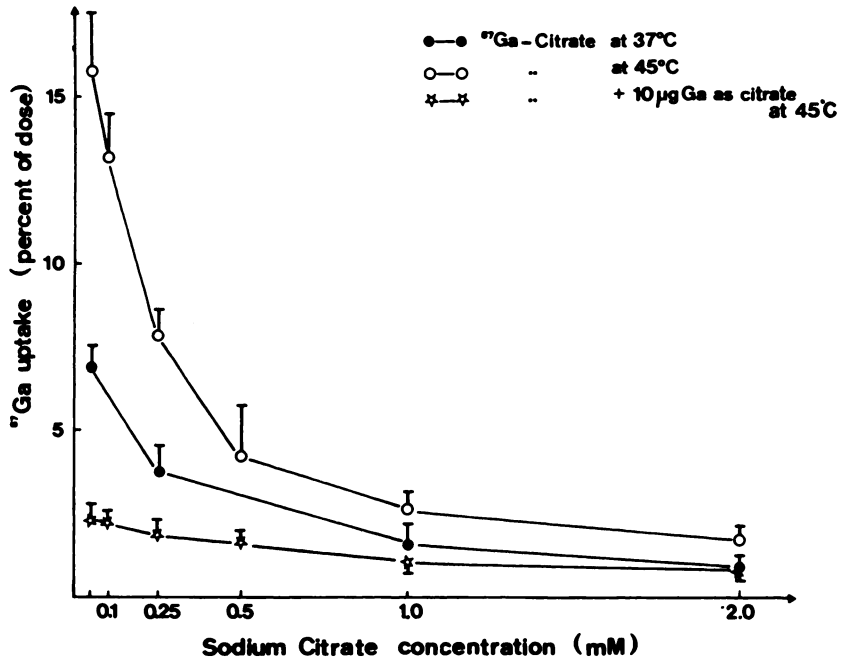

FIGURE 4
Effect of temperature on [^{67}Ga]citrate uptake by Ehrlich ascites cells, and as a function of sodium citrate concentration in the incubation medium (mean of 4 values ± s.d.).

TABLE 2
Effects of Sodium Citrate Concentrations in the Incubation Medium on [^{67}Ga]citrate Uptake by Ehrlich Ascites Cells (mean of 5 values ± s.d.)

Sodium citrate mM	A	B	C
0.1	7.88 ± 0.41	3.70 ± 0.45	5.38 ± 0.68
1.0	8.69 ± 0.14	3.23 ± 0.07	1.73 ± 0.07
0.0	7.65 ± 0.89	3.43 ± 0.28	6.74 ± 0.24

A: 6 × 10^7 cells incubated at 37°C for 30 min with sodium citrate, then washed and incubated with [^{67}Ga]citrate for 30 min.
B: 6 × 10^7 cells incubated at 37°C for 30 min with [^{67}Ga]citrate, then washed and incubated for 30 min with sodium citrate.
C: 6 × 10^7 cells incubated at 37°C for 30 min with sodium citrate plus [^{67}Ga]citrate.
Note that only in C sodium citrate modifies ^{67}Ga uptake.

DISCUSSION

The choice of 30 min as incubation time was made considering that preliminary experiments with Ehrlich ascites and DS sarcoma cells have shown that the inclusion of Trypan blue increases significantly when the incubation time is prolonged to 1 hr (4–5% after 30 min and 6–8% after 1 hr). This increase is an indication of a change in plasma membrane permeability which was also reflected by an equivalent increase of ^{67}Ga uptake (8–12% after 30 min and 14–18% after 1 hr).

Citric acid is a fundamental molecule in the mechanism of cell respiration, a biologic phenomenon in which glycolysis is just a part. The Krebs tricarboxylic acid cycle, in which citric acid is one of the intermediates, is the final common pathway into which carbohydrates, fatty acids, and amino acids are ultimately degraded in catabolism (*7*).

In the presence of citrate ions, a similar pattern of ^{67}Ga uptake has been observed for the different tumor cells assayed. On the other hand, the uptake curves (Fig. 1) appear to have two main components, an initial high uptake that is citrate ion-dependent, and a less significant, and almost unaffected by citrate ion, which resembles the uptake obtained in the presence of gallium carrier.

The [^{14}C]citrate uptake as a function of citrate concentration in the medium indicates that the phenomenon has the characteristics of an unmediated physical diffusion that fits the criterion of lack of saturation kinetics. Contrary to this, [^{67}Ga]citrate uptake presents a curve with a component (a) that decreases proportionally to citrate ion increase in the medium (Fig. 3). This behavior suggests a dilution effect, and as a consequence less [^{67}Ga]citrate is carried across the plasma membrane by the diffusing sodium citrate. On the contrary, when gallium carrier is added this component of the curve (c) is greatly reduced. This phenomenon seems to indicate that at the carrier-free state [^{67}Ga] citrate is carried across the plasma membrane by the physical diffusion of citrate ion. On the other hand, the experimental observation that temperature increases [^{67}Ga]citrate uptake, especially at low citrate concentration (Fig. 4), is in agreement with an earlier report (*8*), and appears to corroborate the hypothesis of [^{67}Ga] citrate carried over by citrate ion diffusion. It is known that temperature increase provokes permeability changes (*9*) that increase physical diffusion and consequently augments ^{67}Ga uptake.

The effects of gallium carrier showing a dramatic decrease of the component (a) of the uptake curve, while tending to a very low and almost steady uptake ((d) in Fig. 3 and Fig. 5) suggest that gallium citrate does not penetrate the cell by physical diffusion. The fact that only 20–30% of the radioactivity is eliminated by EDTA indicates the possibility of a part of the uptake at cell membrane level (lipid double layer) not reached by the chelator. A support for this membrane-binding of gallium is found in the reported inhibition of ATPase by gallium (*10*).

EDTA- and protease-treatment have shown (Table

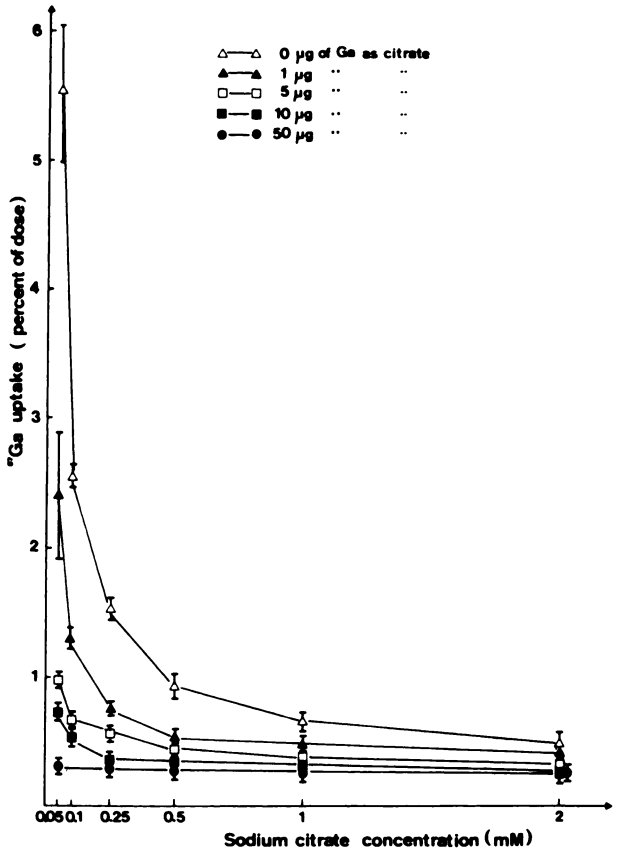

FIGURE 5
Effects of carrier (gallium citrate) on the uptake of [^{67}Ga] citrate by Ehrlich ascites cells, and in the presence of increasing sodium citrate concentration (mean of 3 values ± s.d.).

TABLE 3
In Vivo Distribution of [^{67}Ga]citrate Injected With or Without Sodium Citrate into Rats Bearing a Subcutaneous DS Sarcoma Solid Tumor (Mean Value ± s.d. of Each Group, Significance Evaluated by Student's t-test)

	Blood	Tumor	Liver	Spleen	Kidney	Muscle	Bone
Intraperitoneal + 0.15 m mol citrate 30 min (n = 4)	2.18 ± 0.24*	0.55 ± 0.12*	0.51 ± 0.14	0.32 ± 0.04	0.55 ± 0.07*	0.17 ± 0.01	1.10 ± 0.15
Control group (n = 4)	3.05 ± 0.57	0.86 ± 0.19	0.66 ± 0.12	0.44 ± 0.09	0.84 ± 0.17	0.30 ± 0.11	1.28 ± 0.20
Intraperitoneal + 0.5 m mol citrate 24 hr (n = 7)	0.09 ± 0.02	1.21 ± 0.32	0.74 ± 0.21	0.63 ± 0.17	0.40 ± 0.10	0.15 ± 0.05	2.70 ± 0.32*
Control group (n = 5)	0.10 ± 0.03	1.25 ± 0.11	0.81 ± 0.18	0.76 ± 0.10	0.49 ± 0.04	0.15 ± 0.06	3.23 ± 0.48
Intravenous + 0.07 m mol citrate 24 hr (n = 9)	0.16 ± 0.02*	1.64 ± 0.28*	0.86 ± 0.14	0.77 ± 0.12*	0.56 ± 0.05	0.05 ± 0.01	2.57 ± 0.35*
Control group (n = 6)	0.20 ± 0.04	2.17 ± 0.56	0.93 ± 0.16	0.64 ± 0.09	0.63 ± 0.09	0.07 ± 0.01	3.42 ± 0.47

* $p < 0.05$.

1) that the contribution of glycoproteins present in the glycocalix or cell coat (*11*) to the total ^{67}Ga-binding is a minor one.

Concerning the possible effects of pH on the accumulation of [^{67}Ga]citrate, we must consider that malignant cells living in their genuine milieu are at a pH in the range 6.9–7.2, and only when aerobic tumor glycolysis is stimulated by glucose perfusion, pH differences between cancer and normal tissues in the order of 1.4 units were observed (*12*). Gallium citrate under these conditions of pH (pH ≥ 6.0) is not expected to show any dissociation due to acidity, and if the gallium citrate molecule is susceptible to dissociation at the interior of the cell, a metabolism-induced decrease of citrate concentration modifying the rate of dissociation according to the law of mass action expressed by the equation [Ga-citrate]⇌[Citr.$^{3-}$] + [Ga^{3+}] is the more likely cause. This susceptibility to dissociation and subsequent hydrolysis of the Ga^{3+} are the reasons why injectable solutions of c.f. [^{67}Ga]citrate contain a great excess of sodium citrate (6.8 m*M*).

The experimental findings that ^{67}Ga is bound to different subcellular fractions, and to a variety of molecules, and that an apparent correlation has been observed between ^{67}Ga uptake and chemical composition of various tumors (*13–16*) are explained by the fact that complexes of several biologic molecules, such as nucleic acids, glycosaminoglycans, phospholipids and proteins with magnesium and calcium are able to exchange their cation by ^{67}Ga (*17,18*). To explain this exchange we can use the ionic model of chemical bonding (*19,20*). Accordingly, cations with similar atomic or ionic radii would be expected to bind to the same sites, but the binding would be expected to be stronger when the ion possesses a higher charge or valence. A typical example of the ionic exchange is the replacement of calcium by lanthanides.

Considering the similarity of atomic radii for magnesium and gallium (Mg^{2+}:0.65 Å and Ga^{3+}:0.62 Å) the replacement can be easily explained in terms of that exchange. On the other hand, calcium substitution, calcium radius is much larger (Ca^{2+}:0.99 Å), must be interpreted as the result of a previous process involving steric adjustment (steric fitting) of the ligand to accommodate a cation of different size. The steric fitting by modification of a flexible "hole" may explain the binding of gallium to calcium sites, and in certain cases of calcium to magnesium sites.

Early in 1973, when considering the experimental results of ^{67}Ga uptake by experimental tumors, this isomorphous ionic replacement hypothesis was suggested (*13*), and was extended to the radiolanthanides with the explanation that in the case of c.f. solutions a similar behavior corresponding to radio-gallium should be expected. In spite of the fact that c.f. radiolanthanides are not available, several investigators have demonstrated the uptake of radiolanthanides by tumors (*21–24*).

The in vivo study of the effects of citrate ion on c.f. [^{67}Ga]citrate uptake by tumor presents unavoidable difficulties due to the need to inject a considerable amount of citrate in order to obtain, at the tumor level, concentrations alike to those used in in vitro assays. This technique is complicated by the harmful interaction of citrate with blood (tetany), and by the excretion kinetics of citrate. Concerning this ion, we must point out that it is already present in normal plasma. For humans, the reported concentration values are: total

citrate 0.121 mM, free ions 0.023 m*M*, $NaCitr^{2-}$ 0.016 m*M*, $CaCitr^{-}$ 0.038 m*M* and $MgCitr^{-}$ 0.044 m*M* (*25*). This citrate present in the blood, explains why after the administration of a scintigraphic dose of [^{67}Ga]citrate there is no significative effect of the injected sodium citrate: its amount could give only blood concentrations in the range of 1/200 to 1/500 of that already present in the blood.

A recent publication has shown that concanavalin A increases the uptake of [^{67}Ga]citrate by tumor cells (*26*). This molecule that modifies plasma membrane permeability as a consequence of structural changes due to its binding to certain of its saccharide components is a typical membrane-active agent.

The results of this experimental work emphasize on the role of tumor cell permeability in the accumulation of c.f. [^{67}Ga]citrate. It can be stated that in the case of tumor cells, the influx of ^{67}Ga seems more important to explain the radioactivity accumulation than the nature of the ultimate binding sites.

ACKNOWLEDGMENTS

The authors thank Mr. P. Debas for his excellent technical assistance.

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