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**Abstract**

Using in vivo and in vitro experimental models, the uptake and excretion of 67Ga-citrate in tumor cells and normal cells were studied. The time-lapse accumulation of 67Ga in the tumor of rats bearing Yoshida sarcoma reached its peak 24 h after the administration of 67Ga and gradually decreased thereafter. However, the excretion of 67Ga from the tumor was less than that from normal lung. For culture cells in vitro, the uptake of 67Ga increased with lapse of contact time between 67Ga and the cells, but there was no distinct difference between the results of tumor cells and normal skin fibroblasts. The excretion of 67Ga from the cells tended to decrease with prolongation of the contact time, the excretion from tumor cell being only about 10% after a contact time of 24 h. This indicated a significant delay in excretion in comparison with that of normal skin fibroblasts. This delay in the excretion of 67Ga may be an important factor in the tumor accumulation of 67Ga.

**KEYWORDS:** 67Ga uptake and excretion, malignant cells

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# ACCUMULATION OF RADIOISOTOPES WITH TUMOR AFFINITY
## I. UPTAKE AND EXCRETION OF $^{67}$GA-CITRATE IN MALIGNANT TUMORS AND NORMAL CELLS

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*Abstract.* Using *in vivo* and *in vitro* experimental models, the uptake and excretion of $^{67}$Ga-citrate in tumor cells and normal cells were studied. The time-lapse accumulation of $^{67}$Ga in the tumor of rats bearing Yoshida sarcoma reached its peak 24 h after the administration of $^{67}$Ga and gradually decreased thereafter. However, the excretion of $^{67}$Ga from the tumor was less than that from normal lung. For culture cells *in vitro*, the uptake of $^{67}$Ga increased with lapse of contact time between $^{67}$Ga and the cells, but there was no distinct difference between the results for tumor cells and normal skin fibroblasts. The excretion of $^{67}$Ga from the cells tended to decrease with prolongation of the contact time, the excretion from tumor cell being only about 10% after a contact time of 24 h. This indicated a significant delay in excretion in comparison with that of normal skin fibroblasts. This delay in the excretion of $^{67}$Ga may be an important factor in the tumor accumulation of $^{67}$Ga.

*Key words*: $^{67}$Ga uptake and excretion, malignant cells

Ever since 1969 when it was reported by Edwards and Hayes that $^{67}$Ga-citrate accumulated to a high degree in Hodgkins's disease (1), the tumor accumulation of $^{67}$Ga has been studied from fundamental and clinical aspects by many investigators. The usefulness of $^{67}$Ga as a tumor scanning agent was then established (2–5). Subsequently, the tumor accumulation of other radioisotopes such as $^{111}InCl_3$ (6), $^{169}$Yb (7), $^{57}$Co-Bleomycin (8), $^{99m}$Tc-Bleomycin (9) and $^{201}$Tl (10, 11) was reported. These radioisotopes have also been applied to clinical practice. However, $^{67}$Ga and these other radioisotopes all accumulate in inflammatory foci; (12) moreover, tumor specificity, at lack of it, is still a problem. Hence the search for a superior radioisotope continues.

Tumor accumulation of tumor scanning agents including $^{67}$Ga has been studied by various methods but no definitive conclusions have yet been reached (13). An understanding of the mechanism of tumor accumulation is essential to formulating diagnosis. It is also important in attempting to a new radioisotope.

In this paper, the author studied the difference in excretion of $^{67}$Ga from tumor cells and normal cells using *in vivo* and *in vitro* models. The results are presented here and their significance is discussed.

## MATERIALS AND METHODS

*Radioisotope.* $^{67}$Ga-citrate was obtained from the Daiichi Radioisotope Laboratory. On the assay date, 1 mCi of carrier-free $^{67}$Ga was dissolved in physiological saline containing 1.8 mg sodium citrate, then diluted to the appropriate concentration.

*Animal experiments.* Using male Donryu rats (weight 128-179 g), 0.2 ml of Yoshida sarcoma ascites (cell number: cir. $4\times10^7$ cells) was transplanted subcutaneously into the left femur. Experiments were conducted 5-7 days after this transplantation, during which time the tumor had grown to 1.5-2 cm in diameter. $^{67}$Ga-citrate in a dose of 10 $\mu$Ci per animal was injected intravenously from a tail vein. Six-seven cancer-bearing rats from each group were sacrificed at intervals of 3, 24, 48 and 72 after the injection, and the $^{67}$Ga-acumulation in various organs was measured. The measured values were expressed as % dose/1% body weight for each organ so as to obviate differences due to body weight.

*Culture cells and conditions.* The cells used for the experiment consisted of HeLaS3 cells, ASII cells (14) derived from human ovary embryonic cancer, normal skin fibroblast (normal S. F.) and Yoshida sarcoma cells. The doubling time for each of the 4 kinds of cells was HelaS3 : cir. 19h, ASII : cir. 17h, normal S. F. : cir. 41h and Yoshida sarcoma cells : cir. 17h. HeLaS3, ASII, and normal S. F. cells all proliferated by adhering to the walls of the culture vessel. A $CO_2$ incubator was used to incubate these sells. Yoshida sarcoma cells were cells cultured from Yoshida sarcoma ascites in rats. These cells proliferate while suspended in the medium. For their incubation, the temperature was maintained at 37°C and the culturers were shaken at fixed intervals. Eagle's MEM medium containing 10% fetal calf serum was used in all cultures and the experiments were undertaken at the logarithmic growth phase,

*Experiments using a monolayer culture method.* Cells were planted in 2 ml medium in a plastic Petri dish 35 mm in diameter then incubated for 24-48h at 37°C (Fig. 1). Next carrier-free $^{67}$Ga-citrate usually in the concentration of 1 $\mu$ci/ml-medium was added. Further incubation for 0.5, 3, 6, 12 or 24 h was performed then, while the Petri dish were cooling in ice, each culture was washed 3 times with 2 m cold PBS. To standardize the cell count, the administration time of $^{67}$Ga was made to coincide with this washing time. To measure the uptake of $^{67}$Ga, 1 ml of 0.2% trypsin solution was added, incubation allowed for 20 min at 37°C, then the cells were transferred from the Petri dish to a test tube. To eliminate the effect of $^{67}$Ga attached to the Petri dish, the tube was washed with 7 ml cold PBS and centrifuged at 4°C and 1,000 r.p.m. for 10 min. This was done twice, then the test tube was placed in a well type scintillation counter (window : $93\pm25$ keV) and the activity of $^{67}$Ga in the cells was measured. To measure the excretion of $^{67}$Ga, each culture was washed with cold PBS, fresh medium was

added, and further incubation allowed. The activity of $^{67}Ga$ remaining in the cell was measured during the next 12 h. To prevent resorption of $^{67}Ga$ either adhered to the vessel or previously excreted, the medium was exchanged for a fresh medium at 1, 2, 4, 6, 8 and 10 h.

*Experiments using a suspension culture method.* With a plastic test tube 12 mm in diameter, cells planted in 2 ml medium were subjected to suspension culture and $^{67}Ga$ was added as for the monolayer culture (Fig. 2). Thereafter, each was incubated for 0.5, 1, 3, 6, 12 and 24 h, centrifuged with 7 ml of cold PBS at 4°C, 1,000 r.p.m. for 10 min, and washed 3 times. The uptake of $^{67}Ga$ in the cell was then measured. To measure excretion, each culture was washed with PBS and fresh medium was added. $^{67}Ga$ remaining in the cell during the next 12 h was measured without any exchange of medium.

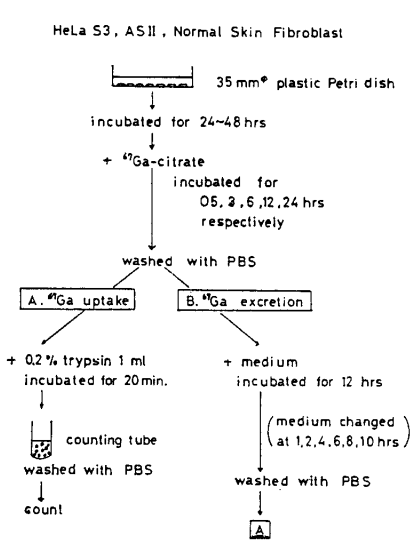


**Fig. 1**

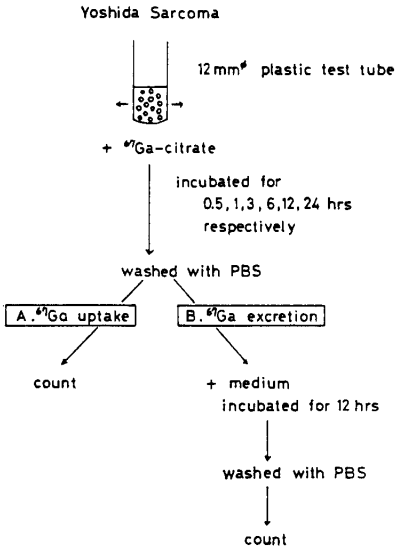


**Fig. 2**

Fig. 1. Schema for experimental methods of Ga-67 uptake and excretion in HeLaS3, ASII and normal skin fibroblasts.

Fig. 2. Schema for experimental methods of Ga-67 uptake and excretion in Yoshida sarcoma.

## RESULTS

*Time-lapse changes of $^{76}Ga$ distribution in the tumor and organs of Yoshida sarcoma-bearing rats.* As shown in Fig. 3, $^{67}Ga$ activity in blood and lung decreased with lapse of time, but rose to a peak for the tumor node and the liver, after which it tended to gradually decrease. The accumulation of $^{67}Ga$ was less in muscle than in the other tissues, being maintained at a fixed level from 3 h to 72 h after administration.

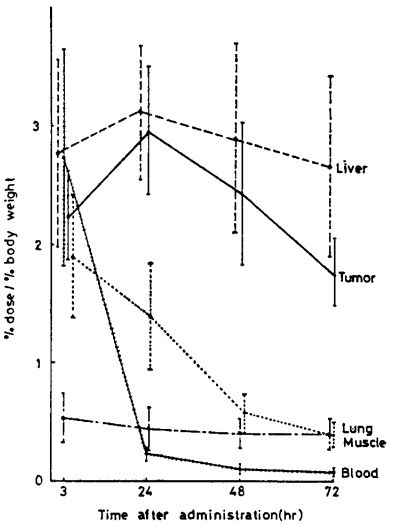


Fig. 3. Time-lapse changes of Ga-67 distribution in the tumor and organs of Yoshida sarcoma-bearing rats. Each point is expressed as mean ± standard deviation for 6-7 rats.

The level of $^{67}$Ga in the tumor node decreased so that, at 72 h, it was 60% of that at 24 h after the administration of $^{67}$Ga. At the same time, in the liver it was about 80%, and in the lung, about 30%, indicating that the excretion of $^{67}$Ga from the tumor node was faster than from the liver, but a marked delay was recognized as compared with the normal lung.

*Effect of cell density and $^{67}$Ga concentration on the $^{67}$Ga-uptake in culture cells.* Fig. 4 shows the relation between cell counts and the uptake of $^{67}$Ga when incubation was carried out for 24 h in medium containing 1 $\mu$Ci/ml-medium of

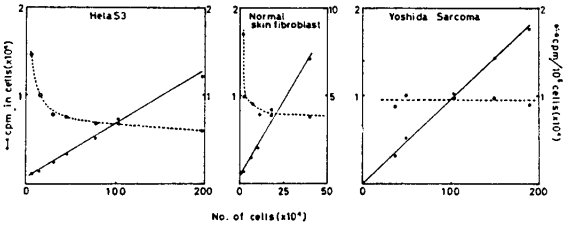


Fig. 4. Gallium-67 uptake by varying numbers of HeLaS3, normal skin fibroblasts and Yoshida sarcoma. Cells were incubated with 1 $\mu$Ci/ml Ga-67 for 24 h at 37°C.

$^{67}$Ga. In the cultures of HeLaS3 and Yoshida sarcoma cells, the uptake of $^{67}$Ga was measured by varying the cell counts up to $2 \times 10^6$ cells per Petri dish or test tube. In the culture of normal S.F., the stationary phase was reached at cir.

$4 \times 10^5$ cells per Petri dish, so uptake of $^{67}$Ga was measured by varying the cell counts up to $4 \times 10^5$ cells per Petri dish. The uptake of $^{67}$Ga increased linearly with increase in cell numbers in every case as shown by the solid line (Fig. 4). However, with HeLaS and normal S. F., the solid line does not pass through the original point. When the cell density was less, the uptake of $^{67}$Ga/$10^6$ cells tended to incrase as shown by the dotted line. With Yoshida sarcoma cells, a straight line passing through the original point was obtained, and irrespective of the cell density the uptake of $^{67}$Ga/$10^6$ cells became almost constant. Cell counts at the time $^{67}$Ga-uptake was measured were: HeLaS3; cir. $50-100 \times 10^4$ cells/dish; normal S. F.: cir. $3-30 \times 10^4$ cells/dish; Yoshida sarcoma: cir. $50-100 \times 10^4$/test tube. The cell counts of ASII were the same as those of HeLaS3.

The relationship between $^{67}$Ga concentration and $^{67}$Ga-uptake in HaLaS3 and normal S. F. cells is shown in Fig. 5. The readings were taken after incubation for 24 h following the addition of $^{67}$Ga. The cell counts were HeLaS3: $48.2 \times 10^4$/dish, normal S.F.: $3.4 \times 10^4$/dish. In both instances, the uptake of $^{67}$Ga increased in proportion to the concentration of $^{67}$Ga from 0.25 to 5 $\mu$ Ci/ml-medium. There was no saturation point for $^{67}$Ga-uptake.

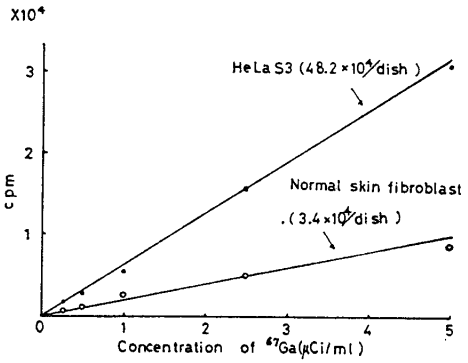


**Fig. 5**

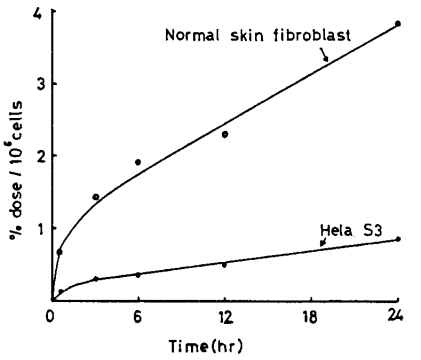


**Fig. 6**

Fig. 5. Effect of various Gallium concentrations on Ga-67 uptake by HeLaS3 and normal skin fibroblasts. Each point is the mean of two experiments.

Fig. 6. Time course of Ga-67 uptake by HeLaS3 and normal skin fibroblasts. Cells were incubated with 1 $\mu$Ci/ml Ga-67 for varying intervals at 37°C. Each point is expressed as %dose/$10^6$ cells and is the mean of two experiments.

*Time-lapse changes for $^{67}$Ga-uptake by culture cells.* The uptake of $^{67}$Ga by HeLaS3 and normal S. F. with lapse of time is shown in Fig. 6. The abscissa indicates incubation time (contact time) after the addition of $^{67}$Ga to the medium. The ordinate indicates the amount of the uptake represented in % of $^{67}$Ga activity per $10^6$ cells. In both cases, the uptake increased rapidly immedeately after

the addition of $^{67}$Ga. Between 3 and 24 h of contact time, the increase was linear. The amount of $^{67}$Ga uptake/$10^6$ cells was greater with normal S.F.

A comparison of the $^{67}$Ga-uptake at 24 h of contact time by HeLaS3 and normal S.F. cells is shown in Table 1. The uptake/$10^6$ cells was $0.83 \pm 0.30$ % with HeLaS3 and $3.55 \pm 0.87$ % with normal S.F. ; that is the uptake for normal S.F. was about 4.3 times greater than that for HeLaS3. However, when the cells in each culture were peeled with trypsin solution and the cell diameters measured by a micrometer, normal S.F. had the diameter cir. 1.6 times larger than that of HeLaS3. Consequently, the $^{67}$Ga-uptake per unit cell volume would be about the same in both cell types.

TAILE 1. COMPARISON OF GA-67 UPTAKE BY HELAS3 AND NORMAL SKIN FIBROBLASTS

| | $^{67}$Ga-uptake[a] (% dose/$10^6$ cells) | Diameter of cell[b] ($\mu$) |
|---|---|---|
| HeLaS3 | $0.83 \pm 0.30$ | $16.75 \pm 3.38$ |
| Normal skin fibroblasts | $3.55 \pm 0.87$ | $27.25 \pm 11.13$ |

*a* Cells were incubated with 1 $\mu$Ci/ml Ga-67 for 24 h, at 37°C. The results are expressed as mean ± standard deviation for 7 experiments (HeLaS3) and 5 experiments (normal skin fibroblast).

*b* The results are expressed as mean ± standard deviation for 50 cells.

Fig. 7 shows the uptake of $^{67}$Ga with lapse of time by Yoshida sarcoma cells. The change in $^{67}$Ga-uptake was not marked between 30 min and 3 h of the contact time, but as the contact time increased from 3 to 24 h, the uptake increased linearly; that is, it was biphasisc. Adsorption to the test-tube used (30 cpm) was negligible. Moreover, when Yoshida sarcoma was allowed to take up $^{67}$Ga in the same manner as HeLaS3 and normal S.F. and incubated in 1 ml of 0.2 % trypsin solution for 20 min, about 50% $^{67}$Ga was liberated in 30 min of contact time. In the period of 3–24 h, however, only 6–26% of $^{67}$Ga was liberated, and as shown by the dotted line, from 30 min to 24 h $^{67}$Ga-uptake increased in approximately a straight line.

*Time-lapse excretion of $^{67}$Ga from culture cells.* Results of the study on $^{67}$Ga excretion with lapse of time in HeLaS3 and normal S.F. are shown in Fig. 8. Taking $^{67}$Ga-uptake by the cells as 100 %, the percentage of $^{67}$Ga remaining in the cells during the period 2 to 12 h after the exchange of the medium was measured. The contact time of $^{67}$Ga and the cell is in parenthesis in the figure. After a contact time of 24 h, HeLaS3 showed a higher remnant percentage suggesting a delay in excretion. Moreover, with a contact time between 0.5 h and 24 h, the remnant percentage at 0.5 h was clearly less, indicating that the excretion of $^{67}$Ga from the cell is related to the contact time.

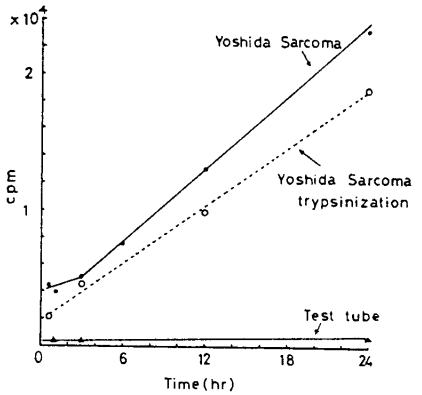


**Fig. 7**

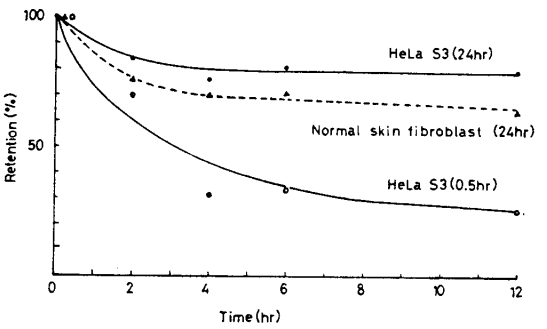


**Fig. 8**

Fig. 7. Time course of Ga-67 uptake by Yoshida sarcoma. Experimental conditions were as in Fig. 6. Each point is expressed as cpm/$10_6$ cells.

Fig. 8. Time-laese excretion of Ga-67 from HeLaS3 and normal skin fibroblasts. Numbers in parenthesis indicate the contact time of Ga-67 and cells.

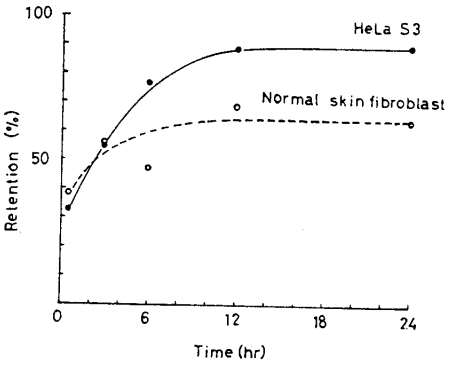


**Fig. 9**

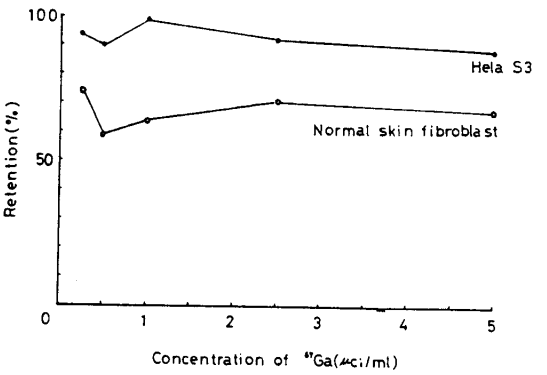


**Fig. 10**

Fig. 9. Effect of contact time on $^{67}Ga$-excretion from HeLaS3 and normal skin fibroblasts. Each point is the mean of two experiments.

Fig. 10. Effect of various Gallium concentrations on excretion of Ga-67 from HeLaS3 and normal skin fibroblasts. Each point is the mean of two experiments.

*Effects of contact time and concentration of $^{67}Ga$ on $^{67}Ga$-excretion from the cell.* Fig. 9 shows the results of the study on the relation between contact time and the remnant percentage of $^{67}Ga$ 12 h later. Both cell groups showed and increase in the remnant percentage as the contact time increased. This became constant at a contact time of 12 to 24 h. In addition beyond a contact time of 6 h, the remnant percentage between the two showed a difference, namely, HeLaS3 showed less excretion than normal S. F.

Fig. 10 shows the results of a comparison between the intracellular remnant percentage of $^{67}$Ga at a contact time of 24 h in the cases where the $^{67}$Ga concentration had been changed from 0.25 to 5$\mu$Ci/ml-medium. In both HeLaS3 and normal S.F., this change in the $^{67}$Ga concentration produced no marked effect on the excretion.

*Comparison of $^{67}$Ga-excretion in various culture cells.* Taking the $^{67}$Ga-uptake at 24 h of the contact time as 100%, the intracellular remnant percentage of $^{67}$Ga 12 h after the medium exchange was compared for various cells (Table 2). In one experiment 2–5 Petri dishes or test tubes were used and the results of 4–6 experiments were summarized for each cell group. The $^{67}$Ga-excretion was $89.0 \pm 7.4$% in HeLaS3, $87.1 \pm 9.7$% in ASII, $63.9 \pm 10.5$% in normal S.F. and in Yoshida sarcoma cells, although the experimental conditions were somewhat different, $89.7 \pm 6.1$%. In HeLaS3, ASII. and Yoshida sarcoma cells, all of which are tumor cells, the intracellular remnant percentage was approximately equal. When HeLaS3 and normal S.F. were compared statistically, the level of significance was less than 1%, indicating a highly significant difference. Moreover, there was also a significant difference between ASII and normal S.F. as the level of significance was less than 5%, indicating a delay in $^{67}$Ga-excretion from tumor cells compared with normal S. F.

TABLE 2. COMPARISON $^{67}$Ga-EXCRETION FROM HeLaS3, ASII, NORMAL SKIN FIBROBLASTS AND YOSHIDA SARCOMA

| | Doubling time (h) | Retention[a] (%) | P values[b] (vs normal skin fibroblast) |
|---|---|---|---|
| HeLaS3 | 19.2 | 89.0 ± 7.4 (6) | < 0.01 |
| AS II | 17.4 | 87.1 ± 9.7 (4) | < 0.05 |
| Normal skin fibroblasts | 40.8 | 63.9 ± 10.5 (5) | |
| Yoshida Sarcoma | 17.2 | 89.7 ± 6.1 (4) | |

*a* The results are expressed as mean ± standard deviation. The numbers in parentheses indicate the number of experiments.

*b* P values were obtained based on paired difference.

## DISCUSSION

From the fact that $^{67}$Ga accumulates strongly not only in tumor tissues but also in inflammatory foci, Ito *et al.* (2) consider that neovascularity and hyperpermeability are important as accumulation mechanisms. They consider that $^{67}$Ga first combines with serum protein (most probably albumin), then approaches the tumor through various vascular routes. These routes increase in size and number secondary to tumor or inflammation and increase in permeability. The resultant overflow from the blood vessels enables the $^{67}$Ga to enter

tumor cells as the ion, which then combines with the cytoplasm where it remains. Although many other factors are involved, it is important to clarify why $^{67}Ga$ remains in a tumor or inflammatory focus for a long period of time. Information on delay in the excretion of $^{67}Ga$ in relation to its accumulation would be helpful, but reports on this point are rare. Paterson *et al.* (15) reported that, in animal experiments, $^{67}Ga$ excretion from lactating mammary glands was about the same as $^{45}Ca$, whereas $^{67}Ga$ excreted from tumor was far less. Moreover, the $^{67}Ga$ remained for a long period of time.

In tumor cells, it was reported (2, 5) that $^{67}Ga$ occurred abundantly in the soluble fraction, but since then, Swartzendruber *et al.* localized its accumulation to lysosomes (16).

Therefore, the author studied *in vivo* time changes in the radioactivity of $^{67}Ga$ in Yoshida sarcoma, liver, lung, muscle and blood specimens. The uptake and excretion of $^{67}Ga$ were studied in cell cultures to exclude vascular factors.

The *in vivo* time-lapse accumulation of $^{67}Ga$ in Yoshida sarcoma node and liver reached a peak 24 h after administration, then gradually decreased. In both tissues, the $^{67}Ga$-accumulation increased despite continued decrease in the blood level of $^{67}Ga$, suggesting an active take up of $^{67}Ga$. In addition, even though the radioactivity of the tumor and the liver decreased gradually 24 h after the administration, it was distinctly higher than for other organs. Decreased $^{67}Ga$ accumulation in the tumor node may reflect rapid growth and degeneration of Yoshida sarcoma during the experimental period. Interstitial fluid in the tumor tissue is more abundant than in normal tissue (17) and excretion of $^{67}Ga$ from this interstitial fluid exceeds that of normal tissue, hence the excretion is not necessarily from tumor cells only. The excretion of $^{67}Ga$ from Yoshida sarcoma cells *in vitro* gave an intracellular remnant percentage of approximately 90% at 24 h of contact time, which was considerably less than that *in vivo*.

Concerning the uptake of $^{67}Ga$ *in vitro*, Glickson *et al.* (18, 19) reported a comparative study of $^{67}Ga$ in various media such as 0.9% NaCl solution and Glucose-Lock's solution using L 1210 cells. They also studies the effects of incubation temperature and pH. Awano and Matsuzawa (20) reported that malignant culture cells undergoing vigorous proliferation ingested a greater amount of $^{67}Ga$ than malignant culture cells not proliferating. The present experiment was conducted with Eagle's MEM medium and at the logarithmic growth phase to allow observations over a long period of time under conditions in which the cells show the most activity.

The time-lapse uptake of $^{67}Ga$ in the culture cells increased along with increase in the contact time as shown in Figs. 7, 8. The change in uptake for Yoshida sarcoma cells without trypsin treatment was less during the first 3 h of contact time, but between 3 h and 24 h of contact time it increased linearly,

showing a biphasic tendency. The fact that about 50% of $^{67}$Ga was liberated by trypsin treatment after 30 min of contact with the Yoshida sarcoma cells suggests that $^{67}$Ga attaches to the cell membrane in the initial stage after $^{67}$Ga addition to the medium and is gradually taken up into the cell thereafter. Tsan *et al.* (21) reported that, in their experiments with human polymorphonuclear leukocytes, the uptake of $^{67}$Ga was maximal at a contact time of 30 min. Trypsin treatment resulted in about 50% of $^{67}$Ga being liberated, so they considered that $^{67}$Ga combines with cell membrane. However, the present experimental results indicate that a longer contact time is necessary for the evaluation of $^{67}$Ga-uptake.

Normal skin fibroblasts at the longarithmic growth phase showed about a 4.3 times greater uptake of $^{67}$Ga than HeLaS3 cells. However, normal skin fibroblasts have a greater cell diameter so the uptake per unit volume in the both cases was approximately the same. Namely, the *in vitro* experiments which excluded vascular factors showed that there was no distinct difference in the uptake of $^{67}$Ga between tumor cells and normal cells.

The excretion of $^{67}$Ga from culture cells tended to decrease as the contact time increased, becoming a constant at 12 h. There was a distinct delay in excretion from HeLaS3 after 6 h of contact time in comparison with normal skin fibroblasts. These results suggest that the shorter the contact time is, the greater is the proportion of $^{67}$Ga liberated. The reason for this is that a greater amount of $^{67}$Ga combines to cell membrane, and uncombined $^{67}$Ga to cell components, so that with longer contact time, the amount of $^{67}$Ga combined to cell components increases and consequently, excretion decreases. Difference in the combination of $^{67}$Ga to the intracellular components of tumor and normal cells may account for the delayed excretion from tumor cells.

Even when the concentration of $^{67}$Ga was altered from 0.25$\mu$Ci/ml-medium to 5$\mu$Ci/ml-medium, the uptake of $^{67}$Ga was practically constant and excretion of $^{67}$Ga was unaffected. One report (22) discribes increased excretion from normal tissues after the administration of stable Ga, hence further studies are required.

The results indicate that the delay in excretion from tumor cells plays an important role in tumor accumulation of $^{67}$Ga.

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