

The Isolation and Characterization of Gallium-binding Granules from Soft Tissue Tumors¹

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SUMMARY

Both rate-zonal and isopycnic-zonal centrifugation experiments indicate that ⁶⁷Ga in rat tumors (RFT tumor and Morris 7777, 5123C, and 7794A hepatomas) and in a mouse lymphosarcoma (P-1798) is associated with lysosomal granules. The particles were identified enzymatically by their acid phosphatase and *N*-acetyl- β -D-glucosaminidase content. Autoradiography of selected gallium-binding granule fractions showed silver grains concentrated over electron-dense single-membrane organelles.

INTRODUCTION

Many different types of tumors in animals and man have an affinity for gallium. It is possible to detect such tumors by scintiscanning techniques using the radionuclide ⁶⁷Ga (5, 8, 11). Elucidation of the mechanism of ⁶⁷Ga uptake by tumor tissue should reveal those factors that control its localization and possibly lead to methods for enhancement of its affinity for cancers.

Intracellular ⁶⁷Ga present in normal and neoplastic tissue 1 to 2 days after i.v. administration has been shown by EM-ARG³ to localize in lysosome-like bodies in a variety of cell types (14). The rate-zonal centrifugation and enzymatic studies presented here provide further evidence of the lysosomal nature of the GBG previously identified by EM-ARG.

MATERIALS AND METHODS

⁶⁷Ga (Oak Ridge National Laboratory, Oak Ridge, Tenn.) was prepared as the neutral citrate and injected i.v. in the tail vein (1 mg citrate per kg). Detection of the ⁶⁷Ga present in gradient fractions was done in a well-type scintillation counter.

The following animals and transplanted tumors were used: male Buffalo rats implanted with Morris 7794A, 5123C, and 7777 hepatomas; male Fischer rats implanted

with RFT tumors (9); and female BALB/c \times DBA/2 F₁ mice with P-1798 lymphosarcomas. Animals were maintained on the same diet and fed *ad libitum*.

Twenty-four to 48 hr after the administration of ⁶⁷Ga (~1 mCi/kg), the animals were lightly anesthetized with ether and the tumor tissues were excised. The tissues were rinsed with 50 ml of 0.25 M sucrose and then homogenized in cold (5°) 0.25 M sucrose (10% w/v) in a Potter-Elvehjem-type homogenizer operating at 1000 rpm (5 strokes), a procedure considered acceptable for release of subcellular organelles with minimal loss of intact particles resulting from the tissue-rupturing technique (4). Nuclei and cell debris were removed from the homogenate by centrifugation in a Sorvall GLC-1 centrifuge for 2500 g-min. Subcellular particles from the homogenates (60 to 100 ml) were then isolated by rate-zonal centrifugation in a B-XXIX zonal rotor (loaned by Dr. N. G. Anderson, MAN Program, Oak Ridge National Laboratory) in a 1-liter gradient of 20 to 30% w/w sucrose using our sequential product recovery technique (2). The rotor effluents were monitored at 260 nm. All operations were carried out at 5°.

A modification of the zonal centrifugation procedure of Leighton *et al.* (12) was also used to isolate lysosomes. Male Buffalo rats bearing Morris 5123C hepatomas were given injections i.v. of the nonionic detergent Triton WR-1339 (Rohm and Haas Co., Philadelphia, Pa.) 2 days before i.v. injection of ⁶⁷Ga. Twenty-four hr after giving ⁶⁷Ga we killed the animals and removed and homogenized the tumors as described above. (A control experiment in which no WR-1339 was administered was also performed.) The brei was then centrifuged for 10,000 g-min to remove the cell debris and nuclei. The supernatant (100 ml) was layered over a 500-ml sucrose gradient (20 to 25%) in an Oak Ridge type B-XXIX zonal rotor. Outboard of the gradient were 3 steps consisting of 300 ml of 35%, 200 ml of 45%, and 227 ml of 55% sucrose. The centrifuge was operated at 25,000 rpm for a total of 4.3×10^8 g-min ($3,750 \times 10^7 \omega_2t$). All operations were carried out at 5°.

Fractions from the centrifugations were assayed for acid phosphatase (EC 3.1.3.2) and *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30) by the automated method described by Beck (1), protein was measured by an automated modified Lowry procedure (6), and sucrose concentrations were determined refractometrically. A method described by Hinton *et al.* (10) was used to correct for sucrose error in the enzyme and protein determinations. Electron microscopy and EM-ARG were performed on the GBG fractions; fixation,

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³The abbreviations used are: EM-ARG, electron microscopic autoradiography; GBG, gallium-binding granules.

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staining, and autoradiography were performed as described previously (14).

RESULTS AND CONCLUSIONS

The zonal profiles of homogenates (cleared of nuclei and cell debris) of 3 different tumors, a poorly differentiated rat tumor (RFT), a mouse lymphosarcoma (P-1798), and a Morris rat hepatoma (7777), are shown in Chart 1. The gradient profiles of the ^{67}Ga activity shown in Chart 1 indicate that the GBG were primarily located in the 6th-stage particle zone. It is apparent that the GBG from each of the tumors sedimented at similar rates in the gradients and therefore had similar sedimentation coefficients. ^{67}Ga recoveries from the rotor varied from 85 to 95%.

To determine whether any of the radioactivity remaining in the starting zone would sediment after longer centrifugation, we removed the 6th-stage particle zone (as in the other centrifugal integrals) and replaced it with an equal volume of 45% sucrose; the rotor was then reaccelerated to 25,000 rpm and held there 17 hr for a centrifugal integral of $42,300 \times 10^7 \omega^2 t$. It is evident from the results of the RFT experiment shown in Chart 1 that only a very small percentage of the ^{67}Ga activity banded on the 45% sucrose cushion after this additional centrifugal integral.

Enzymatic evidence for the lysosomal nature of the 6th-stage GBG is shown in Chart 2 for a Morris 7794A hepatoma. The greatest relative specific activity (percentage of total enzyme activity divided by percentage of total protein) was found in the 6th-stage particle fractions. Lysosomes were, however, distributed in all the particle zones. Recoveries of protein, acid phosphatase, *N*-acetyl- β -

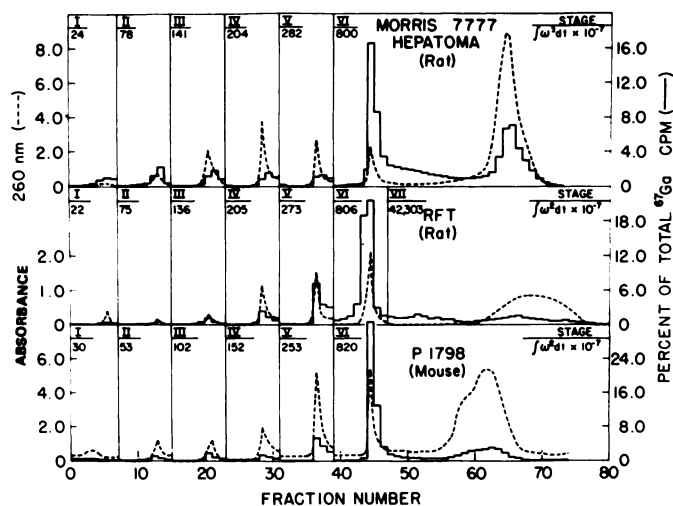


Chart 1. Distribution of ^{67}Ga in subcellular fractionations of 3 different transplanted animal tumors. The particles were separated by a discontinuous rate-zonal centrifugation procedure in an edge-unloading zonal rotor (B-XXIX). The direction of sedimentation is from right to left. The absorbance profile (260 nm) of the rotor effluent is shown as a dashed line. The histograms (solid lines) show the distribution of the ^{67}Ga . The peaks at the right of the charts represent those components that remain in the starting zone.

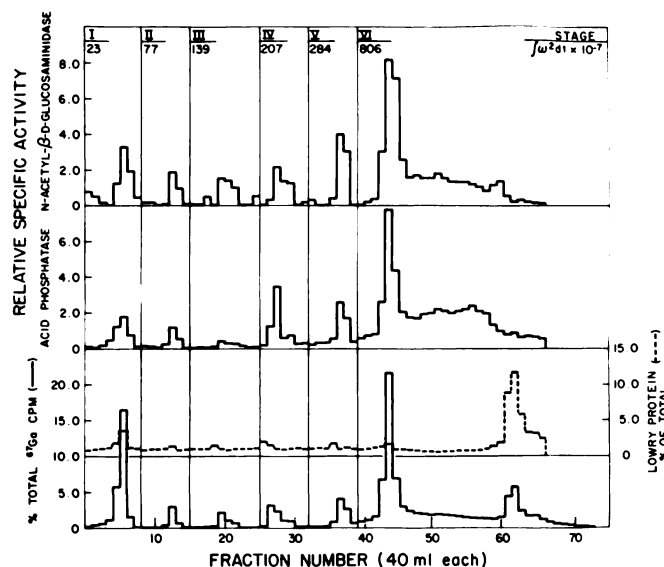


Chart 2. Rate-zonal particle profile of a Morris 7794A rat hepatoma showing the relative specific activities (percentage of total enzyme in fraction divided by percentage of total protein in fraction) of the lysosomal enzymes, *N*-acetyl- β -D-glucosaminidase and acid phosphatase.

D-glucosaminidase, and ^{67}Ga were, respectively, 96, 97, 90, and 104%.

We believe the radioactivity normally remaining in the starting zone after the centrifugal integral of $800 \times 10^7 \omega^2 t$ (Charts 1 and 2) to be mostly non-particle-bound ^{67}Ga , probably arising from broken lysosomes and plasma ^{67}Ga not removed in the rinse procedure before homogenization.

We obtained further evidence for the lysosomal nature of the GBG by isolating "light" lysosomes by isopycnic-zonal centrifugation by the method of Leighton *et al.* (12). The procedure intrinsically depends upon modifying the density of lysosomes in experimental animals. The nonionic detergent Triton WR-1339 is injected 2 to 3 days prior to administration of ^{67}Ga . This material is taken up by lysosomes causing them to become less dense than the mitochondria with which they "normally" band. Hence, one can separate the 2 organelles from each other by isopycnic centrifugation. The density of such altered lysosomes is reported to be 1.13 g/ml (12). The results of a modified version of this procedure are shown in Chart 3. Only a small percentage of the total protein (4%) occurred in the "light" lysosomal zone ($\rho = 1.135$) and the acid phosphatase activity of the particles in this zone was quite high (22%), as was the ^{67}Ga activity (27%) compared to the control experiment (no administered WR-1339) where only 12% of the acid phosphatase activity and 8% of the ^{67}Ga activity were found. A comparison of the relative specific acid phosphatase activities of the light lysosomes regions of the gradients in the 2 experiments [65 (+WR-1339), 17 (-WR-1339)] indicated that the tumor tissue lysosomes did take up Triton WR-1339. The percentage of ^{67}Ga was also greater in the zone where the light lysosomes banded (see above).

It is also evident, however, that the more dense particles contained a large amount of acid phosphatase (27%) and ^{67}Ga activity (21%). Leighton *et al.* (12) found normal as well as light lysosomes in WR-1339-treated animals, which

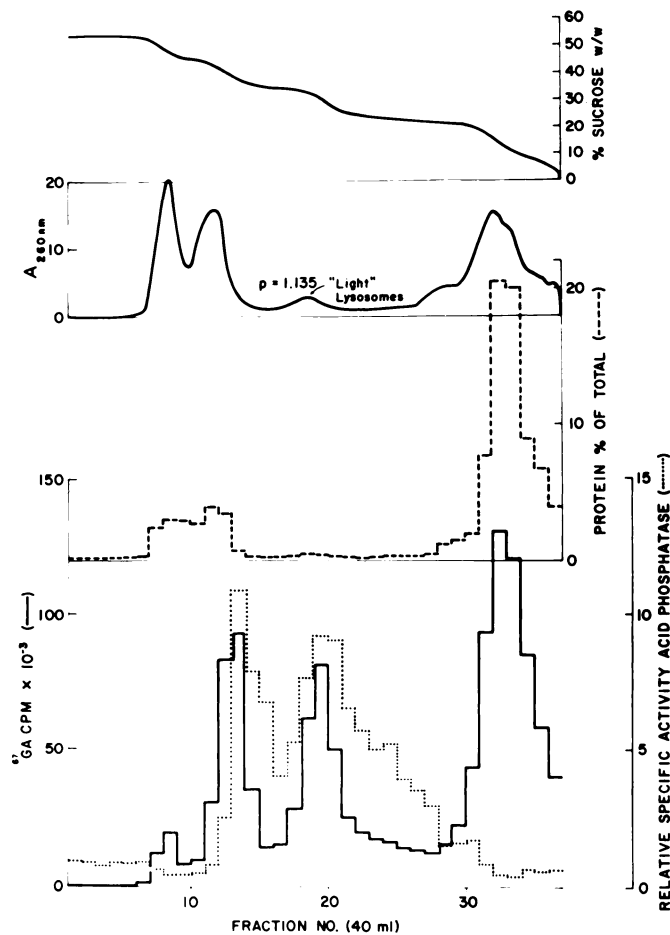


Chart 3. The association of ^{67}Ga with "light" lysosomes isolated by isopycnic-zonal centrifugation from a Morris 5123C rat hepatoma. The upper trace in the chart shows the concentration of sucrose in the rotor as determined from refractive index measurements of the collected samples. Sedimentation of the particles was from *right to left*. The relative specific activity of acid phosphatase (90% recovery) is shown by the *dotted* histogram at the *bottom* of the chart. The total ^{67}Ga radioactivity (82% recovery) of each fraction is shown as a *solid-line* histogram. The recovery of protein was 104%.

could account for the acid phosphatase and ^{67}Ga activity seen in the heavier particles. The large amounts of ^{67}Ga (49%) and acid phosphatase (46%) activity in the soluble "zone" could be accounted for by the rupturing of some of the light lysosomes as a result of increased fragility when they take up the detergent. The control experiment showed only 22% acid phosphatase activity and 10% ^{67}Ga in this zone. Recoveries in this study are indicated in the legend to Chart 3.

Electron microscopic autoradiograms made from thin sections of isolated 6th-stage GBG showed silver grains predominantly localized over small electron-dense organelles limited by single membranes (Figs. 1 and 2). The granules associated with the silver grains were morphologically similar to those identified previously as lysosomes in ^{67}Ga autoradiograms of murine tissues (14). Three distinct regions in the pellets obtained from the 6th-stage fractions were distinguished by ultrastructural study. These were: (a)

mitochondria; (b) vesicles of rough-surfaced endoplasmic reticulum; and (c) single-membrane-limited, electron-dense structures about 0.25 to 0.5 μm in diameter. More silver grains were found over the regions containing the most lysosomes and fewer silver grains were observed over regions of the pellet in which mitochondria and vesicles of rough-surfaced endoplasmic reticulum predominated.

DISCUSSION

Swartzendruber *et al.* (14), using EM-ARG, have shown that intracellular ^{67}Ga present in normal and neoplastic tissue 1 to 2 days after i.v. administration is localized in lysosome-like bodies in a variety of cell types. The subcellular fractionation and enzymatic studies reported here for 4 different transplanted tumors provide further evidence for the lysosomal nature of the organelles previously identified by EM-ARG.

Contrary to the results that we report here, those of Deckner *et al.* (3) with Ehrlich ascites cells and Orii (13) with Yoshida sarcoma have indicated that little or no ^{67}Ga is associated with lysosomes. Orii also reported that no accumulation of ^{67}Ga was observed in liver lysosomes, also contrary to our previous findings (2) as well as those of Haubold and Aulbert (7). Orii's results and those of Deckner *et al.* may, therefore, be due to the disruption of lysosomes in some phase of the fractionation procedures that they used.

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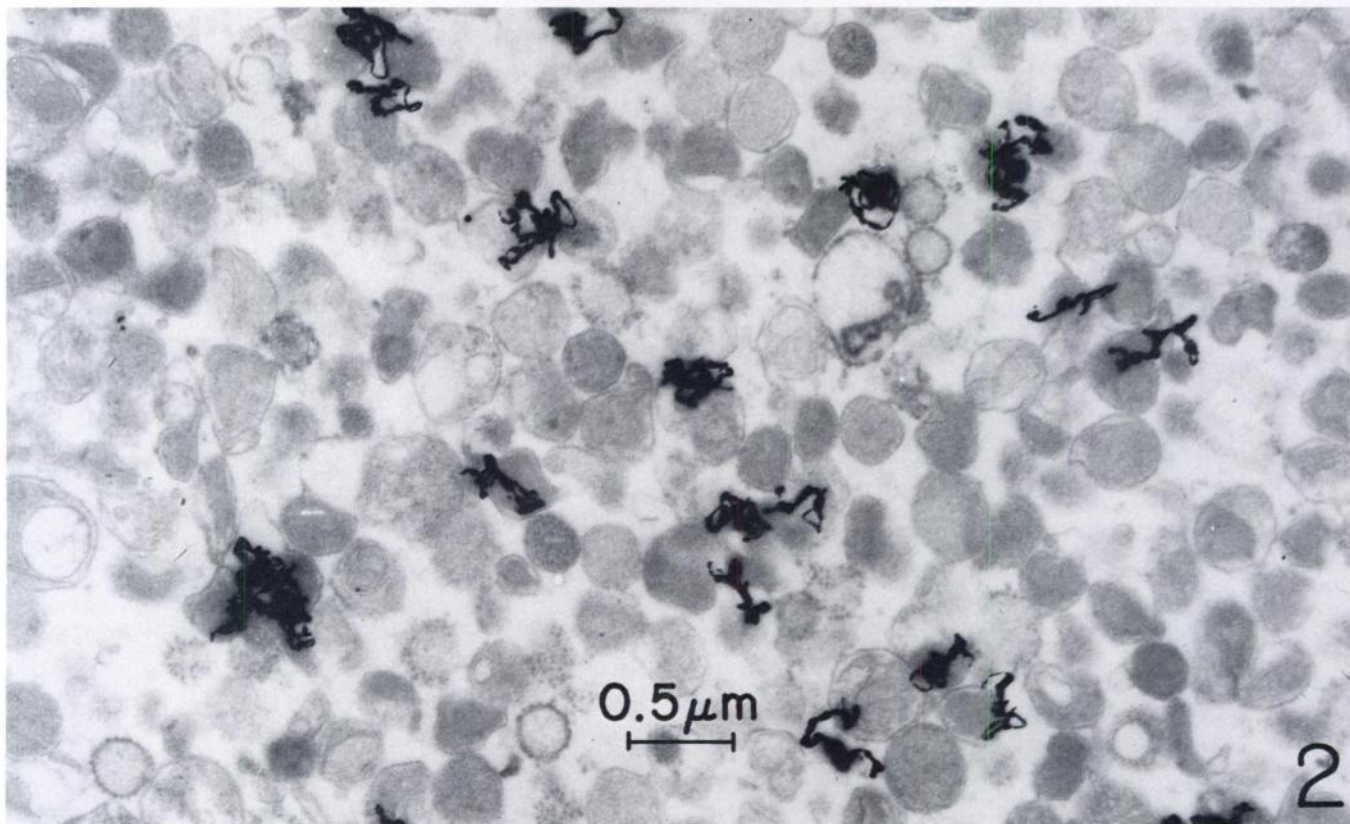
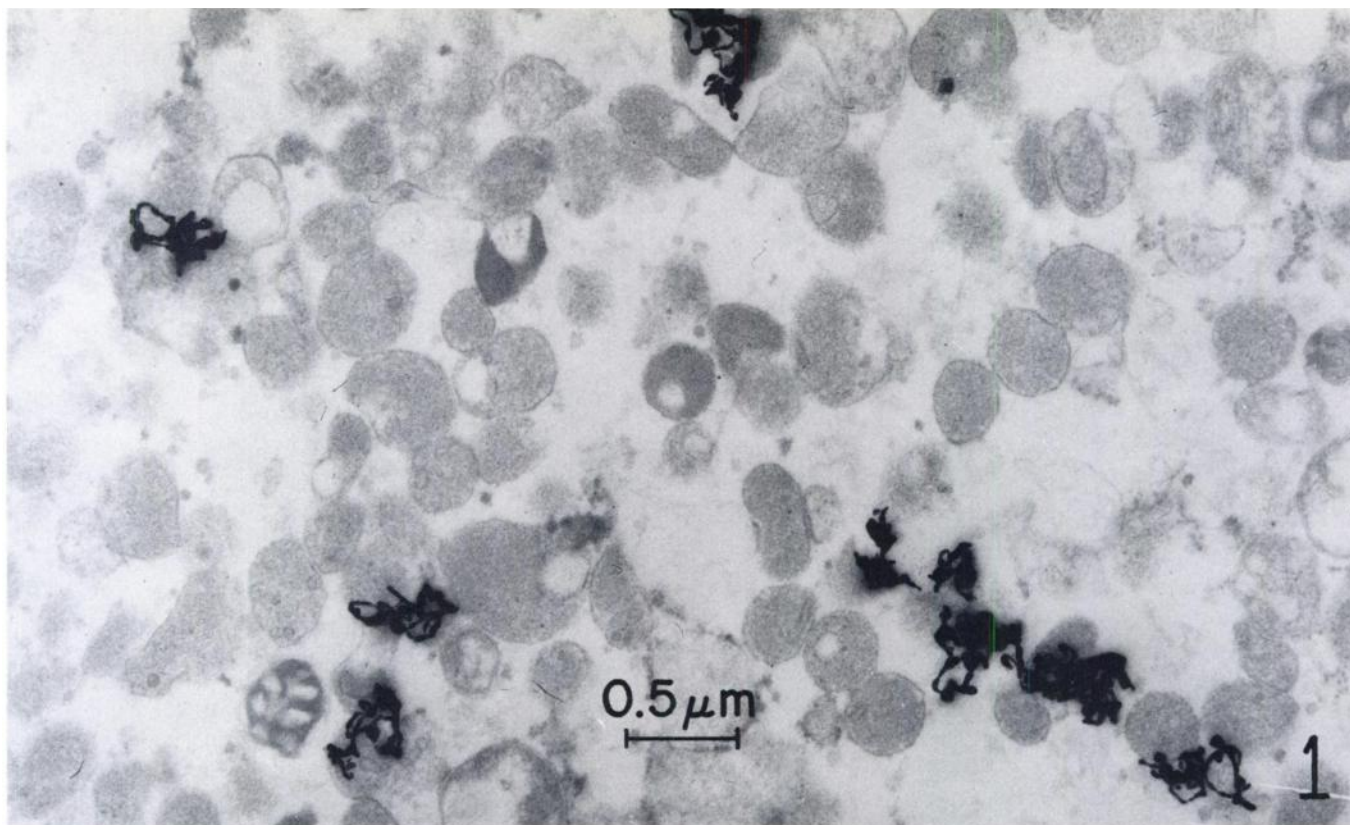


Fig. 1. An electron microscopic autoradiogram of rat tumor (RFT) 6th-stage fraction containing the majority of ⁶⁷Ga activity. This area of the block contained more lysosomes and more silver grains than adjacent regions. $\times 31,000$.

Fig. 2. An electron microscopic autoradiogram of 6th-stage fraction from mouse lymphosarcoma (P-1798) showing many silver grains localized in a region containing many lysosomes. A few mitochondria and vesicles of rough-surfaced endoplasmic reticulum are also present. $\times 29,000$.