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PENETRATION OF SUBSTANCES INTO TUMOR TISSUE — A METHODOLOGICAL STUDY ON CELLULAR SPHEROIDS

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SUMMARY

The penetration of [^{3}H]thymidine, [^{3}H]D-leucine, [^{125}I]albumin, and the drugs [^{3}H]5-fluorouracil and [^{3}H]vinblastine into human glioma spheroids (in vitro tumor models) was studied by a method based on rapid freezing, freeze drying, vapor fixation, wax embedding, dry sectioning, and contact autoradiography. No significant disturbances in the distribution of water soluble substances were observed. Thymidine and D-leucine penetrated the whole spheroids relatively fast, whereas albumin showed reduced penetration. The concentration of albumin was highest at the periphery of the spheroids, but only smaller amounts were detected in the deeper regions. A significant difference between the penetration patterns of the drugs studied was also observed. Fluorouracil penetrated rather freely, but the penetration of vinblastine was limited.

Key words: albumin; cellular spheroids; cytotoxic drugs; leucine; penetration; thymidine.

INTRODUCTION

The penetration of cytotoxic drugs into the poorly vascularized regions of solid tumors influences their effectiveness. Molecular weight, charge, water solubility, and other physicochemical properties of a substance affect the interaction with cells and their extracellular matrix, and thereby the penetration of the substance. In this study we have used three-dimensional spherical cell-colonies, "cellular spheroids," as models of poorly vascularized tumor tissue. Many types of mammalian cells have the ability to form and grow as cellular spheroids. A spheroid consists of an outer layer mainly containing dividing cells and an intermediate layer of resting cells. If the spheroids are big enough, massive necrotic regions develop in the center. This growth pattern is similar to the pattern often seen in nodules of fast-growing malignant solid tumors (1-3).

The aim was to develop a method for following the penetration of substances into the depth of the spheroids. The problem is that most substances of interest are water soluble. In conventional histological techniques the samples are treated with hydrophilic liquids on several occasions, leading to disturbances in the distribution pattern of the water-soluble substances (4). The method described in this work, which is based on rapid freezing in a propane-propene mixture at −193° C followed by freeze drying, vapor fixation, wax embedding, and dry sectioning, does not allow the samples to be in touch with hydrophilic liquids.

Preparation of histological specimens for autoradiography usually is performed under conditions that disturb the distribution of water-soluble material in the samples. We have tested a dry autoradiographic method (contact autoradiography) to overcome this problem. Some other methods to overcome this problem have been described previously (4).

Using these methods we have studied the penetration of two low molecular weight substances (thymidine and D-leucine), a protein (albumin), and two drugs (fluorouracil and vinblastine). The nucleoside thymidine is incorporated in DNA, but D-leucine and albumin do not take part in cell metabolism. The two drugs were chosen

to demonstrate the usefulness of the method for studies of chemotherapeutically interesting substances.

MATERIALS AND METHODS

Chemicals. The radiochemicals used were: [methyl-^{3}H]thymidine, 185 GBq/mmol, D-[4,5-^{3}H]leucine, 37 GBq/mmol, 5-fluoro [6-^{3}H]uracil, 111 GBq/mmol, and [G-^{3}H]vinblastine sulfate, 385 GBq/mmol from the Radiochemical Centre, Amersham, England, and human [^{125}I]serum albumin 4 MBq/ml from Kabi Diagnostica, Nyköping, Sweden. [The unit of radioactivity in the International System of Units is the becquerel (Bq). One becquerel is equal to one disintegration per second. Thus, 1 curie = 3.7×10^{10} becquerels; 1 gigabecquerel (GBq) = 27.027 millicuries (mCi).] The paraformic aldehyde and the xylen were purum from Kebo AB, Stockholm, Sweden. The embedding wax was Ralwax I from R. A. Lamb, London, England, or Paraffin from E. Merck AG, Darmstad, FRG.

Cell culture. The cells used in these experiments were a human glioma cell line called U-118 MG, originally established by Westermark et al. (5). These cells are usually grown as conventional monolayer cultures. In our experiments they were grown as cellular spheroids in minidishes as described previously (3,6). The spheroids were allowed to attach to round cover slips (d 12 mm) 20 to 30 h before each experiment. Each cover slip was then transferred to a small plastic dish (d 3 cm) containing 2 ml medium (Eagle's MEM with 10% fetal bovine serum, penicillin 100 IU/ml, and streptomycin 50 μg/ml).

Administration of test substances. The test substances used were administered by transferring the cover slips with the spheroids to plastic dishes (d 3 cm) containing the test substance in 2 ml fresh medium. The spheroids were then incubated at different times as shown in Table 1.

Incubation in [^{3}H]thymidine was done in the first case (37 kBq/ml for 18 h, *see* Table 1) to test whether the amount of [^{3}H]thymidine incorporated in the DNA of S-phase cells, observed with the present method, was comparable to that previously observed in studies using conventional histological techniques (7). The shorter incubation time (15 min) in [^{3}H]thymidine (1.85 MBq/ml), without washing in nonradioactive medium, was used to determine if any extranuclear, nonincorporated [^{3}H]thymidine could be observed.

TABLE 1

TEST SUBSTANCES, ACTIVITIES, AND INCUBATION TIME USED IN THE EXPERIMENTS

Test Substance	Activity in the Medium	Incubation Time
[^{3}H]Thymidine	37 kBq/ml	18 h[a]
[^{3}H]Thymidine	1.85 MBq/ml	15 min
[^{3}H]D-Leucine	1.85 MBq/ml	0.5, 2, and 5 min
[^{125}I]Albumin	0.67 MBq/ml	20 min
[^{3}H]5-Fluorouracil	0.23 MBq/ml	15 min
[^{3}H]Vinblastine	0.24 MBq/ml	15 min

[a] After the 18 h incubation in radioactive medium the spheroids were incubated for 1 h in medium without radioactivity.

[^{3}H]D-Leucine and [^{125}I]albumin were chosen as examples of substances that are not metabolized by the cells. If the spheroids are supposed to contain only water instead of cell structures, and Einstein's diffusion equation, $x^2 = 2 \cdot D \cdot t$ (where x = diffusion length, D = diffusion constant, and t = time) (8), is used, an approximate calculation of the diffusion time (t) of the test substances into the central parts of the spheroids can be made. For spheroids of 600 μm d, times of about 1 min for a low molecular weight substance such as leucine, and about 15 min for a high molecular weight substance such as albumin, are obtained. The incubation times for leucine and albumin were chosen with the help of these calculations.

The albumin was fractionated by gel filtration on Sephadex G-100 (column size 1.4 × 92 cm) equilibrated in PBS buffer (0.15 *M* NaCl, 0.05 *M* phosphate, pH 7.4). The flow rate, sample volume, and fraction volume were 9 ml/h, 1 ml, and 3 ml, respectively. Each fraction was analyzed for ^{125}I. Only the fraction with monomeric [^{125}I]albumin was used to make sure that no labeled, low molecular weight substances or polymeric albumin were added to the medium.

To check if the albumin was broken down to smaller peptides during the incubation, [^{125}I]albumin was incubated in medium, both with and without spheroids, during different times (2, 24, 48, and 72 h). Thereafter the medium was fractionated on Sephadex G-100 as described above.

In addition to the above substances, the chemotherapeutic drugs 5-fluorouracil and vinblastine were studied. The incubation time was 15 min.

Freezing. After incubation the cover slips were removed with a pair of forceps, and most of the

adhering medium was removed quickly by absorption onto a filter paper. The cover slips were then immersed quickly in a bath of liquid propane-propene mixture, kept cool in liquid nitrogen. This arrangement prevented boiling.

Freeze drying. To avoid thawing of the spheroids, the cover slips were immersed in liquid nitrogen during the transport from the freezing apparatus to the freeze-drying apparatus. Freeze drying was performed at −80 to −70° C, to avoid ice-crystal formation in the spheroids. When all the ice had sublimated (after about 4 to 6 h) from the samples, one could notice a continuous decrease in the pressure corresponding to the maximum capacity of the vacuum pump. When the freeze drying reached that far, the temperature of the sample holder and the preparations were allowed to slowly reach room temperature under continued pumping.

Vapor fixation. The next step in the histologic preparation was vapor fixation in paraformic aldehyde vapor. A closed chamber containing solid paraformic aldehyde was put in an 80° C oven for about 1 h. This gave a paraformic aldehyde-saturated atmosphere in the chamber. Then the samples were put on a net in the chamber and fixation was performed for about 1 h.

Embedding. After fixation the samples were put in embedding molds containing xylen, for 30 min, and thereafter transferred to embedding molds, containing melted wax at 60° C. The melted wax in the molds was changed twice (2 × 30 min) before final casting took place. When the blocks had solidified they were adjusted with a scalpel and the cover slips were removed carefully. The spheroids could now be seen as faint yellow spots in the wax.

Alternatively, embedding without xylen infiltration was tested, the samples being put directly into melted wax (60° C) in a vacuum oven from which air was evacuated. This method, however, harmed the histologic quality, probably due to bubble formation during the air evacuation. No differences could be observed in the penetration patterns when the two embedding methods were compared.

Sectioning. Five-μm-thick sections were prepared with a Leitz Wetzlar microtome. They were dry mounted, using a pair of forceps, on cleaned object plates covered with a very thin layer of glair glycerin, then stretched with a soft brush. The object plates were put on a heating plate to melt the wax for a few seconds and to disperse any air bubbles that might occur. Thereafter they were held at room temperature. The spheroid sections were unwaxed in xylen for 3 × 5 min.

Contact autoradiography. A clean object plate was immersed in photoemulsion (Ilford K5). The emulsion was removed with a clean tissue from the back side, especially near the edges of the front side of the plate, to give a flat surface. The plate was allowed to dry in the dark. Then the plate with the spheroid section and the plate with the dry emulsion were put together, so that the section was pressed tightly to the emulsion. This position was secured by a paper clip. Assuming that the test substance was isotropically distributed, the exposure time needed was calculated from the equation: $G = A \cdot t \cdot E \cdot 432 \cdot 10^{-9}$, where G is the amount of grains over an area of 10 × 10 μm in a spheroid section, A is the activity in the medium in Bq/ml, t is the exposure time in days, and E is the percentage of autoradiographic efficiency. In our experiments we wanted to have a G value of about 50 to get an easily detectable grain density on the film. The efficiency can be assumed to be about 5% for ^{3}H (4), and about 10% for ^{125}I. After the exposure the plates were separated and the autoradiographic plate was developed in Kodak D19 and fixed in Kodafix.

Staining and mounting. The spheroid sections were stained by hematoxylin (an acid solution according to Ehrlich) and mounted with Euparal (Chroma Gesellschaft, Stuttgart, FRG).

Densitometric evaluation. The autoradiogram was photographed (Kodak Panatomic-X) in a light microscope (Leitz Wetzler Orthoplan). The negatives were copied on 17 × 11.5-cm transparent photopaper (Kodalith Ortho type 3) developed in Kodak D72. The grain density was measured with a densitometer (Gelscanner Zeiss KM3) along a line leading from outside the spheroid through the periphery and along the diameter of the spheroid section. Since the grain density was proportional to the concentration of the test substance, this concentration was obtained as a function of distance from the spheroid periphery.

RESULTS

Histology. The gradient in concentration of test substances at the edge of the spheroids was always steep, and inward gradients were sometimes obtained. This indicated that the methods used prevented severe disturbances in the spatial distribution of the test substances. However, the histologic quality in the sections was not as good as

with other methods. In Fig. 1, a section of a glioma spheroid treated as described is compared with a section of a glioma spheroid embedded in methacrylate as described by Carlsson and Brunk (9). The comparison shows that our method gives a more disrupted cellular structure than does the conventional method.

Penetration and incorporation of [^{3}H]*thymidine.* The labeling of proliferative cells due to incorporation of [^{3}H]thymidine, after incubation for

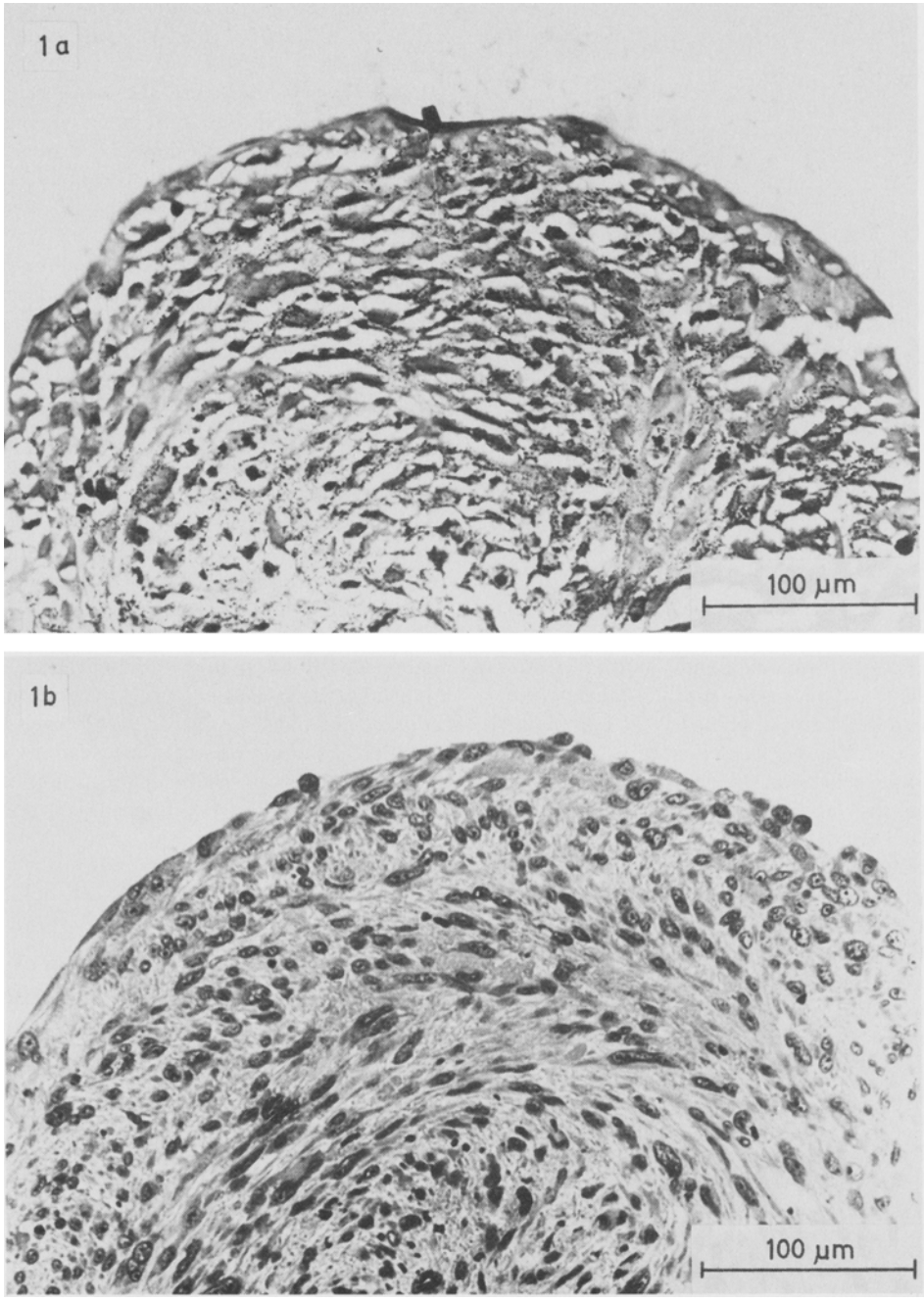

FIG. 1. Comparison between a section of a spheroid prepared by the method described in this paper (*a*) and a section of a spheroid embedded in methacrylate (*b*).

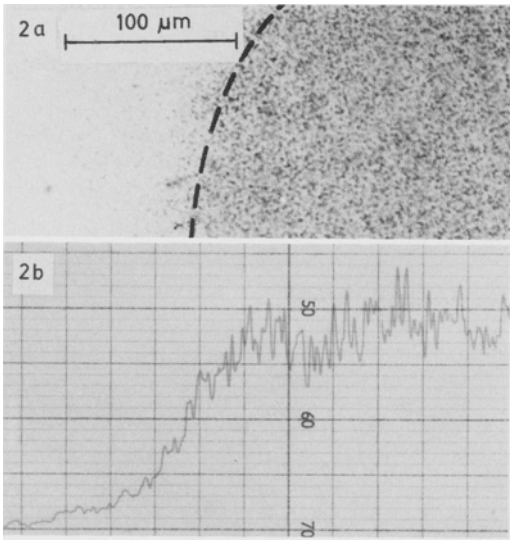

FIG. 2. The penetration pattern of [^{3}H]D-leucine in a spheroid after 5 min incubation. *a*, Grain distribution in the contact autoradiogram. *b*, Grain distribution curve from the densitometric evaluation. The scale is the same in *a* and *b*. The broken line indicates the spheroid periphery.

18 h with 37 kBq/ml and washing for 1 h, were normal in comparison to the labeling seen with conventional histologic and autoradiographic methods (7). This means that the labeling index decreased constantly from nearly 90% in the periphery to almost no labeled cells in the central parts of the spheroids. The amount of grains seen between the labeled cell nuclei was negligible (about 0.4 grains/μm^2).

When spheroids had been incubated for 15 min in medium with [^{3}H]thymidine (1.85 MBq/ml) without washing, labeled nuclei were seen again. However, in this case a significant amount of isotropically distributed, extranuclear [^{3}H]thymidine could also be seen (about 0.15 grains/μm^2).

Penetration of [^{3}H]*D-leucine.* The penetration of [^{3}H]D-leucine in a spheroid after 5 min incubation is reflected in the contact autoradiogram (Fig. 2, *a*), and by a grain distribution curve (Fig. 2, *b*). The grain density was low outside the spheroids. A sharp increase in the grain density was obtained close to the spheroid periphery, but the density was high and at a constant level inside. After 0.5 min incubation a gradient was seen inside the spheroids. After 2 and 5 min of incubation, however, no gradients were seen and the grain density seemed to have reached a stable level, indicating that equilibrium was established within 2 min (Fig. 3).

Penetration of [^{125}I]*albumin.* After 20 min incubation in medium containing [^{125}I]albumin, it was noted that large amounts of grains were confined to the periphery of the spheroids. However, the amount of grains inside were significantly higher than outside (Fig. 4), indicating some penetration.

Penetration studies were also performed after 2 and 18 h incubation in medium with [^{125}I]albumin. The results showed that most of the albumin was confined to the periphery but the concentration in the central regions was increased only slightly.

We attempted to determine if the albumin was broken down to smaller peptides during incubation. After 2 h of incubation, less than 1.5% of the activity appeared as low molecular weight substances. This breakdown of [^{125}I]albumin was independent of whether spheroids were present in the medium or not. This small fraction, if it were isotropically distributed inside the spheroids, would hardly give rise to a measurable grain density. Therefore, the increased grain density seen inside the spheroid in Fig. 4, probably was due to penetration of intact macromolecular [^{125}I]albumin.

Penetration of cytotoxic drugs. Because the method described in this work may have direct application in studies on drug penetration into cellular spheroids, we also present results from our first studies on the penetration of 5-fluorouracil and vinblastine. After 15 min incubation in medium with [^{3}H]5-fluorouracil, this drug

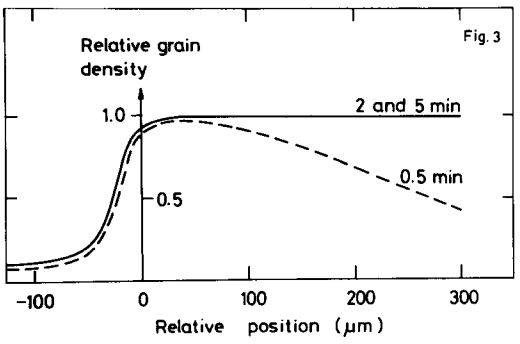

FIG. 3. Idealized grain distribution curves after 0.5, 2, and 5 min incubation in medium with [^{3}H]D-leucine. Zero position on the *abscissa* symbolizes the spheroid periphery. Positive positions are located inside the spheroid.

seemed to be almost isotropically distributed inside the spheroids (Fig. 5). After the corresponding incubation in [^{3}H]vinblastine medium, however, the main part of the vinblastine was present

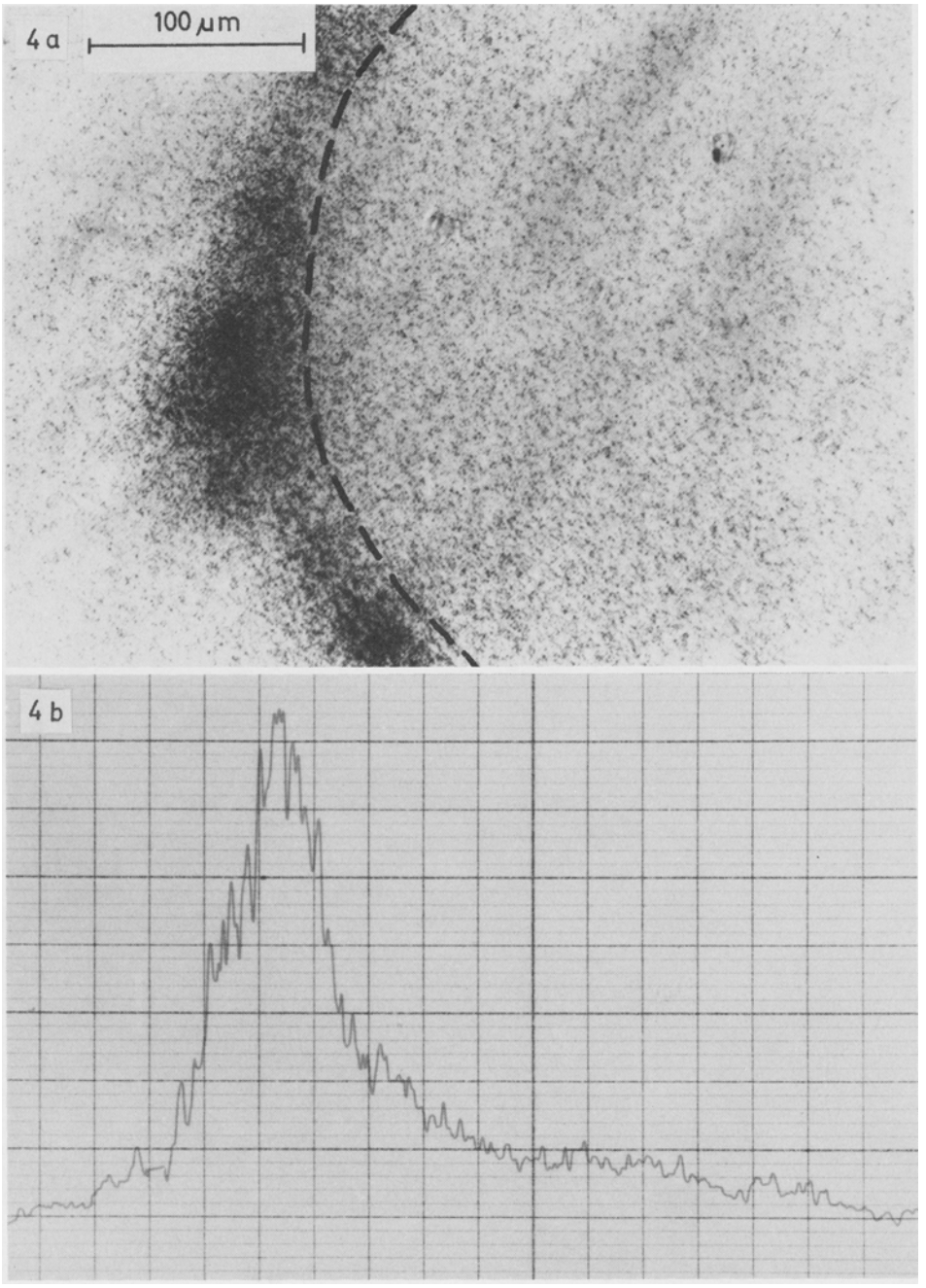

FIG. 4. The penetration pattern of [^{125}I]albumin in a spheroid after 20 min incubation. *a*, Grain distribution in the contact autoradiogram. *b*, Grain distribution curve from the densitometric evaluation. The scale is the same in *a* and *b*. The broken line indicates the spheroid periphery.

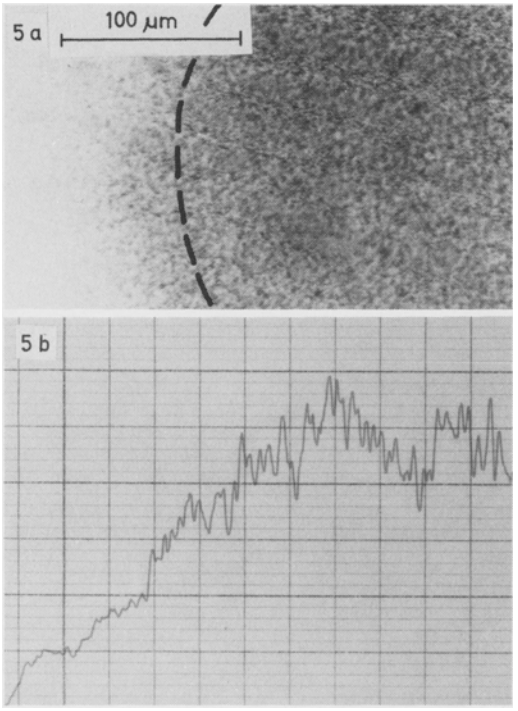

FIG. 5. The penetration pattern of [^{3}H]5-fluorouracil in a spheroid after 15 min incubation. *a*, Grain distribution in the contact autoradiogram. *b*, Grain distribution curve from the densitometric evaluation. The scale is the same in *a* and *b*. The broken line indicates the spheroid periphery.

in the outer cell layers of the spheroid, but only smaller amounts seemed to have reached the central parts (Fig. 6).

DISCUSSION

The methods described in this work allowed studies of penetration of water-soluble substances (e.g., amino acids, drugs, proteins, etcetera) into cellular spheroids. The grain density gradients in the autoradiograms were always relatively sharp outside the spheroids. Inward gradients were obtained in some cases (for example with [^{3}H]D-leucine incubated for 0.5 min), but no outward gradients (highest grain density in the central parts of the spheroids and lowest at the periphery) were observed. This indicated that the histologic and autoradiographic methods described in this work prevented significant disturbances in the distribution of the studied substances. If the distribution had been disturbed during preparation, an outward gradient or no radioactivity at all would have occurred in the spheroids. The contact autoradiography method also had the advantage of allowing a quantitative evaluation using a densitometer since no disturbing cell structures were present in the autoradiogram. The section of the corresponding spheroid could, at the same time, be inspected on a separate glass.

The low molecular weight substances D-leucine and thymidine penetrated relatively quickly into

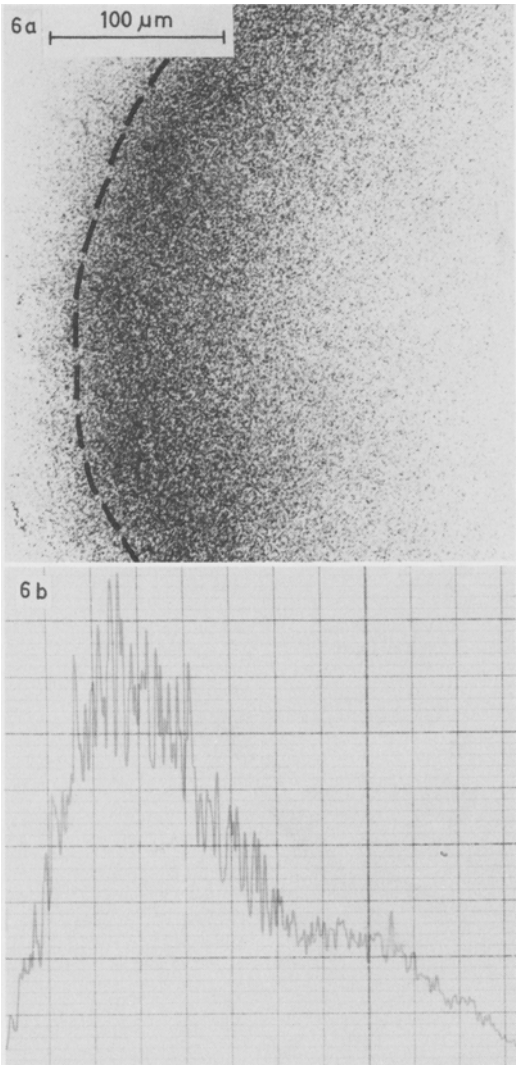

FIG. 6. The penetration pattern of [^{3}H]vinblastine in a spheroid after 15 min incubation. *a*, Grain distribution in the contact autoradiogram. *b*, Grain distribution curve from the densitometric evaluation. The scale is the same in *a* and *b*. The broken line indicates the spheroid periphery.

the spheroids, and an equilibrium seemed to be reached within a few minutes. This is in accordance with the previous findings that thymidine passes easily through the membranes of mammalian cells (10).

Albumin seemed to attach mainly to the periphery of the spheroids (Fig. 4). The distribution along the periphery was, however, somewhat irregular, probably due in part to aggregation during the freeze drying. Some penetration of albumin into deeper regions of the spheroids was also observed. The fact that albumin was seen mostly near the periphery of the spheroids probably is due to restricted diffusion in the extracellular matrix. Large extracellular spaces of unknown composition exist in human glioma spheroids (9). Degradation of albumin molecules by, for example, proteolytic enzymes could not be demonstrated in the gel-filtration experiments. The studies on albumin penetration should be supplemented with studies on other proteins before definite conclusions can be drawn about the penetration of proteins in general.

The knowledge of the penetration properties of cytotoxic drugs in poorly vascularized regions of solid tumors is defective. Such knowledge is, however, very important because these regions are supposed to contain radio-resistant cells caused by hypoxia (11,12) and therefore to be important targets of therapeutic agents. Our studies indicated that fluorouracil penetrated the spheroids rather easily, but the penetration of vinblastine was limited. This is interesting because the serum level of vinblastine is reported to decrease very rapidly in the first phase after drug injection into patients (13). The combination of a rapid decrease in the serum level and bad penetration properties might give rise to a suboptimal concentration of the drug in poorly vascularized tumors. We also intend to study the penetration of vinblastine in other types of spheroids.

Fluorouracil is an antimetabolite, and its primary cytotoxic action is to block thymidylate (and thereby DNA) synthesis (14). Vinblastine is a plant alkaloid, and it acts on the mitotic spindle of the cell (15). The toxic action of the drugs studied might have changed the penetration pattern. Such changes are automatically accounted for in our model system.

The risk of radiation damage influencing penetration, due to the added radioactivity, probably is minor. At the concentrations used, the activity has to be incorporated into macromolecules which then must be exposed for some hours before measurable damage at the cellular level occurs (10).

All studies reported here were carried out using human glioma spheroids. If other types of spheroids (1-3) had been used quite different penetration patterns might have been obtained.

The method described in this work can be used to relate the penetration properties of different substances to their physicochemical properties and biological effects. Such knowledge is important to understand the effects of therapeutically interesting substances (e.g., drugs, immunoglobulins, and hormones) and to optimize their characteristics. Since combination treatments are now common in tumor therapy, it also would be interesting to see whether the penetration properties of different substances are changed after treatment with irradiation or hyperthermia.

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