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PENETRATION OF SUBSTANCES INTO TUMOR TISSUE: A METHODOLOGICAL STUDY WITH MICROELECTRODES AND CELLULAR SPHEROIDS

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SUMMARY

A new method was tested for studies of penetration of substances into tumorlike tissue. The penetration of the ions K^+, Cl^-, and Ca^{2+} through several layers of tumor cells was demonstrated by using double barrelled, ion sensitive microelectrodes with extra thin tip diameters. Spheroids consisting of human glioma, U-118 MG, and human thyroid cancer, HTh-7, cells were used as models of tumor tissue. A microelectrode was inserted into the center of a spheroid. Thereafter, the concentration of the test substance was increased in the surrounding medium. The change in concentration inside the spheroid was recorded and the penetration pattern evaluated. All three types of tested ions penetrated easily through the spheroids. The K^+ ions penetrated most efficiently, and the Ca^{2+} ions showed the slowest penetration. The Ca^{2+} ions penetrated somewhat more slowly in the U-118 MG spheroids (which had rather small extracellular spaces) than in the HTh-7 spheroids (which had larger extracellular spaces). Ion sensitive electrodes, which are easily available, were used in this study only to demonstrate the principle. We hope that the method described can be used for penetration studies of various substances. For example, all substances that can be detected by enzyme microelectrodes could be studied. The main advantage of the method is that the complete penetration pattern can be studied as a function of time in individual spheroids. Previously described methods require histological procedures for each analyzed penetration time.

Key words: calcium; cellular spheroids; chloride; microelectrode; penetration; potassium.

INTRODUCTION

Studies of penetration of different substances into tumor tissue are interesting from many points of view. One obvious question is whether or not therapeutically active substances penetrate efficiently through poorly vacularized tissue (1,2). Another question is whether or not necrosis results from a lack of nutrients with limited penetration capacity or results from accumulation of toxic, catabolic products. The catabolic products might be accumulated due to a limited penetration capacity.

Studies of penetration of ions might not be of direct interest for tumor therapy or tumor biology. In this work ion penetration was used mainly for demonstration of the method. Ion sensitive microelectrodes are easily available and no penetration problems are expected for ions with low molecular weight. Potassium, calcium, and chloride ions are involved in complicated and essential mechanisms in the cell function (3,4) and might therefore be of some interest.

Different physical and chemical properties, such as molecular weight and charge, probably determine the penetration capacity of a substance. For this reason, it might be interesting to study the penetration of ions with low molecular weight. Rather low molecular weight substances such as methotrexate and adriamycin probably have penetration difficulties (1,5).

The penetration patterns were studied in cellular spheroids (6-8). Spheroids consist of aggregated cells in a spherical configuration (Fig. 1). The peripheral cells proliferate intensively and contribute to spheroid growth. Deeper lying cells in the spheroid suffer from poor vascularization from the surrounding culture medium. As a result of this, the degree of proliferation decreases rapidly as a function of depth in the spheroid (8,9). If the spheroids are big enough (0.5 to 1.0 mm, depending on the cell line), massive necrotic regions will develop in the central parts.

Fast growing, solid tumors often develop nodules with necrotic regions, caused by insufficient vascularization. Cells in the intermediate region between the necrosis and the blood vessel suffer in varying degrees from insufficient support from oxygen and other metabolites (10,11). Thus, the cellular spheroids imitate the behavior of nodules in fast growing, solid tumors.

Spheroids have been used recently as a model system in penetration studies. It was found that vinblastine (2) and possibly also methotrexate (1) and adriamycin (5) had a limited capacity to penetrate into the center of the analyzed spheroids. 5-Fluorouracil was found to penetrate much more efficiently (2). All penetration studies were made with histological procedures. The test substance was detected with autoradiography or fluorescence measurements in sections. For each penetration time new spheroids and new sections had to be prepared. In this work we describe a technique by which the complete time pattern of penetration could be studied in individual spheroids.

Materials and Methods

Cell culture. Two human tumor cell lines were used in this study. They were the human glioma cell line U-118 MG (12,13) and the human thyroid cancer cell line HTh-7 (13). Both of these cell lines are usually grown as conventional monolayer cultures. In this study they were cultured as cellular spheroids, using techniques previously described (7,9). The diameter of the spheroids used was 650 ± 150 μm. One day before measurements each spheroid was allowed to attach itself to round cover slips (diam 15 mm, from Lux Scientific Corporation, Munich, FRG). Only cells at the lower end of the spheroids attached themselves to the glass. The cell organization inside the spheroids was not changed on this account, as was seen in sections (Fig. 1). The culture medium used was Ham's F10 with 10% newborn bovine serum, L-glutamine (2 m*M*), pencillin (100 U/ml), and streptomycin (100 μg/ml) (all from Flow Laboratories Swedish AB, Stockholm, Sweden). However, this medium could not be used during the microelectrode measurements in the perfusion chamber. The serum yielded too much foam and some components in the F10 medium poisoned the electrodes. Instead, Locke's solution (NaCl 7.5 g/l, KCl 0.42 g/l, $CaCl_2xH_2O$ 0.31 g/l, glucose 1.0 g/l, HEPES 1.67 g/l, $NaHCO_3$ 0.76 g/l, and NaOH to pH 8.5 at room atmosphere) was used (14).

Microelectrodes. The microelectrodes used were double barrelled ion sensitive electrodes with extra thin tip diameters (about 0.1 μm) manufactured for intracellular measurements. These ion sensitive microelectrodes have been described in detail previously (15,16). Briefly, one channel was filled with an ion exchanger and used for measurements of the ion activity (K^+, Cl^-, and Ca^{2+}, respectively). The other channel was used for membrane potential measurements. The potential signal was used to correct the signal from the ion channel, so that only the signal change due to changed ion activity was recorded from that channel. The potentials also helped us to judge the moment at which the electrode hit the spheroid surface (14). The mean response time was 170, 350, and 250 msec for the potassium, calcium, and chloride electrodes, respectively. There was a linear relation between the exponent of the ion activity and the potential of the electrodes, and the mean steepness per tenfold increase in ion activity was 50.15, 27.4, and 39 mV for the potassium, calcium, and chloride electrodes, respectively (16). All the microelectrodes used were manufactured at the Max-Planck-Institut for Systemphysiology in Dortmund, Federal Republic of Germany.

Experimental set up. The perfusion chamber system is shown in Fig. 2. The pH of the medium was adjusted to about 7.3 by bubbling air containing 5% CO_2 through it. The medium was heated and pumped through the perfusion chamber (≈10 ml/min). The multichannel recorder recorded ion activity, membrane potential, and electrode position. The pH and temperature in the medium were also controlled continuously. By injecting a stain pulse into the medium, the flow pattern in the chamber could be observed in the stereomicroscope (Fig. 2 *b*). This showed irregular flow patterns with strong convection in the upper parts of the medium. However, there

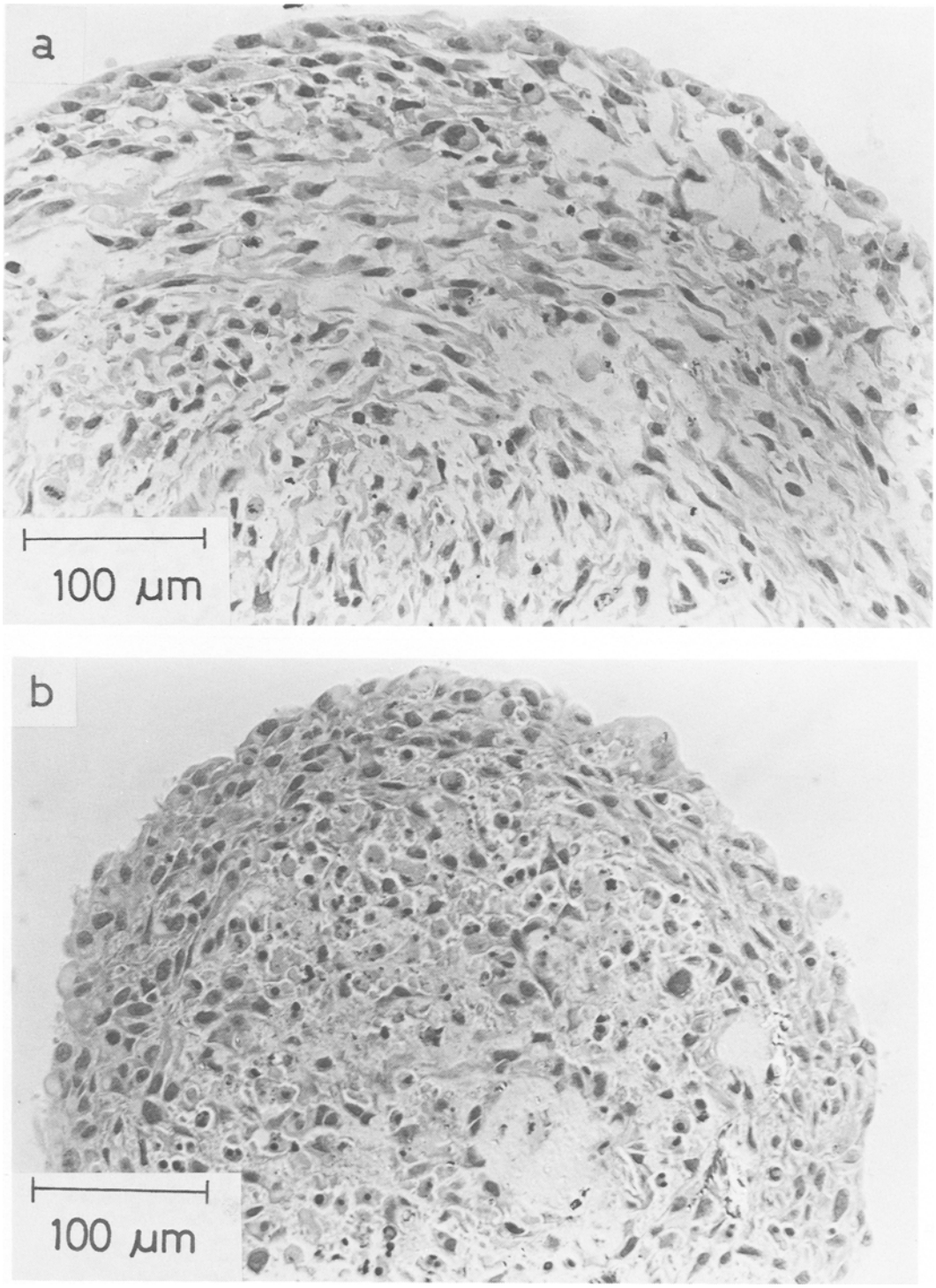

FIG. 1. Three-micrometer sections (stained with hematoxylin) of methacrylate embedded spheroids used in these measurements. *a*, Human glioma, U-118 MG; *b*, human thyroid cancer, HTh-7.

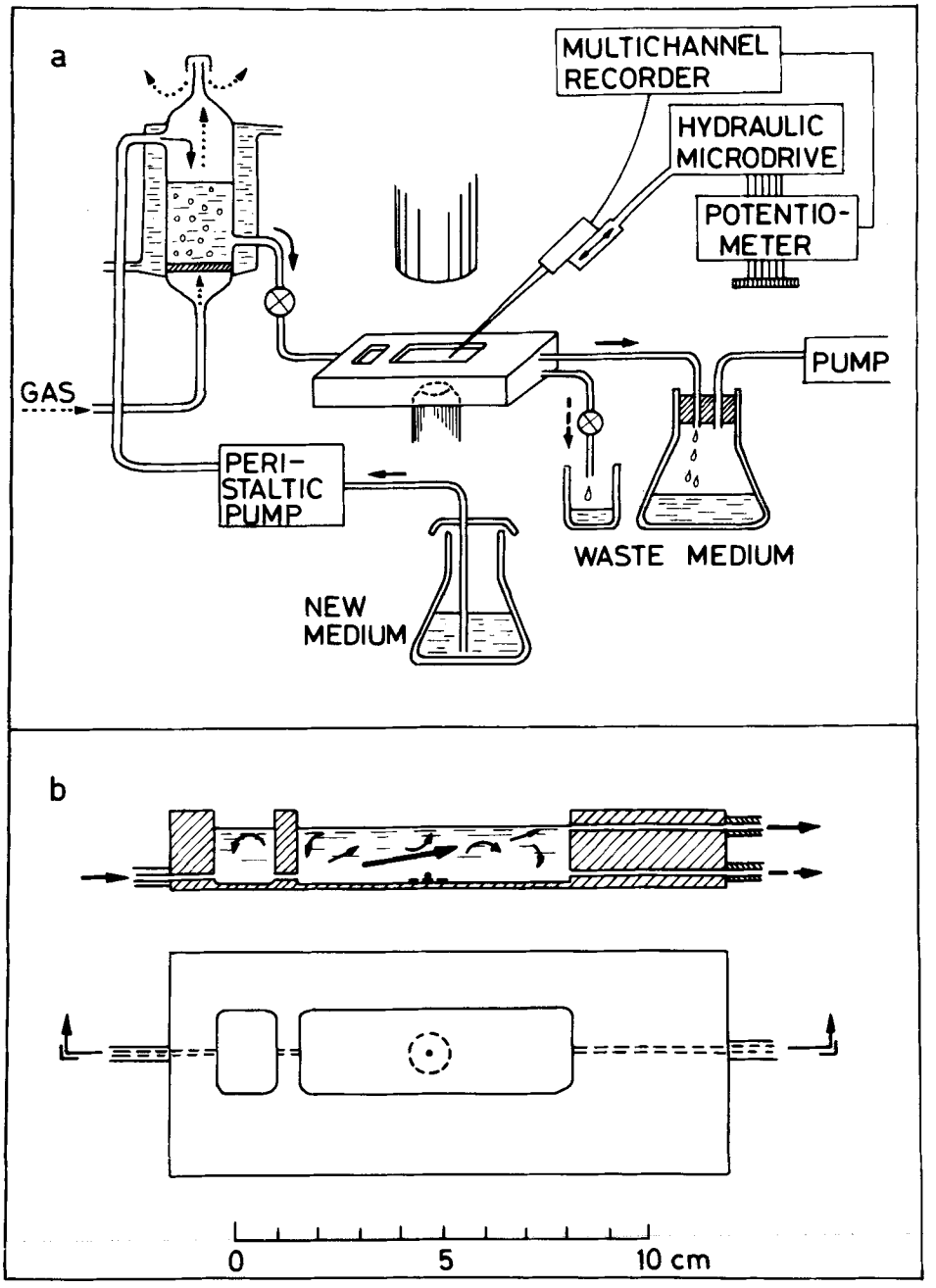

FIG. 2. *a*, The experimental set up for microelectrode measurements. The circulating medium (≈10 ml/min) is heated (37° C) and equilibrated with culture gas. Old medium is sucked out into the waste medium barrels. The valve at the inflow side is used to regulate the flow rate. The valve at the outflow side is opened when the chamber is to be emptied. A microscope lamp is shown above the chamber and an objective below. The objective is connected to a TV camera-monitor system. The fine movements of the microelectrode are made with a hydraulic microdrive. The movements and the electrode signals are registered on a multichannel recorder. *b*, Dimensions of the perfusion chamber. The *arrows* in the chamber indicate the flow directions of the medium, as seen in a stereomicroscope. The spheroid and the thin cover glass are indicated on the bottom of the chamber.

seemed to be a very slow flow, with almost no convection at the bottom of the chamber. All the equipment used is located and all the measurements were performed at the Max-Planck-Institut for Systemphysiology in Dortmund. The experimental set up and the general techniques for microelectrode measurements in cellular spheroids (mainly oxygen measurements) are also described in detail by Acker et al. (14).

Microelectrode measurements. The electrodes were calibrated in well defined ion solutions (10 and 100 m*M* KCl for K^+ and Cl^- electrodes and 0.2 and 2.0 m*M* $CaCl_2$ for Ca^{2+} electrodes) before and after the measurements. In some cases, a drift of both the calibration levels, from the precalibration to the recalibration, could be observed. In these cases, a linear drift all through the measurements was assumed. The drift of the electrodes was less than 4%/h (16). After calibration, the electrode was positioned in an accurate relation to the spheroid. This positioning was made possible by using two independent optical systems working along different axes (14). After final adjustment of the position, the electrode was moved by a hydraulic microdrive (David Kopf Instruments) along an axis through the center of the spheroid. When the electrode tip hit the spheroid surface, a signal was recorded in the potential measuring channel of the double barrelled electrode. The hit position was determined by this signal. The movement was stopped when the electrode tip was located in the central parts of the spheroid. This position was determined by measurement of the spheroid diameter, careful positioning of the electrode tip, and continuous reading of the electrode position on the multichannel recorder during the electrode insertion. The ion activity in the central parts of the spheroid (mainly necrotic region) was recorded and thereafter the concentration of the investigated ion was increased in the surrounding medium. The ion activity increase inside the spheroid could then be read on the multichannel recorder. The ion concentration was increased by injecting an ion solution into the small medium bath to the left in the perfusion chamber (Fig. 2). The injections used were 0.33 ml of 1.5 *M* KCl for K^+, 1.2 ml of 1.5 *M* KCl for Cl^-, and 0.35 ml of 0.15 m*M* $CaCl_2$ for Ca^{2+} measurements. The normal medium flow (≈10 ml/min) was continued all through the measurements.

Between five and seven measurements were made for each ion in each type of spheroid. The corresponding measurements were also made with the electrode tip located about 300 μm beside the spheroid but in exactly the same position in relation to the bottom of the chamber. These measurements gave the time pattern for the ion concentration changes in the medium.

Autoradiography. Most of the spheroids were incubated in medium containing [^{3}H]thymidine (1 μCi/ml, from the Radiochemical Centre, Amersham, England) for 1 h after the measurements. These spheroids were then fixed in glutaric aldehyde (2% glutaric aldehyde in 0.1 *M* sodium cacodylate and 0.1% sucrose, pH 7.2 with HCl) for 1 h. The spheroids were then stored in 70% ethanol. Preparations for light microscopy, autoradiography, and evaluation of labeling index were carried out as described previously (8,9).

Determination of extracellular space. The volumetric fraction of extracellular space was determined for both spheroid types by the Delesse principle (17), using electron microscope photographs. According to this principle, the volume fraction of a component in the tissue is equal to the area fraction of that component, provided that the analyzed sections are randomly selected. This method has been described previously in detail by Carlsson and Brunk (18).

RESULTS

The central parts of the spheroids used in these measurements (650 ± 150 μm diam) consisted of degenerated regions, even though massive necrosis had not yet appeared in the smallest ones. The critical size for appearance of necrotic regions and other growth characteristics of the spheroids have been described previously (13). The ion activities in the central regions of the spheroids before the injection of ion solution were recorded as 14 ± 3 m*M* K^+, 144 ± 34 m*M* Cl^-, and 1.2 ± 0.3 m*M* Ca^{2+}. These values are about the same as the ion activities measured in the surrounding medium.

The calculated increase of ion concentration due to the amount of ions injected into the perfusion chamber (volume about 20 ml) was 25 m*M* for K^+, 90 m*M* for Cl^-, and 2.6 m*M* for Ca^{2+}. However, due to the somewhat irregular flow pattern in the perfusion chamber (*see* Material and Methods and Fig. 2) the concentration was sometimes a little higher or lower near the bottom (in the medium surrounding the spheroid). This gave rise to some variations in the absolute values of the ion activity increases after different injections. Thus, the relative ion activity increases were used instead of the absolute values. The ion activity

measured before an injection was set to zero and the maximum activity measured after the injection was set to one. Drawings from original recordings of ion activity increases and the curves describing the corresponding, relative activity increases are shown in Fig. 3. The irregularities in the original recordings are mainly due to mechanical and electronic disturbances.

The mean rate of the activity increases for the different ions in the different spheroids are shown in Table 1. The inverse of this increase rate gave a measure of the penetration time. The difference

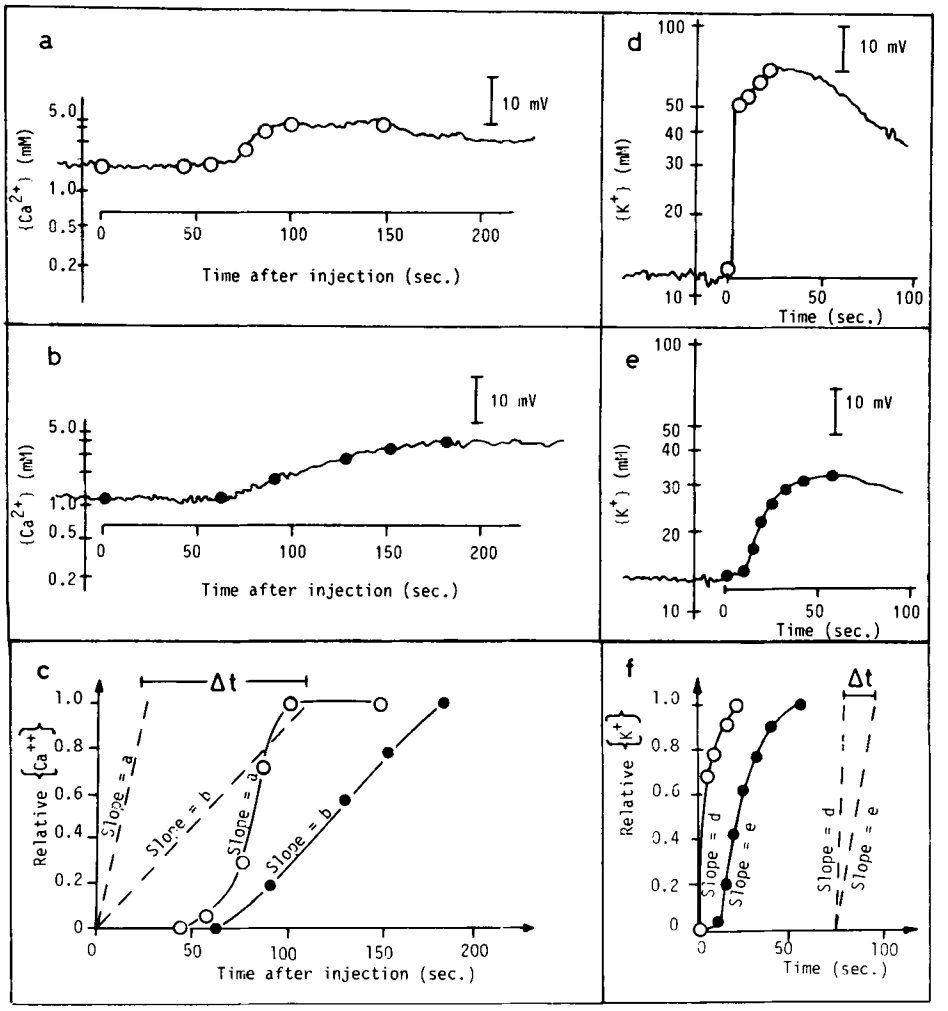

FIG. 3. Drawings from examples of original recordings of the Ca^{2+} (*a* and *b*) and K^+ (*d* and *e*) activity increases after injection of 0.35 ml of 0.15 *M* $CaCl_2$ or 0.33 ml of 1.5 *M* KCl, respectively and the utilization of these recordings for the calculation of the penetration delay, Δt (*c* and *f*). *a* and *d*, Recordings of the ion activity increase with the electrode tip located in the medium, beside the spheroid (*open circles* indicate points used for the construction of the curves in *c* and *f*, respectively). *b* and *e*, Recordings of the ion activity increase with the electrode tip located in the central parts of the spheroids (*solid circles* indicate points used for the construction of the curves in *c* and *f*, respectively). *c* and *f*, Curves describing the increase of the relative ion activity, in the medium (*open circles*) and in the spheroids (*solid circles*), as a function of time after the injection of ion solution. The *dashed lines* have the same slopes as the central parts of the *curves* and the indicated time difference visualizes the penetration delay, Δt. Δt can also be calculated as the difference between the inverse of the slopes ($\Delta t = b^{-1} - a^{-1}$ for Ca^{2+} and $\Delta t = e^{-1} - d^{-1}$ for K^+).

TABLE 1

MAXIMUM INCREASE RATE OF THE ION-ACTIVITY[a]

Measurements Made in	Rate of Ion Activity Increase (Change in Relative Activity/S)		
	K^+	Cl^-	Ca^{2+}
U-118 MG spheroids	0.036 ± 0.008[b]	0.017 ± 0.004	0.009 ± 0.002
HTh 7 spheroids	0.037 ± 0.014	0.022 ± 0.007	0.011 ± 0.001
The surrounding medium	0.214 ± 0.086	0.113 ± 0.041	0.032 ± 0.011

[a] The ion activity increase was measured as a function of time after the injection of ion solution. In general, the increase rates were maximal and rather constant in the range between 0.2 and 0.6 of the maximal activity (*see curves* in Fig. 3). The ion activity increase was evaluated in this part of the curve.

[b] The values are mean (± SD) from five to seven experiments. The significance of the differences between the mean values was tested using the *t*-distribution. The differences between the different types of ions are significant at the 1% level, except for the differences between K^+ and Cl^- in HTh 7 and in the medium, which are significant at the 10% level.

between this time, measured in a spheroid, and the corresponding time, measured in the surrounding medium, was defined as the penetration delay, Δt. A visualization of how Δt was defined is shown in Fig. 3 *c* and *f*.

The penetration delay was shortest for K^+ (about 25 s) for both cell types. It was intermediate for Cl^- (about 40 s for HTh-7 and 50 s for U-118 MG) and longest for Ca^{2+} (about 55 s for HTh-7 and about 90 s for U-118 MG). The Δt values for the three ions in both the spheroid types are shown in Fig. 4. It can be seen that the difference between the Δt values for the three ions is somewhat smaller for HTh-7 than for U-118 MG spheroids.

After the measurements, most of the spheroids were incubated in [^{3}H]thymidine for 1 h. These spheroids were sectioned and studied by light microscopy. It was impossible to see any trace of the electrode in the sections (Fig. 1). During the time in [^{3}H]thymidine medium, the trace probably disappeared. Thus, the insertion of the electrode did not cause any severe mechanical damage. These observations are in accordance with earlier observations (14). In autoradiograms of the spheroid sections it was seen that a normal number of proliferative cells existed in the spheroids after the microelectrode measurements.

The results of the determination of the volumetric fraction of extracellular space in the spheroids (i.e. the volume not consisting of cells in relation to the total spheroid volume) are shown in Fig. 5. The fraction of extracellular space was higher in the thyroid cancer spheroids than in the glioma spheroids. This was especially true in the peripheral regions.

DISCUSSION

The results presented were based on several independent measurements for each combination of ion and spheroid type. The variations within a group of measurements were small, compared with the differences obtained between the groups. The small variations in the slopes and shapes of individual curves that were obtained might have been due to irregularities in the flow pattern in the perfusion chamber.

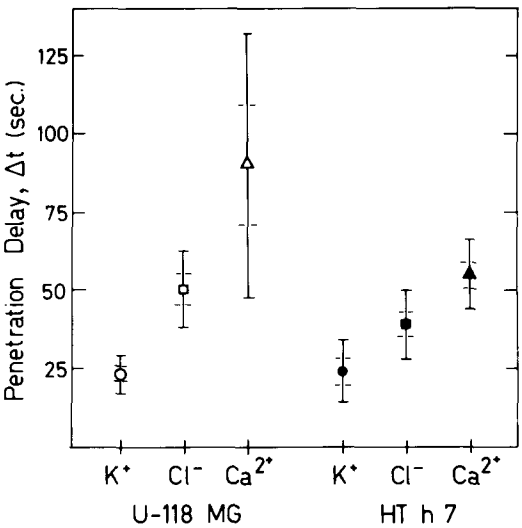

FIG. 4. Penetration delays, Δt, for the three ion types in the spheroids. Mean values ± SD (SE also indicated) of Δt are given for the ions K^+ (*round symbols*), Cl^- (*squares*), and Ca^{2+} (*triangles*) in U-118 MG spheroids (*open symbols*) and HTh-7 (*solid symbols*). Each value is based on five to seven experiments. The difference between the mean values for Cl^- and K^+ is significant on the 1% level for U-118 MG and on the 10% level for HTh 7. The difference between the mean values for Ca^{2+} and Cl^- is significant on the 10% level for U-118 MG and on the 5% level for HTh 7. The difference between the two mean values for K^+ is not significant on the 10% level, nor is the difference between the two mean values for Cl^-. The difference between the two mean values for Ca^{2+}, however, is significant on the 10% level. The significance tests were performed using the *t*-distribution.

If the Einstein-Smoluchowski equation, $x^2=2Dt$ (where x = mean diffusion length, D = diffusion constant and t = time) is used (19), an approximate calculation of the diffusion time of the ions into the central parts of the spheroids can be made. The diffusion coefficients of Ca^{2+} and Cl^- in a dilute solution of $CaCl_2$ are about 0.8×10^{-5} and 1.9×10^{-5} cm²/s, respectively (20). According to the molecular weight the diffusion coefficient should be about 1.3×10^{-5} cm²/s for all three ions. It can be assumed that the diffusion coefficient in the system used in this study was in the range between 0.7×10^{-5} and 2.0×10^{-5} cm²/s for all ions studied. Then, calculations according to the equation above give penetration times of between 25 s ($D = 2.0 \times 10^{-5}$ cm²/s) and 75 s ($D = 0.7 \times 10^{-5}$ cm²/s) to the central parts, assuming a mean radius of 325 μm. All the Δt values presented in Fig. 4 are within the range of the calculated diffusion times. It seems reasonable that the Ca^{2+} ions should have a slower diffusion than the others, because they have a double charge and thereby interact more efficiently with charges on cell surfaces and extracellular matrix components. Such interactions have been studied previously for Ca^{2+} in other systems (4,21). The double charge also leads to Ca^{2+} organizing more water molecules around it. It is difficult to speculate about why K^+ seems to have a faster diffusion than Cl^-. It is reasonable to believe that this has to do with differences in the interaction with charged or polar components. In summary it seems that the main mechanism for the penetration of K^+, Cl^-, and Ca^{2+} ions is free diffusion through the extracellular spaces. Charged and polar groups at the cell surfaces or in the extracellular matrix do not prevent ion diffusion. Charge interactions can at most cause a few seconds delay.

There are different possibilities for a substance to reach the central parts of a spheroid. Some substances might diffuse through the cell membrane, the cytoplasm, and the extracellular spaces. Other substances might diffuse only through the extracellular spaces. The substances may interact with the plasma membranes or other cell organelles, giving a slow penetration. Phenomena such as active or passive transport through the membranes may also alter the penetration pattern. The hydrophobic interior of the cell membranes should present a diffusion barrier for ions. There

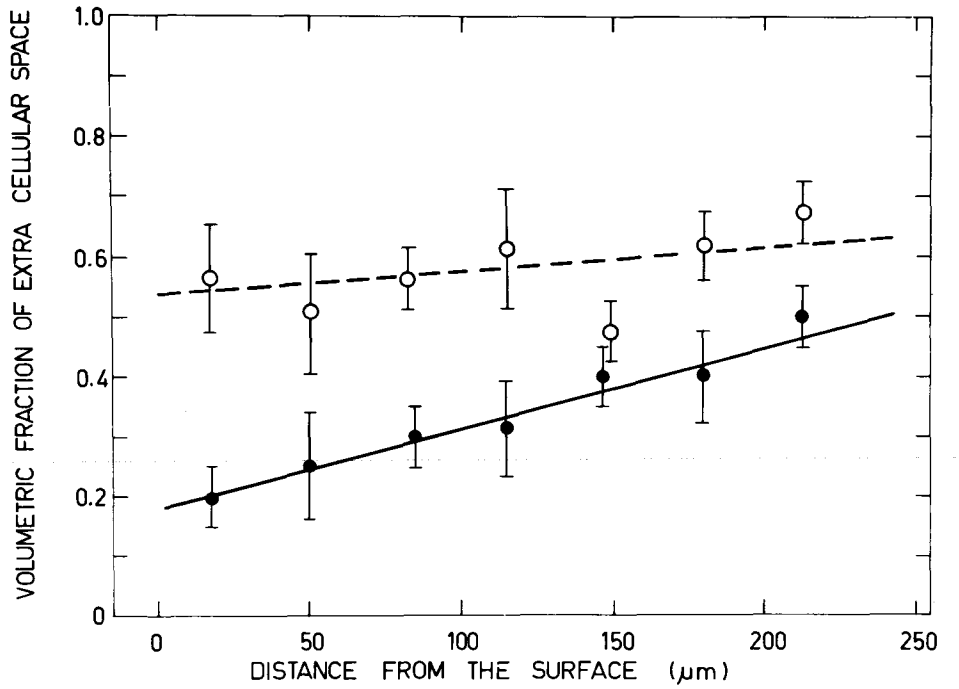

FIG. 5. Volumetric fractions of the extracellular spaces as a function of depth in human glioma U-118 MG (*solid circles* and *continuous line*) and human thyroid cancer HTh-7 (*open circles* and *dashed line*) spheroids. *Error bars* indicate SD of at least 12 repeated measurements.

are, however, possibilities for ions to penetrate the cell membranes by ATP-dependent transport (22,23) or by ion channels (23,24).

The diffusion seemed to be slower in the U-118 MG spheroids than in the HTh-7 spheroids, especially for Ca^{2+}. This is in accordance with the results showing that the U-118 MG spheroids have a denser structure with less extracellular space than the HTh-7 spheroids (Fig. 5). This correlation indicates that microelectrode measurements of ion penetration might be used to investigate changes in the extracellular space caused by different treatments, such as drug treatment, radiation, hyperthermia, hypoxia, et cetera.

This study has presented a new application of microelectrode measurements. Microelectrodes have been used previously to measure membrane potentials, intracellular ion activities (25), and oxygen gradients (26,27) in cellular spheroids. The penetration of substances through poorly vascularized tissue has been studied before by histological and autoradiographical techniques (1,2). In the future, it is possible that microelectrodes sensitive to interesting substances, such as different nutrients and drugs, may be developed (enzyme electrodes). The methods described in this work can then be used to study the penetration properties of these substances in an easy and reproducible way.

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