METHOD FOR THE DETERMINATION OF OXYGEN CONSUMPTION RATES AND DIFFUSION COEFFICIENTS IN MULTICELLULAR SPHEROIDS

W. MUELLER-KLIESER
Department of Applied Physiology, University of Mainz, D-6500 Mainz, Federal Republic of Germany

ABSTRACT A method has been developed for the quantitative evaluation of oxygen tension (P_{O_2}) distributions in multicellular spheroids measured with O_2-sensitive microelectrodes. The experimental data showed that multicellular tumor spheroids in stirred growth media were characterized by a diffusion-depleted zone surrounding the spheroids. This zone was elicited by an unstirred layer of medium next to the spheroid leading to a continuous decrease in the P_{O_2} values from the bulk medium towards the spheroid surface. Theoretical considerations demonstrate that the volume-related O_2 consumption rate, Q, in the spheroids can be assessed by measuring the P_{O_2} gradient in the diffusion-depleted zone outside the spheroids. Accordingly, Krogh's diffusion constant, K_S, in the spheroids can be determined through measuring the P_{O_2} gradient within the spheroids. The results obtained suggest that multicellular spheroids represent useful in vitro tumor models for the experimental and theoretical analysis of the interrelationship among O_2 supply to tumor cells, O_2 metabolism in tumors tissue, and the responsiveness of cancer cells to treatment.

INTRODUCTION

Multicellular spheroids are spherical aggregates of cells that are supplied by diffusion of oxygen and substrates from the surrounding growth medium. Spheroids may serve as an in vitro tissue model because they exhibit many morphological and functional properties of those tissues from which the spheroid cells were originally derived. Multicellular tumor spheroids, e.g., show many characteristics of solid tumors, such as pecularities in cellular proliferation, the development of necrosis or the occurrence of radioresistance during growth. Therefore, tumor spheroids have been widely used to study the interrelationship among metabolism, cell cycle state, and response of tumor cells to cancer treatment with drugs and/or radiation (for a review see Sutherland and Durand, 1976). The results of many of these investigations indicate that oxygen may be critically involved in controlling the responsiveness to treatment. Data regarding the oxygen consumption and oxygen diffusion properties of multicellular spheroids may therefore provide useful information on oxygen metabolism in solid tumors and its correlation with the susceptibility of tumors to various treatment modalities.

The distribution of oxygen tension (P_{O_2}) values in multicellular spheroids was assessed by several investigators using O_2-sensitive microelectrodes (Carlsson et al., 1979; Kaufman et al., 1981; Mueller-Klieser and Sutherland, 1982*a*, *b*, 1983; Mueller-Klieser et al., 1983). The P_{O_2} values obtained during these studies differ considerably due to the use of different cell lines, different culturing conditions, and different measuring techniques. However, all data published to date indicate that the steady state P_{O_2} distribution in multicellular spheroids is determined by the balance between O_2 diffusion from the growth medium into the spheroids and O_2 consumption within the spheroids. A theoretical analysis of steady state P_{O_2} profiles in multicellular spheroids should thus result in a quantitative characterization of O_2 metabolism and O_2 diffusion properties in spheroids. The respective analysis is commonly performed by setting up and integrating the differential equation of diffusion under consideration of appropriate boundary conditions. This has been published previously by several authors for geometrical conditions applicable to spheroids, i.e., O_2 diffusion into a sphere containing O_2 consuming sites (Rashevsky, 1960; Burton, 1966; Thews, 1968; Boag, 1969, 1977; Deakin, 1975; Franko and Sutherland, 1979; Kasche and Kuhlman, 1980; Freyer, 1981). The results show that under steady state conditions the absolute P_{O_2} level within spheroids is determined among other factors by the O_2 consumption rate Q per unit volume and by Krogh's diffusion constant K. Q and K are assumed to be constant and uniform, in these studies. K is related to the oxygen diffusivity, D_{O_2}, through the oxygen solubility, α, according to the equation $K = \alpha \cdot D_{O_2}$, which can be derived using Henry's law. Since both Q and K are not known, neither of these quantities can be unequivocally determined from steady state P_{O_2} profiles in spheroids. A theoretical approach to determining Q from steady state P_{O_2} profiles in spheroids has been published recently (Bush et al., 1982), however, neither numerical results nor data on the accuracy of the method are given to permit further evaluation.

The rationale of the present investigation was to use special properties of steady state P_{O_2} profiles in multicellular spheroids for the determination of Q and K. The profiles referred to were recorded under experimental conditions described in detail in a preceding paper (Mueller-Klieser and Sutherland, 1982*a*). Since these conditions are an essential prerequisite for the applicability of the method suggested, the respective experiments are briefly summarized below.

MATERIALS AND METHODS

Experimental P_{O_2} Profiles

Oxygen tension profiles were recorded with O_2-sensitive microelectrodes in spheroids from EMT6/Ro cells that were derived from a spontaneous mouse mammary sarcoma (Rockwell et al., 1972). The spheroids were cultured in spinner flasks, i.e., in stirred media, according to a technique described in a previous paper (Freyer and Sutherland, 1980). The growth medium was Eagle's basal medium supported with 0.15 (vol/vol) fetal calf serum and was equilibrated with air and 0.03 (vol/vol) CO_2. The measuring device was constructed to provide conditions that match the growth conditions as close as possible with regard to uniform convection in the growth medium surrounding the spheroids and with regard to uniform diffusion of oxygen from the medium into the spheroids (Mueller-Klieser and Sutherland, 1982*a*). The presence of these conditions enables the detection of spherical P_{O_2} distributions that correlate with the histological structure of the cell aggregates. This agreement between O_2 distribution and histological structure is considered a decisive criterion to obtain the necessary equivalent supply conditions during measurement and growth. The respective experiments demonstrate that the P_{O_2} profiles are characterized by an unstirred layer of medium close to the spheroid surface with an oxygen tension below that in the bulk of medium due to oxygen depletion. The thickness of this layer is on the order of 100 to 200 μm and is enlarging with increasing spheroid size. Within the spheroids two types of P_{O_2} profiles were recorded: (*a*) a parabolic profile in small spheroids without necrotic core and (*b*) a steep decrease in P_{O_2} from the spheroid edge towards its center with a central plateau in larger spheroids having necrosis. The decline in P_{O_2} corresponds with the width of the viable rim, whereas the central plateau region with constant P_{O_2} values is generated by the necrotic area, i.e., by the absence of O_2 consumption.

First Integration of the Differential Equation of Diffusion

The differential equation for O_2 diffusion into a sphere with O_2 consumption can be written in polar coordinates for the stationary case, e.g., according to Rashevsky (1960)

$$K\left(\frac{d^2P}{dr^2} + \frac{2}{r}\frac{dP}{dr}\right) - Q = 0, \qquad (1)$$

where P equals the O_2 partial pressure, K equals Krogh's diffusion constant, and Q equals the O_2 consumption rate per unit volume.

The measured P_{O_2} profiles demonstrate that the integration of this equation should be performed for the following regions with respect to O_2 diffusion into the spheroid (see Fig. 1):

(*a*) region M $\infty \geq r \geq g$	bulk of stirred medium without any diffusion gradients;
(*b*) region G $g \geq r \geq s$	diffusion depleted, unstirred layer of medium at the spheroid surface;

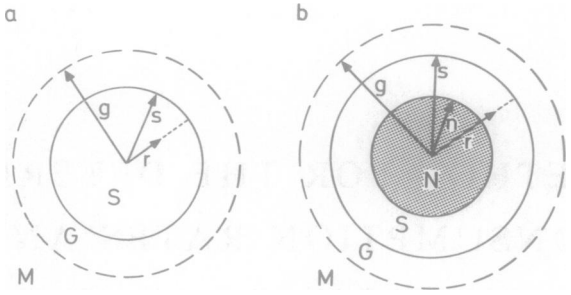

FIGURE 1 Different regions in multicellular spheroids for the integration of the differential equation of diffusion: M represents the stirred growth medium; G, the diffusion-depleted zone; S, the zone of viable cells; and N the necrotic core. (*a*) Spheroids without necrosis and (*b*) spheroids with necrosis.

(*c*) region S $s \geq r \geq 0, n$	the zone of O_2 consuming cells that is identical with the entire spheroid volume if no necrosis is present or that corresponds approximately with the volume of the viable rim in spheroids with central necrosis.
(*d*) region N $n \geq r \geq 0$	the necrotic area with the absence of O_2 consumption.

The respective integral in region M yields a constant P_{O_2} in the medium in all cases considered

$$P(r) = \text{cons} = P_M = P_{O_2} \qquad \text{in the bulk medium.} \qquad (2)$$

Accordingly, a constant P_{O_2} is obtained in the necrotic area, i.e., the P_{O_2} is independent from r, yet is determined by the geometric properties of the spheroid, by Q, by P_M, and by Krogh's diffusion constants, K_M and K_S, in the medium and in the spheroid, respectively.

The first integration of Eq. 1 in region G yields for spheroids with necrosis[1]

$$\frac{dP_G(r)}{dr} = P'_G(r) = \frac{Q}{3K_M} \cdot \frac{s^3}{r^2}; \; g \geq r \geq s, \qquad (3a)$$

for spheroids with necrosis

$$\frac{dP_G(r)}{dr} = P'_G(r) = \frac{Q}{3K_M} \cdot \frac{s^3 - n^3}{r^2}; \; g \geq r \geq s. \qquad (3b)$$

The respective boundary conditions are derived from the assumption that there are no jumps or drops in P_{O_2} at the transitions between regions M, G, S, and N. In addition, the flux of oxygen through the spheroid surface is equal to the diffusive flux into the spheroid, and the flux of oxygen from region S into N is zero. Eqs. 3a and 3b can be solved for Q

$$Q = 3\,K_M\,P'_G(r) \cdot \frac{r^2}{s^3}; \quad g \geq r \geq s \qquad (4a)$$

$$Q = 3\,K_M\,P'_G(r) \cdot \frac{r^2}{s^3 - n^3}; \quad g \geq r \geq s. \qquad (4b)$$

K_M can be considered equal to Krogh's diffusion constant in water. The spheroid diameter is routinely determined with a microscope reticle, and

[1]The letter a indicates the respective equation for spheroids without necrosis, letter b indicates the respective equation for spheroids with necrosis.

the size of the necrotic area can be derived from the shape of the P_{O_2} profile or can be assessed by histological investigations. Thus, Q in the viable zone of the spheroids can be determined from measuring the P_{O_2} gradient in the diffusion-depleted zone surrounding the spheroids.

Respective considerations can be applied to the first integration of Eq. 1 in region S leading to

$$K_S = \frac{Q}{3\,P'_S(r)} \cdot r; \quad s \geq r \geq 0 \tag{5a}$$

$$K_S = \frac{Q}{3\,P'_S(r)} \cdot \frac{r^3 - n^3}{r^2}; \quad s \geq r \geq n. \tag{5b}$$

Eqs. 5a and 5b show that, knowing Q, Krogh's diffusion constant, K_S, in the viable zone of spheroids can be derived from measuring the P_{O_2} gradient $P'_S(r)$ in this area.

Second Integration of the Differential Equation of Diffusion

Knowing Q and K_S, the entire steady state P_{O_2} distribution in multicellular spheroids can be calculated through the second integration of Eq. 1. Under consideration of the respective boundary conditions one obtains

$$P_G(r) = P_M + \frac{\overline{M} \cdot s^3}{g} - \frac{\overline{M} \cdot s^3}{r}, \tag{6a}$$

$$P_G(r) = P_M + \frac{\overline{M} \cdot \overline{V}_S}{g} - \frac{\overline{M} \cdot \overline{V}_S}{r}, \tag{6b}$$

$$P_S(r) = P_M - \frac{\overline{S} \cdot s^2}{K_M}(K_M + 2K_S \cdot \overline{G}) + \overline{S} \cdot r^2; \quad s \geq r \geq 0, \tag{7a}$$

$$P_S(r) = P_M + \overline{V}_S \overline{M}\left(\frac{1}{g} - \frac{1}{s}\right) - \overline{S}s^2 \cdot \left(1 + \frac{2n^3}{s^3}\right) + \overline{S}r^2\left(1 + \frac{2n^3}{r^3}\right); \quad s \geq r \geq n, \tag{7b}$$

$$P_N(r) = P_M + \overline{V}_S \overline{M}\left(\frac{1}{g} - \frac{1}{s}\right) - \overline{S}s^2 \cdot \left(1 + \frac{2n^3}{s^3}\right) + 3\overline{S}n^2; \quad n \geq r \geq 0, \tag{8}$$

with $\overline{M} = Q/3\,K_M$, $\overline{S} = Q/6\,K_S$, $\overline{G} = 1 - s/g$, and $\overline{V}_S = s^3 - n^3$.[2]

RESULTS

The application of the method suggested in 15 EMT6/Ro spheroids yields an O_2 consumption rate ($\bar{x} \pm$ SEM) of $(3.09 \pm 0.31) \cdot 10^{-4}$ $ml_{O_2}\ cm^{-3} \cdot s^{-1}$ and a Krogh's diffusion constant of $(1.87 \pm 0.12) \cdot 10^{-5}$ $ml_{O_2} \cdot cm^{-1} \cdot min^{-1} \cdot atm^{-1}$. In Table I these values are compared with respective data from literature and with the O_2 consumption of single EMT6/Ro cells. It is obvious that Q in EMT6/Ro spheroids is lower than the O_2 consumption rate of EMT6 single cells and higher than Q in solid tumors. Krogh's diffusion constant K in spheroids is comparble with that in tumors. Q in spheroids tends to decrease with increasing spheroid size, whereas K remains constant. This is demonstrated in Table II showing Q as a function of spheroid size. The data indicate that the considerable scattering in the O_2 consumption rates obtained is partially due to a decrease in Q with increasing size particularly in spheroids with small diameters. Considering spheroids larger than 1,000 μm in diameter, Q is less dependent on size. The standard deviation of Q in this more uniform spheroid population is ~17% of the respective mean value.

Once the diffusion constant, K, is known for a certain

TABLE I
VOLUME-RELATED O_2 CONSUMPTION RATE Q ($\bar{x} \pm$ SD) IN SINGLE TUMOR CELLS AS WELL AS Q ($\bar{x} \pm$ SEM) AND KROGH'S DIFFUSION CONSTANT K ($\bar{x} \pm$ SEM) IN MULTICELLULAR TUMOR SPHEROIDS (n = 15) AND IN SOLID TUMORS

$Q \cdot 10^{-4}$	$K \cdot 10^{-5}$	Cell line (reference)
$ml_{O_2} \cdot cm^{-3} \cdot s^{-1}$	$ml_{O_2} \cdot cm^{-1} \cdot min^{-1} \cdot atm^{-1}$	
6.50 ± 0.31	—	exponential phase, EMT6, single cells
5.50 ± 0.45	—	plateau (Freyer, 1981; Mueller-Klieser, W., unpublished observations)
5.40 ± 0.39	—	DS, ascites cells (Mueller-Klieser et al., 1978)
3.09 ± 0.31	1.87 ± 0.12	EMT6 spheroids (present results)
2.50	1.90 ± 0.08	DS-Carcinosarcoma (Vaupel, 1974; Grote et al., 1977)

TABLE II
OXYGEN CONSUMPTION RATE Q IN EMT6 SPHEROIDS OF DIFFERENT DIAMETERS

Spheroid number	Diameter	$Q \cdot 10^4$
	μm	$ml_{O_2} \cdot cm^{-3} \cdot s^{-1}$
1	386	6.44
2	415	4.99
3	426	3.57
4	543	3.53
5	648	3.00
6	703	2.37
7	792	3.16
8	960	2.10
9	1,005	2.73
10	1,228	2.63
11	1,257	2.14
12	1,335	2.99
13	1,531	2.70
14	1,640	1.80
15	1,900	2.26

[2]The factor is proportional to the volume of the viable rim.

type of spheroid, the determination of the P_{O_2} gradient in the diffusion-depleted zone by only a few steady state measurements will provide information on the P_{O_2} distribution in the entire spheroid. Thus, it is possible to easily collect data on the oxygen consumption and on the P_{O_2} distribution in a large number of spheroids.

Using the values for Q and K obtained from Eqs. 4a, b and 5a, b, Eqs. 6a–8 lead to a sufficient approximation of the measured P_{O_2} distribution in multicellular spheroids. Representative examples are shown in Fig. 2. The solid circles are steady state readings from microelectrode measurements, the solid lines represent the P_{O_2} distribution calculated from the Eqs. 6a, b, 7a, b, and 8 both for spheroids without necrosis (Fig. 2 *a*) and for spheroids with necrosis (Fig. 2 *b*). The parameters in the equations mentioned are determined by measuring the P_{O_2} gradients in the diffusion-depleted zone and in the spheroid interior and by deriving Q and K_S from Eqs. 4a, b and 5a, b, respectively. The diameter of each spheroid investigated is measured in an inverted microscope, the diameter of the necrotic area is obtained from histological investigations. The location of the microelectrode with regard to the spheroid is determined from the break in P_{O_2} at the spheroid surface. In general, this break point coincides with the electrode tip touching the spheroid surface (indicated by arrows in Fig. 2), as can be observed in the measuring chamber through a dissecting microscope. The location of the spheroid center is then obtained by correlating the location of the spheroid surface with the diameter.

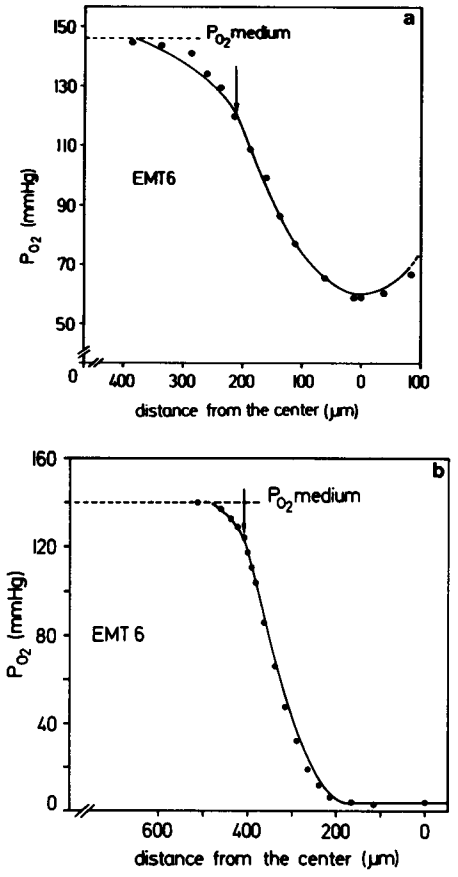

FIGURE 2 Measured P_{O_2} values (●) and calculated P_{O_2} profiles (—) in multicellular spheroids from EMT6/Ro cells (arrows indicate the spheroid surface). (*a*) Spheroid without necrosis (diameter: 426 μm), experiment No. 2/4/81 #4; (*b*) spheroid with necrosis (diameter: 818 μm), experiment No. 5/15/81 #16.

DISCUSSION

The method for the evaluation of P_{O_2} gradients in multicellular spheroids, as presented in this paper, represents a semi-analytical procedure for the determination of Q and K_S in multicell spheroids. The method may be applied to any spheroid subjected to P_{O_2} measurements with microelectrodes in stirred media. The procedure suggested requires only a few steady state measurements of P_{O_2} values and the knowledge of the spheroid diameter and of the thickness of the viable rim. Both the latter values can be assessed by microscopical and histological investigations, respectively. Since these quantities enter the Eqs. 4a and 4b in the third power, they have to be determined fairly accurately, which is possible through multiple measurements. Thus, s and n may be assessed with uncertainties of ±3 μm corresponding with a relative error in s of 0.5% when a spheroid with a diameter of 600 μm is considered. The localization of the microelectrode with regard to the spheroid can be obtained when there is a break in P_{O_2} at the spheroid surface (see Fig. 2). The extent of this break is mainly dependent on the ratio of K_M/K_S. The break in P_{O_2} can be localized with a mean error of ±3 μm by choosing adequate step widths between 5 and 10 μm for steady state readings when approaching the spheroid with the microelectrode. The same procedure also leads to the determination of the P_{O_2} gradients outside and inside the spheroids with relative errors of ~5%. Gradients are obtained through linear interpolation between two P_{O_2} data points. Propagating the errors mentioned, Q and K_S should be obtained with relative errors of 5 to 10%, depending on spheroid size. The values of Q presented exhibit, however, a larger variability mainly due to factors influencing Q, such as spheroid diameter (see Table II) or variations of growth conditions between the routine replenishment of the culture medium. The impact of growth conditions on the oxygenation of spheroids has been demonstrated in detail in a previous paper (Mueller-Klieser et al., 1983).

The accuracy of determining Q and K_S may be improved by fitting the P_{O_2} profile calculated to the experimental data points through variation of Q and K_S. The values for these two variables obtained from the P_{O_2} gradients inside and outside the spheroids and using Eqs. 4a, b and 5a, b may then serve as an initial set of parameters for the fitting procedure.

To eliminate the impact of deviations from spherical symmetry on the results discussed, $P_S(r)$ may be represented by data averaged over spherical surfaces

$$\langle P_S(r) \rangle = \frac{1}{4\pi} \int\int P_S(r, \theta, \phi) \sin\theta d\theta d\phi. \quad (9)$$

If the deviation from spheroid symmetry is small, $\langle P_S(r) \rangle$ may only contain zero- and first-order harmonics and can be calculated as the average of any two values at the opposite ends of the same spheroid diameter. Thus, $\langle P_S(r) \rangle$ can be determined by recording a P_{O_2} profile as the microelectrode entirely penetrates the spheroid on a track through the center. Accordingly, second-order harmonics can be eliminated by averaging over six P_{O_2} values symmetrically placed on the sphere. A regression of the data points obtained against Eqs. 7a and 7b, respectively, gives a value of Q/K_S. K_S may then be derived from the flux boundary condition at the spheroid surface

$$K_M \cdot \frac{\partial P_G}{\partial r} = K_S \cdot \frac{\partial P_S}{\partial r}; \quad r = s. \quad (10)$$

Since K_M is known, K_S can be determined by measuring the P_{O_2} gradients inside and outside the spheroid at the spheroid surface, as suggested by Bush et al. (1982).

Depending on the accuracy required, the procedures described in the two preceding paragraphs may be used to precisely determine the oxygen consumption rate and Krogh's diffusion constant in a few individual spheroids. To gain an estimate of mean values of Q and K_S, however, in a large number of spheroids, the procedure outlined in Materials and Methods may be sufficient and may be applied for reasons of practicability.

Theoretically, the O_2 consumption rate, Q, in spheroids is independent from the absolute P_{O_2} level, P_M, in the medium, since P_M does not appear in the Eqs. 4a and 4b. Therefore, the determination of Q would not be affected by errors in the calibration of the microelectrode with regard to absolute P_{O_2} values, as long as the sensitivity of the probe is known. Also, differences between the P_{O_2} values in the media during growth and during measurement should not influence the Q value to be assessed. However, this may only be true for a certain P_{O_2} range, and a possible regulatory impact of P_{O_2} on the cellular respiration over a wider P_{O_2} range cannot be ruled out either. Regulation of Q below a critical P_{O_2} (e.g., below 2 mmHg) does not seem to play a role in the present investigations, since the P_{O_2} profiles recorded are very steep and, hence, the thickness of cell layers at certain low P_{O_2} values rather small.

Although smaller deviations from spherical symmetry can be eliminated in the way discussed above, the method presented should only be applied to spheroids with an almost perfect spherical shape. Also, the supply conditions regarding convective and diffusive transport in the medium surrounding the spheroids should show spherical symmetry. Thus, experimental effort had to be directed towards creating spherical symmetry in O_2 supply during P_{O_2} measurements in spheroids. It is evident that perfect symmetry may hardly be reached in spheroids that have to be spatially fixed in the streaming growth medium for microelectrode measurements (Mueller-Klieser and Sutherland, 1982a). Deviations between calculated and measured P_{O_2} profiles in multicellular spheroids may therefore be elicited mainly by asymmetries in the outer supply conditions rather than by local variations in O_2 consumption rate and/or in O_2 diffusion properties. The calculated P_{O_2} values may also differ from the experimental data in the outermost part of the diffusion-depleted layer of medium (see Fig. 2 *a*). This has to be expected, since a sudden transition from region M of stirred medium to the unstirred region G has been assumed for calculation. However, the actual transition between the two respective regions is certainly gradual rather than discontinuous. Therefore, the P_{O_2} gradient in region G has to be determined close to the spheroid surface where the medium is definitely not stirred. An indication of the medium being actually stagnant at the spheroid surface can be obtained from the fact that the diffusion-depleted zone cannot be eliminated even through vigorously stirring the medium next to the spheroid.

Despite these restrictions in the theoretical analysis of steady state P_{O_2} distributions in multicellular spheroids, the method presented in this paper enables a quantitative characterization of O_2 metabolism and O_2 supply conditions in multicellular spheroids from measurements with O_2-sensitive microelectrodes. The data obtained show that the O_2 consumption rate of tumor cells is largely influenced by the microenvironmental conditions, since Q is decreasing from the value in single cells to the values measured in spheroids and in solid tumors, respectively. Multicellular spheroids may thus allow the systematic theoretical and experimental investigation of parameters involved in the control of the O_2 metabolism of tumor cells and, hence, in the cellular response to tumor therapy.

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