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Is there a critical tissue oxygen tension for bioenergetic status and cellular pH regulation in solid tumors?

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Abstract. Bioenergetic and metabolic status have been correlated with tissue oxygenation in murine fibrosarcomas (FSaII) of varying sizes (44–600 mm^3). Ratios of β-nucleoside triphosphates to inorganic phosphate (βNTP/P_i) and phosphocreatine to inorganic phosphate (PCr/P_i) ratios derived from ^{31}P nuclear magnetic resonance spectroscopy (NMR) were positively correlated to median tissue O_2 tension (pO_2) values using O_2-sensitive needle electrodes. pH declined during growth with intracellular acidosis being evident in tumors >350 mm^3. Whereas lactic acid formation greatly contributed to this decline in small and medium-sized tumors, adenosine triphosphate (ATP) hydrolysis and slowing down of the activities of pumps involved in cellular pH regulation seem to be major factors responsible for intracellular acidification in bulky tumors. PCr levels decreased at an early growth stage, whilst ATP concentrations dropped in bulky malignancies only, coinciding with a decrease in adenylate energy charge and a substantial rise in the levels of total P_i. On average, median pO_2 values of ca. 10 mmHg represent a critical threshold for energy metabolism. At higher median O_2 tensions, levels of ATP, phosphomonoester (PME) and total P_i were relatively constant. This coincided with intracellular alkalosis or neutrality and stable adenylate ratios. On average, median pO_2 values <10 mmHg coincided with intracellular acidosis, ATP depletion, a drop in energy charge and rising P_i levels.
Key words. Critical O_2 tension; tumor pH; tumor bioenergetic status; tumor oxygenation; tumor ATP; tumor acidosis; tumor hypoxia; ^{31}P-nuclear magnetic resonance spectroscopy.

Introduction

During growth of experimental tumors, structural and functional abnormalities of the tumor vasculature often lead to a progressive decrease in blood flow and hence tissue oxygenation[28,29]. This size dependency has not been observed in most human tumors studied so far[12,27,32]. Many ^{31}P-NMR studies on experimental tumors also showed a decline in high-energy phosphates and pH, and an increase in P_i with increasing tumor size[26]. Some attempts have been made to correlate tumor oxygenation with ^{31}P-NMR parameters within the same cell line. These studies have shown that the pattern of change in the latter parameters with tumor growth is similar to the decline in oxygenation in most experimental tumors[26,30]. Generally, levels of PCr, ATP and P_i in tumors were thought to be related to oxygenation. Low ratios of high-energy phosphates to P_i in these tumors have been observed as a consequence of tissue hypoxia. Commonly these ratios decreased with increasing tumor volumes.

A number of relevant issues still need to be clarified regarding the relationship between energy status and tumor oxygenation: (i) Is there any correlation between tumor pO_2 and high-energy phosphate concentrations comparable to that seen between tumor oxygenation and the ratios of high-energy phosphates to P_i? (ii) What is the impact of the oxygenation status on the ratios of adenylates? (iii) Is there a 'critical' pO_2 in tumor tissue for the development of intracellular acidosis and ATP depletion? These questions are addressed in this article through the correlation of oxygenation data to results obtained with ^{31}P-NMR and/or acid extraction assays.

Tumor oxygenation

Experimental FSaII mouse tumors of varying sizes were used for tissue pO_2 measurements[30]. In addition, pO_2 distributions were measured in the normal subcutis of the hind foot dorsum in tumor-free animals. pO_2 values in the subcutis ranged from 3 mmHg to O_2 partial pressures characteristic for arterial blood, the median pO_2 being 58 mmHg. In FSaII tumors, the median pO_2 decreased exponentially from 30 mmHg in 72 ± 4 mm^3 tumors to 6 mmHg in 558 ± 10 mm^3 tumors (see fig. 1, circles). In the tumor group with volumes <100 mm^3, the tissue pO_2 values ranged from 0 to 75 mmHg, whereas in tumors >500 mm^3 no pO_2 values above 35 mmHg were observed. The fraction of pO_2 readings between zero and 2.5 mmHg increased from 5 to 33% with enlarging tumor mass from 72 to 558 mm^3.

^{31}P-NMR measurements

During growth, FSaII tumors showed a progressive loss of PCr and βNTP with increasing P_i and PME signals.

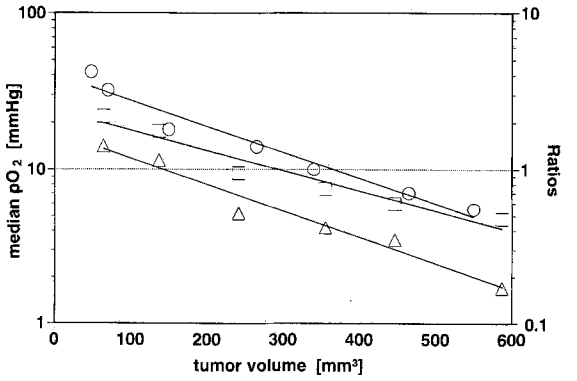

Figure 1. Tumor growth-related changes in mean PCr/P_i (triangles), in mean βNTP/P_i ratios (squares) and in median tumor pO_2 values (circles).

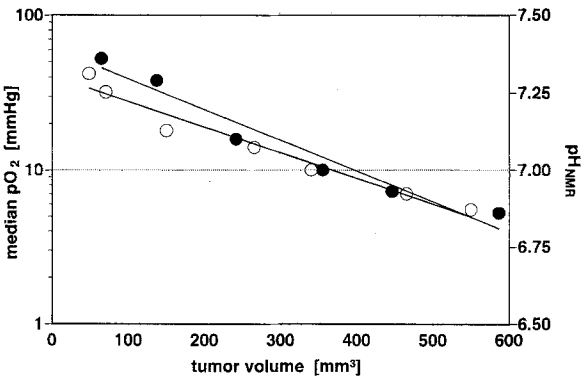

Figure 2. Mean apparent intracellular pH (pH_{NMR}, filled circles) and median pO_2 values (open circles) as a function of tumor volume (FSaII tumors).

The smallest tumors ($\approx$50 mm^3) had a mean PCr/P_i ratio of 1.4 ± 0.2, whereas tumors with volumes >500 mm^3 had an average PCr/P_i ratio of 0.16 ± 0.06 (see fig. 1, triangles). FSaII tumors with an average volume of approximately 50 mm^3 had a mean βNTP/P_i ratio of 2.2 ± 0.2. βNTP/P_i ratios declined exponentially with tumor growth. Tumors with volumes >500 mm^3 had an average βNTP/P_i ratio of 0.36 ± 0.16 (see fig. 1, squares)[30].

The apparent intracellular pH (pH_{NMR}) as measured by ^{31}P-NMR decreased from 7.36 ± 0.08 to 6.88 ± 0.11 with increasing tumor volume from 50 to 580 mm^3 (see fig. 2, filled circles). On average, tumor masses <350 mm^3 exhibited slightly alkaline or neutral intracellular pH values, whereas at tumor volumes >350 mm^3 intracellular acidosis was apparent[30]. The latter condition coincided with median pO_2 values below 10 mmHg (fig. 2, open circles), βNTP/P_i ratios below ca. 0.75 and PCr/P_i ratios below approximately 0.45 (see fig. 1).

Acid extracts of FSaII tumors

Global glucose concentrations decreased from 2.8 ± 0.3 µmol/g in small FSaII tumors to 1.2 ± 0.1 µmol/g in larger malignancies ($2p < 0.0001$), whereas the average tissue lactate level increased from 7 ± 0.2 to 11 ± 0.2 µmol/g ($2p < 0.0001$) with a levelling off at tumor masses >350 mm^3. Changes were significant in tumors >250 mm^3 when values were compared with those obtained from the smallest volume group ($2p < 0.05$)[31].

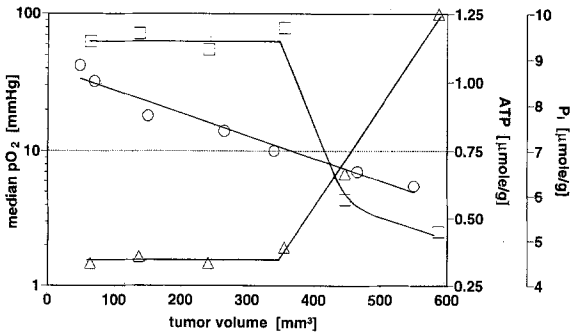

Figure 3. Mean concentrations of ATP (squares) and of total P_i (triangles), and median pO_2 values in FSaII tumors (circles), as a function of tumor volume.

FSaII tumors of varying sizes were assayed in neutralized extracts by high-performance liquid chromatography (HPLC)[31]. Global ATP concentrations were 1.23 ± 0.06 µmol/g in tumors <400 mm^3. In larger tumors there was a drop ($2p = 0.05$) to average ATP levels of 0.45 ± 0.09 µmol/g (see fig. 3, squares). During tumor growth the mean adenosine diphosphate (ADP) concentrations slightly increased (n.s.) from 0.47 ± 0.1 to 0.53 ± 0.1 µmol/g. The average adenosine monophosphate (AMP) content was constant at tumor volumes <350 mm^3 (0.12 ± 0.02 µmol/g). In larger tumors a significant rise ($2p < 0.05$) to average levels of 0.22 µmol/g was observed[31].

Mean PCr concentrations significantly decreased at early growth stages (tumor volumes <250 mm^3) from 1.5 ± 0.1 to 1.1 ± 0.1 µmol/g[31]. Total P_i levels determined with a colorimetric test were almost constant during tumor growth[31]. A substantial increase (from 4.6 ± 0.3 to 10.1 ± 0.7 µmol/g) was found only at tumor masses >350 mm^3, coinciding with the observed decrease in ATP levels and median pO_2 values <10 mmHg (see fig. 3, triangles)[31].

Traditionally, energy charge ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]), phosphorylation potential [ATP]/([ADP] × [P_i]), the sum of adenylate phosphates [ATP] + [ADP] + [AMP], and the fraction of high-energy phosphates ([ATP] + [ADP] + [PCr])/([ATP] + [ADP] + [AMP] + [PCr] + [P_i]) have often been used to give an indication of the energy status of a tissue or organ. However, P_i levels derived from colorimetric measurements yield total P_i (sum of bound, sequestered and free P_i[3]). Since only a minor fraction of the total P_i is available to act as an important substrate or as an effector for cytosolic enzymes, the suitability of energy status parameters which encompass P_i levels needs to be

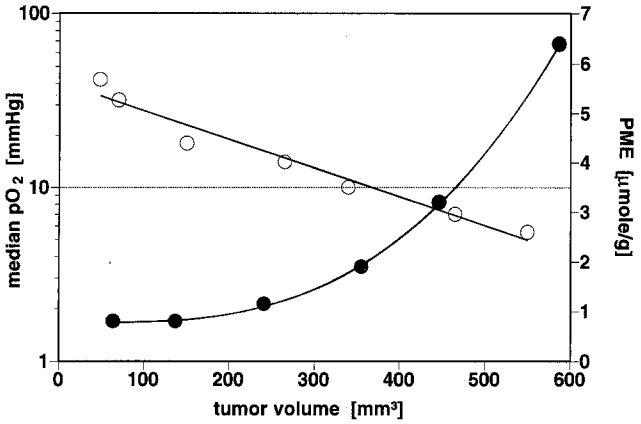

Figure 4. Mean concentrations of PME (filled symbols) and median pO_2 values (open symbols) in FSaII tumors as a function of tumor volume.

reconsidered. The same holds true for ADP, since the latter compound is also partially 'invisible'[3]. Energy status parameters are only meaningful if calculated from 'free' compounds. Previous data derived from classical biochemical extraction procedures are thus difficult to interpret[3,11,31].

Correlations between tumor bioenergetic parameters, pH_{NMR} and tissue oxygenation

In FSaII tumors with median pO_2 values >10 mmHg (i.e. tumor volumes <350 mm³ or ≤1.5% of body weight), levels of ATP, total P_i and PME were relatively constant (see fig. 4, filled symbols). Under these conditions, the nominal intracellular pH is slightly alkaline or neutral. In contrast, in bulky tumors with, on average, median pO_2 values <10 mmHg, a steady decline of the cellular ATP level with a substantial rise of the concentrations of total P_i (fig. 3, triangles) and PME (fig. 4, filled symbols) was seen. This coincides with a drop in the intracellular pH value to below 7.0, that is, cellular acidosis develops in these large tumors. The increase in PME concentrations under these conditions may reflect an intensified membrane phospholipid turnover. From these data, one may conclude that in FSaII tumors, median pO_2 values below 10 mmHg may, on average, reflect tissue hypoxia which is accompanied by a progressive deterioration of the bioenergetic status (ATP depletion due to 'metabolic' hypoxia) with the development of intracellular acidosis.

Interpretation of data

Living cells are known to maintain a 'homeostatically' controlled steady-state ATP concentration[2,11,23]. In FSaII tumors <400 mm³, a relatively constant ATP level is maintained during growth despite a progressive deterioration in tumor oxygenation. Total P_i concentrations also remain constant, whereas PCr levels decrease at early growth stages. This fall in PCr level with growth has been observed previously and was thought to contribute to the maintenance of the ATP level by the creatine kinase reaction[7,11,18]. Other authors, who also observed a decrease in PCr during tumor growth, postulated that the amount of PCr in a tumor is far too small (1–2 µmol/g) to make a significant contribution to the ATP supply. Considering an ATP turnover rate of 5–10% per second, the conversion of all the PCr into ATP in hypoxic cells to maintain the ATP level would take only 10–20 s[16]. In this respect, PCr may be of minor importance for energy metabolism in tumors. The constant level of ATP during tumor growth may be due to an increasing proportion of nonproliferating cells and an intensified glycolysis with cleavage of glucose to lactic acid, a condition which is followed by a drop in intracellular pH.

A decline in ATP concentrations is only found in bulky fibrosarcomas (>400 mm³), coinciding with a substantial increase in total P_i levels. Gerweck et al.[11] have found similar results for the same tumor model, although the increase in P_i level occurred earlier and was somewhat less pronounced. This drop in ATP levels coincides with a progressive intracellular acidosis in this tumor line. Due to this acidification, key enzymes in the glycolytic pathway such as hexokinase and phosphofructokinase are inhibited, and consequently, the fixation of P_i in high-energy phosphates and lactic acid production is blocked. The latter condition is verified by almost constant lactate levels in bulky FSaII tumors[31]. From this, it is concluded that the decline in pH_{NMR} in small tumors may to a large extent be the consequence of aerobic and/or anaerobic glycolysis followed by an accumulation of lactic acid. At later growth stages when intracellular acidosis develops, ATP hydrolysis may be more significantly involved in cellular acidification. In turn, ATP depletion may slow down the activity of the Na^+/H^+-antiport[24] (and most probably HCO_3^-/Cl^--antiport), thus further contributing to intracellular acidosis.

In the cell line studied, there is a strong negative correlation between tumor volume and nominal intracellular pH. Similar findings have been described for a series of tumor lines[1,8,13,15,18,20]. No correlation between increasing tumor size and pH was observed in tumors investigated by others[17,21,22,23].

Under normoglycemic conditions, cellular energy status of FSaII tumors is closely related to the oxygenation status (fig. 1). This finding is in close agreement with earlier data of Gerweck et al.[9], who found a rapid decrease in the (potentially irrelevant) adenylate energy charge in FSaII tumor cells continuously deprived of oxygen. In the latter experiments, the energy charge dropped from 0.94 (under normoxic conditions) to 0.18 within 2 h of hypoxia. In a more recent in vitro study

using cultures of Chinese hamster ovary cells, Gerweck et al.[10] have pointed out that the energy status was not substantially affected by oxygen deprivation as long as glucose was available in the medium. ATP content per cell remained constant in the presence of glucose over the complete range of oxygen levels (pO_2 range from 0.3 to ~7 mmHg).

Calderwood et al.[5] found that energy charge rapidly declined after the concentration of ATP fell below 1 nmol/10^6 cells (1 nmol/10^6 cells approximates 1 μmol/g). In the latter experiments, the phosphorylation potential, which emphasizes the role of P_i as a primary regulator[6], was a poorer indicator of cell viability than energy charge, assuming that AMP is the primary regulator in the energy metabolism[2].

Cellular energy status is related to oxygenation status in a series of solid tumor cell lines[14,19]. So far, only two lines investigated (OWI, a human ovarian carcinoma xenograft line with a high volume fraction of necrotic tissue of 50–70%[18,19], and MCaIV murine mammary tumors with a large hypoxic fraction of 49%[9]) did not show a similar correlation, suggesting that tissue oxygenation and energy metabolism of these lines were independent factors. Further experiments with these cell lines in volume ranges between 50 and 100 mm^3 are still needed to support this suggestion.

On average, median tissue pO_2 values of about 10 mmHg seem to represent a critical 'threshold' for energy metabolism. At higher median O_2 tensions, levels of ATP, PME and total P_i are relatively constant. This coincides with intracellular alkalosis or neutrality and stable energy charge, phosphorylation potential, fraction of high-energy phosphates and sum of adenylate phosphates. At median $pO_2 < 10$ mmHg, intracellular acidosis, ATP depletion ([ATP] < 1 μmol/g), a drop in energy charge, and increased PME and P_i levels are observed in FSaII tumors.

Mean blood flow in 450–600 mm^3 FSaII tumors amounts to ≈3% of cardiac output/g[25]. Assuming a cardiac output of 16 ml/min[4], the rate of blood flow is 0.48 ml/(g · min). An oxygenation status with median pO_2 values <10 mmHg would therefore coincide with average blood flow values ≤0.48 ml/(g · min) in this experimental tumor. This latter flow value is similar to that described by Walenta et al.[33], who found a 'breakpoint' in the correlation between [ATP] and flow at roughly 0.4–0.5 ml/(g · min) for A-Mel 3 tumors in hamsters.

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1 Adams, D. A., Denardo, G. L., Denardo, S. J., Conboy, C. B., and Bradbury, E. M., ^{31}P-NMR analysis of metabolic status in KHJJ tumors. Magnet. Reson. Med. *2* (1985) 419.

2 Atkinson, D. E., Cellular energy metabolism and its regulation. Academic Press, New York 1977.

3 Balaban, R. S., The application of nuclear magnetic resonance to the study of cellular physiology. Am. J. Physiol. *246* (1984) C10–C19.

4 Barbee, R. W., Perry, B. D., Ré, R. N., and Murgo, J. P., Microsphere and dilution techniques for the determination of blood flows and volumes in conscious mice. Am. J. Physiol. *263* (1992) R728–R733.

5 Calderwood, S. K., Bump, E. A., Stevenson, M. A., Van Kersen, I, and Hahn, G. M., Investigation of adenylate energy charge, phosphorylation potential and ATP concentration in cells stressed with starvation and heat. J. Cell. Physiol. *124* (1985) 261–268.

6 Erecinska, M., Stubbs, M., Miyata, Y., Ditre, C. M., and Wilson, D. F., Regulation of cellular metabolism by phosphate. Biochim. biophys. Acta *462* (1977) 20–35.

7 Evanochko, W. T., Ng, T. C., and Glickson, J. D., Application of in vivo NMR spectroscopy to cancer. Magn. Reson. Med. *1* (1984) 508–534.

8 Fu, K. K., Wendland, M. F., Iyer, S. B., Lam, K. N., Engeseth, H., and James, T. L., Correlations between in vivo ^{31}P NMR spectroscopy measurements, tumor size, hypoxic fraction and cell survival after radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. *18* (1990) 1341–1350.

9 Gerweck, L. E., Koutcher, J. A., Zaidi, S. T. H., and Seneviratne, T., Energy status in the murine FSaII and MCaIV tumors under aerobic and hypoxic conditions: an in-vivo and in-vitro analysis. Int. J. Radiat. Oncol. Biol. Phys. *23* (1992) 557–561.

10 Gerweck, L. E., Seneviratne, T., and Gerweck, K. K., Energy status and radiological hypoxia at specified oxygen concentrations. Radiat. Res. *135* (1993) 69–74.

11 Gerweck, L. E., Urano, M., Koutcher, J., Fellenz, M. P., and Kahn, J., Relationship between energy status, hypoxic cell fraction and hyperthermic sensitivity in a murine fibrosarcoma. Radiat. Res. *117* (1989) 448–458.

12 Hoeckel, M., Schlenger, K., Knoop, C., and Vaupel, P., Oxygenation of carcinomas of the uterine cervix: evaluation of computerized O_2 tension measurements. Cancer Res. *51* (1991) 6098–6102.

13 Koutcher, J. A., Alfieri, A. A., Barnett, D. C., Cowburn, D. C., Kornblith, A. B., and Kim, J. H., Changes in ^{31}P nuclear magnetic resonance with tumor growth in radioresistant and radiosensitive tumors. Radiat. Res. *121* (1990) 312–319.

14 Mueller-Klieser, W., Schaefer, C., Walenta, S., Rofstad, E. K., Fenton, B. M., and Sutherland, R. M., Assessment of tumor energy and oxygenation status by bioluminescence, nuclear magnetic resonance spectroscopy and cryospectrophotometry. Cancer Res. *50* (1990) 1681–1685.

15 Ng, T. C., Evanochko, W. T., Hiramoto, R. N., Ghanta, V. K., Lilly, M. B., Lawson, A. J., Corbett, T. H., Durant, J. R., and Glickson, J. D., ^{31}P NMR spectroscopy of in vivo tumors. J. Magnet. Reson. *49* (1982) 271–286.

16 Okunieff, P., Singer, S., and Vaupel, P., Magnetization transfer ^{31}P-NMR to measure metabolic states, dynamic changes and enzyme kinetics. Funktionsanal. biolog. Systeme *20* (1991) 267–292.

17 Rodrigues, L. M., Midwood, C. J., Coombes, R. C., Stevens, A. N., Stubbs, M., and Griffiths, J. R., ^{31}P nuclear magnetic resonance spectroscopy studies of the response of rat mammary tumors to endocrine therapy. Cancer Res. *48* (1988) 89–93.

18 Rofstad, E. K., De Muth, P., and Sutherland, R. M., ^{31}P NMR spectroscopy measurements of human ovarian carcinoma xenografts: relationship to tumor volume, growth rate, necrotic fraction and differentiation status. Radiother. Oncol. *12* (1988) 315–326.

19 Rofstad, E. K., De Muth, P., Fenton, B. M., and Sutherland, R. M., ^{31}P nuclear magnetic resonance spectroscopy studies of tumor energy metabolism and its relationship to intracapillary oxyhemoglobin saturation status and tumor hypoxia. Cancer Res. *48* (1988) 5440–5446.

20 Rofstad, E. K., Howell, R. L., De Muth, P., Ceckler, T. L., and Sutherland, R. M., ^{31}P NMR spectroscopy in vivo of two murine tumor lines with widely different fractions of radiobiologically hypoxic cells. Int. J. Radiat. Biol. *54* (1988) 635–649.

21 Stubbs, M., Bhujwalla, Z. M., Tozer, G. M. Rodrigues, L. M., Maxwell, R. J., Morgan, R., Howe, F. A., and Griffiths, J. R., An assessment of ^{31}P MRS as a method of measuring pH in rat tumors. NMR Biomed. *5* (1992) 351–359.
22 Stubbs, M., Coombes, R. C., Griffiths, J. R., Maxwell, R. J., Rodrigues, L. M., and Gusterson, B. A., ^{31}P-NMR spectroscopy and histological studies of the response of rat mammary tumors to endocrine therapy. Br. J. Cancer *61* (1990) 258–262.
23 Stubbs, M., Rodrigues, L. M., and Griffiths, J. R., Growth studies of subcutaneous rat tumors: comparison of ^{31}P-NMR spectroscopy, acid extracts and histology. Br. J. Cancer *60* (1989) 701–707.
24 Tannock, I. F., and Rotin, D., Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res. *49* (1989) 4373–4384.
25 Tozer, G., Suit, H. D., Barlai-Kovach, M., Brunengraber, H., and Biaglow, J., Energy metabolism and blood perfusion in a mouse mammary adenocarcinoma during growth and following X irradiation. Radiat. Res. *109* (1987) 275–293.
26 Tozer, G. M., and Griffiths, J. R., The contribution made by cell death and oxygenation to ^{31}P MRS observations of tumors energy metabolism. NMR Biomed. *5* (1992) 279–289.
27 Vaupel, P., Oxygenation of solid tumors, in: Drug Resistance in Oncology, pp. 53–85. Ed. B. A. Teicher, Marcel Dekker, New York 1993.
28 Vaupel, P., Physiological properties of malignant tumors. NMR Biomed. *5* (1992) 220–225.
29 Vaupel, P., Kallinowski, F., and Okunieff, P., Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res. *49* (1989) 6449–6465.
30 Vaupel, P., Okunieff, P., Kallinowski, F., and Neuringer, L. J., Correlations between ^{31}P-NMR spectroscopy and tissue O_2 tension measurements in a murine fibrosarcoma. Radiat. Res. *120* (1989) 477–493.
31 Vaupel, P., Schaefer, C., and Okunieff, P., Intracellular acidosis in murine fibrosarcomas coincides with ATP depletion, hypoxia and high levels of lactate and total P_i. NMR Biomed. *7* (1994) 128–136.
32 Vaupel, P., Schlenger, K., Knoop, C., and Hoeckel, M., Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O_2 tension measurements. Cancer Res. *51* (1991) 3316–3322.
33 Walenta, S., Dellian, M., Goetz, A. E., Kuhnle, G. E. H., and Mueller-Klieser, W., Pixel-to-pixel correlation between images of absolute ATP concentrations and blood flow in tumors. Br. J. Cancer *66* (1992) 1099–1102.