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# RELATIONSHIP BETWEEN TUMOUR OXYGENATION, BIOENERGETIC STATUS AND RADIOBIOLOGICAL HYPOXIA IN AN EXPERIMENTAL MODEL

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**Tumour oxygenation and bioenergetic status were measured in the same tumour and these results related to radiobiological hypoxia. A C3H mouse mammary carcinoma grown in the feet of CDF1 mice was used. Bioenergetic status was assessed by $^{31}$P MRS using a SISCO 7 Tesla magnet, oxygen measurements were done by a polarographic electrode and the hypoxic fraction was determined from direct analysis of the radiation dose–response data. During all examinations restrained, non-anaesthetized mice were allowed to breathe either 100% oxygen, carbogen, normal air, carbon monoxide (CO) at 75, 220, or 660 ppm or had blood flow occluded by clamping. Results showed a significant correlation between the radiobiological hypoxic fraction and % $pO_2 \leqslant 5$ mmHg under the different treatment conditions, whereas no correlation was found between beta nucleosidetriphosphate/inorganic phosphate ($\beta$-NTP/Pi) ratio and either the hypoxic fraction or the % of $pO_2$ values $\leqslant 5$ mmHg under the different treatment conditions. In conclusion, oxygen electrode measurements were sensitive to changes in tumour hypoxia whereas the bioenergetic status alone seemed to be a less precise measure of hypoxia in this tumour model. Furthermore, the present study demonstrated that tumour cells in vivo can actually maintain the bioenergetic status during a period of severe hypoxia.**



It is well accepted that hypoxic cells exist in solid tumours and that they contribute to resistance to radiotherapy (1, 2). Attempts to identify radiobiological hypoxia have involved a whole range of indirect methods as, for example, measurements of intercapillary distance, vascular density, cryospectrophotometric measurements of intracapillary haemoglobin oxygen saturations, immunohistochemical detection of nitroimidazole based hypoxic cell markers, estimation of tumour metabolic activity by high performance liquid chromatography, ATP bioluminescence or $^{31}$P NMR (nuclear magnetic resonance spectroscopy), and oxygen measurements by polarographic oxygen electrodes (for review, see (3)).

Determination of tumour oxygenation by oxygen electrodes is perhaps the most direct method for trying to identify tumour hypoxia. Previous results in experimental tumours have shown a correlation between radiobiological hypoxia and tumour oxygenation measured by oxygen electrodes within the same tumour line during different treatment conditions, whereas no correlation was found between these parameters across tumour sublines (4, 5). Furthermore, these results documented that oxygen electrode measurements were sensitive to even small changes in tumour oxygenation within one tumour model (4). For clinical use the method has been reported as reliable and feasible (6, 7), and preliminary results have suggested that oxygen electrode measurements of human tumours can predict the outcome of radiotherapy (8–10). However, larger trials need to confirm the predictive value of this method.

Assessment of tumour bioenergetic status by $^{31}$P MR spectroscopy has been suggested as another clinically ap-

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plicable albeit indirect method to measure hypoxia in tumours (3). However, a direct relationship between in vivo $^{31}P$ NMR energy measurements and radiation response has not been shown. An attempt to show a relationship between bioenergetic status and radiobiological hypoxia was made in experimental tumour models (11–16), but most of these studies used tumour volume as a variable. In some of the tumour models it was found that increasing tumour volume correlated with a decrease in the bioenergetic status and a rise in the hypoxic fraction in tumours of corresponding sizes (11–15). In other tumour models the bioenergetic status was independent of tumour size (11, 13). Furthermore, a correlation between $^{31}P$ NMR energy measurements and hypoxic fraction across tumour sublines was not found (13). These discrepancies make it unclear whether or not the bioenergetic status is an appropriate indicator of hypoxia in tumours.

The aim of the present study was to compare the oxygen electrode measurements with $^{31}P$ NMR spectroscopy in the same tumour under different treatment conditions, and to compare these results with the radiobiological hypoxic fraction measured in comparable groups of animals under similar treatment conditions.

## Material and Methods

*Tumour model.* A C3H mouse mammary carcinoma was grown in the feet of 10- to 14-week-old CDF1/Bom (C3H/tif♀ × DBA/2♂) male mice. The derivation and maintenance of the tumour has been described previously (17). Treatments were carried out when tumour volume reached 200 $mm^3$ as determined by the formula $\pi/6 \times D_1 \times D_2 \times D_3$, where the Ds represent the three orthogonal diameters. This tumour location was convenient as irradiation could be applied without involvement of critical normal tissue in the field. Furthermore, it allowed all experiments to be performed in non-anaesthetized mice, restrained in a plastic jig with the tumour bearing foot loosely taped to the jig to avoid occluding the blood supply.

*Protocol and treatment conditions.* Each tumour was examined by in vivo $^{31}P$ MRS followed by oxygen electrode measurements within 6 h. Mice were allowed to breathe either 100% oxygen, carbogen (95% $O_2$ + 5%$CO_2$), or different concentrations of carbon monoxide (CO) at 75, 220 or 660 ppm ( ± 5%). Gas was administered through a nozzle placed over the jig at a flow of 2.5 l/min. The pretreatment breathing time was 10 min for oxygen and carbogen and 45 min for CO, the flow being maintained during subsequent treatments. For the CO studies blood samples were collected from the orbital sinus at each treatment session and analyzed for carboxyhaemoglobin (HbCO) on a blood gas analyzer (Radiometer OSM3) calibrated for mouse blood. To achieve hypoxia the blood supply to the tumour was compromised by tightening a rubber tube around the tumour-bearing leg for 5–20 min before and during measurement. An average of 5 mice per group were used for the $pO_2$ and NMR studies and an average of 8 mice per dose group for the hypoxic fraction estimation.

*Bioenergetic status.* Assessment of tumour bioenergetic status was done by $^{31}P$ MR-spectroscopy using a 7 Tesla SISCO spectrometer with 18 cm horizontal bore. In all experiments a custom probe (SISCO) and a homebuilt two-turn surface coil with 8 mm inner diameter were used, tuned to phosphorous. The probe was placed over the tumour and spectra were acquired at 121.5 MHz. Frequency modulated (adiabatic) pulses (90° pulse over tumour volume by use of 3 ms hyperbolic secant pulse, repetition time 2 s) ensure a homogeneous excitation of the whole tumour volume, while the signal transmitted by the coil will be more heterogeneous but still coming from the entire tumour volume. All spectra were collected using the following acquisition parameters: 256 averages with 4680 data points over a spectral with of 12 MHz, total sampling time per spectrum being 8.5 min. Examples of representative spectra are shown in Fig. 1. In general the signal-to-noise ratio for α-NTP was above 8 when measuring in tumour. Processing of each spectrum included 20 Hz linebroardning. Further baseline correction was not required. The spectra were analyzed by integrating resonance areas with a Lorentzian line fitting program (FID-SPEC, SISCO version 7.1). The bioenergetic status, expressed as the β-nucleosidetriphosphate/inorganic phosphate (β-NTP/Pi) ratio obtained from each tumour, was calculated for further analysis. The temperature around the mice was kept stable at 24°C by flowing heated air through the magnet bore during all measurements.

*Tumour oxygenation.* Measurements of tumour $pO_2$ were done using a polarographic oxygen electrode (Eppendorf $pO_2$ Histograph, Germany). The method has been described in detail previously (6, 7, 18). Briefly, the oxygen probe was inserted one mm into the tumour and from here automatically moved through the tumour in a stepwise pattern, with a forward step of 0.7 mm followed by a backward step of 0.3 mm, this giving a 0.4 mm distance between each measurement. This procedure was repeated in three to six tracks per tumour, yielding from 53 to 108 measurements per tumour (median of 72). For further analysis of oxygenation status the percentage of $pO_2 \leq 5$ mmHg was calculated from the raw data for each tumour.

*Hypoxic fraction.* Comparable groups of mice were treated as described above, except from incubation of the CO gas mixtures, which was done in a sealed box, flushed with CO at a flow rate of 5 l/min for 35–45 min before and during irradiation. Local irradiation was given to the tumour-bearing foot by a conventional therapeutic 250 kV x-ray machine at a dose rate of 2.3 Gy/min. To secure homogeneity of the radiation dose, tumours were immersed in a water bath at room temperature with 5 cm of water between the x-ray source and the tumour. Irradia-

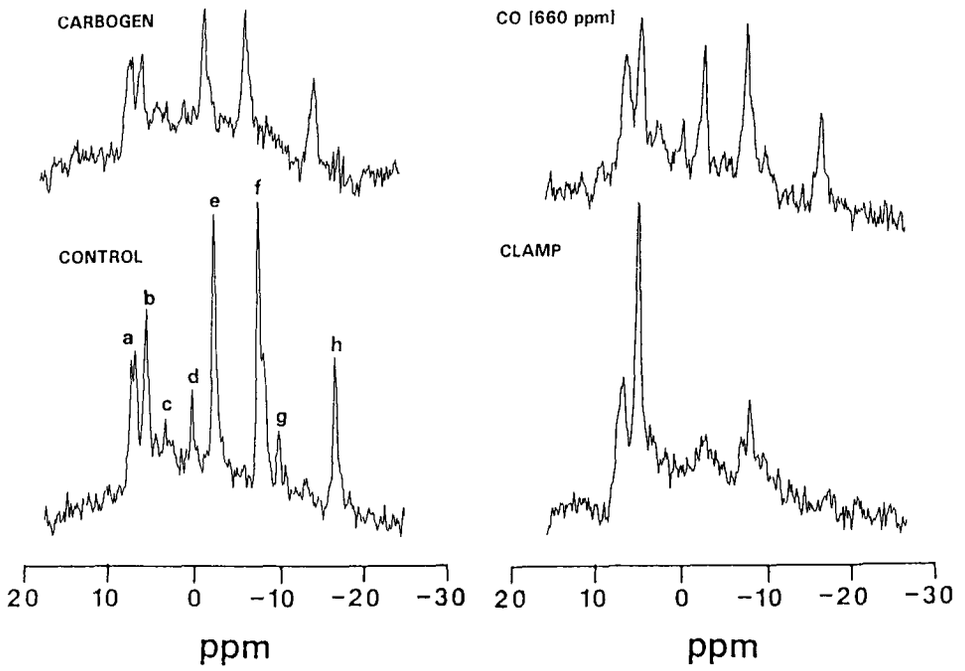


*Fig. 1.* Representative examples of $^{31}P$ spectra obtained from individual tumours, under different treatment conditions. Peak assignments include a) phosphomonoesters; b) inorganic phosphate; c) phosphodiesters; d) phosphorcreatine; e) γ-nucleoside triphosphates and γ-nucleoside diphosphates; f) α-nucleoside triphosphates and α-nucleoside diphosphates; g) diphosphodiesters; and h) β-nucleoside triphosphates.

tion of hypoxic tumours was done by clamping 5 min before and during the treatment period. The hypoxic fractions were determined from direct analysis of the radiation dose–response data, obtained under clamped and unclamped conditions, as described previously (19).

*Statistical analyses.* The experimental data of each group of treated animals were calculated as means, standard error of the mean and standard deviation and compared by Student's t-test. Univariate linear regression analysis was used to test for correlation as the line was fitted by the mean values of each group. The estimation of hypoxic fraction from tumour control data and its confidence intervals derived from direct analysis, as described by Bentzen & Grau (19). A significance level of 5% was used for all data.

## Results

Fig. 1 shows examples of representative spectra obtained from individual tumours either after clamping for 5–20 min, breathing carbogen and CO (660 ppm) or from an untreated control tumour. Only peak ratios between the spectra are comparable and not individual peak intensities as these spectra are sampled from different tumours. The quality of the spectra varies as seen by the differences in signal-to-noise ratios. The peak intensity of phosphocreatine (Pcr) was in general very low, maximum S/N ratio being 3, which indicates a negligible contribution of signal from the underlying normal tissue.

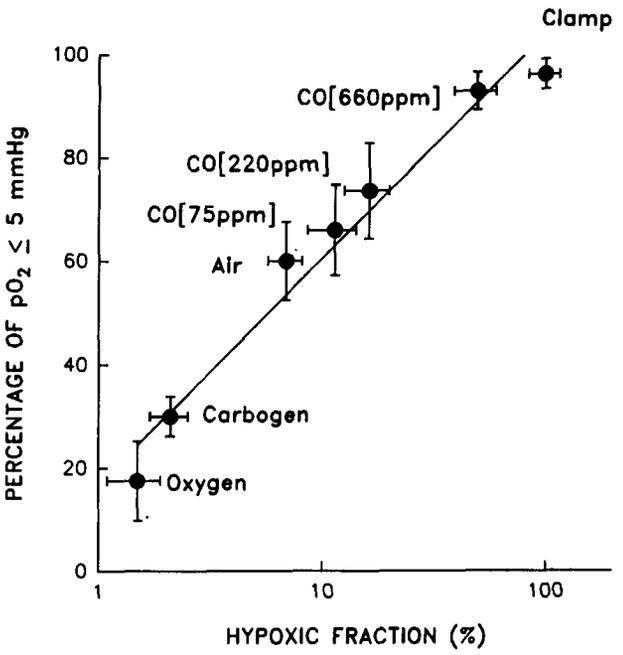


*Fig. 2.* The relationship between the hypoxic fraction and oxygen electrode measurements under various treatment conditions. Points show means (±SEM).

The relationship between the hypoxic fraction measured under the different treatment conditions and the percentage of $pO_2$ values ≤ 5 mmHg under identical conditions is shown in Fig. 2. The correlation between these two

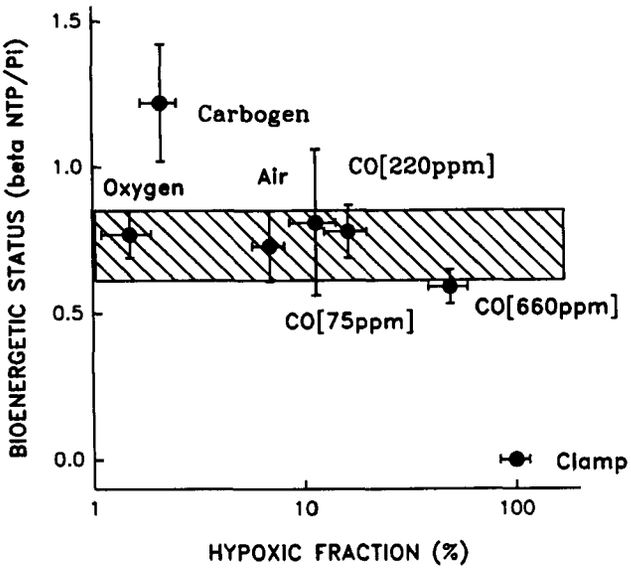


*Fig. 3.* The bioenergetic status as a function of the hypoxic fraction under various treatment conditions. Points show means (±SEM). The shaded area represents the bioenergetic status of normal air breathing tumours.

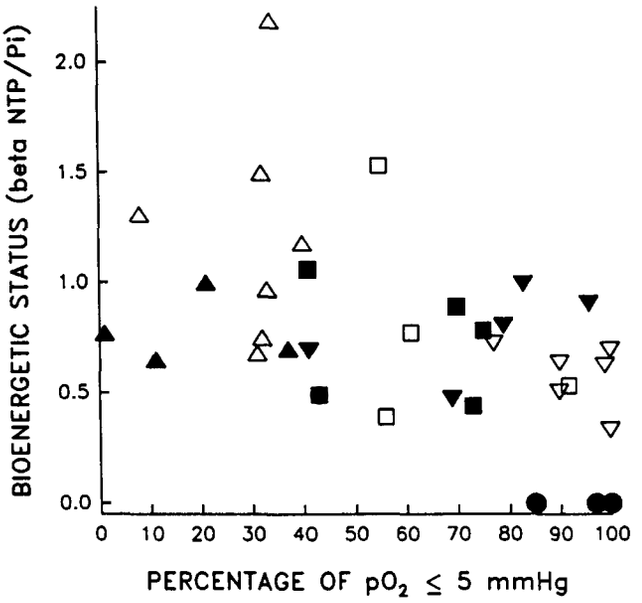


*Fig. 4.* The relationship between bioenergetic status and tumour oxygenation in individual tumours during clamping (●), breathing air (■), carbogen (△), oxygen (▲), CO at 660 ppm (▽), 220 ppm (▼) or 75 ppm (□).

parameters was highly significant ($r = 0.98$, $p < 0.001$) in this tumour model.

In Fig. 3 the relationship between the hypoxic fraction under the various treatment conditions and the $\beta$-NTP/Pi ratio under equal conditions is shown. When mice inhaled increasing concentrations of CO up to 660 ppm the radiobiological hypoxic fraction increased from 7% to 49%, while the bioenergetic status did not show any significant change. Only clamping decreased the bioenergetic status significantly. On the other hand carbogen breathing improved the energy status, although this difference was not significant.

The results of both the percentage of $pO_2 \leqslant 5$ mmHg and the $\beta$-NTP/Pi ratio measured in individual tumours during all different treatment conditions are shown in Fig. 4. Carbogen breathing was the only treatment that caused a rise in the bioenergetic status and a reduction in the percentage of $pO_2 \leqslant 5$ mmHg from that seen in air breathing animals. The decline in oxygen availability when breathing increasing doses of CO (75, 220 and 660 ppm) was followed by an increase in the percentage of $pO_2 \leqslant 5$ mmHg from 55% to 91%, whereas the $\beta$-NTP/Pi ratios were not significantly different from that of normal air breathing mice. When clamping the tumour the bioenergetic status came down to zero and the percentage of $pO_2$ increased from 59% to 96%.

## Discussion

The present study has demonstrated that the bioenergetic status in a tumour measured by $^{31}P$ MRS is only sensitive to extreme changes in the tumour oxygenation status, such as occurs after total occlusion of blood flow or as a consequence of breathing carbogen. The extreme changes in tumour bioenergetic status were consistent with large $pO_2$ changes from oxygen electrode measurements and also from estimation of the radiobiological hypoxic fraction. The largest improvement in tumour oxygenation status was achieved by breathing 100 % oxygen for 10 min before and during the measurement procedure, but a similar oxygen breathing schedule had no effect on the energy metabolism in this tumour model. This may be explained by 10 min being an insufficient pretreatment breathing time to see a change in the energy metabolism. Previous reports have shown that 100% oxygen breathing over longer time scales causes an increase in the NTP/Pi and Pcr/Pi ratios (16, 20). However, the aim of the present study was to compare bioenergetic status with the radiobiological hypoxic fraction data. Several studies have shown that breathing high oxygen content gas produces maximum radiosensitization within 2 min and that this is maintained for at least 20 min (21, 22), hence our rationale for using 10-min preirradiation and pretreatment breathing intervals.

When allowing mice to breathe CO at (75, 220 and 660 ppm) the bioenergetic status was stable within the range of air breathing control animals, while the oxygenation status gradually decreased and the level of radiobiological hypoxia increased. The results indicate that measurement of the bioenergetic status by $^{31}P$ MRS is a less precise and less sensitive measure of small changes in hypoxia. A significant correlation between the radiobiolog-

ical hypoxic fraction and percentage of $pO_2 \leq 5$ mmHg under the different treatment conditions was found. This is in agreement with another set of data previously published from our laboratory (4).

The hypoxic effect of CO inhalation is due to less $O_2$ availability in the blood and to a reduction in blood flow. Previous results using the same C3H tumour model have shown that breathing CO reduces the blood flow significantly in a dose-dependent manner (23). In that particular study the blood perfusion was found to be reduced to 20% of that of control tumours by inhalation of CO (660 ppm) (23). In the present study oxygen electrode measurements documented that inhalation of CO (660 ppm) caused severe hypoxia of the tumour even though the energy metabolism was unaffected. The reason for this is not clear, but it is likely that the supply of glucose and other nutrients is sufficient in spite of the very low blood flow. The relationship between tumour cell bioenergetic status and other relevant metabolic parameters, such as pH, lactate and glucose during severe hypoxia is currently being investigated in this tumour model. The relationship between tumour oxygenation and energy metabolism was elaborated recently by Vaupel et al. (24), who found a lower limit of blood perfusion, above which the tumour energy remained stable provided that glucose could be recruited. In addition, an in vitro study by Gerweck et al. (25) described that the availability of glucose made the energetic status of tumour cells resistant to changes in oxygen supply, whereas in the absence of glucose the energetic status was affected by changes in oxygenation.

In conclusion, oxygen electrode measurements were sensitive to changes in tumour hypoxia, whereas the bioenergetic status alone seemed to be a less precise and less sensitive measure of hypoxia in this tumour model. The relationship between tumour oxygenation and energy metabolism seems to be complex and dependend on several factors, such as tumour size, blood flow, oxygen supply and glucose availability, and all parameters should be taken in consideration if using the bioenergetic status as an indirect measure of tumour hypoxia. This study also demonstrated that tumour cells in vivo can maintain the bioenergetic status during severe hypoxia.



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