

IS MISONIDAZOLE BINDING TO MOUSE TISSUES A MEASURE OF CELLULAR pO_2 ?

DEBORA J. VAN OS-CORBY,* CAMERON J. KOCH and J. DONALD CHAPMAN

Department of Radiation Oncology, Cross Cancer Institute, and Department of Radiology and Diagnostic Imaging, University of Alberta, Edmonton, Alberta, Canada, T6G 1Z2

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Abstract—Misonidazole (MISO), a hypoxic cell radiosensitizer, forms covalently-linked adducts to cellular molecules as a result of bioreductive metabolism, a process which is strongly dependent upon oxygen concentration. MISO binding to liver tissue taken from air-breathing mice was three to five times greater than binding to other normal tissues. The relative binding of [^{14}C]MISO to various mouse tissue cubes *in vitro* was measured by autoradiography as a function of defined oxygen concentrations, and standard curves (binding rate vs oxygen concentration) were generated. The oxygen concentration for half-maximum binding as well as the maximum and minimum binding rates (grains per 100 μm^2) observed for liver tissue were not significantly different from those measured for brain or heart tissue. These results, along with previously published data on MISO binding to isolated hepatocytes *in vitro*, suggest that the elevated binding to liver *in vivo* may result, in part, from the organ existing at a significantly lower pO_2 than other normal tissues. They also suggest that this drug adduct procedure could be developed as a sensitive method for the quantitative measurement of tissue pO_2 at the cellular level.

Misonidazole (MISO) has been shown to radiosensitize selectively, hypoxic mammalian cells both *in vitro* [1] and *in vivo* [2]. Metabolic studies with radioactively labeled drug show accumulation of activity in these cells within both the acid-soluble and -insoluble fractions [3]. The binding rate is 20–50 times higher in the absence of oxygen than in its presence [3]. These data, plus the observation that [^{14}C]MISO adducts are released from the cells with a half-life of 50–55 hr [4], suggest that sensitizer adducts may be useful as markers for hypoxic cells in solid tumors.

Studies by Garrecht and Chapman [5] measured the distribution of [^{14}C]MISO in Balb/C mice bearing EMT-6 tumors after both single and multiple injections of drug. By both liquid scintillation and autoradiographic procedures it was determined that the level of ^{14}C retained in tumors known to contain 30% hypoxic cells [6] was 4–15 times greater than that retained in all other normal tissues, except for liver. The level of ^{14}C retained in liver tissue was comparable to that retained in the tumors. The higher accumulation of ^{14}C in the tumors was heterogeneous at the microscopic level and was attributed to a significant fraction of chronically hypoxic tumor cells. The higher binding of [^{14}C]MISO to liver tissue was homogeneous at the microscopic level and was not explained. Since hypoxic cell sensitizers tagged with an appropriate label are being developed as markers for treatment-resistant cells in tumors, any excessive binding to a normal tissue, such as liver, could limit their usefulness.

Previous studies from this laboratory [7] measured the hypoxic and aerobic binding rates of [^{14}C]MISO to isolated mouse hepatocytes, mouse hepatoma cells, V79 Chinese hamster fibroblasts and EMT-6 tumor cells *in vitro*. The low rate of binding to mouse hepatocytes observed in short-term culture *in vitro* suggested that mouse hepatocytes do not contain a unique or elevated complement of bioreductive enzymes that could account for the elevated binding of [^{14}C]MISO observed *in vivo*.

We now describe studies in which the rates of binding of [^{14}C]MISO to intact mouse tissue cubes in short-term tissue culture were measured as a function of various oxygen tensions. These data suggest, as did the isolated cell results, that liver tissue metabolizes MISO at rates similar to, and with a similar oxygen dependence as, other normal tissues. This would imply that the higher binding to liver *in vivo* is a result of either reduced oxygen tension or some metabolic property only present *in vivo*. As well, the data suggest that the sensitizer adduct technique can be usefully developed as a quantitative tool for the determination of tissue pO_2 at the cellular level.

MATERIALS AND METHODS

Animal and tumor procedures. Female Balb/C mice, weighing 19–22 g, were supplied by the Small Animal Breeding Unit of the University of Alberta. Mice were housed at a maximum of five animals per cage, fed Rodent Chow (Ralston Purina Co.), given water *ad lib.*, and maintained on a 12-hr light/dark cycle.

[^{14}C]Misonidazole [1-(2-nitro-1-imidazole)-3-methoxy-2-propanol], labeled at C-2 of the imidazole ring, was provided by Dr. W. E. Scott of Hoffmann-

* Address all correspondence to D. J. Van Os-Corby, M.Sc., Department of Radiation Oncology, Cross Cancer Institute, University of Alberta, 11560 University Ave., Edmonton, Alberta, Canada T6G 1Z2.

La Roche (Nutley, NJ) or purchased from the Stanford Research Institute (Palo Alto, CA). Its specific activity was 230 $\mu\text{Ci}/\text{mg}$.

EMT-6 mouse fibrosarcoma (supplied by Dr. J. M. Brown, Stanford University, 1978) was maintained in culture by alternate passage *in vivo* and *in vitro*. For tumor initiation, 2×10^6 EMT-6 cells in 0.05 ml Waymouth's medium (GIBCO) were injected subcutaneously in the animal's flank with a Tuberculin 1-ml syringe (Becton Dickinson & Co.) and a 26-gauge $\frac{3}{8}$ in. Yale needle (Becton Dickinson & Co.). After 14 days, the tumors had grown to diameters of 1–2 cm. Mice whose tumors showed surface necrosis were not used in labeling experiments.

[^{14}C]Misonidazole binding to tissue cubes *in vitro*. Donor mice were killed by cervical dislocation, and

the liver, kidney, heart, spleen, brain, and tumor were rapidly removed and placed in sterile petri dishes containing 10 ml of ice-cold Spinner minimal essential medium [MEM with low bicarbonate, 20 mM 4-(2-hydropyethyl)-1-piperazine-ethane-sulfonic acid (HEPES), 10% fetal calf serum and penicillin-streptomycin]. Using a sterile razor blade, the tissue samples were cut into cubic fragments with sides of 1–2 mm. Tissue cubes (10 cubes/dish) were transferred to 50 mm Pyrex petri dishes (Corning) containing 5 ml of Spinner MEM and 50 μM [^{14}C]MISO. These were placed in specially designed leak-proof aluminum chambers [8] along with a petri dish filled with distilled water to provide humidity. For chambers gassed with pure nitrogen, the distilled water was replaced with a 0.1 M solution of sodium carbonate and sodium dithionite, which acts as a

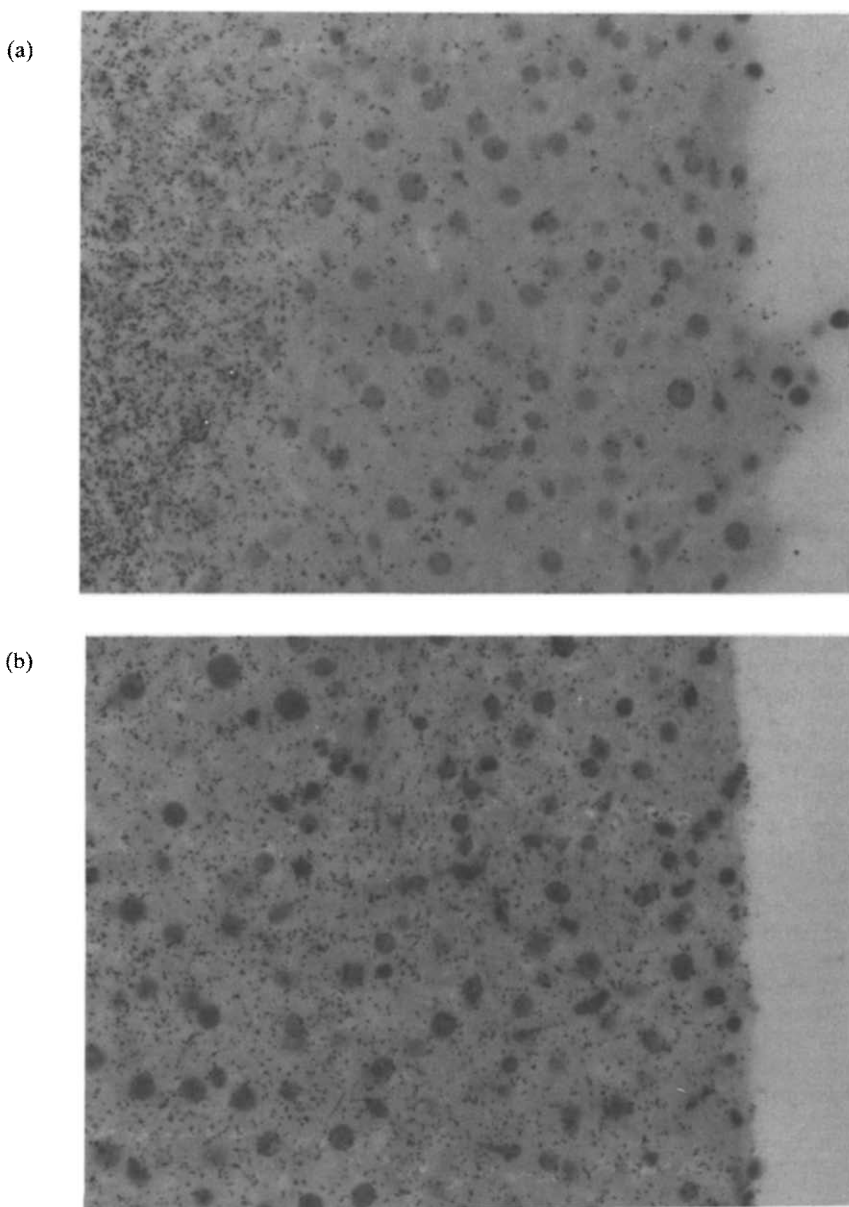


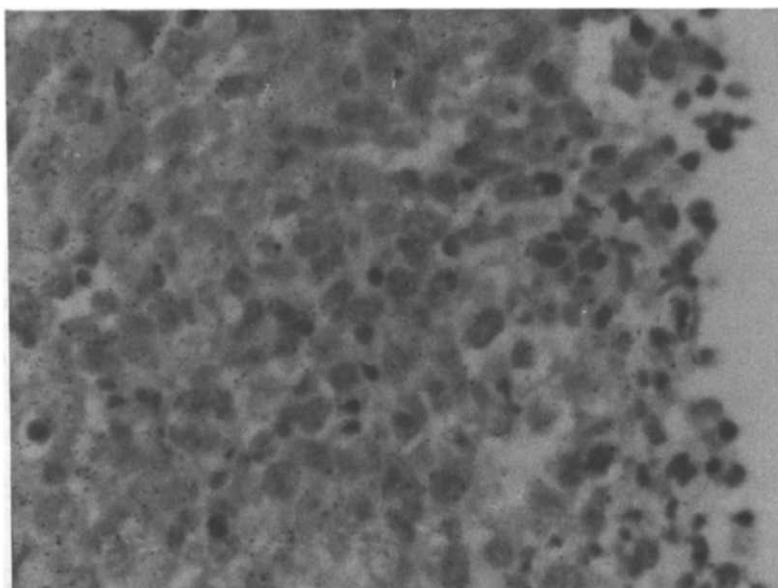
Fig. 1. Grain density at surface of mouse liver cube incubated in (a) 30% oxygen, and (b) nitrogen. Magnification: 422 \times . Cube surface is located at right edge of figure.

reducing agent to scavenge trace concentrations of oxygen from the chamber. The chambers were sealed and connected to a manifold which, in turn, was connected to a vacuum pump, a precision vacuum gauge, and cylinders of pure oxygen and pure nitrogen. The chambers could be evacuated to a given absolute pressure (always well above the vapour pressure of water) and refilled with either oxygen or nitrogen depending on whether oxygen levels higher or lower than air were desired [9]. Typically, all chambers were equilibrated at 32% oxygen and then a series of gas changes were made, each one of which decreased the oxygen concentration by 0.316, giving values of 32%, 10%, 3.2% . . . , 0.1% and pure nitrogen. This process was monitored continuously using a special measuring chamber, also connected

to the manifold, with a leakproof ultra-torr fitting (Cajon) into which was sealed a Clark oxygen sensor (manufactured by one of the authors, C.J.K.).

The sealed chambers were placed on a reciprocating shaker table in an environmental chamber at 37°, which operated at 70 cycles/min. It required approximately 30 min for the medium in the petri dishes to equilibrate to 37°. Incubation was continued for an additional 3 hr after which the final percent oxygen in the gas phase of each chamber was measured by transferring a portion of its atmosphere to the special measuring chamber, initially containing zero oxygen. Since the initial oxygen concentration in the measuring chamber was zero, and since the fraction of gas transferred from the experimental

(a)



(b)

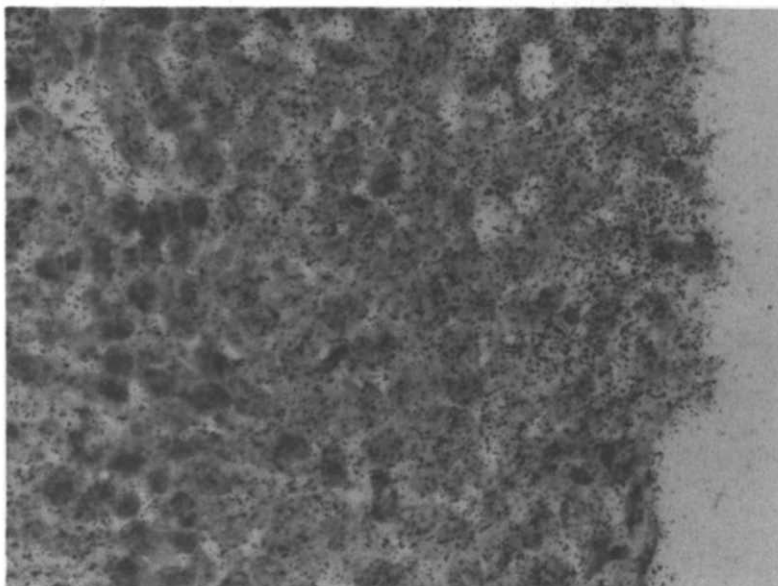


Fig. 2. Grain density at surface of EMT-6 tumor cube incubated in (a) 30% oxygen, and (b) nitrogen. Magnification: 422 \times . Cube surface is located at right edge of figure.

chamber was known, the actual pO_2 content of the experimental chamber could be determined.

After incubation in the presence of [^{14}C]MISO, the tissue culture dishes were removed and placed on cold metal trays. The tissue cubes were washed three times with saline and two times with 10% buffered formalin and, then, fixed in formalin for 24 hr. Fixed tissue cubes were embedded in paraffin, and 4- μm serial sections were obtained.

As a control, 10 μl of the radioactive medium from each culture dish was added to a glass scintillation vial containing 15 ml of Scintillation fluid (Scintiverse I, Fisher), and the ^{14}C activity was determined in a Beckman LS 7000 Liquid Scintillation Counter.

Dehydrated histological sections were dewaxed and stored at 4° in distilled water. They were then processed for autoradiography using a modification of the procedure outlined by Rogers [10]. NTB-2 Liquid Nuclear Track Emulsion (Kodak) was melted at 45°, diluted with an equal volume of distilled water, and poured into a plastic dipping pot maintained at 45°. Dewaxed slides were gently shaken to remove excess water, dipped into the melted emulsion, wiped, and placed on cold metal trays to gel. After 20 min, the slides were transferred to glass holders, kept in a light-tight box for 1.5 hr and, then, dried in silica gel for 2 hr. When dry, the slides were wrapped tightly in cloth and stored in a foil-wrapped can. Excess moisture was prevented from accumulating by placing a silica gel filled petri dish in the bottom of the can. Emulsion coated slides were exposed at 4° for 4–14 days, developed in D19 (Kodak) for 3 min, dipped in 3% acetic acid stop bath (Kodak), and fixed for 3 min in Kodak fixer. All solutions were maintained at 17° during the developing procedure. Slides were rinsed in cold running water for 4 min, wiped, and air dried for 24 hr before staining with hematoxylin and eosin.

Grains were scored under oil immersion using a 10 \times 10 μm grid aligned perpendicularly to the edge of each tissue cube. Grain densities over the outermost cell layers (approximately 2 cell diameters) were determined for each oxygen concentration by averaging at least 100 individual counts and subtracting the mean background grain density.

[^{14}C]MISO binding to mouse tissues *in vivo*. Air-breathing female Balb/C mice bearing EMT-6 tumors were injected i.p. with [^{14}C]MISO (10 $\mu g/g$ body). Peak plasma concentrations of 60 μM MISO were observed within 15–20 min and, since the drug had a plasma half-life of 30–40 min, additional doses of 5 $\mu g/g$ [^{14}C]MISO were administered at 45, 90 and 135 min after the initial injection to approximate a continuous tissue exposure of $\sim 50 \mu M$ drug over 3 hr. Unbound drug was allowed to clear from the plasma and tissues of the mice over 21 hr, and the animals were killed by cervical dislocation [5]. Brain, liver and tumor tissue samples were fixed and processed for autoradiographic analysis by procedures similar to those described above. The distribution of grains over the tissue sections was determined under oil immersion using a 10 \times 10 μm ocular grid. Grains were counted in 1200 μm^2 areas at random throughout tissue sections, and background grains were subtracted from all values.

RESULTS

The binding patterns of [^{14}C]MISO to liver and tumor tissue cubes *in vitro* in both nitrogen and 30% oxygen environments are shown in Figs. 1 and 2. In 30% oxygen, few grains were observed at the surface of each tissue cube, but the number of grains increased with depth within each cube. This increase in binding of [^{14}C]MISO correlated with the expected intracellular oxygen concentrations that were determined by both oxygen diffusion into, and oxygen consumption by, cells within each tissue specimen. For tissue cubes labeled in N_2 there was relatively uniform binding of [^{14}C]MISO up to the cells at the cube surface. This indicates that the intact surface cells had not been damaged seriously by the procedures that produced the tissue specimens, and that the diffusion of radiolabeled drug was not limiting. The [^{14}C]MISO binding rates to the outer cell layers were measured as a function of oxygen concentration since oxygen concentrations at the tissue/medium interface could be reasonably well controlled.

Standard binding curves were constructed for mouse liver, spleen, heart, brain, kidney and EMT-6 tumor by plotting the amount of [^{14}C]MISO bound (grains per 100 μm^2) to the outermost cell layers of the tissue cubes versus the concentration of oxygen in the gas phase of the incubation chambers. These data are shown in Fig. 3, panels a–f. Each graph contains the data from two, and in the case of liver three, independent experiments. The standard error is shown for each determination. Since MISO binding to mammalian cells *in vitro* is linear with time [4], the values of grains per 100 μm^2 after 3 hr of labeling shown in Fig. 3, a–f can be considered to be relative rates of binding.

The absolute values of grains per 100 μm^2 for brain, liver and heart were similar at all oxygen concentrations studied. Tumor tissue showed binding rates similar to liver but, in extreme hypoxia, had a significantly higher capacity for binding [^{14}C]MISO than all the other tissues studied. Table 1 summarizes the maximum and minimum binding rates and the concentration of oxygen at which the binding was half-maximum ($C1/2$) for all six tissues. The $C1/2$ values obtained for brain, liver, heart and tumor tissues were similar at 0.25 to 0.40% O_2 . Kidney and spleen had significantly higher $C1/2$ values (1.0 to 1.3% O_2), although the maximum binding rates were similar to those of the other normal tissues studied. Kidney tissue was distinguished from the other tissues by displaying a high [^{14}C]MISO binding rate at 30% O_2 .

The binding of [^{14}C]MISO to brain, liver and EMT-6 tumor in air-breathing mice was used to estimate the pO_2 of these tissues *in vivo*. Brain and liver exhibited uniform labeling *in vivo*. Average grains per 1200 μm^2 for brain, liver and densely labeled or sparsely labeled tumor are shown in Table 2. We have assumed that the densely labeled regions of mouse tumor are indicative of chronic hypoxia and have expressed the binding rates to other tissues relative to hypoxic tumor, assigned a value of 1.0. Data for two independent experiments are shown in Table 2 and have been averaged. To relate these *in vivo* binding data to the standard curves in panels a,

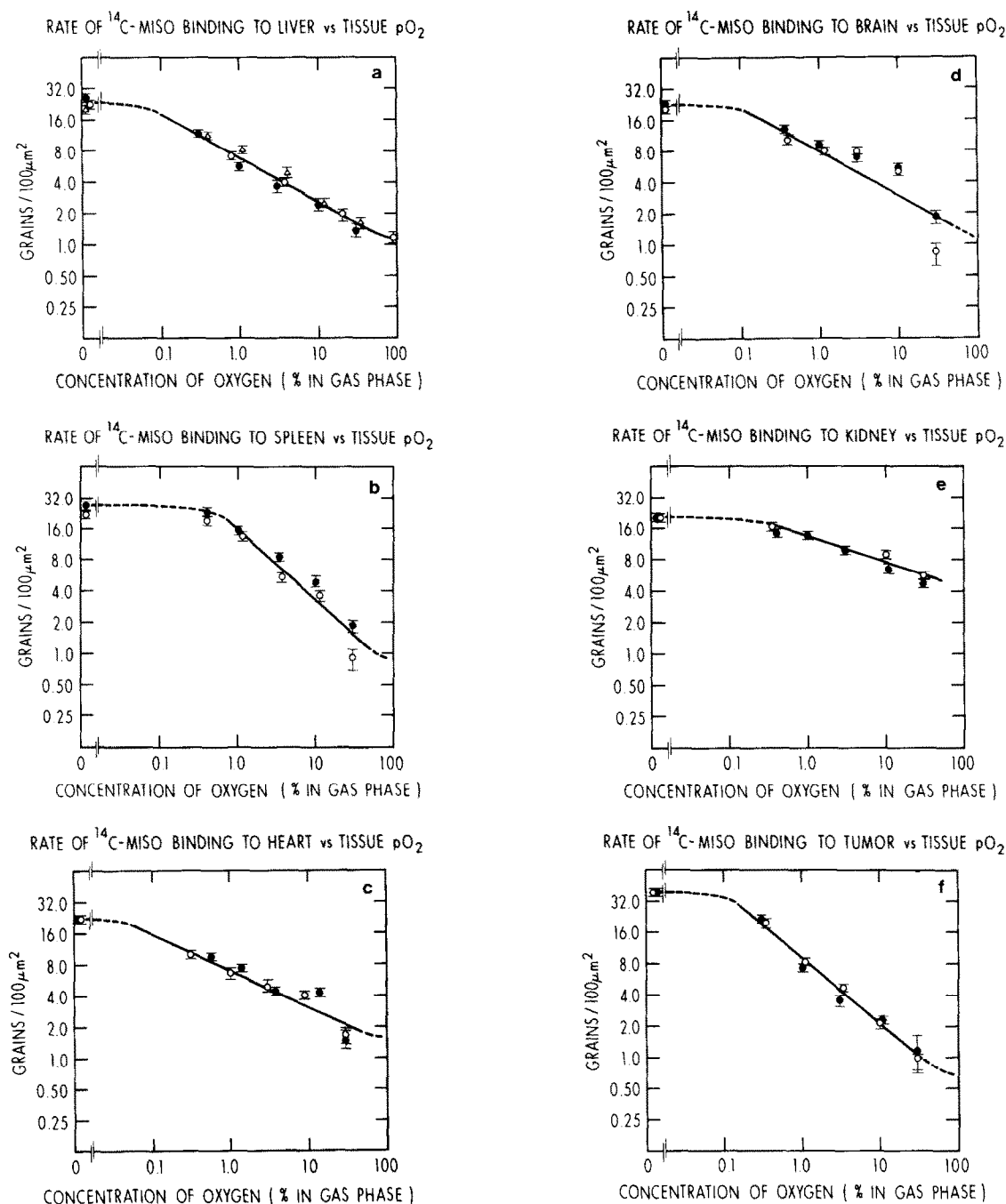


Fig. 3. Rate of $[^{14}C]$ misonidazole binding to various mouse tissues vs tissue pO_2 . Key: (a) liver, (b) spleen, (c) heart, (d) brain, (e) kidney, (f) tumor. Data from two or three (liver) individual experiments are plotted. The different symbols (\bullet \circ \triangle) represent independent determinations performed with the same tissue specimens, and standard error is indicated for each point.

d and f of Fig. 3, the densely labeled tumor values were equated to 40 grains/100 μm^2 , the maximum binding observed to tumor cubes *in vitro* labeled in N_2 (Table 1). The rates of binding to brain, liver and lightly labeled tumor *in vivo* were estimated by multiplying each ratio (Table 2) by a factor of 40 to obtain the average grains per 100 μm^2 area. These values were then fitted on the appropriate standard

curves (Fig. 3, a, d and f), and the pO_2 for each tissue was determined. As outlined in Table 2, the *in vivo* pO_2 values determined using this method were: liver, $\sim 1.0\%$ (7.6 mm Hg); brain, $\sim 20\%$ (152 mm Hg); lightly labeled (aerobic) tumor, $\sim 10\%$ (76 mm Hg); and densely labeled tumor, $\sim 0.1\%$ (0.76 mm Hg). For densely labeled tumor this procedure can only approximate a tissue pO_2 since a

Table 1. Half-maximum (C1/2) values for mouse tissues estimated from standard binding curve

	Hypoxic binding rate* (grains/100 μm ²)	30% O ₂ Binding rate (grains/100 μm ²)	C1/2 (%)
Brain	22.5	1.5	0.4
Kidney	21.5	6.0	1.0
Liver	24.0	1.5	0.3
Tumor	40.0	1.0	0.3
Spleen	27.5	1.5	1.3
Heart	24.0	1.5	0.25

* Assuming hypoxic binding as maximum and 30% binding as minimum.

maximum binding of 40 grains/100 μm² area is expected at an oxygen concentration of 0.1% (0.76 mm Hg) and less.

DISCUSSION

The binding of [¹⁴C]MISO to mammalian cells in culture is a metabolic process that is strongly dependent upon intracellular oxygen concentration [4]. The data presented in this paper demonstrate this to be the case for cells within various mouse tissues. The relative rates of binding (grains per 100 μm²) of MISO to five normal mouse tissues and to EMT-6 tumor as a function of oxygen concentration have been measured for the first time (see Fig. 3, a-f). The striking feature of these data is the similarity between both the rates of binding and the inhibitory effect of oxygen concentration on this process in all the tissues studied. There are some minor differences in [¹⁴C]MISO binding shown in Table 1. All of the normal mouse tissues studied showed similar values for the maximum amount of [¹⁴C]MISO bound under severely hypoxic conditions. EMT-6 tumor tissue was unique in showing a ~70% higher amount of MISO bound in extreme hypoxia compared to that observed for cells in the other tissues. This result is consistent with *in vitro* studies with single cells which showed that the rate of binding of [¹⁴C]MISO to EMT-6 tumor cells was greater than that of hamster fibroblast cells, mouse hepatoma cells and mouse hepatocytes [7]. Although EMT-6 tumor cells have a relatively large volume, and consequently a larger number of cellular biomolecules to which partially reduced MISO can covalently bind, other factors, in addition to their larger volume and/or mass [3], are probably involved

in the higher rate of binding to these cells. In the studies presented in this paper, [¹⁴C]MISO binding was estimated using equal cross sectional areas of tissue instead of attempting to determine binding per cell. The amount of [¹⁴C]MISO bound to mouse tissues in medium equilibrated with atmospheres containing 30% oxygen was twenty times lower than that measured under extreme hypoxia, with the exception of kidney. Kidney tissue in short-term tissue culture exhibited only a 3 to 4-fold lower rate of binding at this oxygen tension compared to binding under severe hypoxic conditions. Why [¹⁴C]MISO binding was four to five, times more efficient in kidney tissue compared to the other mouse tissues at these high oxygen concentrations is not understood but is currently under investigation. For brain, liver, heart and tumor tissue, oxygen tensions of 0.3% (2.28 mm Hg) were seen to reduce the rate of [¹⁴C]MISO binding to approximately one-half of that observed under severely hypoxic conditions. This was not the case for mouse kidney and spleen for which oxygen concentrations of 1.0 to 1.3% (7.6 to 9.8 mm Hg) were required to reduce the rate of binding to half the observed maximum.

In spite of these minor differences in the amounts of ¹⁴C bound and the inhibitory effects of various oxygen concentrations on the binding process, reasonable reproducibility was observed among independent determinations of relative binding rates. These data suggest that MISO adducts to cellular biomolecules are products of some uniform cellular reduction process.

In this regard, many scientists have investigated the reduction of various nitroimidazole drugs by both water radiolysis products and single enzymes [11-15]. The nitro group of MISO can be reduced to an

Table 2. Estimated pO₂ levels for mouse brain, liver and EMT-6 tumor

	Expt. 1 (grains/ 1200 μm ²)	Ratios	Expt. 2 (grains/ 1200 μm ²)	Ratios	Mean (expt. 1 and 2)	Grains/ 100 μm ²	Estimated pO ₂
Brain	21 + 2.2	0.06	10 + 1.1	0.05	0.05	2.4	20% (152 mm Hg)
Liver	112 + 23.7	0.34	29.8 + 3.0	0.16	0.25	10.0	0.7% (5.3 mm Hg)
Tumor dense label	329 + 43.9	1.0	181.6 + 16.5	1.0	1.0	40	<0.1% (0.76 mm Hg)
Tumor light label	26.4 + 5.3	0.08	6.6 + 1.2	0.03	0.05	2.3	10% (76 mm Hg)

Data are for two independent experiments and have been averaged.

amine by a 6-electron reduction process that likely involves the nitro radical anion (1-electron reduction), the nitroso (2-electron reduction), and the hydroxyl amino (4-electron reduction) intermediates. Under oxygenated conditions, MISO reduction is inhibited and reversed by electron transfer to substances in the cell with greater electron affinity, such as molecular oxygen. Some recent evidence suggests that in the absence of oxygen the hydroxyl amino intermediate is the reactive reduction product of MISO that can covalently bind to various biomolecules. Other studies show that it is an intact MISO molecule which becomes covalently linked with cellular biomolecules through such a mechanism [16].

The studies reported in this manuscript suggest that mouse brain, liver, heart, and EMT-6 tumor tissues have similar "reductive capacities" for MISO. Mouse kidney and spleen tissue can be considered to have somewhat higher "reductive capacities" for this drug, since approximately 4-fold higher concentrations of oxygen are required to inhibit MISO adduct formation in them compared to the other four tissues. These studies suggest that the MISO "reductive capacity" of kidney tissue is the highest of all tissues studied, since significant MISO binding is observed in cells equilibrated with 30% oxygen environments.

Biodistribution studies of [¹⁴C]MISO in tumor-bearing Balb/C mice show that significantly higher levels of ¹⁴C are retained in liver tissue compared to most other normal tissues [5]. The data in Table 2 (from two independent experiments) show the relative amounts of [¹⁴C]MISO retained in brain, liver, and both sparsely and densely labeled regions of EMT-6 tumor. This study suggested that the oxygenation status of brain tissue *in vivo* would be relatively high at approximately 20% O₂, that the average oxygen concentration in sparsely labeled areas of EMT-6 tumor was approximately 10% O₂, that the average oxygen tension over the parenchymal cells of liver was approximately 1% O₂ and that the oxygen concentration in the densely labeled areas of EMT-6 tumors was equal to or less than 0.1% O₂. The precision of the sensitizer adduct technique for estimating tissue pO₂ at the cellular level should be confirmed by alternative techniques. Unfortunately, most alternative methods are complex and invasive and have their own limitations. It should be noted that the estimate of intracellular pO₂ at the surface of the tissue cubes by the microelectrode measurements is a maximum value. Oxygen consumption by the surface cell layer could result in up to a 3-fold lower intracellular pO₂ at extremely low oxygen levels. For most oxygen concentrations studied in this paper the estimated error between the surface intracellular and medium pO₂ is less than 50%.

We could speculate from these data that the oxygen concentration in the livers of air-breathing mice is several-fold lower than that of other normal tissues. Although the liver is a well vascularized organ, it is supplied by a mixture of arterial and portal blood in the ratio of 25:75% [17]. The pO₂ of blood in the hepatic artery has been measured to be 12.5% oxygen (95 mm Hg) and to fall to only 3.9%

(30 mm Hg) in the hepatic vein, whereas the pO₂ of blood in the portal vein has been measured to be 5.9% (45 mm Hg) [17]. Studies in which the hepatic arteries were ligated described a lack of hepatic necrosis and adequate function for long periods of time when the liver was supplied with only venous blood [18, 19]. Studies in which the sensitivities to ionizing radiation of cellular DNA in animal tissues irradiated both *in vivo* and *in vitro* were measured [20] indicate that the sensitivity of liver DNA is similar to that of other normal tissues when irradiated *in vitro* but, when irradiated *in vivo*, liver DNA is found to be significantly more resistant. Meyn and Jenkins [20] have suggested that this radio-resistance of cellular DNA may indicate that the pO₂ of liver cells *in vivo* is significantly lower than that of other tissues. There is a growing awareness that several mammalian cells from both normal and malignant tissues can grow at faster rates and plate with higher efficiencies if cultured *in vitro* at reduced oxygen tensions [21–24]. These results imply that cells from some tissues have adapted to environments of lower pO₂.

There are additional studies which suggest that other normal tissues exist at low oxygen tension. In a recent review, Hendry [25] describes an increase in the radiosensitivity of tissues in animals breathing oxygen at one atmosphere, or greater, pressure during irradiation compared to their sensitivities in air. He notes that the findings could be explained if the critical normal cells were at an oxygen tension of approximately four times lower than venous blood. Some tissues that have been shown to be moderately hypoxic using this procedure include the neuraxis in rats and the laryngeal human cartilage [25]. In addition, Bohlen [26] has reported microelectrode measurements of pO₂ in the rat intestinal mucosa of less than 2 mm Hg (0.2% oxygen) for cells at the base or apex of the villus.

A study by Lammertsma *et al.* [27] in which positron emission tomography was used to determine *in vivo* measurements of regional blood flow, oxygen utilization, oxygen extraction ratios and fractional blood volumes in human brain tumors and surrounding cerebral tissues found that there was no relationship between oxygen supply and utilization within the tumors studied. In all cases, the blood supply to tumor was in excess of their metabolic requirements for oxygen. This suggests that human brain tumors are over perfused, and it is not known whether this could be true also for some normal tissues. If over perfusion of liver does occur, it may explain why measurements of pO₂ of its blood supply are higher than the estimates of liver cell pO₂ determined by this sensitizer adduct technique.

We have no evidence indicating that the tissue preparation techniques are selectively more damaging to liver tissue than to other normal tissues studied. Furthermore, neither hepatocytes in short-term tissue culture nor a mouse hepatoma cell line show elevated capacities for metabolizing and/or binding [¹⁴C]MISO *in vitro* [7]. We believe, therefore, that the elevated binding of [¹⁴C]MISO to liver in air-breathing mice is a result, in large part, of that organ residing at an oxygen tension several-fold lower than that of other normal tissues. It is difficult,

however, to rule out the possibility that the disruption and fragmentation of liver tissue into cubes for these short-term tissue culture studies damaged liver cell function uniquely or that specific substrate requirements were not met by the standard culture medium. If this were the case, one would conclude that some additional factor could modulate the MISO binding rates and their dependence upon oxygen concentration. These possibilities are under current investigation.

The technique of sensitizer adduct formation to cells *in vivo* appears to be capable of monitoring tissue pO_2 at the cellular level. The construction of standard binding curves as a function of oxygen tension is required for each tissue to be investigated, and it is a laborious procedure. Nevertheless, this drug metabolism procedure has potential for application in the study of several tissue disorders such as ischemia, stroke, and necrosis and has been successfully used to measure the hypoxic fraction of several human cancers [28].

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