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Lithium phthalocyanine: A probe for electron paramagnetic resonance oximetry in viable biological systems

(in vivo electron paramagnetic resonance/oxygen)

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ABSTRACT Lithium phthalocyanine (LiPc) is a prototype of another generation of synthetic, metallic-organic, paramagnetic crystallites that appear very useful for *in vitro* and *in vivo* electron paramagnetic resonance oximetry. The peak-to-peak line width of the electron paramagnetic resonance spectrum of LiPc is a linear function of the partial pressure of oxygen (pO₂); this linear relation is independent of the medium surrounding the LiPc. It has an extremely exchange-narrowed spectrum (peak-to-peak line width = 14 mG in the absence of O₂). Physicochemically LiPc is very stable; its response to pO₂ does not change with conditions and environments (e.g., pH, temperature, redox conditions) likely to occur in viable biological systems. These characteristics provide the sensitivity, accuracy, and range to measure physiologically and pathologically pertinent O₂ tensions (0.1–50 mmHg; 1 mmHg = 133 Pa). The application of LiPc in biological systems is demonstrated in measurements of pO₂ *in vivo* in the heart, brain, and kidney of rats.

The purpose of this article is to describe a technology based on electron paramagnetic resonance (EPR or equivalently, electron spin resonance, ESR) that can significantly improve the ability to measure the partial pressure of O₂ (pO₂) under biologically pertinent conditions *in vitro, in vivo*, and potentially in human subjects. This article focuses on a prototype of a class of crystalline paramagnetic probes, lithium phthalocyanine (LiPc), and aims at providing sufficient detail to facilitate the use of these probes in viable biological systems. The critical capabilities of this technology are the ability to measure pO₂ at the levels (usually <40 mmHg and can be as low as 0.1 mmHg; 1 mmHg = 133 Pa) and sites (e.g., in tissues *in vivo* and inside cells) needed to understand biological processes.

pO₂ is one of the most important variables in many physiological, pathological, and therapeutic processes. As the terminal acceptor in the electron transport chain, O₂ plays a critical role in cellular metabolism. Reactions of O₂ with biological substrates such as autoxidizable metabolites and xenobiotics, unsaturated lipids, etc. lead to products (e.g., O₂⁻, H₂O₂, OH·, organic peroxides, aldehydes) that are involved in many pathological processes, such as lipid peroxidation, ischemia–reperfusion injury, generation of cytotoxic products by leukocytes during inflammatory response, carcinogenesis, freezing damage, and aging (1–5). pO₂ is an especially important variable affecting the response of cells to ionizing radiation and cytotoxic chemotherapeutic agents.

The existing methods for measuring pO₂ in cells and tissues—e.g., the Clark electrode (6), fluorescence quenching (7), O₂ binding to myoglobin and hemoglobin (8), chemilu-

minescence (9), phosphorescence quenching (10), and spin label oximetry (11), are useful but have certain limitations, especially when used *in vivo*. Measurement of pO₂ by the Clark electrode is the most frequently used method, but it is technically difficult to use in tissue because (*i*) it may damage the tissue during insertion; (*ii*) accurate measurements are very difficult below 10 mmHg of O₂; and (*iii*) it may change pO₂ in the region of the electrode because it consumes O₂. Myoglobin is present only in muscle tissue and is a useful probe for pO₂ only in a limited concentration range. Fluorescence quenching also is limited due to technical difficulties. Although the phosphorescence technique can detect pO₂ as low as 1.5 × 10⁻² mmHg, it is restricted to measurement of O₂ at the surface, with a maximum sampling depth of 1 mm under normal conditions (12). Spin label oximetry with EPR, especially when combined with the capabilities to use EPR *in vivo* also is very promising, but it is not sensitive at pO₂ <10 mmHg, and the nitroxides can undergo bioreduction to EPR-inactive species (13).

The paramagnetic metallophthalocyanines are organic compounds with semiconductor properties and can have EPR signals that are extraordinarily sensitive to oxygen (14, 15). Metallophthalocyanines can have very favorable biological properties, including stability, very low toxicity, and rapid response to pO₂. We have described some of the properties of LiPc in a preliminary report (16).

MATERIALS AND METHODS

Materials. LiPc was synthesized by a reported method (17). 2,2,6,6-Tetramethylpiperidine-*d₁₆*-1-¹⁵N-oxyl-4-one (¹⁵N-PDT) was purchased from MSD Isotopes. Other chemicals were purchased from Sigma. Cell culture medium and serum were purchased from GIBCO.

EPR Measurements. The spectra in tissues were obtained by using a modified Varian E-15 EPR spectrometer, equipped with a home-made low-frequency (1.2 GHz, L-band) microwave bridge (18). Some calibrations and studies of physiochemical properties of LiPc were done by using a Varian E-12 EPR spectrometer (9.2 GHz, X-band). Typical settings for the spectrometers were as follows: magnetic field, 3210 G (X-band)/392 G (L-band); microwave power, 15-decibel attenuation; modulation frequency, 10 kHz or 100 kHz, depending on the line width of the spectrum. Modulation amplitude was set at less than one-third of the EPR line width. Temperature control at X-band was achieved by a Varian gas flow system using a N₂/O₂ mixture. At L-band, the temperature of anesthetized rats and mice (37 ± 2°C, measured by

a rectal probe) was regulated by an infrared lamp. The EPR spectra on both spectrometers were collected by using the software EW (Scientific Software, Normal, IL) installed on an IBM AT computer. For measurements of spin density, the EPR spectrum was recorded at X-band and double integrated. α-α-Diphenyl-β-picrylhydrazyl was used as a secondary standard (19).

Calibration of Line Width with pO_2. A single crystal of LiPc in various media was drawn into an 8-cm-long gas permeable Teflon tube (Zeus Industrial Products, Raritan, NJ, 0.623-mm i.d.; wall thickness, 0.038 $\pm$ 0.004 mm). This Teflon tube was folded twice and inserted into a quartz EPR tube open at both ends. Samples were maintained in the cavity (Varian TE_{102}) at 37 $\pm$ 0.2°C. pO_2 in the perfusing gas was monitored and measured by a Clark electrode (Microelectrodes, Londonderry, NH), calibrated by vigorously stirring 100 ml of distilled water equilibrated with O_2 and/or N_2. The quantitative dependence on pO_2 of the EPR spectrum was obtained by measuring the peak-to-peak line width (LW) as a function of pO_2 in the perfusing gas, with LW defined as the difference in magnetic field between the maximum and minimum of the first-derivative recording of the signal. The resulting calibration curve was fitted to a first-order regression equation, which then was used to convert values of LW measured in biological systems into appropriate values of pO_2.

Relationship Between %O_2, pO_2, and [O_2]. At least three different units are used in the literature for the expression of oxygenation, %O_2, pO_2, and [O_2]. Each may be appropriate for specific conditions, and often they are interconvertible. For example, one atmosphere (760 mmHg) of air (20.95% O_2/78.08% N_2) is equivalent to a pO_2 = 159/pN_2 = 593 mmHg gas mixture. At 37°C, air in equilibrium with pure distilled water or olive oil will result in solutions containing 224 or 1074 μM [O_2], respectively. Therefore in water at 37°C, 1%, 7.6 mmHg, and 11.2 μM of O_2 are equivalent. The pO_2 will be used in this paper.

Preparation of Sonicated LiPc. LiPc was sonicated in distilled water for variable times (0.1–3 hr). The final size of the LiPc crystals depends on the length of sonication. For example, 1-μm-diameter crystals were obtained after 2 hr of sonication at room temperature in an ultrasonic bath (model 8854, Cole-Parmer).

Implantation of LiPc in Tissues. The LiPc was introduced into tissue through a 26-gauge needle with a fine glass rod as plunger.

Measurement of pO_2 in Rat Heart. Adult rats were anesthetized (sodium pentobarbital at 40 mg/kg of body weight, i.p.), intubated, and subjected to a left thoracotomy. Crystals of LiPc ($\approx$100 μm) were implanted into the left ventricular wall. The animal was placed in the gap of the magnet with the L-band detector (coupled loop) positioned over the heart. In the experiment with isolated hearts, LiPc crystals were placed into the wall of the left ventricle immediately after removal, and the still-beating heart was placed directly on the surface of a loop gap detector (18).

Measurement of pO_2 in Skeletal Muscles of Mice. A single crystal of LiPc was inserted in the gastrocnemius muscle of an anesthetized (ketamine HCl, 0.125 mg/g of body weight, i.p.) adult mouse. The leg of the animal was then centered on the detector. When required, blood flow was restricted by a ligature around the upper leg.

Cell Preparation. Chinese hamster ovary (CHO) cell cultures were maintained as monolayers and subcultured three times a week. Monolayers of the cells were grown to confluence in McCoy's 5A medium/10% of fetal bovine serum/1% of penicillin/streptomycin in a 37°C incubator with 5% CO_2. Twenty-four hours before the experiment, the cells were transferred to a flask at a density of 10^5 cell per ml. Cells were washed with serum-free medium before use.

RESULTS

Calibration Curve of LiPc. LW and spin density of the LiPc crystal were measured at 37°C as a function of pO_2 in various environments. Fig. 1 demonstrates that the increase in LW was a linear function of pO_2 throughout the experimental region; the slope of the line was 6.1 mG/mmHg of O_2, and the intercept (LW in the absence of O_2) was 14 mG. The spin density, 9.2 $\times$ 10^{19} spin per g, was not affected by pO_2. The results also show that the broadening effect of oxygen is independent of the surrounding medium—i.e., independent of the solubility of O_2.

Investigation of Physicochemical Properties. LiPc was insoluble in all the solvents that we tested. The shape and integrated intensity of the EPR spectrum of LiPc were not affected when LiPc was treated with various solutions and conditions: in H_2O for 35 days, in H_2O at a temperature of 100°C, in H_2O at low pressure (<100 mmHg), in 100 mM $K_3Fe(CN)_6$ (a paramagnetic broadening agent), in 30% (wt/wt) H_2O_2 (an oxidizing agent) for 4 days, in 30 mM ascorbic acid (a reducing agent), in buffer solutions with pH of 2 and 12, and sonication for 2 hr. Although the same was true when LiPc was in triolein (a model for lipid environments) for 8 hr, a gradual decrease in LW was seen after 3 days.

The response time of LiPc to changes of pO_2 was measured in the gaseous state (Fig. 2) and in the presence of a solvent. In both cases, the change of the EPR signal was very fast, suggesting that the rate probably is limited by the diffusion rate of O_2 in the medium. This result was confirmed by observing that the response times of LiPc and a nitroxide, 2,2,6,6-tetramethylpiperidine-d_{16}-1-^{15}N-oxyl-4-one (^{15}N-PDT; 0.1 mM), were identical when a hexane solution containing both ^{15}N-PDT and LiPc in the same sample tube was perfused by a gas that was changed rapidly from air to N_2. The responses of nitroxides to changes in pO_2 are instantaneous because they arise from collision with O_2 (11).

The effect of O_2 on broadening the EPR line of LiPc is proportional to pO_2, independent of the total pressure of other gases (Table 1).

Between 30° and 50°C, LW was independent of temperature in the absence of O_2. When LiPc was perfused with 159 mmHg of O_2 (air), a linear decrease in LW was seen by increasing the temperature. The temperature effect coeffi-
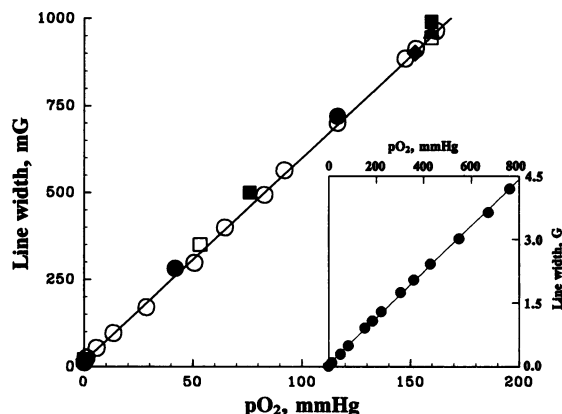

FIG. 1. Calibration of LW of the EPR spectra of LiPc to pO_2 under various conditions: removed from mouse leg muscle after 30 days implantation and washed with 5% sodium hypochlorite (□), in ethyl alcohol (■), sonicated in phosphate-buffered saline (●), in gas phase (○), in brain tissue (▲), in oleic acid (◆), and in CHO cells with respiration inhibited with NaCN (◇). (Inset) Response of LW in gas phase up to 760 mmHg (one atmosphere of 100% O_2). Measurement of all types of samples included data points at pO_2 of 159 mmHg (air) and 0 mmHg (N_2). Some data points are overlapping and are not readily visible. Data with brain tissue were obtained with an L-band spectrometer, and the rest were obtained at X-band.
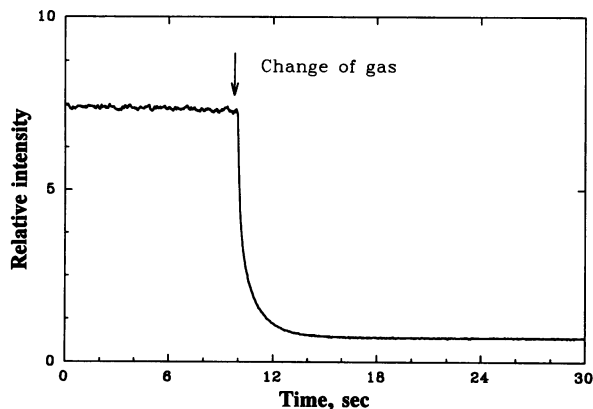
FIG. 2. Response time of LW of LiPc to pO$_2$ changes. The magnetic field was set at top of the EPR spectrum, and the peak height was monitored at X-band as a function of time before and after the perfusing gas passing around a LiPc crystal was changed from N$_2$ to air.

cient was related to pO$_2$ and was calculated to be 0.06 mG·°C^{-1}·mmHg^{-1}, a factor too small to affect the measurement of pO$_2$ under most experimental conditions likely to be used with biological samples.

Interaction with Viable Biological Systems. The stability of the response of LiPc to pO$_2$ in gastrocnemius muscles of mice *in vivo* was studied by measuring the EPR spectra before and after the blood flow was restricted. The initial LWs were 170 mG and 70 mG, respectively. The same procedure was repeated 24 hr later with similar results (Fig. 3). By 14 days, LW without restriction of the blood flow was 90 mG and again decreased to 70 mG with restriction of the blood flow. To determine the cause of these changes in responsiveness to changes to pO$_2$, the LiPc was removed from the muscle 30 days after implantation and reexamined before and after treating the crystals with 5% sodium hypochlorite and/or H$_2$O$_2$ to remove tissue residues from the surface. The response of LW to pO$_2$ was completely restored by this treatment (Fig. 1).

Colony formation at 9 days by CHO cells was used to evaluate the potential toxicity of LiPc *in vitro*. Survival rates of plated CHO cells after 24-hr exposure to various concentrations (1, 10, 20, and 40 µg/ml) of LiPc (diameter <1 µm) were not significantly different from the control. When the unincorporated LiPc was not removed from the medium for 9 days, the survival rate was significantly reduced at the higher LiPc concentrations. No toxicity was observed when larger crystals (>20 µm) were used under the same conditions.

Illustrative Biological Applications. Fig. 4 illustrates the capability to measure very low pO$_2$ (<1 mmHg) and to distinguish between small differences in pO$_2$. A crystal of LiPc was inserted into the wall of a freshly removed rat heart, the preparation was placed on the surface detector, and EPR spectra were recorded repetitively with a scan time of 30 sec. The heart continued to beat, leading to rapid hypoxia. This result illustrates the potential of using peak height, instead of

Table 1. Effect of N$_2$ on LW of the X-band EPR spectra of LiPc

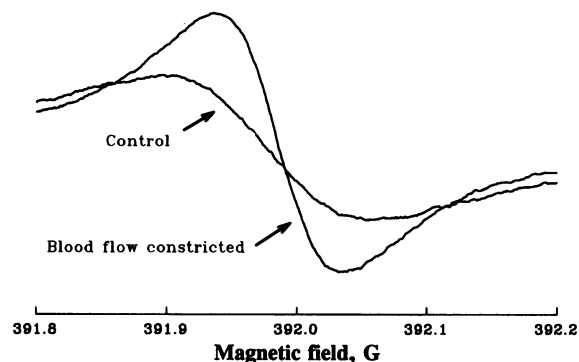| pN$_2$, mmHg	pO$_2$, mmHg	LW, mG
0.0	0.0	14.2 ± 1
10.0	0.0	14.4 ± 2
120.0	0.0	14.0 ± 2
760.0	0.0	14.1 ± 2
0.0	10.0	116 ± 2
10.0	10.0	115 ± 2
760.0	10.0	117 ± 2

FIG. 3. L-band EPR spectra in the mouse gastrocnemius muscle *in vivo* 24 hr after implantation. The response to changes of pO$_2$ is indicated by narrowing of LW and increase of line height when blood flow to the leg was restricted by a ligature around the mouse upper leg. The corresponding pO$_2$ before and after constriction of blood flow were 24.3 and 7.7 mmHg, respectively.

LW, to calculate pO$_2$. This approach yields much better sensitivity to pO$_2$, as peak height is much more sensitive to changes of pO$_2$ than LW.

The ability to measure pO$_2$ *in vivo* in the rat heart is shown in Fig. 5, which shows that myocardial pO$_2$ increased with an increase of pO$_2$ in the respiratory gas.

Another area of potential widespread application of the technique is the measurement of pO$_2$ in the brain. In this example, crystals of LiPc were placed in the brain of an anesthetized rat. Seven days later we measured the steady-state pO$_2$ in the unanesthetized animal and the effect of two different anesthetics on pO$_2$ (Fig. 6).

The ability to measure pO$_2$ in an abdominal organ is illustrated in Fig. 7 with LiPc placed in the cortex of the rat kidney. This maneuver was experimentally simpler than the studies in the heart because only respiratory motion was involved. The reoxygenation of the kidney, presumably due to retrograde flow into the renal artery, was studied immediately and then reexamined several days later by leaving the crystal in place. pO$_2$ increased gradually with time; by day 4, pO$_2$ was close to the level before ligation of the aorta.

DISCUSSION

Intensity of EPR Signal of LiPc. These results indicate that LiPc provides sufficient signal/noise ratio to make biologically relevant measurements of pO$_2$ *in vivo*. The enhanced signal/noise ratio, relative to nitroxides, is due to several factors. (*i*) The LW of the EPR spectrum of LiPc in the absence of oxygen is very narrow (14 mG) compared with perdeuterated nitroxides (150–500 mG) and conventional nitroxides (1000–2000 mG). (*ii*) The EPR spectrum of LiPc is
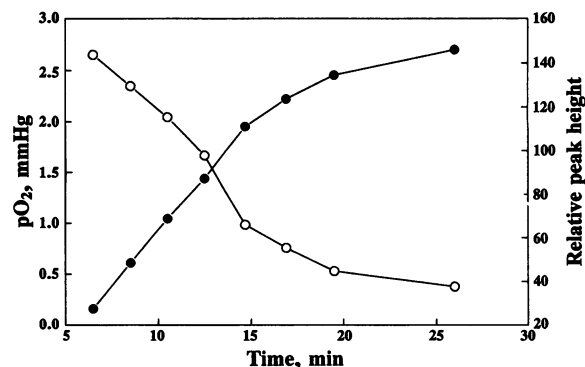

FIG. 4. Measurement of oxygen depletion in a freshly isolated rat heart. ○, pO$_2$; ●, peak height.
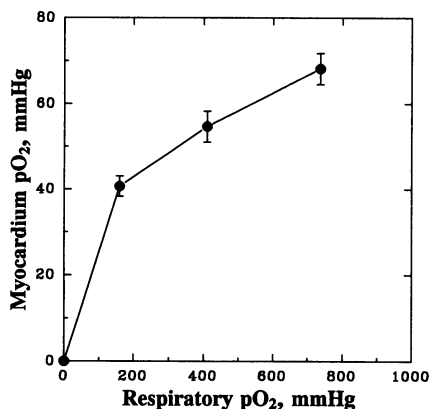
FIG. 5. Measurement of pO_2 in rat myocardium *in vivo*. A small LiPc crystal was placed in the left ventricular myocardium of a ventilated rat, and EPR spectra were recorded repeatedly with a coupled loop positioned over the heart while pO_2 in the respiratory gas was varied.

a single line instead of the typical three lines of nitroxides. These two factors increase the signal/noise ratio 300-fold relative to perdeuterated nitroxides, assuming an equal number of free spins. In addition, LiPc has a very high spin density, 9.2×10^{19} spin per g, which makes it possible in many applications to work with a single crystal.

Sensitivity and Specificity to pO_2. For LiPc, LW in N_2 and air is 14 and 1000 mG, respectively, giving a unit sensitivity in aqueous solution at 37°C of 6.1 mG/mmHg. It is feasible to detect changes of 1–3 mG for lines as narrow as 14 mG, indicating that measurement of pO_2 with an accuracy of <1 mmHg can be achieved. Under experimental conditions in which one can use changes in peak height, rather than changes in LW, an accuracy of 0.1 mmHg can be achieved. It is worth noting that LiPc is more than two orders of magnitude more sensitive to pO_2 than nitroxides, and nitroxides have been used successfully to measure intra- and extracellular pO_2 at levels of 10 mmHg (20). The relationship of LW of LiPc to pO_2 is linear throughout the entire range of pO_2 and, therefore, also can be used at very high levels of pO_2, while maintaining its desired sensitivity.

The broadening effect of O_2 results from simple Heisenberg spin–spin interaction, without complications due to adsorption or bonding because the effect of O_2 fully, rapidly, and reproducibly changed with alteration of pO_2. The lack of an effect of N_2 is consistent with this mechanism. The lack of an effect on the response of LiPc of being in different media, including media with markedly different oxygen solubilities,
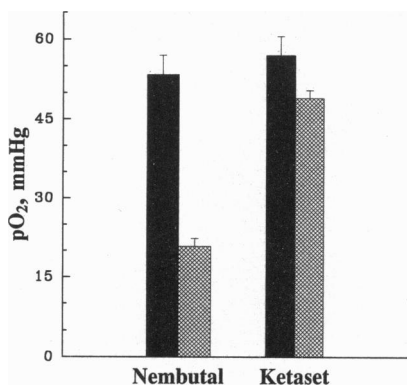

FIG. 6. Measurement of pO_2 in the rat brain. Measurements were made before (black bar) and after (hatched bar) the i.p. administration of Nembutal and Ketaset. LiPc was inserted into the brain 7 days earlier. Values are means ± SDs for >10 measurements.
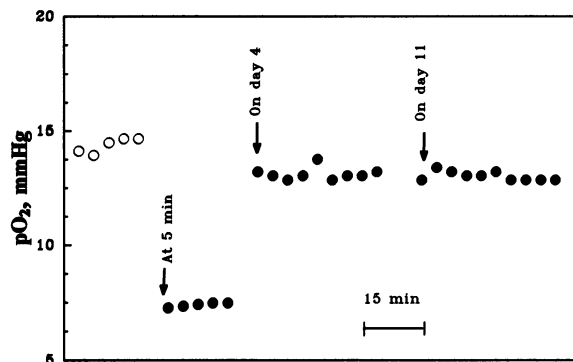

FIG. 7. Measurement of pO_2 *in vivo* in the kidney cortex of a rat before (o) and after (•) aortic ligation.

combined with a lack of an effect of the presence of N_2 indicates that the response of LiPc reflects the pO_2. This may be a significant experimental advantage in circumstances where there may be ambiguity as to the solubility of oxygen in the environment under study.

Biological Inertness. The physicochemical stability of LiPc, which includes resistance to oxidation, reduction, extremes of pH, and the presence of other paramagnetic materials suggest that it should be inert in tissues. The direct measurement of biological effects by colony-forming ability of cells and histological studies of tissues containing implanted LiPc (data not shown) confirm this expectation. This biological inertness clearly is a significant advantage for the use of LiPc in experimental systems and would be consistent with its clinical use.

The results also indicate, however, that the response to pO_2 in tissues is not always stable. When LiPc was implanted in skeletal muscle of mice, a gradual diminution of the response to pO_2 was observed after 3 days. This result appears due to deposition of material in and around the LiPc crystal that impedes the access of O_2 to the paramagnetic centers. This explanation is consistent with the fact that the access of O_2 is via a small opening in the crystal (15). There are two pieces of evidence supporting this explanation: (*i*) the EPR signal intensity of implanted LiPc remained unchanged for 30 days, and (*ii*) the responsiveness to O_2 was restored when the implanted LiPc was removed from the animal and washed with 5% sodium hypochlorite or H_2O_2. The time span for the usefulness of LiPc *in vivo* may depend on the type of tissue. In the experiments with rat brain and kidney, the response of LiPc to pO_2 was maintained for the duration (7 days) of the experiments.

Implications of Illustrated Biological Applications. Our results indicate that there is a wide variety of experimental situations in which LiPc could find immediate applications. Because LiPc has a very fast response time to changes in pO_2, it may be used to detect and follow O_2 consumption in isolated systems. Because of its extreme sensitivity to low pO_2, it can report levels of O_2 that cannot be measured with most other methods; examples in this category include pO_2 in acute ischemia in organs and hypoxia in tumors, where pO_2 is a critical parameter in understanding the phenomena. The measurement of pO_2 by LiPc in combination with NMR spectroscopy may be especially useful, making it feasible to relate NMR spectra from phosphorus, carbon, or hydrogen nuclei of metabolites to the actual pO_2 in the tissue under study. Because this technique measures pO_2 at the crystal, it is possible to use multiple crystals to measure pO_2 in several places of interest simultaneously.

Similarly this technique should be readily applicable to *in vitro* systems. Here one can take advantage of the flexibility in the size of crystals, such as using larger crystals, which

cannot be taken up by cells, to measure extracellular pO_2 or using very small crystals, which can enter the cell by phagocytosis, to measure intracellular pO_2. LiPc also can be combined with other paramagnetic probes, such as nitroxides, to measure extra- and intracellular pO_2 simultaneously.

Because of the flexibility in size, one can measure pO_2 in a very localized area by using a single crystal *in vivo* or measure the average pO_2 in small animals, such as mice, by using small-sized particles injected into the blood stream of the animal.

Based on the various media we have studied, we anticipate that in most systems in which LiPc is likely to be used, the calibration will be medium-independent.

Cautions and Potential Limitations. Although LiPc seems an excellent probe to measure and follow pO_2 under many experimental conditions, it may lose its response to pO_2 over time, and, therefore, it is essential that the stability of the response to pO_2 be investigated in experiments in which the LiPc will be used for >24 hr after administration. This precaution is especially important in lipid-rich tissues, in view of the effect on the calibration of LiPc with pO_2 in triolein, in which the response of LiPc to pO_2 decreased after 3 days. The reason for the effect was not clear, but it might be the same as suggested for the effect in skeletal muscle: triolein molecules or their oxidative products may block the channel by which O_2 enters the crystal.

Because of the unusually narrow LW of LiPc (14 mG in the absence of O_2) and its susceptibility to microwave saturation, some special instrumental considerations are required to avoid distortion of the spectrum, which may include aspects that are not familiar to many investigators (21). The modulation frequency needs to be decreased from the usual 100 kHz to 25 kHz or less to reduce broadening from side bands, the homogeneity of the magnets may be limiting for the very narrow lines that occur at very low pO_2, and physiological movements of the animal may move it to regions with different magnetic fields.

Conclusion. LiPc in combination with a low-frequency EPR spectrometer equipped with an appropriate detector has the properties necessary to measure pO_2 in tissues and cell systems with a sensitivity and ease that has not been available previously. The methodology should be especially useful to measure rapid changes in pO_2 and low levels of pO_2 in short-term experiments.

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