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Forum Review

In Vitro and *In Vivo* Measurement of pH and Thiols by EPR-Based Techniques

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ABSTRACT

***In vitro* and *in vivo* measurements of pH and thiols provide critical information on physiology and pathophysiology of living organisms, particularly related to oxidative stress. Stable nitroxides of imidazoline and imidazolidine types provide the unique possibility of measuring local values of pH and glutathione content in various biological systems, including *in vivo* studies. The basis for these applications is the observation of specific chemical reactions of these nitroxides with protons or thiols, followed by significant changes in the electron paramagnetic resonance (EPR) spectra of these probes, measured by low-frequency EPR techniques. The applications of some newly developed pH and SH probes in model systems of pharmacological interest, biological fluids, tissues, and cells as well as *in vivo* studies in isolated hearts and in the gut of living animals are discussed.** *Antioxid. Redox Signal.* 6, 667–676.

INTRODUCTION

LOCAL pH AND THIOL CONTENT, particularly glutathione (GSH) concentration, are among the most important parameters in the biochemistry of living organisms. Many of the vital activities of cells depend on the pH inside cellular organelles, transmembrane pH gradients, and the pH at the surface of membranes or proteins (60). Likewise, the roles of vital SH groups and intracellular GSH, particularly in the maintenance of intracellular "redox state," are also widely appreciated (65). In certain stress conditions, *e.g.,* inflammation, high exercise levels, interruption of normal blood supply, or biochemical shock, the body's ability to regulate its pH and GSH content, at least locally, may be compromised. Tissue acidosis and redox state are dominant factors in inflammation (4, 27, 72, 73) and in tumors (24, 26, 45, 68) and after ischemia (28, 70, 79). In addition, the delivery, absorption, and pharmacological effectiveness of drugs can be altered by changing the pH and redox state of their local environment (1, 57, 69, 76). It can be seen therefore that pH and GSH measurement *in vivo* may be of considerable clinical relevance.

Recently Kuppusamy *et al.* (45) used indirect evaluation of GSH content by *in vivo* electron paramagnetic resonance (EPR) spectroscopy and imaging to demonstrate a central role of GSH in the reduction of the nitroxide in a murine tumor model. However, up to now there is no direct, noninvasive method for GSH detection in living tissues.

As for *in vivo* pH measurements, ^{31}P-nuclear magnetic resonance (NMR) has proven to be the most suitable noninvasive approach. Nevertheless, pH assessment using ^{31}P-NMR and inorganic phosphate has its own limitations, including lack of resolution (about 0.2–0.3 units and even less at lower pH), the fact that inorganic phosphate concentrations vary with metabolism and ischemia, and its chemical shift depends on ionic strength (19, 46, 62, 67). Because of these problems, exogenous pH probes are being designed for ^{31}P-NMR spectroscopy (11, 62) to improve detection of acidosis in isolated perfused organs. In addition, several NMR approaches for pH detection utilizing ^{1}H-NMR (71), ^{19}F-NMR

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(61), and contrast-enhanced magnetic resonance imaging (63) have been reported. Recently developed EPR-based spectroscopic and imaging approaches, in combination with function-directed pH- and SH-sensitive spin probes (7, 15, 26, 31, 32, 38), open another unique possibility for following early stages in the changes of body chemistry *in vivo* that are associated with certain stressful or pathological conditions. The pH-sensitive nitroxides, since they are exogenous probes, have certain advantages over exogenous NMR probes, due to the much higher sensitivity of EPR.

In vivo *EPR-based spectroscopy and imaging*

Despite formidable technical problems, significant progress has been made in the development of *in vivo* EPR techniques during the last decade. Low-frequency L-band EPR spectrometers, designed to decrease non-resonant losses and increase depth of microwave penetration, have become commercially available. Moreover, even lower-frequency (down to 300 MHz) home-made radiofrequency-EPR spectrometers, as well as instrumentation for spatial and spectral-spatial EPR imaging of radicals, have been constructed in several laboratories and have effectively been used for free radical spectroscopy and imaging (5, 16, 18, 23, 30, 38, 42, 47, 66). Along with the progress in low-frequency radiofrequency-EPR, other techniques such as longitudinally detected electron spin resonance (LODESR) (58), proton electron double-resonance imaging (PEDRI), and dynamic nuclear polarization (DNP) (49, 51) have been developed for *in vivo* applications. The EPR and PEDRI techniques have been used to image free radicals in living tissues, including heart (39, 43, 44, 80) and stomach (15, 17, 38) in rats. However, their potential is still far from maximally defined, in part, because of the need for development of new specific function-directed spin probes.

Molecular pH-sensitive spin probes

The possibility of accurate determination of pH values by EPR is based on early findings that EPR spectra parameters of stable nitroxides of the imidazoline and imidazolidine types, namely, the hyperfine (hf) splitting constant, a_N, and the *g*-factor, depend on the pH of the surrounding solution (29, 33). It is possible to use the a_N constant as a sensitive indicator of pH in the range of about 1.5 pH units above and below the p*K* value of the nitroxides. Here we discuss both the current biological applications of spin pH probes and the development of new probes that are particularly designed for *in vivo* studies, namely, with higher spectral sensitivity, stability towards reduction, and usefulness for pH monitoring in physiological range of pH, and for potential intracellular targeting of the probe.

Molecular SH-sensitive spin probes

The EPR method for SH group detection is based on the use of thiol-sensitive paramagnetic disulfides (35, 37), which participate in reactions of thiol-disulfide exchange, similar to Ellman's reagent, commonly used for thiol determination by an optical method (8). Here we discuss the applications of two different paramagnetic SH probes, particularly with respect to the possibility for *in vivo* detection of thiols.

SPIN pH PROBES

Manifestation of pH effect in the EPR spectra of imidazoline and imidazolidine nitroxides

The ionizable molecular pH probes have spectroscopically distinguishable protonated (RH^+) and unprotonated (R) forms with their relative fraction depending on pH, namely, $[RH^+]/[R] = [H^+]/K_a$, where K_a is the equilibrium constant of the reaction of probe protonation or dissociation. Figure 1 illustrates the EPR spectra of a spin probe of the imidazoline type, R1 (Scheme 1), and its deuterated analog, R1*, in aqueous solutions, pH ≈ p*K* (p$K = -\log K_a$), at various microwave frequencies. The presence of two forms of the probes with different hf splittings ($\Delta a_N \approx 1$ G) and *g*-factors ($\Delta g \approx 0.002$) is more evident for the substituted R1* compound because of narrow lines (Fig. 1a*–c*). Note that the contribution of the *g*-factor is significant only at higher frequency (X-band, 9.9 GHz), resulting in the asymmetry of the pH effect for low- and high-field EPR spectral components (Fig. 1a and a*), while the L-band EPR spectrum (1.2 GHz) demonstrates a very symmetric spectral pattern (Fig. 1b and b*). The asym-

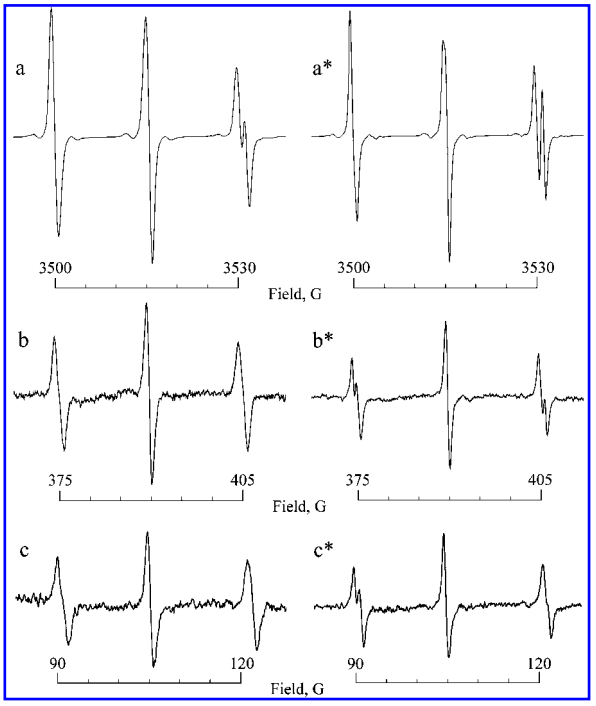
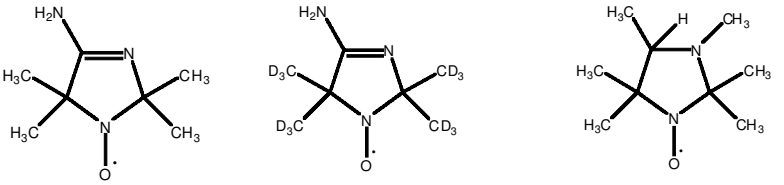


FIG. 1. The EPR spectra of an aqueous solutions of 0.5 m*M* nitroxides R1 and R1* at pH 6.1 measured at 9.9 GHz (a and a*), 1.2 GHz (b and b*), and 300 MHz (c and c*). The experimental volumes were 50 μl, 0.5 ml, and 20 ml, respectively. The spectra were detected with an X-band Bruker EMX spectrometer (Bruker Instruments, Karlsruhe, Germany) and home-built L-band and 300 MHz spectrometers (EPR Center at The Ohio State University, Columbus, OH, U.S.A.) using the same modulation amplitude (0.2 G) and detection time (2 min) for the sensitivity comparison. EPR spectra of the substituted radical (*) are depicted at two times lower scale and show about a twofold increase in signal-to-noise ratio due to decrease in the linewidths.



SCHEME 1. Chemical structures of the imidazoline radical, R1; its deutero-substituted analog, R1*; and the imidazolidine radical, R2.

metry of the spectra at lower frequency (300 MHz, Fig. 1c and c*) has a completely different origin and is due to the Breit–Rabi effect (9). The value of the p*K* of the radical is the main characteristic of the "molecular pH meter" that determines the range of pH sensitivity to be about 3 pH units, centered on the p*K*. The pH determination may be done from calculation of $[RH^+]/[R]$ ratio by spectra computer simulation or by using titration curve for certain spectral parameters, *e.g.,* hf splitting or spectral peak intensities ratio (32). The pH dependence of hf splitting, a_N, measured as a distance between low- and central field components, was proven to provide a simple and sensitive approach for pH measurement by X-band EPR spectroscopy (32). However, using hf splitting for pH determination by L-band and 300 MHz spectroscopy is less preferable because of the narrow range of its pH sensitivity (Fig. 2c, squares). Therefore application of peak intensity ratio or computer analysis of spectral shape based on superposition of two triplet spectra of protonated and unprotonated forms of the radical is recommended for pH determination by these EPR techniques. Substituted probes have an advantage in spectral resolution and signal-to-noise ratio while being less available and more costly. Interestingly, both signal-to-noise ratio and the range of pH sensitivity of hf splitting can be significantly increased by spectra detection at high modulation amplitude (Fig. 2). Indeed, improvement in signal-to-noise ratio due to application of the substituted radical R1* compared with R1 is significant only at lower modulation amplitude (about two times at 0.2 G modulation, Fig. 1), while it becomes less significant at higher modulation (about 1.5 times at 0.5 G modulation) and does not improve signal-to-noise at a modulation amplitude larger than 1 G. On the other hand, the measurement of EPR spectra at a high modulation amplitude of 2 G results in about two and four times signal-to-noise improvement compared with L-band EPR spectra detected at 0.5 G and 0.2 G modulation, respectively (Fig. 2a and b). This enhancement in the signal-to-noise is by the cost of broadening of the spectral lines and corresponding loss of spectral resolution. Fortunately, it also results in smoothing the pH titration curve for hf splitting, a_N (see Fig. 2b), making this parameter a highly sensitive pH marker. The methodical approach described could be important for applications *in vivo* where fundamental sensitivity is much lower.

Up to the present time we have developed a wide set of pH-sensitive nitroxides, with differing ranges of pH sensitivity, labeling groups, and lipophilicity (31, 32).

In vitro *applications of spin pH probes*

Biological EPR applications at X-band are mostly limited by *in vitro* or *ex vivo* studies because of high non-resonant absorption of the excitation frequency by aqueous samples. Therefore spin pH probes have been used for the studies of the samples whose volume does not exceed 200 µl. Monitoring transfer of protons across lipid bilayers of liposomes caused by a transmembrane pH gradient had been one of the first applications of the pH measurement by EPR (2, 34). Interestingly, the observation of the different rates of proton transport across phospholipid membranes upon the establishment of the gradient of different acids (34) allowed us to measure the membrane permeability coefficient for the undissociated forms of the acids, p_{HX}, including that for

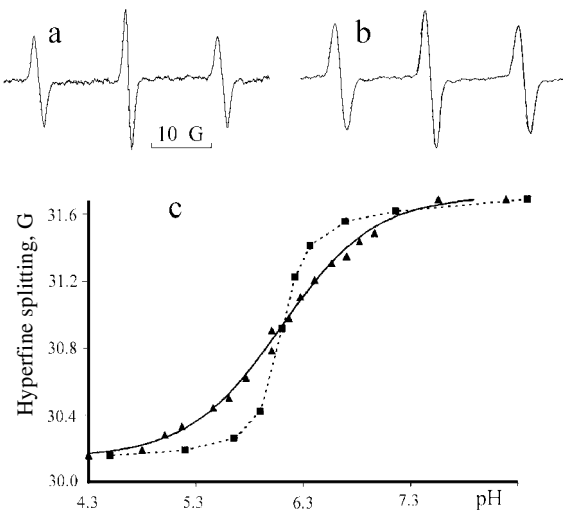


FIG. 2. The L-band EPR spectra (a and b) and pH dependence of hf splitting (c) for the radical R1 measured at different modulation amplitudes. The EPR spectra of the 0.5 m*M* aqueous solutions of the radical R1 were detected at pH 6.1, modulation amplitude 0.5 G (a) or 2 G (b), microwave power 10 mW, sweep widths 50 G, center field 389 G, acquisition time 56 s, and time constant 0.1 s. Spectrum b is depicted at two times lower scale and shows 2.1 and 4.2 times increase in signal-to-noise ratio compared with Spectrum a and the spectrum shown in Fig. 1b, respectively. c: The pH dependence of hf splitting for the radical R1 was measured as the distance between low- and high-field components of L-band EPR spectra obtained at modulation amplitude 0.5 G (■) and 2 G (▲).

HNO_3 ($pK = -1.62$; $p_{HX} = 1.4 \times 10^{-4}$ cm/s). Because of the importance of NO_x species in cellular biology, and discussion of nonenzymatic nitric oxide formation from nitrite in acidic environments or upon ischemic or metabolic acidosis (64, 79), there appears to be significant interest in the studies of nitrite transfer through the biomembranes *via* transmembrane diffusion of the undissociated acid HNO_2 ($pK_{HNO_2} = 3.3$). This transport facilitates proton transfer across the biomembrane and therefore can be studied using membrane-impermeable pH-sensitive nitroxides (2, 3, 34).

The other *in vitro* applications of spin pH probes include studies of water-in-oil ointments (41), proteins and proteinaceous matrix (10, 36), and biodegradable polymers and phospholipid membranes (10, 36, 53, 54). Recently Kroll *et al.* applied spin pH probes for non-invasive direct and depth-specific measurement of pH values within rat and human skin (the latter obtained from cosmetic surgery) (42) to study influence of drug treatment on the microacidity.

In vivo *applications of spin pH probes*

Spin pH probes offer a unique opportunity for non-invasive pH measurement in living animals using low-field EPR-based techniques [for reviews, see Khramtsov *et al.* (38) and Mäder (52)]. L-band EPR spectroscopy (1.2 GHz) was shown to be a valuable tool for *in vivo* monitoring of biodegradable drug delivery systems (55) and pH measurement in mice (17). Recently we applied spin pH probes for the studies in living rats (15, 38) using LODESR (58) and field-cycled DNP (48) with its imaging analogue, field-cycled PEDRI (FC-PEDRI) (49, 50), operating at an EPR frequency as low as 120 MHz.

Figure 3 displays an FC-PEDRI image from a living rat with gastric infusion of the probe R2 (Scheme 1) in deionized water (15). The image obtained with FC-PEDRI is used to determine the probe localization, supporting its presence in the stomach, as shown in Fig. 3d. The EPR-based spectroscopy component of FC-PEDRI and the LODESR spectra provide information on the environmental acidity (Fig. 4). The changes in the distance between low- and high-field components of the spectra, after addition of 0.1 *M* sodium bicarbonate, clearly demonstrate the action of this particular antacid. Oral administration of some of the pH-sensitive radicals has been found to improve the ability of the probe to stay in the stomach for ~1 h, in the radical form, while more hydrophobic radicals tend to be absorbed through the membranes of the stomach walls, therefore losing the EPR signal (15, 38).

Figure 5 demonstrates a typical EPR spectrum of the pH probe, R1, in the isolated working heart (39), located directly in the resonator of the L-band EPR spectrometer. The EPR spectrum is significantly affected by ischemic acidosis and corresponds to pH 6.5 after 5 min of ischemia. This result is in agreement with the data observed by a ^{31}P-NMR technique that demonstrates similar acidosis at this time point (79). Note that the reduction of the radical into diamagnetic products (mostly to corresponding hydroxylamine) limits its de-

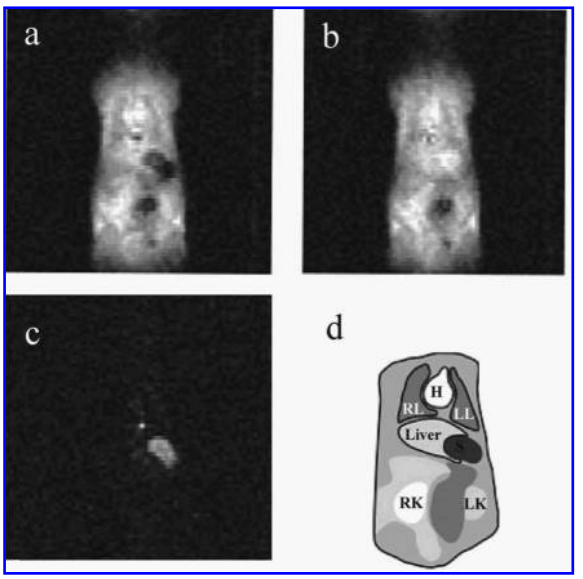


FIG. 3. FC-PEDRI image of a rat intubated 30 min previously with 3.5 ml of 7 m*M* R2 solution: obtained (a) with ESR irradiation off and (b) with ESR irradiation on. c: The difference between (a) and (b). **d:** The diagram demonstrates the anatomy. RL and LL, right and left lung, respectively; H, heart; S, stomach; RK and LK, right and left kidney, respectively.

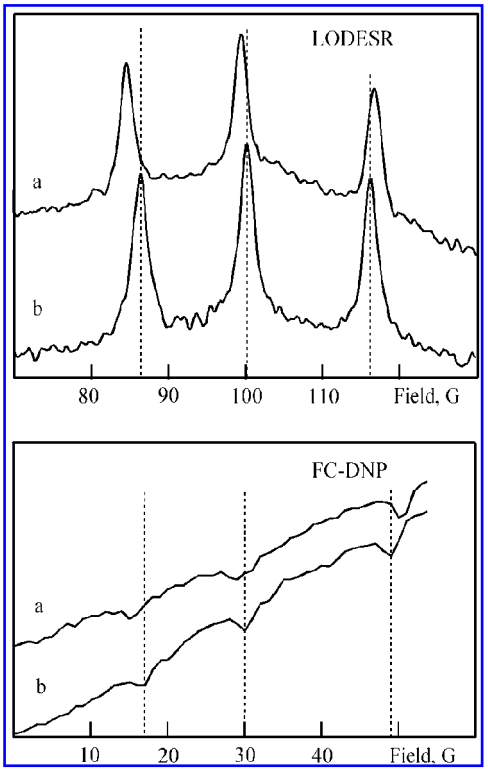


FIG. 4. LODESR (304 MHz; top panel) and FC-DNP (119 MHz; bottom panel) spectra from rats given 3.5 ml of 7 m*M* R2 solution in 0.1 *M* $NaHCO_3$ solution (Spectrum a) or in deionized water (Spectrum b). The LODESR spectra were collected approximately 10 min and the FC-DNP spectra 15 min after dosing. In addition to the narrowing of the splitting arising from acidification of the agent when given in water alone, there is a small difference in splitting between the two techniques, arising from the different measurement frequencies. In each case a dotted line is extended from each peak of Spectrum b to aid the eye.

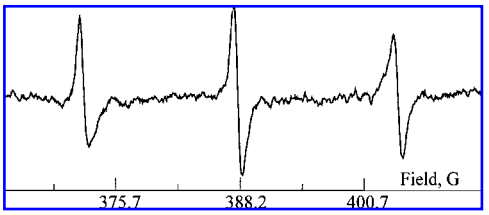
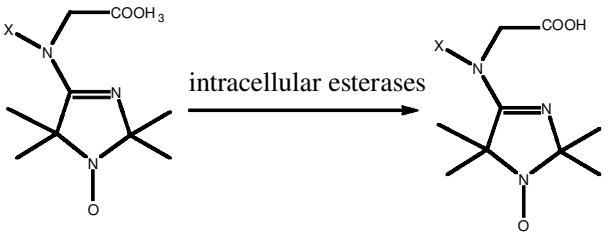


FIG. 5. L-band EPR spectrum of R1 (see Scheme 2) in isolated perfused rat heart (infused 1 min prior to ischemia through a side arm to achieve final radical concentration of about 50 μ*M*) after 5 min of ischemia. The spectrum is detected at a modulation amplitude of 0.5 G and shows superposition of two forms of the radical (protonated and unprotonated) resulting in disturbance of low- and high-field components and inequality of spectral peak intensities, as well as pH-dependent changes in the distance between these components. EPR-determined pH = 6.5 ± 0.1.



SCHEME 2. Expected hydrolysis of the ester derivatives of the imidazoline radicals, R3 (X = Me) and R4 (X = Ph), into the corresponding carboxy derivatives, R5 and R6, inside the cells. This strategy maximizes the potential for intracellular localization of the spin pH probes.

tection by EPR for the time window of about 20 min after a single infusion of the R1 as a bolus. Recently we described the synthesis of new series of pH-sensitive nitroxides with increased stability towards reduction due to the substitution of methyl groups in the vicinity of the -NO fragment by more bulky ethyl groups (40).

Intracellular and extracellular targeting is another important direction in the development and biological applications of spin pH probes. For this purpose we proposed synthesis of pH-sensitive nitroxides with ester groups for its intracellular targeting (74). Similar to other molecular probes with ester groups, *e.g.,* fluorescent probes (20), these nitroxides will presumably accumulate intracellularly after hydrolysis of the ester bond by endogenous intracellular esterases (Scheme 2). This is supported by preliminary data obtained with carboxy-derivative R5 (Scheme 2, X = Me). The EPR-measured p*K* of carboxyl group of R5 was found to be as low as 2.5, resulting in ionic character of this compound at physiological pH and its low transmembrane permeability through the bilayer of phosphatidylcholine liposomes. Note that application of an intracellular targeted probe with the ^{14}N-O fragment and an extracellular membrane-impermeable probe with the ^{15}N-O fragment provides possibility to detect both signals because their EPR spectra do not overlap (Fig. 6) and, therefore, opens the possibility of simultaneous detection of intracellular and extracellular pH.

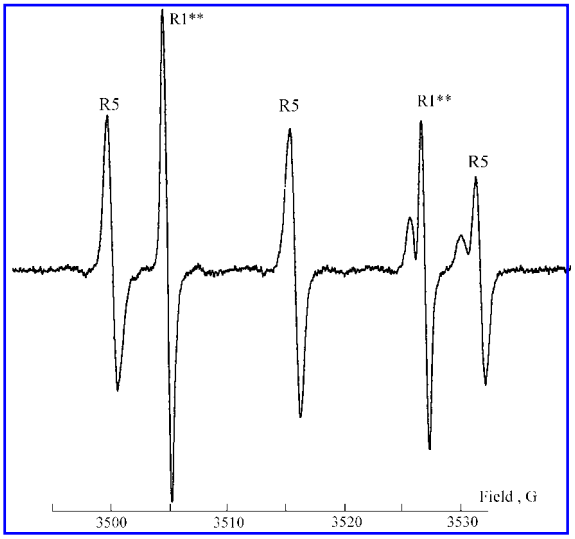


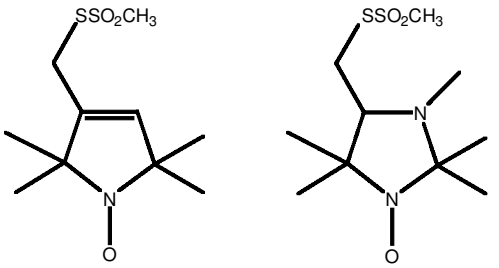
FIG. 6. X-band EPR spectrum of the mixture of the radicals R5 and ^{15}N-1 substituted radical R1 in 0.1 m*M* sodium phosphate buffer, pH 6.7. The doublet spectrum of the ^{15}N isotopically substituted probe, R1**, and triplet spectrum of the R5 radical with the ^{14}N-O fragment do not overlap, allowing simultaneous application of these spin probes for pH determination.

SPIN SH PROBES

Basis of the method of spin SH probes

The usefulness of the application of the paramagnetic disulfide, methanethiosulfonate spin label R7 (Scheme 3), in assaying reactive thiol groups in proteins was first demonstrated by Berliner *et al.* (6). The approach utilizes cysteine-directed spin labeling using this highly reactive thiol-specific reagent. One of the disadvantages of the approach is the requirement of the purification of the labeled protein from the unreacted radical. The other obstacle for thiol detection using disulfide R7 is insensitivity of its EPR spectrum towards the reaction with low-molecular-weight thiols.

The thiol-specific reagent R7 was found to be the most commonly used label in site-directed spin labeling, a powerful tool for monitoring the structure and dynamics of both soluble and membrane proteins (25). Recently a new methanesulfonate spin label, R8 (Scheme 3), with a pH-sensitive imidazolidine radical fragment (*cf.* R8 with imidazolidine radical R2, Scheme 1) has been synthesized (21). The reaction of the R8 with SH group of human serum albumin results in the formation of a pH-sensitive spin-labeled protein (Fig. 7), showing the ability of the label both to modify SH



SCHEME 3. Chemical structures of the methanethiosulfonate spin label R7 (6) and R8 (21).

groups of the proteins and to report local pH in the site of modification. To our opinion, the application of pH-sensitive thiol-specific reagents in site-directed spin labeling could provide new information on local polarity and electrostatic properties within the protein matrix.

The more direct EPR method for the detection of both low- and high-molecular-weight thiols is based on the application of biradical disulfide reagents, RSSR, where R represent an imidazoline (35) or imidazolidine (37) radical fragment (Scheme 4). These paramagnetic disulfides react with thiols, splitting the disulfide bond, resulting in characteristic changes of the EPR spectra (Figs. 8 and 9). The measurement of relative changes of the intensities of monoradical (Im) or biradical (Ib) components allows quantitative determination of GSH. The significant difference in the reactivity of the imidazoline biradical, R_1SSR_1, and the imidazolidine biradical, R_2SSR_2, with thiols (Table 1) allows different experimental approaches for their application.

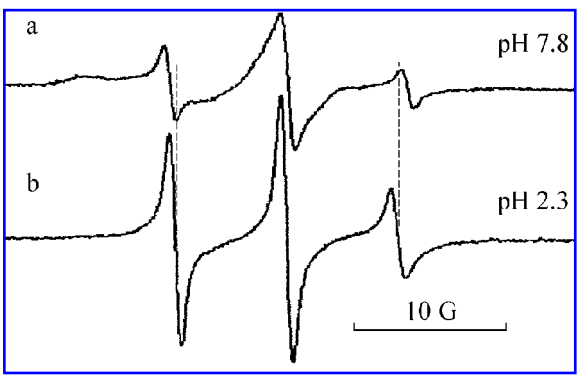


FIG. 7. X-band EPR spectra of human serum albumin solution labeled with the label R8 [modification degree 0.96, protein concentration 50 μ*M*; for labeling procedure see Khramtsov *et al.* (37)]. The observed pH-dependent spectral changes were reversible. Spectrum a represents the unprotonated form of the radical bound to protein (pH 7.8, a_N = 15.80 G), and Spectrum b is characteristic for the protonated form (pH 2.3, a_N = 14.60 G). The higher mobility of the protonated label bound to albumin may indicate its weaker association with the protein surface due to electrostatic repulsion of the positively charged radical fragment from the surface of positive electric potential [ϕ ~30 mV for human serum albumin at pH from 2.5 to 3 (36)]. The dotted lines are extended from low- and high-field components of Spectrum b to aid the eye.

TABLE 1. BIMOLECULAR RATE CONSTANTS AND CHARACTERISTIC REACTION TIME OF THE DISULFIDE BIRADICALS WITH GSH (31)

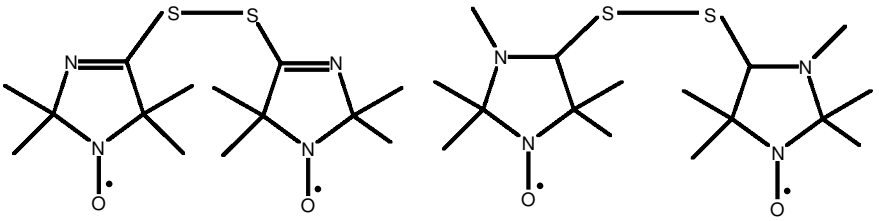
	k_f *with GSH at pH 7.0*	$\tau \sim 1/k_f$*[GSH] at 1 mM GSH*
R_1S-SR_1	~5 × 10^3 M^{-1} s^{-1}	0.2 s
R_2S-SR_2	0.26 M^{-1} s^{-1}	60 min

The fast reactive R_1SSR_1 probe can be applied only in a "static" approach, which measures the fraction of the biradical split by the reaction with thiols. This approach unambiguously requires an excess of the probe over thiols and results in consumption of total thiols under measurement, therefore interfering with normal cell function and making it impossible for *in vivo* use. The slow reactive R_2S-SR_2 probe, however, can be applied in both "static" and "kinetic" approaches. The latter measures the rate of the biradical disulfide bond splitting, and therefore insignificant fraction of thiols may be consumed during measurement.

The biradical R_1S-SR_1 has been used to measure GSH content in blood cells (14, 35), Chinese hamster ovary cells (75), murine neuroblastoma and malignant melanoma cells (12), and tumor HeLa cells (13). Noninvasive measurement of intracellular GSH using disulfide SH probes is based on the dominant contribution of GSH in the reaction with biradicals, fast diffusion of the probe across the cellular membrane, and its comparatively low reduction. The sensitivity of the method is sufficiently high to perform the measurements in very few (~100) cells (75). EPR studies of thiols in human and rat blood have demonstrated an increased level of oxidized GSH in the plasma under oxidative stress conditions (14, 77). The EPR assay for measurement of thiols in the blood is a sensitive and convenient method for fast analysis, which does not require complicated procedures for sample preparation. Nohl *et al.* (59) have applied the biradical, R_1SSR_1, for determination of thiol levels in isolated perfused hearts during ischemia/reperfusion. The authors measured release of the monoradicals into the perfusate, due to the reaction of the biradical disulfide with tissue thiols, and observed a decrease in the GSH level following ischemia/reperfusion.

The disadvantages of the "static" approach are the requirement of excessive amounts of the label, RS-SR, over thiols, resulting in the "consumption" of critical SH groups and subsequent irreversible damage to the system. The application of the "kinetic" approach using the disulfide reagent, R_2S-SR_2, allows a decrease of label concentration and therefore minimizes its possible toxicity. For the first time the application of the R_2S-SR_2 in combination with "kinetic" approach provides a tool that can be used for real-time measurements of the GSH redox state that is not damaging to the tissue being studied. The reasonable value of characteristic time, τ, for the reaction of the biradical R_2S-SR_2 with GSH at physiological GSH concentrations and neutral pH (see Table 1 and Fig. 9) allows its application *in vivo*. Indeed, Fig. 9 demonstrates a convenient time window for the biradical, R_2S-SR_2, reaction

SCHEME 4. Chemical structures of the disulfide biradical labels.



R_1SSR_1 R_2SSR_2

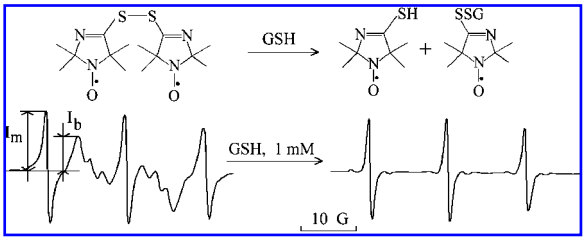


FIG. 8. X-band EPR spectrum of 0.1 m*M* solution of R_1SSR_1 in 0.1 *M* phosphate-buffered saline buffer, pH 7.4, and its transformation upon treatment with GSH, and the scheme of the corresponding reaction of thiol-disulfide exchange responsible for observed spectral changes. The measurement of relative changes of the intensities of monoradical (Im) or biradical (Ib) components allows quantitative determination of GSH.

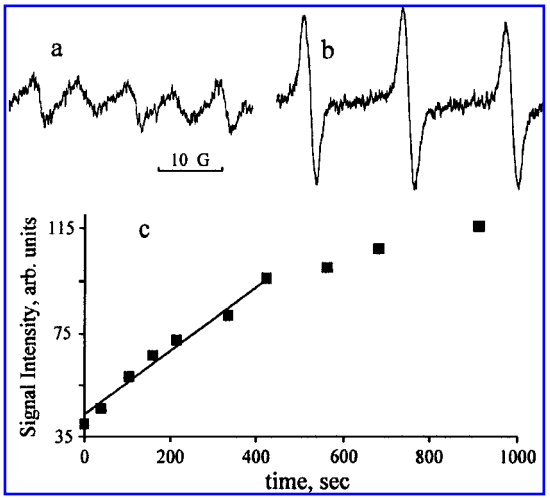


FIG. 9. L-band EPR spectra of 100 µl of blood taken from a Sprague-Dawley rat measured 10 s (a) and 700 s (b) after addition of 5 µl of the biradical R_2SSR_2 (final concentration of the label 50 µ*M*, acquisition time 10.5 s). c: The time dependence of the low-field component of the spectra allows linear approximation in the time range less than 400 s, which can be used for estimation of GSH concentration.

with GSH in untreated rat blood, as well as its relative stability towards reduction (less than 10% of the radical was reduced during a 15-min incubation in blood). Note that the R_2S-SR_2 radical freely penetrates cellular membranes [lipophilicity coefficient = 240 (37)], reacting preferably with intracellular GSH, while the reaction with protein thiols seems to be very slow, *e.g.,* rates constants for the reaction of R_2S-SR_2 with SH groups of human serum albumin and hemoglobin are hundreds of times less compared with that for GSH (37). Therefore, taking into account the dominant contribution of GSH in the experimental kinetics shown in Fig. 9, we calculated the GSH concentration in rat whole blood (located mostly in the erythrocytes) to be equal to 0.95 ± 0.2 m*M,* in reasonable agreement with literature data and with the data obtained by optical methods (8, 22).

Recently, we applied low, nontoxic concentrations of R_2SSR_2 and the kinetic EPR approach for a demonstration of increased GSH oxidation in an animal model of stress-sensitive arterial hypertension (56, 78). It was found that the concentration of GSH in blood of the hypertensive rats is significantly lower compared with that of normotensive Wistar rats. These data support an elevated thiol oxidation and free radical mechanism involved in the pathogenesis of this animal model of hypertension (52, 78).

The possibility of applying the kinetic EPR approach *in vivo,* using a redox-sensitive nitroxide, was recently demonstrated by Kuppusamy *et al.* (45), allowing redox mapping of a tumor in "radiation-induced fibrosarcoma (RIF-1)-tumor bearing" mice. The authors showed a central role of GSH in the reduction of the nitroxide. The GSH level was significantly higher in the tumor tissue compared with normal tissue (26). However, it is necessary to note that the applied probe is not GSH-specific and is not even thiol-specific, but rather sensitive to any reducing compound. Therefore, application of an SH-sensitive probe, the biradical R_2SSR_2, which entails a similar kinetic EPR approach, might provide even more specific information.

CONCLUSIONS

It is apparent that functional spin probes with EPR spectra sensitivity to concentrations of physiologically relevant species have become an important field of biomedical EPR applications. In this respect imidazoline and imidazolidine nitroxides were found to be the most promising probes providing the possibility to measure critical intracellular parameters, namely, local concentrations of protons (pH) and thiols.

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ABBREVIATIONS

DNP, dynamic nuclear polarization; EPR, electron paramagnetic resonance; FC-PEDRI, field-cycled proton electron double-resonance imaging; GSH, glutathione; hf, hyperfine; LODESR, longitudinally detected electron spin resonance; NMR, nuclear magnetic resonance; PEDRI, proton electron double-resonance imaging.

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