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Forum Review

Use of Electron Paramagnetic Resonance Spectroscopy to Evaluate the Redox State *In Vivo*

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ABSTRACT

The aim of this article is to provide an overview of how electron paramagnetic resonance (EPR) can be used to measure redox-related parameters *in vivo*. The values of this approach include that the measurements are made under fully physiological conditions, and some of the measurements cannot be made by other means. Three complementary approaches are used with *in vivo* EPR: the rate of reduction or reactions of nitroxides, spin trapping of free radicals, and measurements of thiols. All three approaches already have produced unique and useful information. The measurement of the rate of decrease of nitroxides technically is the simplest, but difficult to interpret because the measured parameter, reduction in the intensity of the nitroxide signal, can occur by several different mechanisms. *In vivo* spin trapping can provide direct evidence for the occurrence of specific free radicals *in vivo* and reflect relative changes, but accurate absolute quantification remains challenging. The measurement of thiols *in vivo* also appears likely to be useful, but its development as an *in vivo* technique is at an early stage. It seems likely that the use of *in vivo* EPR to measure redox processes will become an increasingly utilized and valuable tool. *Antioxid. Redox Signal.* 9, 1757–1771.

OVERVIEW

THE GOAL OF THIS ARTICLE is to indicate how the unique capabilities of EPR can be used very effectively to follow redox status/events *in vivo*. This will be done in the context of a general overview and two more detailed complementary minireviews of the current status of the use of *in vivo* EPR to measure free radicals and thiols. The article also seeks to delineate areas where developments are needed and the potential pathways to achieve them.

The ability to measure redox status and redox processes *in vivo* is very attractive for several reasons. It has been clearly established that redox-active species are intrinsically involved in many physiological and pathophysiological processes. The roles of these species include being essential intermediates in key physiological reactions and, in many cases, being directly involved in the causal chain leading to pathology. The redox state is an important parameter for many key events in cell signaling (13). Also, therapeutic approaches for many types of pathophysiology involve redox reactions (84).

Whereas many useful insights can be achieved by measurements in model systems and cell cultures, the complexity of redox processes and physiology makes it difficult to understand fully the redox processes that occur *in vivo* without making direct measurements with the intact system. The dynamics of the vascular system, metabolism in different organs, and the complex web of oxidants and antioxidants, cannot be modeled readily. There are few techniques that are capable of making such measurements *in vivo*, but fortunately EPR can follow many of the most important aspects of these complex processes.

Some of the redox-active species are free radicals, and for these, EPR is the most direct and unambiguous method to measure them. Under some circumstances the concentration of these species or their magnetic properties may make it difficult to

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measure them directly with EPR. Therefore, a technique termed "spin trapping" has been developed in which a detecting molecule (usually termed a "spin trap") is added to the system, which, upon reaction with a free radical, can form a product that is another free radical that is considerably more stable than the initial radical (Fig. 1). This provides at least a qualitative indication of the presence and often the type of free radical, and sometimes also may provide quantitative information. Under appropriate conditions, the resulting EPR-active product has spectral features that enable the original radical to be identified. As described below, this technique has been applied for efficient detection and identification of many different types of free radicals *in vivo*.

Although the "spin trap" is different, the same principle has been used to identify and, often, to quantitate nitric oxide, taking advantage of its free radical nature. In the case of nitric oxide, the trapping usually occurs by the localization of the unpaired electron in a nitrogen–metal complex.

Thiols have a key role in redox reactions *in vivo*. Redox equilibria between thiols (the reduced state) and disulfides (the oxidized state) are integrally involved in many processes such as cell signaling and enzymic mechanisms. Many redox processes affect the status of thiols and disulfides. Some thiols, such as glutathione, are by themselves key effectors. The structure of proteins can be profoundly affected by changes in thiols and disulfides. Therefore, methods to measure some of the key parameters *in vivo*, including the amount of glutathione and other small thiols, their oxidized derivatives, and thiols and disulfides in macromolecules could be extremely valuable. As noted below in detail, currently such measurements are very difficult to make, but EPR techniques appear to have the capability to obtain much of the needed data.

It also is potentially possible to detect the presence of free radicals and other redox-active species by following the status of paramagnetic molecules that are redox-active. This usually is done with nitroxides, measuring the changes in their intensity as a function of time and location. A reversible reduction of the paramagnetic nitroxide group to the nonparamagnetic hydroxylamine group is the principal redox reaction *in vivo*. The distribution of the nitroxides, and hence the compartment that they sample, can be selected by using appropriate structures; for example, charged nitroxides injected into the vascular system will stay in the vascular system; nitroxides whose structures enable them to cross the blood brain barrier can enter the brain; lipophilic nitroxides will localize in lipid-rich domains, including membranes. This can be a powerful tool but also requires considerable planning and controls to attain an unambiguous result. The rate of disappearance of the paramagnetism from a region can be due to a number of factors, including reduction of the nitroxide, reoxidation of the hydroxylamine to the paramagnetic nitroxide, excretion of either the nitroxide or hydroxylamine, and irreversible reactions that destroy the nitroxide group.

There are a number of other capabilities of *in vivo* EPR related to measurements of redox status that are not covered in this paper, but should be mentioned here because of their importance. The most important one for the subject of this paper is the ability to measure oxygen sensitively and repeatedly. The amount of available oxygen is clearly a critical factor for most redox processes, and *in vivo* EPR provides some unique capabilities to measure it, as illustrated in another article in this special issue (High Spatial Resolution Multi-Site EPR Oximetry of Transient Focal Cerebral Ischemia in the Rat; B Williams *et al.*). Other relevant capabilities of *in vivo* EPR that have implications for redox reactions include measuring a number of parameters that are difficult to measure *in vivo* by other means, such as: the structure of membranes (especially fluidity), pH, charge, viscosity, and motion of the macromolecules.

Finally, it is important to note that while *in vivo* EPR is indeed a very powerful technique that has been used extensively and successfully for the types of measurements discussed in this paper, there also are some important potential limitations that one needs to bear in mind. The *in vivo* EPR technique itself has some technical limitations in regards to sensitivity and the depth at which measurements can be made (the two, of course, are closely related). In the most typical *in vivo* EPR spectrometers, operating at 1,200 MHz, the depth that can be probed for most uses is ~10 mm. Other spectrometers have been used successfully with frequencies as low as 300 MHz, but as the frequency decreases the sensitivity also decreases. The specificity of EPR for species with unpaired electrons, while usually considered an important asset, of course does limit the measurements to only molecules with unpaired electrons. Because of the paucity of such molecules *in vivo*, most uses of *in vivo* EPR require the addition of paramagnetic materials or their precursors (*e.g.,* spin traps). There are some potential perturbations from these substances, but in animal systems the perturbations are usually minor and, if considered carefully, impose few limits on their effective use. In studies with human subjects, however, the paramagnetic materials need to undergo the same time consuming and costly process, as is required for any drug to demonstrate that they are safe and efficacious for use in humans. The time and cost for this process may limit their use in clinical medicine despite the inherent low toxicity of most of these materials. While the measurements of free radicals either by direct observation or with spin traps usually provide data that can be interpreted readily, there are some important caveats. Free radical intermediates can be generated by a variety of processes, and therefore it is essential to understand the pathways that can occur in the context of the specific experiment that is carried out. It is important to recognize that spin traps can be converted to species that are identical to radical adducts through nonradical paths. It also is possible for the spin traps to perturb the process that they are being used to follow, especially if the amount of radicals that is trapped is high; the latter can be especially important in measuring nitric oxide where the spin traps can significantly alter the pathophysiology by effectively scavenging a significant portion of the nitric oxide. The interpretation of data on redox status by the use of nitroxides can be quite complex because of the many paths for reduction and oxidation of nitroxides and their potentially complex metabolism, distribution, and excretion. The measurement of thiols *in vivo* also can be quite complex because of potential perturbations from the consumption of free thiol groups. While this list of potential limitations seems long and formidable and also probably is incomplete, there are very effective and practical ways to overcome them. This can be done by the same process that is needed for virtually all experimental studies, for example:

1. One needs to understand the potential perturbations that can occur;
2. Appropriate controls need to be included to determine the occurrence and potential effects of the perturbations;
3. Confirmatory experiments, often using other methods need to be carried out.

With such precautions, which really are simply good experimental techniques, *in vivo* EPR can be a very effective and powerful technique for following redox-related processes *in vivo*, bringing many unique capabilities to these important types of measurements.

IN VIVO SPIN TRAPPING

The interest in free radicals in biological systems became prominent with the postulates by Michaelis that free radical intermediates should occur in many biochemical processes (67). Over the succeeding years there has been a vast growth of interest in the subject and many theories and some data linking free radicals to many diseases and biological processes such as aging have been established (14). While many aspects of the field remain controversial, it is clear that the progress in defining the pathophysiological roles of free radicals and resolving the controversies will be significantly advanced by measurements of free radicals under biologically pertinent conditions. The development of *in vivo* EPR provides an opportunity for making such measurements.

Most of the free radicals that occur in functioning biological systems are short-lived and occur in low concentrations. This makes their direct detection by EPR challenging and in most cases impossible. Less direct methods based on other parameters, such as measurements based on the oxidation or reduction of a dye which leads to fluorescence, chemiluminescence, or changes in absorbance spectra have been developed. However, the application of these methods for measurements *in vivo* is difficult, and further these methods often lack specificity for measuring free radicals and in the identification of the specific free radicals that are involved.

With the development of the spin trapping method, it became possible to use EPR spectroscopy or imaging to detect short-lived free radicals in chemical and biological systems with the potential for high specificity and identification of the radicals that are involved. Spin trapping is a technique in which a spin trap (usually a nitrone or nitroso compound) reacts with a free radical to produce a nitroxide (the product usually is termed a radical adduct or spin adduct), whose stability is considerably greater than that of the parent free radical (34). Fig. 1 illustrates the spin trapping method with the spin trap DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide) as an example.

The nitrone or nitroso compounds do not have unpaired electrons and therefore are not detected by EPR, but the radical adduct formed is EPR active. The stability of the radical adduct often allows its accumulation to detectable levels. The EPR spectra of the radical adducts often exhibit a hyperfine splitting pattern that is characteristic of the trapped radical. The EPR-spin trapping technique now has become an established tool in the detection of free radicals occurring in chemical and biological systems.

However, the radical adducts detected by spin trapping do not necessarily represent the primary radicals formed in the system, because further reactions can occur if the initial radical is very reactive (*e.g.,* the hydroxyl radical, which reacts with all organic molecules at the rate of diffusion). The quantification of the free radicals that are generated is difficult because the changes in the EPR signal intensity of the radical adduct reflect the processes of both free radical generation and decay; both of these can vary due to several factors such as oxidation or reduction of free radicals or radical adducts in the biological systems. In addition, there are a number of other factors that should be considered when carrying out spin trapping experiments to be able to interpret the results correctly. These include (a) the rate of production of primary and secondary radicals; (b) the rate of spin trapping of the various radicals generated, and (c) the rate of decomposition of the radical adducts. Recently, techniques such as high performance liquid chromatograph (HPLC), gas chromatography/mass spectroscopy (GC/MS), liquid chromatography/mass spectroscopy (LC/MS) have been developed for chemical identification of all the radicals formed (120). While these methods require an *ex vivo* sample collection and analysis, they can be very useful complementary techniques for *in vivo* EPR spin trapping.

The extension of spin trapping to enable direct measurements *in vivo* became feasible with the development of low frequency EPR (≤1,200 MHz) spectrometers with sufficient sensitivity (20, 23, 38). This ability to make the measurements immediately under physiological conditions has the potential to significantly increase our understanding of physiological and/or pathological roles of free radicals in biology and medicine. In this section, we have summarized results of spin trapping done directly *in vivo* (which also include some results with *ex vivo* detection) and have indicated potential areas which warrant further development.

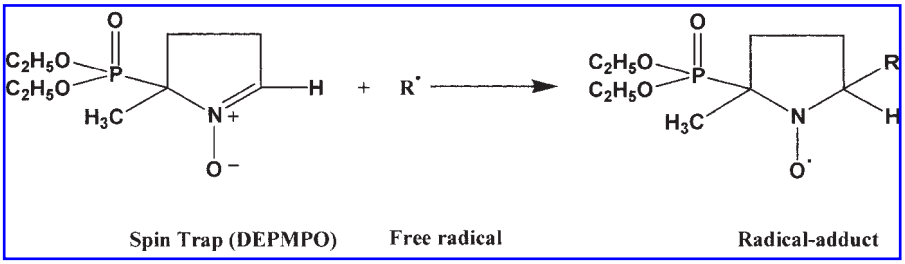

FIG. 1. Spin trapping.

Spin trapping of carbon-, oxygen-, and sulfur-centered radicals

Spin trapping of carbon-centered radicals. The direct detection and quantification of carbon-centered radicals has proven to be difficult as they have half-lives in microseconds and can react with oxygen at a diffusion controlled rate to form secondary radicals that are oxygen centered. The use of spin traps therefore has had an important impact on determining the role of carbon-centered radicals in toxic effects of many substances. Most of the investigations have used *ex vivo* detection to characterize *in vivo* radical production. Knecht *et al*. provided first evidence of ethanol-derived radical adducts of POBN (alpha-4-pyridyl-1-oxide *N-tert*-butyl nitrone) in deer mice fed with diets containing alcohol (49). The 1-hydroxyethyl radical adduct of POBN was detected in bile samples and was positively identified using ^{13}C-substituted ethanol. To increase the sensitivity, a deuterated PBN (phenyl-*N-tert*-butyl nitrone) analog was used to detect lipid-derived radicals after acute alcohol administration in rats (79). Lipid-derived radicals have been detected after acute and chronic administration of ethanol using spin trapping, and the radical concentration increased further after acute administration of ethanol to alcohol-fed rats (80). The free radicals generated from ethanol have been observed directly *in vivo*, using high doses of irradiation (23). To date, this seems to be the only successful study directly detecting free radicals generated from ethanol in living animals.

The toxicity of methanol also is thought to be directly related to the generation of carbon-centered radicals (39). In non-human primates and humans, it has been shown that methanol is metabolized to toxic metabolites by alcohol dehydrogenase (33). However, there is no report on direct *in vivo* detection of methanol-derived free radical metabolites. Kadiska *et al*. detected the carbon-centered radical (CH_2OH) in both bile and urine samples after 2 h of acute methanol poisoning in rats (40). Spin trapping also has been used to detect and identify free radicals produced after formate poisoning in rats. Formic acid and methanol have a common mechanism of toxicity, because formic acid is a metabolic end product of methanol, which leads to acidosis through the inhibition of cytochrome c oxidase (58). Carbon dioxide anion radical adducts have been identified using the POBN spin trap in bile and urine samples of rats subjected to sodium formate (16).

The hepatotoxicity of CCl_4 also is believed to be largely due to carbon-centered radicals ($\cdot CCl_3$) produced from the metabolic activation of the halocarbon by P450. The $\cdot CCl_3$ radical forms adducts with hepatic molecules to oxidize glutathione and protein thiols and induce lipid peroxidation (114). PBN-CCl_3, PBN-CO_2, and lipid-derived radicals have been detected *in vitro* (35) and *ex vivo* (81), both in chloroform extracts of rat liver and in bile samples using spin trapping. Sentjurc *et al.* modified the extraction procedure to obtain a radical adduct signal with relatively good signal-to-noise ratio (91). Different oxidizing agents were used to detect different adducts; however, one potential disadvantage of the reoxidation procedure is the possibility of initiation or catalysis of free radical chemical reactions *ex vivo* in the presence of spin traps, which could lead to artifacts. Recently, nitric oxide has been implicated in CCl_4-induced acute liver injury and chronic liver injury (69).

Spin trapping of oxygen-centered radicals (reactive oxygen species, ROS). Many pathological conditions are associated with ROS-mediated events (12, 50, 105). The direct biochemical effects of ROS that can damage cells include lipid peroxidation, oxidative modification of proteins, and alterations in DNA (64). ROS, especially superoxide, have recently been implicated to have an important role in activating signal transduction (13). This has further augmented the need to develop and employ methods that can measure ROS more sensitively and quantitatively.

Postischemic reperfusion injury is often attributed to ROS; however, most of the evidence supporting the role of free radicals remains indirect. After two inconclusive reports (2, 77), Bolli *et al*. (6) unequivocally demonstrated the production of free radicals by the intracoronary infusion of the spin trap in the intact dog subjected to coronary occlusion and subsequent reperfusion. Stable oxygen- and carbon-centered radicals were detected in the blood. Using the same method, the production of free radicals in the intact rabbit kidney subjected to an ischemia–reperfusion was detected by Pincemail *et al*. (74). It also has been demonstrated that the damage mainly occurs at the time of reperfusion of ischemic tissue (72). Janzen et al. combined EPR and GC/MS methods to obtain improved identification of the radicals trapped in *in vitro* and *in vivo* systems (36). The combination of these two methods helped to identify adducts that were not detected by EPR due to their fast reduction in biological systems.

Oxidative reactions lead to secondary neuronal and cerebrovascular damage following traumatic brain injury (88). Using *ex vivo* techniques, spin trapping of free radicals *in vivo* has been reported to occur in the brain, spleen, liver, and lung after such injuries (54, 101). Hydroxyl radicals have been detected in a rat brain occlusion model using spin trapping with POBN (73).

The first successful direct measurement of spin trapped hydroxyl radicals *in vivo* was reported by Halpern *et al*. using POBN and a very high dose of X-ray irradiation (3000 Gy) of tumors in mice treated with ethanol (23). Timmins *et al*. succeeded in obtaining direct *in vivo* detection of hydroxyl radicals in mice under more generally applicable conditions, with stimulation of hydroxyl radical formation by administration of 5-aminolevulinic acid and ferrous ammonium sulfate, using DEPMPO as the spin trap (104). A very useable signal/noise was achieved in this experiment. When DMPO was used as the spin trap under the same experimental conditions, the hydroxyl radical adduct was not detectable. This is consistent with the better stability of the hydroxyl adduct of DEPMPO as compared to that of DMPO (the rate of spin trapping of hydroxyl radicals should be very similar for both spin traps). This is a good example that illustrates the key role of the type of spin traps on the stability of the stable adducts, which can determine the success of spin trapping studies.

Capani *et al*. investigated the ROS release in the neostriatum of new born rats subjected to acute perinatal asphyxia (PA) followed by various periods of reoxygenation (9). These experiments were carried out *ex vivo* using neostriatum homogenized with the spin traps (*N-tert*.-butyl-alpha-phenylnitrone and diethylenetriamine-pentacetic acid) in phosphate-buffered saline. The complexes formed were extracted with ethyl acetate and analyzed on X-band EPR spectrometer. A significant release of ROS was detected at 20 min of PA after 5 min of reoxygena-

tion. Direct *in vivo* detection of ROS during ischemia/reperfusion can advance the understanding of the mechanism and the role of ROS generation in the injury and can facilitate the development of more effective treatments.

Spin trapping of sulfur-centered radicals. The formation of free radical intermediates ($SO3^{\cdot-}$, $SO4^{\cdot-}$) from autoxidation or enzyme-initiated oxidation of sulfite contributes to the mechanism of sulfite-induced toxicity (78). Direct *in vivo* spin trapping has successfully detected sulfite radicals using low frequency EPR (1,200 MHz, L-band) (59). The positive results with these initial studies *in vivo* were facilitated by the fact that the radical adduct of sulfite is relatively stable. It should be noted, however, that the spin trapping of sulfite radicals using nitrone spin traps such as DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) or DEPMPO can also result from a nonradical addition pathways (76). Although the detailed mechanism of sulfite-induced toxicity is unclear, it is believed that one important event involves the sulfur trioxide anion radical, generated during oxidation of the sulfite.

Reactive sulfur species also can be generated by oxidation of GSH or GSSG in the presence of a variety of ROS, such as peroxides, glutathione, and peroxynitrite (57). Langley–Evans *et al*. investigated sulfur dioxide-induced changes in GSH concentrations and GSH-related enzyme activities in tissues of rats exposed to varying levels of the gas (55). They concluded that the sulfitolysis of GSSG does occur *in vivo* during sulfur dioxide exposure and that sulfur dioxide, even in the absence of a pulmonary injury, is a potent glutathione-depleting agent. Phenyl hydrazine is a well-known hemolytic agent that induces non-indigenous redox processes in erythrocytes. One of the reactive intermediates is the hemoglobin thiyl free radical, which forms a relatively stable DMPO radical adduct in rats (62). The DMPO-thiyl radical adduct has been detected with X-band EPR from rat blood (63). The findings of *ex vivo* studies have been confirmed by direct *in vivo* detection of the DMPO-hemoglobin thiyl radical adduct (38).

Nitric oxide (NO)

Spin trapping of nitric oxide. Nitric oxide (NO) is a highly reactive short-lived free radical which plays a key role in diverse biological processes: neurotransmission, regulation of vascular tone, molecular oxygen transport, regulation of prostaglandin production, and immune-host defense (61). The first *in vivo* NO detection was reported by Komarov *et al*. (51) in the tail of the endotoxin-shocked mice using S-band EPR. Since then, several investigators have succeeded in direct detection of NO with spin trapping in a mice model of septic shock using L-band EPR (4, 32, 37). The spin trapping results, along with the pharmacokinetic data, indicate that NO is produced primarily in the upper abdomen near the liver; this has been confirmed also by EPR imaging (30). Direct *in vivo* detection of NO in brain was reported by Kaneko *et al*. in an animal model of epileptic seizures (41). James *et al.* demonstrated the simultaneous measurement of NO and pO_2 directly in septic mice *in vivo* (37). Jackson *et al*. extended this approach to measure pO_2 at two different locations in the liver along with the simultaneous measurement of NO after the mice were subjected to PR-39, a proline-rich antibacterial peptide (32). Using 1000 MHz EPR with a 3.4 cm birdcage resonator; the application of *in vivo* EPR for the measurements of pO_2 and NO in endotoxin shock was demonstrated.

The ability of *in vivo* EPR to provide both spectral and spatial information improves the understanding of the pathophysiology of NO. The use of EPR to measure tissue pO_2 and NO simultaneously *in vivo* could be particularly powerful when used in conjunction with simultaneous monitoring of circulatory hemodynamics.

Status of spin traps for use in vivo

In the following paragraphs, we summarize the new generation of spin traps that have been synthesized recently and highlight areas that warrant potential improvement.

Nitrone and nitroso spin traps. The redox potential of a series of nitroxides is in the range of 0.5–0.9V (18), which suggests that in spin trapping experiments in biological systems, a major reaction pathway could be the reduction of nitroxides (radical adducts) to EPR-silent hydroxylamine derivatives. The stability of the radical adduct can be enhanced by delocalizing the unpaired electron density over the molecule by including high electron affinity groups in the spin trap and by groups that can provide steric hindrance. This approach has been used to design and develop new spin traps. In 1995, the synthesis of DEPMPO with an electron withdrawing dialkoxyphosphoryl group in the β-position was a major step forward for the trapping of superoxide radicals (18). By substituting the methyl group of DMPO by a butoxyl group; BMPO (5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide) has been synthesized and the superoxide adduct of this spin trap was found to be more stable then DMPO (122). The methyl substituents of DMPO have been further modified to obtain EMPO (5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide) and its N^{15}-labelled derivative (98). More recently, DEPPEPO (2-(diethoxyphosphoryl)-2-phenethyl-3,4-dihydro-2H-pyrrole-1-oxide) has been synthesized, which provided a similar half-life of superoxide adduct as DEPMPO but has the advantage of being more lipophilic and solid at room temperature (115). It is easy to purify solid spin traps (such as BMPO, DEPPEPO) by recrystallization, and the spin trap could be stored even at room temperature.

Karoui *et al*. reported DECPO (2,2-diethoxycarbonyl-3,4-dihydro-2H-pyrrole-1-oxide), a DMPO type di-ester nitrone which trapped superoxide approximately nine times faster than BMPO (42). Allouch *et al.* synthesized DEEPyON (*N*-[(1-oxidopyridin-1-ium-4-yl)-methylene]-1, 1-bis(ethoxycarbonyl) ethylamine *N*-oxide, a diester nitrone), and DEEPN (*N*-benzylidene-1, 1-bis(ethoxycarbonyl)ethylamine *N*-oxide) (1). Stolze *et al*. synthesized and investigated the superoxide radical adduct stability of different 5,5-disubstituted EMPO-derived spin traps (98, 99). The stability of the adducts in solution varied from $<$1–25 min, depending upon the substituents with spectral features similar to that of DMPO radical adducts.

In the linear nitrone series, more efficacious phosphorylated analogues such as PPN (*N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide) and PyOPN (*N*-[(1-oxidopyridine-1-ium-4-yl) methylidene]-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide) have been developed (109). Linear ester nitrones

also yielded long-lived superoxide radical adducts (96). In addition, these could be easily synthesized with high purity and yield adducts with simpler EPR spectra. Karoui *et al*. have shown that the spin trapping in presence of methyl- β-cyclodextrin results in a sevenfold increase in the half-life time of the DEPMPO-OOH adduct *in vitro*, and this adduct was protected partially from reduction by glutathione peroxidase and by ascorbate anion (43). However, this approach has not been tested *in vivo*.

The signal intensity of the resulting radical adducts can be enhanced by using an isotopic substitution for hydrogen and nitrogen atoms of the spin traps. Deuterium has a magnetic moment of approximately 1/7 that of hydrogen but higher multiplicity; this will result in an overall linewidth reduction by a factor of four. The reduction in the hyperfine splitting from 3 to 2 by replacing ^{14}N with ^{15}N can provide an additional enhancement of signal intensity by a factor of 1.5. A number of isotope-labeled spin traps have been synthesized to date (106, 121). The signal intensity, however, not only depends on the spectral linewidth, and the total number of lines, but also on the half-life of the adducts, the rate constant of the spin trapping reaction, and on factors such as reduction by antioxidants and degradation by reactions catalyzed by transition metals.

It also is desirable to have spin traps that can accumulate at the defined site of free radical generation to compete with oxygen or other cellular components for reaction with the radicals and enhance spin trapping efficiency. Several nitrones derived from PBN have been synthesized for targeting mitochondria (70), cell membranes (10), and lipid membranes (26) via noncovalent interactions. Recently, a polyclonal antibody that can recognize the protein radical adduct of DMPO as an antigen has been reported (15). The localization of the spin traps in the cytosol or in the cell membrane could be achieved by altering the lipophilic properties of the spin traps. Although DEPMPO has outstanding spin trapping properties (half-life $\sim$ 15 min for superoxide adduct at physiological pH), it has a limited application in lipophilic systems (partition coefficient $\sim$0.06) (97). To facilitate the detection of superoxide and other free radicals in lipid environment (within or close to the surface of the mitochondria or cell membrane), a series of lipophilic derivatives of EMPO was recently reported by Stolze *et al*. (98). Liu *et al*. reported that *N*-4-(iodoacetamido)-benzylidene]-*N*-tert-butylamine N-oxide is a spin trap that can target sulfhydryl containing biomolecules such as glutathione (GS) and bovine serum albumin (BSA), and the resulting bioconjugates can efficiently trap free radicals (60). This approach demonstrated that, with the help of a covalent link or molecular recognition, the targeting for polypeptides makes possible the transport of a spin trap to the areas of interest in which the monitored radical species is site-specifically generated.

It also is essential to understand the interactions of the spin traps with functioning biological systems. Khan *et al*. evaluated the stability of hydroxyl, methyl, and sulfite radical adducts and the effects of DMPO, CMPO, EMPO, BMPO, and DEPMPO spin traps on colony formation and oxygen consumption rate of functioning CHO cells (44). Results indicate that the extent of the effects observed up to 50 m*M* concentrations are unlikely to compromise many studies. Experiments carried out with two cell lines (CHO and 9L cells) indicate that the structure of the spin trap that is used with a particular cell line might be critical for the experiments. Recently, the toxicity of EMPO derivatives has been investigated in cultured human lung carcinoma (A549), breast carcinoma (SKBR3), colon carcinoma (SW480), and human fibroblast (F2000) cell suspensions (82). Based on the results, the spin traps can be listed as follows for their toxicity: t-BuMPO > BuMPO > s-BuMPO > PrMPO > i-PrMPO $\sim$ DEPMPO $\sim$ EMPO. Fuchs *et al*. evaluated the acute dermatotoxicity of the PBN, POBN, DMPO, DEPMPO, DDC, and MGD spin traps (19). DMPO, POBN, DDC, and MGD were clinically nonirritant at all concentrations tested (50–500 m*M*), and no delayed-acute inflammatory reaction was observed.

Such data are needed to predict potential physiological or pathological effects associated with the use of these spin traps and to design experiments that overcome these unwanted side effects. Each type of application is likely to need preliminary studies of stability and toxicity to determine which spin trap is the most appropriate for the experimental goal. These newly synthesized derivatives provide the users with a range of choices depending on the target environment.

Spin traps for nitric oxide (NO). The spin trapping methods for NO have somewhat limited sensitivity, and detection is possible only at high levels of NO production. Because of the large amount of traps that are required, the effect of these complexes on the concentrations of NO need to be considered for appropriate interpretation of the spin trapping results. There also is a clear need to understand how the iron-DTC (dithiocarbamate) traps work and to develop improved NO traps for *in vivo* and clinical applications. Some attempts have been made to improve the sensitivity; for example, reducing agents and albumin were added to increase the availability and stability of the complex (107), and the tissue containing lipophilic NO complex was extracted by organic solvent and concentrated (110). But these methods are not suitable for use in living animals. The iron complex of MGD (*N*-methyl-D-glucamine dithiocarbamate) has been used as a hydrophilic spin trap for endogenously produced NO. However, Fe(II)-MGD undergoes very complex redox chemistry, which is likely to result in an inaccurate assessment of local production of NO (108).

Yoshimura *et al*. developed highly water soluble spin traps using Fe-DTCS (*N*-dithiocarboxysarcosine) and were successful in imaging endogenous NO in LPS (lipopolysacharide)-treated animals (119). Recently, two new spin trap formulations based on Fe(II)-DETC have been developed: (a) a lipid-based carrier system stabilized by lecithin and (b) inclusion complexes in hydroxypropylcyclodextrin (11). The sensitivity of the NO detection significantly increased using the lipid-based carrier systems or inclusion complexes as compared to the standard spin trap agents. Most of the studies have provided very useful data on the *in vivo* production of NO but only limited insights into its mechanism. Development of well-defined metal complexes with a variety of biodistribution profiles that are both stable and slow to exchange the metal, have a high selectivity and optimum affinity for NO but lack toxic side effects, is certainly needed.

Summary of current state of direct measurements of free radicals in vivo with spin trapping. Considerable progress has been made in the development of spin traps that provide high spectral resolution of carbon-, oxygen-, and

sulfur-centered free radicals with spin adducts that are stable *in vivo*. The basis for these successes was the production of a high concentration of the radicals, and the use of spin traps with better stability of the resulting radical adducts. While experiments under conditions in which there are large amounts of free radicals have been quite useful, at present, we believe that the *in vivo* spin trapping method does not yet have the sensitivity to be used routinely to detect reactive oxygen, carbon, and nitrogen species at steady state physiological concentrations in living animals.

The measurement of *in vivo* free radicals in the clinic depends on the development of spin traps that can be used safely in human subjects and the success of spin trapping in animal models. Once this is established and the spin traps have been demonstrated to be safe for use in human subjects, this method will have very attractive potential clinical applications. Nonetheless, spin traps have been successfully used on body fluids and tissue samples (27, 106). We believe that the future of *in vivo* spin trapping lies in the hands of chemists who can synthesize spin traps that can result in more persistent radical adducts with greater spectral resolution between different radical adducts, are less toxic, and can accumulate in relevant sites and cell compartments. It also depends on biologists who can design feasible experiments in which the data on spin trapped radicals provides crucial information.

REDOX STATE OF THIOLS

The redox state of thiols is an important property of prokaryotic and eukaryotic cells, and it is associated with all major biological processes (92). The redox couple of glutathione, GSH, and its disulfide form, GSSG, is considered to be the major regulator of the intracellular redox state (90). GSH is an important factor in controlling the redox status of thiols in proteins, and therefore it may affect protein mediated switching ("protein nanoswitches") in intracellular signaling pathways. Glutathione also plays a crucial role in antioxidant defense mediated through enzymes and is responsible for the detoxification of hydrogen peroxide. The redox status of GSH is related to many physiological processes that involve other nonprotein thiols as well as their symmetric and mixed disulfides (72, 90). All these interrelated roles of thiols raise the need for simultaneously monitoring their levels in organisms, since certain thiol redox state-associated patterns may identify normal/abnormal metabolic processes. So far, the thiol redox state has been measured either as total cellular thiol content (100), by its main redox buffering components GSH and GSSG (86, 103), or by simultaneous determination of the concentrations of protein and nonprotein thiols and their symmetric and mixed disulfides (72). Because it can readily be sampled, the oxidation–reduction status of blood glutathione is especially used in investigations involving oxidative stress and free radical pathologies that occur in other tissues. Low GSH, high GSSG, and a lower GSH/GSSG ratio have been found in blood from patients with various pathologies (86, 89, 117). However, the use of the redox status of GSH in the blood as an indicator of disease risk in humans has recently been questioned because of the extremely high variability in the reported data for GSH and GSSG in the blood, which might reflect problems with the assays that are used (86).

Methods of thiol detection

Fluorometric, photometric, and chromatographic assays for GSH/GSSG measurements all require sample preparation, often involving chemical modification of the thiols, and are quite laborious in practice (17, 72, 86, 103, 116). The use of these approaches is mostly limited to *in vitro* or *ex vivo* detection of thiols, due to the invasiveness required and/or the limited depth of penetration of light. Magnetic resonance approaches for thiol detection have greater potential for use *in vivo*. The NMR approaches normally detect endogenous GSH but have limitations due to low sensitivity and difficulties in assignment of spectra because of overlapping of numerous resonances (102). The recently developed fluorinated disulfide analog of Ellman reagent for ^{19}F NMR detection of thiols so far is limited to use *in vitro* due to a lack of sensitivity and the need for complex spectra analysis (75).

EPR has a potential advantage over NMR, possessing more than three orders of magnitude higher intrinsic sensitivity to the probe concentration. Previously, however, the low depth of microwave penetration into aqueous samples and the absence of endogenous thiol-sensitive probes significantly limited the ability of using EPR for detection of thiols *in vivo*. The recent development of low-field EPR techniques now makes feasible EPR measurements in isolated organs and living small animals. Kuppusamy et al. demonstrated the capability of L-band (1200 MHz) EPR spectroscopy to follow the tissue redox state *in vivo*, supporting a central role of GSH in the reduction of nitroxides and the presence of increased GSH content in a murine tumor model (52). In the following sections we discuss several thiol-specific paramagnetic probes, their *in vitro* applications, and their potential use *in vivo*.

Thiol-specific spin labels. The traditional thiol-specific reagents include such reactive functionalities as maleimides, iodoacetates, organomercurials, alkylthiosulfonates, and disulfides. The first nitroxide analog of the maleimide, label **R1,** was proposed by Griffith and McConnell (22), who demonstrated its specific binding to the thiol groups of the proteins in their pioneering work in the EPR spin labeling field. Both five- and six-membered maleimide and iodoacetamido nitroxides of piperidine, pyrrolydine, and pyrroline types (**R1–R3**, Fig. 2) were applied in numerous biophysical applications as thiol-specific spin labels. Maleimide and iodoacetamide spin labels react (22, 94) with thiols at or below room temperature in the physiological pH range (pH 6.5–8.0) to yield chemically stable thio-ethers. Among the applications are specific thiol labeling of sarcoplasmic reticulum ATPase (111), human and horse hemoglobin (66), sugar-H(+) symporter, GalP, in cell membranes of *Escherichia coli* (65, 94), and bovine cardiac and rabbit skeletal myosins (95). However, at an elevated pH they can react with amines or may be hydrolyzed to nonreactive products, therefore compromising labeling specificity. Isothiocyanate- (25), alkylhalide- (29), indanedione- (83), and even organomercurial-containing nitroxides (29) were synthesized and may have advantages in some specific applications for the labeling of thiols. For example, it was observed that the isothiocyanate-based spin label is more sensitive to the domain orientation in myosin head than the widely used maleimide spin label (25).

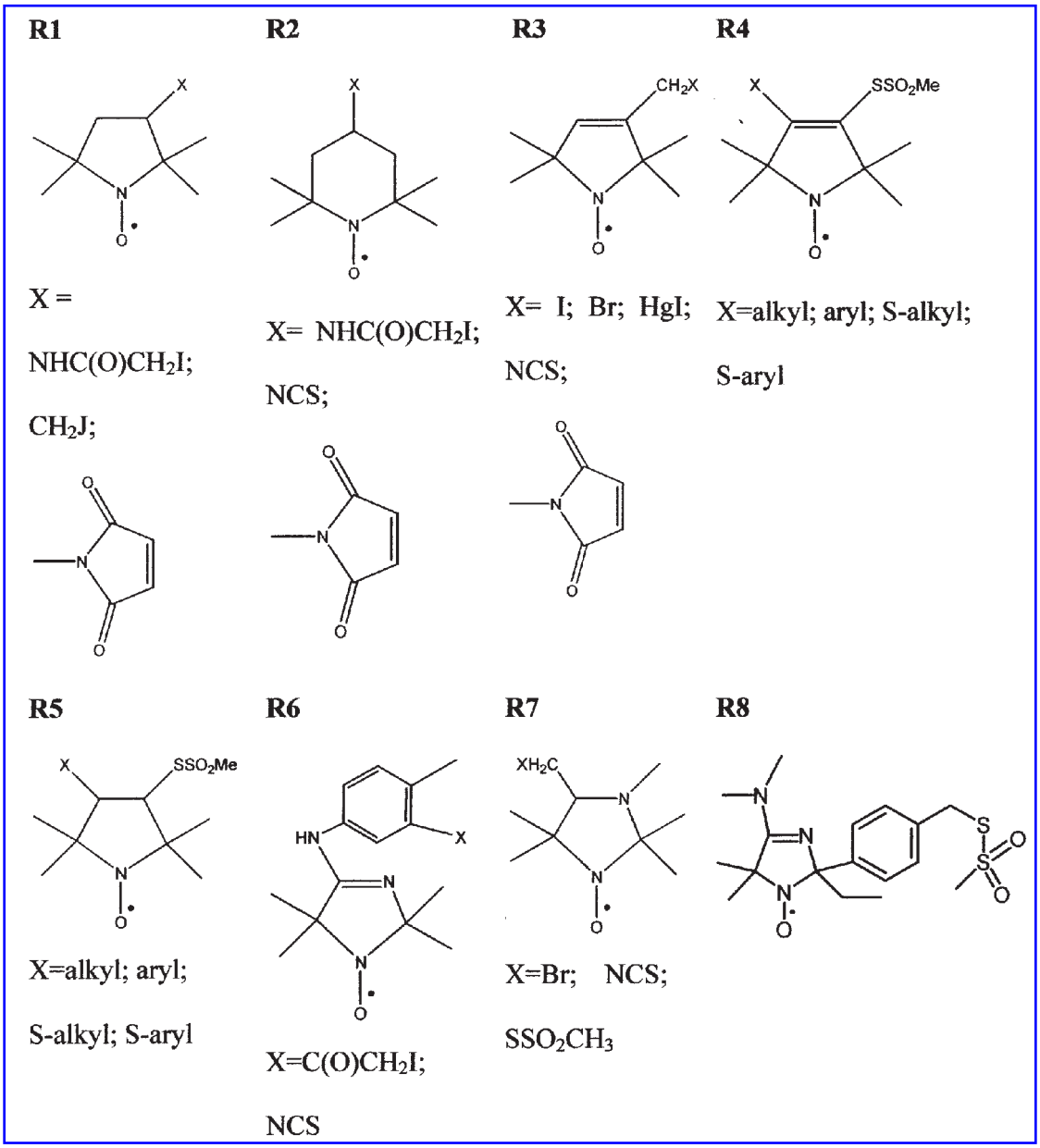

FIG. 2. Chemical structures of thiol-specific nitroxide reagents.

The above labels are in general slow to react and therefore require long reaction times and a large excess of reagent. The alkylthiosulfonate spin labels developed by Hideg *et al.* (5, 29), **R4** and **R5,** are distinguished by their extremely rapid reactivity under mild conditions, high selectivity for sulfhydryl groups, ability to undergo quantitative and complete conversion to the disulfide without requiring a large excess of reagent, and the general reversibility of the formation of disulfide bonds upon the addition of thiols such a β-mercaptoethanol or dithiothreitol. The synthesis of the methanethiosulfonate spin label, **R4** (X = H) (5), greatly facilitated the development of an important area of spin labeling applications, site-directed spin labeling, SDSL (31).

Multiple labeling, for example, using both iodoacetamido and methanethiosulfonate labels (24), and the use of perdeuterated (or/and ^{15}N substituted) labels (68) enhance the resolution of the probing of local conformational changes in proteins. The pH-sensitivity of the EPR spectra (46) of nitroxides of the imidazol type provides additional advantageous functionality for spin labeling applications. Thus, thiol-targeted labels **R6–R8** (Fig. 2) provide a tool for probing local electrostatics of the biological macromolecules (45, 46, 68, 93). Figure 3A demonstrates the pH dependence of the nitrogen hyperfine splitting of the imidazolidine methanethiosulfonate label **R7** (X = SSO_2CH_3). An attachment of this label to the protein sulfhydryl groups allows the probing of the local electrostatics by EPR (45). The use of high-field EPR with pH-sensitive methanethiosulfonate labels opens a new possibility for the investigation of site-directed spin-labeled proteins, as was demonstrated for the label **R7** (X = SSO_2CH_3) (68, 93). Recently, an imidazolidine label **R8** was synthesized which has a pK_a value close to the

physiological range (pK_a = 5.7 for the label alone and pKa = 6.15 when bound to the glutathione) (87), therefore extending the arsenal of the pH-sensitive methanethiosulfonate labels.

Measurement of thiols by EPR. The use of thiol-specific nitroxide labels allows the determination of the accessible thiol groups in various macromolecular structures, such as human plasma low-density lipoproteins and erythrocyte membranes (53). The EPR characterization of the protein sulfhydryl groups is based on the immobilization of the nitroxide upon binding to the macromolecular structure. The measurement of low-molecular-weight thiols, such as GSH, using thiol-specific nitroxides, is difficult due to the small EPR spectral changes of the label upon binding. This drawback was overcome by the development of the biradical disulfide labels, which have significant EPR changes upon reaction with low-molecular-weight thiols (47, 48).

Disulfide biradicals of imidazoline (**R_1SSR_1**) (48), imidazolidine (**R_2SSR_2**) (47) and pyrroline (**R_3SSR_3**) (28) types have been synthesized (Fig. 4).

Disulfide biradicals (RSSR), being paramagnetic analogs of widely used Ellman's reagent (17), react with thiols (BSH) via the reversible reaction of thiol–disulfide exchange:

$$\text{RS-SR} + \text{BSH} <=> \text{RSSB} + \text{RSH} \qquad \text{[Eq. 1]}$$

The principal advantage of the biradical disulfide reagents over monoradical labels is the large EPR spectral changes that accompany the reaction [1] even with low-molecular-weight thiols (46) such as GSH (Fig. 5).

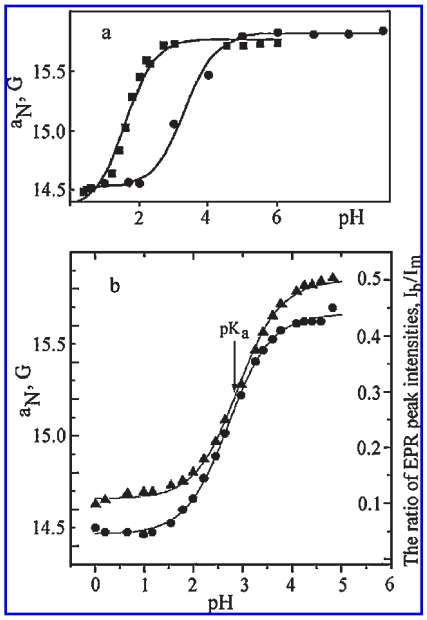

FIG. 3. pH-induced variation of the isotropic hyperfine constant, a_N, measured (A) for the label R7 (X = SSO_2CH_3) alone (■) and attached to the cysteine (●). *Solid lines* are Henderson–Hasselbalch titration curves with the pK_a values 1.58 and 3.21 for the free and attached labels, respectively (93). (**B**) pH-induced variation of the isotropic hyperfine constant a_N (■), and the ratio of the EPR peak intensities (▲) (see Fig. 5) measured for the disulfide biradical label R_2SSR_2 (Fig. 2). *Solid lines* are Henderson–Hasselbalch titration curves with the pK_a value 2.8 (46).

EPR spectra of the biradical disulfides

EPR spectra of the biradicals R_1SSR_1 and R_2SSR_2, shown in Figs. 5A and D, are significantly affected by the spin exchange between two radical fragments. The EPR spectrum of the R_1SSR_1 was interpreted in terms of a single effective conformation of the biradical with exchange integral, $J = 3.5\ a_N$ (48). The EPR spectrum of the R_2SSR_2 demonstrates the contribution of several conformations with an averaged exchange integral $J \approx a_N$ (47). The disulfide bond of the biradical R_1SSR_1 decomposes spontaneously in a few seconds in alkaline medium (pH > 11), forming two monoradicals, while in acidic medium (pH < 6) it is stable for days. The rate of spontaneous disulfide bond splitting at physiological pH is low ($\tau_{1/2} \approx 18$ h in 10 m*M* sodium phosphate, pH 7.5, 23°C) and is not critical for most biological applications. The disulfide bond of the biradical R_2SSR_2 is extremely stable in an aqueous solution over a wide pH range from 1 to 12 for days or weeks. The EPR spectral parameters of the R_2SSR_2 are pH sensitive with pK = 2.8 (47) (Fig. 3B), therefore opening an additional possibility for using this disulfide label as a pH-sensitive one. Recently the imidazolidine methanethiosulfonate label **R7** (X = SSO_2CH_3) was used for site-directed electrostatic measurements (68, 93). Note that the R_2SSR_2 and **R7** (X = SSO_2CH_3) form identical adducts, R_2SSB, in the reaction with thiol BSH. However, the disulfide biradicals have an advantage in EPR visualization of the labeling reaction, allowing one to follow by EPR both R_2SSR_2 decay and R_2SSB formation.

Titration of the EPR spectra of the RSSR with GSH

Figure 5 demonstrates the typical changes of the EPR spectra of the biradicals R_1SSR_1 and R_2SSR_2 upon the addition of thiols. The decrease of biradical spectral components and the simultaneous increase of the monoradical ones is consistent with the splitting of the disulfide bond of the biradical and the formation of two monoradicals with conventional triplet EPR spectra according to reaction [1]. A double integration of the EPR spectra of the biradicals obtained at different concentrations of GSH indicates the preservation of the integral intensity of the EPR signal, indicating that there was negligible reduction of the nitroxide by GSH under these conditions. The maximal increase of the intensity of the monoradical components in the presence of excess of GSH (Fig. 5) is ~15 times for the R_1SSR_1 and 5 times for the R_2SSR_2. The dependencies of the relative changes of the peak intensities of the monoradical ($\Delta I_m/I_m^0$) and biradical ($\Delta I_b/I_b^0$) components (Fig. 5) of the EPR spectra on the ratio of the concentration of thiols and biradical is determined by calibration, and they are linear at low concentrations of the thiols ([BSH] ≤ 0.3 $[RSSR]_0$). Therefore, the following simple equations might be used for the determination of [BSH] from the spectral peak intensity changes:

$$[\text{BSH}]/[\text{RS-SR}]_0 = -(I_b - I_b^0)/I_b^0; \text{ or } [\text{BSH}]/[\text{RS-SR}]_0 = (I_m - I_m^0)/(I_m^{max} - I_m^0) \qquad \text{[Eq. 2]}$$

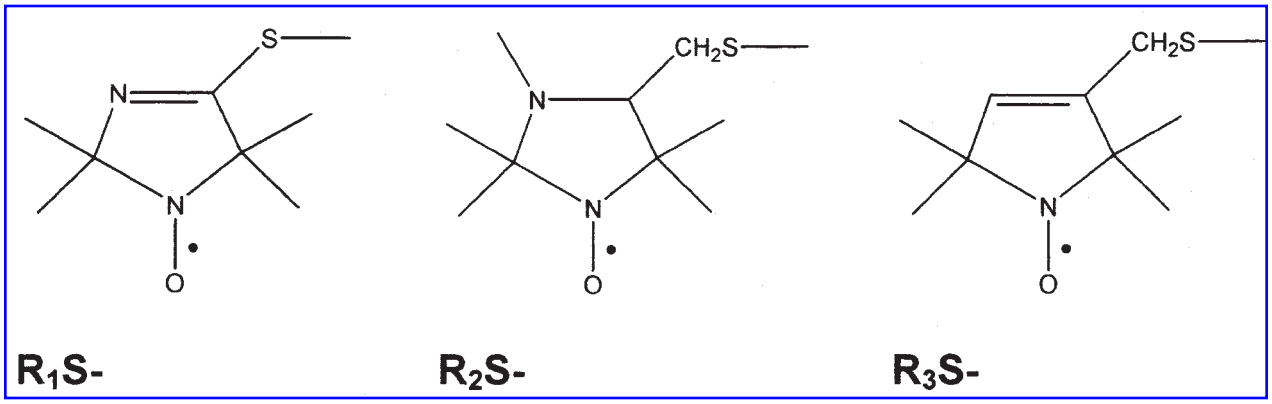

FIG. 4. Structures of the paramagnetic fragment, R_iS-, of the disulfide biradical reagents R_iSSR_i.

where $[RSSR]_0$ is the initial concentration of the biradical; I_b^0, I_m^0 and I_b, I_m are spectral peak intensities of the biradical and monoradical components before and after the addition of thiols, respectively; I_m^{max} is the maximal increase of the monoradical spectral peak intensity observed in the presence of excess thiols after the complete breakage of the disulfide bond of the biradical. Since the fraction of the reacted biradical is equal to the thiols consumed in the reaction, the ratio $\Delta I_b/I_b^0$ does not require calibration. Use of the ratio $\Delta I_m/I_m^0$ has the advantage of much higher sensitivity (Fig. 5) while its absolute value depends on the nature of the thiol and requires calibration. In the case of GSH and cysteine, similar thiol-induced EPR changes were observed (46) supporting the very small influence of the nature of the low molecular weight thiol BSH on the magnetic resonance parameters of the resulting monoradical RS-SB. However, the structure of the larger BSH molecules might influence the $\Delta I_m/I_m^0$ calibration.

Reversible labeling of protein sulfhydryls by biradical disulfides

The disulfide biradicals **R_1SSR_1** and **R_2SSR_2** have been used for the reversible labeling of accessible SH groups of human serum albumin (HSA) and human hemoglobin (47, 48), NADPH-cytochrome P-450 reductase, acetylcholinesterase

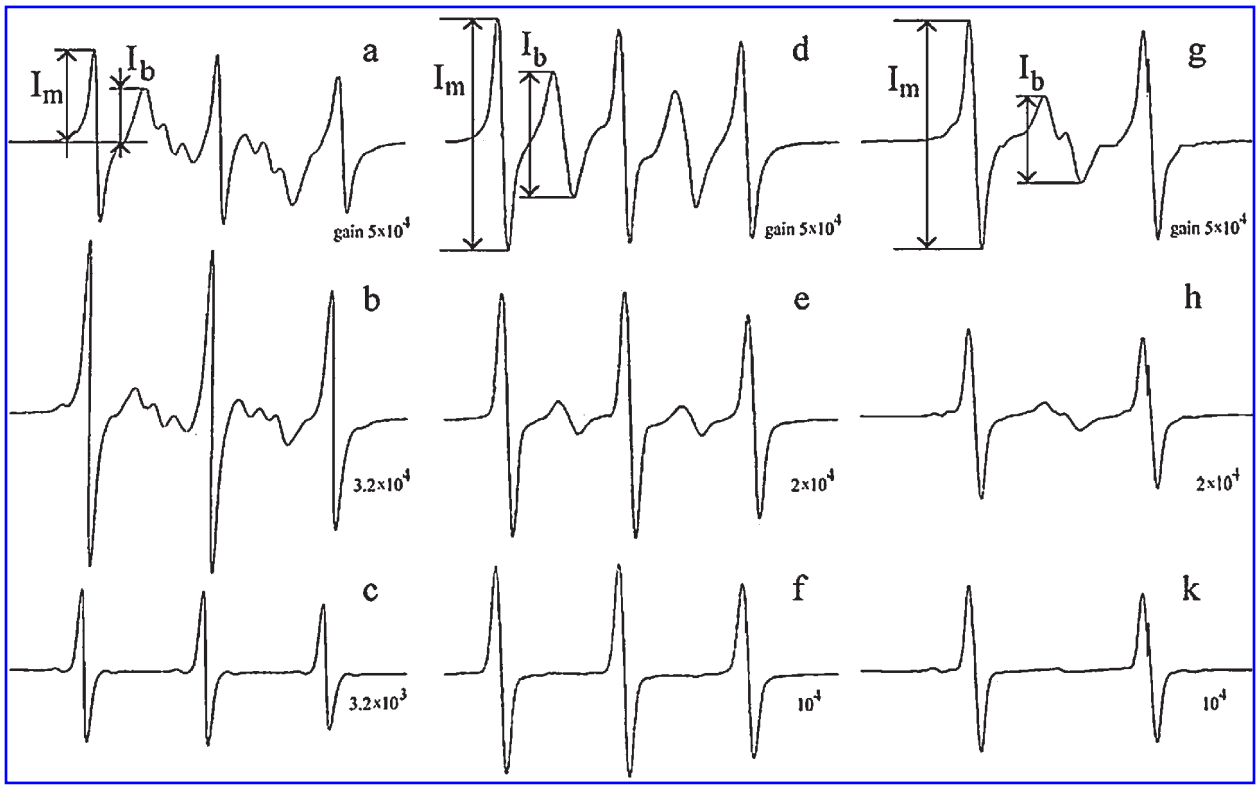

FIG. 5. X-band EPR spectra of 0.1 *M* aqueous solutions of the disulfide labels R_1SSR_1 (a, b, c), R_2SSR_2 (d, e, f), and ^{15}N-substituted $R^*_2SSR^*_2$ (g, h, k) alone and after various additions of the GSH: 7.5 μM (b), 30 μM (e), 10 μM (h), and 1 mM (c, f, k). The spectrometer settings were as following: total scan range, 50 G; microwave power, 20 mW (**a–f**) or 10 mW (**g–k**); modulation amplitude, 0.8 G (**a–f**) or 0.5 G (**g–k**); and gain indicated near the spectra.

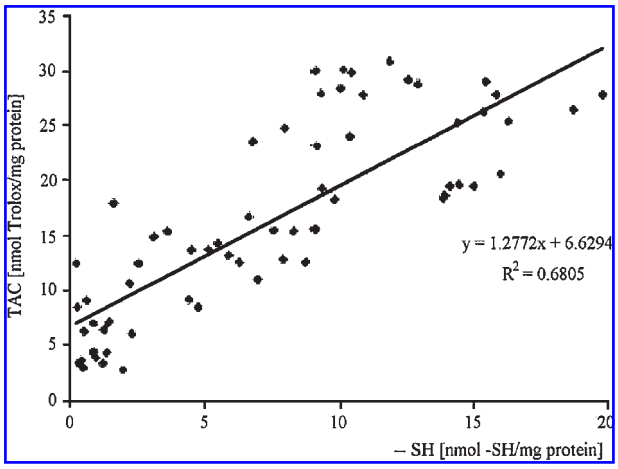

FIG. 6. Correlation between the thiol content and total antioxidant capacity (TAC) of extracts of various cell lines (pooled data for control and treated cells). From Balcerczyk and Bartosz (3) with kind permission from Taylor & Francis Group.

from *Torpedo californica*, and alcohol dehydrogenase from *Thermoanaerobium brockii* (112). The reversible modification of SH groups of enzymes by disulfide biradicals allows the study of the influence of the disulfide reagent-induced structural changes of the protein on enzymatic activity. Additionally, by taking advantage of the biradicals, one can follow the kinetics of protein labeling directly by measurement of the kinetics of the decrease of the biradical component, I_b, and/or the increase of the monoradical component, I_m (Fig. 5). For instance, the treatment of alcohol dehydrogenase with o-phenantroline removes the Zn^{2+} atom from its active site and results in an increase of the rate of the protein labeling by biradical $\mathbf{R_1SSR_1}$ (112). The author interpreted these data as indicating the appearance of an additional SH-group that became accessible for interaction with the biradical disulfide, which therefore probably is a ligand of Zn^{2+}.

Measurement of intracellular glutathione/thiols

The biradical $\mathbf{R_1SSR_1}$, being a lipophilic compound, diffuses easily across cellular membranes where it undergoes fast reactions with intracellular thiols. This biradical reacts, particularly with glutathione, at physiological pH within a few seconds, therefore providing a fast and reliable EPR approach for determination of GSH in optically nontransparent samples. The authors (3, 7, 8, 48, 113) used the $\mathbf{R_1SSR_1}$ label to measure GSH and/or total thiols in various cells and cellular homogenates. There were good correlations between the content of intracellular thiols and the total antioxidant capacity for the various cell lines (3) (Fig. 6). The authors (3) used GSH to calibrate spectral changes of the $\mathbf{R_1SSR_1}$, which is in agreement with a dominant contribution of the GSH in the intracellular pool of the fast-reacting thiols. Another way to calibrate the $\mathbf{R_1SSR_1}$ spectral changes is to measure maximal spectral intensity, I_m^{max}, observed upon excess addition of the thiol-containing sample, which corresponds to complete disappearance of the biradical (see Eq. [2]). The sensitivity of the EPR approach allows detection of the intracellular GSH in a few hundred cells (113).

EPR measurements of thiols in blood, tissues, and isolated organs

The EPR approach using the biradical $\mathbf{R_1SSR_1}$ has been applied for the measurements of GSH in erythrocytes, cysteine in blood plasma, and total thiol content in the blood (48, 117). The EPR studies of the thiols in human and rat blood show an increased level of the oxidized GSH in the blood plasma under oxidative stress conditions, including some human pathologies such as kyphoscoliosis (117). The application of the above approach to thiol measurements in the isolated perfused rat hearts showed GSH depletion related to ischemia/reperfusion oxidative damage of the myocardium (71). The content of the protein sulphydryl groups in the myocardium was estimated from the EPR spectra of the tissue homogenate obtained after washing away the unbound radical and treatment by potassium ferricyanide to reoxidize reduced nitroxides (71).

Noninvasive measurement of thiols using disulfide R_2SSR_2 and its potential for in vivo use

The fast reaction of the biradical $\mathbf{R_1SSR_1}$ with GSH and other low-molecular-weight thiols is the basis of its application for EPR detection of thiols. This "static" EPR approach, however, while being simple and reliable for *in vitro* systems, cannot be used *in vivo* because vital thiols are consumed in the reaction with the label. The biradical $\mathbf{R_2SSR_2}$ reacts with thiols significantly slower, with the bimolecular rate constant for the reaction [1] with GSH being equal to 0.26 $M^{-1}s^{-1}$ (47) at pH 7.0 (cf., with $k_1 \approx 5 \times 10^3$ $M^{-1}s^{-1}$ for the $\mathbf{R_1SSR_1}$ (48)). This complicates the use of the static EPR approach using the $\mathbf{R_2SSR_2}$ reagent due to a possible incomplete reaction with the thiols and, therefore, requires a longer incubation time. On the other hand, this time scale could be used to follow the kinetics of the reaction, making feasible a "kinetic" approach for measuring thiols *in vivo*. The advantage of the kinetic approach is

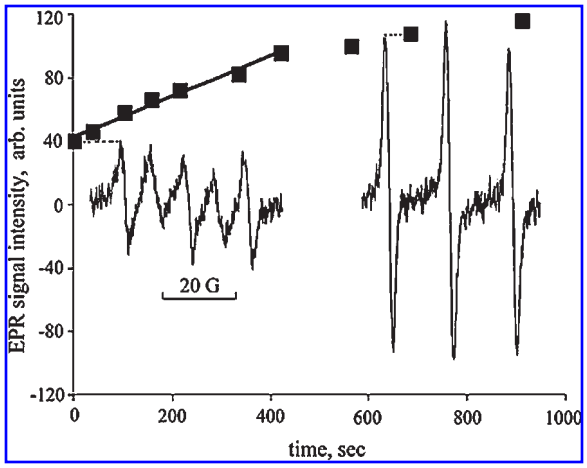

FIG. 7. The kinetics of the increase of the monoradical spectral peak intensity, Im, of the biradical R_2SSR_2 measured in blood from a Sprague–Dawley rat (45). The spectra are L-band EPR spectra of the 0.1 ml blood sample measured immediately (*left*) and 700 sec after (*right*) addition of 5 μl of the biradical to achieve a final concentration 50 μM. Acquisition time 10.5 s.

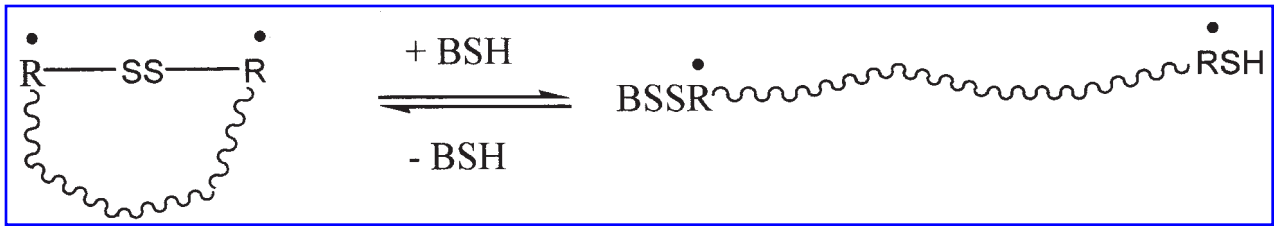

FIG. 8. Hypothetical biradical disulfide structures with the radical fragments participating in intramolecular thiol–disulfide exchange. The intensity of the spin exchange between monoradical fragments in general will be affected by the redox state of the disulfide bond of the label, and, therefore, provides sensitivity to the redox properties of the environment.

the possibility for the use of lower concentrations of the label, $[R_2SSR_2]<<[GSH]$, minimizing consumption of thiols and possible toxicity. Figure 7 demonstrates the kinetics of the increase of the peak intensity, I_m, of the biradical $\mathbf{R_2SSR_2}$ measured by L-band EPR spectroscopy in rat blood. The initial part of the kinetics in a range of <400 s allows a linear approximation, which can be used to estimate the GSH concentration (45). ($\mathbf{R_2SSR_2}$ freely penetrates erythrocyte membranes reacting preferably with GSH, while its reaction with the protein sulfhydryls is extremely slow (47)).

Low nontoxic concentrations of the label $\mathbf{R_2SSR_2}$ were used to measure thiols in rat blood, demonstrating an increased depletion of GSH in an animal model of stress-sensitive arterial hypertension (118). The findings suggest that oxidative stress may play a significant role in the pathogenesis of stress-sensitive hypertension in this rat strain resulting, in part, in an increased level of GSH oxidation (118).

The $\mathbf{R_2SSR_2}$ label may be easily adopted for *ex vivo* analysis of the blood samples. The label has the potential for *in vivo* use, but there are some limitations due to sensitivity, probe redistribution in the body, and reduction to the EPR silent hydroxylamine. We recently synthesized the derivative of the $\mathbf{R_2SSR_2}$ label, with the substituted ^{15}N isotope in the radical N-O fragment (85). This label, $\mathbf{R^*_2SSR^*_2}$, has a simplified EPR spectrum, which enhances sensitivity and simplifies interpretation (Fig. 5, G–K), and, therefore, has a higher potential for use *in vivo*, particularly for imaging applications.

One of the interesting future directions of the thiol-specific probes for *in vivo* EPR applications could be the development of disulfide probes with structures that allow intramolecular thiol–disulfide exchange as shown schematically in Fig. 8.

These probes would have advantages compared with the disulfide labels $\mathbf{R_iSSR_i}$, allowing use of simple "static" EPR measurements of the ratio between reduced and oxidized forms of the label, but not requiring excess label, therefore avoiding significant thiol consumption. It would provide the possibility of monitoring changes in the redox state of thiols in real time by *in vivo* EPR. Recently the above design of the disulfide label with intramolecular thiol–disulfide exchange was realized for a fluorescent redox sensitive probe (56).

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ABBREVIATIONS

BMPO, 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; DECPO, 2,2-diethoxycarbonyl-3,4-dihydro-2H-pyrrole-1-oxide; DEEPyON, *N*-[(1-oxidopyridin-1-ium-4-yl)-methyldene]-1, 1-bis (ethoxycarbonyl)ethylamine *N*-oxide, a diester nitrone; DEEPN, *N*-benzylidene-1, 1-bis(ethoxycarbonyl)ethylamine *N*-oxide; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DEPPEPO, 2-(diethoxyphosphoryl)-2-phenethyl-3,4-dihydro-2H-pyrrole-1-oxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTCS, *N*-dithiocarboxysarcosine; EMPO, 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; LPS, lipopolysaccharide; MGD, *N*-methyl-D-glucamine dithiocarbamate; PA, perinatal asphyxia; PBN, phenyl-*N*-*tert*-butyl nitrone; POBN, alpha-4-pyridyl-1-oxide *N*-*tert*-butyl nitrone; PPN, *N*-benzylidene-1-diethoxyphosphoryl-1-methyl-ethylamine *N*-oxide; PyOPN, N-[(1-oxidopyridine-1-ium-4-yl) methylidene]-1-diethoxyphosphoryl-1-methylethyamine *N*-oxide.

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