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METABOLISM OF PHENYL AND ALKYL SPIN ADDUCTS OF PBN IN RAT HEPATOCYTES. RATE DEPENDENCE ON SIZE AND TYPE OF ADDEND GROUP

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The decay of the EPR signal of phenyl and alkyl spin adducts of PBN has been monitored in rat hepatocytes. The rate of decay depends on the length of the hydrocarbon chain as follows: *n*-hexyl > *n*-dodecyl > *n*-octadecyl. The rate of signal decay is faster in the membrane phase than in the aqueous phase for the phenyl spin adduct.

KEY WORDS: α-Phenyl-N-*tert*-butyl-nitrone, PBN, EPR, spin adduct, spin labeling, hepatocyte, metabolism.

INTRODUCTION

Spin traps and spin trapping techniques are extensively used in chemistry and biomedical research[1-3]. In order to improve the usefulness of the spin trapping method, we have begun to study the mechanism of decay of the EPR signal of spin adducts.[4,5] In this communication, we report on the decay rates of phenyl and alkyl spin adducts of α-phenyl-N-*tert*-butyl nitrone (PBN) in rat hepatocytes. We have found that the EPR signal decay rates for PBN spin adducts depend on the relative hydrophobicities of the spin adducts. This result is similar to that observed for nitroxide spin labels.[6-8] In addition, it was possible to determine separate decay rates for the phenyl spin adduct (Ph-SA) of PBN located either in the aqueous or membrane region of the hepatocyte using the same spectrum. In this case, Ph-SA functions as a spin probe or spin label much as it does in the study of multimodal inclusion within the cyclodextrin cavity.[9]

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MATERIALS AND METHODS

Phenyllithium (2M), *n*-hexylmagnesium bromide, dodecylmagnesium chloride (1M), and PBN were obtained from Aldrich Chemical Company. Thin layer chromatographic (TLC) silica gel plates were from Merck and ascorbate oxidase from Sigma. All other chemicals used were of reagent grade.

Male Wistar rats (Charles River, Canada) weighing 100–120 g, were housed in individually suspended stainless steel metabolic cages in a temperature and humidity controlled room, illuminated from 8:00 to 20:00 hours. The rats were fed standard laboratory chow for at least 2 days before use and were fasted overnight prior to use for experiments.

For hepatocyte isolation, the rats were euthanized with carbon dioxide, the livers quickly removed and perfused by an open *in situ* method previously described.[10] Cells were counted and diluted to $20 \pm 2 \times 10^6$ cells/ml. Cell viability was estimated by the Trypan Blue exclusion test and only hepatocytes with viability of $90 \pm 2\%$ were used. Hepatocytes were stored under oxygen at 0°C and used for EPR measurements within a few hours.

The preparation of alkyl spin adducts in substantial quantities has been described before.[9] Either organolithium or organo-Grignard reagents were used for addition to PBN. The resulting hydroxylamine of the alkyl adduct can be oxidized to the nitroxide spin adduct. The following general procedure was used. Approximately 0.2 ml of a 2M organolithium or organo-Grignard reagent was added to a cooled vessel under nitrogen containing dry nitrogen-saturated benzene or hexane. The reaction mixture was vigorously agitated for 3 min and washed with 1% aqueous sodium bicarbonate. A small amount (two drops) of 10 μM aqueous cupric sulfate was added to the benzene or hexane layer and bubbled with oxygen for 3 min. The benzene or hexane layer was separated, washed with 1 ml water, dried over magnesium sulfate and filtered. The crude reaction mixture was separated on preparative TLC silica gel plates (20×20). When benzene/hexane/pyridine (60:50:1) mobile phase was used, yellow spots due to the alkyl spin adduct of PBN could be observed (based on EPR characterization). These spots were scraped from the plate, extracted with benzene and filtered. Samples used for decay experiments were prepared daily although stock solutions could be stored under nitrogen at −70°C. When the treated TLC plates were observed, either in an iodine tank or by using a UV-light, retention times could be obtained (see Table I).

EPR measurements were made with a Bruker ER-200D EPR spectrometer. Approximately 0.1 mM spin adduct in benzene solution was evaporated to dryness using nitrogen gas. The hepatocyte suspension was added and gently mixed. This dispersion was drawn into a glass capillary tube, sealed at the bottom with wax and placed in a quartz tube in an oxygen atmosphere. The initial concentration of the spin adduct was 0.01–0.05 mM. The tube was placed in the EPR cavity and the rate of spin adduct signal decay recorded by setting the magnetic field on the top of the desired peak in the spectrum. Decay curves could be obtained by setting the field sweep to zero. Temperature was controlled at room temperature or 37°C. The period of time from mixing spin adduct with hepatocytes to initial recording of spectrum was about 2 min. The decay was assumed to be first order and rates were calculated from initial slopes of curves which were plotted for 15–30 min.

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TABLE I
Retention times of alkyl spin adducts of PBN from thin layer chromatography

$Ph-CH(R)-N(O\cdot)-C_4H_9$		benzene hexane pyridine 60:50:1	benzene hexane methanol 50:50:1
R	Source	R_f*	R_f*
phenyl	PhLi	0.32	0.46
n-hexyl	$C_6H_{13}MgBr$	0.50	0.61
n-dodecyl	$C_{12}H_{25}MgCl$	0.56	0.71
n-octadecyl	$C_{18}H_{37}MgBr$	0.64	0.75
–	PBN	0.15	0.15

*R_f = A/B where A is advance of solvent front and B is position of component of interest.

RESULTS AND DISCUSSION

The EPR spectra of alkyl spin adducts obtained in benzene from straight chain addend groups consist of triplets of doublets with slightly diminishing nitrogen and β-H hyperfine splitting constants (hfsc's) as the length of the hydrocarbon chain increases (see Table II). The parameters for the phenyl adduct are even smaller, consistent with the electron-withdrawing effect of the sp^2 carbon atom of the phenyl ring on the nitroxyl function.

In hepatocytes, the EPR spectra show line broadening which is dependent on the branch of the nitrogen-14 triplet scanned; i.e. the high field group of lines is broadened most, the center field group is broadened least and for the low field group of lines, broadening is intermediate (see Figure 1). This effect is most clearly seen with the phenyl adduct since all lines are still relatively sharp. Immobilization in this case is not severe and the tumbling rate is considered to be near the fast rate limit. However, in the case of the *n*-hexyl adduct, all lines appear to be broadened more extensively and no resolution of separate components is possible in the high field wing. A detailed analysis of these spectra has not been attempted at this time.

TABLE II
Hyperfine splitting constants (in gauss) of phenyl and alkyl spin adducts of PBN*

$Ph-CH(R)-N(O\cdot)-C_4H_9$	Benzene		Phosphate Buffer (pH 7.4)	
R	a_N	a_β^H	a_N	a_β^H
phenyl	14.35	2.64	16.00	4.20
n-hexyl	14.52	3.35	16.20	3.40
n-dodecyl	14.47	3.30	16.12	3.42
n-octadecyl	14.40	3.20	–†	–†

*recorded at 37°C, ±0.05C.
†insoluble.

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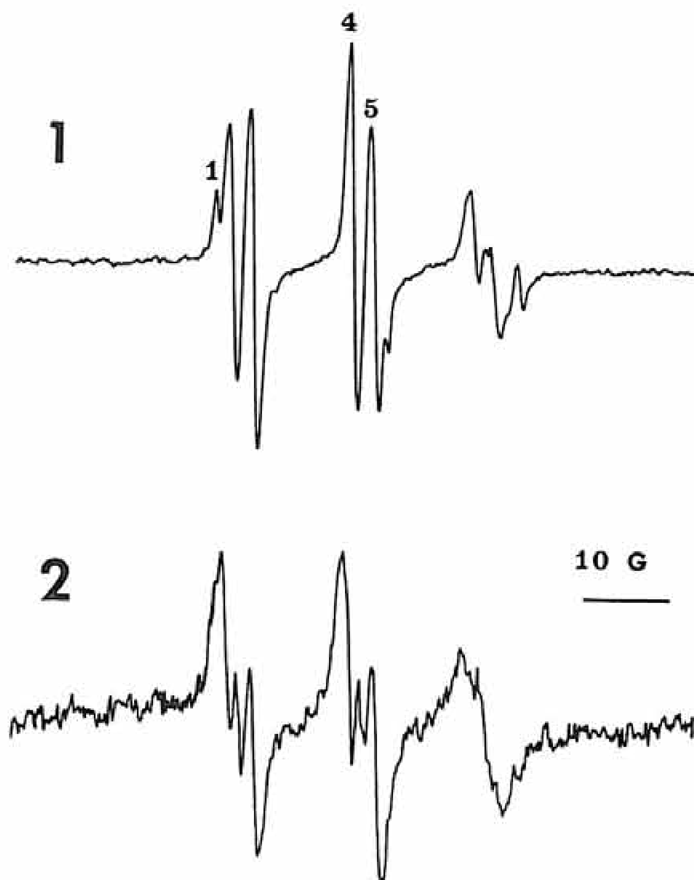

FIGURE 1 EPR spectra obtained from PBN spin adducts added to rat hepatocytes: Top, Phenyl Adduct; Bottom *n*-Hexyl Adduct.

Of further interest is the observation that separation of different components of the spin adduct solution occurs so that some portion is in a more polar region of the system and the remaining portion is located in a less polar region. This situation is most obvious in the case of the phenyl adduct. For example, in Figure 1 a portion of the spectrum is due to a nitroxide with $a_N = 14.90$ and $a_\beta^H = 2.90$ G and the remainder is from a nitroxide with $a_N = 15.96$ and $a_\beta^H = 4.10$ G based on computer simulation. Since the former is indicative of the phenyl spin adduct in a hydrocarbon-like environment slightly more polar than benzene, this portion of the spin adduct is assigned to the membrane phase. The EPR parameters for the latter are essentially the same in magnitude as those found in phosphate buffer and are thus assigned to the phenyl adduct in an aqueous phase. The separation of the phenyl spin adduct of PBN into two regions of a heterogeneous system has been reported before in the case of SDS micelles.[11]

In this study, we have followed the decay rate of the EPR signal of each component as a function of time. Thus, if the signal intensity is monitored either on peak

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TABLE III
Decay rates of phenyl spin adduct of PBN in rat hepatocytes depending on location*

EPR Line/Temp	1st Line (Aqueous phase)	5th Line (Membrane phase)
24°C	7.6 ± 1.9 (3)	22.2 ± 4.2 (3)
37°C	23.8 ± 2.5 (4)	67.0 ± 5.4 (3)

* 20 ± 2 × 10^6 cells/ml, 90 ± 2% viability; rates quoted as mean ± standard deviation (number of experiments).

1 or on peak 5, the ratio is approximately 3 at both 24° or 37°C (Table III). Since peak 1 is mostly spin adduct in the aqueous phase and peak 5 is mostly spin adduct in the membrane phase, it is possible to say that decay occurs faster in the membrane phase of hepatocytes than in the aqueous phase. Whether the detected spin adducts are located inside or outside the cell cannot be ascertained from the spectra or from the decay data.

In addition, we have found that the decay rates of the alkyl spin adducts of PBN depend on the length of the alkyl chain. Thus, when the decay rates of *n*-hexyl, *n*-dodecyl and *n*-octadecyl adducts of PBN are compared, a decrease in rate of decay is found in this series:

n-hexyl 8.0 ± $1.4^{(4)}$; *n*-dodecyl 2.4 ± $0.4^{(3)}$; *n*-octadecyl $0^{(3)}$; (at 24°C using the same conditions as in Table III and numbers in parentheses are number of trials).

The spin adducts listed do not decay in either benzene or phosphate buffer during the same time interval as studied with rat hepatocytes. Although the mechanism of EPR signal loss is not known, it is assumed that reduction to the hydroxylamine is the major route based on preliminary rat liver microsomal work.[12] In rat hepatocytes, the addition of ascorbate oxidase causes a lowering of the rate of signal decay using frozen/thawed hepatocytes. For the phenyl adduct, the decrease in decay rate is about 3X and for the hexyl adduct, the rate is lowered by approximately 13X. This result implicates ascorbic acid as a component of the spin adduct decay mechanism under the conditions chosen for these experiments.

In conclusion, it is likely that reduction of spin adducts occurs both in the aqueous phase as well as in the membrane phase. Although the rate of decay is faster in the membrane phase, an optimum region for reaction seems to be indicated since increasing the length of carbon chain produces a diminution of rate of decay.

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