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Binding of Spin-Labeled Local Anesthetics to Lobster Nerves

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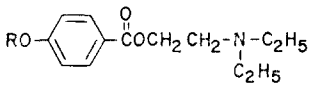
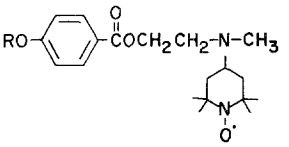
Summary. The hyperfine coupling constant of spin-labeled local anesthetics, 2-(N-methyl N-(2,2,6,6-tetramethylpiperidinooxyl)) ethyl 4-alkoxybenzoates, showed these compounds to partition between the aqueous exterior and the hydrocarbon phase of the membrane. Increased partitioning into the hydrocarbon phase of the membrane was in the order: hexyloxy > butoxy > ethoxy. Since these compounds are known to have different durations of anesthesia in the same order, this suggests that durations of activity and ability to partition into the hydrocarbon region of the membrane are related.

Local anesthetics block the action potential of nerve axons by preventing the transient increase in permeability of sodium to the axonal membrane [14, 16]. The first step in the production of local anesthesia is believed to be an interaction of the drug with the lipid bilayer [15, 19]. Since most local anesthetics are basic tertiary amines with pK_a values of 7 to 9, at physiological pH they exist in both charged and uncharged forms. Narahashi [13] and others have shown that the charged form is responsible for nerve block activity and exerts its effect from inside of the membrane. However, whether these compounds are completely buried in the bilayer, partially or completely exposed to the outside, is unknown.

A technique which is ideally suited to study these possibilities is that of spin labeling as developed by McConnell and colleagues [10] and Jost *et al.* [7]. It is based on line shape changes of the electron spin resonance spectrum of the nitroxide free radical. In situations where the radical can tumble unrestricted as in solution, three narrow symmetrical lines result. As the molecular motion of the radical decreases the lines become broad and asymmetrical. In addition, since the spectrum of the nitroxide radical is dependent on the dielectric constant of its solvent environment, it can give information concerning the polarity of its binding site. Although other techniques are more capable of giving quantitative data on binding, such as radioactive labeling, they cannot give information concerning the molecular properties of the binding sites.



Intracaine derivatives
(2-(N,N-diethyamino)ethyl 4-alkoxybenzoates)



Spin labelled analogues
(2-[N-methyl N-(2,2,6,6-tetramethylpiperidinooxyl)]ethyl 4-alkoxybenzoates)

$R = CH_2\ CH_3$
$R = (CH_2)_3\ CH_3$
$R = (CH_2)_5\ CH_3$

Fig. 1. Spin-labeled local anesthetics, 2-[N-methyl N-(2,2,6,6-tetramethylpiperidinooxyl)] ethyl 4-alkoxybenzoates, and the nonspin-labeled compound, 2-(N,N-diethylamino) ethyl 4-alkoxybenzoates (commercial name: intracaine)

In a previous paper [4] we reported the synthesis and pharmacological testing of spin-labeled local anesthetics, 2-(N-methyl N-(2,2,6,6-tetramethyl-piperidinooxyl)) ethyl 4-alkoxybenzoates (Fig. 1). The spin-labeled derivatives were shown to have anesthetic properties very close to those of the non spin-labeled compounds. For this series of local anesthetics the duration of anesthesia was shown to increase on elongating the hydrocarbon chain [4]. We now report experiments utilizing the spin-labeled local anesthetics in combination with nerve membrane. Our results suggest these compounds partition between the external aqueous environment of the membrane and its hydrocarbon-like interior. Also, those compounds which partition in favor of the hydrocarbon environment have considerably longer duration of activity.

Materials and Methods

Biological

The walking leg nerve of the lobster *Homarus americanus* was carefully dissected out and washed with ice cold Ringer's (NaCl 457 mM, $CaCl_2$ 25 mM, KCl 10 mM, Na_2SO_4 4 mM, $MgCl_2$ 8 mM, pH 7.4 Tris-HCl buffer). Next, it was immersed in the solution of choice for the appropriate time, then washed with Ringer's. In experiments aimed at determining the affinity of the drug for the nerve, after spin labeling, the nerve

was washed with 60 ml of Ringer's, then allowed to agitate for 1.5 hr in 200 ml of Ringer's, and finally, 12 hr in a fresh change of 200 ml of Ringer's.

The ESR spectra were recorded in a quartz tissue cell. Unless noted otherwise all experiments were performed at room temperature.

Chemical

The spin-labeled local anesthetics were synthesized and tested for drug activity as previously described [4]. They will be referred to as R2C for the ethoxy, R4C for the butoxy and R6C for the hexyloxy derivatives (Fig. 1). A 0.5 mM solution of each drug was heated to 60 °C for 1 min in Ringer's. The solution was allowed to return to room temperature before use. Phospholipases A and C were obtained from Calbiochemicals (bee venom) and Worthington (*Clostridium welchii*), respectively. Both enzymes were made up in Ringer's and used without prior heating.

The spin-labeled local anesthetics had no effect on the hydrolysis action of phospholipase A as measured by the production of fatty acids using the titration technique of Dole and Meinertz [3]. Under similar experimental conditions phospholipase C was also active [17]. Osmium tetroxide was from Alpha Inorganic chemicals and made up in S-Collidine buffer, pH 7.4. Nerves were soaked for 5 min at room temperature in a 1% solution. Methylenedichloride and hexane (spectro-grade) were purchased from Matheson, Coleman and Bell, and were degassed with nitrogen before use. They were used in a ratio of 1 to 1.

ESR spectra were recorded with a Varian E-3 spectrometer at a power setting of 5 mW.

Results and Discussion

Model System Measurements

To properly interpret the ESR spectra of the spin-labeled local anesthetics when in combination with the axonal membrane, we first made measurements on model systems. The ESR spectra of R4C in Ringer's and degassed methylenedichloride-hexane are shown in Fig. 4*A*, *B*. Similar spectra were obtained from R2C and R6C; these data are summarized in Fig. 2. Note the hyperfine coupling constant (A_n) which decreased from

	Ringer's	Hexane-methylene dichloride	Lobster Nerve
$R=CH_2CH_3$	16.7	15.3	16.7
$R=(CH_2)_3CH_3$	16.7	15.2	—
$R=(CH_2)_5CH_3$	16.7	15.2	15.2

Fig. 2. Hyperfine coupling constants of the spin-labeled local anesthetics in lobster Ringer's, methylenedichloride-hexane (1:1), and with the walking leg nerve

16.7 gauss to 15.2 gauss in going from Ringer's to methylenedichloride-hexane. Similar solvent dependent A_n values have been reported previously [7].

Axonal Membrane Measurements

Bathing the lobster walking leg nerves for 1 hr in Ringer's containing the spin-labeled local anesthetics at a concentration of 0.5 mM, and washed with 30 ml of Ringer's gave rise to spectra shown in Fig. 3. For R2C, the nitroxide radical gave three narrow lines with a hyperfine coupling constant of 16.7 gauss. This is similar to the spectrum of the compound in Ringer's alone. Thus, this drug is tumbling very rapidly in a polar environment when combined with the walking leg nerve. Identical treatment of nerves with R4C local anesthetic gave a composite spectrum (Fig. 3). We interpret the high field peak (noted with the arrow) to arise from one population of drug molecules tumbling rapidly in a polar environment. The remaining population is situated in a nonpolar hydrocarbon-like environment with weakly restricted motion. This is more clearly shown by mildly warming the sample. Warming the nerve to 40 ± 5 °C has a twofold effect: 1) the compound completely partitions in favor of the hydrocarbon region as noted by the absence of the solution component; and 2) it converts the weakly immobilized component to one with a high degree of mobility. The A_n value of this spectrum is 15.2 which is identical to that for R4C in methylenedichloridehexane. Similar mobility and partitioning effects using other lipid-soluble spin probes have been reported [11, 12].

The spectrum of compound R6C (Fig. 3) is similar to that of R4C. However, note the absence of the high field solution peak. This would be expected if the compound partitioned more favorably into the hydrocarbon region of the membrane. This spectrum has a hyperfine coupling constant of 15.2 gauss which is identical to that of the compound in methylene dichloride-hexane. Thus, this series of local anesthetics shows an increased partitioning into the hydrocarbon region of the membrane in the order: R6C > R4C > R2C.

Whether these compounds are in the hydrocarbon interior of the membrane near the aqueous interface, or deeply buried in the bilayer is unknown. Preliminary work from this laboratory, however, based on the ability of the spin-labeled drug's nitroxide radical to quench 1-anilinonaphthalene-8-sulfonate fluorescence (D. Koblin *et al.*, *to be published*) suggests a superficial location in the hydrocarbon phase. The NMR data of Hauser *et al.* [6] and Cerbon [2] support this conclusion.

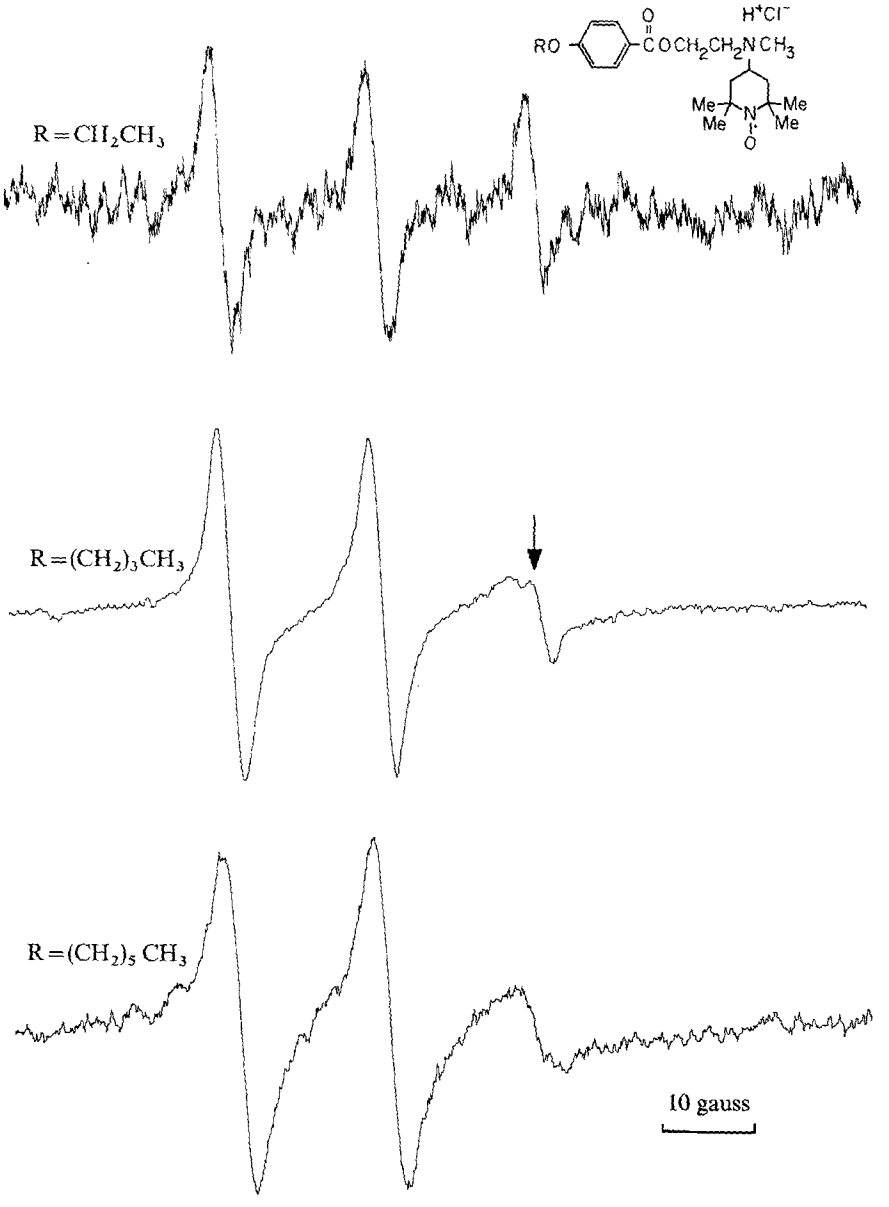


Fig. 3. ESR spectra of a series of 2-[N-methyl, N-(2,2,6,6-tetramethylpiperidinooxyl)] ethyl 4-alkoxybenzoates with the walking leg nerve of the lobster *Homarus americanus*

Simulated Spectra in Model System

To test our hypothesis that these probes are partitioning between an aqueous and hydrocarbon environment we tried simulating the spectrum for R4C. One capillary tube was filled with Ringer's containing R4C and

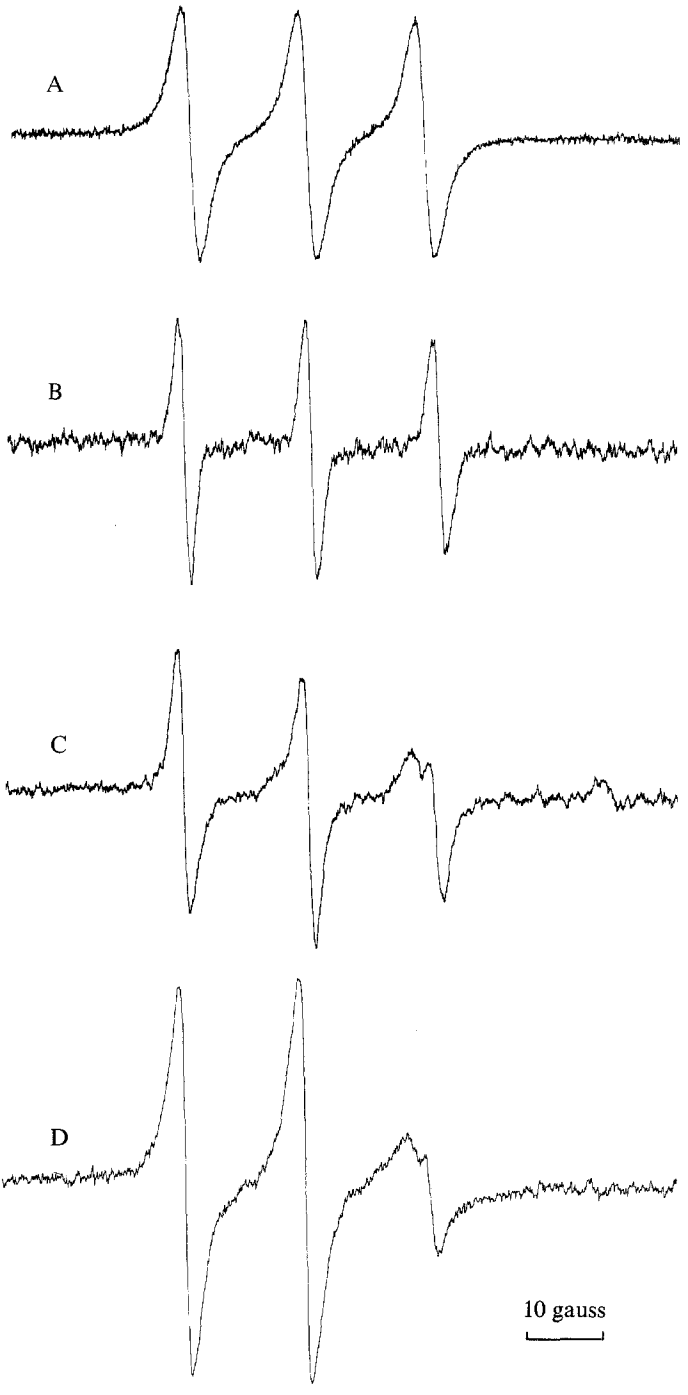


Fig. 4. ESR spectra of the butoxy spin-labeled analog (*A*) in a capillary tube containing methylenedichloride-hexane, (*B*) in a capillary tube containing Ringer's, (*C*) in two separate capillary tubes, one containing methylenedichloride-hexane and the other Ringer's, (*D*) with the walking leg nerve

another with methylenedichloride-hexane. Both tubes contained the drug at a concentration of 0.5 mM. Next, they were inserted singly in the ESR cavity and the spectra recorded (Fig. 4*A*, *B*). Finally, they were simultaneously inserted in the cavity and the spectrum recorded (Fig. 4*C*). As can be seen the similarity in the simulated spectrum and that of the nerve (Fig. 4*D*) are striking.

Washing Experiments

Further support for different aqueous-hydrocarbon partitioning of these compounds in the membrane comes from washing experiments. As shown in Fig. 5, the ease with which these compounds wash off the nerve with Ringer's is: R2C > R4C > R6C. These results would be expected if there is increased partitioning of these compounds into the hydrocarbon region of the membrane. The order of this increased partitioning is R6C > R4C > R2C. Our data agrees with Bennett and coworkers [1] who studied the ability of the non spin-labeled analogs of R2C and R4C to block the action potential of the frog sciatic nerve. They showed that the ease of reversing the blocking effect by washing was: R2C > R4C.

Control for Hydrolysis

Local anesthetics are known to be hydrolyzed by esterases present in biological materials [8]. Thus, it is necessary to make certain the ESR spectra are reflecting the environment of the intact drug molecule, and are not due to unattached nitroxide radical produced by hydrolysis. That this is not the case was shown by incubating a nerve with 4-[N,-2-hydroxyethyl), N-methylamino]-2,2,6,6-tetramethylpiperidinooxy. This compound is one part of the expected hydrolysis product of the intact local anesthetic and contains the nitroxide radical. The ESR spectrum of the compound in the presence of the nerve has three sharp peaks with a hyperfine coupling constant of 16.7 gauss. Since this value is identical for the compound in Ringer's alone, it is unlikely that hydrolysis of the ester linkage is affecting our results.

Osmium Tetroxide, Phospholipases A and C

To further locate these compounds in the axonal membrane, reagents that attack different regions of phospholipid molecules were tried. Nerves spin-labeled with R6C were incubated with either phospholipase A or C. Both enzymes over concentration ranges of 0.5 mg/ml to 3 mg/ml after 1 hr of incubation at 37 °C had no effect on the ESR spectrum. These results

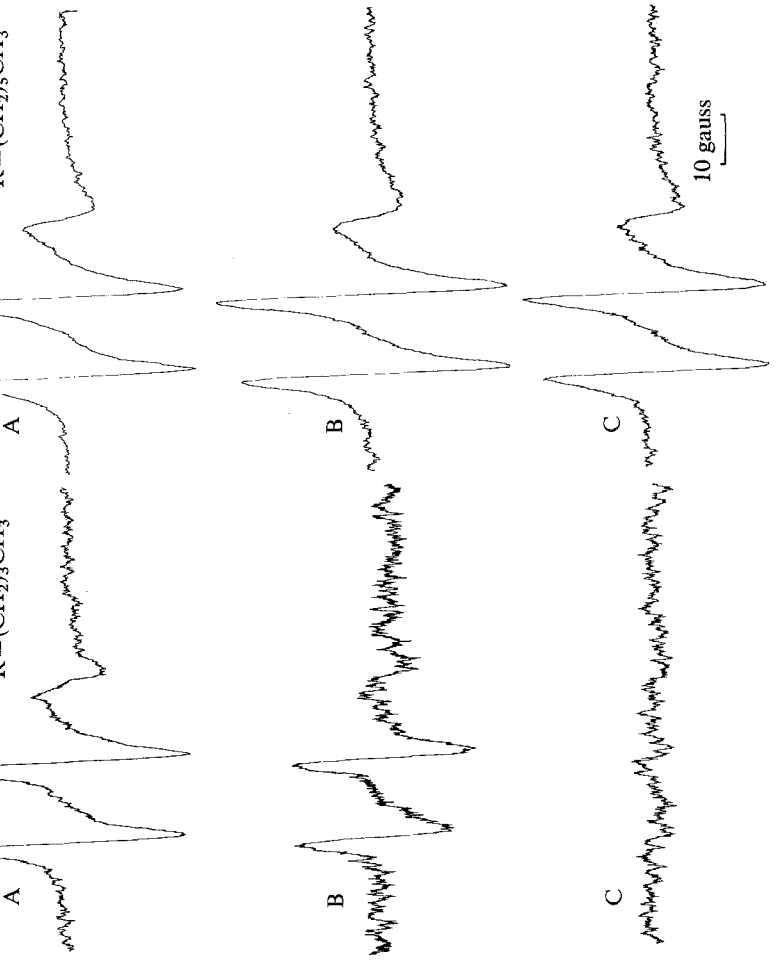


Fig. 5. ESR spectra of lobster walking leg nerves spin-labeled with R2C, R4C or R6C and then (*A*) washed with 60 ml of Ringer's, (*B*) agitated for 1.5 hr in 200 ml of Ringer's, (*C*) agitated for 12 hr in 200 ml of Ringer's

are consistent with our previous findings since: 1) phospholipase C is known to produce little perturbation to the lipid bilayer of the membrane [17]; and 2) phospholipase A, while known to cause structural changes in the bilayer [18], probably does not alter its internal dielectric constant. This is supported by previous work which has shown that neither product of hydrolysis, fatty acids or lysolecithin, are removed from the membrane [5, 18]. Thus, the solvent environment surrounding the spin-labeled local anesthetic should be minimally affected.

The lack of effect on the ESR spectrum of R6C with phospholipase A and C is in striking contrast to the results obtained with osmium tetroxide. As shown in Fig. 6, osmium tetroxide converted the weakly immobilized spectrum to a composite spectrum consisting of: 1) a strongly immobilized component, as represented by the low and high field peaks separated by approximately 64 gauss; and 2) a rapidly tumbling radical. While the chemistry of osmium tetroxide reacting with biological membranes is not known in detail, it has been shown that osmium tetroxide is capable of reacting with unsaturated double bonds to cross link adjacent fatty acyl residues of phospholipid molecules [9]. This is probably responsible for the severe restriction of molecular motion of R6C observed in our spectra and agrees with our other data which shows this compound to be located in a hydrocarbon-like environment. It is interesting to note that osmium tetroxide has been shown to have similar effects on spin-labeled fatty acids [7].

Conclusion

We have presented data which suggest that the spin-labeled local anesthetics partition into the hydrocarbon region of the membrane in the order of R6C > R4C > R2C. That these local anesthetics interact directly with the hydrocarbon region of the membrane was supported by the hyperfine coupling constants which, for R2C, was 16.7 and decreased to 15.2 for R6C. As shown in our model system, A_n values of 16.7 are typical of the nitroxide-labeled drugs in aqueous solvents, while A_n values of 15.2 are typical of the nitroxide radical in hydrocarbon solvents. Thus, in combination with the axonal membrane, R2C was primarily located in the aqueous exterior of the membrane while R6C was shown to be primarily in the hydrocarbon region.

These interpretations are further supported by simulated spectra of R4C where the partition between aqueous and hydrocarbon environments were nearly identical to that observed with R4C in the presence of the lobster nerve.

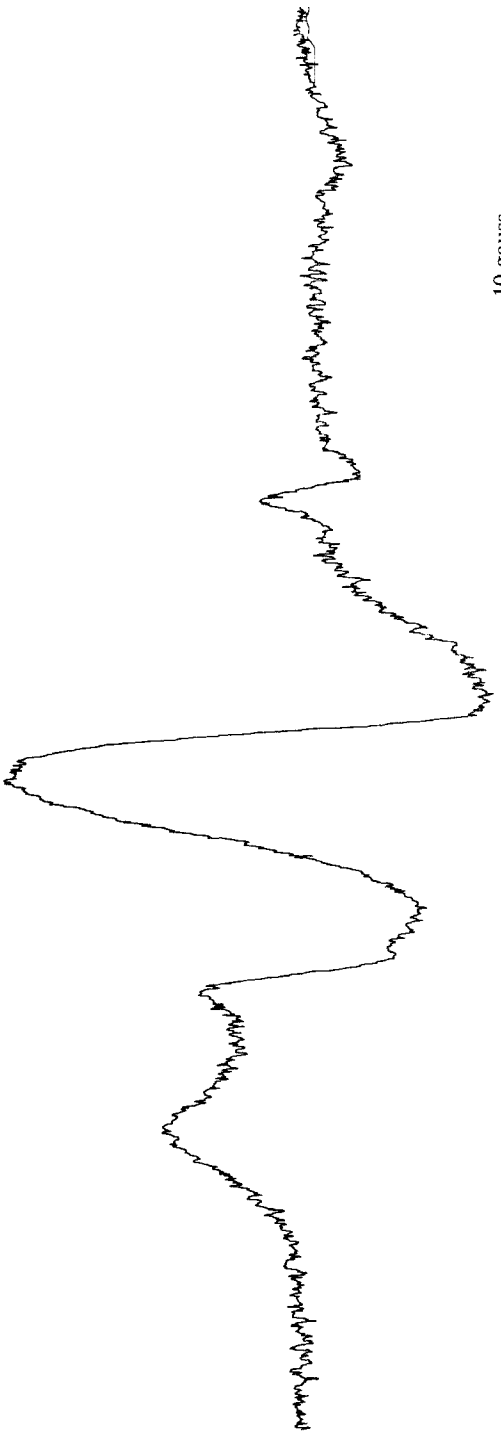


Fig. 6. ESR spectrum of a walking leg nerve spin-labeled with the hexyloxy derivative and then treated with a 1% solution of osmium tetroxide for 5 min at room temperature

Washing experiments showed R2C to have a weak affinity for the axonal membrane, R4C had an intermediate binding affinity and R6C had a strong binding affinity. This would be expected if the compounds partition into a hydrophobic region of the membrane in the order of R6C > R4C > R2C.

Osmium tetroxide severely restricted the molecular motion of the nitroxide radical. Again, this is in agreement with these drugs being in the hydrophobic region of the membrane since osmium tetroxide is known to cross link unsaturated fatty acyl molecules of the membrane phospholipids [9].

In a previous communication, the spin-labeled local anesthetics were shown to have different durations of anesthesia [4]. The order of increasing duration was: R6C > R4C > R2C. In this report we have presented evidence which suggests that the duration of anesthesia of these compounds is related to their ability to dissolve in the hydrocarbon region of the membrane as shown by the partition order: R6C > R4C > R2C. Thus, those compounds which partition in favor of the hydrocarbon region of the membrane have a considerable longer duration of activity.

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