*SPIN-LABEL STUDIES OF THE EXCITABLE MEMBRANES OF NERVE AND MUSCLE**

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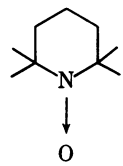
The present paper describes the results of an exploratory study of the structure of a number of biological membranes in which we used the spin-label[1] 2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPO),



This paramagnetic probe gives a characteristic resonance spectrum when dissolved in low-viscosity liquid-like hydrophobic regions of membranes of nerve and muscle. These liquid-like hydrophobic regions were also found in phospholipid vesicles in solution, but were not detected in a number of other systems, such as membranes from erythrocytes. The effects of cholesterol, calcium ion, local anesthetics, and other substances in changing the solubility of TEMPO in phospholipid vesicles indicate that this solubility is sensitive to the conformational state of the lipids and may therefore be useful for studies of conformational changes in lipid regions of biological membranes.

Insofar as there is overlap, conclusions reached from the present work are in excellent accord with earlier X-ray and nuclear resonance studies of membranes and lipid-water systems.

Experimental.—The preparation of the TEMPO spin label has been described previously.[2] Vagus nerves were excised from rabbits anesthetized with urethane (1.6 gm/kg of a 25% solution) administered in the marginal ear vein. Nerves were cleaned and desheathed under a microscope. The Locke's solution (without glucose) used for the vagus was given by Armett and Ritchie.[3] We are greatly indebted to Dr. David Nachmansohn and Dr. W. D. Dettbarn for suggesting the vagus and for instructions on its preparation.

Unmyelinated walking leg nerves were obtained from *Homarus americanus* (Maine lobster) and bathed in fresh, filtered seawater. Skeletal muscle was obtained from *Rana pipiens* and bathed in frog Ringer's solution. Mitochondria isolated from *Neurospora crassa* were a gift from Dr. D. Woodward. Sarcoplasmic vesicles from rabbit skeletal muscle were a gift from Dr. G. Inesi. Glycerinated muscle fibers prepared from rabbit psoas muscle were a gift from Dr. M. Morales. Human erythrocyte membranes were prepared according to Dodge *et al.*[4] from outdated human blood obtained from the San José Red Cross Blood Bank. Purified soybean phosphatides (asolectin) were purchased from Associated Concentrates, Woodside, New York.

Nerve fibers approximately 3 cm long were contained in no. 16 Teflon tubing ($\sim$ 2-mm inside diameter) through which the solution of interest flowed while the paramagnetic resonance was observed. The fibers were tied at each end with filaments from nylon stockings. The Teflon tubing was held in a Varian microwave resonance cavity with a Lucite or Kel-F support, which permitted the Teflon tubing to be compressed gently so as to minimize excess surrounding fluid.

Results.—The paramagnetic resonance spectrum obtained from a vagus nerve that had been soaked for five minutes in a Locke's solution containing 5×10^{-4} *M* TEMPO is shown in Figure 1. This treatment had no apparent effect on the electrical activity of the nerve. The spectrum is readily interpreted as the sum of two spectra; one arises from radicals in aqueous solution and the other from radicals in a *low-viscosity fluid* hydrophobic region. The spectrum in Figure 1 can be duplicated by recording the spectrum of a dual sample, one a solution of TEMPO in water and the other a solution of TEMPO in dodecane. In Figure 1, signals *A* and *B* arise from TEMPO in the hydrophobic and aqueous environments, respectively. In the two other hyperfine components, the signals from the labels in the two environments are not resolved. The difference between the spectra in the aqueous and hydrophobic environments is due to small solvent effects on the isotropic hyperfine interaction and average *g*-factor.[1]

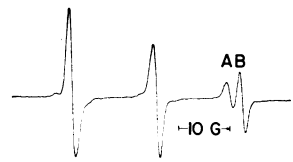


FIG. 1.—The paramagnetic resonance of the spin-label TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) in the liquid-like hydrophobic region of the rabbit vagus nerve (*A*) and in surrounding aqueous solution and axoplasm (*B*). The spin-label signals from these environments coincide for the other two hyperfine components of the spectrum.

The line shape of signal *A* shows that TEMPO must be tumbling in the hydrophobic fluid environment at a very rapid rate, with a correlation time in the order-of-magnitude range 10^{-9} to 10^{-11} sec.[1] The TEMPO dissolved in the nerve fiber is in reversible equilibrium with the TEMPO dissolved in Locke's solution; the relative signal intensity A/B decreases as the volume of Locke's solution bathing the nerve is increased.

Although the vagus is largely unmyelinated,[7] we do not know to what extent the hydrophobic component of the spectrum arises from TEMPO in the axonal membranes, and to what extent it arises from TEMPO in various neuroglia. Indeed, from the early X-ray work of Schmitt, Bear, and Palmer,[8] one expects that the myelin may contain fluid hydrophobic regions. The excitable membranes of muscle, however, are apparently free of interfering cells and are believed to be very similar to the axonal membrane.[9] Intact muscle, as well as isolated sarcoplasmic vesicles,[10] give spectra with TEMPO that are equivalent to those obtained from the vagus. Glycerinated muscle fibers (muscle fibers with the membrane system removed) show no binding of TEMPO. We find also that the unmyelinated walking leg nerves of *Homarus Americanus* exhibit the same binding and spectra for TEMPO as do the vagus nerve and muscle membrane. Of course, in each case, the relative intensities of signals *A* and *B* depend on the amount of Locke's or Ringer's solution bathing the preparation. Signals *A* and *B* are not quite as well separated for the walking leg nerves of *Homarus americanus* as they are in Figure 1 for the case of the rabbit vagus. At the concentrations of TEMPO used in our experiments (5×10^{-4} *M*), the ratio of signal intensities A/B is independent of TEMPO concentration. In the hydrophobic regions where TEMPO dissolves, the local TEMPO concentration must be low, certainly less than 10^{-2} *M;* otherwise, there would be spin-spin

broadening of the spin-label resonance signals. This rigorously excludes the possibility that TEMPO itself forms a fluid lens[5] or droplet[6] in the hydrophobic region of the membrane.

Concentrated (i.e., packed) whole human erythrocytes, erythrocyte ghosts, and *Neurospora* mitochondria show no binding of the label: we observe a simple hyperfine triplet due to label in aqueous solution. Our failure to find highly fluid hydrophobic regions in erythrocytes is consistent with the recent nuclear resonance studies of Chapman, Kamat, de Gier, and Penkett,[11] who have found the lipid methylene proton signals to be very broad in erythrocyte membranes. TEMPO shows no affinity for the lipid-binding protein systems investigated. Thus, bovine serum albumin and erythrocyte ghosts extracted with 90 per cent aqueous acetone show no binding of the label.

Concentrated whole human serum, however, shows an interaction with TEMPO very similar to that of the excitable membranes. Presumably, the label binds to the lipoprotein fraction of serum.

When a nerve is treated with ether prior to labeling, the binding of TEMPO is not affected. When the nerve is treated with ether-ethanol (1:1 by volume), the nerve completely loses its binding capacity for the label. This is consistent with the idea that TEMPO is dissolved in a phospholipid region, since it is well known that ether is a poor phospholipid extractant, whereas ether-ethanol is a very effective solvent for this purpose.[12]

Luzzati *et al.*[13] have emphasized the liquid nature of phospholipid hydrocarbon chains in lamellar structures in solution. In order to determine whether or not TEMPO distributes between water and bilayers of phospholipid in the same manner as it does between water and excitable membranes, vesicles of soybean phosphatides (asolectin) were prepared according to Papahadjopoluos and Miller.[14] When TEMPO is added to a solution of these vesicles, a spectrum essentially identical to that in Figure 1 is obtained. These vesicles have been well studied and it is believed that the phospholipids are arranged in a bilayer.[13, 14] Vesicles prepared from phosphatidyl serine, phosphatidyl ethanolamine, and egg yolk lecithin behave in the same way as asolectin vesicles with respect to TEMPO binding.

The spectra described above for TEMPO-labeled excitable membranes and phospholipid vesicles are to be contrasted with spectra obtained from TEMPO in solutions containing micelles of sodium dodecyl sulfate and sodium deoxycholate. The spectra of TEMPO in these micellar solutions show a simple isotropic splitting that is an average of that for a hydrophobic environment and that for an aqueous environment. Similar results for other spin labels in sodium dodecyl sulfate micellar solutions have been obtained previously by Waggoner, Griffith, and Christensen.[15] In micelles of sodium oleate, the two signals analogous to A and B in Figure 1 are just barely resolved.

We have found that some compounds increase the solubility of TEMPO in the hydrophobic regions of excitable membranes *and* phospholipid vesicles. For example, a tenfold increase in signal A and a concomitant decrease in signal B are observed when a low concentration ($\sim$10 mM) of butanethiol is added to a labeled solution of phospholipid vesicles or excitable membranes. We suggest

that this effect is due to a local disordering or "melting" of the hydrophobic regions of the membranes, which increases the "fluid" volume available to TEMPO. The enhancement of the hydrophobic TEMPO signal appears to be especially large with thiols, but can be easily detected with other lipid-soluble compounds such as octylamine or ethylbenzene. The local anesthetic tetracaine also enhances the solubility of TEMPO in hydrophobic regions. For example, a solution of tetracaine of the order of 20 mM enhances the solubility of TEMPO in phospholipid vesicles and in the walking leg nerve by a factor of the order of 2–5.

We have found that the incorporation of cholesterol in phospholipid vesicles decreases the solubility of TEMPO in the hydrophobic region. This result is consistent with studies of molecular areas in mixed films of cholesterol and lecithin, which show an enhanced packing of the film when both molecules are present.[16]

Also in accord with this picture are the nuclear resonance results of Chapman and Penkett,[17] who have found that methylene proton motions in lecithin suspensions are strongly inhibited by the addition of cholesterol. We have observed that gramicidin S likewise decreases the incorporation of TEMPO in the hydrophobic regions of phospholipid vesicles. We may therefore imagine that cholesterol, gramicidin S, and perhaps other large, rigid hydrophobic molecules—including proteins—tend to "freeze" the otherwise fluid hydrophobic regions and decrease the fluid volume available to TEMPO. The addition of Ca^{++} to suspensions of phospholipid vesicles also increases the tight packing of the bilayer, as judged by the reduced solubility of TEMPO in the hydrophobic region. Vagus nerves bathed with an isotonic KCl solution (in an effort to displace Ca^{++} from the excitable membrane) showed a very small ($\sim$5%) but statistically significant increase in hydrophobic TEMPO binding.

Summary.—(1) Certain excitable membrane systems—the vagus nerve of the rabbit, the walking leg nerve of *Homarus americanus*, and the excitable membrane of muscle—contain liquid-like hydrophobic regions of low viscosity.

(2) Extraction experiments with the rabbit vagus indicate that these hydrophobic regions of low viscosity are due to lipid components of the membranes.

(3) A number of systems showed no detectable binding of the TEMPO spin label. These include, for example, whole erythrocytes, erythrocyte ghosts, acetone-extracted erythrocyte ghosts, bovine serum albumin (soln.), and mitochondrial membranes from *Neurospora*.

(4) It is suggested that molecules that dissolve or intercalate in the hydrophobic region of an excitable membrane may have one of two possible effects: (*a*) A molecule such as butanethiol, ethylbenzene, or tetracaine can interact with the membrane so as to increase the volume of the fluid hydrophobic region and thus increase the binding of the TEMPO spin label. (*b*) A molecule such as cholesterol or gramicidin S may intercalate in the membrane, increase the packing or ordering of the molecules, and decrease the volume of the fluid hydrophobic region available to TEMPO.

(5) The low-viscosity hydrophobic regions of the excitable membranes and the phospholipid vesicles are very similar to one another with respect to the

TEMPO spectrum, the binding of TEMPO, and the effects of other substances on the binding of TEMPO. Thus, the excitable membranes must contain either phospholipid bilayers similar to those present in phospholipid vesicles, or other components with very similar hydrophobic regions.

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