SPIN-LABELING TECHNIQUES FOR STUDYING MODE OF ACTION OF PETROLEUM HYDROCARBONS ON MARINE ORGANISMS

WILLIAM T. ROUBAL[1] AND TRACY K. COLLIER[2]

ABSTRACT

Spin-labeling studies of membrane-contaminant interaction are being conducted by biochemists at the Northwest Fisheries Center in Seattle, Washington. The aim of these studies is to gain a better understanding of the mode of action of hydrocarbon contaminants at the molecular level. Basic spin-labeling theory together with experimental results are presented and discussed. Spin-labeling holds great promise not only for environmental studies but also for drug research, toxicology, and pharmacology as well.

The interaction between contaminants and living systems commences when contaminants combine with so-called active sites in living tissue. Active sites are varied in nature, but often they are groups of molecules assembled in a special fashion such as those which comprise membranes and associated enzymes or other biopolymers.

Although the exact nature of contaminant-host interaction may not be known in each and every case, experimental data from biochemical/biophysical studies allow us to draw certain conclusions about interactions. With detailed investigations, collected data may even allow us to draw a fairly accurate picture concerning the molecular basis of physiological changes which contaminants are able to induce.

Admittedly, investigations such as these are difficult to perform. The molecular complexity of living systems defies ready characterization, and it is even more difficult to relate alterations in molecular organization to the subsequent physiological changes wrought by this contaminant-host interaction.

Recent years have seen an upsurge of interest in membranes and how membrane structure is modified when invaded by such things as drugs and insecticides. The reasons are several: membranes house cells, control the influx and efflux of nutrients and metabolites; membranes control and form the basis for the transmission of electrical signals (nerve impulses) when nerve receptor sites are stimulated. Any major alteration to normal membrane structure may be expected to play some role in animal physiology, especially if the contaminated membrane is associated with neural function or other viable life processes.

Membranes consist of a sandwich of phospholipids, sterols, and proteins; individual membranes are microscopically thin. The thinness and complexity of membranes make their study most difficult. One way of characterizing membranes is by measuring their electrical properties (conductance, resistance, capacitance, etc.). However, with respect to contaminant-host interaction, it is more desirable to be able to deduce structural features such as lipid fluidity, protein-lipid interaction, and arrangements of constituents in dynamic tissue preparations. This, then, precludes the use of electron microscopy or other methods which are incompatible with maintaining tissue in a viable unchanged condition.

THE THEME OF THIS REPORT—SPIN-LABELING

Several years ago, it was observed that certain free radical derivatives of fatty acids could be introduced into membrane preparations without unduly disturbing the natural arrangement of native membrane constituents (Jost et al. 1971). Furthermore, it was shown that these free radicals would associate with and align in membranes

[1]Northwest Fisheries Center, National Marine Fisheries Service, NOAA, 2725 Montlake Boulevard East, Seattle, WA 98112.
[2]Northwest Fisheries Center, National Marine Fisheries Service, NOAA, 2725 Montlake Boulevard East, Seattle, WA 98112, and The College of Fisheries, University of Washington, Seattle, WA 98195.

Manuscript accepted July 1974.
FISHERY BULLETIN: VOL. 73, NO. 2, 1975.

much the same way as do the nonradical natural fatty acids present in membranes (Libertini et al. 1969; Hubbell and McConnell 1969; Schreier-Muccillo et al. 1973). By using appropriate instrumentation, it was found that these radicals could be used as submicroscopic probes (or labels) for investigating membrane structure. This has become known as spin-labeling, and forms the underlying theme of this paper. This report describes our work on contaminant-host interaction in fingerling salmon. To show how these studies were performed, we need to arm ourselves with some basic background information. Let us review briefly some very important points concerning radicals, electrons, and nuclei.

THE FOUNDATION OF SPIN-LABELING

Free Radicals

Many free radicals are known or have been isolated. Most, as we know, are quite reactive chemically, and unless conditions conducive to their formation and stabilization (trapping) are maintained, radicals normally disappear once formed. Radical reactivity stems from the fact that radicals, by definition, are molecules which contain one or more unpaired electrons. Two radical partners normally pair together to yield end products with the normal complement of two electrons per chemical bond. This is the usual covalent bond and is characteristic of organic compounds.

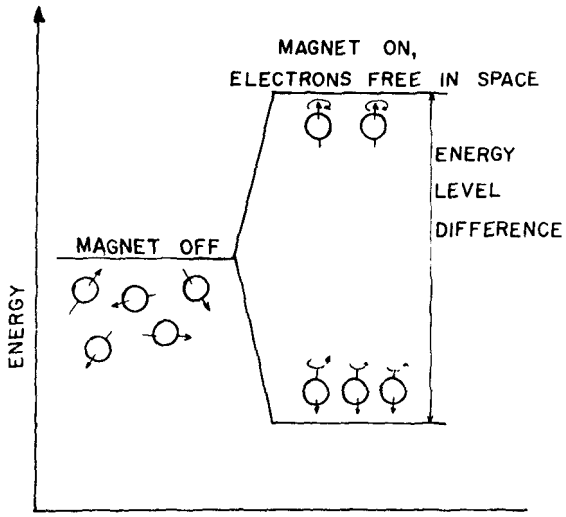

FIGURE 1.—Resonance condition for isolated electrons. In the absence of external field, energy levels are indistinguishable. When a magnetic field is applied, two levels (and only two levels, by quantum mechanical restrictions) are populated by electrons. By employing X-band microwaves (9 GHz) with the proper energy ($h\upsilon = g\beta H$; see for instance, Roubal 1972), flipping between levels occur and the absorption of microwave energy is observed.

There is one class of free radicals, the nitroxides, which are stable under many of the usual laboratory conditions. Nitroxide stability derives from resonance and other contributing factors, but we need not discuss these here. Many nitroxides are relatively easy to synthesize, provided the necessary starting intermediates (some of which are rare) are at hand.

The important point to be made is the fact that nitroxides can be used to characterize biological systems. Nitroxides so used are called spin-labels; *spin* from the fact that it is the unpaired electron(s) (which is/are spinning) which forms the basis for the *label* or probe. Spin-labeling might just as conveniently be called spin-probing.

Spinning Electrons and Their Magnetic Properties

All electrons are in a state of motion; they all spin about on their axis. Spinning electrons are therefore moving charges of electricity. Thus electrons are magnetic. Spinning electrons therefore are influenced by an external magnetic field such as produced by a solenoid or electromagnet. When a sample of free radicals is placed between the poles of an electromagnet, the spin of the electron is described as clockwise or counterclockwise and is depicted in Figure 1.

Of immediate consequence is the fact that one spin condition is more stable than the other, and is so indicated by the reference to the energy of the system as shown. Although one population level is more stable than the other, the temperature of the system is always great enough to insure that the higher level contains just about as many electrons as the lower level. This is something akin to placing two bar magnets end to end. If they are aligned N-S N-S, we know from everyday experience that the interaction will be attractive and stable. If, on the other hand, we try to force them N-S S-N, we know again from experience that this is an unstable situation and requires an expenditure of energy (heat in the case of electrons, physical in the case of magnets) to maintain

them aligned in this fashion. Apart from this, however, the analogy extends no further, for in the realm of electrons and nuclear phenomena, quantum mechanical postulates hold and the common experience of our everyday world does not pertain.

The important point we want to make here is that we can induce an electron with one spin rotation to flip over and assume the alternate rotation. Energy is required to do this. To achieve flipping, the sample is irradiated (while between the poles of the electromagnet) by microwaves with a frequency of 9 gigahertz (GHz). Thus we see that what we are really talking about is just another type of spectroscopic method. The presence of free radicals is detected by measuring the loss of microwave energy. The actual instrument used for such studies is called the electron paramagnetic resonance (EPR) spectrometer (also called the electron spin resonance/ESR/spectrometer).

At this point we must consider other factors which contribute to the total magnetism of the system. Remember that the unpaired electron is not merely floating freely about in space. It belongs to a molecule. In fact it is coupled to a nucleus, and in the case of nitroxides, to a nitrogen nucleus, which is itself a magnetic entity. Thus in the presence of an external field, the nuclear magnetism can couple with the external field and alter the magnetism immediate to the electron. We must now consider this situation, called hyperfine splitting (hfs).

Hyperfine Splitting (hfs)

Simply stated, it is found that the nitrogen magnetism can add to, subtract from, or be orthogonal (no interaction) to the external field. This is depicted in Figure 2.

Note that the situation of Figure 1 is now modified. The original resonance (flipping) condition is broken down into three resonances (hfs). The flipping from one level to the other is depicted by the double ended arrows A, B, and C. Certain restrictions are placed on flipping, and we find that only those shown are allowed. All levels are equally populated, and an actual EPR spectrum of a nitroxide in dilute solution is shown in Figure 3.

Spin-Label Spectra

While the spectrum of Figure 3 tells us most conclusively that we are dealing with a nitroxide,

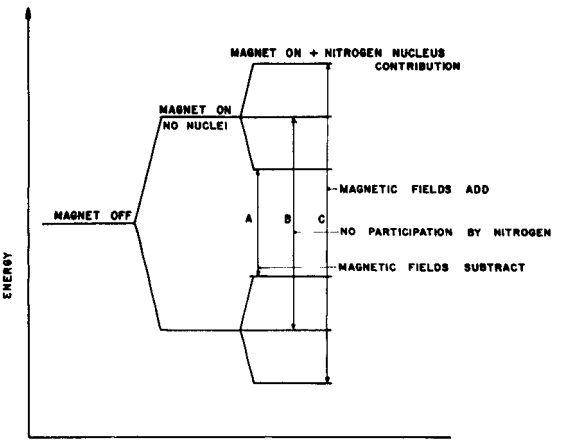

FIGURE 2.—Resonance condition for the case of one electron interacting with a nitrogen-N^{14} nucleus. Due to quantum mechanical restrictions, only those transitions shown are allowed.

it serves no further purpose other than a possible quantitation of the amount of radical (number of spins) present. If all we ever measured were three sharp, hyperfine lines, we could not use nitroxides as spin-labels.

Fortunately, when a nitroxide is placed in an actual biological system, the hyperfine lines are modified both in shape, intensity (relative height), and in spacing. These spectral modifications are environment dependent, and it is this dependency which makes nitroxides valuable as probes for characterizing biological systems.

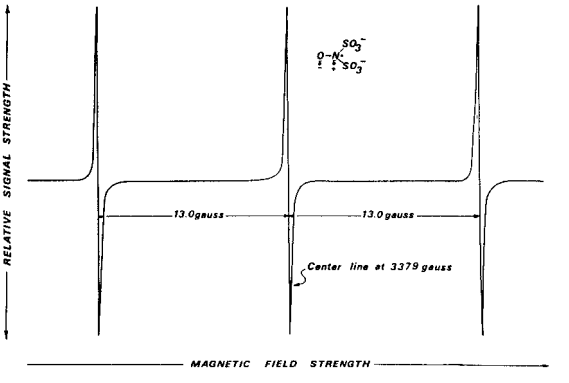

FIGURE 3.—EPR spectrum of the nitroxide compound potassium peroxylamine disulfonate (Freemy's salt) (10^{-3}M) in water at room temperature. Three hyperfine lines of equal intensity, and spaced 13 gauss apart, distinguish nitroxides in water.

It is sufficient to simply state that the unpaired electron is localized on nitrogen and this localization resides primarily in a p-orbital on nitrogen. p-Orbitals are dumbbell-shaped electron density regions in space, and the magnetic properties associated with an electron in such an orbital depends on the so-called tumbling frequency of the electron (how fast the p-orbital assumes a random distribution of orientations while part of the nitroxide in a biological system). An example of two limiting situations is shown in Figure 4. Spectrum 4A was recorded for a nitroxide in liquid glycerol, while 4B is for the frozen solution. Spectrum 4A tells us that moderate fast tumbling prevails, while 4B shows tumbling to be essentially quenched. The reader is referred to the literature (Roubal 1972) for a more thorough discussion on the dependency of spectral characteristics on tumbling frequency (label mobility).

Intermediate mobilities are characterized by a family of spectra. Examples are to be found in the recent spin-labeling study of a hapten combining site of trout antibody by Roubal et al. (1974). Using appropriate mathematical manipulations of recorded spectra, one can measure tumbling frequencies with accuracy. These frequencies together with other derived data provide quantitative characterization of labeling studies.

MEMBRANES AND MEMBRANE PROPERTIES

The importance of membranes in biological roles of living systems cannot be overemphasized. Membranes, as mentioned, are responsible for neural function. Membranes participate in ion-binding and in governing tissue permeability. Associated with membranes, especially mitochondrial membranes, are a variety of enzymes. Cytochrome oxidase and other electron transport enzymes are membraneous in nature. Membrane-bound enzymes require the proper conditions such as lipid fluidity, proper phase transition temperatures, and lipid-protein interactions for their function. The participation of membranes in neural control is well documented. Neural membranes contain molecular size pores which mediate sodium/potassium transport. The exact nature of these pores has not been delineated completely, but several lines of evidence suggest that pores consist of a cagelike arrangement of protein which spans the membrane from the inner to the outer surface. Membranes are considered to be the basis of life itself.

Membranes consist principally of proteins and lipids. Carbohydrates comprise 0-10% of the membrane mass. Lipids account for about 40% of the mass, and the balance is protein. Membranes are a matrix of lipids and proteins arranged in a bimolecular leaflet (Singer 1972; Green 1972), illustrated in Figure 5. The little circles represent phospholipid headgroups (choline, ethanolamine, serine, phosphatidic acid, etc.) while proteins are indicated by the larger "islands." Interspersed with the fatty acid tails (squiggly lines) are sterols and lesser tissue lipid components. Typical membranes are about 100 Å thick.

Membrane lipids are amphiphilic—provided

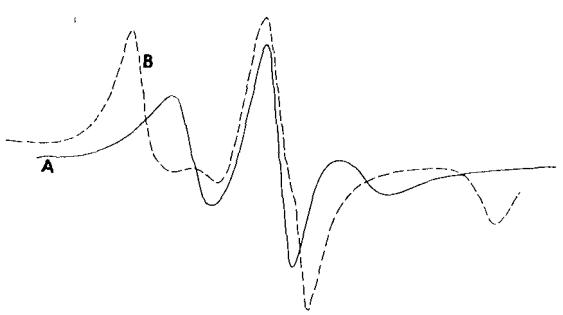

FIGURE 4.—Label II (see text) in glycerol.
A. In liquid glycerol at room temperature.
B. Frozen in glycerol at liquid nitrogen temperature.
Label mobility (effect of environment) is calculated by measurements of line widths, heights, intensity ratios, and spacing.

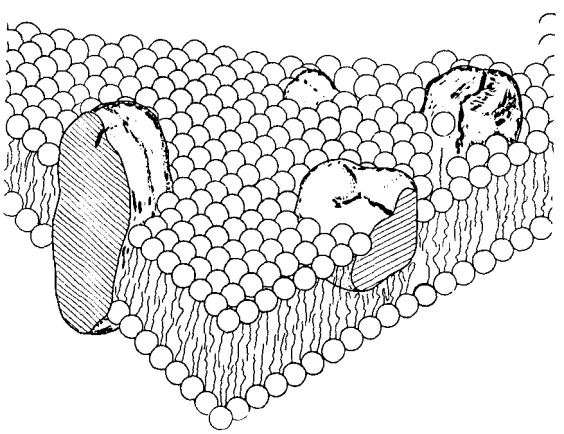

FIGURE 5.—Membrane bilayer leaflet. Small circles represent phospholipid headgroups. Squiggly lines are fatty acid tails. Large islands represent membrane protein. From Singer 1972. (Courtesy of S. J. Singer and New York Academy of Sciences.)

with polar headgroups and nonpolar tails of carbon-hydrogen chains. Thermodynamically, and for other reasons as well, the greatest stability for membrane structure results when membrane constituents are arranged as shown; polar surfaces are exposed to aqueous environments, and the fatty acid tails are tucked away out of contact with water.

QUESTIONS ABOUT CONTAMINANT-HOST INTERACTION

We would like to answer the following questions concerning membrane-contaminant interaction: a) are certain regions of membranes affected or is the whole membrane affected when invaded by contaminant? b) if the effect is localized, where is the localization? c) are there differences in membrane perturbations when treated on the one hand by paraffins, and aromatics on the other? d) can the differences, if they exist, be related to anything presently known about the toxicology of any of these contaminants?

EXPERIMENTAL METHODS AND RESULTS

The study was performed in two steps. First, an in vivo feeding study was undertaken. Here we used spin-labeled hydrocarbons (Roubal 1974) and fed them to fish. Second, an in vitro study was employed using excised tissue. In order to restrict our investigations of membranes (in vitro) to specified regions, the series of labels I, II, and III (Roubal 1974a) (Figure 6) were synthesized by the EPR group of this Center. The positively charged quarternary nitrogen of label I directs this portion of the label to the polar membrane surface, which in turn insures that the nitroxide nitrogen is situated at or very near to the membrane surface. The carboxyl groups of labels II and III direct the carboxyl end of these amphiphiles to the membrane surface, but now the nitroxide nitrogen lies some 12-15 Å below the membrane surface in II, and deep into the hydrophobic membrane interior in III. Thus we can "look" at the membrane's surface, subsurface, and interior.

In the feeding study, spin-labeled hydrocarbons were incorporated into fish food and fed to coho salmon, *Oncorhynchus kisutch*, fingerlings in a 2-day feeding study. Within an hour, or even less, after onset of intake of food by fish, the blood showed EPR activity. Using radiotracers, we have shown this activity to be associated with blood lipoproteins and albumins (Roubal 1974b), with lipoproteins making the greatest contribution to hydrocarbon transport in blood.

After an induction period of about a day, blood-associated labels (Roubal 1974b) slowly transfer to neural tissue and flesh. Weight for weight, the greatest concentrations are to be found in the spinal cord, lateral line nerve bundles, and brain. The nature of the EPR line shapes indicated that the invasion of hydrocarbon is site selective. All labeled paraffins appeared to intercalate with membrane in such a way that the nitroxide mobility is little impeded. In direct contrast to this, mobility of aromatics appeared to decrease. This in vivo study suggests that paraffins associate with molecularly fluid portions of membrane fatty acids, while aromatics associate with the more structured and rigid regions of membranes. In order to clarify these possible differences, an in vitro study was undertaken using labels I, II, and III (Roubal 1974a).

Neural tissue from untreated fish was carefully excised and placed in cold, 0.1 M phosphate buffer, pH 7.4 at 4°C, isotonic in NaCl. A sonicated dispersion of label, complexed to bovine serum albumin was added and allowed to transfer to neural membrane (overnight at 4°C). Membranes were then inserted directly into the EPR spectrometer, and the spectra were recorded immediately. After

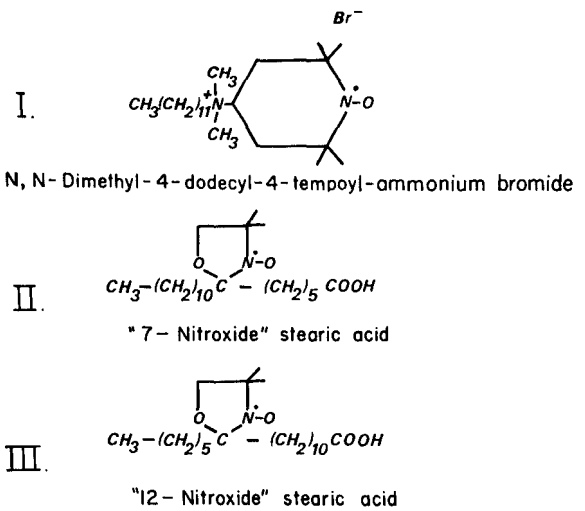

FIGURE 6.—Lipid-intercalating spin-labels.
Label I reports on surface conditions.
Label II reports on subsurface conditions.
Label III reports on interior conditions.

the spectrum for one sample was obtained, the tissue was returned to a large volume of fresh, cold buffer to which test hydrocarbon (in separate tests) was incorporated via sonication. Final hydrocarbon concentration in buffer was 15-25 ppm. Hydrocarbons included benzene, toluene, ethyl benzene, hexane, heptane, octadedecane, and cyclohexane. Actual uptake of paraffin hydrocarbon by tissue membrane, as measured by gas-liquid chromatography (GLC), was on the order of 1 ppm. Aromatics were present in higher amounts (5-10 ppm). Tissue was exposed to hydrocarbon in buffer for 1 h. At the end of this period, tissues were withdrawn, rinsed well, and the EPR spectra were re-recorded.

A comparison of the in vitro spectra for controls with those same samples after hydrocarbon treatment provided evidence that a differentiation in binding sites for paraffins and aromatic compounds does indeed exist (Figure 7).

DISCUSSION

We can explain rather easily the preference of paraffins seeking the interior of membranes—paraffins are nonpolar, very soluble in neutral hydrocarbons, such as those which comprise the hydrogen-carbon chains (or tails) of phospholipid fatty acids. Hence, thermodynamically, system stability is enhanced by mutual interaction of paraffin hydrocarbon with lipid tails.

Aromatics, on the other hand, are unique, for in addition to their ready solubility in many organic environments, aromatic compounds are fashioned from conjugated double bond systems with pi-electron unsaturation. These factors give aromatics the ability to form quasi-chemical complexes with other molecules which can act in electron acceptor-donor roles.

The surface of the membrane contains many different sites, both polar, nonpolar, and electron-interactive. It appears that some of these sites contain the necessary properties which make binding of aromatics possible. A charge-transfer mechanism (Kier 1971) may direct aromatics away from the membrane interior to the surface.

These site preferences for paraffins and aromatics may account in part for the differences we observe for retention of these substances in living tissue. For instance, in other studies (Roubal 1974b) we have shown via GLC, spin-labels, and radiotracers that paraffins are retained in living tissue for long periods of time; aromatics are not. What is more, paraffins are relatively nontoxic, while aromatics generally are quite toxic, even at low levels.

The molecular basis for physiological phenomena is associated in a very direct way with important membrane properties. Ion-binding, lipid protein (enzyme) interaction, lipid phase-transition temperatures, and lipid fluidity are all involved in one way or another. Membrane disor-

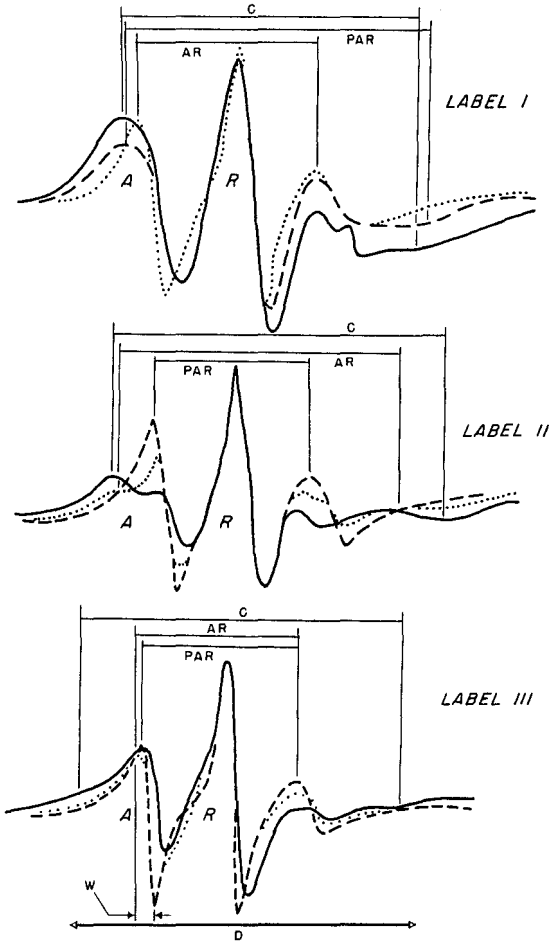

FIGURE 7.—Spin-label spectra of aromatic-treated and paraffin-treated coho salmon spinal cord (SC).
SC + Label I (+ treatment).
SC + Label II (+ treatment).
SC + Label III (+ treatment).
Changes in the A/R (line height) ratio, narrowing of peak widths (W), and shifts in distance D to lesser values provide data for characterizing influence of treatments (Roubal 1972; Roubal 1974a). C, spinal cord control.
AR, treatment by aromatics.
PAR, treatment by paraffins.

ganization is considered to involve alterations in these properties. Accordingly, on invasion of membrane primarily by aromatics, surface perturbations as indicated by spin-labeling spectra reflect changes in ion-binding properties of phospholipid headgroups, enzyme activity, and permeability changes.

From the standpoint of membranes, paraffins are tolerated up to a point, by shunting them into internal reaches of membrane, away from active metabolic processes. Our spin-labeling studies show lipid fluidity to be altered when tissue is exposed to paraffins. These changes are rather diffuse, however, and not associated with any one portion of the membrane interior. Such alterations however, could be operative in altering ion transport.

We contend, therefore, that aromatic-membrane interaction is of paramount concern. This is especially true when behavioral/physiological patterns are to be explained. Additional insight into these areas will necessitate further biophysical studies—both spin-labeling and broad-based electrophysiological studies.

FUTURE OUTLOOK ON SPIN-LABELING

Spin-labeling was first described only as recently as about 1968. Since then, a vast array of labels have been described. New instrumentation has evolved, and the technique has grown from a tool of limited application to one of major importance. For many biochemical and biophysical studies, the technique stands prominently above other methods. Applied to drug studies, pharmacology, immunology, cancer research, enzymology, and protein structure studies, spin-labeling promises to play an ever growing role. Environmentalists, biologists, zoologists, and food scientists now apply this tool to their studies. The future of spin-labeling is bright indeed.

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