Biochimica et Biophysica Acta, 550 (1979) 369—383
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BBA 78261

AZETHOXYL NITROXIDE SPIN LABELS

ESR STUDIES INVOLVING THIOUREA CRYSTALS, MODEL MEMBRANE SYSTEMS AND CHROMATOPHORES, AND CHEMICAL REDUCTION WITH ASCORBATE AND DITHIOTHREITOL

TERRY D. LEE, G. BRUCE BIRRELL, PAMELA J. BJORKMAN and JOHN F.W. KEANA
Department of Chemistry, University of Oregon, Eugene, OR 97403 (U.S.A.)
(Received June 20th, 1978)

Key words: Azethoxyl nitroxide; Nitroxide; Spin label; Boundary lipid; ESR; (Model membrane)

Summary

Trans- and *cis*-azethoxyl nitroxides 1, 2, 3 and 4 can be trapped in the cavities of thiourea crystals. The presence of a single gauche conformation on either side of the pyrrolidine ring within the crystals was indicated by the ESR spectra. Rotation about the long molecular axis then corresponds approximately to *y*-axis motion of the nitroxide moiety. Proxyl nitroxides in which the nitroxide group is located on the penultimate carbon of long chain lipids can also be trapped and were shown to adopt the azethoxyl conformation in the thiourea crystals.

The measured ΔA values ($A_{\parallel} - A_{\perp}$) of oriented egg lecithin multilayers containing *trans-* and *cis*-azethoxyl nitroxides 1 and 2 were quite small, consistent with the unique orientation of the nitroxide principal axes with respect to the long axis of the molecule. The ΔA values for a series of lipids bearing a label near the terminus of the chain were very similar to that of 1, showing that the azethoxyl conformation is likely the predominant one in these labels in orienting systems.

Computer simulations of the ESR spectra of 1 and 2 in egg lecithin vesicles provided values for molecular orientation and motion parameters consistent with those expected from a consideration of molecular models in the extended (all trans) conformation.

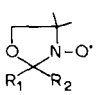
Azethoxyl nitroxides have also proven useful in the investigation of motion restricted (boundary) lipid in a lipid-protein system. Thus, the values (69 ± 10%) for the amount of boundary lipid in the chromatophore membranes from *Rhodopseudomonas sphaeroides* as determined using *trans-* 2 and *cis-* 2 are in good agreement with values using 16-doxylstearic acid (64 ± 3%). The fact that all three labels show about the same fraction of boundary lipid in this system

indicates that the lipid binding sites are relatively insensitive to the geometry of the lipid chain. Also, both 1 and 2 appear to be able to detect a third lipid environment not seen with the doxyl fatty acid. The apparent fluidity of this component lies between that of boundary and bilayer lipid. The unique orientation of the nitroxide principal axes with respect to the long molecular axis in azethoxyl nitroxides 1 and 2 allows detection of hindrance to rotation about the long molecular axis, in contrast to the analogous doxyl and proxyl fatty acids.

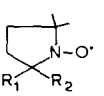
Comparative reduction studies using ascorbate and dithiothreitol indicate that azethoxyl nitroxides are slightly more resistant toward reduction than proxyl nitroxides and much more resistant than doxyl nitroxides.

Introduction

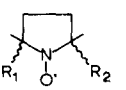
Electron spin resonance (ESR) spectroscopic studies with nitroxide-labeled lipids have made a major contribution to the understanding of the structure and function of biological membranes [1]. The proven utility of the spin labeling method has prompted the continued development of new nitroxide spin labels with improved or alternative chemical and spectroscopic properties [2]. Recently, we reported the synthesis and some properties of a new series of minimum steric perturbation nitroxide lipid spin labels which we term azethoxyl nitroxides [3,4]. Azethoxyl nitroxides are 2,5-disubstituted 2,5-dimethylpyrrolidine-*N*-oxyl nitroxides. Since three of the five atoms of the pyrrolidine ring are integrated into the hydrocarbon chains in these nitroxides, the steric bulk of the nitroxide moiety is significantly reduced over that of a doxyl [5] or proxyl [6] nitroxide. Additionally, both *cis*- and *trans*-azethoxyl nitroxides may be prepared. Molecular models suggest that the geometry in the region of the pyrrolidine ring in the cis isomers is similar to that of a cis carbon-carbon double bond, while the geometry in the trans series resembles quite well that of a saturated hydrocarbon chain. Thus, azethoxyl nitroxides have some unique properties, especially suitable for studies of the role that fatty acid chain geometry plays in membrane systems.



A doxyl nitroxide



A proxyl nitroxide



An azethoxyl nitroxide

Although similar to proxyl nitroxides in chemical properties [3], azethoxyl nitroxides differ from proxyl (and doxyl) nitroxides in at least one important way, namely, the orientation of the nitroxide principal axes of the A and g tensors with respect to the long molecular axis. Both proxyl and doxyl nitroxide derivatives of fatty acids have A_{zz} and g_{zz} parallel to the long axis of the molecule (Fig. 1a). With azethoxyl nitroxides, however, the long molecular axis (extended conformation) does not correspond to any of the principal axes but rather lies between the y and z axes (Fig. 1b and 1c). While ESR spectral line shapes for oriented doxyl spin labeled long chain lipids are well understood [7], it is not immediately obvious what effect the off-axis orientation of

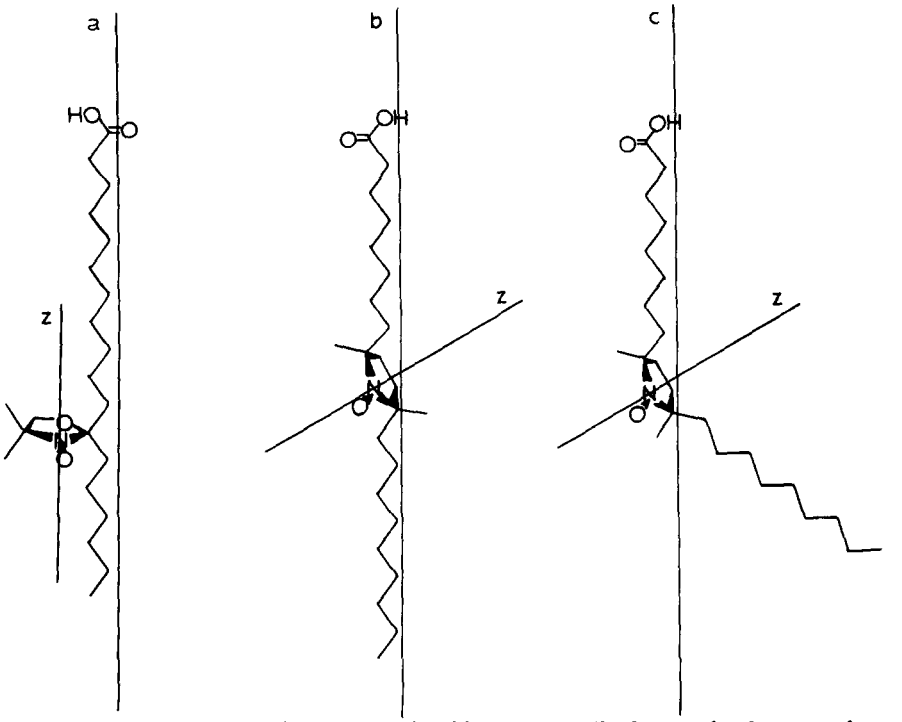


Fig. 1. Orientation of the nitroxide *z* axis with respect to the long molecular axes of representative nitroxides (a) 12-doxylstearic acid (9); (b) *trans*-10-azethoxylheneicosanoic acid (1), and (c) *cis*-10-azethoxylheneicosanoic acid (2).

azethoxyl nitroxides has on the spectral line shapes.

In the present paper we report on the ESR spectra of representative azethoxyl nitroxides which have been incorporated into the following systems: the cavities of thiourea crystals, egg lecithin multilayers, egg lecithin vesicles and chromatophores isolated from the photosynthetic bacterium, *Rhodopseudomonas sphaeroides*. We also examine differences that exist between cis and trans isomers of a given azethoxyl nitroxide. Lastly, the comparative behavior of azethoxyl, proxyl and doxyl nitroxides toward the reducing agents sodium ascorbate and dithiothreitol is described.

Experimental

Spin labels. The structures of the spin labeled molecules used in this paper are shown in Chart 1. Azethoxyl derivatives 1—4 [3,4] and proxyl derivatives 5 and 11 [8] were prepared as previously described. Proxyl nitroxides 6—8 were synthesized [9] using the general method of Keana et al. [6]. Doxyl nitroxides 9 and 10 were purchased from Syva Corp. All of the above showed a single spot by analytical thin layer chromatography (silica gel). ESR spectra were recorded on a Varian E-line ESR spectrometer interfaced with a Varian 620L/100 computer and equipped with a Field/Frequency lock and temperature regulator.

Thiourea inclusion crystals. Thiourea inclusion crystals were grown as follows. To 5 ml of a saturated solution of thiourea in methanol was added a

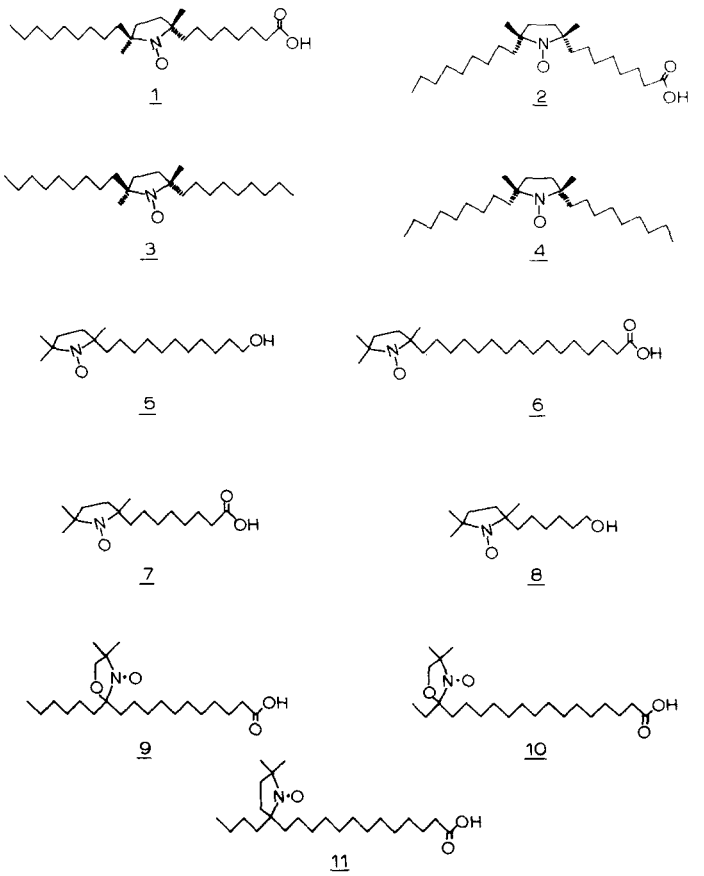


Chart 1

solution of 0.5 mg of the nitroxide and 200 mg of camphor (used as a diluent) in 3 ml of methanol. The solution, in a 10 ml Erlenmeyer flask, was allowed to evaporate at 8°C. After 4—5 days, large crystals formed. A crystal was placed on a teflon crystal mount and aligned in the cavity of the ESR spectrometer. Spectra were taken with the laboratory magnetic field perpendicular and parallel to the crystal needle axis.

Egg lecithin multilayers and vesicles. Egg lecithin multilayers were prepared using a modification of the method of Jost et al. [7]. To a solution of 5 mg of egg lecithin in 0.25 ml of 95% ethanol was added 1 μl of a $2.0 \cdot 10^{-2}$ M solution of the spin-labeled lipid in 95% ethanol. The resulting solution was placed on a 14 × 60 × 0.1 mm glass cover slip which was prescored with a diamond pencil into four 3.5 × 60 mm sections. The solvent was allowed to evaporate and then the slide was broken into four strips. The two outside sections of the intact slide were discarded thus essentially eliminating the disorder associated with the edge of the multilayer. The remaining two were placed back to back in a 5 mm NMR tube which had been previously flushed with wet N_2. The slides were held in place with a plug of glass wool and the tube was inverted through a rubber stopper over a saturated aqueous solution of $NH_4H_2PO_4$ (rel. humidity

=93% [10]). After 24 h, the tube was removed, capped, and placed in the cavity of the ESR spectrometer. Spectra were taken with the magnetic field parallel and perpendicular to the normal of the glass slide. Duplicate samples were prepared for each run.

Vesicles were prepared by the addition of 6.0 mg of egg lecithin and 0.01 mg of the spin labeled lipid to 0.1 ml of 10^{-3} M Tris buffer (pH 7.4) (0.25 M in sucrose). The suspension was bath sonicated for 10 min and then probe sonicated for 1 min.

Chromatophore preparation. Cells of the photosynthetic bacterium *Rps. sphaeroides* strain Ga [11] were grown on photosynthetic medium M22 of Sistrom [12] with malic acid replacing lactic acid and supplemented with 0.1% (w/v) casamino acids. The cells were grown under a light intensity of 100 ft-candles and were harvested according to the method of Fraker and Kaplan [13]. The whole cells were passed through a French pressure cell twice at 20 000 lb/inch^2; the resulting crude chromatophores were then purified by sucrose density gradient centrifugation [13] and dialyzed against 0.1 M sodium phosphate (pH 7.6, containing 1 mM EDTA and 0.25 M sucrose). The purified chromatophores contained 0.33 mg of phospholipid (determined by the method of Lowry and Tinsley [14]) per mg protein (determined by the method of Lowry et al. [15]).

A composite ESR spectrum was obtained as follows. The spin label (3 μg) dissolved in ethanol was placed in the bottom of a glass homogenizer. The solvent was evaporated by a stream of nitrogen. The chromatophores (containing 1.6 mg of protein), suspended in the phosphate/EDTA buffer (pH 7.5), were added and the mixture was homogenized and then centrifuged for 1 h at 74 000 $\times g$. The pellet was drawn into a capillary and placed in an ESR sample tube. Lipid-depleted protein (0.12 mg phospholipid/mg protein) was obtained by the method of Yu et al. [16]. The spin labels were incorporated in the lipid depleted proteins as before except 2 μg of spin-labeled lipid were added to 2 mg of protein.

Reduction studies. Comparative reduction studies were done in 0.1 M aqueous phosphate buffer, pH 7.5, which was 0.25 M in sucrose and 1 mM in EDTA. The initial nitroxide concentration for every run was $1.1 \cdot 10^{-4}$ M. Under these conditions, no ESR spectral evidence of nitroxide-nitroxide interactions (i.e., micelle formation) was observed. The solutions were prepared by adding 0.2 ml of the buffer to a 1—1.7 μl aliquot of a 95% ethanol stock solution of the nitroxide already in the sample tube. Next, a 10 μl aliquot of either sodium ascorbate (0.22 M or 2.2 M) or dithiothreitol (2.2 M) dissolved in the buffer was added to the sample tube and mixed thoroughly by bubbling a vigorous stream of nitrogen through the solution for 1 min. The rate of ESR signal decay was monitored by observing the low field peak height for a period of 20 min. The data were collected using a computer and then replotted so that the initial signal in each run was scaled to 100%. The time between addition of the reducing agent and the start of the computer collection varied from 2 to 2.5 min. Despite the simple design of the experiment, a number of technical difficulties were encountered. Oxygen had to be carefully excluded since it not only affected the peak width and g values, but was also capable of reoxidizing the nitroxides once they had been reduced. With the dithiothreitol reductions,

it was necessary to avoid prolonged contact of the reagent solutions with the syringe needles used to transfer them in order to obtain consistent results. Despite other occasional unexplained inconsistencies, reproducible results were obtained and these are summarized in Table III.

Results and Discussion

Thiourea inclusion crystals

Thiourea inclusion crystals provide a unique environment for the study of spin labeled species. The crystals have hexagonal cavities approx. 7 Å in diameter extending along the 6 fold symmetry axis [17,18]. A long chain guest molecule bcomes oriented so that its long axis is parallel to the needle axis of the crystal, essentially eliminating any rotational motion other than that about the long axis. Consequently, the ESR spectrum of a nitroxide trapped within the cavity will be a function of the orientation of the crystal relative to the magnetic field of the spectrometer.

Attempts to orient long chain doxyl and proxyl nitroxides such as 9, 10 and 11 in crystals of thiourea were not successful, undoubtedly owing to the steric size of the nitroxide moiety in these spin labels. By contrast, *trans-* and *cis-*azethoxyl nitroxides 1, 2, 3, and 4 are readily oriented in the crystals, constituting direct evidence of the smaller steric size of these new labels. As expected, ESR spectra (Fig. 2) of the included nitroxides are strongly dependent on the orientation of the crystal in the laboratory magnetic field. The one exception is that the spectra are isotropic to rotations about the needle axis with the magnetic field in the plane perpendicular to this axis.

The hyperfine splitting (in gauss) expected when the laboratory magnetic

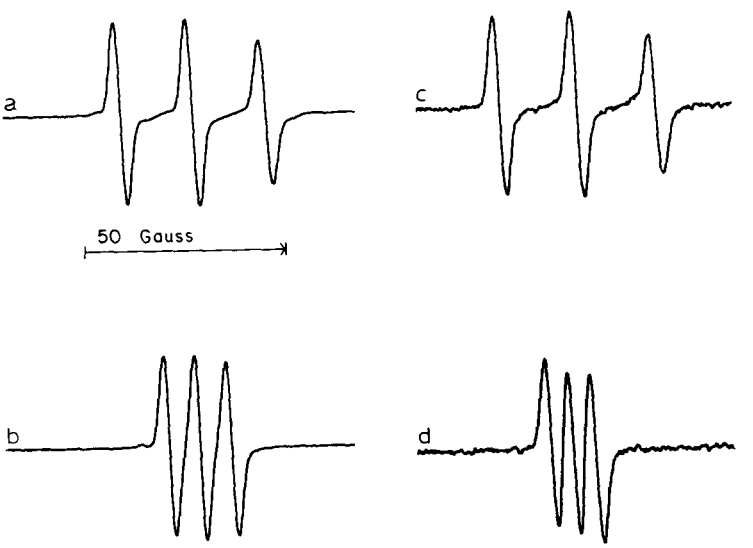


Fig. 2. 9.5-GHz ESR spectra of azethoxyl derivatives 3 and 4 trapped in the cavities of thiourea inclusion crystals; (a) trans isomer 3 with $H_{\perp}$ to the needle axis; (b) trans isomer 3 with $H_{\parallel}$ to the needle axis; (c) cis isomer 4 with $H_{\perp}$ to the needle axis, and (d) cis isomer 4 with $H_{\parallel}$ to the needle axis.

field is parallel and perpendicular to the needle axis of the thiourea inclusion crystal can be termed $A_{\parallel}$ and $A_{\perp}$. If one makes the assumption that (a) the long molecular axis is parallel to the crystal needle axis and (b) rotation is rapid about the long molecular axis within the crystal [19], values for $A_{\parallel}$ and $A_{\perp}$ may be calculated using Eqns. 1 and 2 [20]:

$$A_{\parallel} = A_{zz}\cos^2\theta + A_{yy}(1 - \cos^2\theta) + (A_{xx} - A_{yy})\sin^2\theta\cos^2\psi \quad (1)$$

$$A_{\perp} = \tfrac{1}{2}A_{zz}(1 - \cos^2\theta) + \tfrac{1}{2}A_{yy}(1 + \cos^2\theta) + \tfrac{1}{2}(A_{xx} - A_{yy})(1 - \sin^2\theta\cos^2\psi) \quad (2)$$

where A_{zz}, A_{yy} and A_{xx} are the principal A values for the nitroxide, θ is the angle between the needle axis of the crystal and the nitroxide z axis, and ψ is the angle between the needle axis of the crystal and the nitroxide x axis *.

Values for θ and ψ may be estimated for a given conformation from molecular models. For all conformations examined, $\psi \cong 90°$. For a *trans*-azethoxyl nitroxide having an all trans (extended) side chain conformation, $\theta \cong 60°$ (Fig. 3a). Using these values and the principal A values of 2-doxylpropane (A_{xx} = 5.9 G, A_{yy} = 5.4 G, A_{zz} = 32.9 G) [7] in Eqns. 1 and 2, the motionally averaged values of $A_{\parallel}$ and $A_{\perp}$ are calculated to be 12.3 G and 16.0 G, respectively.

These values, however, differ substantially from the experimentally observed values of $A_{\parallel}$ and $A_{\perp}$ shown in Table I for *trans*-azethoxyl nitroxides 1 and 3. A closer inspection of the molecular models reveals the likely explanation. Introduction of a single gauche conformation in the alkyl chains on each side of the pyrrolidine ring results in a structure in which the average plane of the ring is more in line with the long axis of the molecule, i.e., $\theta \cong 80°$ (Fig. 3b). This value is consistent with that (θ = 78—81°) calculated from the experimentally determined $A_{\parallel}$ and $A_{\perp}$ using Eqns. 1 and 2 (ψ = 90°). Interestingly, models also suggest that the cross-sectional diameter of the *trans*-azethoxyl molecule is reduced somewhat by imposing the two gauche conformations, with the result likely being a better fit into the thiourea cavity.

cis-Azethoxyl nitroxides 2 and 4 are also included in thiourea crystals, although the ESR signal strength of comparable size crystals is substantially weaker than that from the trans isomers. Observed $A_{\parallel}$ and $A_{\perp}$ for 2 and 4 are indicative of nearly pure y-axis motion (θ = 90°, Table I). It is apparent from Fig. 3c that the imposition of one gauche conformation on each side of the pyrrolidine ring gives rise to a linear shaped structure where $\theta = \psi = 90°$, and the average plane of the ring (and consequently the y axis) approximately parallels the long molecular axis *.

In order to investigate the behavior of pyrrolidine nitroxides near the terminus of the chain, ESR spectra for 12-proxyltridecanol (5) and 17-proxyloctadecanoic acid (6) trapped in thiourea crystals were measured. The results are given in the last two entries of Table I and correspond to the values obtained for the *trans*-azethoxyl derivatives. Clearly, within the cavity of a

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* Following the usual convention, the x axis of a nitroxide group lies along the N-O bond; the z axis lies along the 2p orbital on nitrogen; the y axis is perpendicular to the xz plane.

** Evidence for the presence of gauche conformations on either side of the double bond in unsaturated phospholipids has been recently summarized (see, Huang, C.-H. (1977) Lipids, 12, 348—356).

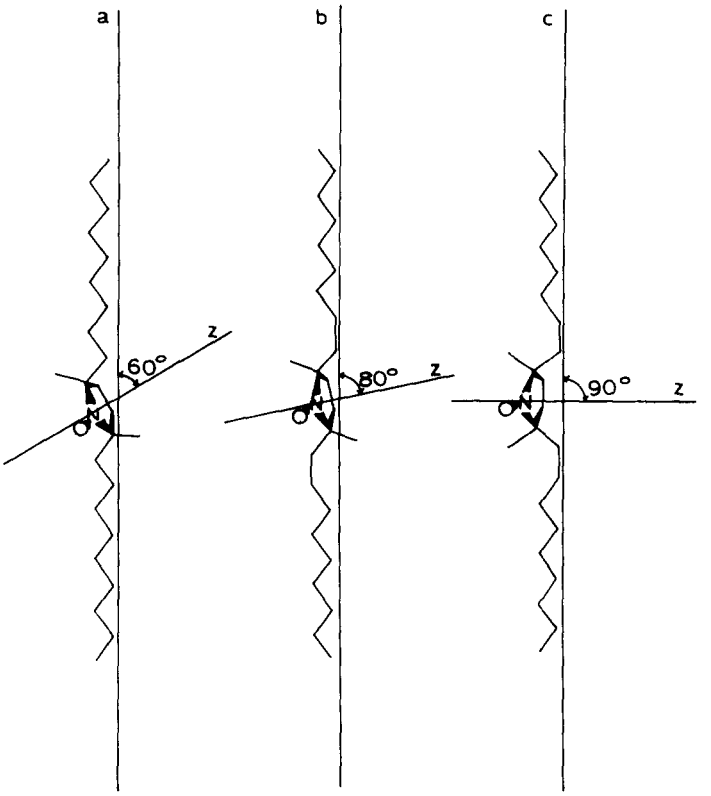


Fig. 3. Conformations of azethoxyl derivatives 3 and 4: (a) trans isomer 3 in all trans extended conformation; (b) trans isomer 3 in double gauche conformation, and (c) cis isomer 4 in double gauche conformation.

thiourea crystal, a proxyl nitroxide near the end of the chain adopts an azethoxyl conformation.

Oriented egg lecithin multilayers

Oriented egg lecithin multilayers have been used extensively as a model

TABLE I

EXPERIMENTAL PRINCIPAL A VALUES AND CORRESPONDING VALUES OF θ FOR NITROXIDES IN THIOUREA CRYSTALS

Nitroxide	H parallel to needle axis		H perpendicular to needle axis	
	A(G)	θ(deg.)	A(G)	θ(deg.)
1	6.5	78	18.7	78
2	5.5	87	19.4	90
3	6.0	81	18.9	80
4	5.5	87	19.5	90
5	6.0	81	19.0	81
6	6.0	81	19.0	81

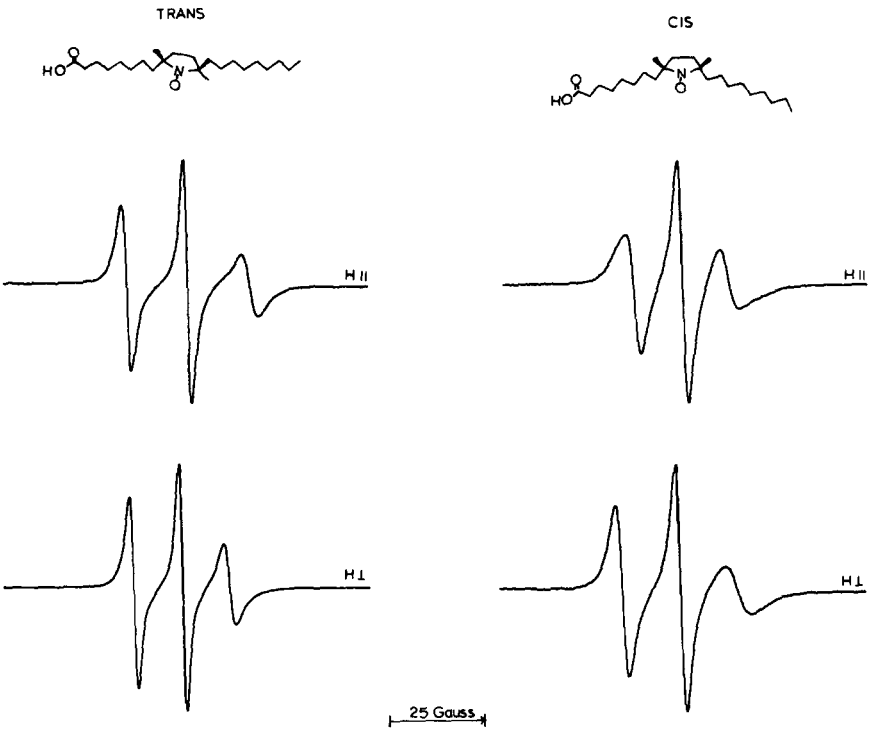


Fig. 4. 9.5-GHz ESR spectra of *trans*- and *cis*-azethoxyl derivatives 1 and 2 in oriented egg lecithin multilayers hydrated by equilibration at 93% relative humidity. Spectra were recorded with the laboratory magnetic field parallel and perpendicular to the direction normal to the surface of the multilayer.

system for phospholipid membrane studies [21,22]. It was therefore of interest to characterize the behavior of representative azethoxyl nitroxides 1 and 2 in this system. The ESR spectra for azethoxyl acids 1 and 2 in egg lecithin multilayers oriented on a glass slide are reproduced in Fig. 4. The most striking feature is the similarity between spectra taken with the laboratory magnetic field perpendicular to and parallel to the normal of the glass slide (see also Table II). Thus, the A value anisotropy ($\Delta A = A_{\parallel} - A_{\perp}$) for both isomers is quite small ($\Delta A_{trans} = 3.4$ G; $\Delta A_{cis} = -2.3$ G) in comparison to that observed in the thiourea crystals, or, for example, with 7-doxylstearic acid ($\Delta A \sim 10$ G) or 12-doxylstearic acid ($\Delta A \sim 5$ G) under similar conditions [7].

The more fluid environment in the multilayer would be expected to accommodate better the extended conformations of 1 and 2 which apparently are not allowed in the rigid thiourea crystal environment. Rotation about the long molecular axis in this conformation (Fig. 3a) would tend to average partially all the principal tensor elements, with the consequence being little spectral anisotropy. The degree of ordering in the multilayer system is inherently less than that in the thiourea crystals, which also helps to explain the nearly isotropic spectra for 1 and 2 in the multilayers (Fig. 4).

Proxyl nitroxides 6, 7 and 8 were also incorporated into the egg lecithin multilayer system (Table II). Because the proxyl nitroxide group is located on the penultimate carbon atom of the chain in these spin labels, the nitroxide

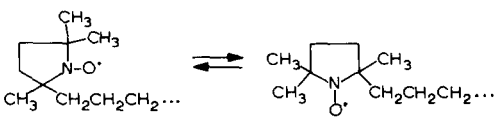
TABLE II

A VALUES FOR NITROXIDES IN ORIENTED EGG LECITHIN MULTILAYERS

Parallel and perpendicular refer to the orientation of the laboratory magnetic field relative to the normal of the glass slide.

Nitroxide	Parallel A(G)	Perpendicular A(G)	ΔA ($A_{\parallel} - A_{\perp}$)
1	16.6	13.2	3.4
2	13.5	15.8	−2.3
6	15.6	13.4	2.2
7	14.5	14.9	−0.4
8	14.5	14.9	−0.4

portion of the molecule could behave as a proxyl nitroxide A or, alternatively, by a rotation about the adjacent C-C bond, the nitroxide moiety could adopt the azethoxyl arrangement B in which the total chain length would appear to be two atoms longer than in the proxyl arrangement.



A B

It is seen from the data in Table II that, as the location within the multilayer of the penultimate proxyl nitroxide group is moved closer (6 vs. 7 vs. 8) to the lamellar plane formed by the hydrated polar ends of the lecithin molecules, there is little change in ΔA. By way of comparison ΔA values for a label in the 7- and 12-position of stearic acid in a similar system are 10.3 G and 5.4 G, respectively [7]. Since the ΔA values for 6, 7 and 8 are more like those for azethoxyl nitroxides 1 and 2, we conclude that when the proxyl group (or doxyl group) is located on the penultimate carbon atom of a long chain, the molecule prefers to adopt the sterically smaller azethoxyl arrangement in orienting systems.

This observation introduces a note of caution in the interpretation of order parameters for fatty acids or phospholipid spin labels in membrane systems. As Seelig points out [23], the order parameters are related to the molecular geometry of the spin label. Therefore, when one encounters the situation where the nitroxide label experiences a change in conformation (proxyl → azethoxyl) as it is systematically moved to the end of the chain, then the calculated order parameters for the individual spin label molecules may not be meaningfully compared with one another. In other words, on the same scale, one should not compare order parameters for a 12-doxyl group on a C-18 chain to that of a 17-doxyl group on a C-18 chain. The latter will, of course, show less anisotropy, but this may be at least in part because at the end of the chain, it can assume the azethoxyl conformation for which relatively little anisotropy is observed.

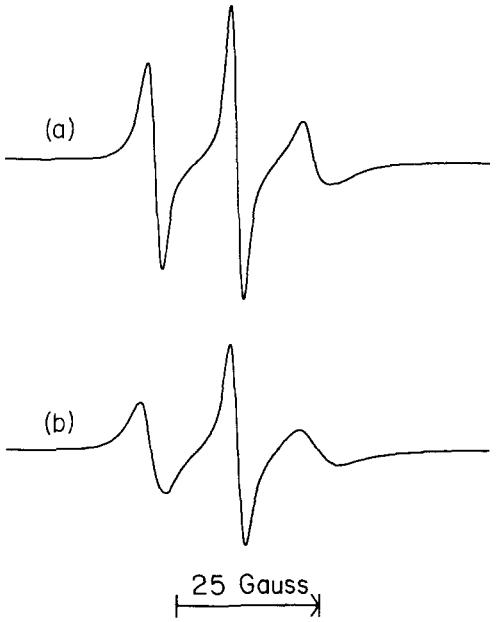


Fig. 5. 9.5-GHz ESR spectra of (a) trans isomer 1 and (b) cis isomer 2 ($1 \cdot 10^{-4}$ M) in egg lecithin vesicles (60 mg/ml in 10 mM Tris buffer, pH 7.4) at 25°C, scaled to the same total double integral.

Egg lecithin vesicles

ESR spectra of lipid spin labels in phospholipid multilayers deposited on glass slides reflect orientation effects [21,22] as well as those due to molecular motion. In order to eliminate complications arising from orientation effects, spectra were also obtained of the *cis*- and *trans*-azethoxyl labels in phospholipid vesicles. ESR spectra of isomers 1 and 2 in egg lecithin vesicles at 25°C are shown in Fig. 5. These spectra are scaled to the same value of the double integral of the first derivative spectrum. Differences in peak height therefore reflect real lineshape differences between the two isomers in this model system.

Spectral simulations of these data were performed using the program of Libertini et al. [24]. The ESR input parameters were the motion-averaged parameters $A_{\parallel}^{I}$ and $A_{\perp}^{I}$ calculated by the method of Van et al. [19]. For these simulations θ is defined as the angle between the nitroxide principal z axis and the long molecular axis and ψ is the angle between the nitroxide principal x axis and the long molecular axis. Best fits were observed when $\psi = 90 \pm 5°$ for either isomer. For trans isomer 1 best fits were observed when $\theta = 50°$, while for cis isomer 2, $\theta = 57°$. These values of θ and ψ are quite close to those expected from a consideration of molecular models in the extended conformation (cf. Fig. 3a). The simulations also suggest that the ESR spectra of both labels are relatively insensitive to wobble * [19] of the long chains. This is to be expected since, with long chain azethoxyl nitroxides, rotation about the long axis already partially averages all the principal A values (see above)

* Wobble refers to random fluctuations of the angle between the long molecular axis of the nitroxide chain and the normal to the bilayer plane.

resulting in little remaining spectral anisotropy. Thus, additional averaging resulting from wobble of the lipid chains would not be expected to introduce further dramatic changes in the ESR spectra.

Of course, this relative insensitivity to wobble is in marked contrast to the behavior [7] of long chain doxyl fatty acids, where the doxyl group is located somewhere in the middle of the chain. Here, rotational motion about the long molecular axis averages only the already nearly identical (and small) x- and y-axis components since the long molecular axis in these doxyl nitroxides is parallel to the z axis. Introduction of wobble then begins to average in the large z-axis component, resulting in dramatic changes in the calculated spectra.

Chromatophores

Lipid spin labels have played a key role in the detection and quantitation of different lipid environments within a biological membrane or model system. For example, integral membrane proteins such as cytochrome oxidase have been shown by the spin labeling method to be surrounded by a strongly immobilized layer of lipid which has been termed boundary lipid [25]. In the past, those nitroxide spin labels showing the greatest ESR spectral differences between protein-bound spin label and spin label located in the more fluid bilayer region have been spin labels in which the nitroxide group is located near the end of the hydrocarbon chain rather than near the polar head group. This is because doxyl or proxyl nitroxide moieties located near the end of a long chain end up somewhere near the rather fluid center of the phospholipid bilayer, giving rise to a narrow ESR line shape which is nearly isotropic. Immobilization of such a label in the boundary lipid results in a spectrum which resembles the rigid glass limit with characteristically broad lines. On the other hand, when the nitroxide label is near the polar head-group of the phospholipid bilayer, wobbling motion is more restricted and the resulting line shape differences between bilayer and boundary spectral components are less evident [26]. Interestingly, although azethoxyl acids 1 and 2 have the nitroxide moiety near the middle of the chain, their ESR spectra in oriented systems resemble those of terminally located doxyl or proxyl nitroxides. It was therefore of importance to evaluate the suitability of centrally located azethoxyl nitroxides for boundary lipid studies.

The system which was chosen for these studies is the chromatophores isolated from the photosynthetic bacterium *Rps. sphaeroides*. This system had already been partially characterized using 16-doxylstearic acid 10 [27]. The ESR spectrum for 10 diffused into the chromatophores as they are obtained from the bacterium clearly shows the presence of two components (see Fig. 6a). A spectrum of the nitroxide bound to the protein was obtained by adding 10 to the membrane proteins which had been lipid depleted (Fig. 6b). Using computer spectral titration [28], a difference spectrum was obtained (Fig. 6c) that was typical for the nitroxide 10 in a fluid bilayer. By this method, the percentage of the lipid which was immobilized in the original spectrum was found to be 64 ± 3%.

The corresponding spectra obtained using the *trans*- and *cis*-azethoxyl acids 1 and 2 are shown in Fig. 7. The quantity of the bound component in each instance was determined to be 69 ± 10%. The greater error range is due in

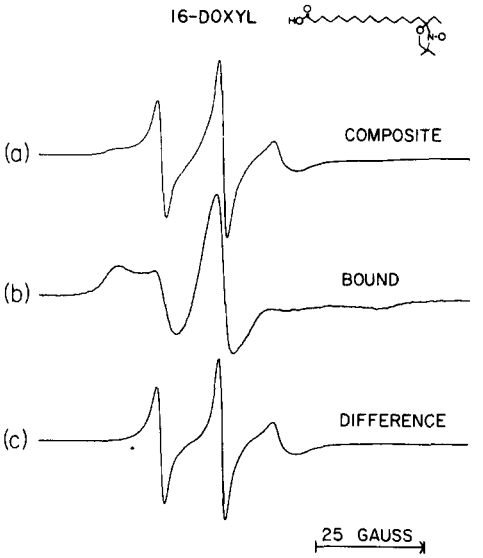


Fig. 6. ESR spectra of 16-doxylstearic acid (10) in the chromatophores of *Rps. sphaeroides* used to determine the amount of lipid immobilized by the protein. The top ESR spectrum (composite) is that obtained by diffusing 10 into the chromatophores. The center spectrum is the bound component obtained from lipid-depleted chromatophores. The bottom spectrum is the difference spectrum obtained by spectral subtraction of the bound spectrum from the composite spectrum.

part to a third component (see arrow Fig. 7) that is clearly present in the difference spectrum of the cis isomer and is evident to a lesser extent in the spectrum of the trans isomer. Apparently, the azethoxyl labels are sensitive to a region in the membrane where the fluidity is intermediate to that of fluid bilayer lipid and boundary lipid. This might be additional evidence for a layer of mo-

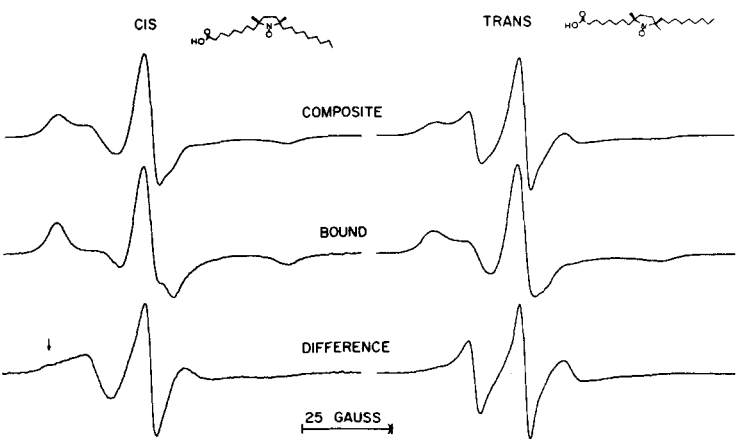


Fig. 7. ESR spectra of *cis*- and *trans*-azethoxyl acids 2 and 1 in the chromatophores of *Rps. sphaeroides* analogous to those for 16-doxylstearic acid shown in Fig. 6. The top spectra are those obtained by diffusion of the labels into the chromatophores. The center spectra are the bound components obtained from lipid-depleted chromatophores. The bottom spectra are difference spectra obtained by spectral subtraction of the bound spectra from the corresponding composite spectra. The arrow points to an additional component in the difference spectrum of the cis isomer.

tionally restricted lipid surrounding that bound by the protein [29,30].

It is striking that all three nitroxide spin labels, 16-doxylstearic acid 10, *trans*-azethoxyl nitroxide 1 and *cis*-azethoxyl nitroxide 2, show about the same fraction of boundary lipid in the chromatophore system despite the fact that these labels differ considerably in overall molecular shape. One may conclude, therefore, that the nitroxide fatty acid binding sites in this system are relatively insensitive to the geometry of the lipid chain.

It is interesting to note that the ESR spectra of both the *cis*- and *trans*-azethoxyl nitroxides show a pronounced z component in the chromatophore spectra. A z component is the large ($2A_{max}$) splitting observed, for example, in nitroxide rigid glass spectra and is due to those nitroxides for which the z axis happens to be approximately parallel to the laboratory magnetic field. The only way in which the z component may be observed with azethoxyl nitroxides is if rotation about the long molecular axis becomes hindered on the ESR time scale. Such hindered rotation about the long molecular axis of long chain doxyl or proxyl nitroxides cannot be easily detected, since the large z axis component is not averaged by rotation.

Reduction of azethoxyl nitroxides with sodium ascorbate and dithiothreitol

At times, a troublesome problem associated with the spin labeling method is the loss of ESR signal due to reduction of the nitroxide. Preliminary experiments with the reducing agent phenylhydrazine in deuteriochloroform [31] indicated that in that system, azethoxyl nitroxides were more resistant toward reduction than doxyl or proxyl nitroxides. In order to determine whether this was the case also in aqueous systems, we have investigated the relative ease of reduction of representative azethoxyl, doxyl and proxyl nitroxides by sodium ascorbate [32] and dithiothreitol in 1.0 M aqueous phosphate buffer (pH 7.5). The course of the reductions was monitored by observing the change in peak height of the low field ESR line with time.

Azethoxyl acid 1 shows the greatest resistance to reduction by sodium ascorbate. The difference between azethoxyl derivative 1 and proxyl derivative 11 is not nearly as great as the difference between 11 and doxyl acid 9. When the concentration of the sodium ascorbate was increased to 0.11 M, the signal

TABLE III

AVERAGE (4 RUNS) PERCENT ESR SIGNAL REMAINING FROM A $1.1 \cdot 10^{-4}$ M SOLUTION OF NITROXIDE TREATED WITH SODIUM ASCORBATE OR DITHIOTHREITOL FOR 20 MIN

Nitroxide	Reducing agent	Percent signal remaining (±3%)
1 (azethoxyl)	sodium ascorbate (11 mM)	93
11 (proxyl)	sodium ascorbate (11 mM)	88
9 (doxyl)	sodium ascorbate (11 mM)	5
1	sodium ascorbate (0.11 M)	70
11	sodium ascorbate (0.11 M)	53
9	sodium ascorbate (0.11 M)	0
1	dithiothreitol (0.11 M)	93
11	dithiothreitol (0.11 M)	93
9	dithiothreitol (0.11 M)	76

for 9 was totally gone by the time the sample tube was put into the spectrometer.

The rate of reduction of any of the nitroxides by dithiothreitol is significant only at higher concentrations of reducing agent. There is no significant difference in the rate of reduction between the proxyl and azethoxyl derivatives. Even the doxyl nitroxide is quite resistant to this particular reagent. We may conclude, based on the limited data presented, that in a given experiment where nitroxide reduction is to be avoided, the azethoxyl nitroxides are more likely to be successful than proxyl or doxyl nitroxides.

Acknowledgement

This research was supported by research grants GM CA 24951 and CA10337 from the U.S. Public Health Service. We thank Drs. O.H. Griffith and P.C. Jost for valuable advice and discussion.

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