*SPIN-LABELED MITOCHONDRIAL LIPIDS IN NEUROSPORA CRASSA**

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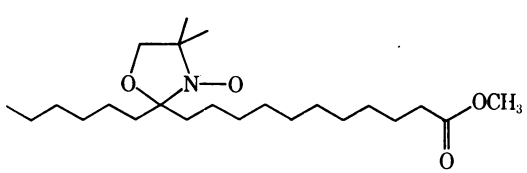
The current interest in membrane models and membranes has increased the search for new probing techniques. Recently, spin labeling has been explored as a technique for investigating micelles.[1, 2] Although micelles are a rather primitive type of membrane model, the system is simple and forms a logical starting point for more ambitious studies. The main result of the micelle work was the demonstration that a water-insoluble molecule, in this case a nitroxide free radical, remains highly mobile when solubilized by a micelle. It also became clear during the course of this work that nitroxide spin labels would be useful as probes of more complex membranelike systems.

The chemistry of nitroxide free radicals is now well understood and a wide choice of probes may be synthesized by known procedures. Electron spin resonance (ESR) is the main spectroscopic tool for observing the probes. The analysis of the ESR spectra when the nitroxide probes are tumbling at intermediate rates or aggregating in solution is still an active field of investigation. Even though the analysis is not yet complete, it is obvious from the work of many laboratories that the ESR spectrum contains a large amount of information. The number of probes, the rotational mobility of the probes, the polarity of the environment, and the state of aggregation of the probes are types of information that can, in principle, be obtained from the ESR data. A number of investigations of proteins have already been published[3] and a good review of the chemistry of nitroxides and the spin-labeling technique is available.[3]

At least three important questions remain unanswered from the earlier work. (1) How can one incorporate a nitroxide into the lipid portion of a membrane in a meaningful way? (2) Is the nitroxide moiety sufficiently stable to remain paramagnetic in a living system? (3) Will the system survive in the presence of nitroxide free radicals? The present paper is an attempt to answer these questions for one specific case. The approach in this work was to allow an organism to take up a lipid spin label. The fate of the spin label in the membrane-rich mitochondria was then followed by chemical and ESR methods. The organism chosen for this study was *Neurospora crassa*, the common bread mold.

Experimental.—Synthesis of the 12-nitroxide methyl stearate spin label: The 12-nitroxide methyl stearate (I) was prepared from 12-keto methyl stearate by the procedure of Keana, Keana, and Beetham[4] for converting ketones into stable nitroxide free radicals. The details of this synthesis are described elsewhere.[5]



I

Radioactive stearic acid: Stearic-1-^{14}C acid, specific activity 9.19 mc/mmole, was obtained from the Nuclear-Chicago Corporation. An analysis by gas chromatography on the methyl ester showed that 99% of the counts occurred in the peak corresponding to methyl stearate, and therefore the compound was used without further purification. A stock solution containing 1 μc/10 μl labeled sodium stearate was prepared for the experiments described below by neutralizing the acid with one equivalent of Na_2CO_3 and diluting to the appropriate concentration.

Culture conditions: Neurospora crassa, no. 74-OR8-1a of the Oak Ridge *Neurospora* culture collection, was kindly supplied by Dr. Patricia St. Lawrence. All cultures of *Neurospora* were grown for 72 hr in liquid medium at 34°C with constant shaking. A loop inoculum of the hyphae was routinely added to 200 ml of the nitrogen base minimal medium.[6] The nitroxide stearate (35 mg ± 5 mg) was dissolved in 0.1 ml ethanol, and the solution was added to the medium either at the time of inoculation or after 60 hr of growth. Upon addition of the alcohol solution of the nitroxide, small orange droplets were visible on the surface of the medium, but after several hours of growth these droplets disappeared. In the corresponding experiments involving ^{14}C-labeled sodium stearate in addition to, or in place of, the nitroxide-labeled stearate, 1 μc (10 μl) of the radioactive stock solution and 35 mg ± 5 mg of unlabeled sodium stearate in 0.1 ml of 50% aqueous ethanol were added at the time of inoculation or after 60 hr of growth. In all cases the hyphae were harvested by filtration after a total growth period of 72 hr. Typical yields were 4–8 gm of *Neurospora.* The differences in yield are presumably due largely to variations in the size of the loop inocula. The variation was not correlated with the presence of label. In fact, the presence of the nitroxide did not appreciably affect the yield.

Estimation of the amount of ^{14}C label and spin label remaining in the growth medium after 72 hr: After the *Neurospora* had been harvested, the growth medium was analyzed to obtain a crude measure of the uptake of labels. This was done only for the samples in which the ^{14}C-labeled or spin-labeled stearate was added immediately after inoculation. The growth medium was first adjusted to pH 6.5 and was then extracted with light petroleum to remove the remaining lipids. After evaporation of the light petroleum extract to dryness, a portion of the sample was analyzed directly for ^{14}C content by scintillation spectrometry. To estimate the amount of spin label, another portion of dried lipid was saponified and analyzed by gas chromatography as described below.

Mitochondria and lipid preparation: Mitochondria were isolated according to the procedure of Luck and Reich.[7] The lipids were extracted from the mitochondrial pellets by the method of Folch, Lees, and Stanley.[8]

Separation of the mitochondrial lipids into the three main classes: The separation of the lipids into phospholipids, free fatty acids, and neutral lipids was accomplished with a special thin-layer chromatographic plate. To prepare the plate, a standard 20 × 20-cm glass plate coated with neutral Silica Gel-G (Brinkman Instruments) was streaked across the center with a 0.5 *M* methanolic KOH solution. This procedure left a basic strip approximately 2.5 cm wide across the entire plate, and thus the plate was divided into three distinct regions. The total lipids were dried under vacuum, and 5–20 mg of the solid was taken up in diethyl ether and applied near the bottom of the thin-layer plate. Reagent grade diethyl ether was used to develop the chromatogram. The phospholipids, of course, remained near the bottom of the plate. The free fatty acids migrated until they reached the basic strip, and the neutral lipids migrated through the bottom region and the basic strip into the third region of the plate. The separation was checked on similar plates with known phospholipids (phosphatidyl ethanolamine and phosphatidyl choline), known fatty acids (palmitic, stearic, oleic, linoleic, and linolenic acids), known neutral lipids (olive oil glycerides), and mixtures of all three classes of lipids. In an additional control experiment, mixtures were made of the methyl ester nitroxide with whole *Neurospora* phospholipids, with whole *Neurospora* free fatty acids, with whole *Neurospora* neutral lipids, and with the three lipid fractions combined. Each of these mixtures was then separated by this thin-layer technique. In all cases the nitroxide migrated as a neutral lipid.

In the separation of the lipids, the three areas of the thin-layer plate were scraped into separate sintered glass funnels and eluted with 150 ml of the appropriate solvents. The eluent for the phospholipid fraction was reagent grade methanol. Diethyl ether was used to elute the neutral lipids, and diethyl ether containing 2% formic acid was used to elute the free fatty acids. After elution, the samples were evaporated to dryness and each was dissolved in 1.0 ml $CHCl_3$.

Gas chromatographic analysis: The phospholipid fraction was saponified, and the resulting fatty acids were methylated by modifications of standard procedures. The esters were dissolved in carbon disulfide and injected into a Varian Aerograph model 600D preparative unit equipped with a $^1/_4$-in. × 10-ft aluminum column packed with 20% diethylene glycol succinate on 60/80 mesh acid-washed Chromosorb W. The injection port temperature was 250°C, the column temperature was 180°C, and nitrogen was used as the carrier gas. The detector used was a flame-ionization detector fitted with a stream splitter. This column gives good separation of the methyl esters of various C_{16} and C_{18} saturated and unsaturated fatty acids. The peaks were calibrated with a standard mixture of fatty acids, no. K104, obtained from the Applied Science Laboratories. Each peak was collected by standard methods of tracer analysis. One symmetrical peak with a relatively long retention time was observed for the nitroxide stearate. The corresponding fraction collected was not, however, paramagnetic. Apparently, the somewhat severe column conditions were responsible for an unidentified structural change in the spin label, but this peak could be used as an indication of whether the nitroxide was appearing in C_{18} unsaturated fatty acids or in C_{16} fatty acids, saturated or unsaturated.

Radioassay of ^{14}C: Tracer analysis was carried out by scintillation spectrometry employing toluene:PPO and an internal quenching standard in a Beckman model 200 scintillation spectrometer. For the data of Table 1, the three lipid classes were analyzed directly without saponification or methylation of the lipids.

ESR measurements: All ESR spectra were recorded with a Varian V4502 or Varian E-3 X-band spectrometer. Low-temperature spectra were obtained with a Varian V4540 variable-temperature apparatus. A Varian C1024 time-averaging computer was used to enhance weak signals. Microwave power incident on the ESR cavity was always less than 5 mw to prevent saturation effects.

Results.—Uptake of ^{14}C-labeled and spin-labeled stearate from the growth medium: Less than 5 per cent of the original ^{14}C counts remained in the medium after removal of the *Neurospora* at the end of the 72-hour growth period. The nitroxide remaining in the growth medium at the end of the 72-hour period was also estimated to be less than 5 per cent of the original amount added. Thus, the *Neurospora* appears to take up nearly all the labeled stearate added at the beginning of the 72-hour growth period.

Incorporation of ^{14}C-labeled and spin-labeled stearate into mitochondrial lipids: The ratio of the amount of label found in the phospholipid fraction to the amount found in the neutral lipid fraction is given in Table 1 for two different experimental conditions. Thus, Table 1 gives the relative distribution of labels between these two mitochondrial lipid fractions. The free fatty acid fraction also contained ^{14}C label and spin label, but quantitative recovery of this fraction was experimentally more difficult; for this reason, the free fatty acid values were not used for direct comparisons between experiments. Furthermore, no detailed analysis of the phospholipids was attempted. The main constituents were, however, phosphatidyl ethanolamine and phosphatidyl choline.[9]

Distribution of ^{14}C-labeled and spin-labeled fatty acids found in mitochondrial lipids: The mitochondrial lipids from experiments in which the label was added immediately after inoculation were analyzed by gas chromatography. The

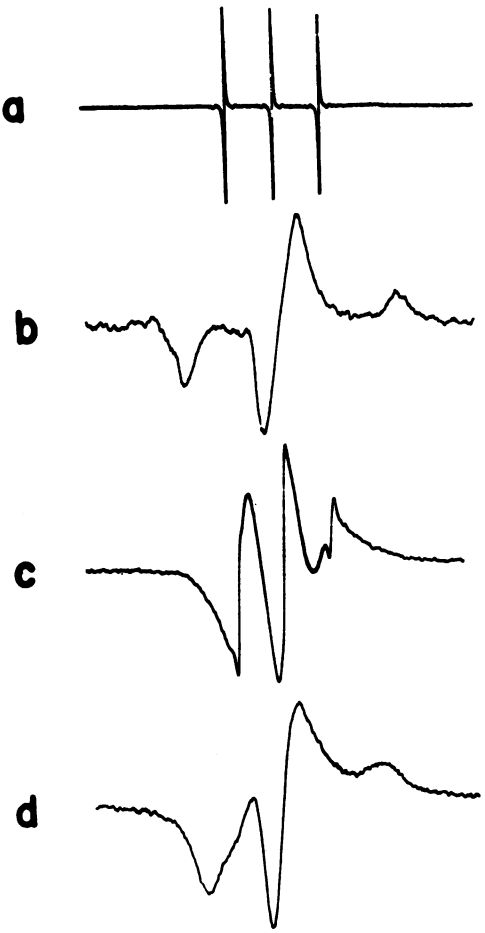


FIG. 1.—ESR spectra of (*a*) 10^{-4} *M* nitroxide IV in water at room temperature, (*b*) 10^{-4} *M* nitroxide IV in ethylene glycol at −65°C, (*c*) spin-labeled *Neurospora* mitochondria at room temperature, and (*d*) spin-labeled *Neurospora* mitochondria at −15°C. The distance between the outermost lines of spectrum (*a*) is 31 gauss. All four spectra have the same horizontal scale; however, each has a different vertical scale.

results for the ^{14}C-labeled lipids, given in Table 2, show that this label appears in both saturated and unsaturated C_{16} and C_{18} fatty acids.

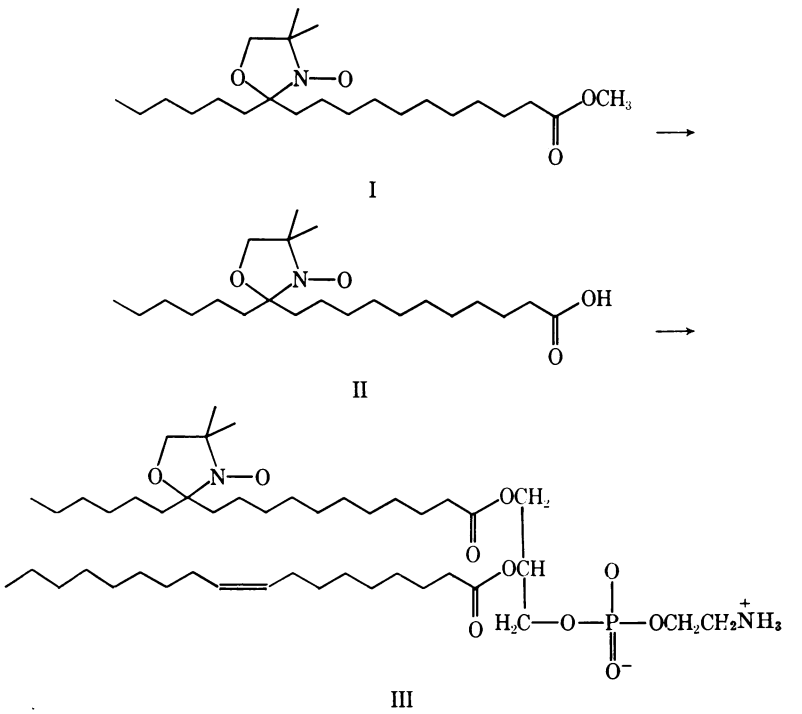
In the case of the spin-labeled lipids, only one peak was observed in the gas chromatogram and this corresponded to nitroxide methyl stearate. Thus, there was no detectable amount of spin label occurring in either unsaturated or saturated fatty acids, other than stearic acid, after 72 hours of *Neurospora* growth.

ESR results: ESR was used to determine the spin concentration of the mitochondrial phospholipid fraction obtained from *Neurospora* grown 72 hours in the presence of the spin label. If an average phospholipid molecular weight of 1000 is assumed, the measured spin concentration corresponds to approximately 1–2 per cent of the fatty acid chains having a spin label. The ESR spectra of the whole mitochondria and some useful reference spectra are given in Figure 1.

Discussion.—As mentioned in the introduction, these experiments are an attempt to discover (1) how one can incorporate a nitroxide into a membrane in a meaningful way, (2) whether the nitroxide moiety is sufficiently stable to remain

paramagnetic in a living system, and (3) whether the system will survive in the presence of nitroxide free radicals.

Concerning the first problem, several results suggest that *Neurospora crassa* incorporates the spin-labeled stearate into the lipid fraction much as it would a normal stearate molecule. Thus, as in the case of the ^{14}C-labeled stearate, only a small fraction of the spin label is recovered from the growth medium after removal of the *Neurospora*. Furthermore, Table 1 shows that a significant proportion of the spin label is found in the phospholipid, as compared with the spin label found in the neutral lipid fraction. This result is important because it indicates that the spin label is not just physically adsorbed onto the cells or dissolved in the hydrophobic regions of the cells. Some of the spin label has been enzymatically coupled with glycerol moieties to form phospholipid molecules. This is depicted by the over-all reaction:



The phosphatidyl ethanolamine III is, of course, given only as a typical phospholipid, and other spin-labeled phospholipids are undoubtedly present.

The next point to consider is whether the nitroxide is sufficiently stable to remain paramagnetic in *Neurospora*. The attention here is focused on the $\rangle N—O$ group since the unpaired electron responsible for the paramagnetism (and the ESR signal) is localized in this region of the spin label. The spin label would be unstable in this respect if, for example, it were consumed as a general carbon source by the organism or if it were reduced by some enzyme. Almost all the

TABLE 1. *Incorporation of labels into mitochondrial phospholipid and neutral lipid fractions.*

Time (hr)	Expt.	Spin Label ($M \times 10^5$) PL	NL	PL/NL	^{14}C Label (cpm) PL	NL	PL/NL
0–72	(*a*)	0.8	2.1	0.4	2819	3378	0.8
	(*b*)	1.6	2.1	0.8	2590	2371	1.1
	(*c*)	0.9	2.2	0.4	2688	2866	0.9
60–72	(*d*)	12	2.5	5	1650	1035	1.6
	(*e*)	26	0.6	42	1982	2430	0.8
	(*f*)	18	6.0	3	3140	4206	0.7

PL, recovered phospholipid fraction; NL, recovered neutral lipid fraction, for six experiments. The entry "0–72 hr" indicates that the ^{14}C and spin labels were added to the same culture immediately after inoculation, and *Neurospora* were harvested after 72 hr. Similarly, "60–72 hr" indicates the simultaneous addition of the two labels 60 hr after inoculation, with *Neurospora* harvested after an additional 12 hr of growth. The unusually low value for the NL spins in experiment (*e*) is not readily explained, but is apparently not accounted for by poor recovery of the NL fraction as judged by the ^{14}C label. ^{14}C cpm were determined on 0.1-ml sample of the $CHCl_3$ solution of each fraction.

TABLE 2. *Distribution of ^{14}C in fatty acids of mitochondrial lipids after 72 hours in growth medium.*

Fatty acid	Type	^{14}C Label (%)
Less than 16 carbons	—	1.6
Palmitic	16:0	2.0
Palmitoleic	16:1	1.2
Stearic	18:0	6.6
Oleic	18:1	47.2
Linoleic	18:2	29.4
Linolenic	18:3	11.9

The sodium salt of stearic-1-^{14}C acid was added to the growth medium immediately after inoculation with hyphae. The type of fatty acid as well as the common name of the acid is listed. The notation 18:1 means, for example, an 18-carbon chain containing one double bond. The percentage figures given represent the distribution of counts in the fatty acids of the total lipids (neutral lipids, free fatty acids, and phospholipids) obtained from the mitochondria of *Neurospora crassa*. All numbers are believed accurate to within ±5%.

spin label is taken up by *Neurospora* after 72 hours. The percentage of spin labels remaining in *Neurospora* after 72 hours is difficult to estimate because these labels are undoubtedly widely distributed throughout the cell. The nitroxide stearate must be reasonably stable, however, because strong ESR signals are obtained from the mitochondrial lipids. The spin-labeled probe accounts for several per cent of the phospholipid fatty acid chains, which is by no means a trace amount.

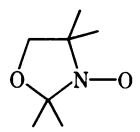
The data of Table 1 show that the ratio of spin labels in the phospholipid fraction to spin labels in neutral lipid fraction is higher in experiments in which exposure of the label is limited to 12 hours. The analogous ratios for the ^{14}C label show no such difference with time. The possibility exists that incorporation of the nitroxide label into different lipid classes may affect the relative probabilities of loss of signal with time. Perhaps this could result from availability for enzymatic reduction of the paramagnetic $\diagdown\!\!\diagup$N—O portion of the label. In any case, from these experiments one can conclude that the nitroxide is sufficiently stable for spin-labeling experiments but that there may be an optimum time of exposure for the label.

The third problem can also be solved from the data. *Neurospora* obviously survived quite well in the presence of the nitroxide free radical since the presence

of spin label did not appreciably affect the amount of *Neurospora* produced. This is somewhat surprising since the nitroxide was present in approximately 5×10^{-4} *M*. This is, in terms of labeling studies, a reasonably high concentration.

There are two other observations worth noting. First, the hydrocarbon chain of the recovered spin label *appears* to remain unchanged. This is in contrast to normal stearate molecules, which, of course, undergo many enzymatic modifications. Table 2 shows that after 72 hours nearly half the original stearate recovered occurs as an unsaturated derivative, oleic acid. Only 6 per cent of the original stearic acid is found unmodified after 72 hours. The conclusion that the nitroxide stearate remains unaltered can be made with more certainty when unsaturated and other saturated fatty acid spin labels are available to calibrate the gas chromatograph. At present the conclusion remains tentative but highly probable, since only one new peak appears in each of the gas chromatograms of the spin-labeled mitochondrial lipids and this is identical in appearance to the peak obtained from the nitroxide stearate.

Secondly, the appearance of the ESR spectrum obtained with the probe in various environments is of some interest at this point, even though results on spin-labeled lipid-protein binding studies using this nitroxide stearate are not yet available. The ESR spectrum of a typical nitroxide free radical in a nonviscous solvent such as water consists of three sharp lines of nearly equal height (Fig. 1*a*). The ESR spectrum in water is quite insensitive to the structure of the radical. The particular nitroxide used to obtained Figure 1*a* was



IV

This radical was chosen because it approximates the spin-label portion of the spin-labeled stearate. (The spin-labeled stearate itself is essentially insoluble in water, but when dissolved in ethanol, it has a spectrum nearly identical to IV in ethanol.)

As the tumbling rate of the nitroxide is slowed, the three lines of the spectrum begin to broaden unequally, and at low temperatures a rigid glass spectrum is observed (Figure 1*b*). Spectra *a* and *b* represent the two extremes observed. Nitroxides tumbling at intermediate rates exhibit partially broadened spectra. The isolated spin-labeled mitochondria (Fig. 1*c*) is readily recognized to be an intermediate case. As the temperature of the mitochondria is lowered (Fig. 1*d*), the spectrum approaches the rigid glass limit. Analyses of these and related spectra are in the very primitive stages. Spectra similar to that of Figure 1*c*, however, are observed when the spin-labeled phospholipid fraction is dissolved in membranelike lecithin aggregates in water. The rotational motion of the spin-labeled phospholipid is obviously quite hindered.[10] From the ESR spectra it should prove possible to analyze the degree of molecular motion and perhaps the relative orientations of the labels in various types of membranes.

Summary.—A novel nitroxide-labeled fatty acid ester was synthesized for spin-labeling studies of membranes and introduced into the growth medium of *Neurospora crassa*. The spin-labeled stearate was taken up by the organism to the same extent as stearic acid. The spin label was found in the mitochondrial neutral lipids, free fatty acids, and phospholipids. Although much of the spin label may have been destroyed, significant quantities survived. There was apparently no enzyme-catalyzed unsaturation or shortening of the recovered lipid spin label. Conversely, the growth of *Neurospora* was not appreciably altered by the presence of the spin label. The spin-labeling experiments were accompanied by control experiments with ^{14}C-labeled stearic acid.

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[1] Waggoner, A. S., O. H. Griffith, and C. R. Christensen, these PROCEEDINGS, **57**, 1198 (1967).

[2] Waggoner, A. S., A. D. Keith, and O. H. Griffith, *J. Phys. Chem.*, in press.

[3] Hamilton, C. L., and H. M. McConnell, in *Structural Chemistry and Molecular Biology*, ed. A. Rich and N. Davidson (San Francisco and London: W. H. Freeman and Co., 1968).

[4] Keana, J. F. W., S. B. Keana, and D. Beetham, *J. Am. Chem. Soc.*, **89**, 3055 (1967).

[5] Waggoner, A. S., T. J. Kingzett, O. H. Griffith, and A. D. Keith, manuscript in preparation.

[6] Vogel, H. J., *Microbial Genet. Bull.*, **13**, 42 (1956).

[7] Luck, D. J. L., and E. R. Reich, these PROCEEDINGS, **52**, 872 (1964).

[8] Folch, J., M. Lees, and G. H. Sloane Stanley, *J. Biol. Chem.*, **226**, 497 (1957).

[9] Determined by thin-layer chromatography with standard staining techniques.

[10] Superimposed on spectrum *c* are three relatively sharp lines due to a small fraction of the nitroxide spin labels whose motion is not greatly hindered. The superimposed spectrum is particularly noticeable on the right-hand peak of *c*.