Biochimica et Biophysica Acta, 943 (1988) 477–484
Elsevier

BBA 74098

Kinetics of enzyme-mediated reduction of lipid soluble nitroxide spin labels by living cells

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(Received 26 April 1988)

Key words: Nitroxide spin label; Spin label; Enzymatic reduction; Kinetics; Doxylstearic acid; ESR; (Mouse cell); (Hamster cell)

Nitroxide spin labels can be reduced to the corresponding hydroxylamines in cells. The selective action of inhibitors, and thermal and chemical inactivation demonstrate that the reduction of nitroxides in cells is an enzymatic or enzyme-mediated process. The kinetics of reduction of doxylstearates are affected by the position of the doxyl moiety along the stearic acid chain. The doxyl moiety of 5-doxylstearate is close to the membrane surface, and its reduction is first order with respect to the nitroxide, whereas the doxyl moieties of 10- and 12-doxylstearate are in the membrane hydrocarbon region and their reduction is a zero-order process. The reduction of 16-doxylstearate which usually has a mixture of first- and zero-order kinetics becomes zero order with addition of an extracellular broadening agent, potassium trioxalatochromiate(III). These results suggest that the rate of reduction of doxyl moieties is controlled by their accessibility to reducing equivalents, i.e., the rate-limiting step for the reduction of the doxyl moiety deep in the membrane is the diffusion of reducing equivalents within or into the membrane. The reduction of doxylstearates in cells is inhibited by rotenone but not antimycin A, cyanide, propyl gallate or SKF-525A. It appears that the reduction of doxylstearates takes place at the level of the ubiquinone in the respiratory chain in mitochondria in these cells.

Introduction

In the past, the reduction of nitroxides by living systems has been considered a liability for the spin label technique and has limited its use as a means to study the molecular dynamics of cells [1–4]. However, recent work has shown that the reduction of nitroxides to their corresponding hydroxylamines is reversible and can be modified, by the presence or absence of oxygen [5]. These observations, combined with the knowledge of the effectiveness of nitroxides as relaxers of water and lipid protons [7,8], has led to the concept of nitroxides as metabolically responsive contrast or imaging agents for in vivo NMR [9–12] or ESR [13,14] imaging and spectroscopy.

There have been a number of studies on the reduction of nitroxides in microsomes [15–17], mitochondria [18], *Escherichia coli* membrane vesicles [19], rabbit spermatozoa [12], and whole animals [21,22] which relate the reduction process to enzyme activity [15–20], non-enzymatic activity [21,22], sulfhydryl groups [23,24], and ascorbate

Abbreviations: PDT, 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl. See Table I for further nomenclature.

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[25,26]. The large number of postulated mechanisms for nitroxide metabolism stems from the large number of systems and types of nitroxides in which this process has been studied and it is possible that all the above mechanisms may play greater or lesser roles in any given system under study. It appears that major variables that affect the rate of reduction may include the type of tissue, cell, or cell fraction, the lipid solubility of the nitroxide, the ring structure of the nitroxide, and the environmental conditions, especially the concentration of oxygen and pH.

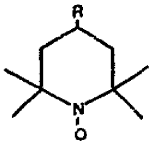
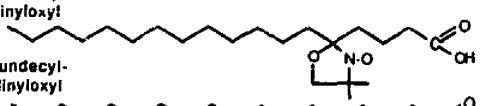
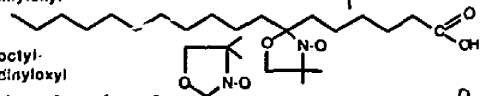
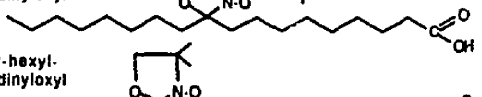
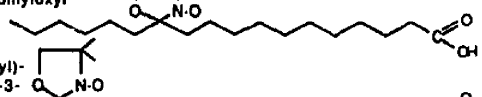
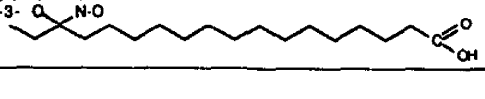
In view of the above considerations, we have begun a systematic study of the reduction of nitroxides at the cellular level which includes determination of the possible major sources of nitroxide metabolism and their resultant products, the kinetics of the reduction process, and the location of the reduction process within the cell. In this report, we present our results on the reduction of the lipid-soluble doxylstearic acid nitroxides in two established mammalian cell lines (mouse thymus-bone marrow and Chinese hamster ovary cells). We have concentrated initially on these nitroxides because they are used extensively as spin labels to probe membrane dynamics and they also appear to be among the most useful series of nitroxides for the study of redox metabolism via their reduction and reoxidation in cells. Our results show that the mitochondria are the major source of the reduction of lipid-soluble nitroxides in these cells and that the kinetics of reduction are dependent on the depth at which the doxyl moiety is located in the membrane.

Materials and Methods

Cell. Mouse thymus-bone marrow (TB) cells were obtained from Professor P. Wong of the University of Illinois, cloned from a single cell. TB cells were prepared as described previously [27]. Chinese hamster ovary (CHO) cells were obtained from Dr. L. Hopwood, Medical College of Wisconsin, Milwaukee and prepared as described

TABLE I

STRUCTURE OF NITROXIDES STUDIED

Acronym	Chemical name		Structure
Tempone	4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl	R: =O	
Tempol	4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl	R: OH	
Tempo	2,2,6,6-tetramethylpiperidine-1-oxyl	R: H	
5-DS	2-(3-carboxylpropyl)-2-tridecyl-4-4'-dimethyl-3-oxazolidinyloxyl		
7-DS	2-(5-carboxylpentyl)-2-undecyl-4-4'-dimethyl-3-oxazolidinyloxyl		
10-DS	2-(8-carboxyloctyl)-2-octyl-4-4'-dimethyl-3-oxazolidinyloxyl		
12-DS	2-(10-carboxyldecyl)-2-hexyl-4-4'-dimethyl-3-oxazolidinyloxyl		
16-DS	2-(14-carboxyltetradecyl)-2'-ethyl-4-4'-dimethyl-3-oxazolidinyloxyl		

in another report [5]. Cells were washed with serum-free medium before used.

Chemicals. All nitroxides except the one described below, were purchased from Molecular Probes (Junction City, OR) and used without further purification. 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-[1-^{15}N]oxyl ([^{15}N]PDT) was purchased from MSD Isotopes (St. Louis, MD). The acronyms, full chemical names, and structures of the nitroxides used in these studies are shown in Table I. Inorganic chemicals were of analytical grade. SKF-525A was a gift from Smith Kline French laboratories (Philadelphia, PA). Rotenone, antimycin A and propyl gallate were purchased from Sigma Chemical Co. (St. Louis, MO). Malonate was purchased from Eastman Kodak Co. (Rochester, NY).

Labeling. Cells (10^8 per ml of approx. 4.5 mg of protein per ml) were labeled with lipid soluble nitroxides as follows: An appropriate aliquot of a nitroxide in ethanol was pipetted into a 6×50 mm glass culture tube and dried to make a uniform film of nitroxide on the side of the tube. The cell suspension (100 μl) was then added, vortexed intermittently for 0.5 to 2 minutes, and removed for ESR studies. These procedures did not alter cell viability, as measured by exclusion of Trypan blue, and labeled cells are capable of replicating normally in vitro.

ESR. All spectra were taken on a Varian E-109E ESR spectrometer. The cells were mixed with a 10% Dextran solution to retard settling of the cells. The cavity (Varian TE 102) was mounted so the sample was horizontal to prevent cells from settling out of the sensitive volume of the cavity. Samples were drawn into a gas-permeable Teflon tube (Zeus Industries, Raritan, N.J.) and inserted into a quartz ESR tube open at each end. Experiments were performed at 37 °C using a Varian gas flow temperature controller. The time-dependent changes in the concentration of nitroxides were monitored by setting the magnetic field of the spectrometer at the peak of the midfield line of ESR spectrum of the nitroxide and turning the field sweep to zero (Fig. 1A). A modulation amplitude of 5 Gauss was used to broaden the peak purposely, to minimize the effect of any drift of the magnetic field or line-width changes during the course of measurements. After the experiments

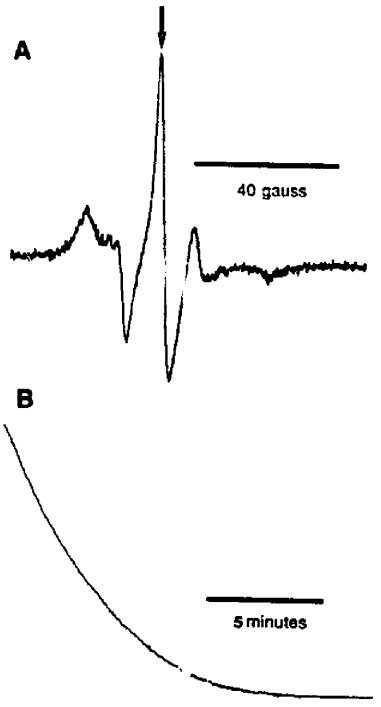


Fig. 1. ESR Spectrum of 5-doxylstearate in mammalian cells and measurement of rate of reduction. The sample was 10^7 TB cells in 100 μl with approximately 2 nmol of 5-doxylstearate in a gas-permeable tube at 37 °C. Time-dependent changes in the concentration of the nitroxide were obtained by setting the recorder at the peak of the midfield ($m = 0$) line of the ESR spectrum (1A). When the sample was perfused by nitrogen, the decrease of the line height was recorded and the rate of reduction was calculated from the initial slope (1B).

were performed cell viability was measured by 0.4% Trypan blue exclusion. Typically 95–99% of the cells were able to exclude the dye and were considered viable. Rates of reduction were calculated from the initial slopes (Fig. 1B) by digitizing the data on a Houston HI-PAD digitizer. Data were collected, stored, and manipulated with an IBM PC computer [28].

Measurement of concentrations of nitroxides and hydroxylamines. The concentrations of nitroxides in the membrane were measured by double integration of the ESR spectra and calibrated against a standard solution of Tempol. Then the peak heights were correlated with the concentrations of the nitroxides in the membrane. The kinetics of the reduction process were derived from the time-dependent changes of the peak

heights of the ESR spectra of the nitroxides. The concentrations of hydroxylamines were measured by oxidizing them back to the corresponding nitroxides by [^{15}N]PDT, an isotopic variant of perdeuterated Tempone [5]. [^{15}N]PDT is membrane permeable and is an efficient oxidizer of membrane soluble hydroxylamines but will not oxidize other reduction products such as amines (Chen et al., unpublished data). It reoxidizes 100% of the hydroxylamines from reduced doxylstearates, as compared to ferricyanide [29] which in our hands reoxidized only 80–90%. The ESR spectrum of [^{15}N]PDT has two lines and, therefore, the middle line of conventional [^{14}N]nitroxides can be distinguished easily from the spectrum of [^{15}N]PDT.

Results

Our general aims were to determine (1) if the reduction of nitroxides was enzymatic or nonenzymatic; (2) the products of the reduction of doxylstearates in TB and CHO cells; (3) the kinetics of reduction, quantitatively under well controlled conditions; and (4) the site of reduction of nitroxides in cells.

1. Products of reduction

In order to identify the products of the reduction of doxylstearates in cells, we used [^{15}N]PDT to study systematically the oxidation of reduced 5-, 7-, 10-, 12-, and 16-doxylstearates by TB and CHO cells under hypoxic conditions. After complete reduction of 5 nmol of one of the above doxylstearates by 10^7 cells in 100 μl at 37°C, 50 nmol of [^{15}N]PDT were added into the sample. By comparing the middle peak of ESR spectrum of the nitroxide before reduction by cells and after oxidation by [^{15}N]PDT, we determined that we had recovered 100% of the signal.

2. Inactivation of enzymes

We investigated the role of enzyme mediated processes in the reduction of nitroxides by observing the effects of enzyme-inactivating treatments on the rates of reduction. (We use the term enzyme-mediated to indicate that our results do not differentiate between direct reduction by enzymes and enzymatic production of a reducing intermediate that diffuses to the nitroxides.) Reduction rates were determined at the temperatures shown in Fig. 2 for untreated cells, or cells which had been heated to 70°C for 10 min. The difference between these curves determines that part of the reaction which is heat sensitive. Above 50°C the two curves were essentially identical while at lower temperatures there was less activity in the heated cells, with the maximum difference occurring at about 40°C. Trichloroacetic acid treatment affected reduction rates in a manner similar to those shown in Fig. 2.

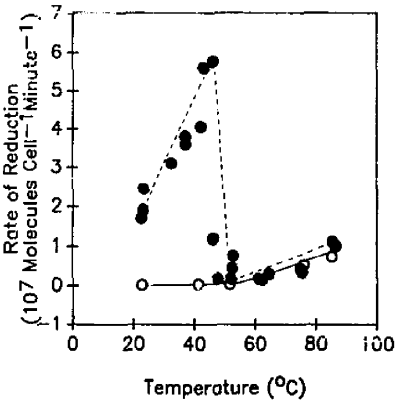


Fig. 2. Effect of temperature on the rate of reduction of nitroxides by cells. TB cells (10^7 in 100 μl) were incubated either at the measurement temperature (●) or at 70°C for 10 min (○) and then labeled with 4 nmol of 5-doxylstearate. The labeled cells were then placed in the spectrometer at the indicated temperature and the reduction rate in the absence of oxygen was measured.

3. Kinetics of the reduction of doxylstearates

As shown in Fig. 1B, the amount of 5-doxylstearate decreases exponentially as a function of time, whereas it decreases linearly for 10-doxylstearate (Fig. 3). When the decrease of the amount of 5-doxylstearate is plotted on a semilogarithmic scale (Fig. 4), a straight line is obtained whose slope is equal to the first-order rate constant. These results indicate that, with respect to nitroxide, the reduction of 5-doxylstearate follows first-order kinetics, whereas the reduction of 10-doxylstearate follows zero-order kinetics. The rate of reduction of 12-doxylstearate is very close to that of 10-doxylstearate and also follows zero-

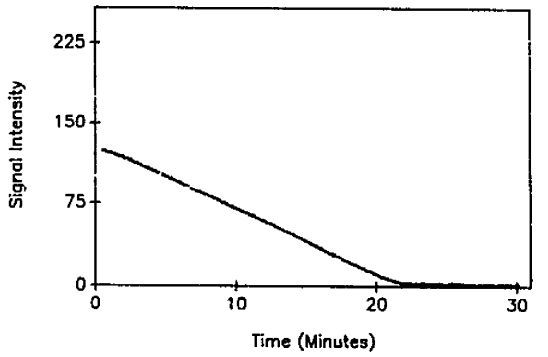


Fig. 3. Reduction of 10-doxylstearate by mammalian cells. The experimental conditions were identical with those described in Fig. 1 except 10-doxylstearate was used, the signal intensity is in arbitrary units.

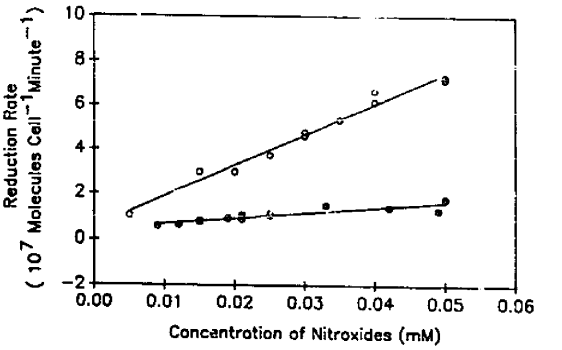


Fig. 5. Concentration dependence of the rate of reduction of 5- (○) and 10-doxylstearates (●) in cells. The concentrations of 5- and 10-doxylstearates are bulk concentrations as described in the Results. The straight lines are least squares fits of the data.

order kinetics. In the case of 7-doxylstearate, the kinetics are between those of 5- and 10-doxylstearate.

Our kinetic analysis was verified by plotting the rates of reduction versus the bulk concentrations of the nitroxides (Fig. 5). (We use the term bulk concentration to indicate the concentration that would occur if the lipophilic nitroxides were homogeneously distributed throughout the sample; in reality the nitroxides are located almost exclusively in the lipid-rich areas of the cells, principally membranes.) As shown in Fig. 5, the rate of reduction of 5-doxylstearate in CHO cells is linearly proportional to its bulk concentration below 0.05 mM. However, the rate of the reduction of 10-doxylstearate is almost independent of the nitroxide concentration (Fig. 5). Similar results were seen in TB cells.

Fig. 6 indicates that the reduction of 16-doxylstearate has complex kinetics which appear to be a mixture of first and zero order. The high-field line of 16-doxylstearate in TB cells is composed of two components, a broad signal characteristic of slow isotropic motion which would occur in the disordered hydrocarbon region of membranes, and a narrow signal characteristic of rapid isotropic motion which is characteristic of a nitroxide in an aqueous environment (Fig. 7A). By addition of potassium trioxalatochromiate(III), an extracellular broadening agent [30], the 'aque-

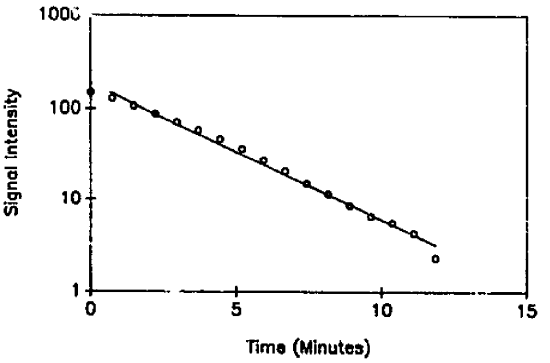


Fig. 4. Semi-log plot of data in Fig. 1B. The straight line is a least-squares fit of the digitized data from Fig. 1B. The signal intensity is in arbitrary units.

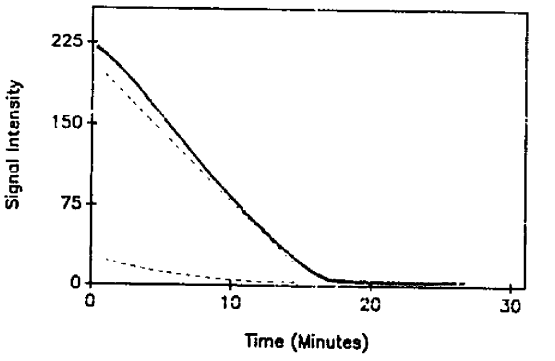


Fig. 6. Reduction of 16-doxylstearate by mammalian cells. The experimental conditions were identical with those described in Fig. 1 except 16-doxylstearate was used. The dashed lines illustrate schematically that the complex kinetics for the reduction of 16-doxylstearate appear to be a mixture of first and zero order. The signal intensity is in arbitrary units.

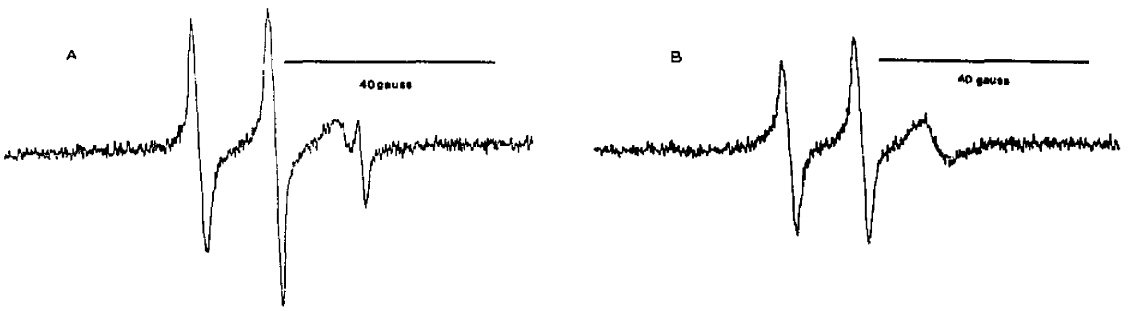


Fig. 7. ESR spectra of 16-doxylstearate in TB cells. A narrow signal can be seen near the high-field line of the spectrum of the control sample (A). By addition of 55 mM potassium trioxalatochromiate(III), an extracellular broadening agent, the narrow signal from the aqueous component is broadened away and leaves only the membrane component (B).

ous' component is broadened away and leaves only the 'hydrocarbon' component (Fig. 7B). Under these conditions, the rate of reduction follows zero-order kinetics (Fig. 8). This result indicates that the 'aqueous' and 'hydrocarbon' components are not in rapid equilibrium and that the rate-limiting step for reduction of the 'hydrocarbon' component is diffusion of reducing equivalents to it.

4. Effects of the position of doxyl moiety on the rate of reduction

As shown in Fig. 9, the rates of reduction are affected by the position of the doxyl moiety along the fatty acid chain. The rate of reduction of 5-doxylstearate is greater than that of 7-doxylstearate and still greater than that of 10- and 12-doxylstearates. The exception is the rate of

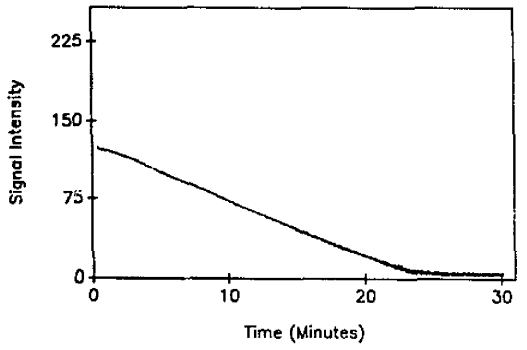


Fig. 8. Reduction of 16-doxylstearate in the presence of the extracellular broadening agent. These reduction kinetics were obtained by monitoring the decay of the midfield peak of the spectrum in Fig. 7B. The signal intensity is in arbitrary units.

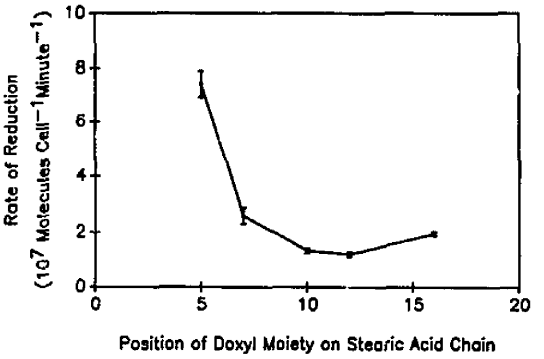


Fig. 9. Rate of reduction as a function of the position of the doxyl-moiety. TB cells were labeled with the same amount of 5-, 7-, 10-, 12-, and 16-doxylstearates, as measured from double-integrated spectra. Experimental conditions and methods are the same as those in Fig. 1.

TABLE II

EFFECTS OF INHIBITORS ON RATE OF REDUCTION

TB cells were labeled with 5 nM 5-doxylstearate. The concentration was 5 mM for malonate and cyanide; 0.05 mM for antimycin A, SKF-525A, and propyl gallate; and 0.2 mM for rotenone. The units of the rates are 10^7 molecules·cell^{-1}·min^{-1}. The results are given as mean ± S.E. (number of independent measurement in parentheses).

Inhibitors	Rates of reduction
Control	8.8 ± 2.5 (10)
Rotenone	1.8 ± 0.5 (4)
Malonate	8.3 ± 1.5 (4)
Rotenone + malonate	1.9 ± 0.6 (4)
Antimycin A	6.7 ± 0.6 (4)
Cyanide	10.0 ± 0.8 (4)
SKF-525A	9.2 ± 1.2 (4)
Propyl gallate	6.3 ± 1.2 (4)

reduction of 16-doxylstearate, which is greater than that of 10- or 12-doxylstearates.

5. Effect of inhibitors on the reduction of a doxylstearate

Table II, rotenone decreased the rate of reduction of 5-doxylstearate significantly, whereas malonate, antimycin A, cyanide, or the microsomal inhibitors propyl gallate or SKF-525A had little effect on the rate of reduction.

Discussion and Conclusions

1. Products of reduction

The identity of the products of reduction of nitroxides in biological systems has been the subject of much debate [15–17,21–22,26]. Using ferricyanide, we and others were not able to obtain complete recovery of the reduced nitroxides and the previously reported uncertainties as to the products of cellular reduction of nitroxides may have arisen, in part, from the use of oxidizing agents which had similar efficiencies. We recently have shown that [^{15}N]PDT can oxidize hydroxylamines back to nitroxides (Chen et al., in preparation) by hydrogen atom exchange [6] but will not regenerate nitroxides from amines or other products. The use of [^{15}N]PDT is advantageous in oxidizing hydroxylamines in membranes because it partitions into the lipid regions of the sample and comes in contract with the doxyl moieties in the membranes. Using [^{15}N]PDT, we recovered 100% of the ESR signal lost during the reduction of doxylstearate. This demonstrates that the products of the reduction of doxyl-stearates in TB and CHO cells are the corresponding hydroxylamines. There is no evidence for the formation of amines or any other products from these nitroxides under our experimental conditions.

2. Inactivation of enzymes

The data on thermal inactivation of the reduction of nitroxides suggests that reduction of nitroxides is primarily enzyme-mediated, yet another possibility is that reducing agents might be consumed during the heating process. This seems less likely in view of the selective action of inhibitors (Table II), and chemical inactivation by trichloroacetic acid. Therefore, we conclude that reduction of doxylstearates in these cells is principally enzyme-mediated.

3. Kinetics analysis

Our data show that the rate of reduction of 5-doxylstearate, whose doxyl moiety is close to the membrane surface, follows first-order kinetics, whereas the rates of reduction of 10- and 12-doxylstearates, whose doxyl moieties are deep in the membrane, follow zero-order kinetics. These results suggest that the rate of reduction of the doxylstearates is controlled by the accessibility of the doxyl moieties to the reducing equivalents. Since the reduction of 5-doxylstearate follows first order kinetics and is dependent on concentration (Figs. 1, 4 and 5), this suggests that 5-doxylstearate and the reducing equivalents are in frequent contact. The rate-limiting step for the reduction of the doxyl moieties deep in the membrane can be diffusion of either the doxylstearates toward to the surface of the membrane or the reducing equivalents into the membrane. As shown in Fig. 5, the rate of reduction of 10-doxyl stearate is almost independent of the concentration of the nitroxide molecules in the membrane. Therefore, the principal rate-limiting step for the reduction of the doxyl moiety deep in the membrane appears to be the diffusion of reducing equivalents from the surface into the hydrocarbon region of the membrane.

4. Effects of the position of doxyl moiety along the fatty acid chain on reduction rate

We now can provide an explanation on the effects of the position of doxyl moiety on the rate of reduction (Fig. 9). The slower reduction rates of 10- and 12-doxylstearates compared to that of 7-doxylstearate and the fastest rate seen with 5-doxylstearate are connected with the source of reducing equivalents being near the surface of the membrane. The increase of the rate of reduction of 16-doxylstearate compared to that of 10- or 12-doxylstearates seems to be caused by the presence of some 16-doxylstearate in the aqueous phase of the sample (Fig. 7A). As shown in Fig. 8, when the reduction of the membrane component of the 16-doxylstearate signal was selectively followed by broadening away the aqueous component with potassium trioxalatochromiate(III), the rate was similar to that of 10- or 12-doxylstearates. This

suggests that the doxyl group of 16-doxylstearate partitions between the hydrophobic interior of the membrane and the aqueous compartment of the sample and that in TB cells, the two components of 16-doxylstearate in their two compartments are not in rapid equilibrium. The zero-order kinetics for reduction of the 10- and 12-doxylstearates indicates that the reducing equivalents are generated close to the membrane surface.

5. Effects of inhibitors

It has been suggested from the studies in isolated rat liver mitochondria [18] and in intact rabbit spermatozoa [20] that the reducing equivalents responsible for the reduction of nitroxides have a redox potential that indicates the involvement of ubiquinone in the respiratory chain. In other reports [15–17], the responsible enzyme was suggested to be cytochrome *P*-450 in liver microsomes. As shown in Table II, the reduction of 5-doxylstearate in living TB cells is inhibited by rotenone but is relatively insensitive to malonate, antimycin A or cyanide. In addition, SKF-525A and propyl gallate, inhibitors for cytochrome *P*-450 and cytochrome *P*-450 reductase, have little effect on the reduction. We have previously shown that both cyanide and antimycin can eliminate the dependence of reduction of lipophilic nitroxides on intracellular oxygen concentration (Chen, K. et al., unpublished results). Based on the above results, it appears that the reduction of doxylstearates in these cells principally takes place at the level of the ubiquinone in the mitochondrial respiratory chain.

Acknowledgements

We thank Dipali Apte for obtaining some of the preliminary results and Maoxin Wu for preparing cell cultures. This work was supported by NIH grants GM 35534, and GM 34250 and used the facilities of the University of Illinois ESR Center which is supported by grant RR 01811 from NIH.

References

1 Berliner, L.J. (1976) Spin Labeling Theory and Application, Academic Press, New York.
2 Berliner, L.J. (1979) Spin Labeling Theory and Application II, Academic Press, New York.
3 Swartz, H.M. and Swartz, S. (1983) in Methods of Biochemical Analysis (Glick, D., ed.), pp. 207–323, John Wiley and Sons, New York.
4 Morse, P.D., II. (1985) in Structure and Properties of Cell Membranes (Benga, G., ed.), pp. 195–236, CRC Press, Boca Raton, FL.
5 Chen, K. and Swartz, H.M. (1988) Biochim. Biophys. Acta, in press.
6 Schwartz, M.A., Parce, J.W. and McConell, H.M. (1979) J. Am. Chem. Soc. 101, 3592–3595.
7 Bennett, H.F., Brown, R.D., III, Koenig, S.H. and Swartz, H.M. (1987) Magn. Reson. Med. 4, 93–111.
8 Bennett, H.F., Swartz, H.M., Brown, R.D., III and Koenig, S.H. (1987) Invest. Radiol. 22, 502–507.
9 Brasch, R.C., London, D.A., Wesbey, G.E., Tozer, T.N., Nitecki, D.E., Williams, R.D., Doemeny, J., Tuck, L.D. and Lallemend, D.P. (1983) Radiology 147, 773–779.
10 Brasch, R.C. (1983) Radiology 147, 781–788.
11 Swartz, H.M., Chen, K., Pals, M., Sentjurc, M. and Morse, P.D., II. (1986) Magn. Reson. Med. 3, 169–174.
12 Swartz, H.M. (1988) in Advances in Magnetic Resonance Imaging (Feig, E., ed.), Ablex Publishing Co., Norwood, NJ, in press.
13 Berliner, L.J. and Fujii, H. (1985) Science 227, 517–519.
14 Bacic, G., Demsar, F., Zoltnic, Z. and Swartz, H.M. (1987) J. Magn. Reson. Med. Biol. in press.
15 Stier, A. and Reitz, I. (1971) Xenobiotica 1, 499–500.
16 Stier, A. and Sackmann, E. (1973) Biochim. Biophys. Acta 311, 400–408.
17 Rosen, G.M. and Rauckman, E.J. (1977) Biochem. Pharmacol. 26, 675–678.
18 Quintanilha, A.T. and Packer, L. (1977) Proc. Natl. Acad. Sci. USA 74, 570–574.
19 Baldassare, J.J., Robertson, D.E., McAfee, A.G. and Ho, C. (1974) Biochemistry 13, 5210–5214.
20 Chapman, D.A., Killian, G.J., Gelerinter, E. and Jarrett, M.T. (1985) Biol. Reprod. 32, 884–893.
21 Couet, W.R., Eriksson, M.G., Tozer, T.N., Tuck, L.D., Wesbey, Nitecki, D. and Brasch, R.C. (1984) Pharm. Res. 1, 203–209.
22 Couet, W.R., Eriksson, U.G., Brasch, R.C. and Tozer, T.N. (1985) Pharm. Res. 69–72.
23 Giotta, C.J. and Wang, H.H. (1972) Biochem. Biophys. Res. Commun. 46, 1576–1580.
24 Chen, K.-Y. and McLaughlin, M.G. (1985) Biochim. Biophys. Acta 845, 189–195.
25 Perkins, R.C., Bath, A.H., Wilkerson, L.S., Serafin, W., Dalton, L.R., Park, C.R. and Park, J.H. (1980) Proc. Natl. Acad. Sci. USA 77, 790–794.
26 Eriksson, U.G., Brasch, R.C. and Tozer, T.N. (1987) Drug Metab. Dispos. 15, 155–160.
27 Swartz, H.M., Sentjurc, M. and Morse, P.D., II. (1986) Biochim. Biophys. Acta 888, 82–90.
28 Morse, P.D., II. (1987) Biophys. J. 51, 440a.
29 Kaplan, J., Canonico, P.G. and Caspary, W.J. (1973) Proc. Natl. Acad. Sci. USA 70, 66–70.
30 Berg, S.P. and Nesbitt, D.M. (1979) Biochim. Biophys. Acta 548, 608–615.