Human Erythrocyte Membrane Permeability and Nitroxyl Spin-Label Reduction

ULF G. ERIKSSON[‡], THOMAS N. TOZER[‡x], GEORGE SOSNOVSKY[§], JAN LUKSZO[§], AND ROBERT C. BRASCH[*]

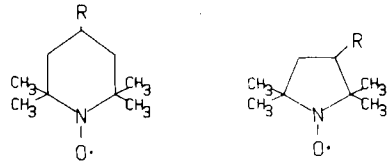
Received September 16, 1985, from the [‡] *Department of Pharmacy, School of Pharmacy, University of California San Francisco, San Francisco, CA 94143*, the [§] *Department of Chemistry, University of Wisconsin–Milwaukee, Milwaukee, WI 53201*, and the [*] *Contrast Media Laboratory, Department of Radiology, School of Medicine, University of California San Francisco, San Francisco, CA 94143.* Accepted for publication January 14, 1986.

Abstract □ Nitroxyl spin labels are paramagnetic compounds that have demonstrated utility as contrast enhancing agents in proton magnetic resonance imaging. The time-course of contrast enhancement depends on distribution and elimination of these agents. Reduction, resulting in formation of the diamagnetic hydroxylamine, is the major metabolic pathway observed in vivo. This bioreduction has implications for the design of contrast agents and for understanding their imaging behavior. Bioreduction has been shown to occur, at least in part, intracellularly. As such, cell membrane permeability to nitroxyl spin labels may influence their bioreduction. In this study, this influence was examined using eight nitroxyl derivatives and the human erythrocyte suspension as a model biomembrane system. Ionizable weak acids and bases were found to equilibrate rapidly across the erythrocyte membrane with half-times of equilibration ranging from <10 s to 1.6 min. These derivatives had low octanol:buffer distribution coefficients and were extensively ionized at the pH of the system (7.0). A strong acid, a phosphate ester, and a quaternary amine derivative were excluded by the cell membrane. Reduction of nitroxyl spin labels by the erythrocyte was shown to occur intracellularly. Except for the impermeable probes, the reduction rate was slow in comparison with the membrane penetration rate. The structural dependence of reduction rate was unrelated to penetration rate but correlated well with that observed in other reducing systems, namely, ascorbic acid solution and rat tissue homogenates.

Nitroxyl spin labels have an unpaired electron and are, therefore, paramagnetic and potentially useful as contrast-enhancing agents in proton magnetic resonance imaging.[1] The strong magnetic moment of the unpaired electron causes paramagnetic relaxation enhancement of protons in a magnetic field and consequently an increase in the image intensity. Contrast enhancement has been demonstrated in animal studies to be diagnostically useful in defining renal functional abnormalities,[2] brain abscesses,[3] and tumors.[4]

The degree of contrast depends on the distribution and elimination of nitroxyl spin labels in vivo. Reduction of the nitroxyl moiety, which has been observed in the dog[5,6] and in the rat,[7] is the major metabolic pathway. This bioreduction results in the formation of a diamagnetic compound, presumably the hydroxylamine, which lacks ability to give proton relaxation enhancement. Elucidating the factors that control bioreduction is important to the understanding of imaging behavior and to optimizing the diagnostic value of nitroxyl spin labels. Several mechanisms for bioreduction that may be influenced by membrane permeability, have been suggested.[8] For example, enzymatic reduction by electron transport systems has been observed in microsomal[9,10] and mitochondrial[11] subcellular fractions. Also, nonenzymatic reduction by reducing agents, such as ascorbic acid, has been demonstrated.[12]

In this study, the permeability of the human erythrocyte membrane to nitroxyl spin labels of varying polarities is examined. Octanol:buffer distribution coefficients were determined and used as indices of the polarities of the labels, and the erythrocyte membrane served as a model biomembrane.



1. Piperidines

2. Pyrrolidines

1a, R = OH

1b, R = NH_2

1c, R = COOH

1d, R = $^{+}N(CH_3)_3\ I^-$

1e, R = OPO_3H_2

2a, R = OH

2b, R = NH_2

2c, R = COOH

Experimental Section

Materials—Compounds **1a**, **1b**, and **2c** were obtained from Sigma (St. Louis, MO), while **1c**, **2b**, and **2a** were synthesized as described elsewhere.[13] Compound **1e** was obtained from Aldrich Chemical Company (Milwaukee, WI).

N,N,N,-Trimethyl(2,2,6,6,-tetramethyl-1-oxyl-4-piperidinyl)ammonium iodide (1d)—Methyl iodide (0.648 g, 4.0 mmol) was added in one portion at 25°C to a dimethylformamide solution (3 mL) of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**1b**) (0.171 g, 1.0 mmol), obtained from Sigma (St. Louis, MO) and 4-hydroxy-2,2,6,6-tetramethylpiperidine (0.312 g, 2.0 mmol), synthesized as described previously.[14] The mixture was stirred until a homogeneous solution was obtained, then left for 3 h at 25°C. The crystalline material was collected by filtration and washed with anhydrous ether. Recrystallization from anhydrous ethanol (~50 mL) gave 0.18 g (52%) of **1d** in the form of orange crystals, mp 241–242°C (dec.) (lit.[15] 210–214°C); MS: m/z = 215 ($M^+ + 1$).

Anal.—Calc. for $C_{12}H_{26}N_2IO$: C, 42.23; H, 7.75; N, 8.12. Found: C, 42.48; H, 7.68; N, 8.21.

The nitroxyl spin labels were stored as 25–85 mM aqueous solutions at 4°C. Under these conditions, the nitroxyl moiety was found to be stable (no detectable change) for a month as measured by electron paramagnetic resonance spectroscopy.

An electron paramagnetic resonance spectrometer (model E-104A, Varian Instrument Division, Sunnyvale, CA), with a 0.04-mL flat cell placed in the microwave cavity, was used to measure the concentration of the nitroxyl spin label. The concentration was estimated from the peak-to-peak height of the first derivative of the low-field line of the nitroxyl triplet spectrum.

0022-3549/86/0400-0334$01.00/0

Octanol:Buffer Distribution Coefficients—Two milliliters of nitroxyl spin-label solution (0.2–1.0 mM) in 0.067 M phosphate buffer (pH = 2.5 or 7.0) was mixed with 2 mL of 1-octanol and left to equilibrate in a rocking shaker overnight. The nitroxyl spin-label concentrations in the octanol and water phases were measured by electron paramagnetic resonance spectroscopy using reference solutions in octanol and water, respectively. The distribution coefficient was calculated by dividing the nitroxyl concentration in the octanol phase by that in the buffer phase.

Reduction in Human Erythrocyte Suspension and Lysate—Blood was drawn from a healthy volunteer on the day of the experiment. The blood was centrifuged at 1000 × *g* for 5 min and the plasma was discarded. The erythrocytes were separated from plasma because plasma also has some reducing activity.[5] The erythrocytes were then washed three times by repeated resuspension in one volume of buffer; centrifuging as above and exchanging supernatant fluid with fresh buffer. The buffer consisted of 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 3.5 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 10 mM dextrose. The erythrocytes were suspended in buffer at an hematocrit of 50% after washing. Protein content in erythrocyte lysate and in supernatant fluid from erythrocyte suspensions was determined.[16] A low protein content in supernatant fluids compared with lysate (0.6–0.8%) showed that lysis of cells in the erythrocyte suspension virtually did not occur. Erythrocyte lysate was prepared by centrifuging the erythrocyte suspension, discarding the supernatant fluid, and adding the same volume of distilled water to lyse the cells hypotonically. The erythrocyte concentration in lysate was the same as in the erythrocyte suspension. Determinations of oxygen partial pressure and pH were performed on an automatic blood gas analyzer (Corning 175, automatic pH/blood gas system, Corning Medical and Scientific, Medfield, MA) in buffer (pO_2 = 187 ± 34 mm Hg; pH = 7.0 ± 0.04), erythrocyte suspension (pO_2 = 53 ± 18 mm Hg; pH = 7.1 ± 0.06), and erythrocyte lysate (pO_2 = 66 ± 28 mm Hg; pH = 7.0 ± 0.06). Essentially no differences were observed between determinations made just after preparation and after completion of experiments. A small volume (20–100 μL) of the diluted stock solution of the nitroxyl spin label was added to each 2-mL sample of erythrocyte suspension and lysate. The sample was rapidly vortexed and injected into the flat cell of the electron paramagnetic resonance spectrometer. The low-field peak of the nitroxyl spectrum was recorded at 1-min intervals for 30 min. The peak width was determined at 5 and 30 min and found to remain constant. The decrease in peak height was therefore not due to peak broadening. Peak broadening can be caused by immobilization of the spin label (binding to cell membrane or proteins) or by an increase in concentration of other paramagnetic species[17] (for example, changes in oxygen concentration). The initial concentration of nitroxyl spin label was 0.01 mM and the experiments were performed at room temperature (22.5 ± 1°C). Potassium ferricyanide was added (2 mM final concentration) to the samples at the end of the experiment and the low field peak was recorded and compared with freshly prepared standards.

Determination of Penetration Rate—Nitroxyl spin labels in the intracellular fluids were selectively measured and monitored over time in erythrocyte suspension by the addition of potassium ferricyanide, an extracellular quenching agent.[18] The paramagnetic exchange between the nitroxyl moiety and ferricyanide ion causes broadening of the spectrum of the nitroxyl spin label only in extracellular fluids, because the ferricyanide ion does not enter cells. Ferricyanide ion is considered the most ideal quenching agent; it does not penetrate the cell membrane and no other interaction with the cell has been reported.[18–21]

The erythrocyte suspension was prepared as described above. A portion was centrifuged at 1000 × *g* for 5 min and the supernatant fluid was exchanged with a 200 mM potassium ferricyanide solution. The nitroxyl spin label under study was added to make a total concentration of 0.1 mM. The electron paramagnetic resonance spectrum was first recorded in the erythrocyte suspension without ferricyanide ion and the magnetic field was set on the low-field peak of the spectrum so that the height of the peak (signal) could be continuously monitored with time. After removing this sample, the nitroxyl spin label was added to an erythrocyte suspension containing ferricyanide ion. After mixing, the suspension was injected into the flat cell within the spectrometer and the intracellular electron paramagnetic resonance signal was monitored with time. For the permeable probes, the intracellular signal reached an upper limit in less than eight min at which time a complete nitroxyl spectrum was recorded.

Results

Distribution Coefficients—The distribution coefficients between 1-octanol and buffer are shown in Table I for buffer pH values of 2.5 and 7.0.

Reduction of Nitroxyl Spin Labels by Erythrocyte Suspension and Lysate—The loss of the electron paramagnetic resonance (EPR) signal in erythrocyte suspension and lysate is presumably caused by the reduction of the nitroxyl spin label to their corresponding hydroxylamines. This conclusion is based on the observations that the peak width remained constant during the experiment and that the original signal was recovered on addition of potassium ferricyanide, an oxidizing agent (see the *Experimental Section*). Reductions of the piperidine derivatives, **1c**, **1a**, and **1b**, in erythrocyte suspension and lysate are shown in Fig. 1A. These derivatives show little or no difference in reduction rates between erythrocyte suspension and lysate. This indicates that penetration does not limit the rate of reduction. The reduction rates for the piperidine and pyrrolidine derivatives showed the same ranking: carboxylic acid derivatives < hydroxyl derivatives < amine derivatives. This structural dependence of reduction rates was unrelated to membrane permeability for these derivatives. Quantitation of penetration was not conducted for the pyrrolidine derivatives because 90% or more remained after 30 min, i.e., they all were more stable than the piperidine derivatives.

Figure 1B shows that the reduction rates of **1e** and **1d** are much faster in erythrocyte lysate than in erythrocyte suspension. This indicates that the rate of reduction in erythrocyte suspension is limited by membrane penetration. The slow reduction in erythrocyte suspension may be explained by leakage of a reducing agent from the cell. All derivatives were stable (no detectable decrease in electron paramagnetic resonance signal in 30 min) in buffer solution as well as in supernatant fluid separated from a freshly prepared erythrocyte suspension. However, when supernatant fluid was separated from an erythrocyte suspension prepared 8 h earlier, reducing activity, comparable with that in erythrocyte suspension, was observed in the supernatant fluid of **1d** and **1e** (11 and 2% reduction, respectively; see Fig. 1B).

Determination of Penetration Rate—The time-course of penetration is illustrated in Fig. 2 for the carboxylic acid derivatives. The half-times of equilibration were 0.6 min for **1c** and 1.6 min for **2c**. The penetration rates for the amine and alcohol derivatives were too rapid to be determined. Half-lives of equilibration must be <10 s because full equilibration was obtained at the time of the first measurement (~30 s following addition of the nitroxyl spin label). The derivatives **1e** and **1d** did not give any intracellular signal within 60 min, indicating that the erythrocyte membrane is impermeable to these probes.

The intracellular EPR signal was followed until it reached an apparent maximum value. Because reduction of the spin label in this time period was negligible (see Fig. 1), the maximum represents the final equilibrium. The intracellular

Table I—Distribution Coefficients Octanol:Buffer[a]

Spin Label	pH 2.5[b]	pH 7.0
1a	3.9	4.2
1b	0.0084	0.043
1c	18.6	0.019
1d	<0.0004	0.0004
1e	0.046	0.01
2a	2.1	1.7
2b	0.064	0.25
2c	10.3	0.008

[a] Average of at least two determinations that differed by ≤20%. [b] The pH of the phosphate buffer.

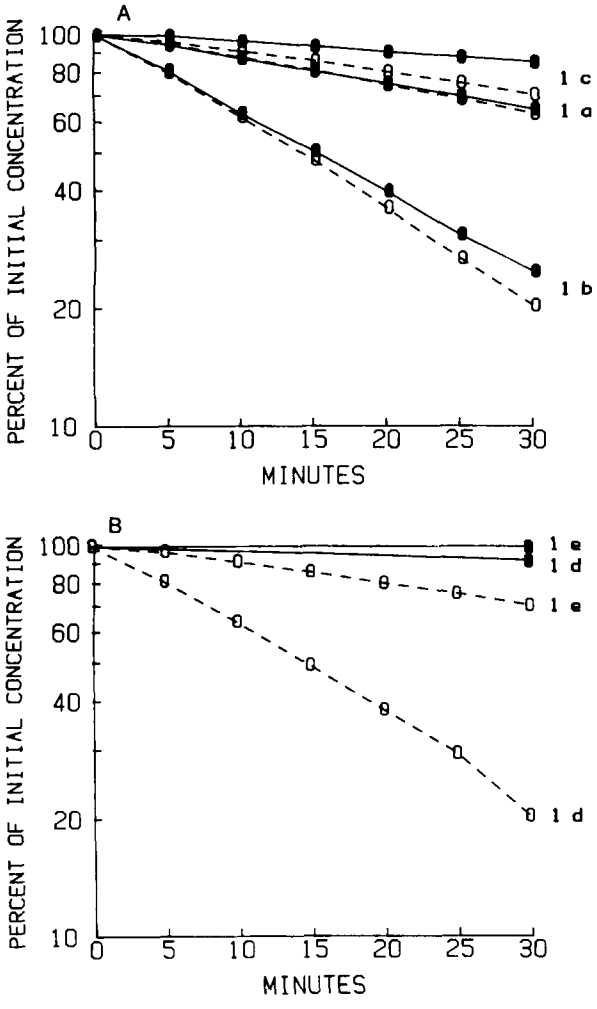


Figure 1—*Differences in reduction rates between erythrocyte suspension (—) and erythrocyte lysate (– –) indicate penetration limitations. Panel A: nitroxyl spin labels with little or no observed differences; panel B: nitroxyl spin labels with penetration limitations. Each data point is an average of at least three experiments; lines connect successive points. Initial nitroxyl derivative concentration was 0.01 mM. The CV (not shown) increased from 1–2% at the first time point to a maximum 15% at the last. Experiments were run at room temperature (22.5 ± 1°C).*

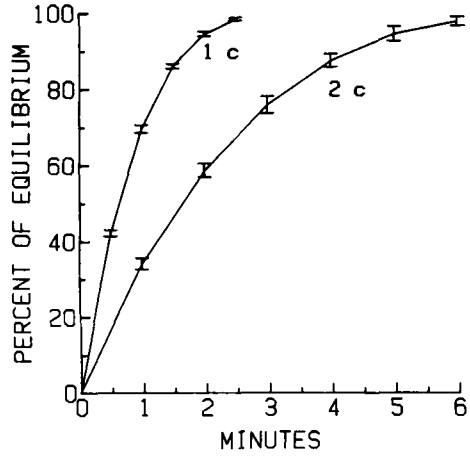


Figure 2—*The carboxylic acid derivatives enter the erythrocyte at measurable rates. Half-times of equilibration were 0.6 min for* **1c** *and 1.6 min for* **2c**. *The intracellular electron paramagnetic resonance signal is expressed as a percent of the equilibrium value reached in <8 min. The bars represent SDs of data from three experiments.*

EPR signal at equilibrium was expressed as a percent of the total (intracellular and extracellular) EPR signal, determined without the quenching agent.

The carboxylic acid derivatives gave a stronger intracellular signal (33% for **1c** and 29% for **2c**) than the hydroxyl derivatives (16% for **1a** and 13% for **2a**). The amine derivatives gave an even weaker intracellular signal (4% for **1b** and 2% for **2b**) than the hydroxyl derivatives. From this information, the intracellular pH of the erythrocyte can be estimated.[20] Assuming that the hydroxyl derivatives equilibrate to the same intracellular and extracellular concentrations, the cell volume accessible to these spin labels is ~15%. This is about that expected for a hematocrit of 50% because only 50% of the packed cell volume represents cell space (if the cells are perfect spheres) and only part of the cell space (containing a high concentration of hemoglobin) is accessible.

We can then use the data for the carboxyl and the amine derivatives to estimate the pH gradient across the cell membrane. The extracellular pH was measured (7.1 ± 0.06) and from this the intracellular pH was estimated to be 7.8 ± 0.1. This observation is in agreement with a previous study[22] in which the intracellular pH of erythrocytes was found to be higher (7.8) at low temperatures (4°C) and in the presence of impermeable anions.

Discussion

Octanol:Buffer Distribution Coefficients—Distribution coefficients were determined as indices of lipophilicity of the nitroxyl derivatives. Lipophilic compounds are expected to penetrate the phospholipid structure of the cell membrane faster than hydrophilic ones, unless specific carriers facilitate the transport.[23]

The most hydrophilic derivatives, **1e** and **1d**, have the lowest distribution coefficients as expected from their charge. Their inability to cross the erythrocyte membrane is therefore supported by their high hydrophilicity.

For the carboxylic acid and amine derivatives, the distribution coefficients are low but, unlike **1e** and **1d**, are pH–dependent (Table I). Previously determined pK_a values for **1b** and the pyrroline analogue of **2c** were 9.37 and 4.4, respectively.[19] Thus, the relatively rapid penetration rate for these derivatives may be explained by the lipophilicity of the uncharged form, which is measured for the carboxylic acid derivatives at pH 2.5.

Reduction of Nitroxyl Spin Labels by Erythrocyte Suspension and Lysate—This approach to studying membrane permeability of the erythrocyte based on the reducing capability of the cell was first reported by Ross and McConnell.[24] Reduction of the nitroxyl moiety is assumed to occur inside the cell and can, therefore, be limited by the membrane permeability to the nitroxyl derivative. When the rate constant for reduction inside the cell is greater than that for transport into the cell, then the rate of reduction in the erythrocyte suspension represents the penetration rate. Because the susceptibility of nitroxyl spin labels to reduction varies with structure, it is important to determine the reduction rate in both erythrocyte suspension and lysate. The reduction rate in lysate is assumed to be the same as that inside the intact erythrocyte.

In this study, the assumptions of this model were validated. The impermeable probes, **1e** and **1d**, are not reduced indicating that reduction occurs intracellularly. For the permeable probes, reduction was found to be slow in comparison with penetration in that reduction rates were essentially the same in erythrocyte suspension and lysate. Recent studies of erythrocyte membrane permeability have utilized this model and assumed that penetration was slow in comparison

with reduction for these probes.[25-28] Our findings show that this assumption was incorrect and that their results pertain more to reduction susceptibility than to membrane permeability.

The relationship between structure and reduction rate in erythrocytes for different derivatives is the same as that found in ascorbic acid solution[13] and rat tissue homogenates.[29] Pyrrolidine derivatives were more stable than piperidine derivatives. The substituent also influenced stability: the carboxylic acids were more stable than the alcohols which were more stable than the amines. Ascorbic acid reduces nitroxyl spin labels to their corresponding hydroxylamines[30] and has been suggested to be the reducing agent in bovine erythrocytes.[31] In a study of the reduction of nitroxyl spin labels in mice erythrocytes, it was concluded that sulfhydryl-containing compounds, such as cysteine, were responsible for the reduction.[32] These small molecular weight-reducing agents could conceivably leak out of the cell and explain our observation of the appearance of reducing activity in the supernatant fluid.

Determination of Penetration Rate—The extracellular quenching agent potassium ferricyanide provides a means of selectively measuring the intracellular electron paramagnetic resonance signal. This technique, described in a review by Mehlhorn and Packer,[18] has been utilized for determination of cell volume, electrochemical and pH gradients across cell membranes, and membrane permeability.[19-21] The 200 mM ferricyanide concentration creates a hypertonic environment, but the estimated cell volume is not apparently different from the expected value. Furthermore, the time of exposure to ferricyanide ion is relatively short and therefore changes in membrane properties were assumed not to occur.

Using this approach, penetration can be determined even when it is faster than the reduction process. The limiting factor is how soon the intracellular signal can be monitored after addition of the nitroxyl spin label. In our studies this was ~30 s. Half-times of equilibration shorter than 10 s cannot be determined by this system. The carboxylic acid derivatives had intermediate penetration rates with the more lipophilic derivative, **1c**, penetrating the cell membrane faster than **2c**.

In pharmacokinetic studies in the dog,[5,6] **2c** was found to be eliminated more slowly (lower clearance) than a succinic acid piperidine derivative. This difference in elimination was attributed to pyrrolidine derivatives being more slowly reduced than piperidine compounds as observed in ascorbate solution. However, the slower membrane permeability rate of **2c** could also be a factor limiting its bioreduction in vivo.

Conclusions

Permeability of the human erythrocyte membrane to eight charged and uncharged nitroxyl derivatives was assessed using two approaches: by rates of reduction in lysate and cell suspension, and by appearance in intracellular fluids in the presence of the extracellular quencher, potassium ferricyanide. The two techniques gave observations that correlated well with each other: the erythrocyte membrane was impermeable to two probes, but freely permeable to the other six. This information is useful for the design of contrast agents in proton magnetic resonance imaging depending on the desired distribution and application. Permeable agents have the advantage of entering cells and may potentially serve as probes of intracellular reduction–oxidation metabolism. Differences in binding and reduction rates among tissues are bases for contrast enhancement with these agents. If reduction in vivo is primarily intracellular, those agents with slow membrane penetration rates may be used advantageously as extracellular contrast agents because relatively smaller doses would be required in comparison with nitroxyl spin labels that penetrate cells rapidly.

Acknowledgments

This research was supported by grant AM 31937 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases of the National Institutes of Health and by Schering, A. G., Berlin, F.R.G. One of us (G.S.) would like to thank the graduate school of the University of Wisconsin–Milwaukee for the J. D. and D. Shaw Award in 1983, 1984, and 1985.

References and Notes

1. Brasch, R. C. *Radiology* **1983**, *147*, 781.
2. Brasch, R. C.; London, D. A.; Wesbey, G. E.; Tozer, T. N.; Nitecki, D. E.; Williams, R. D.; Doemeny, J.; Tuck, L. D.; Lallemand, D. P. *Radiology* **1983**, *147*, 773.
3. Brasch, R. C.; Nitecki, D. E.; Brant-Zawadzki, M.; Enzmann, D. R.; Wesbey, G. E.; Tozer, T. N.; Tuck, L. D.; Cann, C. E.; Fike, J. R.; Sheldon, P. *A.J.N.R.* **1983**, *4*, 1035.
4. Ehman, R. L.; Wesbey, G. E.; Moon, K. L.; Williams, R. D.; McNamara, M. T.; Couet, W. R.; Tozer, T. N.; Brasch, R. C. *Mag. Res. Imag.* **1985**, *3*, 89.
5. Couet, W. R.; Eriksson, U. G.; Tozer, T. N.; Tuck, L. D.; Wesbey, G. E.; Nitecki, D.; Brasch, R. C. *Pharm. Res.* **1984**, 203.
6. Couet, W. R.; Eriksson, U. G.; Brasch, R. C.; Tozer, T. N.; *Pharm. Res.* **1985**, 69.
7. Griffeth, L. K.; Rosen, G. M.; Rauckman, E. J.; Drayer, B. P. *Invest. Radiol.* **1984**, *19*, 553.
8. Rauckman, E. J.; Rosen, G. M.; Griffeth, L. K. in "Spin Labelling in Pharmacology"; Holtzman, J. L., Ed.; Academic Press: Orlando, 1984; pp 175–190.
9. Rosen, G. M.; Rauckman, E. J.; Hanck, K. W. *Toxicol. Lett.* **1977**, *1*, 71.
10. Rosen, G. M.; Rauckman, E. J. *Biochem. Pharmacol.* **1977**, *26*, 675.
11. Quintanilha, A. T.; Packer, L. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 570.
12. Perkins, R. C.; Beth, A. H.; Wilkerson, L. S.; Serafin, W.; Dalton, L. R.; Park, C. R.; Park, J. H. *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 790.
13. Couet, W. R.; Brasch, R. C.; Sosnovsky, G.; Lukszo, J.; Prakash, I.; Gnewuch, C. T.; Tozer, T. N. *Tetrahedron* **1985**, *41*, 1165.
14. Sosnovsky, G.; Konieczny, M. *Z. Naturforsch.* **1978**, *33B*, 792.
15. Rauckman, E. J.; Rosen, G. M.; Hord, W. W. *Org. Prep. Proced. Int.* **1977**, *9*, 53.
16. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
17. Nordio, P. L. in "Spin Labeling: Theory and Applications", vol. 1; Berliner, L. J., Ed.; Academic Press: New York, San Francisco, London, 1976; pp 29–36.
18. Mehlhorn, R. J.; Packer, L. *Ann. NY Acad. Sci.* **1983**, *414*, 180.
19. Mehlhorn, R. J.; Probst, I. *Methods Enzymol.* **1982**, *88*, 334.
20. Quintanilha, A. T.; Mehlhorn, R. J. *FEBS Lett.* **1978**, *91*, 104.
21. Mehlhorn, R. J.; Candau, P.; Packer, L. *Methods Enzymol.* **1982**, *88*, 751.
22. Minakami, S.; Tomoda, A.; Tsuda, S. In "Erythrocyte Structure and Function"; Brewer, G. J., Ed.; Alan R. Liss Inc.: New York, 1975; pp 149–162.
23. "The Movement of Molecules Across Cell Membranes"; Stein, W. D., Ed.; Academic Press: New York, 1967; pp 65–126.
24. Ross, A. H.; McConnell, H. M. *Biochemistry* **1975**, *14*, 2793.
25. Bartosz, B. *Biochim. Biophys. Acta* **1981**, *664*, 69.
26. Bartosz, G.; Gwozdzinski, K. *Am. J. Hematol.* **1983**, *14*, 377.
27. Gwozdzinski, K.; Bartosz, G.; Leyko, W. *Radiat. Environ. Biophys.* **1981**, *19*, 275.
28. Gwozdzinski, K.; Bartosz, G.; Leyko, W. *Radiat. Environ. Biophys.* **1983**, *22*, 53.
29. Couet, W. R.; Brasch, R. C.; Sosnovsky, G.; Tozer, T. N.; *Mag. Res. Imag.* **1985**, *3*, 83.
30. Sosnovsky, G.; Konieczny, M. *Synthesis* **1977**, 619.
31. Bartosz, G.; Gwozdzinski, K.; Wagner, J. *Biomedicine* **1981**, *35*, *185*.
32. Giotta, G. J.; Wang, H. H. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 1576.