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Pharmacokinetics and bioreduction of nitroxides with potential utility as contrast agents in proton magnetic resonance imaging

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PHARMACOKINETICS AND BIOREDUCTION OF NITROXIDES WITH POTENTIAL UTILITY AS CONTRAST AGENTS IN PROTON MAGNETIC RESONANCE IMAGING

by

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DISSERTATION

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Dedicated to:

My parents, John and Sylvia Eriksson

and my sister, Pia.

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Grodd, W.; Paajanen, H.; Eriksson, U. G.; Revel, D; Ogan, M.; Engelstad, B.; Brasch, R. C. : Comparison of ionic and non-ionic contrast media for renal enhancement in NMR imaging. *Invest. Radiol.,* **20,** S43 (1985).

Paajanen, H.; Grodd, W.; Revel, D.; Ogan, M.; Tozer, T. N.; Eriksson, U. G.; Engelstad, B.; Brasch, R. C. : MRI contrast enhancement of experimentally induced abcesses. A comparison of Gd-DTPA and a new non-ionic nitroxide spin label. *Invest. Radiol.,* **20,** S43 (1985).

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PHARMACOKINETICS AND BIOREDUCTION OF NITROXIDES WITH POTENTIAL UTILITY AS CONTRAST AGENTS IN PROTON MAGNETIC RESONANCE IMAGING.

Abstract

Nitroxides are organic stable free radicals that have potential utility as contrast enhancing agents in proton magnetic resonance imaging (MRI). The pharmacokinetics and metabolic fate, important determinants of the time-course of MRI contrast enhancement, of three nitroxide derivatives were examined. A piperidine, TES (N-succinyl-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl), and two pyrrolidine nitroxide derivatives, PCA (3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl) and TAP (2,2,5,5-tetramethyl pyrrolidine-1-oxyl-3-carbonic acid-(2,3-dihydroxy-1-hydroxymethyl)-amide), were selected because these derivatives were concurrently evaluated in animals and demonstrated to be useful as MRI contrast agents. Intravenous doses of 0.1 and 2.5 mmol/kg of all three derivatives were administered to dogs. TAP was also administered to rats at the same mmol/kg doses. Recoveries of the unchanged nitroxides and their corresponding hydroxylamines in urine collected during 24 hours after administration accounted for 83 to 98 percent of the dose. Renal clearances, estimated in the dog for PCA and TAP, were similar to the glomerular filtration rate (approximately 4 ml/min-kg). The corresponding hydroxylamines, which lacks contrast-enhancing activity, were quantified in urine after oxidation using electron spin resonance spectroscopy and high performance liquid chromatography assays. Reduction of the nitroxide moiety to the corresponding amine or any other metabolism were not observed. These observations were

confirmed for PCA by administering the tritium-labeled compound. Reduction, examined *in vitro* in dog plasma and urine, ascorbic acid solution, rat tissue homogenates and human erythrocytes, was slower for the pyrrolidine derivatives, PCA and TAP, than for the piperidine derivative, TES. Nitroxide reducibility *in vitro* may be of predictive value for selecting a more stable nitroxide derivative. Reduction in rat tissues were most rapid in liver and kidney. In these tissues, the reduction was, in part, due to reduction by ascorbic acid. Enzymatic bioreduction, that also appeared to occur, may be limited by cell membrane permeability to nitroxides. This hypothesis was supported by the slow bioreduction in the dog and the slow penetration of the erythrocyte membrane observed for TAP compared to PCA. The best candidate for use as an MRI contrast agent appeared to be TAP because of its low metabolic clearance, presumably due to low reducibility and slow cell membrane penetration, and high fraction of the dose excreted unchanged

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List of Abbreviations

Nitroxide Derivatives

6-OH	4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl
6-NH_2	4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl
6-COOH	4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl
TES	N-succinyl-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl
CAT	N,N,N-trimethyl-(2,2,6,6-tetramethyl-1-oxyl-4-piperidinyl) ammonium iodide
TPH	4-phosphonoxy-2,2,6,6-tetramethylpiperidine-1-oxyl
5-OH	3-hydroxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl
5-NH_2	3-amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl
PCA	3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl
TAP	2,2,5,5-tetramethyl pyrrolidine-1-oxyl-3-carbonic acid-(2,3-dihydroxy-1-hydroxymethyl)-amide

Other Abbreviations

AAOx	Ascorbic acid oxidase
DTPA	Diethylenetriaminepentaacetic acid
ESR	Electron spin resonance
MRI	Magnetic resonance imaging
NADPH	ß-Nicotinamide adenine dinucleotide phosphate (reduced form)
NEM	N-Ethylmaleimide
L, mL and µL	Liter, milliliter and microliter

PHARMACOKINETICS AND BIOREDUCTION OF NITROXIDES WITH POTENTIAL UTILITY AS CONTRAST AGENTS IN PROTON MAGNETIC RESONANCE IMAGING.

Overview and Goals of Dissertation Research

Nitroxides have potential utility as contrast enhancing agents in proton magnetic resonance imaging (MRI) used for clinical diagnosis. The unpaired electron of the paramagnetic nitroxide moiety can decrease the relaxation times of tissue protons. The decrease in relaxation times and the resulting increase in image intensities are related to the nitroxide concentration in tissues.

Contrast enhancement can increase the diagnostic benefit of MRI by facilitating the differentiation of isointense, but histiologically different, tissues. Contrast enhancement also permits better localization and characterization of lesions and may provide information about physiologic function. Contrast agents can serve to reduce the time necessary to complete an MRI examination. In animal studies, nitroxides have been demonstrated to enhance kidneys, areas of blood-brain barrier damage, inflammatory lesions and tumors.

In this dissertation research, the pharmacokinetics and metabolic fate of a piperidine and two pyrrolidine nitroxide derivatives were examined *in vivo* in the dog and the rat. Biodistribution and elimination of nitroxides, determinants of the contrast enhancement, are of importance for the evaluation of nitroxides as MRI contrast agents. These three nitroxide derivatives were selected because they were concurrently being tested for MRI contrast enhancement in animals by our collaborators, Dr. Robert C. Brasch and coworkers (Contrast Media Laboratory, Department of Radiology, University of California San

Francisco). Nitroxides are low-molecular weight (200 to 300 Daltons) compounds with a general chemical structure that stabilizes the unpaired electron of the nitroxide moiety, i.e., they are stable free radicals. These compounds are used as probes in biologic electron spin resonance spectroscopy (ESR) studies but have no previous history as pharmaceuticals. Consequently, only limited information was previously available on the biodistribution and elimination of nitroxides.

For the three nitroxides studied, reduction of the nitroxide moiety was observed in the dog and the rat. This bioreduction resulted in formation of the diamagnetic hydroxylamine that lacks proton relaxation enhancing activity. Consequently, bioreduction of nitroxides influences their imaging properties and is of importance for their development as MRI contrast agents. Observations of reduction of nitroxides in biologic systems have been reported previously. Both nonenzymatic reduction, caused by small molecular weight reductants such as ascorbic acid and sulfhydryl compounds, and enzymatic reduction mechanisms have been suggested.

In addition to the studies *in vivo*, reduction of nitroxides was examined *in vitro* to elucidate the factors that may influence bioreduction. Structural dependence of reducibility of nitroxides was evaluated in ascorbic acid solution, rat tissue homogenates and human erythrocytes. The reducing activities of different tissue homogenates were compared. The relative contributions of small molecular weight reductants to the reduction in rat liver and kidney are examined in protein-free preparations of these tissue homogenates. As cell membrane permeability to nitroxides may limit the access of nitroxides to intracellular reductants, the structural dependence of nitroxide membrane penetration was examined using the human erythrocyte as a model cell.

The dissertation begins with a literature review of magnetic resonance imaging, the use of nitroxides as MRI contrast agents and accumulated data about their chemical and biologic properties. In the next chapter, the general experimental methods used in the experiments are described. The main body of the dissertation is divided into two sections. The pharmacokinetic information obtained in the dog and the rat is presented in Section I and the results of the studies *in vitro* of the bioreduction of nitroxides are presented in Section II.

Chapter 1. Background

The principles of magnetic resonance, the basis for MRI and the contrast enhancement produced by nitroxides, are briefly summarized herein to give a background for the reader. Literature data on the chemical properties, toxicity and *in vivo* fate of nitroxides, important properties for the application of nitroxides as MRI contrast agents, are also reviewed.

1.1 Proton Magnetic Resonance Imaging.

MRI is a relatively new diagnostic imaging technique that utilizes the nuclear magnetic resonance (NMR) of proton nuclei in tissues to generate tomographic images. Following the first reports of the phenomenon (Bloch et al., 1946; Purcell et al., 1946), NMR spectroscopy has become an indispensible analytical tool in chemistry and biochemistry. *In vivo* NMR techniques include spectroscopy of various nuclei (e.g., ^{1}H, ^{19}F, ^{23}Na and ^{31}P) and production of tomographic images in living systems. Since the first two-dimensional proton-NMR images were produced in the early 1970s (Lauterbur, 1973), magnetic resonance imaging has developed rapidly. The utility of MRI has been established and the technique was approved for clinical use in 1983 by the Food and Drug Administration.

In MRI, cross-sectional images are produced of living subjects similar to those obtained by x-ray computed tomography (CT). MRI is considered a non-invasive imaging modality that gives excellent soft-tissue contrast without the use of ionizing radiation. Multiplanar images (transverse, sagittal and coronal), obtained by MRI, often equal or exceed CT images in quality and diagnostic

value. The principles of NMR spectroscopy and MRI are briefly discussed in this chapter. The reader is referred to available text books and recent reviews for a more complete description of NMR spectroscopy (Abragam, 1961; Slichter, 1978; Becker, 1980; James, 1975) and MRI (Kaufman et al., 1981; Gore et al., 1981; Wells, 1982; Bottomley, 1982; Andrew, 1983; Partain et al., 1981; 1983; James and Margulis, 1985).

Both NMR spectroscopy and MRI utilize the phenomenon of magnetic resonance (Andrew, 1983; Wehrli, 1984). Important differences between them occur in the sample type and magnetic field requirements. In conventional NMR spectroscopy the sample is small, relatively pure and homogeneous, while in MRI the sample is large and heterogeneous, e.g., the human body. The magnetic field must be uniform in NMR spectroscopy but in MRI the magnetic field is made non-uniform in a controlled manner to obtain spatial information.

The hydrogen nucleus (or proton) is used in MRI because of its high biologic abundance which, together with its high isotopic abundance and strong magnetic moment, gives a stronger signal and therefore a higher signal-to-noise ratio than other nuclei (Table 1.1). The NMR sensitivities for other nuclei of biologic interest are several orders of magnitude lower because of weaker magnetic moments and lower biologic abundance.

To produce an image, it is necessay to be able to differentiate the NMR signals produced by protons at different locations in the sample. As mentioned above, controlled magnetic field gradients are used to modify a static magnetic field and thereby obtain spatial information from the NMR signal. The strength of the magnetic field, that varies along the field gradients across the sample, determines the resonance frequency for a nucleus (Larmor frequency). Because the magnetic field is different depending upon the location within the sample, the resonance frequency can be translated to a particular position in

Table 1.1 *Magnetic resonance properties of some biologic relevant nuclei.* [a]

Nucleus	Spin quantum number	Magnetogyric ratio (MHz/Tesla)	Relative [b] sensitivity	Relative [c] concentration in mammalian tissue	Relative [d] overall tissue NMR sensitivity
^{1}H	1/2	42.38	1	1	1
^{2}H	1	6.53	0.409	1.5×10^{-4}	6.2×10^{-5}
^{13}C	1/2	10.71	0.251	1.0×10^{-3}	2.5×10^{-4}
^{19}F	1/2	40.05	0.941	6.7×10^{-5}	6.3×10^{-5}
^{23}Na	3/2	11.26	1.320	7.9×10^{-4}	1.0×10^{-3}
^{31}P	1/2	17.23	0.405	3.5×10^{-3}	1.4×10^{-3}
^{39}K	3/2	1.99	0.233	4.5×10^{-4}	1.1×10^{-4}

(a) From Kramer (1981) and references therein.

(b) NMR sensitivity relative to hydrogen (^{1}H) for an equal number of nuclei at constant frequency.

(c) Average concentrations in mammalian tissues relative to hydrogen.

(a) Overall NMR sensitivity relative to hydrogen (accounting for differences in magnetic moment and isotopic and biologic abundances).

the sample. This process is called frequency encoding and is made feasible with the use of computers.

Image intensities in tissues depend on parameters intrinsic to the sample as well as extrinsic parameters that are under instrument control. This dependence is illustrated by the following equations that show how image intensity is determined for the two most common pulse sequences used in clinical MRI, namely the inversion recovery and spin echo pulse sequences (Wehrli et al., 1984a, 1984b).

Pulse sequence	*Image intensity (I) equation*	
Inversion recovery	$I = N \cdot f(v) [1 - 2\exp(-TI/T_1) + \exp(-TR/T_2)]$	Eq. 1.1
Spin echo	$I = N \cdot f(v) [1 - \exp(-TR/T_1)] \exp(-TE/T_2)$	Eq. 1.2

In these equations, N, f(v), T_1 and T_2 are the intrinsic parameters and TI, TR and TE are the extrinsic parameters

Several intrinsic parameters determine the image intensity in MRI, while in CT, tissue attenuation of the x-rays is the only determinant of image intensity. The intrinsic parameters are the tissue concentrations of protons (N), the longitudinal (T_1, spin-lattice) and the transverse (T_2, spin-spin) relaxation times of protons. The movement of nuclei (f(v)), e.g. flow of blood in a vessel, also modifes the intensity. The relaxation times describe the return of the magnetic moment of nuclei to the lower energy state after excitation by radiofrequency radiation at the resonant (Larmor) frequency. The variation in the concentration and the relaxation times of these protons are the basis for the good soft tissue contrast obtained by MRI. Proton concentration (N) usually varies little between

soft tisses, for example, the proton concentrations of different brain tissues were reported to be 75 to 95 % of the proton concentration in cerebrospinal fluid (Wehrli et al., 1984b). However, the relaxation times of brain tissues are sufficiently different to produce contrast, for example, between white and gray matter (Wehrli et al., 1984b). The relaxation times are sensitive to changes in the environment of tissue protons and may indicate changes due to disease states, for example, the relaxation times of protons in tumorous tissues have been demonstrated to be different from normal tissues and also to vary between different tumor types (Damadian, 1971; Mills et al., 1984; Araki et al., 1984).

Contrast between tissues is optimized by changing the extrinsic parameters (Wehrli et al., 1984a, 1984b), i.e., the type of pulse sequence and the timing of pulses (TR = time between repetition of pulse sequence; TI = interpulse delay for the inversion recovery pulse sequence; TE = spin echo delay time for the spin echo pulse sequence). The resultant variability in contrast differences is an additional advantage of MRI compared to CT. Practically, patient compliance and cost considerations limit the number of pulse sequences and timing variations that can be tried for a given diagnostic examination. The time to create an image is 5 to 20 minutes depending upon the number of times that the pulse sequence is repeated. The NMR signal is averaged for the accumulated signal of repeated pulse sequences (each starting 0.5 to 2 seconds apart, TR).

Image intensities of rat liver, kidney, muscle and fat tissues are shown in Figure 1.1. The values were calculated for two spin echo pulse sequences, by substituting literature values of the proton relaxation times in these tissues (Cameron et al., 1984) into Equation 1.2 for image intensity and expressing the intensities relative to that in liver. As mentioned above, the proton concentrations of soft tissues are high and similar between tissues. The proton

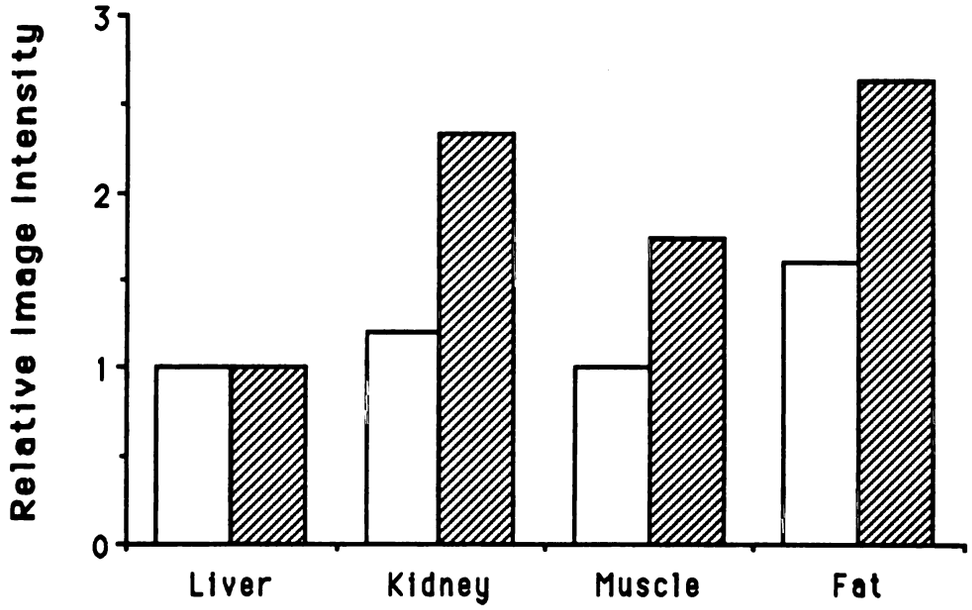


Figure 1.1 The image intensities of four tissues, normalized to the image intensity of liver. Literature values of the relaxation times in rat liver, kidney, muscle and fat tissues (Cameron et al., 1984) were substituted into Equation 1.2 for the image intensity of the spin echo pulse sequence. Relative image intensities of the tissues are shown for two pulse sequencies; TR = 500 msec and TE = 28 msec (open bars); TR = 2000 msec and TE = 56 msec (striped bars). For the calculations, the proton concentrations were assumed to be similar for these tissues. Because of differences in proton relaxation times, the image intensities of fat and kidney can be made 2 to 3-fold higher than the image intensity of liver depending upon the time parameters used (TR and TE). The relative image intensities, that are greater for the longer TR and TE, illustrate that changing the time parameters of the pulse sequence may improve the contrast between tissues.

concentrations (N) were therefore set to be the same for all four tissues to calculate the relative intensities.

The majority of the NMR signal arises from protons in water and lipids (Bottomley, 1982). The water content of most soft tissues is about 70 to 85 % (Cameron et al., 1984). With a molecular weight of 18 and a density of about 1, the proton concentrations then are 80 to 90 mmol per gram of tissue. In fatty tissues, the water content is only about 15 % (Cameron et al., 1984, Kamman et al., 1984) but the proton concentration is still high (83 to 124 mmol/g) due to the high concentration protons in lipids. Consequently, the differences in image intensities between the tissues are determined by the relaxation times. Liver tissue has low image intensity because the spin-spin relaxation time (T_2) is relatively short. Protons in fatty tissues have relatively short T_1 and long T_2 relaxation times, a combination that gives high image intensity. The intensities of liver and muscle are similar for the pulse sequence with short TR and TE. However, using a pulse sequence with longer TR and TE, muscle is easily differentiated from liver. This illustrates that the contrast of images can be influenced by extrinsic instrument parameters.

1.2 Pharmaceutical contrast enhancement for MRI

Contrast agents may increase the diagnostic utility of MRI in several ways. For example, they can increase contrast between isointense but histologically-different tissues. Contrast agents may provide information about physiologic function of organs, show changes in tissue perfusion, help to identify and characterize pathologies such as tumors and abcesses. The use of a contrast agent may also decrease the time necessary for an MRI examination

by reducing the number of pulse sequences required for obtaining a good-quality image.

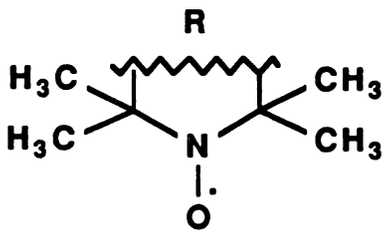
Potential approaches for contrast enhancement of MRI have been summarized by Brasch (1983) and the research in this field has recently been reviewed (Ogan and Brasch, 1985; Engelstad and Brasch, 1985). MRI is a relatively new imaging modality and the development of contrast media is still in its infancy but the rationale for using contrast media in MRI is similar to that for the more established imaging modality, x-ray computed tomography. An ideal contrast agent should be: chemically stable in a form that is ready for administration; inexpensive and readily available; water soluble; well tolerated in diagnostic doses; rapidly and completely excreted; be highly effective, i.e., give contrast enhancement at a low dose to keep the injection volume small.

Image intensity may be modified by a pharmaceutical that changes the proton density (N) or the relaxation times (T1 and T2), as is apparent from the equations for image intensity in MRI. The potential of effecting contrast by altering proton density is limited because the proton density is inherently high and varies little between soft tissues (Wehrli et al., 1984a,1984b; Kamman et al., 1984; Cameron et al., 1984). Oral administration of lipid solutions (Newhouse et al., 1982; Alfidi et al., 1983) or dehydration of tissues (Hricak et al., 1983) are examples of approaches that have been investigated.

The relaxation times, however, vary considerably between different tissues (Wehrli et al., 1984a; Cameron et al., 1984) and are sensitive to changes in the physio-chemical environment of the protons e.g. changes in viscosity and temperature (Brasch, 1983). Consequently, a more promising approach to obtain pharmaceutical contrast enhancement in MRI is the use of paramagnetic compounds that shorten the relaxation times of protons.

Paramagnetic compounds with potential application as contrast agents for MRI (Brasch, 1983; Ogan and Brasch, 1985; Engelstad and Brasch, 1985) are listed in Table 1.2. Proton relaxation occurs by interaction with local magnetic fields generated by unpaired electrons or nuclei with nonzero spin quantum numbers. Paramagnetic compounds, that have unpaired electrons, are more effective in inducing proton relaxation than nuclei of diamagnetic species because the magnetic moment of the unpaired electron is 657 times stronger than that of the proton nuclei.

Table 1.2 *Paramagnetic substances with potential use as MRI contrast agents.*

Transition metals	**Mn^{2+}, Fe^{3+}, Ni^{2+}, Cr^{2+}, Cu^{2+}**
Lanthanide metals	**Gd^{3+}, Dy^{3+}, Ho^{3+}, Eu^{3+}**
Nitroxides	

Transition and lanthanide metals, which have several unpaired electrons and therefore are highly paramagnetic and effective relaxation enhancers, have demonstrated utility as MRI contrast agents (Runge et al., 1983; Runge et al.,

1984a, b; Weinmann et al., 1984; Engelstad et al., 1984). However, these metals are relatively toxic and must be administered as complexes to be tolerated. Gadolinium (Gd^{3+}), an ion with seven unpaired electrons, is the strongest paramagnetic proton relaxer among these metals (Weinmann et al., 1984). The paramagnetic relaxation rates are expected to be directly proportional to the concentration of the paramagnetic compound (James, 1975). To obtain the paramagnetic relaxation rates, the relaxation rates (obtained as the inverse of the spin-lattice, T_1, and spin-spin T_2, relaxation times) of a solution without any solute are subtracted from the respective relaxation rates observed in a solution containing the paramagnetic species. The slope of the linear regression of the paramagnetic relaxation rate versus concentration is the *relaxivity* of the compound. The spin-lattice and spin-spin relaxativities in water of the diethylenetriaminepentaacetic acid complex of gadolinium (Gd-DTPA) are 4.5 and 5.7 sec^{-1} mM^{-1}, respectively (Weinmann et al., 1984). Gd-DTPA, effective at doses as low as 0.01 mmol/kg (Weinmann et al., 1984), has been extensively studied for MRI contrast enhancement (Ogan and Brasch, 1985). This contrast agent has been used in humans and is presently undergoing clinical trials in USA and Europe (Carr et al., 1984a,b; Steiner et al., 1984).

Another class of paramagnetic compounds with potential as MRI contrast agents is the nitroxides. These compounds are stable organic free radicals with a general chemical structure, depicted in Table 1.2, that stabilizes the unpaired electron of the nitroxide moiety. The paramagnetic relaxivities of water soluble small-molecular weight nitroxide derivatives that have one nitroxide moiety per molecule are approximately ten to fifteen-fold lower than for Gd-DTPA (Ehman et al, 1986, Lovin et al., 1985). However, the nitroxides have properties that make them attractive as potential MRI contrast agents.

Nitroxides have been shown to have low toxicity (Afzal et al., 1984) indicating that high doses can be safely administered. The LD_{50} values in rats of nitroxides and Gd-DTPA are 15 to 20 mmol/kg and 10 mmol/kg, respectively (Afzal et al., 1984; Weinmann et al., 1984). Furthermore, nitroxides are chemically versatile and can be modified to improve their paramagnetic relaxativity. For example, compounds with two nitroxide moieties per molecule have been shown to be more effective than those with only one (Ehman et al, 1986). The molecular weight also influences the relaxivity, larger molecules being more effective than small-molecular weight compounds (Lovin et al., 1985). The relaxivity of nitroxides appeared to be higher in plasma than in water in contrast to Gd-DTPA for which the relaxivity is approximately the same in both plasma and water. Biodistribution and elimination of nitroxides*in vivo* may also be varied by changing the chemical structure. Consequently, nitroxides can be chemically designed to satisfy different diagnostic applications. Animal studies have demonstrated an increased diagnostic yield of MRI by the use of nitroxides. For example, identification of blood-brain barrier damage (Brasch et al., 1983b), enhancement of bladder and kidney tissues to assess kidney function (Brasch et al., 1983a; Lamarque et al., 1986), and localization and characterization of tumors (Ehman et al., 1985).

1.3 Chemical Properties of Nitroxides

Nitroxides were first synthesized in the early 1960's (Baird and Thomas, 1961; Hoffmann and Henderson, 1961; Baird, 1962) and applied as probes in biologic electron spin resonance (ESR) spectroscopy studies (Stone, 1965; Ohnishi and McConnell, 1965). The chemical properties of nitroxides have been summarized in several reviews (McConnell and Gaffney McFarland, 1970;

Rozantsev and Sholle, 1971a,b; Keana, 1978, 1979, 1984; Gaffney, 1976). The different classes of nitroxides, which have been synthesized for use as probes in ESR studies, are shown in Figure 1.2 An unusual property of these organic free radicals is their stability. Free radicals are generally considered to be reactive, short-lived intermediates in chemical and biologic reactions. However, nitroxides are stable and can be stored for extended periods of time either in dry form or in solution (aqueous or organic). "Nitroxide", the standard name (Chemical Abstracts) that is most commonly used, refers to the general chemical structure of these organic stable free radicals (Figure 1.2). In analogy to the nomenclature for free radicals, a more correct term is "nitroxyl" (suffix "-yl"). These compounds are also often referred to as "nitroxide spin labels" or "nitroxyl spin labels", because of their use as labels or membrane probes in ESR studies. In MRI imaging, these compounds are not used for labeling purposes but as contrast agents and the common term "nitroxide" is therefore used hereafter.

Reactions involving the paramagnetic nitroxide moiety are shown in Table 1.3 (Gaffney, 1976 and references therein). These reactions are of particular interest because the unpaired electron of the nitroxide moiety, the active part of the molecule, is responsible for proton relaxation enhancement and thus for MRI contrast enhancement.

Disproportionation (reaction 1.) can easily be prevented by fully substituting the two carbons next to the nitrogen with methyl or other carbon-containing groups so that no alpha-hydrogens are available. Formation of the nitrone is then hindered. Reaction with other free radicals (reaction 2.) may occur *in vivo* and has been postulated as a mechanism for the radiosensitizing effect observed for nitroxides (Cadet and Teoule, 1982, Sridar, 1982). Nitroxide

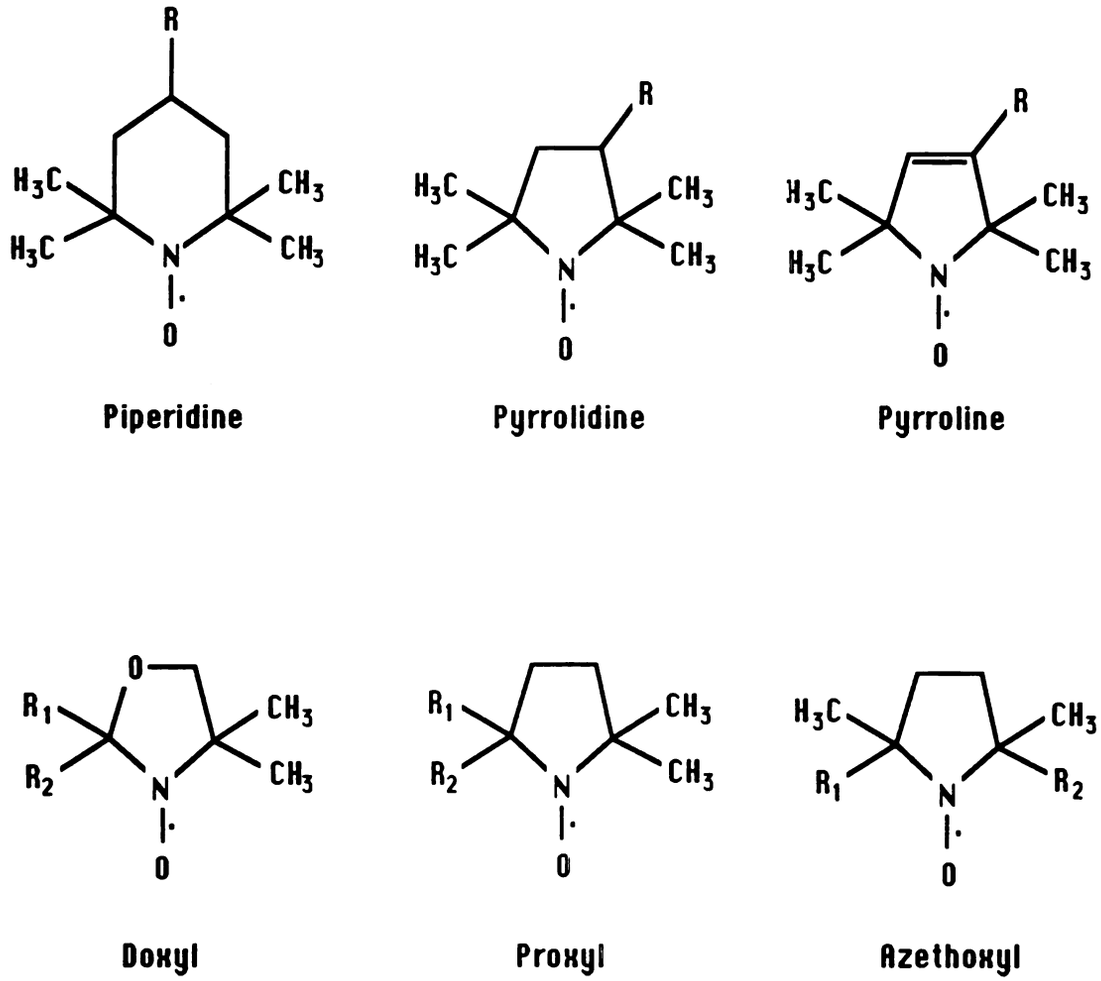
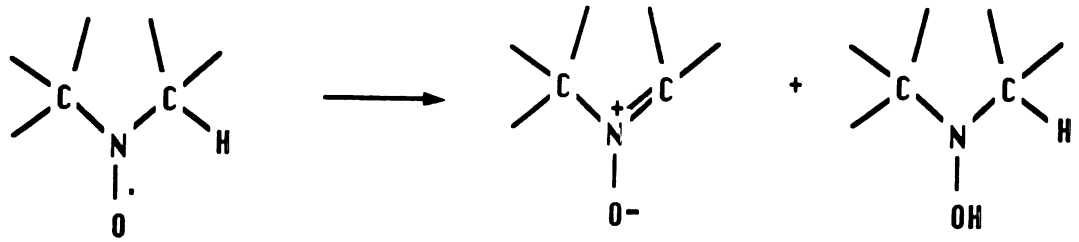


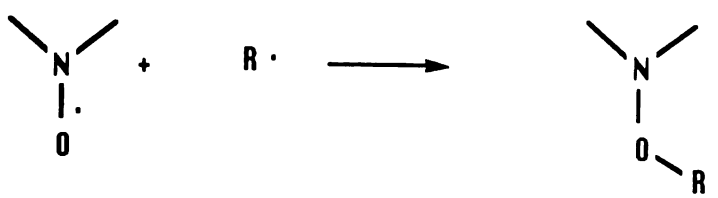
Figure 1.2 Nitroxide derivatives that have been synthesized as ESR probes for labeling of biologic macromolecules or incorporation into membranes (Keana, 1979). The derivatives fall into these major classes for which R is any group and R_1 and R_2 are any combination of carbon-containing groups.

Table 1.3 *Chemical Reactions of the Nitroxide Moiety.*

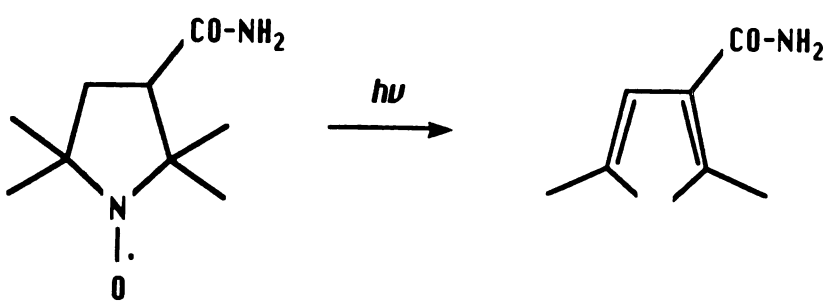
1. Disproportionation



2. Free radical reaction

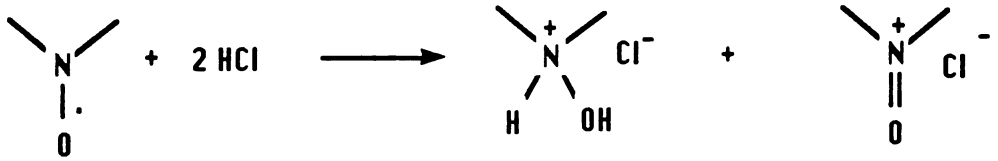


3. C - N bond cleavage

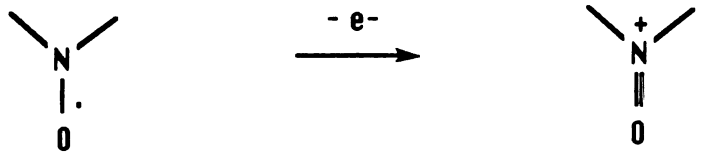


(continued on next page)

4. Reaction with acid

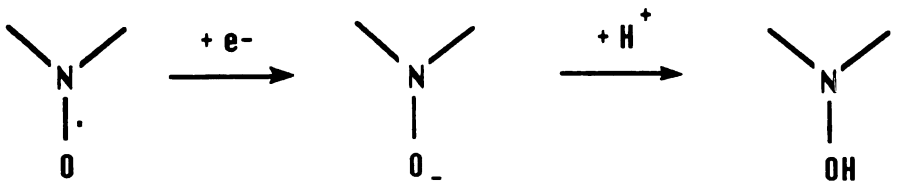


5. Oxidation



Immonium oxide ion

6. Reduction



derivatives of pharmacologic compounds, such as cancer chemotherapeutic agents, have been reported to have lower toxicity and the hypothesis is that the nitroxide moiety acts as a scavenger of toxic free radicals formed *in vivo* (Emanuel et al.,1976; Sosnovsky and Li, 1985 a, b, c; Emanuel, 1985; Claycamp et al., 1986). Photolysis and reaction with acid (reactions 3. and 4.) are mainly of interest for the synthesis and storage of nitroxides.

Oxidation of the nitroxide moiety (reaction 5.) can be achieved by relatively strong oxidizing agents such as bromine or chlorine resulting in formation of the reactive immonium oxide ion (Rozantsev, 1971b). Unless nitroxide oxidation occurs in an inert solvent, the immonium oxide ion is unstable and is rapidly reduced by aldehydes, ketones and tertiary amines. Even water slowly reduces the immonium oxide ion. The corresponding hydroxylamine or nitroxide is formed, presumably by a radical mechanism (Rozantsev and Sholle, 1971), as a result of the reduction of immonium oxide ion.

Reduction of the nitroxide moiety to the hydroxylamine has been observed in biologic systems (Rauckman et al., 1984). This one-electron reduction can be accomplished by relatively weak reductants, such as ascorbic acid, and is easily reversed by spontaneous oxidation in air (Rozantsev, 1971b). In our pharmacokinetic studies, the hydroxylamine was oxidized to the corresponding nitroxide and then detected with ESR or UV spectroscopy (Chapter 2). The further two-electron reduction to the amine requires stronger reducing conditions, including the presence of a catalyst, such as Raney nickel (Rozantsev, 1971b). The oxidation of the amine back to the nitroxide form is also more difficult and requires strong oxidizing conditions, such as hydrogen peroxide combined with sodium pertungstate as a catalyst (Rozantsev, 1971a).

1.4 ESR spectroscopy

ESR spectroscopy, a powerful and versatile tool in biological research, has not gained the same wide use as NMR spectroscopy (Holtzman, 1984). Nitroxides have been used as probes in ESR studies for a multitude of different applications. For example, nitroxides can be used for examination of the structure of cell membranes or proteins and for examination of drug interactions with enzymes or other macromolecules (Holtzman, 1984). ESR spectroscopy is a sensitive and selective method for detection of nitroxides This spectroscopic technique was used in our studies: to determine nitroxide concentrations in biological samples; to monitor the reduction kinetics in ascorbic acid solutions, rat tissue homogenates and erythrocytes; and to determine erythrocyte membrane permeability to nitroxides.

In ESR spectroscopy, the interaction between the electron magnetic moment of paramagnetic species and the magnetic field is observed. The principles are the same as for NMR spectroscopy for which the interactions between the nuclear magnetic moment and the magnetic field are observed (Nordio, 1976). The magnetic moment of the electron, which has a spin of 1/2, has two allowed positions, parallel and antiparallel to the magnetic field. The same is true for the magnetic moment of the proton that has a spin quantum number of 1/2. At the same magnetic field strength of 3400 G, that often is used in ESR spectrometers, the resonance frequency of the electron and the proton are 9.5 GHz and 14.5 MHz, respectively. The difference in resonance frequencies is due to the almost 700-fold stronger magnetic moment of the electron. As a consequence of this strong magnetic moment of the electron and the fact that most compounds are diamagnetic, i.e., have paired electrons, ESR spectroscopy is a highly sensitive and selective method for detection of

paramagnetic species. The ESR spectra of the nitroxides, shown for the nitroxide derivative PCA (3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl) in Figure 1.3 (spectrum A), have 3 absorption peaks due to coupling of the magnetic moment of the electron with the magnetic moment of the nitrogen nucleus, an atom with three possible magnetic quantum numbers (-1, 0 and +1). The areas under the absorption peaks are proportional to the nitroxide concentration. The first derivative of the absorption peaks, often referred to as lines of the spectrum, are recorded by the ESR spectrometer. The peak-to-trough heights of the spectral lines can be used as measures of nitroxide concentration if the peak widths stay constant.

The line widths are proportional to the spin-spin relaxation rate and may change when the mobility of the nitroxide moiety is restricted or when other paramagnetic species are present (Freed, 1976; Jost and Griffith, 1976). The second ESR spectrum in Figure 1.3 (spectrum B) illustrates the effect of line broadening that occurs in the presence of the paramagnetic metal, ferric ion, in potassium ferricyanide. This could be interpreted as disappearance of the nitroxide, e.g., due to reduction. In experiments in which the nitroxide concentration is monitored over time by measuring the peak-to-through height of the lines of the ESR spectrum, it is important to determine that the line widths stay constant during the period of examination.

1.5 Toxicity of nitroxides

Toxicity is an important consideration for the use of nitroxides as diagnostic agents. Clearly, it is necessary for a contrast agent to have a wide margin of safety and essentially no side effect.

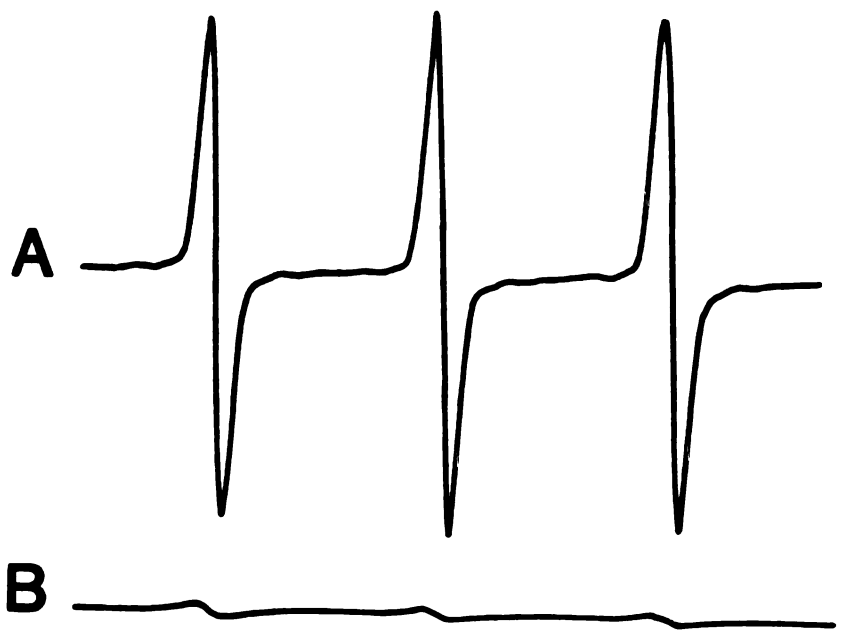


Figure 1.3 The ESR spectra of the nitroxide, shown for PCA at a concentration of 0.1 mM in an aqueous solution of lysed erythrocytes, has three sharp absorption peaks (spectum A). The first derivative of the absorption peaks are recorded by the ESR spectrometer. Addition of the paramagnetic anion, ferricyanide (200 mM), causes essentially complete loss of the ESR spectrum due to broadening of the line widths (spectrum B).

Nitroxides have an unpaired electron and are therefore free radicals. Free radicals are usually reactive chemical species produced in metabolism of endogenous compounds and xenobiotics, for example, oxygen radicals (superoxide anion, hydroxyperoxy radical, hydroxyl radical), have been related to the development of disease states, e.g., cancer, and to the process of aging (Cerutti, 1985; Halliwell and Gutteridge, 1985; Mehlhorn et al., 1985; Mehlhorn and Cole, 1985; Proctor and Reynolds, 1985; Marx, 1987). Formation of relatively unstable nitroxides from the metabolism of aromatic amines and subsequent redox cycling of these species to cause formation of reactive oxygen species have been suggested as a possible mechanism for the observed cancinogenicity of aromatic amines (Stier et al., 1980). The reactivity of free radicals, which is believed to be the cause of the different types of cell damage, is an important difference between the stable nitroxide derivatives (shown in Figure 1.2) and most other free radicals. Consequently, it is not appropriate *a priori* to assume that nitroxides and reactive free radicals have the same toxic behavior.

Nitroxides have been investigated for use as radiosensitizers to selectively increase the effect of x-ray radiation on tumors (Cadet and Teoule, 1982; Sridhar, 1982). In the presence of nitroxides more effective killing of hypoxic bacterial and mammalian cells by ionizing radiation was demonstrated *in vitro* (Emmerson and Howard-Flanders, 1964; Millar et al., 1985). The ability of nitroxides to react with free radicals was suggested as an explanation for the radiosensitizing effect of nitroxides (Wold and Brustad, 1973, 1975). This theory was supported by the observation that nitroxide derivatives more reactive towards free radicals were more effective radiosensitizers (Emmerson et al., 1971, Blackett et al., 1974). Nitroxides were believed to react with free radicals formed in important molecules, such as DNA, and therefore cause cell death.

However, *in vivo* no radiosensitizing effect of nitroxides was observed presumably because of rapid reduction of nitroxides *in vivo* (Hewitt and Blake, 1970; Hill et al., 1975; Schimmack et al.,1976; Couet et al.,1985c). Nitroxides are also believed to act as scavengers of free radicals produced by cancer chemotherapeutic agents and therefore suppress the toxic effects of these agents. Cancer chemotherapeutic agents labeled with nitroxides have been shown to have decreased toxicity and increased effect compared to the parent compounds (Emanuel et al.,1976; Sosnovsky and Li, 1985a,b,c; Emanuel, 1985; Claycamp et al., 1986). The reactions of nitroxides with free radicals formed *in vivo* and the potential effects of such reactions is difficult to predict and may or may not have any relation to the potential toxicity of nitroxides. In a recent study, a piperidine nitroxide derivative was shown to enhance the mutation frequency caused by oxidative stress in a *Salmonella typhimurium* cell line (TA104) which is a sensitive indicator of oxidative damage (Sies and Mehlhorn, 1986).

Only limited information is available on the toxicity of nitroxides*in vivo* . Acute toxicity was demonstrated for a piperidine nitroxide derivative that produced tranquilizing effects after administration of 0.5 and 2.0 mmol/kg and killed the mice within 10 minutes after administration of 3 mmol/kg (Hewitt and Blake, 1970). In a recent study, the toxicity and mutagenicity of two nitroxide derivatives, that have potential utility as MRI contrast agents, were evaluated. These nitroxides, a piperidine and a pyrrolidne derivative, had low acute toxicity in the rat (Afzal et al.,1984). The LD_{50} values for intravenous doses were approximately 15 mmol/kg which is an order of magnitude higher than the doses used for MRI contrast enhancement (0.15 to 3 mmol/kg) (Brasch et al., 1983, 1984; Ehman et al., 1985; Lamarque et al., 1986). The mutagenicity of the nitroxides and their corresponding reduced forms, the hydroxylamines and

amines, were also tested by examining the ability of these compounds to cause sister chromatid exchanges or mutations in Chinese hamster ovary cells (Afzal et al.,1984). No such activities were observed for either the parent compounds or their corresponding reduced forms, the hydroxylamines and the amines. This study indicates that these nitroxide derivatives can be safely administered but the toxicity has to be carefully examined for each nitroxide derivative before human use.

1.6 Pharmacokinetics of nitroxides

Biodistribution, metabolism and excretion of nitroxides are important determinants of the MRI contrast enhancement produced by nitroxides. A few studies of a preliminary nature have been published on the pharmacokinetics and metabolic fate of nitroxides because of the potential utility of nitroxides as radiosensitizers and free radical scavengers. Selected piperidine nitroxide derivatives were shown to be eliminated with half-lives of 1 to 2 minutes or less after administration to normal or tumor-bearing mice and rats (Blackett et al., 1974; Hill et al., 1975; Schimmack et al., 1976; Dodd et al., 1976; Zhdanov et al., 1979). Biodegradation of nitroxides was also observed *in vitro* in tissue homogenates and in erythrocytes (Blackett et al., 1974; Hill et al., 1975; Schimmack et al., 1976). The disappearance of nitroxide concentration, monitored with ESR spectroscopy, in these biologic systems was assumed to be due to reduction of the nitroxide moiety to the corresponding hydroxylamine. Oxidative treatment and exposure to air resulted in an increase in the ESR signal (Emanuel et al., 1976; Voronina et al., 1975). Because such treatment has been shown to oxidize the hydroxylamine to the corresponding nitroxide

(Rozantsev, 1971b), this observation indicates that the hydroxylamine, formed as result of a one-electron reduction of the nitroxide moiety, was present.

1.7 Bioreduction of nitroxides

The reduction of nitroxides is of particular interest for the use of nitroxides as contrast agents in MRI. As mentioned in the previous section, reduction of nitroxides has been observed *in vivo* and *in vitro* (Rauckman et al., 1984) in biologic systems. The hydroxylamine, which is assumed to be the reduced species, is formed as a result of a one-electron reduction and is therefore diamagnetic. The proton relaxation enhancing activity, that is responsible for the increase in MRI image intensity, is lost when the paramagnetic nitroxide moiety is reduced.

The cause of bioreduction is not well understood but reduction of nitroxides have been demonstrated in a multitude of different biologic systems that support both nonenzymatic and enzymatic mechanisms (Chumakov et al., 1971; Giotta and Wang, 1972; Kaplan et al., 1973; Blackett et al., 1974; Schimmack et al., 1976; Rosen and Rauckman, 1977; Rosen et al., 1977; Quintanilha and Packer, 1977; Perkins et al., 1980; Rauckman et al., 1984). Endogenous, small molecular-weight reductants have been suggested to cause nonenzymatic bioreduction of nitroxides. Ascorbic acid was shown to be responsible for nitroxide reduction in avian muscle tissue (Perkins et al., 1980). Small molecular-weight sulfhydryl-containing reductants, such as glutathione and cysteine, were concluded to cause nitroxide reduction observed in several different biologic systems (crab and lobster walking nerves, mice erythrocytes, neuroblastoma cells) (Giotta and Wang, 1972; Chen and McLaughlin, 1985).

Enzymatic reduction of nitroxides has been observed in microsomal (Rosen and Rauckman, 1977; Rosen et al., 1977) and mitochondrial subcellular fractions of rat liver (Quintanilha and Packer, 1977). Cytochrome P450 was concluded to be the nitroxide-reducing system in hepatic microsomes because the reduction was found to be: saturable; NADPH-dependent; inducible by phenobarbital; and competitively inhibited by specific antagonists of cytochrome P450 (Rosen and Rauckman, 1977; Rosen et al., 1977). In mitochondria, the respiratory chain was shown to reduce nitroxides (Quintanilha and Packer, 1977). Ubiquinol was suggested to be the reducing site in the respiratory chain because of the effects of different inhibitors of the respiratory chain. This mechanism was also reported to explain the nitroxide reduction observed in intact rabbit spermatozoa (Chapman et al., 1985). Reduction by electron transport systems has also been observed in non-mammalian cells such as bacteria (Baldassare et al., 1974; Maruyama and Ohnishi, 1974) and yeast cells (Nakamura and Ohnishi, 1972). Nitroxides incorporated in the cell membrane of E. coli, that has an electron transport system similar to that found in the membrane of mammalian mitochondria, were found to be reduced and this reduction was completely inhibited by reagents that bind covalently to sulfhydryl groups (Baldassare et al., 1974). As in mammalian mitochondria, ubiquinol was suggested to be the electron donor.

In summary, the reduction of nitroxides may occur by several enzymatic and nonenzymatic mechanisms. The relative contributions of these mechanisms to the bioreduction of nitroxides *in vivo* have not been demonstrated. Bioreduction may affect the extent and time course of MRI contrast enhancement by nitroxides. Consequently, the mechanisms of bioreduction and the factors that influence this process are of interest for the development of nitroxides as MRI contrast agents.

Chapter 2. General Methods

The particular nitroxide derivatives used in the *in vivo* and *in vitro* studies are presented in this chapter. Assay methods, including ESR spectroscopy and HPLC, and general procedures and experimental protocols are described. Methods for specific experiments are described in subsequent chapters.

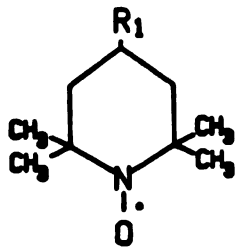
2.1 Nitroxides

The structures, code names and sources of the piperidine and pyrrolidine nitroxide derivatives used in the studies are shown in Table 2.1 (see Abbreviations for chemical names).

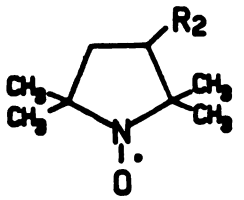
Synthesis of CAT:

Methyl iodide (0.648g, 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 (0.171g, 1.0 mmol), and 4 hydroxy-2,2,6,6-tetramethylpiperidine (0.312g, 2.0 mmol), synthesized as described previously (Sosnovsky and Konieczny, 1978). The mixture was stirred until a homogeneous solution was obtained, then left for 3 hours 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 CAT in the form of orange crystals, mp 241-242°C (dec.); MS: m/z = 215 (M^+ +1). Elementary analysis for $C_{12}H_{26}N_2IO$: C, 42.23; H, 7.75; N, 8.12 (calculated) and C, 42.48; H, 7.68; N, 8.21 (found).

Table 2.1 *Structures and Sources of the Piperidine and the Pyrrolidine Nitroxide Derivatives Used in the Studies.*



Piperidine (R1)



Pyrrolidine (R2)

Code Name	Substituent	M.W.	Source
6-OH	R1=OH	172	Sigma [a]
6-NH_2	R1=NH_2	171	Sigma [a]
6-COOH	R1=COOH	200	Synthesized [b]
TES	R1=NH-$(CH_2)_2$-COOH	271	Synthesized [c]
CAT	R1=$^+N(CH_3)_3$ I^-	341	Synthesized [d]
TPH	R1=OPO_3H_2	252	Aldrich [a]
5-OH	R2=OH	158	Synthesized [b]
5-NH_2	R2=NH_2	157	Synthesized [b]
PCA	R2=COOH	186	Eastman [a]
TAP	R2=CO-NH-CH(CH_2OH)-CH(OH)-CH_2OH	289	Schering [a]

(a) Sigma Chemical Co (St. Louis, MO); Aldrich Chemical Co. (Milwaukee, WI); Eastman Kodak Co. (Rochester, NY); Schering A.G. (Berlin, FRG).

(b) Couet et al., 1985.

(c) Kosman et al., 1969.

(d) Synthesis is described in this chapter.

2.2 ESR spectroscopy for analysis of nitroxide concentration

ESR spectroscopy was used as an assay method to: measure nitroxide concentration in biological samples; monitor the kinetics of nitroxide reduction in ascorbic acid solutions and tissue homogenates; and examine membrane permeability and bioreduction of nitroxides in human erythrocytes.

The ESR spectrometer (Model E-104A,Varian), operated at a field strength of 3400 G and a frequency of 9.5 GHz, was equipped with a 0.04 mL flat cell placed in the microwave cavity. A volume of approximately 1 to 2 mL of the particular sample was injected into the flat cell with a disposable plastic syringe. The peak-to-trough height of the first derivative of the low field line of the nitroxide spectrum was used as a measure of the ESR signal. This line was selected to avoid potential interference from the ESR spectrum of the ascorbic acid ion radical that overlaps with the mid-field line of the nitroxide spectrum (Bielsky, 1982). In some cases the full spectrum was recorded. All ESR spectra were recorded at room temperature (21 ± 1 °C). For the kinetic studies, the peak width was determined to ensure that no broadening occurred during the experiment.

2.3 HPLC methods for analysis of nitroxide concentration

HPLC methods were developed for determination of nitroxide concentrations in plasma, blood and urine samples collected in the *in vivo* studies in the dog and the rat. These methods were complementary to the ESR assay in that potential metabolites, in particular products of conjugation of the water-soluble side chains, could be separated from the unchanged nitroxide. Such metabolites, in which the nitroxide moiety is intact or reduced to the

hydroxylamine and for which the ESR spectrum of the metabolites in their oxidized form is expected to be similar to the unchanged nitroxide, would be measured as the nitroxide in the ESR assay. In the HPLC assay, the nitroxides and potential metabolites could be separated and detected with ultraviolet spectroscopy. The nitroxide moiety has an absorption maximum at about 245 nm.

The HPLC systems consisted of the following:

Columns: Stainless steel columns packed with μBondapack Phenyl (5μm particle size, Waters Associates) or C18 material (10 μm particle size, Alltech), 250 x 3.2 mm and 250 x 4.6 mm, respectively. A precolumn, packed in the laboratory with C18 material (40 x 3.2 mm) using 30-38 μm particle size material or a commercially-available precolumn (30 x 4.6 mm) with 10 μm particle size C18 material (Brownlee Labs, MPLC Guard Cartridge), was used to protect the analytical column.

Injectors: A manual Model U6K or an automatic WISP 710B injector (Waters Associates) were used.

Pump: The mobile phase was pumped with a Model A-60-S precision pump (Eldex Laboratories Inc., Menlo Park, CA) at a flow rate of 2.5 to 2.7 mL/min that produced a back pressure of 2800 to 3000 psi, .

Detector: The effluent was monitored at 245 nm with variable wavelength detectors, either a Model SF 770 (Kratos Analytical Instruments)

with an integrator (Model 3390A, Hewlett Packard) or a Varichrom with a Model 20 recorder (Varian).

The mobile phases consisted of absolute HPLC-grade methanol mixed with 20 mM phosphate buffer (pH 2.2 or 7.4). The particular composition of the mobile phase and the column used for each nitroxide derivative and its respective retention time are listed in Table 2.2. The analyses were performed at room temperature. Sample preparations for specific individual studies are described in the subsequent chapters.

2.4 Oxidation of the hydroxylamine

The hydroxylamines of the corresponding nitroxides are formed by a one-electron reduction of the nitroxide moiety. The hydroxylamine is diamagnetic and therefore not detectable with ESR spectroscopy. It is also not detected in the HPLC assays in which ultraviolet absorption is utilized. The only chromophore, the nitroxide moiety, is lost as a consequence of reduction. The hydroxylamine was therefore oxidized to its corresponding nitroxide. For samples that were assayed for nitroxide concentration before and after oxidative treatment, the hydroxylamine concentration was obtained from the increase in ESR signal after oxidative treatment. The nitroxide concentration determined after oxidation is a sum of the nitroxide and the hydroxylamine present in the sample. Different oxidative treatments, previously reported to oxidize the hydroxylamine (for review see Rozantsev and Sholle, 1971a; Keana, 1979), were evaluated.

Table 2.2 *HPLC Systems and Retetion Times of the Nitroxides Studied In Vivo.*

Nitroxide	Sample	Mobile phase	Retention time
TES [a]	Plasma	25 % methanol, pH 2.2	4.8 min
TES [a]	Urine	15 % methanol, pH 2.2	10.5 min
PCA [a]	Plasma	25 % methanol, pH 2.2	4.7 min
PCA [a]	Urine	15 % methanol, pH 2.2	10.0 min
PCA [b]	Urine	20 % methanol, pH 2.2	8.0 min
TAP [c]	Urine/Blood	10 % methanol, pH 7.4	5.1 min

(a) Column: μBondapack Phenyl (Chapter 3).

(b) Column: C18, Alltech (Chapter 4).

(c) Column: C18, Alltech (Chapter 5).

1. Autoxidation

Hydroxylamines have been reported to be oxidized in the presence of oxygen (Rozantsev and Sholle, 1971a). Simply exposing the samples to air therefore results in oxidation of the hydroxylamine. This treatment was used for blood and plasma samples. The samples were diluted five fold in methanol and the resulting denatured proteins were precipitated by centrifugation at 1000 x *g* for 5 min. Precipitation of proteins was necessary for the HPLC assay and methanol facilitates the autoxidation because oxygen has a higher solubility in methanol than in water (10.3 mM in methanol versus 1.3 mM in water at 22 °C; International Critical Tables, vol. 3, 1928, pp. 254-262). For some samples the oxidant m-chloroperoxybenzoic acid was added to the methanolic supernatant fluid (final concentration of 20 mM) to ensure complete oxidation. The samples were left at room temperature for 24 hours before measurement with ESR. For the HPLC assay, the methanol was evaporated under a flow of nitrogen gas and the samples were reconstituted in mobile phase before injection.

2. Oxidation with hydrogen peroxide

A 30 % solution of hydrogen peroxide, shown to oxidize the hydroxylamine (Rozantsev and Sholle, 1971a), was added to urine and plasma samples. The urine samples were diluted several-fold in distilled water before measurement were made. Hydrogen peroxide could not be used for oxidative treatment of blood and tissue homogenates because addition of hydrogen peroxide caused oxygen production and formation of foam that interfered with the ESR measurement.

3. Oxidation with potassium ferricyanide

The hydroxylamine can be oxidized by addition of potassium ferricyanide (Rozantsev and Sholle, 1971a). Potassium ferricyanide was added to a final concentration of 2 mM to the samples just before measurement with ESR spectroscopy. The ferric cation is paramagnetic and causes broadening of the lines of the nitroxide ESR spectrum. At this low concentration (2 mM) the line broadening was minor. Both samples and standard solutions were treated similarly. This method was used for blood and urine samples. Ferricyanide was not used for oxidative treatment in tissue homogenates because rapid destruction of the nitroxide was observed upon addition of the oxidant. This destruction has been observed previously and was suggested to be related to the presence of sulfhydryl groups (Graceffa and Seidel, 1980).

4. Oxidation with cupric sulfate

Copper cation in cupric sulfate, also shown to oxidize the hydroxylamine (Schwartz et al., 1979), did not cause any destruction of the nitroxide in tissue homogenates or line broadening of the ESR spectrum at the 2 mM concentration used for oxidation. As with potassium ferricyanide, cupric sulfate was added just before measurement with ESR spectroscopy. All methods appeared to completely oxidize the hydroxylamine, assessed by oxidation of samples prepared in ascorbic acid solution and rat tissue homogenates, but this relatively rapid and simple method was preferred.

2.5 Experimental Protocols of Studies *In Vivo*

Pharmacokinetics and metabolic fate of the nitroxides were examined in the dog and the rat. The dog was selected as an animal model because large, multiple blood samples could be collected and the dogs could be used repeatedly for comparisons of different derivatives and doses of nitroxides. Pharmacokinetic information was also obtained in the rat because the bioreduction of nitroxides was studied *in vitro* in rat tissue homogenates. The observations in both a small (rat) and a large animal species (dog) were extrapolated to predict the pharmacokinetics in humans. Furthermore, the dog and the rat were used as an animal models in concurrent MRI studies by Dr. Robert C. Brasch and coworkers (Contrast Media Laboratory, Department of Radiology, School of Medicine, University of California San Francisco). The pharmacokinetic and metabolic studies of nitroxides were performed in collaboration with the Contrast Media Laboratory where the potential utility of nitroxides as MRI contrast agents were evaluated.

Experimental Design of Dog Experiments

Three male beagles (Marshall Research Animals, North Rose, NY) weighing 10 to 15 kg were used in all but one preliminary study, in which a male mongrel dog weighing 30 kg was used. After positioning the dog in a sling and frame, catheters were placed in a superficial limb vein (Angiocath No. 2818, The Deseret Company, Sandy UT) and in the urinary bladder (Swan-Ganz, Monitoring catheter Edwards Lab. Inc., Anasco, Puerto Rico) for collection of blood and urine, respectively. To keep the blood sampling catheter patent during the experiment and to maintain urine flow and hydration, the dog was

given a continuous drip infusion of normal saline into the venous catheter at a flow rate of 25 to 50 mL/hour. After collection of blank urine and blood samples, the nitroxide was administered into a superficial vein, of a leg different from that of blood sampling, over approximately one minute with an intermittent infusion set (Butterfly INT, Abbott Hospitals, Inc.). Following the injection, the line was flushed with 10 mL of normal saline. Blood samples (2 to 5 mL) were collected, in Vacutainer tubes (Becton and Dickinson) containing potassium oxalate and sodium fluoride, for six hours at time intervals of 5 to 10 min within the first 30 min, 15 to 30 min for the next 90 min and every 30 min for the last four hours. Urine was collected for six hours via the urinary catheter. At the end of the experiment, the bladder was flushed with 20 mL normal saline and allowed to empty before the volume of the collected urine was recorded and a sample saved for subsequent analysis. The catheters were removed at the end of the six hours and the dog was transferred to a metabolic cage, where urine was collected for another 18 hours in most experiments. The urine was usually collected in three separate fractions, namely, 0 to 2, 2 to 6, and 6 to 24 hours after administration.

Experimental Design of Rat Experiments

In the rat, two different protocols were used for the *in vivo* experiments, one protocol (Protocol 1) was used to determine the pharmacokinetic parameters in blood and a second protocol (Protocol 2) was used to examine the urinary recovery of the nitroxide and its metabolites.

Protocol 1: Male Sprague-Dawley rats (Simonsen Laboratories) weighing 250 to 300 g were used. For administration of the nitroxide, a catheter (Intramedic polyethylene tubing, inner and outer diameters of 0.58 mm

and 0.97 mm, Clay Adams) filled with normal saline containing heparin, 50 I.U. per mL (heparinized saline), was placed in the left jugular vein. For blood collection another catheter, of the type used for drug administration, was placed in the right carotid artery. The catheters were externalized at the back of the neck and the incision was closed with surgical clips. The rats were anaesthetized with ether during the 45 to 60-minute period required for insertion of the catheters. While recovering from anaesthesia and during the experiment, the animals were kept in a cage with free access to food and water. An isotonic solution of the nitroxide, filtered through a 0.2 μm filter (Acrodisc, Gelman Sciences Inc.), was administered via the vein catheter over one minute. Following the injection, the catheter was flushed with 0.3 mL of heparinized saline. Blood samples (0.5 mL) were collected in tubes, containing potassium oxalate and sodium fluoride (Vacutainer, Becton and Dickinson), at 5,10, 20, 30, 40, 60, 90 and 120 minutes after the low dose and at 10, 20, 40, 60, 90, 120, 180, 240, 300 and 360 minutes after the high dose. After collection of each blood sample the arterial catheter was flushed with 0.3 mL of heparinized saline. The arterial catheter contained a known volume of heparinized saline which caused a dilution of each blood sample. A dilution factor, based on this volume and the total volume (saline plus blood) withdrawn, was used to calculate the blood nitroxide concentration.

Protocol 2: For collection of urine, the nitroxide was injected via a tail vein as a bolus over one minute to male rats (source and body weight same as above). The rats were kept in metabolic cages with free access to food and water for 24 hours after injection. Six hours and again 24 hours after injection, the urine was collected and the metabolic cage was rinsed with distilled water. For each collection period, the urine and water rinses were combined; the total volumes were recorded; and the samples were frozen for subsequent analysis.

Plasma Protein Binding:

Protein binding of the nitroxides in rat and dog plasma was determined by ultrafiltration. Plasma was spiked with nitroxide at concentrations of 0.1 mM and 1.0 mM and incubated for 30 minutes. The spiked plasma samples (500 µL) were transferred to sample reservoirs of the ultrafiltration devices (Micropartition System MPS-1, Scientific Systems Division, Amicon Corporation, Danvers, MA), and centrifuged for 15 min at 1000 x *g*. The nitroxide concentrations in the ultrafiltrates were measured by ESR spectroscopy and compared to standard solutions in water. The fraction unbound was calculated from the ratio of nitroxide concentrations in ultrafiltrate and plasma.

2.6 Experimental Protocols of Studies *In Vitro*

The kinetics of reduction of nitroxides was examined in ascorbic acid solution, rat tissue homogenates and human erythrocytes.

Reduction in ascorbic acid solutions

The time courses of reduction of the nitroxides in ascorbic acid solutions were determined by ESR spectroscopy at room temperature (22 ± 1°C) at different initial concentrations of the nitroxide and of ascorbic acid. Solutions of ascorbic acid were prepared freshly in 67 mM phosphate buffer adjusted to pH 7.4 (with 1 M NaOH) on the day of the experiment. Solutions of the nitroxide and ascorbic acid were mixed so that the desired initial concentration for each was achieved. After mixing for 5 seconds with a vortex agitator, the sample was

injected into the flat cell of the ESR spectrometer and was not removed until the end of the experiment. The peak-to-trough height of the first derivative of the low-field line of the nitroxide ESR spectrum was continuously recorded for 5 to 15 min.

Reduction in rat tissue homogenates

Male Spragues-Dawley rats (Simonsen Laboratories) weighing 250 to 350 g were used for preparation of tissue homogenates. After the animal was killed by decapitation, the tissue (brain, heart, kidney, liver, lung or muscle) was immediately removed, weighed and homogenized with a Potter-Elvehjem tissue grinder in 3 mL of isotonic saline or phosphate buffer (67mM, pH 7.4) per g of tissue. The reduction of nitroxide derivatives in rat tissue homogenates was measured by ESR spectroscopy. After addition of the nitroxide at an inital concentration of 0.025 to 0.25 mM, the low field peak was monitored for 10 to 60 minutes.

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 x *g* for 5 min and the plasma was discarded. The erythrocytes were separated from plasma because plasma was found to reduce nitroxides. The erythrocytes were then washed three times by repeated resuspension of one volume of packed cells in an equal volume of isotonic buffer (pH 7.4); centrifuging as above and exchanging supernatant fluid with fresh buffer. The erythrocytes were suspended at an hematocrit of 50 % after washing. Erythrocytes were lysed by centrifuging the erythrocyte

suspension, discarding the supernatant fluid, and adding the same volume of distilled water to lyse the cells hypotonically (i.e., the erythrocyte lysate and the suspension were prepared to contain the same amount of cells). Nitroxide reduction in erythrocyte suspension and lysate was monitored by ESR spectroscopy at room temperature, as described above. The nitroxide derivatives were added to erythrocyte suspension and lysate at initial concentrations of 0.01 mM.

Determination of Erythrocyte Membrane Permeability to Nitroxides

Using an extracellular quenching agent, the nitroxides in the intracellular fluids of erythrocytes were selectively measured and monitored over time. The paramagnetic ferricyanide ion causes broadening of the nitroxide ESR spectrum in extracellular fluids. The intracellular signal is not broadened because the ferricyanide anion does not penetrate the cell membrane. This technique has been used to examine membrane permeability, determine cell volume and intracellular pH (Mehlhorn and Packer, 1983).

The erythrocyte suspension and lysate were prepared as described for the experiment in which nitroxide reduction was examined. A portion of the erythrocyte suspension was centrifuged at 1000 x *g* for 5 min and the supernatant fluid was exchanged with a 200 mM potassium ferricyanide solution. The quenching agent was also added to a portion of the erythrocyte lysate to make a final concentration of 100 mM. The ESR spectrum was recorded for erythrocyte suspension and lysate, with and without the quenching agent, after addition of the nitroxide under study to make a total concentration of 0.1 mM. To monitor the penetration rate, the ESR spectrum was first recorded in erythrocyte suspension without ferricyanide ion and the magnetic field of the

ESR spectrometer was set on the low-field peak of the nitroxide spectrum so that the height of this peak (signal) could be continuously monitored. The same nitroxide was then added to another sample of the erythrocyte suspension that contained the quenching agent, potassium ferricyanide. This sample was rapidly vortexed, injected into the flat cell of the ESR spectrometer, and the intracellular ESR signal was monitored with time. For the permeable nitroxide derivatives, the intracellular signal reached an upper limit in less than eight minutes at which time a complete ESR spectrum was recorded. In the time frame of these experiments, the nitroxide reduction by erythrocytes were found to be negligible.

Section I

Pharmacokinetics and Metabolic Fate of Nitroxides.

In this section data from pharmacokinetic studies of selected nitroxides are presented. These nitroxides were concurrently being studied in animals by our collaborators, Dr. Robert C. Brasch and coworkers (Brasch et al., 1983a, 1983b; Wesbey et al., 1984; Ehman et al., 1985) to determine the diagnostic utility of using nitroxides as MRI contrast agents. Initial pharmacokinetic studies, presented in this chapter, were performed in a mongrel dog using two prototypic nitroxide derivatives, TES and PCA (see Chapter 2 for chemical name and structure). These derivatives, representatives of the piperidine (TES) and pyrrolidine (PCA) classes of nitroxides, were selected because of their high water solubility, stability and availability. High water solubility is a desired property that facilitates administration of relatively large intravenous doses required for MRI contrast enhancement (0.1 to 2 mmol/kg; Brasch et al., 1983a, 1983b; Wesbey et al., 1984; Ehman et al., 1985). After the initial study, a more complete evaluation, presented in Chapter 4, of the pharmacokinetics and metabolic fate of PCA and TES was performed in three beagles. More attention was then given to the pyrrolidine derivative, PCA, that appeared to be more stable *in vivo* and therefore more useful as an MRI contrast agent. Based, in part, upon observations of the comparative pharmacokinetics of the two nitroxide classes, a nonionic pyrrolidine nitroxide derivative was synthesized to incorporate the higher stability of pyrrolidine derivatives and the lower osmolarity of nonionic x-ray contrast media. The last chapter in this section, presents the results of the pharmacokinetics and metabolism obtained in the dog and the rat for this nonionic derivative.

Chapter 3. Preliminary Pharmacokinetics and Metabolic Fate of TES and PCA in a Mongrel Dog.

In the preliminary pharmacokinetic study in a mongrel dog, presented in this chapter, ESR spectroscopic and HPLC assay methods for determination of nitroxide concentrations in plasma and urine and for identification of metabolites were developed. The dog was chosen as the animal model because it was being used simultaneously in MRI imaging studies (Brasch et al., 1983a, 1983b, Wesbey et al., 1984) and because multiple blood samples of the volumes necessary (3 to 4 ml) for analysis would not be possible in small animals. Furthermore, the dog could be used repeatedly for comparison of different nitroxide derivatives and doses.

Materials and Methods

Chemicals

The nitroxides, TES and PCA, were stored as aqueous solutions buffered to pH 7.4 with 67 mM phosphate buffer at concentrations of 0.25 mM and 0.8 mM, respectively. The solutions were passed through a 0.2 μm filter (Acrodisc, Gelman Sciences Inc.) prior to intravenous administration.

Experimental Design

The male mongrel dog weighing 30 kg was given 0.55 mmol/kg of TES or PCA on two separate occasions at least two weeks apart. Details of the

experimental procedures are described in Chapter 2. In these studies urine was collected for six hours.

Each nitroxide was studied twice; the samples obtained in the two experiments were treated differently: one protocol was used to determine the pharmacokinetic parameters, calculated from plasma nitroxide concentrations; the second protocol was used to determine the amount of nitroxide and its metabolites in plasma and excreted in urine.

Protocol 1. Determination of Pharmacokinetic Parameters

Plasma, separated from blood samples by centrifugation at 1000 x *g* for 1 min, was injected into the flat cell of the ESR spectrometer within 5 minutes of sampling for determination of the nitroxide concentration. After collection of the last plasma sample, 100 μL of a 30 % hydrogen peroxide solution was added to 2 mL of each of the plasma samples and then left for 24 hours before repeating the analysis with ESR spectroscopy to determine the sum of the concentrations of the nitroxide and its corresponding hydroxylamine. The oxidation of the hydroxylamine appeared to be complete because no further increase in nitroxide concentration was seen when the samples were kept for 48 hours before the analysis. Standards in blank plasma, freshly prepared for each experiment, were treated identically to the samples. Urine samples were diluted in distilled water to obtain nitroxide concentrations below 1.5 mM. Above this concentration line broadening was observed due to the high paramagnetic concentration. Before analysis with ESR spectroscopy, the urine samples were treated with hydrogen peroxide, as described above for plasma samples, to oxidize the hydroxylamine.

Protocol 2. Examination of Metabolic Fate

Blood was centrifuged as above immediately after collection. Plasma and urine were stored at - 20 °C and analyzed within 3 days of each experiment, although storage for up to one month produced no detectable change in the nitroxide concentration. Samples were thawed and brought to room temperature on the day of analysis. Plasma samples were prepared by mixing 1 mL of plasma with 3 mL of methanol to precipitate proteins. After centrifugation, 20 μL of the methanolic supernatant fluid was directly injected into the HPLC system (described in Chapter 2) and 2 mL was injected into the flat cell of the ESR spectrometer. The methanolic supernatant fluid of the plasma samples were also measured 24 hours after addition of 100 μL of a 30 % solution of hydrogen peroxide to 2 mL of each sample. Urine was measured after dilution in methanol (a dilution factor of 10 to 100 depending on the particular urine sample) and oxidative treatment with hydrogen peroxide, as described above for the plasma samples.

The concentrations of TES and PCA remained constant in the samples for at least 24 hours after preparation. Concentrations of TES and PCA as low as 50 μM could be determined with the HPLC assay and less than 0.5 μM with the ESR assay. The standard curves were linear for both methods up to 1.5 mM; the average coefficients of correlation were 0.985 and 0.998 for the HPLC and ESR assays, respectively.

Pharmacokinetic Analysis

Total body clearance (CL) was calculated as CL = Dose/AUC, where AUC is the area under the plasma-concentration-versus-time curve. The AUC

was calculated using the trapezoidal rule until the last measured concentration, C(last), and extrapolated to infinity by adding C(last)/k. The elimination rate constant (k) and the corresponding half-life were estimated by linear least squares regression of the logarithm of the plasma concentration with time in the terminal phase of the decline. The volume of distribution was calculated as V = CL/k.

Results

Stability in Plasma and Urine

The stabilities of TES and PCA in fresh dog plasma and urine were examined by monitoring their concentrations by ESR spectroscopy at room temperature. PCA was stable in fresh dog plasma for at least one hour at concentrations as low as 0.1 μM while TES was unstable under the same conditions. For example, at initial concentrations of 1 μM and 1 mM, 20 % and 3 % decreases in the concentration of TES, respectively, were observed. Both TES and PCA were unstable in fresh dog urine. The results obtained in dog urine collected on different occasions, showed greater variability but the decrease in nitroxide concentration was always greater in urine than observed in dog plasma. TES was always less stable than PCA. As an example, only 1 % of TES remained after 5 minutes while 65 % of PCA remained after 15 minutes at an initial urinary nitroxide concentration of 1 mM.

The stabilities of TES and PCA were also examined in plasma and urine samples collected after injection of the nitroxides to the dog. In plasma, an increase of the concentration was observed with time for both TES and PCA. For example, a plasma sample collected 2 hours after administration of PCA

and immediately injected into the flat cell of the ESR spectrometer showed a 20 % increase in PCA concentration when the sample was retained in the flat cell for 30 minutes. Increases in TES and PCA concentrations were also observed in urine samples. About 5 hours after dilution of urine samples with methanol, the nitroxide concentrations increased to values 5 to 50-fold higher than those measured directly after dilution. These increases indicate that the respective hydroxylamines are present in the plasma and urine samples. Spontaneous oxidation of hydroxylamines in the presence of air have been previously reported (Rozantsev and Sholle, 1971a). The nitroxide is restored as a result of the oxidation and is detected with ESR spectroscopy.

Pharmacokinetics of TES and PCA

Pharmacokinetic parameters calculated from nitroxide concentrations in plasma, determined with ESR spectroscopy as soon as possible after collection, are presented in Table 3.1. The half-lives of decline of the concentrations determined after oxidative treatment were 44 and 85 minutes for TES and PCA, respectively. Figure 3.1 shows the time courses of the concentrations of PCA determined before and after oxidation (see Tables A3.1 and A3.2 in the Appendix for the plasma concentration data for TES and PCA). The urinary recoveries, determined after oxidative treatment of urine collected for six hours after administration, were 86 and 82 % of the dose for TES and PCA, respectively.

Table 3.1 *Pharmacokinetic Parameters in a Mongrel Dog.*

	Clearance [a] (mL/min)	Volume of Distribution [b] (L/kg)	Half-life [c] (min)
PCA	185	0.41	43.5
TES	214	0.21	18

[a] Determined from Dose/AUC.

[b] Volume of distribution was calculated as V=CL/k where k is the slope of the terminal decline of the logarithm of nitroxide concentrations in plasma versus time.

[c] From least squares fit of the decline after 30 min.

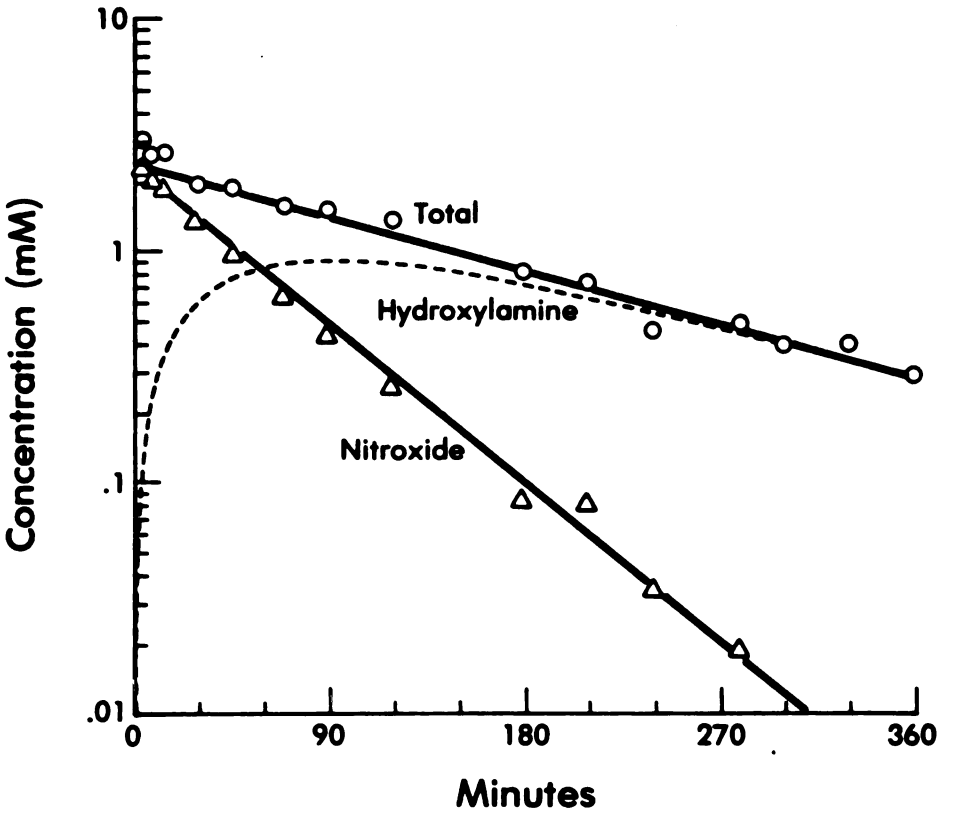


Figure 3.1 Decline of PCA concentration in plasma with time in the dog, when concentration is measured as soon as possible after sampling (nitroxide, triangles) and after oxidative treatment with hydrogen peroxide (total, circles). Linear least square regressions of both sets of data are shown (solid lines). The arithmetic difference (dashed line), corresponds to the hydroxylamine of PCA.

Metabolic Fate of TES and PCA

The HPLC chromatograms of plasma and urine showed no peaks other than that corresponding to the nitroxide and those of endogenous compounds observed in blank samples. The hyperfine ESR spectra of the nitroxides in plasma and urine samples, collected after administration to the dog, were not different from those obtained by adding the nitroxides to blank plasma and urine. The nitroxide concentrations determined in plasma and urine by ESR spectroscopy were virtually the same as those determined with the HPLC assay (Tables A3.3 and A3.4 in the Appendix), as shown in Figure 3.2 for PCA concentrations in plasma. These observations indicate that the unchanged nitroxides or their corresponding hydroxylamines were excreted in urine. The methanol treatment of plasma samples appeared to oxidize the hydroxylamines. The PCA concentrations in plasma determined after oxidation as described in the protocol for the pharmacokinetic study were close (± 20 %) to those obtained in the methanolic supernatant fluids for the metabolic studies while small increases in general were observed for TES.

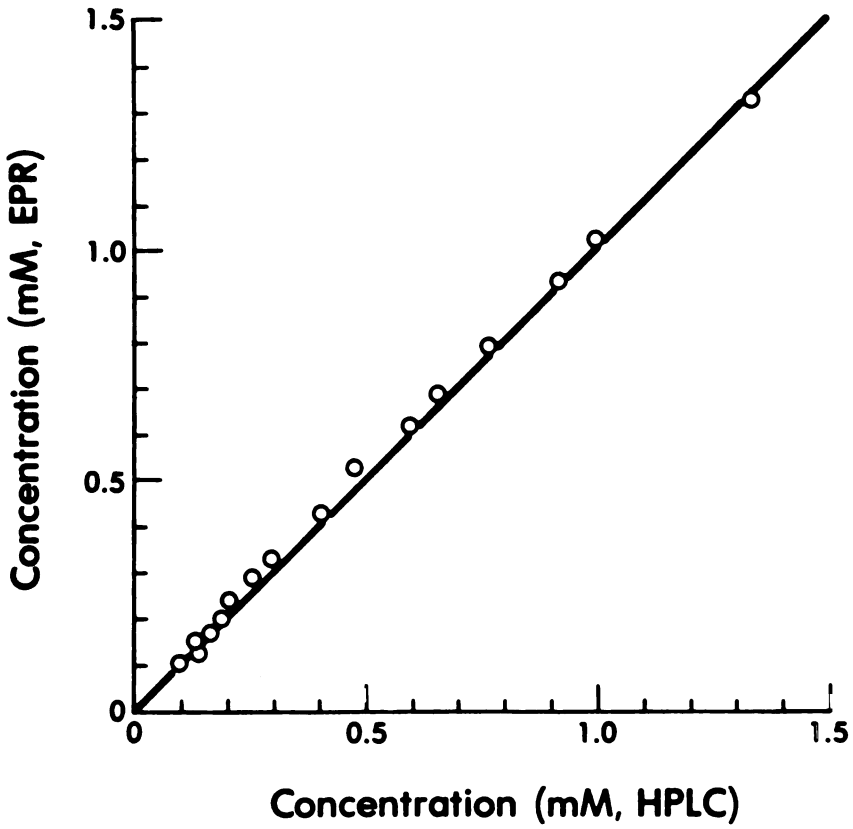


Figure 3.2 The concentrations of PCA in methanolic supernatant fluids of dog plasma samples, measured by ESR spectroscopy, are plotted against the corresponding concentrations, determined by the HPLC assay. The straight line, with an intercept of zero and a slope of one, shows the perfect relationship.

Discussion

The nitroxides appeared to be reduced *in vivo* to their corresponding hydroxylamines. The hydroxylamine is diamagnetic and therefore not detected by the ESR assay. It is also not detected in the HPLC assay because the only chromophore in these compounds, the nitroxide moiety, would be lost as a consequence of the reduction. However, the formation of the hydroxylamine is supported by the following indirect evidence. The nitroxide concentrations in plasma and urine samples increased with time after collection, as determined by ESR, which indicates a spontaneous oxidation of the corresponding hydroxylamines in the presence of air. Such oxidation of hydroxylamines has been previously reported to occur (Rozantsev and Sholle, 1971a). Dilution of the samples in methanol appeared to facilitate the autoxidation. Oxidation of hydroxylamine upon extraction with organic solvents have been observed (Keith at al., 1970; Maruyama and Ohnishi, 1974) and may be attributed to a higher solubility of oxygen in these solvents. The solubility of oxygen is 10.3 mM in methanol and 1.3 mM in water at 22 °C (International Critical Tables, vol. 3, 1928, pp. 254-262). Oxidative treatment of the samples with hydrogen peroxide appeared to have the same effect as methanol dilution of the samples indicating that the sponataneous oxidation of the hydroxylamine was essentially complete. Hydrogen peroxide is known to oxidize hydroxylamines to their corresponding nitroxides (Rozantsev and Sholle, 1971a). It is interesting to note that hydrogen peroxide did not increase the concentration of PCA any more than methanol treatment alone did, while small further increases were observed for some of the TES plasma samples. This indicates that the hydroxylamine of PCA is more readily oxidized than that of TES. In the stability tests in plasma and urine, PCA appeared to be more stable than TES.

Assuming that this decay is due to reduction of the nitroxide to the hydroxylamine, this observation suggests that TES is reduced more easily than PCA.

The reduction of the nitroxides and the spontaneous oxidation of their corresponding hydroxylamine observed *in vitro* in plasma and urine samples are stability problems that complicate sample analysis. It can be concluded that the HPLC assay, as well as any other technique that requires protein precipitation or extraction into organic solvents, cannot be used for quantitative determination of nitroxides. However, the determination of nitroxide concentration by ESR spectroscopy which can be performed rapidly before any significant reduction or oxidation can occur makes this technique opportune for pharmacokinetic studies of nitroxides.

ESR spectroscopy is a sensitive and selective method for detection of nitroxides. No changes in the hyperfine ESR spectra were observed indicating that the parent nitroxides were present in plasma and urine; however the ESR spectra of different nitroxide derivatives often overlap (Chapter 2). Metabolic transformation of the nitroxides (or their corresponding hydroxylamines) may therefore produce compounds that have ESR spectra that cannot be differentiated from those of the original nitroxides. For example, a conjugate of the carboxylic acid side chain in either TES or PCA could potentially be formed *in vivo*. The HPLC assay is expected to separate such metabolites and is therefore complementary to ESR. However, no metabolite peaks were observed in the HPLC chromatograms and the nitroxide concentrations were essentially the same as those determined with the ESR assay, as shown in Figure 3.2. Consequently, if metabolites other than the corresponding hydroxylamine are formed they must cochromatograph with the nitroxide or be present at concentrations below detection limits. A stable glucuronide

conjugate is unlikely because such a metabolite would be more polar than the parent compound and should have been observed by the HPLC assay. Because the urinary recoveries were high (86 and 82 % for TES and PCA, respectively), it can be concluded that most of the administered doses were eliminated in urine within the six hour period of urine collection as the unchanged nitroxides and their corresponding hydroxylamines.

Other metabolites in urine not detected by the ESR or HPLC assays or elimination via other routes (e.g., bile excretion) contribute less than 20 percent to the elimination. For example, the potential formation of the corresponding amines cannot be excluded. The amine can be formed by a further two-electron reduction of the hydroxylamine. The oxidative methods used in the present studies are inadequate to oxidize the amine form (Rozantsev and Sholle, 1971a) and the amine would not be detected by either the ESR or the HPLC assay.

The fairly complete and rapid elimination of TES and PCA in urine is compatible with the use of these compounds as contrast agents. Retention of the compounds *in vivo* may increase the risks for toxicity. Furthermore, it is desirable that the contrast agent be eliminated relatively shortly after completion of the MRI examination to avoid interference with future examinations.

Comparisons of the plasma concentrations before and after oxidation indicate that both nitroxides were extensively reduced *in vivo*, as shown in Figure 3.1 for PCA. Three hours after administration of the 0.55 mmol/kg dose, the hydroxylamine was virtually the only form present *in vivo* for both TES and PCA. For both compounds, the elimination of the nitroxide was more rapid than that of the hydroxylamine at this dose. Furthermore, TES appeared to be eliminated more rapidly than PCA.

The pharmacokinetic parameters were calculated from concentrations measured as soon as possible after sample collection. Indeed, because of the propensity of the hydroxylamine to be autoxidized to the nitroxide, it is possible that TES and PCA plasma concentrations may have been overestimated, especially at later times when the concentration of the hydroxylamine is much greater than that of the corresponding nitroxide.

Reversible metabolism complicates pharmacokinetic analysis. In this case, clearance and volume of distribution determined after administration of a single dose have limited value (Wagner et al., 1981). Hence, to validate the pharmacokinetic analysis used for calculation of clearances and volumes of distribution, it was necessary to establish whether the hydroxylamine was reoxidized to the nitroxide *in vivo*. From the present observations in the dog, the oxidation of the hydroxylamine is unlikely to be of importance *in vivo* because the terminal half-lives of the nitroxides and their corresponding hydroxylamines are different. If reoxidation of the hydroxylamine occurs *in vivo* the concentrations of the hydroxylamine and the nitroxide are expected to decline in parallel when equilibrium between the two species is established. In Figure 3.1, the PCA concentration appears to decline to levels less than one percent of the hydroxylamine without evidence of parallelism in the two curves. The same observation was made for TES. This indicates that the hydroxylamines of PCA and TES are not readily reoxidized *in vivo*, i.e., the metabolism does not appear to be reversible within this time frame.

The renal clearances of the unchanged nitroxide and the corresponding hydroxylamine or the fraction of the dose excreted unchanged could not be calculated from the data obtained in these studies. The urinary nitroxide concentrations were only determined after oxidative treatment. This measurement is the sum of the nitroxide and the hydroxylamine. Determination

of the nitroxide concentration before oxidation was not meaningful because of the potential reduction of the nitroxide and autoxidation of the hydroxylamine in urine during collection. Such reduction and oxidation was observed in urine *in vitro* (see Results).

The extent of reduction to the hydroxylamine is of particular interest for the use of nitroxides as MRI contrast agents. The diamagnetic hydroxylamine does not have an unpaired electron and therefore does not produce proton relaxation enhancement. The higher clearance and shorter half-life of TES in the dog compared to those of PCA may indicate that TES is reduced more rapidly than PCA *in vivo*. This is supported by the stability studies because TES appeared to be reduced more rapidly than PCA in plasma and urine. Good MRI contrast enhancement of tissues such as kidney and brain have been obtained with TES at doses of 0.15 to 3.0 mmol/kg (Brasch et al., 1983a, 1983b). However, PCA, which is reduced more slowly than TES, may be effective as a contrast agent at a lower dose and give an effect that persist for a longer time period.

Chapter 4. Pharmacokinetics of TES and PCA in the Dog.

In this study, the pharmacokinetics of TES and PCA were examined in more detail in three beagles. Dose dependencies of the pharmacokinetic parameters were examined by administering intravenous doses of 0.1 and 2.5 mmol/kg. Doses within this range have been shown to produce contrast enhancement in MRI (Brasch et al., 1983a, 1983b; Ehman et al., 1985; Lamarque et al., 1986). More attention was given to PCA because of its higher stability *in vivo* (Chapter 3). In particular, the metabolic fate of the nitroxide moiety in PCA was examined. This was important to determine because the paramagnetic nitroxide moiety is responsible for the enhancement of image intensity by nitroxides in MRI.

Methods

Experimental Design of Pharmacokinetic Experiments

Three male beagles were given 0.1 and 2.5 mmol/kg of TES and PCA intravenously on separate occasions at least two weeks apart. Details of the experimental procedures are described in Chapter 2. TES and PCA were stored at 4 °C as aqueous solutions buffered to pH 7.4 with 67 mM phosphate at concentrations of 0.25 and 0.85 mM, respectively. The solutions were passed through a 0.2 μm filter (Acrodisc, Gelman Sciences, Inc.) prior to administration.

An intermittent infusion set was used for the injection of the nitroxides as described in Chapter 2 except for the high dose of TES which was infused (quadruple syringe infusion/withdrawal pump, Model 600-930, Harvard

Apparatus Co. Inc., Dover, MA) simultaneously into two different veins due to the large volume (110 to 150 mL) injected. The line was flushed with 10 mL of normal saline after injection.

Urine was collected as described in Chapter 2 except that, in most experiments, the urine was collected in only two separate fractions, 0 to 6 and 6 to 24 hours after injection. In two particular studies, urine was collected in three separate fractions: 0 to 2; 2 to 6; and 6 to 24 hours.

Analysis of Samples

Blood samples were always assayed by ESR spectroscopy for nitroxide concentrations within 5 minutes of their withdrawal as described in Chapter 2. A standard curve was prepared from spiked blood samples measured directly after their preparation. For the dose of 0.1 mmol/kg, blood was collected in plastic syringes and directly injected into the flat cell of the ESR spectrometer. For the dose of 2.5 mmol/kg, blood was collected in plastic syringes, immediately transferred to tubes (Vacutainer # 6428, Becton and Dickinson) containing potassium oxalate and sodium fluoride, and diluted 5 times with distilled water before measurement. In one particular experiment (PCA 2.5 mmol/kg), nitroxide concentrations were measured in both plasma and blood.

The blood samples were treated to oxidize the hydroxylamine present. After the last blood collection, 0.5 mL of each sample was mixed with 2 mL of methanol, vortexed for 10 sec and centrifuged at 1000 x *g* for 1 min. The methanolic supernatant fluid was transferred to a closed vial and kept 1 to 2 days before measurement. The hydroxylamine concentration was calculated from the difference between the nitroxide concentration measured after

oxidation (sum of nitroxide and hydroxylamine) and that measured before oxidation (immediately after collection).

Urine was oxidized by addition of 50 μL of 30 % hydrogen peroxide to 100 μL of urine. After 24 hours, the mixture was diluted 30 to 100 times with distilled water and analyzed for nitroxide concentration by ESR spectroscopy.

Protein Binding

Protein binding of the nitroxides in plasma was determined at 37 °C by ultrafiltration as described in Chapter 2.

Pharmacokinetic Analysis

Total body clearance (CL) was calculated as CL = Dose/AUC, where AUC is the area under the plasma-concentration-versus-time curve extrapolated to time infinity. The AUC, volume of distribution, elimination rate constant and corresponding half-life were estimated as described in Chapter 3.

The amount of nitroxide in urine $Ae(t_1\text{-}t_2)$, collected during a particular time interval and determined by ESR spectroscopy after oxidative treatment, is the sum of the nitroxide and the corresponding hydroxylamine. The amounts excreted as the nitroxide and the hydroxylamine, respectively, are products of their respective renal clearances and AUC values. This is described in the following equation:

$$Ae(t_1\text{-}t_2) = CL_R(Nit) * AUC(Nit,t_1\text{-}t_2) + CL_R(Hyd) * AUC(Hyd,t_1\text{-}t_2)$$

Where CL_R(Nit) and CL_R(Hyd) are the renal clearances of the nitroxide and the hydroxylamine, respectively, and AUC(Nit,t_1-t_2) and AUC(Hyd,t_1-t_2) are the AUC's of the nitroxide and the hydroxylamine, respectively. The amount recovered in urine (Ae(t_1-t_2)) and the AUC values for the nitroxide and the hydroxylamine can be determined but both renal clearances are unknown. In two particular studies, urine was collected in two fractions, 0 to 2 and 2 to 6 hours after administration of 0.1 mmol/kg of PCA. For these studies the equation above can be written for each collection period and two equations with two unknowns (the renal clearances) are obtained. The equations can then be simultaneously solved to estimate the renal clearances of the nitroxide and the hydroxylamine.

The metabolic clearances were calculated from the difference between total and renal clearances. The fraction of total elimination that occurs by urinary excretion of the unchanged nitroxide was calculated from the ratio of renal clearance and total clearance.

Study in a Dog Using Tritium-Labeled PCA

Tritium Labeling of PCA. PCA was randomly labeled with tritium by microwave discharge in the presence of tritium gas as described previously (Chiu and Peng, 1979). Briefly, about 1 mg of PCA in 0.05 mL of deuterated chloroform was dried onto a solid support and exposed to carrier-free tritium gas at low pressure (5 Torr) in an evacuated microwave cavity for 20 min. The labeled sample was extracted with concentrated ammonium hydroxide. After lyophilization of the solvent, the tritium-labeled PCA was purified by preparative thin-layer chromatography on silica gel (Analtech GF, 5 cm x 20 cm x 250 μm) with the eluent, chloroform:methanol:acetic acid (90:9:1). The PCA-band was

removed from the plate and eluted from the silica gel with chloroform:methanol (90:10). An analytical HPLC system, described in Chapter 2, was used for further purification and determination of the final product (specific activity approximately 7 x 10^{12} Bq/kg). The column effluent, monitored at 245 nm, was collected in 1-minute fractions for 15 minutes and assayed for radioactivity by liquid scintillation counting (Mark III liquid scintillation system, Model 6880, Searle Analytic Inc., Des Plaines, IL).

Dog Study. Unlabeled (1.3 mmol) and tritium-labeled PCA (1.11 x 10^6 Bq) were dissolved in 7 mL of phosphate buffer (67 mM, pH 7.4), filtered through a 0.2 μm filter (Acrodisc, Gelman Sciences Inc.), and injected intravenously over 1 minute to a beagle weighing 13 kg (Dog #1). After injection, the catheter was flushed with 5 mL of normal saline. No blood samples were collected but the experimental design of the dog experiment was otherwise the same as that described in Chapter 2. Urine was collected via a bladder catheter for six hours. During this time, the bladder was flushed hourly with 10 mL of normal saline. At six hours, the bladder was flushed with 20 mL of normal saline and the urinary and venous catheters were removed. The dog was transferred to a metabolic cage in which the urine was subsequently collected in three additional fractions: 6 to 24, 24 to 48 and 48 to 72 hours. The volume of each fraction was recorded and samples were saved for analysis.

Analyses of Samples . The injection solution and urine samples were assayed for radioactivity by liquid scintillation counting. The urine collected in the first six hours ("fraction 1") was also assayed with ESR spectroscopy for nitroxide concentration as described in Chapter 2. This urine sample was diluted 10-fold before measurement and potassium ferricyanide (2 mM final

concentration) was added to oxidize any hydroxylamine present. The injection solution was used as a reference; the amount of PCA in urine was expressed as percent of injected dose.

Samples of the injection solution and urine fraction 1 were also injected into the HPLC system, described in Chapter 2, to separate potential metabolites. The urine (fraction 1) was diluted by an equal volume of mobile phase, containing potassium ferricyanide (4 mM); 200 µL of this mixture was injected onto the column. The injection solution, diluted in blank dog urine (250-fold dilution), was further diluted with the mobile phase and assayed as was the urine sample. The 1-minute fractions of the column eluent, collected for 15 minutes, were assayed for radioactivity by liquid scintillation counting.

Results

The pharmacokinetic parameters calculated for TES and PCA are presented in Tables 4.1 and 4.2, respectively. A typical example of a plot of nitroxide concentration in blood versus time is shown in Figure 4.1 for PCA at two different doses in the same dog. The blood concentrations of PCA and TES for all experiments are listed in Tables A4.1 to A4.12 in the Appendix.

The blood concentrations of PCA and the ratio of the blood and plasma concentrations obtained during the same experiment are reported in Table 4.3.

PCA did not appear to be bound to plasma proteins. The unbound fractions in plasma, averages of duplicate determinations at 0.1 mM and 1.0 mM of PCA, were 1.0 at both concentrations.

The average urinary recoveries at 24 hours for TES and PCA after oxidative treatment (nitroxide plus hydroxylamine) are presented in Table 4.4. The percent of the dose excreted in urine from 6 to 24 hours after administration

Table 4.1 *Pharmacokinetic Parameters of TES in Dogs.*

	Clearance [b] (mL/min-kg)	Volume of Distribution [c] (L/kg)	Half-life [d] (min)
		0.1 mmol/kg [a]	
Dog #1	27.1	0.33	8.5
Dog #2	26.3	0.37	9.8
Dog #3	29.9	0.26	6.1
Mean ± S. D. [e]	27.8 ± 1.9	0.32 ± 0.06	8.1 ± 1.9
		2.5 mmol/kg [a]	
Dog #1	12.2	0.32	18.1
Dog #2	11.2	0.30	16.2
Dog #3	12.3	--- [e]	---
Mean ± S. D. [f]	11.9 ± 0.6	---	---

[a] Dose administered intravenously

[b] Determined from Dose/AUC.

[c] Volume of distribution calculated from CL/k.

[d] Calculated as 0.693/k, where k was obtained from the least square regression of the natural logarithm of the concentration versus time, after 10 min.

[e] The half-life continued to decrease with time in Dog #3, therefore, no values for this parameter or the volume of distribution were estimated.

[f] Mean ± standard deviation of the parameters of the three dogs.

Table 4.2 *Pharmacokinetic Parameters of PCA in Dogs.*

	Clearance [b] (mL/min-kg)	Volume of Distribution [c] (L/kg)	Half-life [d] (min)
		0.1 mmol/kg [a]	
Dog #1	12.4	0.41	22.8
Dog #2	13.5	0.40	20.6
Dog #3	13.6	0.46	23.4
Mean ± S. D. [e]	13.2 ± 0.7	0.42 ± 0.03	22.3 ± 1.5
		2.5 mmol/kg [a]	
Dog #1	10.0	0.47	32.9
Dog #2	11.5	0.46	28.6
Dog #3	12.2	0.49	27.6
Mean ± S. D. [e]	11.2 ± 1.2	0.48 ± 0.01	29.7 ± 2.9

[a] Dose administered intravenously

[b] Determined from Dose/AUC.

[c] Volume of distribution calculated from CL/k.

[d] Calculated as 0.693/k, where k was obtained from the least square regression of the natural logarithm of the concentration versus time, after 10 min.

[e] Mean ± standard deviation of the parameters of the three dogs.

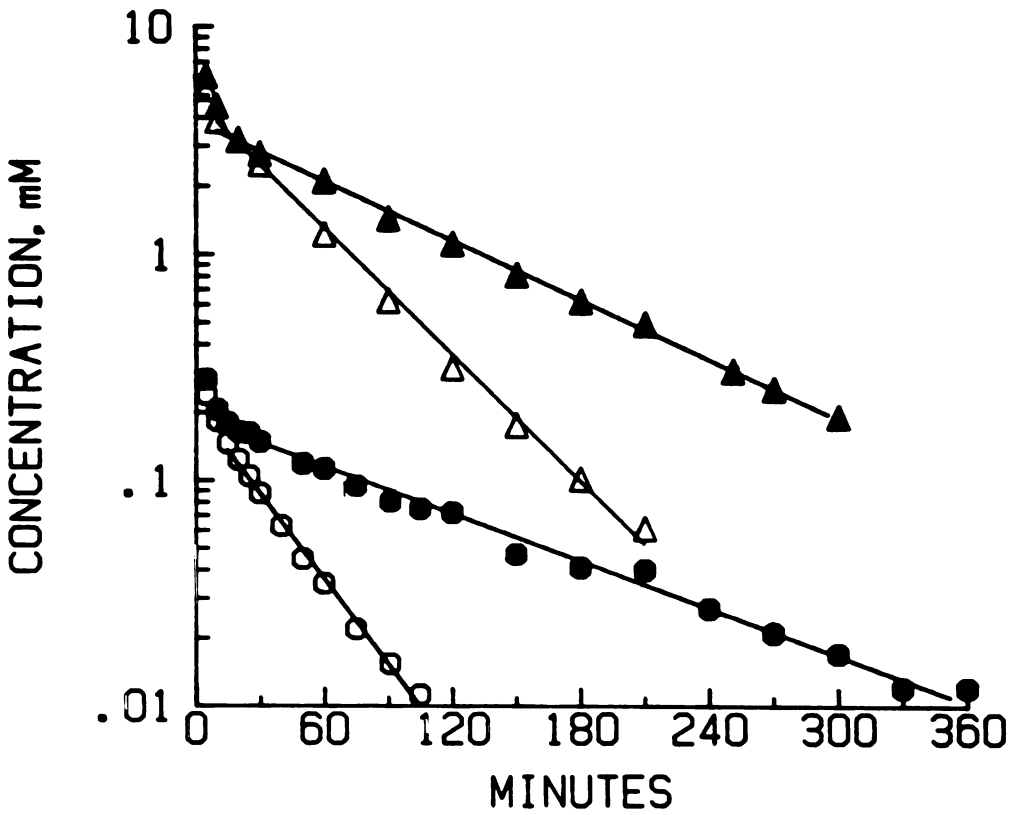


Figure 4.1 Blood PCA concentrations with time in Dog #1. The concentration was measured as soon as possible after sampling (open symbols) and after oxidative treatment (closed symbols). Circles represent the 0.1 mmol/kg dose and triangles the 2.5 mmol/kg dose. Linear least square regression of the two data sets beginning at 10 minutes are shown (solid lines).

Table 4.3 *PCA Blood Concentration and Blood to Plasma Concentration Ratio.*

Sampling time [a] (min)	Blood Concentration (mM)	B/P [b]
5	6.09	0.89
10	4.49	0.89
20	3.19	0.85
30	2.50	0.88
60	1.22	0.84
90	0.62	0.89
120	0.31	0.89
150	0.17	0.77
180	0.10	1.00

[a] Time after injection of 2.5 mmol/kg of PCA a Dog #1.

[b] Ratio of PCA concentrations in blood and plasma.

Table 4.4 *Urinary Recovery of PCA and TES.* [a]

	PCA		TES	
Dose (mmol/kg) [b]	0.1	2.5	0.1	2.5
Mean ± S. D. [c]	83.4 ± 8.6	89.8 ± 5.5	86.9 ± 11.7	88.1 ± 16.4

[a] Average values determined by ESR spectroscopy following oxidative treatment of urine collected for 24 hours after administration.

[b] Dose administered intravenously.

[c] Mean recovery ± standard deviation in three dogs.

was less than 10 % for both TES and PCA. Urinary recoveries for the individual experiments are given in Tables A4.1 to A4.12 in the Appendix.

The terminal half-lives of the decay of PCA concentrations measured after oxidative treatment were 96.5 ± 13.0 min and 74.3 ± 5.1 min following the low and high doses, respectively. The estimates of the metabolic and renal clearances of PCA after administration of the 0.1 mmol/kg dose to two different dogs, are presented in Table 4.5. The excretion of unchanged PCA, as a percent of the adminstered dose, was estimated to be 42 % in Dog #1 and 43 % in Dog #3. The renal clearance of the hydroxylamine was found to be equal to 3.4 mL/min-kg in Dog #1 and 4.0 mL/min-kg in Dog #3. Assuming that urinary excretion is the only route of elimination for the hydroxylamine of PCA, the volume of distribution of the hydroxylamine can be estimated as the ratio of its renal clearance and elimination rate constant. The volumes of distribution estimated in this manner were 0.42 and 0.53 L/kg in Dog #1 and Dog #3, respectively.

Table 4.5 *Renal and Metabolic Clearances of PCA.* [a]

	Dog #1	Dog #3
Renal clearance (mL/min-kg)	5.2	5.8
Metabolic clearance (mL/min-kg)	7.2	7.8

[a] Calculated in two dogs after intravenous administration of PCA at a dose of 0.1 mmol/kg.

The purity of the tritium-labeled PCA used for injection to Dog #1 was determined with HPLC. Greater than 98 % of the radioactivity eluted with the same retention time as PCA.

The radioactivity recovered in urine, collected in the first six hours after administration, was 90.3 % of the injected dose. Another 3.2 % was collected within the 6-24 hour interval and 0.3 % was collected between 24 and 48 hours. The urine of the third day had a background level of radioactivity. The total urinary recovery of radioactivity was 93.8 %.

Eighty-eight per cent of the dose was recovered in the first 6-hour urine sample, when assayed by ESR spectroscopy. Components in the urine (fraction 1) were separated by HPLC and the resulting chromatogram had one major radioactive peak (93.5 % of the total radioactivity injected into the HPLC system) with the same retention time as PCA (Figure 4.2). The remaining radioactivity coeluted with the solvent front. The injection solution diluted in blank urine gave a similar HPLC chromatogram with 87.1 % coeluting with PCA and the remainder coeluting with the solvent front (Figure 4.2).

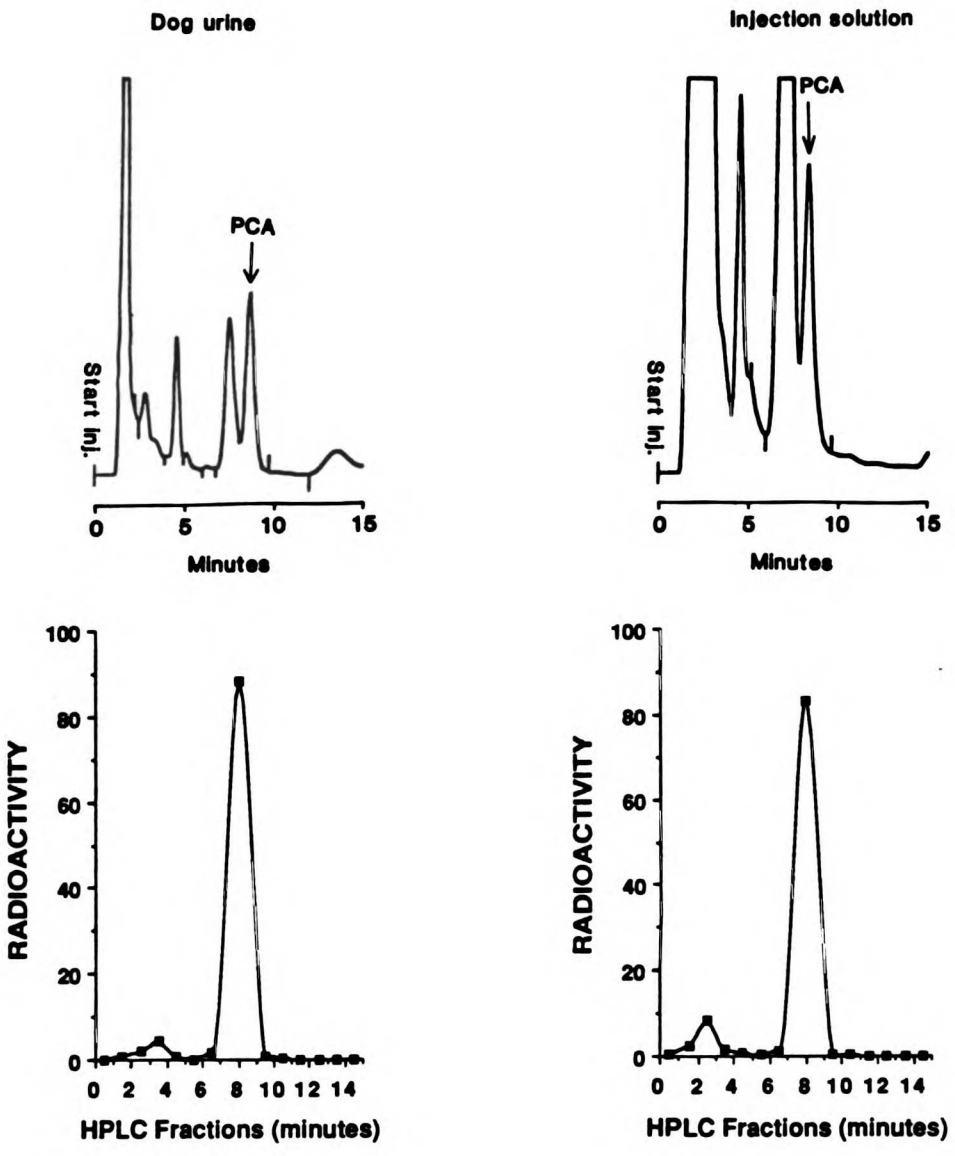


Figure 4.2 The HPLC chromatograms of samples of dog urine (left panels) and a injection solution (right panels) that was used as a reference. The top panels show the ultraviolet detection (245 nm). The bottom panels show the amount of radioactivity, as a percent of the total radioactivity injected into the HPLC, in each 1-minute fraction. The dog urine sample was obtained during the first six hours after administration of the PCA. The reference solution was a sample of the solution that was administrated to Dog #1. This sample was diluted in dog urine and treated as a sample of dog urine.

Discussion

ESR spectroscopy was used as the assay method for determination of nitroxide concentrations in blood and urine. The selectivity of this method was established in the preliminary study in a mongrel dog (Chapter 3). The spontaneous oxidation of the hydroxylamine to the nitroxide in plasma and urine samples was noted previously. To reduce the risk of such oxidation, centrifugation was avoided by measuring nitroxide concentration in blood rather than in plasma and the time between sampling and ESR measurement was limited as much as possible.

The pharmacokinetic parameters of PCA showed only minor difference with dose (Table 4.2). The average value of clearance decreased 15 % while the average volume of distribution and half-life increased 13 % and 33 %, respectively, on increasing the dose. These changes are negligible compared to the 25-fold increase in dose administered. With TES, clearance decreased more than 2-fold at the higher dose (Table 4.1). The average values for volume of distribution and half-life, calculated from Dog #1 and Dog #2 after administration of TES at the higher dose, were 0.31 L/kg and 17.1 min. The half-life decreased with time in Dog #3, therefore no values for this parameter or the volume of distribution were obtained. The clearance was estimated assuming that the AUC beyond the last sample was negligible. The experiment was not repeated because of the expense of the larger dose. No model for the dose dependency of the pharmacokinetic parameters of TES was proposed or tested.

Bioreduction *in vivo* appeared to be slower for PCA than for TES. This was supported by the higher stability in plasma and urine, and lower clearance of PCA compared to TES (Chapter 3). The greater stability towards reduction

favors the use of PCA, as an MRI contrast agent, because the reduction results in formation of the diamagnetic hydroxylamine that lacks contrast enhancing activity. Consequently, PCA was subsequently given more attention.

PCA showed no appreciable binding to plasma proteins. This finding is consistent with the lack of broadening of the peaks of the ESR spectrum of PCA in plasma. Binding to macromolecules decreases the mobility of the nitroxide in solution which causes broadening of the peaks or even more dramatic changes in the spectra (Freed, 1976). Such spectral changes have, for example, been suggested as a method to estimate the extent of plasma protein binding of nitroxide-labeled drugs (Chou et al., 1981).

An experiment was conducted in which both blood and plasma concentrations of PCA were measured after administration of 2.5 mmol/kg (Table 4.3). The observation of a practically constant ratio (0.88 ± 0.06) of blood and plasma PCA concentrations, even at early times after injection, suggests that PCA penetrates rapidly into red blood cells. Because the ratio is close to one, the pharmacokinetic parameters calculated from blood and plasma measurements are similar. Thus, the pharmacokinetic parameters of PCA obtained in the beagles for blood can be compared to those obtained previously in a mongrel dog for plasma (Chapter 3). The clearance in the beagles was about two-fold higher than that observed in the mongrel dog while the volume of distribution was about the same in both the beagles and the mongrel dog.

Analysis of blood nitroxide concentrations after oxidative treatment (nitroxide plus hydroxylamine) were conducted with PCA to assess the pharmacokinetics of the metabolite, the corresponding hydroxylamine. Because PCA was eliminated faster than the hydroxylamine, the nitroxide concentrations measured at later times (about 2 hours after injection)

represented only the hydroxylamine (Figure 4.1). Therefore, the terminal half-lives calculated from these concentrations were half-lives of the hydroxylamine. The average values of the PCA-hydroxylamine half-life, 96.5 ± 13.0 and 74.3 ± 5.1 min for the low and high doses, respectively, are in good agreement with that (85 min) previously observed in plasma for the 0.55 mmol/kg dose in the mongrel dog (Chapter 3).

The relative importance of the reduction process in the overall elimination of PCA was a question of great interest in our search for nitroxides more resistant to reduction *in vivo*. Because PCA is reduced in urine, it was only possible to determine the amount of PCA excreted as a sum of the unchanged (nitroxide) and the reduced form (hydroxylamine). Consequently, renal clearance could not be determined directly. However, by collecting urine in two different fractions and measuring the area under the blood-concentration-time curve for both the nitroxide and its hydroxylamine in each collection period, the renal clearance of both the unchanged and reduced form could be estimated as described in Methods (see Pharmacokinetic analysis). From these estimates of the renal clearance of PCA in two dogs at the low dose (Table 4.5), it appears that approximately 40 % of the administered dose is excreted unchanged in urine. The remaining 60 % of the dose (metabolic clearance) is assumed to be reduced to the hydroxylamine which then is excreted in urine. The renal clearances of PCA and its hydroxylamine are similar and close to the estimated clearance by glomerular filtration in the dog (4 ml/min-kg) (Ladd et al., 1956).

The large urinary recoveries (84 to 90 %) for TES and PCA indicated that almost all of the administered dose was excreted in urine as either unchanged nitroxide or the hydroxylamine (Table 4.4). In the previous study in a mongrel dog, TES and PCA were found to be extensively reduced *in vivo* to their

corresponding hydroxylamines. Using HPLC for separation and ultraviolet spectroscopy for detection, no other metabolites containing the nitroxide moiety were observed (Chapter 3). Thus, the ESR measurements were specific for the nitroxide administered. In this study, tritium-labeled PCA was synthesized to examine if the remaining 10 to 15 % of the dose missing in the urine represented metabolites not detectable by ESR or ultraviolet spectroscopy. In particular, the formation of the corresponding amine, a two-electron reduction product of the hydroxylamine, and cleavage of the ring structure were anticipated. The products of such reduction and ring-cleavage would not be detected with either the ESR or HPLC assays.

The total recovery of radioactivity in urine (93.8 %) after administration of 0.1 mmol/kg (specific activity 4.58×10^9 Bq/kg) was similar to the urinary recovery (83.4 ± 8.6 %) observed in the dogs after administration of only unlabeled PCA. Again, most of the dose was recovered in urine collected in the first six hours after administration. The urinary recovery of this urine fraction was about 90 percent of the dose. Virtually the same recovery was determined for this urine sample when it was analyzed for radioactivity with liquid scintillation counting, 90.3 %, and for nitroxide concentration with ESR spectroscopy, 88%. The HPLC analysis of this urine sample showed that most of the radioactivity coeluted with PCA. From these observations it is concluded that PCA was excreted unchanged or as its corresponding hydroxylamine. Furthermore, the oxidative treatments used appeared to completely oxidize the hydroxylamine.

The corresponding amine of PCA appeared not to be formed in the dog. The treatment with hydrogen peroxide or potassium ferricyanide, used for oxidative treatment of the urine samples, are known to cause oxidation of the hydroxylamine (Rozantsev and Sholle, 1971a). Oxidation of the corresponding

amine, however, requires stronger oxidizing conditions such as long periods of treatment (days) with hydrogen peroxide in the presence of sodium tungstate (Rozantsev and Sholle, 1971a). Indeed, on treating 2,2,5,5-tetramethyl-3-pyrrolidine-carboxamide (0.5 mM), the corresponding amine of PCA-amide, with potassium ferricyanide at room temperature, less than 0.1 % was converted to the nitroxide in 24 hours. The conditions for the oxidative treatment was the same as those used for the samples obtained from the dog experiment (see Methods).

There was no evidence of formation of polar metabolites, such as conjugates of the carboxylic acid group, of either PCA or its hydroxylamine. Such metabolites are expected to be excreted in urine. The small fraction of the dose not recovered could be explained by: incomplete recovery of urine; elimination via nonrenal pathways (e.g. feces); or retention of PCA in the animal.

The lack of biotransformation of the nitroxide moiety in PCA, except for reduction to the hydroxylamine, is expected to apply to other nitroxide derivatives. Biotransformation at other locations on the molecule may occur, of course, although such was not observed for the carboxylic acid group in PCA in the dog.

Chapter 5. Pharmacokinetics in the Rat and the Dog of a Nonionic Nitroxide MRI Contrast Agent.

In this study the pharmacokinetics and metabolic fate of a new nonionic nitroxide derivative are examined in the dog and the rat. Predictions of the pharmacokinetics in humans was made from the data obtained in both small (rat) and large (dog) animal species.

This nitroxide derivative was given the code name TAP, referring to the trihydroxyl-amide-pyrrolidine structure (2,2,5,5-tetramethyl pyrrolidine-1-oxyl-3-carbonic acid-(2,3-dihydroxy-1-hydroxymethyl)-amide) (see Chapter 2 for structure). TAP was chosen because this derivative was expected to be better tolerated and to be more stable *in vivo* than the previously-evaluated nitroxide derivatives, PCA and TES (Chapters 3 and 4).

Toxicity is an important consideration for the use of nitroxides as MRI contrast agents which has been evaluated by our collaborators in the Contrast Media Laboratory. PCA and TES, nitroxide derivatives with side-chains that are ionizable at physiologic pH (carboxylic acid groups), were shown to be well tolerated with LD_{50} values of approximately 15 mmol/kg in the rat (Afzal et al., 1984). The polar side chain confers water solubility that facilitates intravenous administration of doses required for contrast enhancement (0.15 to 2.0 mmol/kg) (Brasch et al., 1983a, 1983b; Ehman et al., 1985; Lamarque et al., 1986). Nonionic iodinated compounds for x-ray contrast enhancement have been found to be less toxic than ionizable ones (Speck et al., 1983; Higgins, 1984). The toxicity is believed to be related to the osmotic load which is lower for nonionic compounds. Multiple nonionic hydroxyl groups were therefore selected over groups ionized at physiologic pH to increase water solubility. The

pyrrolidine nitroxide derivative, PCA, was shown to be reduced more slowly than the piperidine derivative, TES, in the dog (Chapters 3 and 4). A pyrrolidine derivative was therefore chosen over the less stable piperidine derivatives. TAP has been shown in preliminary studies to enhance magnetic resonance images of renal tissues and sterile abcesses in the rat (Grodd et al., 1985; Paajanen et al., 1985). Urographic contrast enhancement of TAP was observed at a lower dose and appeared to persist longer than for TES and PCA (Grodd et al., 1985).

Materials and Methods

The pyrrolidine nitroxide derivative, TAP, obtained from Schering, Berlin (see Chapter 2 for structure), was used in the pharmacokinetic studies in the rat and the dog. The nitroxide was kept at 4 °C in dry form or in phosphate buffer (67 mM, pH 7.4).

Experimental Design

Dog experiments : Two male beagles (Marshall Research Animals, North Rose, NY), weighing 11.5 and 14.5 kg were each given low and high (0.1 and 2.5 mmol/kg, respectively) intravenous doses of TAP, on separate occasions at least 2 weeks apart. A 0.8 M solution of TAP (in 67 mM phosphate buffer, pH 7.4), first filtered through a 0.2 μm filter (Acrodisc, Gelman Sciences), was injected over 1 min. The injection volumes were about 2 mL and 50 mL for the low and high doses, respectively. Details of the experimental procedures are described in Chapter 2.

Rat experiments : The rats received 0.1 and 2.5 mmol/kg intravenous doses of TAP. Two protocols were used: to determine pharmacokinetic parameters in the rat from blood nitroxide concentrations; and to determine urinary recovery of drug and metabolites. Details of the experimental procedures are described in Chapter 2.

Analysis of Samples

First measurement of TAP concentration in blood : The blood samples were: collected in disposable syringes; transferred to tubes (Vacutainer #6428, Becton and Dickinson) containing potassium oxalate and sodium fluoride; and then analyzed directly (within 5 min of collection) by ESR spectroscopy as described in Chapter 2. Except for the low-dose experiments in the dogs, the blood was diluted in distilled water before measurement.

Second measurement of TAP concentration after oxidative treatment : After the initial measurement, the blood samples were treated to oxidize the hydroxylamine present and assayed again for nitroxide concentration with ESR spectroscopy. Oxidation was performed by two methods as follows. Method 1, used for dog blood samples: Two mL of methanol were added to 0.5 mL of each sample to precipitate proteins and facilitate autoxidation by air. To ensure complete oxidation, the oxidant m-chloroperoxybenzoic acid was added (final concentration 20 mM) to the methanol supernatant fluid which was left for 24 hours before measurement. Method 2, used for rat blood samples: The oxidant cupric sulfate was added (final concentration 2 mM) about 5 to 10 min before measurement. The two methods of oxidation were found to give similar results for selected dog blood samples and were therefore assumed to be equivalent in

terms of oxidation of the hydroxylamine. The latter method using cupric sulfate was faster and thus preferred. The concentration of the hydroxylamine was calculated from the difference between the second and first measurements.

Analysis of urine samples : Urine samples were analyzed with ESR spectroscopy after oxidative treatment. This measurement is a sum of the nitroxide and the hydroxylamine present in the sample. Two methods, found to give similar results for selected samples of dog urine, were used for oxidative treatment of the urine samples. For the dog urine samples hydrogen peroxide was used as described in Chapter 4. The rat urine samples were diluted ten to 1000-fold in distilled water, depending upon the particular sample, to an expected final concentration of 0.01 to 0.5 mM of TAP. The oxidant potassium ferricyanide was added to a final concentration of 2 mM before measurement.

Additionally, the urine samples were assayed with HPLC to separate potential metabolites, particularly conjugates of the hydroxyl groups of the side chain, from TAP. The samples were prepared in the same way for both the ESR and HPLC assays.

Determination of protein binding

Protein binding of TAP in rat and dog plasma was determined at room temperature by ultrafiltration as described in Chapter 2.

Pharmacokinetic analysis

An open two-compartment model was fitted to the blood-concentration-versus-time data (weighting 1/Y) using nonlinear regression computer program (Huang

et al., 1984). The clearance, half-life of the terminal elimination phase and volumes of distribution were obtained by this computer program. Three parameters were determined that describe drug distribution. These parameters are proportionality constants that relate the concentration to the amount of drug in the body; directly after injection, Vc; during the terminal elimination phase, Varea; and at steady-state during constant rate infusion, Vss (Gibaldi and Perrier, 1982). In the dogs, the renal and non-renal clearances of TAP and the renal clearance of the hydroxylamine were estimated as described in Chapter 4.

Results

The time courses of the concentrations of TAP and its corresponding hydroxylamine in blood for representative experiments in the dog and in the rat are shown in Figures 5.1 and 5.2, respectively. The pharmacokinetic parameters of TAP, estimated using the model, are presented in Table 5.1. TAP concentrations in blood measured in each individual experiment are listed in the Appendix (Tables A5.1 to A5.4 and Tables A5.5 to A5.10 for the dog and rat experiments, respectively).

Urinary recoveries of the sum of TAP and its hydroxylamine are shown for both the dog and the rat in Table 5.2. The urinary recoveries for each collection period are listed in the Appendix for the dog (Tables A5.1 to A5.4) and rat (Table A5.11) experiments. The HPLC assay of rat and dog urine samples revealed no peaks other than that of TAP and those observed in blank samples. Concentrations in rat and dog urine determined by HPLC correlated well with the concentrations determined by ESR spectroscopy (see Table A5.12 in the Appendix). Linear correlation of the concentrations obtained by the two

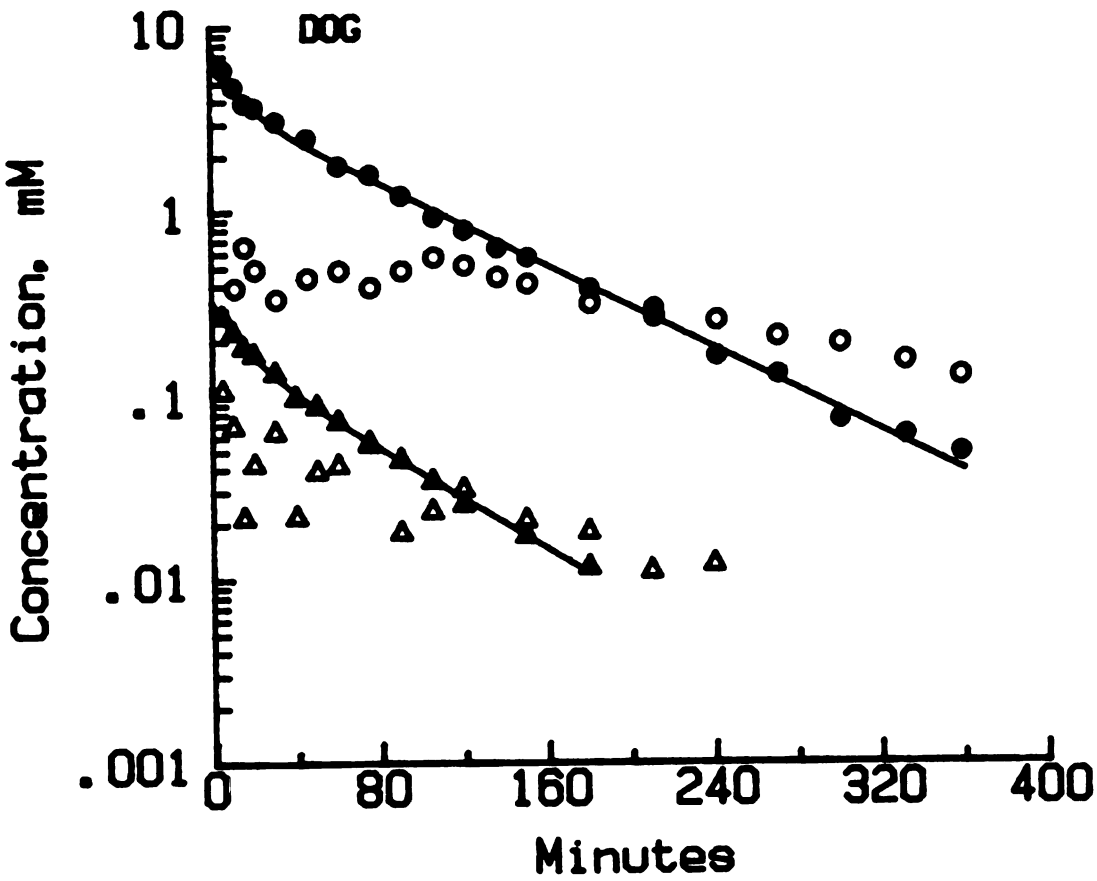


Figure 5.1 Blood concentrations of TAP (closed symbols) and the hydroxylamine metabolite (open symbols),formed by reduction, after intravenous administration of 0.1 mmol/kg (triangles) and 2.5 mmol/kg (circles) doses to Dog #1. The solid lines show the fit of the two-compartment model to the data. TAP concentrations for both doses could be essentially superimposed when corrected for dose, suggesting that the pharmacokinetic parameters of TAP are constant in this dose range.

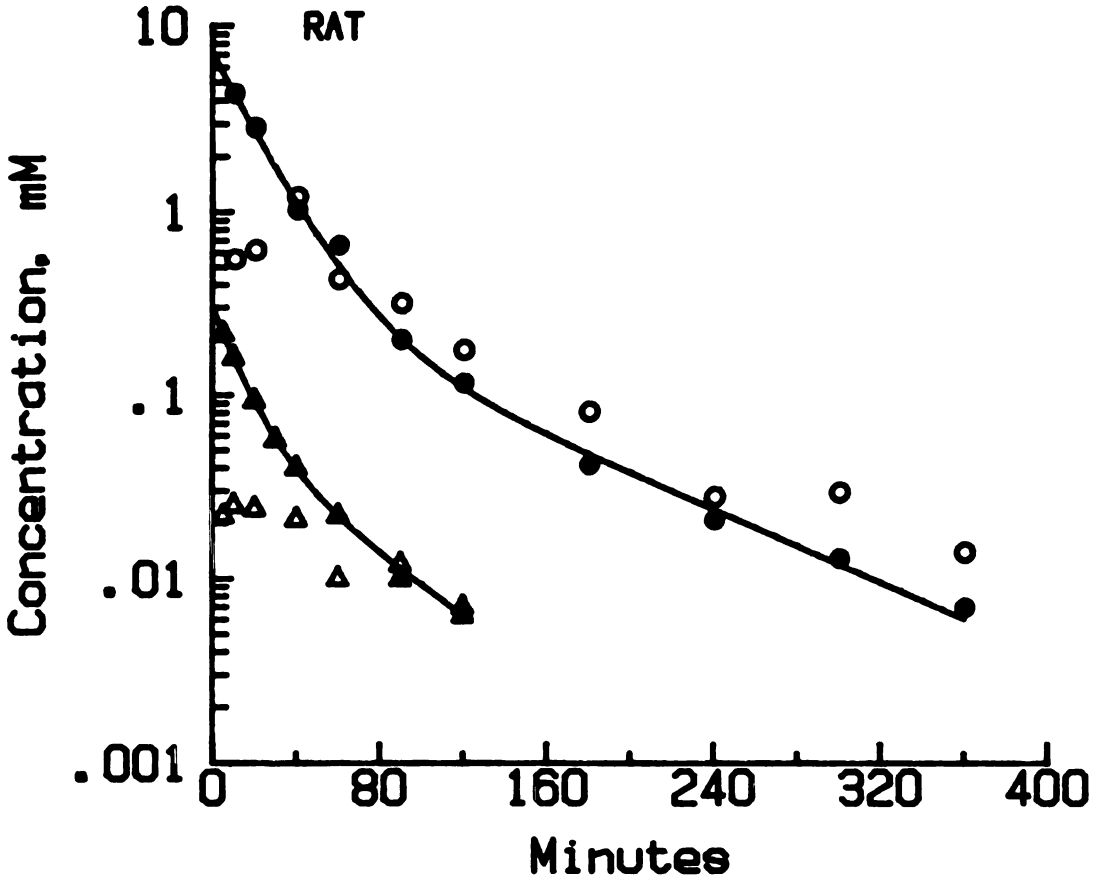


Figure 5.2 Blood concentrations of TAP (closed symbols) and the hydroxylamine (open symbols), formed by one-electron reduction of TAP, after intravenous administration of 0.1 mmol/kg (triangles) and 2.5 mmol/kg (circles) doses to rats for two representative experiments. The solid lines show the fit of the two-compartment model to the data. As for the dog experiment (Figure 5.1), TAP concentrations for both doses could be essentially superimposed when corrected for dose, suggesting that the pharmacokinetic parameters of TAP are constant in this dose range.

Table 5.1 *Pharmacokinetic Parameters of TAP.*

	Dose (mmol/kg)	Clearance (mL/min-kg)	Vc (L/kg)	Vss (L/kg)	Varea (L/kg)	Half-life [a] (min)
Dog #1	0.1	7.4	0.30	0.42	0.47	44.0
	2.5	7.1	0.33	0.50	0.55	53.6
Dog #2	0.1	6.3	0.31	0.35	0.38	41.9
	2.5	6.0	0.26	0.40	0.44	50.5
Rat [b]	0.1	15.4 ± 2.0	0.33 ± 0.01	0.49 ± 0.04	0.67 ± 0.14	30.2 ± 6.1
	2.5	15.3 ± 1.4	0.42 ± 0.13	0.60 ± 0.13	1.11 ± 0.21	50.4 ± 9.1

[a] The elimination half-life is estimated from the second phase of the biexponential decline.

[b] The parameters for the rat are average values ± S.D. for three experiments.

Table 5.2 *Urinary Recovery of TAP.* [a]

Collection period (hours)	Dog #1		Dog #2		Rat [b]	
	Low [c]	High	Low	High	Low	High
0 - 6	78.8	89.0	82.2	87.2	88.4 ± 4.3	91.0 ± 1.6
6 - 24	5.6	4.6	0.3	6.1	3.2 ± 0.2	7.1 ± 3.0
Totals	84.4	93.6	82.5	93.3	91.7 ± 4.3	97.6 ± 1.9

[a] The urinary recoveries are expressed as percentages of the dose administered.

[b] Mean ± standard deviation for three experiments.

[c] Urinary recovery after administration of 0.1 mmol/kg ("Low") and 2.5 mmol/kg ("High") doses.

assays gave correlation coefficients of 0.999 and 1.000 for the rat and dog samples, respectively. The slopes of the regressions (95 % confidence intervals were 0.987 to 1.043 and 0.931 to 0.949, respectively) were close to one, indicating an almost perfect relationship, and the intercepts (95 % confidence intervals were -0.498 to 0.486 and -0.553 to 0.715, respectively) were not different from zero.

The renal clearances in Dog #1 and Dog #2, respectively, were estimated to be 3.5 and 3.8 mL/min-kg in the low dose experiments and 6.3 and 4.6 mL/min-kg in the high dose experiments. The fractions of TAP excreted unchanged were estimated to be 0.47 and 0.60, respectively, after the low dose, and 0.89 and 0.77, respectively, after the high dose.

The fraction unbound of TAP in plasma was 0.96 ± 0.06 and 0.98 ± 0.06 in the dog and 1.01 ± 0.03 and1.03 ± 0.02 in the rat at 0.1 mM and 1.0 mM concentrations, respectively.

Discussion

In both the rat and the dog, biexponential declines of the blood concentrations of TAP were observed. The central distribution volume (normalized for body weight, L/kg), which determines the initial TAP concentrations, is similar for both animal species and independent of dose. The clearance (mL/min-kg) was about two-fold higher in rats than in dogs. No appreciable differences were observed in the clearance values determined after the low and high doses in either species.

The terminal half-lives were longer and the volumes of distribution (L/kg, relating the concentration to the amount in the body during the elimination phase after distribution is complete) were larger after the high dose compared

to that after the low dose in both animal species. However, this apparent dose dependency is probably only an artifact. The terminal half-lives are probably underestimated for the low-dose experiment because the lower detection limit of the assay is reached before the terminal elimination phase is established. This was more noticeable in the rat in which the initial distribution phase lasted longer and distribution was more extensive than in the dog (Figs. 5.1 and 5.2). The calculated volumes of distribution in the dog, however, were similar indicating that the pharmacokinetics in the dog could have been relatively well-described by a one-compartment model. In both animals, the time courses of TAP concentration for the low and high-dose experiments are essentially identical when the concentrations are normalized to account for the 25-fold difference in dose, a range which includes doses used to obtain contrast enhancement in animals (Brasch et al., 1983a, 1983b; Ehman et al., 1985; Lamarque et al., 1986). These observations appear to support no dose-dependence in either elimination or distribution of TAP in this dose range. These results are in contrast to dose dependencies observed previously in the rat for piperidine nitroxide derivatives (Griffeth et al., 1984). In these previous studies, both clearance and volume of distribution were reported to be lower after doses of 1.75 mmol/kg than after doses of 0.01 mmol/kg.

The blood concentration of TAP was measured by ESR spectroscopy within 5 minutes of collection to avoid interference from the hydroxylamine. Autoxidation of the hydroxylamine to the nitroxide occurs *in vitro* in the presence of oxygen (Chapters 3 and 4). Consequently, the TAP concentrations in blood may have been overestimated. At later times, when the concentration of the hydroxylamine represents a large percentage of the total blood concentration (TAP and hydroxylamine), the autoxidation is of greater concern

because oxidation of even a small fraction of the hydroxylamine present can cause an important overestimation of the TAP concentration.

Maximum concentrations of the hydroxylamine were reached by the time of the first measurement. Variability in the hydroxylamine concentration was large because these values were determined as the difference between the measurements after (TAP + hydroxylamine) and before (TAP) oxidation. This was most apparent during the first hour after administration of TAP when the differences were small compared to the absolute values of the two concentration measurements.

As mentioned above, the hydroxylamine is relatively easily reoxidized *in vitro* and this may also occur *in vivo*. Such reoxidation would be expected to produce parallel decline of TAP and the hydroxylamine on semilogarithmic plots. In the dog, the hydroxylamine appears to decline more slowly than TAP. This observation and the rapid achievement of peak concentrations of the hydroxylamine indicate that oxidation *in vivo*, if it does occur within the six-hour period, is negligible. However, in the rat the decline of the hydroxylamine more closely parallels that of TAP. Definite determination of the contribution of reversible bioreduction, presumably by administration of the hydroxylamine, was not carried out because of the rapid autoxidation of the hydroxylamine in solution. Reoxidation *in vivo* could not be excluded based on the results in the dog and the rat for TAP. However, in the previous studies in the dog (Chapters 3 and 4), nonparallel declines were observed for both TES and PCA even when the concentrations of the corresponding hydroxylamines were one to two orders of magnitude higher than those of the corresponding nitroxide. In the pharmacokinetic analysis, reversible metabolism was assumed to be negligible.

Urinary recoveries within 24 hours in dogs and rats ranged from 83 to 98 percent of the dose. The ESR assay, used for determination of the urinary

recovery, selectively detects the nitroxide moiety. The recoveries include nitroxides and compounds that are oxidized to form nitroxides. Similar high urinary recoveries were observed for TES and PCA in the dog (Chapters 3 and 4). In contrast to the results for these derivatives, a previous pharmacokinetic study in the rat of several piperidine nitroxide derivatives reported smaller urinary recoveries (Griffeth et al., 1984). Furthermore, the urinary recoveries reported were lower after administration of 0.01 mmol/kg than after administration of 1.75 mmol/kg (2 to 6 % and 20 to 60 %, respectively).

The small percentage of the dose (about 5 - 10 %) not accounted for as TAP or its hydroxylamine is most likely due to incomplete collection of urine or incomplete oxidation of the hydroxylamine. In addition, TAP or its metabolites could be excreted via other routes, e.g., biliary excretion, or remain in the animal after 24 hours. Furthermore, the small fraction unaccounted for may be due to formation of metabolites that escape detection by the assay methods used. For example, the oxidative treatments used for the urine and blood samples are known to cause formation of the nitroxide from hydroxylamine but oxidation of the corresponding amine, potentially formed by a two-electron reduction of the hydroxylamine, requires stronger oxidizing conditions with a catalyst present (see Discussion in Chapter 4). Cleavage of the ring structure could also result in metabolites that would not be oxidized to the nitroxide form and therefore not be detected by either the ESR or HPLC assays. However, such metabolites were not observed for PCA (Chapter 4).

The ESR assay is a sensitive and selective method for detection of nitroxides but ESR spectra of different nitroxide derivatives often overlap. As discussed for PCA and TES in Chapter 3, potential metabolites of TAP, formed by metabolic transformation of the side chain, with the nitroxide moiety intact or reduced to the hydroxylamine would be measured together with TAP in the ESR

assay. Thus, the urinary recoveries were also determined by an HPLC assay. In this assay TAP may be separated from potential metabolites such as glucuronides or other conjugates. In the HPLC chromatograms of rat and dog urine, no peaks other than those corresponding to unchanged TAP and endogenous compounds were observed. Furthermore, the concentrations of TAP in urine determined by HPLC were essentially the same as those determined by ESR spectroscopy. These observations suggest that only unchanged TAP and its hydroxylamine were excreted in urine.

Strongly polar metabolites, such as glucuronic acid conjugates, would be expected to have short retention times in the reverse-phase HPLC system. Such metabolites may coelute with the solvent front and their respective peaks in the HPLC chromatogram could be hidden under the peaks of polar endogenous compounds. This is unlikely because TAP concentrations determined by the HPLC assay were not significantly different from those determined by the ESR assay. Furthermore, no nitroxide ESR spectrum could be detected in the eluate collected during the first three minutes, a time period that includes the solvent peak of the chromatogram, after injection of a rat urine sample into the HPLC system. These observations support the conclusion that no stable conjugates of TAP were excreted in urine.

Renal clearance of TAP in the dog was estimated indirectly using the method described in Chapter 4 because TAP could not be separated from its hydroxylamine in the urine. Reductants in urine may reduce TAP in the bladder or during collection of urine and autoxidation of the hydroxylamine may also occur. Therefore, the urine was treated to oxidize the hydroxylamine before assay; the urinary recovery thus represents both the nitroxide plus its hydroxylamine. The estimated renal clearances of TAP are close to the glomerular filtration rate (approximately 4 mL/min-kg) in the dog (Ladd et al.,

1956). No protein binding of TAP was observed in dog plasma. The nonrenal clearance of TAP , i.e., bioreduction, calculated as the difference between the total and renal clearances, was relatively small (about 2 mL/min-kg). Hence, the majority of TAP (47 to 60 % and 77 to 89 % of the dose for the low and high dose experiments, respectively) was excreted unchanged. These observations indicate that TAP is cleared renally at the rate of glomerular filtration and that bioreduction of TAP is relatively slow.

Renal clearance could not be estimated for the rat experiments by the method used for the dog experiments because most of the dose recovered was obtained in the first urine collection period (0 to 6 hours after administration). Similar to dog plasma, no protein binding was observed in rat plasma. Unless TAP is extensively reabsorbed, the renal clearance of TAP is expected to be at least equal to the glomerular filtration rate which in the rat is about 10 mL/kg-min (Harvey and Malvin, 1965). Assuming that the renal clearance of TAP in the rat is 10 mL/min-kg, the non-renal clearance is about 35 % or less of the total clearance (15 mL/min-kg) and the fraction of TAP excreted unchanged is about 0.65. The fraction excreted unchanged can also be estimated if the following assumptions are made: the renal clearances of TAP and its hydroxylamine are the same; and the hydroxylamine is excreted directly in urine. Comparing the AUC-values for the blood concentrations of TAP and its hydroxylamine, the fraction excreted unchanged then is estimated to 0.68 ± 0.11 (average ± standard deviation for all six experiments in the rat). These estimates indicate that the nonrenal clearance, presumably due to bioreduction, in the rat is low compared to the renal clearance of TAP which agrees with the estimates in the dogs.

This relatively slow bioreduction suggests that the time course of MRI contrast enhancement by TAP should be determined more by the

biodistribution than the reduction of TAP. Compared to nitroxides with more rapid bioreduction, TAP may give contrast enhancement at lower doses and the effect may persist longer.

Assuming that the elimination and distribution of TAP in humans and in the two animal species are similar, the pharmacokinetic parameters of TAP observed in the rat and the dog, normalized for body weight, can be used to make predictions of the expected pharmacokinetic parameters in humans. The average glomerular filtration rate is approximately 125 mL/min (or 1.8 mL/min-kg) in a normal healthy individual (Seldin and Giebish, 1985). If the renal clearance of TAP in humans is similar to the glomerular filtration rate and this clearance is about 70 % of the total clearance, as estimated in the rat and the dog, then the total clearance of TAP in humans is predicted to be about 2.6 mL/min-kg. The corresponding half-life is predicted to be about 2 hours if the volume of distribution is similar to those observed in the rat and the dog (0.5 L/kg). From these predictions it appears that only a moderate decrease in TAP concentrations would occur within the time interval of a MRI examination (5 to 20 min). A dose of TAP would be essentially completely eliminated about 8 hours after administration. The high urinary recovery, the large fraction excreted unchanged in urine and the absence of dose dependencies for TAP in the rat and the dog are desirable properties for its use as a MRI contrast agent.

Section II

Bioreduction of Nitroxides Examined *In Vitro*

Bioreduction of the nitroxides to their corresponding hydroxylamines was the only metabolism observed *in vivo* in the dog and the rat for TES, PCA and TAP. Because the hydroxylamine lacks contrast-enhancing activity, the bioreduction was of particular interest for the evaluation of these compounds as MRI contrast agents. In this section, factors that influence bioreduction of nitroxides are examined *in vitro*. In the first two chapters the reducibility of nitroxides is evaluated in rat tissue homogenates and ascorbic acid solution. Under the hypothesis that cell membrane permeability to nitroxides limits their intracellular bioreduction, human erythrocyte membrane permeability to nitroxides was determined. These studies are presented in the last chapter of this section.

Chapter 6. Nitroxide Reduction in Rat Tissue Homogenates and Ascorbic Acid Solution

In this chapter, the influence of chemical structure on the reduction of the nitroxide moiety is examined *in vitro* in rat tissue homogenates and ascorbic acid solution. The rat was selected as an animal model for these studies. The reduction rates in homogenates of different tissues are compared to identify tissues with high reducing activity. Reduction by ascorbic acid was examined because it is an endogenous reductant demonstrated to reduce nitroxides to their corresponding hydroxylamines *in vitro* (Paleos and Dais, 1977; Sosnovsky and Konieczny, 1977).

Materials

TES, PCA and TAP, used in these studies (see Chapter 2 for structures), were stored at 4 °C as aqueous solutions (50 mM) buffered to pH 7.4 with 67 mM phosphate buffer. L-Ascorbic acid was obtained from Sigma Chemical Co. (St.Louis, MO). Diethylenetriaminepentaacetic acid (DTPA) was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Reduction in Rat Tissue Homogenates

Rat tissue homogenates were prepared in isotonic saline or phosphate buffer (67 mM, pH 7.4) as described in Chapter 2. To assure that the homogenates were fresh, only one tissue was obtained from each rat. The homogenate was prepared immediately from the particular tissue (liver, kidney,

heart, muscle, lung or brain). The nitroxide was added to the homogenate less than 10 min after the animal was sacrificed and the experiment was stopped one hour after addition of the nitroxide.

Reduction in Ascorbic Acid Solution

The time courses of reduction of the nitroxide derivatives by ascorbic acid were measured by ESR spectroscopy at room temperature at different concentrations of the nitroxides (0.1 to 10 mM) and ascorbic acid (0.1 to 50 mM) as described in Chapter 2. The solutions were prepared in 67 mM phosphate buffer and adjusted to pH 7.4 with 1 M NaOH, if necessary.

Results

Reduction in Rat Tissue Homogenates

Reduction of TES and PCA in various rat tissue homogenates is shown in Figures 6.1 and 6.2. Rat liver and kidney homogenates showed highest reducing activity for both TES and PCA. The average data for the reduction of TES, PCA and TAP in liver and kidney homogenate at pH 7.4 are summarized in Table 6.1.

Reduction in Ascorbic Acid Solution

The observed reduction rates in ascorbic acid solutions were dependent on the concentration of ascorbic acid as shown in Figure 6.3. Pseudo-first order kinetics of the nitroxide reduction was observed after addition of a metal-chelating agent to the ascorbic acid solution. Second-order rate constants of reduction in ascorbic acid were: 0.45 ±0.04 $mM^{-1}min^{-1}$ for TES; 0.0042 ± 0.0001 $mM^{-1}min^{-1}$ for PCA; and 0.018 ± 0.002 $mM^{-1}min^{-1}$ for TAP. Without the metal-chelating agent added to the buffer, the reduction by ascorbic acid appeared to be more rapid initially and then approach an equilibrium value (Figure 6.4). When ascorbic acid was in great excess, the reaction would slowly continue until no ESR signal of the nitroxide was detected. At lower concentration of ascorbic acid, the ESR signal would increase at later times indicating spontaneous oxidation of the hydroxylamine in the presence of air.

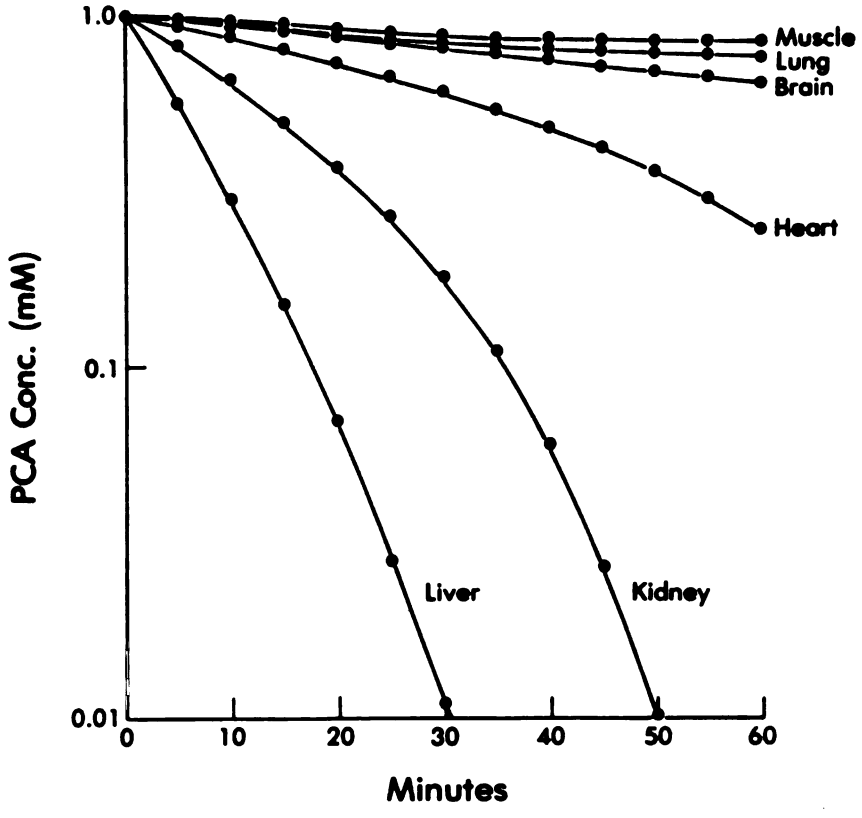


Figure 6.1 Decline of PCA concentration (initially 1 mmol/kg tissue) in rat tissue homogenates diluted with 3 volumes of normal saline per gram of tissue. The lines connect points for identification purposes. Note the large difference in the reductive activity between homogenates of liver and kidney and those of brain, lung and muscle .

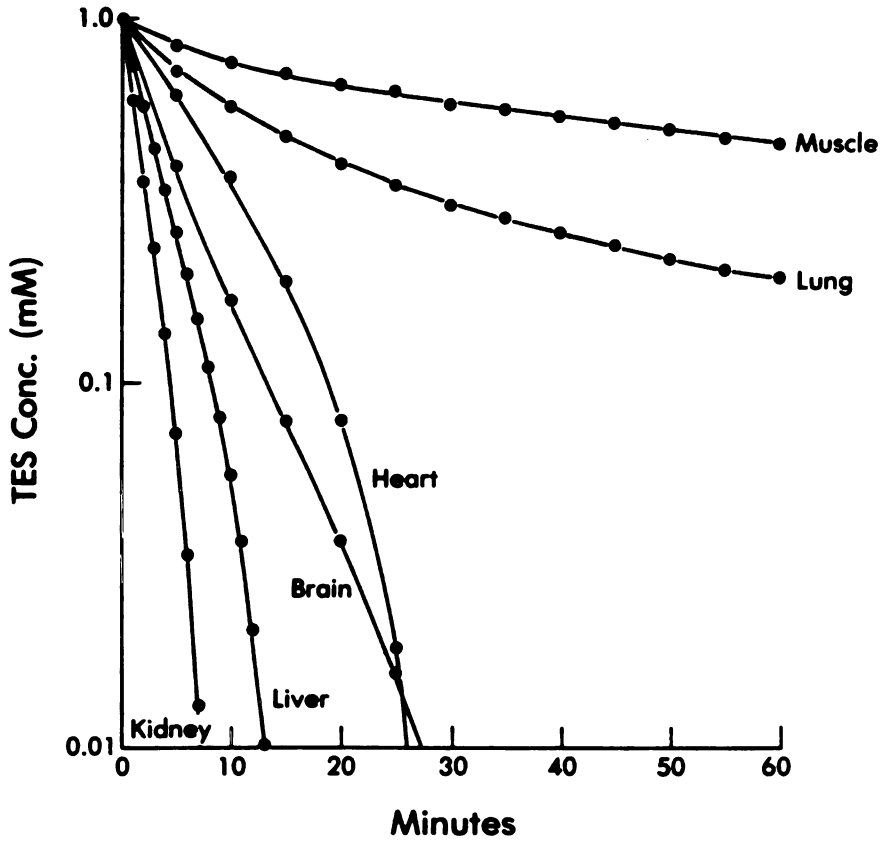


Figure 6.2 Decline of TES concentration (initially 1 mmol/kg tissue) in rat tissue homogenates diluted with 3 volumes of normal saline per gram of tissue. The lines connect points for identification purposes. As with PCA (see Figure 6.1), the reductive activity in liver and kidney homogenates greatly exceeded those of lung and muscle, but there appears to be relatively more activity in brain for TES than observed for PCA (Figure 6.1).

Table 6.1 *Reduction of TES, TAP and PCA in rat liver and kidney.* [a]

	TES		TAP		PCA	
Time	Liver	Kidney	Liver	Kidney	Liver	Kidney
2	50.3 ± 8.7	40.8 ± 1.2	79.0 ± 1.6	79.4 ± 5.8	89.1 ± 0.6	88.7 ± 0.4
4	21.3 ±10.0	14.8 ± 0.8	62.9 ± 0.6	61.3 ± 6.6	78.5 ± 2.5	78.2 ± 1.3
6	7.3 ± 5.7	4.2 ± 0.4	48.3 ± 1.1	47.0 ± 6.7	68.6 ± 1.8	68.1 ± 1.5
8	1.9 ± 2.0	0.7 ± 0.2	37.4 ± 0.4	30.6 ± 6.2	60.0 ± 2.3	61.3 ± 2.3
10			28.5 ± 0.6	27.8 ± 5.8	52.8 ± 2.7	53.7 ± 1.9
15			13.9 ± 1.4	14.1 ± 4.3	37.4 ± 2.6	38.4 ± 2.4
20			6.7 ± 0.6	6.8 ± 2.7	26.5 ± 2.7	27.5 ± 2.5
25			2.9 ± 0.6	3.1 ± 1.5	18.4 ± 2.4	19.0 ± 2.6
30			1.2 ± 0.5	1.4 ± 0.8	12.7 ± 1.9	12.9 ± 2.2

[a] The values are expressed as percent remaining of the initial ESR signal, obtained by extrapolating the data back to the time the nitroxide was added to the homogenate (time zero). The mean ± standard deviation of three different homogenates are shown.

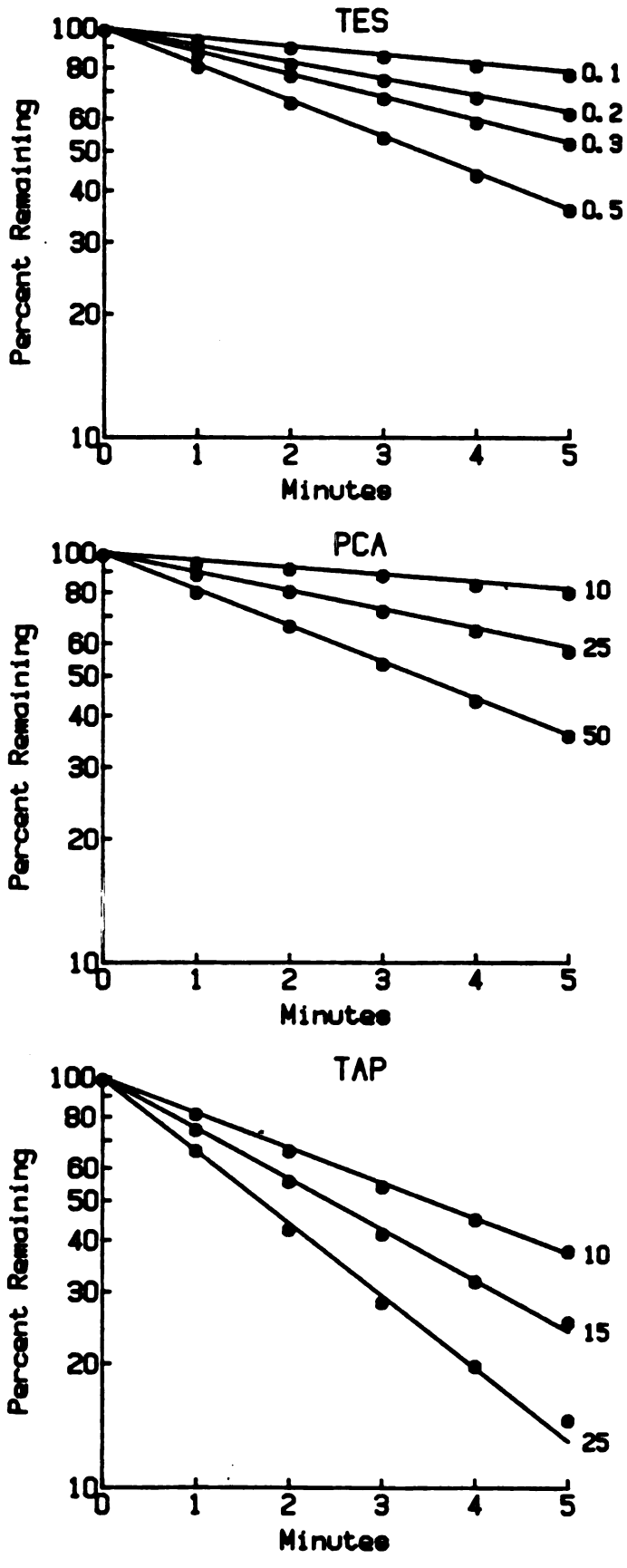


Figure 6.3 The decline of the concentration of the nitroxides depends on the initial concentration of ascorbic acid added. The difference in reducibility between the piperidine derivative, TES (top panel), is demonstrated by the lower ascorbic acid concentrations compared to those used for the pyrrolidine derivatives, PCA (middle panel) and TAP (bottom panel). The initial concentration of the nitroxides were 0.125 mM and the ascorbic acid concentrations (mM) are shown in the graph. The solid lines were obtained by linear least square regression of the data during the first 4 min.

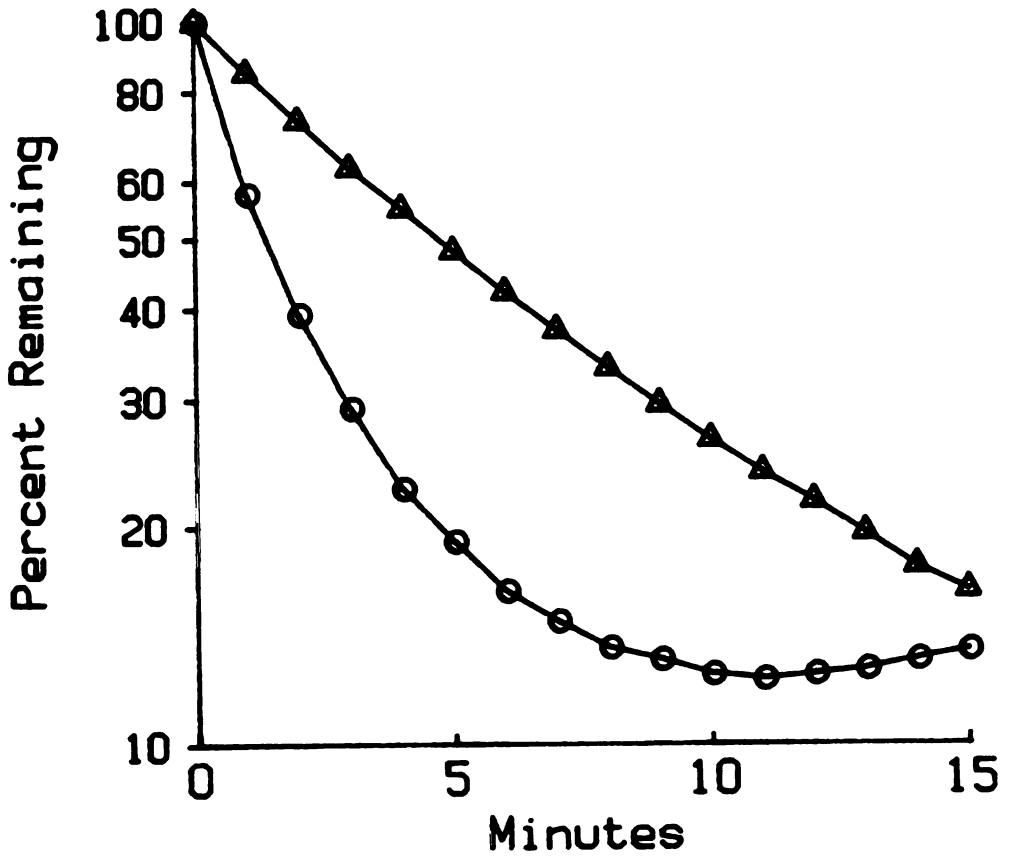


Figure 6.4 Reduction of TES in ascorbic acid solution in the presence (circles) and the absence (triangles) of the metal-chelating agent, DTPA. The concentrations of TES and ascorbic acid were 0.24 mM and 0.38, respectively, in both experiments. The lines connect points for purposes of identification.

Discussion

Reduction in Rat Tissue Homogenates

Liver and kidney homogenates showed the greatest reducing activity, lung and muscle the least (Figures 6.1 and 6.2). Similar interorgan differences in reducing activity have been reported previously for a piperidine nitroxide derivative (Schimmak et al., 1976). Averages of data obtained in three different rats for the liver and kidney homogenates showed relatively little variability (Table 6.1). The interanimal differences were small compared to interorgan differences. The ability of the tissues to reduce the nitroxides depended on the freshness of the homogenate. A decrease of the reducing activity of the tissue homogenates was observed with time after preparation. This decrease was slow and the reducing activity was stable within the first hour after preparation. Consequently, the homogenates were prepared freshly for each experiment and the nitroxides were always added at approximately the same time.

Exposure of the sample to air during the experiments resulted in restoration of the ESR signal. This is an indication that the hydroxylamine is formed as a result of the reduction of the nitroxide. Spontaneous oxidation of nitroxides in the presence of air has been reported (Rozantsev and Sholle, 1971a) therefore, to minimize air exposure, the homogenate was not removed from the flat cell of the ESR spectrometer during the experiment. Enzymatic oxidation of hydroxylamines to their corresponding stable nitroxides has also been proposed. Such oxidation was observed in rat (Rosen and Rauckman, 1977), rabbit (Stier and Reitz, 1971) and pig (Rauckman et al., 1978) liver microsomes. This enzymatic oxidation of the hydroxylamine was in all studies reported to be dependent on oxygen. In a recent study, the nitroxide reduction

in cultured mammalian cells (derived from thymus and bone marrow cells of mice) was more rapid in anoxic cells than in normally oxygenated cells. The reduction was reversed (ESR signal restored) upon reintroduction of oxygen to the cells. It appears from these studies that reversible oxidation of nitroxides may occur *in vivo* but it is difficult to interpret these results because of the rapid spontaneous oxidation of the hydroxylamine *in vitro* in the presence of air.

In all tissues, TES was reduced more rapidly than PCA. The reduction rates of TAP in liver and kidney was more rapid than for PCA. However, reduction of the pyrrolidine derivatives, TAP and PCA, was slower than that of TES, a piperidine derivative. These observations indicated a structural dependence of the reduction of nitroxides. A study in our laboratory, in which the reducibility of ten nitroxide derivatives was determined, demonstrated that pyrrolidine derivatives were more stable than piperidine derivatives in rat kidney homogenates (Couet et al., 1985b). The substituents of the nitroxides also influenced the stability in rat tissue homogenates. Carboxylic acid derivatives were more stable than hydroxyl derivatives which, in turn, were more stable than amine derivatives. This agrees well with the results of the present study because PCA (carboxylic acid derivative) was more stable than TAP (hydroxyl derivative). The effect of the substituent may be related to its charge at physiologic pH.

The structural influence of reduction may indicate selectivity of enzymes that catalyze the reaction, varying sensitivity to a chemical reductant or a combination of both mechanisms. Liver and kidney, tissues rich in enzymes, showed high reducing activity. The reduction of TES in brain homogenate is almost as rapid as that in liver and kidney. Nitroxide reduction in brain tissue may be due to chemical reaction with a reductant. For example, ascorbic acid has been demonstrated to reduce nitroxides *in vitro* (Paleos and Dais, 1977;

Sosnovsky and Konieczny, 1977). Consequently, the rapid reduction of TES in rat brain may be explained by the relatively high concentration of ascorbic acid (2.0 to 2.8 mmol per kg of tissue; Hornig,1975) in this tissue. However, the ascorbic acid concentration of rat tissues correlates poorly with their reducing activity. For example, the ascorbic acid concentration in brain is 4 to 5 times higher than in heart tissue, yet these tissues reduce TES at about the same rate.

Reduction in Ascorbic Acid Solution

The reduction in ascorbic acid solution is a simple but biologically relevant system for evaluating the relative reducibility of nitroxide derivatives. Ascorbic acid is an endogenous reductant that transfers electrons to enzymes and affects metabolism of endogenous compounds (Ginter et al., 1982; Levine, 1986) and xenobiotics (Zannoni et al., 1982). Deficiency of ascorbic acid is known to cause scurvy but the physiologic role of this vitamin is controversial.

Reduction of nitroxides *in vivo* may, at least in part, be due to ascorbic acid in tissues (Rauckman et al., 1984). For example, reduction of nitroxides in avian muscle tissues was demonstrated to be caused by ascorbic acid (Perkins et al., 1980). Ascorbic acid has been shown to reduce nitroxides *in vitro* (Paleos and Dais, 1977; Sosnovsky and Konieczny, 1977). As a result of this reduction the corresponding hydroxylamine is formed and ascorbic acid is oxidized to dehydroascorbic acid. Each mol of ascorbic acid reduces two mol of nitroxide (Paleos and Dais, 1977). A mechanism for ascorbic acid oxidation has been proposed in which ascorbic acid is oxidized in two one-electron steps, first to the ascorbic acid radical and then further to dehydroascorbic acid (Iyanagi et al., 1985). The first step was estimated to be rate limiting. Reduction rate in ascorbic acid of nitroxide complexed in cyclodextrin appeared to be the

same as the reduction rate observed for free nitroxide which suggests that the reduction occurs by electron transfer and not by proton transfer (Ebel et al., 1985). The rate of the reduction has been reported to be first-order in both nitroxide and ascorbic acid concentration (Kocherginskii et al., 1981; Craescu et al., 1982, Ebel et al., 1985).

In our hands, first-order decay did not always occur. An initial rapid decrease in ESR signal was observed followed by a much slower process (Figure 6.4). The reaction seemed to approach an equilibrium but the reaction continued to completion when ascorbic acid was in large excess. This was observed when the sample was left in the flat cell of the ESR spectrometer over a longer period of time (24 hours). Consumption of the product of ascorbic acid oxidation may explain that observation. In aqueous solution, dehydroascorbic acid is irreversibly hydrolyzed to 2,3-diketogulonic acid (Paleos and Dais, 1977).

Addition of a metal chelating agent (DTPA) appeared to influence the kinetics of the reaction and a characteristic pseudo-first order decline was observed when the ascorbic acid was added in excess (Figures 6.3 and 6.4). This observation indicates that metal-ion catalyzed reactions may occur and interfere with the reduction. Metal-ions have been reported to catalyze autoxidation of ascorbic acid (Martell, 1982) and hydroxylamine (Schwartz et al., 1979). In the present study, the reduction of nitroxides by ascorbic acid as well as the oxidation of ascorbic acid and the hydroxylamine appear to be catalyzed by metal ions. Introducing air into a sample, by repeatedly withdrawing the sample from the flat cell of the ESR spectrometer and then injecting it again, caused an increase in ESR signal indicating that the hydroxylamine is autoxidized. After reduction in ascorbic acid, the ESR signal

could be completely restored by oxidative treatment of the samples using hydrogen peroxide, cupric sulfate or potassium ferricyanide (see Chapter 2).

The second-order reduction rate constant of TES, a piperidine derivative, was 25 and 100 times higher than those determined for the pyrrolidine derivatives, TAP and PCA, respectively. This agrees with previous studies that demonstrated that piperidine derivatives were reduced more rapidly than pyrrolidines (Kocherginskii et al., 1981; Craescu et al., 1982; Keana and VanNice, 1984).

The reduction rates of twenty nitroxide derivatives in ascorbic acid solution were examined in our laboratory (Couet et al., 1985a). In that study as well as the previously mentioned study on reduction in tissue homogenates (Couet et al., 1985b), all the pyrrolidine derivatives were more stable than any of the piperidine derivatives, irrespective of the substituents of the derivatives. As in the rat tissue homogenates, the nitroxide substituent also influenced the stability. Derivatives with substituents negatively charged at physiologic pH (the solutions were buffered to pH 7.4) were more stable than derivatives with neutral substituents, which in turn were more stable than derivatives that were positively charged. The influence of the charge of the nitroxide on the reduction rate has been attributed to reaction with the monoanionic form of ascorbic acid (Kocherginskii et al., 1981; Craescu et al., 1982). This conclusion was based upon the observed pH dependence of the reaction. The rank order of stability in ascorbic acid solution was the same as that observed in tissue homogenates which agrees with the observation for TES, TAP and PCA in the present study.

A study, examining the reduction of 2,6-dichlorophenolindophenol and ferricyanide by ascorbic acid, indicated that the reduction rates were influenced by the difference in reduction potentials between oxidant and the reductant in agreement with the theory for outer sphere electron transfer reactions (Iyanagi

et al., 1985). The reduction potential of a piperidine nitroxide derivative was found to be higher than those of two pyrroline nitroxide derivatives (Chan and Brucie, 1977). Except for the double bond between carbons 3 and 4, the structure of pyrroline nitroxides is the same as for pyrrolidine derivatives (see Chapter 2 for structure). The stability in ascorbic acid of a pyrroline derivative was demonstrated to be similar to that of pyrrolidine derivatives (Couet et al., 1985a). Consequently, there appears to be a correlation between the reduction potential and the reduction rate of nitroxides in ascorbic acid.

In conclusion, the nitroxides are reduced in rat tissue homogenates and ascorbic acid solution to their corresponding hydroxylamines. The reduction is influenced by the chemical structure of the nitroxide derivatives; the same rank order of stability was observed in both rat tissue homogenates and ascorbic acid solutions. Reducibility in these systems appeared to be mostly influenced by the ring structure of the nitroxides and to a lesser degree by the charge of the substituent. The relative stabilities *in vitro* of TES and PCA observed in these systems are in agreement with their relative clearances in the dog (Chapters 3 and 4). Therefore, determination of the stability of nitroxides *in vitro* may be an indication of the stablity *in vivo* and is a useful test for the design of more stable nitroxides.

Chapter 7. Nonenzymatic Bioreduction of Nitroxides in Rat Liver and Kidney

Enzymatic and nonenzymatic mechanisms for bioreduction of nitroxides have been postulated (Rauckman et al., 1984). Enzymatic reduction by electron transport systems has been observed in microsomal (Rosen and Rauckman, 1977; Rosen et al., 1977) and mitochondrial (Quintanilha and Packer, 1977) subcellular fractions of rat liver and mitochondria of rabbit spermatozoa (Chapman et al., 1985). Nonenzymatic reduction with endogenous reductants, such as ascorbic acid in avian muscle (Perkins et al., 1980) and sulfhydryl compounds in several biologic materials (crab and lobster walking nerves, mice erythrocytes, neuroblastoma cells) (Giotta and Wang, 1972; Chen and McLaughlin, 1985) has been demonstrated.

The objective of this study was to determine the contribution and nature of nonenzymatic reduction in rat liver and kidney, mammalian tissues with high reducing activity (Chapter 6). Reduction of TES and PCA, particularly by ascorbic acid and sulfhydryl compounds, was examined in protein-free preparations obtained by heat treatment and ultrafiltration of rat liver and kidney homogenates.

Materials and Methods

Chemicals

PCA and TES, used in these studies (see Chapter 2 for structures and source), were stored at 4 °C as aqueous solutions (50 mM) buffered to pH 7.4

with 67 mM phosphate buffer. The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) : L-ascorbic acid, N-ethylmaleimide (NEM), ß-nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), and ascorbic acid oxidase.
2,6-Dichlorophenolindophenol and diethylenetriaminepentaacetic acid (DTPA) were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Heat-resistant reducing activity

Liver and kidney homogenates were prepared in phosphate buffer as described in Chapter 2. The phosphate buffer, used here and in subsequent experiments, was 67 mM in phosphate (from mixing equimolar solutions of monobasic potassium phosphate and dibasic sodium phosphate to obtain pH 7.4) and contained 0.1 mM DTPA, a metal chelating agent added to prevent metal-catalyzed autoxidation. The agent was found to stabilize the reducing activity in the protein-free preparations of rat liver and kidney homogenates. The liver or kidney homogenate was heated to 100 °C for 10 min in a water bath. Denatured protein and cell debris were precipitated by centrifugation at 1000 x*g* for 5 min, and the supernatant fluid was then tested for reducing activity as described in Chapter 2. The ESR signal was followed for 15 min after adding PCA or TES at initial concentrations of 0.025 mM and 0.25 mM, respectively. PCA was added at a 10-fold lower initial concentration than TES because the pyrrolidine derivatives have been found to be reduced at a slower rate (Chapter 6; Couet et al., 1985a, 1985b) and it would therefore be more difficult to observe any decrease in ESR signal within 15 minutes. A longer incubation time is complicated by an observed time-dependent loss of reducing activity.

Ultrafiltrable Reducing Acitivity

Liver and kidney homogenates were prepared in phosphate buffer as described in Chapter 2. For preparation of kidney ultrafiltrate, the organs (approximately 4 g of kidney) of two rats were used. The homogenate was divided into 12 test tubes and centrifuged at 1000 x *g* for 20 min. The supernatant fluid from each of six of the test tubes was transferred to the sample reservoirs of ultrafiltration devices (Micropartition System MPS-1, Scientific System Division, Amicon Corp., Danvers, MA) and centrifuged at 1000 x *g* for 30 min. The resulting ultrafiltrate was diluted with an equal volume of phosphate buffer. Each of the six remaining test tubes of whole tissue homogenate was centrifuged together with the ultrafiltration devices. The homogenate in these tubes was then diluted with an equal volume of phosphate buffer and vortexed. Both the ultrafiltrate and the whole tissue homogenate were tested for reducing activity as described in Chapter 2 by following the ESR signal for 15 min after adding PCA (0.025 mM) or TES (0.25 mM). Experiments were performed with and without addition of 0.5 mM NADPH.

Oxidation of TES and PCA Hydroxylamines

The oxidant cupric sulfate was used to oxidize the hydroxylamines (Chapter 2). After a 15-minute incubation of the nitroxides (initial concentrations were 0.25 mM for TES and 0.025 mM for PCA) with tissue homogenates (prepared as described above and diluted two-fold in phosphate buffer), the samples were diluted ten-fold in buffer and cupric sulfate (final concentration of

0.5 mM). The ESR signal was measured for each sample and compared to that of standards in the same concentration of cupric sulfate.

Inhibition of TES Reduction by Ascorbic Acid Oxidase and N-ethylmaleimide

The contribution of ascorbic acid and sulfhydryl compounds was examined by incubating the supernatant fluids and ultrafiltrates with either ascorbic acid oxidase (30 units/mL), N-ethylmaleimide (30 μmol/mL), or both. The supernatant fluids and ultrafiltrates were prepared from rat liver and kidney as described above and then incubated with the inhibitors for 15 min. The reduction was monitored by following the ESR signal for 15 min after adding TES at an initial concentration of 0.25 mM.

After preparation of the supernatant fluids, reductions were measured in the following order: supernatant fluid + phosphate buffer (control); supernatant fluid + ascorbic acid oxidase; supernatant fluid + N-ethylmaleimide; repeat of control; and finally supernatant fluid + ascorbic acid oxidase + N-ethylmaleimide. The order for the ultrafiltrates was the same except the first control was omitted. The final dilution factors of the tissues were 5.3 for the supernatant fluids and 8 for the ultrafiltrates.

All the reduction experiments described above were performed at room temperature (21 ± 1°C). The reduction was quantified by least squares regression of the logarithm of the ESR signal for the first 4 min and by extrapolating back to the time when TES or PCA was added. The reduction rate constants were determined as the negative slope of this regression. The ESR signal was expressed as per cent of ESR signal extrapolated to time zero.

Determination of Ascorbic Acid Concentration

Ascorbic acid content in heat-treated supernatant fluids and ultrafiltrates was determined using a modification of a previously-published spectrophotometric method (Omaye et al., 1979). Briefly, an aliquot of the sample (75-200 µL) was diluted in 10 % phosphoric acid to a final volume of 600 µL to which 300 µL of a mixture of 0.1 M citric acid and 0.2 M dibasic sodium phosphate (the mixture had a pH of 4.0 and contained 1 mg/mL N-ethylmaleimide) was added. Thirty seconds after adding 300 µL of a solution of 2,6-dichlorophenolindophenol (0.1 mg/mL), the absorption at 520 nm was measured.

Determination of Protein Concentration

Protein concentrations in tissue homogenates, supernatant fluids, and ultrafiltrates were determined by the method of Lowry et al. (1951).

Results

Reduction rates were more rapid for TES than for PCA in protein-free preparations (supernatant fluids and ultrafiltrates) of rat liver and kidney (Table 7.1). No reduction was observed within 15 minutes for PCA in the ultrafiltrates. Liver preparations reduced TES more rapidly than kidney preparations. The estimated reduction rate constants for TES were similar in supernatant fluids and in ultrafiltrates when correction for the difference in dilution was made.

Reduction of TES and PCA in whole tissue homogenate was NADPH-dependent (Figure 7.1). Addition of NADPH to ultrafiltrates did not alter the reduction rates, and no reduction was caused by NADPH itself. TES was reduced more rapidly than PCA in whole tissue homogenates (Figure 7.1).

Oxidative treatment of the tissue homogenates with cupric sulfate after reduction led to restoration of the ESR signal for both TES and PCA. The restored ESR signals, expressed as per cent of standards, were 99.3 ± 1.8 and 102 ± 2.2 in liver homogenates and 105.6 ± 2.0 and 104.7 ± 7.8 in kidney homogenates (mean ± SD of at least 3 experiments) for TES and PCA, respectively.

The protein concentrations in liver and kidney, respectively, were 52 ± 5 mg/mL and 43 ± 6 mg/mL in whole homogenates, 2.8 ± 0.2 mg/mL and 3.6 ± 0.2 mg/mL in supernatant fluids, and 1.0 ± 0.3 mg/mL and 1.2 ± 0.4 mg/mL in ultrafiltrates. All values refer to a original tissue concentration of 1 g/mL of solution and are averages (± SD) of at least six determinations.

Table 7.1 *Reduction rate constants in protein-free preparations of rat liver and kidney.* [a]

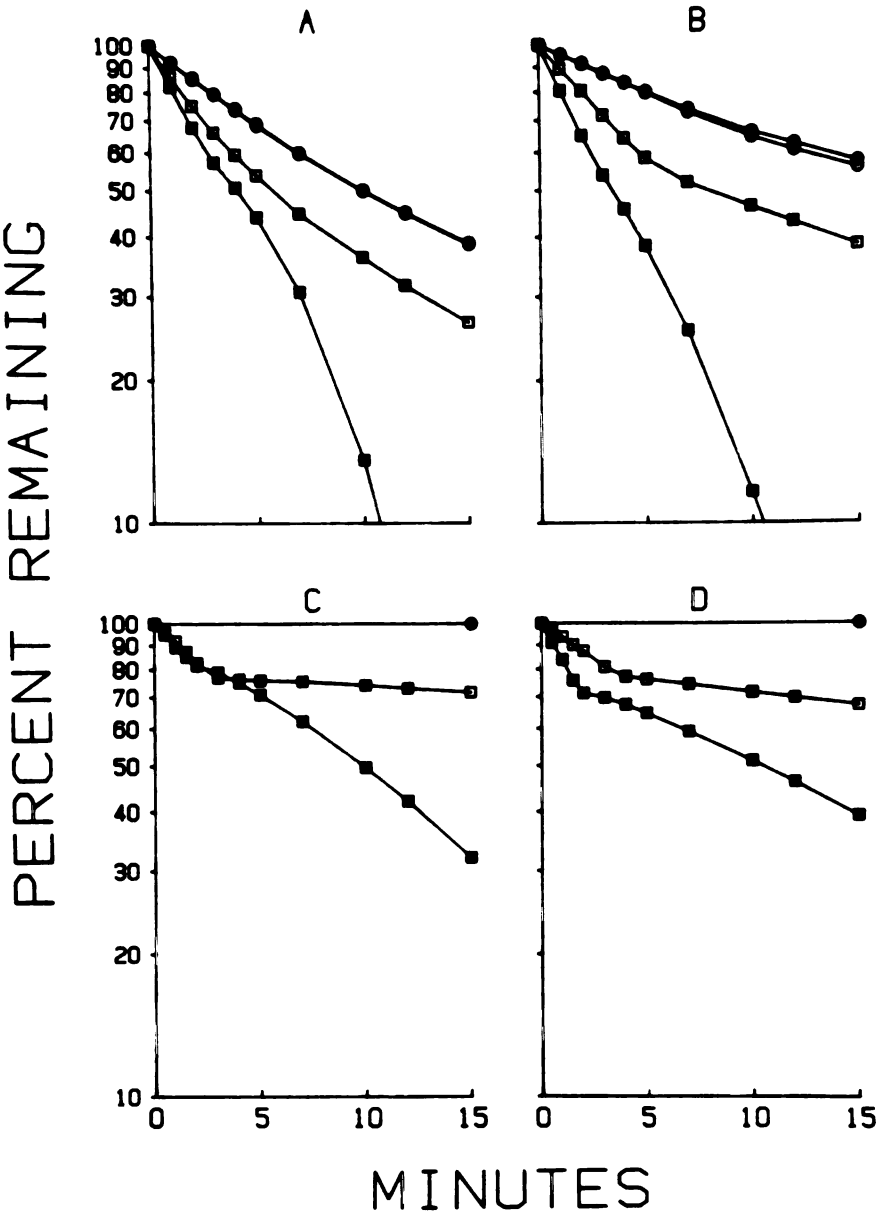
	Liver		Kidney	
	Supernatant Fluid	Ultrafiltrate	Supernatant Fluid	Ultrafiltrate
TES	0.12 ± 0.01 (n = 7) [b]	0.09 ± 0.01 (n = 5)	0.07 ± 0.01 (n = 8)	0.05 ± 0.002 (n = 4)
PCA	0.002 ± 0.001 (n = 3)	N. R. [c]	0.003 ± 0.001 (n = 4)	N. R. [c]

[a] Reduction rate constants (min^{-1}) were estimated during the first 4 minutes of reduction and are expressed as average ± SD of at least three experiments. The reduction rate constants in supernatant fluids were corrected for the difference in dilution factor (reduction rate constant divided by the dilution factor) to make them comparable to those in ultrafiltrates. All reduction rate constants apply to an 8-fold dilution of the tissue. The initial concentration was 0.025 mM for PCA and 0.25 mM for TES.

[b] Number of experiments.

[c] No reduction (N. R.) was observed within 15 minutes.

Figure 7.1 Semilogarithmic plot of the time course of reduction of TES (panels A and B) and PCA (panels C and D) in ultrafiltrates (*circles*) and whole tissue homogenates (*squares*) of rat liver (panels A and C) and kidney (panels B and D). The percent remaining of the initial ESR signal is expressed as the mean of three sequential experiments in an ultrafiltrate or tissue homogenate preparation. The standard deviation of the means ranged from 0.5 % for the early time points up to 6.5 % at the late time points. Upon addition of 0.5 mM NADPH (closed symbols), the reduction rates for both TES and PCA in the whole tissue homogenates were increased compared to those in buffer alone (open symbols). However, the reduction rates in the ultrafiltrates were unchanged by the addition of NADPH (the curves overlap in all but panel B). No reduction was observed for PCA in the ultrafiltrates. The initial concentrations were 0.25 mM, except for PCA in the ultrafiltrates (0.025 mM). The dilution factor was 8 (1g of tissue/8 mL) for the ultrafiltrates and homogenates.


A
B
C
D
PERCENT REMAINING
100
90
80
70
60
50
40
30
20
10
0
5
10
15
MINUTES

The effect of adding ascorbic acid oxidase and N-ethylmaleimide on the reduction of TES in liver and kidney supernatant fluid was examined to determine the contributions of ascorbic acid and sulfhydryl compounds, respectively. Ascorbic acid oxidase causes essentially complete inhibition of reduction while N-ethylmaleimide has only a minor effect (Figure 7.2). When both ascorbic acid oxidase and NEM were added, TES reduction was completely inhibited and the ESR signal actually increased 1 to 2 % over 15 minutes. This minor increase in ESR signal could be due to the presence of a small amount of hydroxylamine in the stock solution of TES which is autoxidized upon dilution.

The effect of adding ascorbic acid oxidase and NEM on the reduction of TES in liver and kidney ultrafiltrates (Figure 7.3) is similar to that found in supernatant fluids (Figure 7.2).

Assuming that TES reduction in liver and kidney supernatant fluids and ultrafiltrates was due only to ascorbic acid, the observed reduction rates were divided by the second-order reduction rate constants obtained in freshly-prepared ascorbic acid solutions (0.45 ± 0.04 $mM^{-1}min^{-1}$; see Chapter 6) to estimate the concentrations of ascorbic acid in rat liver and kidney (Table 7.2). The ascorbic acid concentrations are expressed as mmol/kg of original undiluted tissue wet weight. Determinations made spectrophotometrically are also shown in Table 7.2.

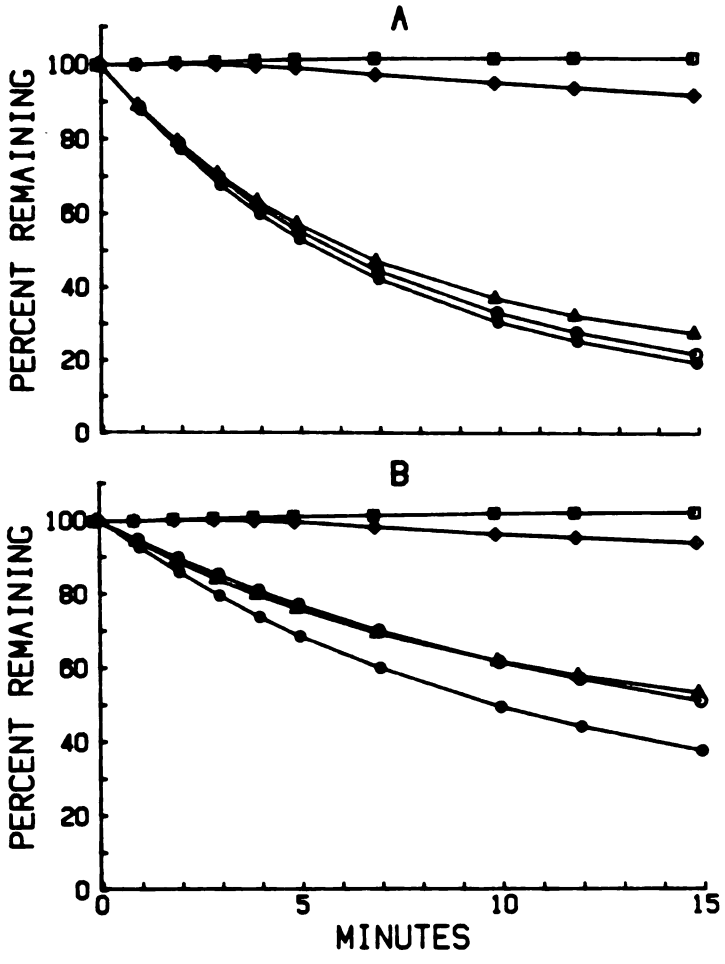


Figure 7.2 The effect of NEM and AAOx on the time course of TES reduction (0.25 mM initial concentration) in supernatant fluids prepared from heat-treated homogenates of rat liver (panel A) and kidney (panel B). The amounts remaining (percent of initial ESR signal) are mean values from three different preparations (SD ranged from 0.2-3.5 %). In each experiment, TES reduction was tested in the following order : supernatant fluid without inhibitor (control, *closed circles*); supernatant fluid + AAOx (*diamonds*); supernatant fluid + NEM (*triangles*); repeat of control (*open circles*); and supernatant fluid + AAOx + NEM (*squares*). The final dilution factor of the supernatant fluids was 5.3 (1 g of tissue/5.3 mL).

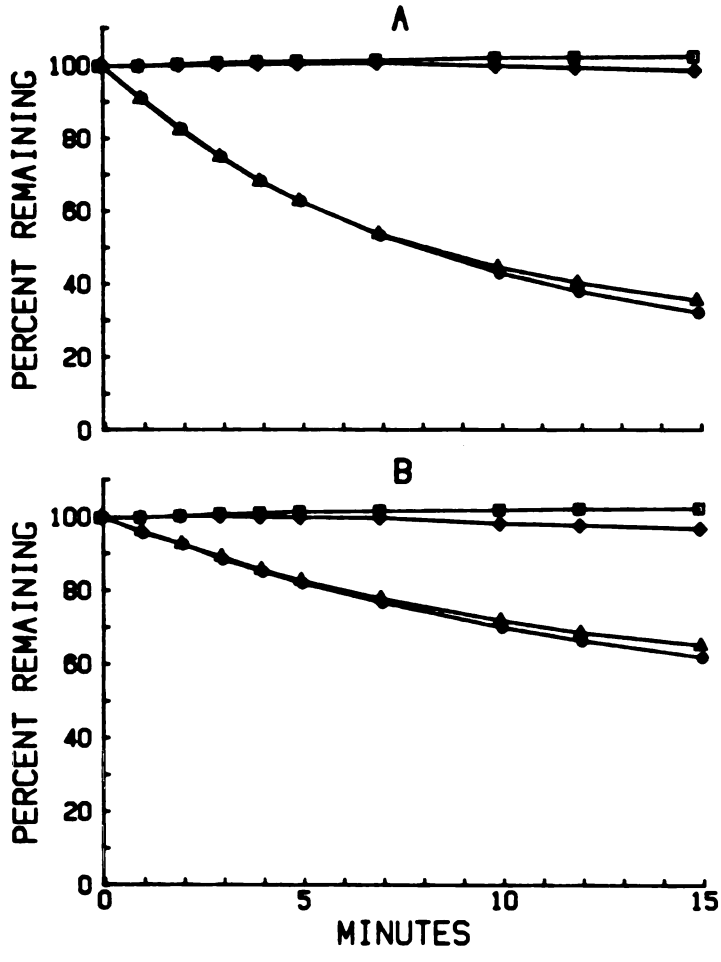


Figure 7.3 The effect of NEM and AAOx on the time course of TES reduction in ultrafiltrates prepared from homogenates of rat liver (panel A) and kidney (panel B). The percent remaining of initial ESR signal (0.25 mM) at each time point is the mean of at least three preparations (SD ranged from 0.1-6 %). TES reduction was tested in the following order: ultrafiltrate + AAOx (*diamonds*); ultrafiltrate + NEM (*triangles*); ultrafiltrate without inhibitor (*closed circles*); and ultrafiltrate + AAOx + NEM (*squares*). The final dilution factor of the ultrafiltrates was 8 (1g of tissue/8 mL).

Table 7.2 *Ascorbic acid concentration in rat liver and kidney.* [a]

	Supernatant Fluid		Ultrafiltrate	
Tissue	Method A [b]	Method B [c]	Method A [b]	Method B [c]
Liver	2.2 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	1.9 ± 0.2
Kidney	1.2 ± 0.1	1.3 ± 0.1	0.8 ± 0.03	1.0 ± 0.3

[a] All values are mean ± SD of at least three determinations. The concentrations of ascorbic acid in supernatant fluids and ultrafiltrates are expressed as mmol/kg of wet tissue used.

[b] Estimated from rate constants of TES reduction.

[c] Determined by spectroscopy.

Discussion

The loss of ESR signal of the nitroxides in rat tissue homogenates is assumed to be caused by the one-electron reduction of the nitroxide moiety to the hydroxylamine. This is based on the observation of constant peak width of the nitroxide ESR spectral lines and on restoration of the ESR signal upon addition of the oxidant, cupric sulfate.

Rat liver and kidney were used because the previous study (Chapter6) demonstrated that homogenates of these tissues reduced TES and PCA more rapidly than those of brain, heart, lung or muscle. Furthermore, piperidine derivatives have been found to be reduced more rapidly than pyrrolidine derivatives both in rat tissue homogenates (Couet et al., 1985b) and by ascorbic acid (Couet et al., 1985a). TES and PCA were used in the present study as representatives of the piperidine and pyrrolidine derivatives, respectively.

Heat treatment of tissue homogenates should remove reducing activity due to macromolecules and heat-sensitive reductants. The measured protein concentrations show that most of the proteins are precipitated and removed from the supernatant fluids. The supernatant fluids from both both liver and kidney reduce TES, suggesting that small molecular weight, heat-resistant reductants are involved. The reduction is more rapid, indicating a higher concentration of reductants, in liver than in kidney for TES. In contrast, PCA reduction is slow in both liver and kidney supernatant fluids. TES reduction rate constants in supernatant fluids were about 30-60 % of those in whole liver and kidney homogenates.

The ultrafiltrates reduce TES at rates comparable to those in supernatant fluids when correction for dilution is made. The protein concentrations determined in ultrafiltrates are close to the minimum detectable by the Lowry

method, indicating that the ultrafiltration is efficiently removing macromolecules. Again, reduction is more rapid in liver than in kidney for TES, while no reduction is observed for PCA in ultrafiltrates of either tissue. Initial reduction rate constants of TES in ultrafiltrates are about 25-50 % of the values in whole liver and kidney homogenates, indicating that small molecular weight reductants account for a major portion of the reduction of TES.

The observations that the reduction rates appeared to increase as the nitroxide concentration decreased in whole liver and kidney homogenates and the NADPH dependence of this reduction indicate that enzymatic processes may be involved. Rat liver microsomes have been reported to reduce nitroxides enzymatically (Rosen and Rauckman, 1977; Rosen et al., 1977). The reduction in these studies was found to be saturable, NADPH-dependent, inducible by phenobarbital, and competitively inhibited by specific antagonists of cytochrome P-450. It was concluded that cytochrome P-450 was the enzyme responsible for bioreduction of nitroxides in rat liver microsomes. The respiratory chain of rat liver mitochondria has also been reported to reduce nitroxides (Quintanilha and Packer, 1977). The reduction in mitochondria was dependent on electron donors such as succinic acid and lactic acid. NADPH can also donate electrons to the respiratory chain. From the effect of inhibitors of the respiratory chain, the authors suggested that ubiquinol in the electron transport chain was the site of nitroxide reduction (Quintanilha and Packer, 1977). The same mechanism was proposed for the nitroxide reduction observed in rabbit spermatozoa (Chapman et al., 1985).

One of the objectives of this study was to estimate the contribution of the nonenzymatic pathway to the total reduction in whole tissue homogenates. This evaluation is complicated because of the different reduction kinetics between the protein-free preparations and the whole tissue homogenates. As indicated,

the reduction in whole tissue homogenates may be concentration-dependent. It is clear, however, that the contribution of nonenzymatic reduction to the total reduction is larger for the piperidine derivative, TES, than for the pyrrolidine derivative, PCA. Also, reduction in whole liver and kidney homogenates is more rapid for TES than for PCA.

Addition of ascorbic acid oxidase prevents TES reduction in supernatant fluids and ultrafiltrates. Ascorbic acid has been suggested to be responsible for bioreduction of nitroxides in avian muscle (Perkins et al., 1980). The effect of ascorbic acid oxidase, an enzyme that oxidizes ascorbic acid to dehydroascorbic acid and reduces oxygen to water (Dawson and Magee, 1955), on TES reduction indicates that ascorbic acid accounts for most of the nonenzymatic bioreduction in rat liver and kidney.

Sulfhydryl compounds, such as cysteine and glutathione, have also been reported to reduce nitroxides in vitro (Morrisett and Drott, 1969) and in biological systems (Giotta and Wang, 1972). N-Ethylmaleimide is a sulfhydryl-blocking agent that binds covalently to sulfhydryl groups and is therefore expected to inhibit reduction caused by sulfhydryl compounds (Brewer and Riehm, 1967). This agent, by itself, had essentially no effect on TES reduction in supernatant fluids and ultrafiltrates. The observed lack of inhibition by N-ethylmaleimide suggests that sulfhydryl compounds are not responsible for reduction of TES in these fluids.

In a previous study in our laboratory the effects of ascorbic acid oxidase and N-ethylmaleimide on TES reduction in whole rat liver homogenate were examined (Couet et al., 1985b). It was shown that N-ethylmaleimide, by itself, caused a substantial inhibition of reduction and complete inhibition when combined with ascorbic acid oxidase. From these findings it was concluded that large molecular weight sulfhydryl groups were involved because N-

ethylmaleimide did not inhibit TES reduction in methanol extracts of rat liver homogenates in which macromolecules are separated out. Also, TES was found to be stable for at least 60 minutes in a 5 mM glutathione solution and only slowly reduced in 5 mM cysteine solution. Glutathione concentration in rat liver is 7-8 mM (Tateishi, et al., 1974), which means that the concentrations in the supernatant fluids and ultrafiltrates should be 1-2 mM or less and would therefore not cause any reduction. A recent *in vitro* study suggested that sulfhydryl reduction of nitroxides require the presence of superoxide anion radical (Finkelstein et al., 1984). Glutathione and cysteine may therefore reduce nitroxides *in vivo* where superoxide anion-producing enzyme systems are operating. Mouse neuroblastoma cells have been reported to reduce nitroxides, and nonprotein-bound sulfhydryl groups were thought to be responsible for the reduction (Chen and McLaughlin, 1985).

Ascorbic acid concentrations in rat liver and kidney, estimated from TES reduction rate constants, agreed well with values reported in the literature (Hornig, 1975), namely 1.4-2.3 and 0.9-1.1 mmol/kg of tissue in liver and kidney, respectively, and with the spectrophotometrically determined values (Table 7.2). This confirms that ascorbic acid is the reductant responsible for the nonenzymatic reduction in rat liver and kidney. The rate constants were estimated during the initial phase (first 4 minutes) of reduction. This was felt to be appropriate because ascorbic acid is consumed and at later times the reaction loses its first order character (Figure 7.1).

Ascorbic acid concentrations in human liver (0.6-0.9 mmol/kg) and kidney (0.3-0.9 mmol/kg) are similar to those in rat liver and kidney (Hornig, 1975). The ascorbic acid reduction rates *in vitro* (Chapter 6) indicate that ascorbic acid may be important *in vivo* for bioreduction of TES, but not of PCA. Furthermore, these results suggest that administration of ascorbic acid can be

used to modulate the effect of TES on the intensities of magnetic resonance images.

Ascorbic acid accounted for 5 % or less (estimated from PCA reduction rate in ascorbic acid solution; Chapter 6) of the reduction of PCA in whole tissue homogenates which indicates that PCA reduction is related to macromolecules. If PCA reduction, as suggested (Rosen and Rauckman, 1977; Rosen et al., 1977; Quintanilha and Packer, 1977), is caused by electron transport systems of microsomal and/or mitochondrial origin, a change in the metabolic activity in tissues could then influence the contrast-enhancing effect of PCA.

In summary, nonenzymatic bioreduction in rat liver and kidney homogenates occurs by ascorbic acid. This mechanism is of major importance for TES, a piperidine nitroxide derivative. The ascorbic acid reduction of PCA, on the other hand, is slow at physiologic concentrations, and PCA reduction may therefore be related to macromolecular constituents of tissues.

Chapter 8. Membrane Permeability and Bioreduction of Nitroxides

Both bioreduction and biodistribution of nitroxides, important determinants of the contrast enhancing effect of nitroxides in MRI, may be influenced by cell membrane permeability. Bioreduction of nitroxides appears to occur, at least in part, intracellularly (Chapter 7). This intracellular bioreduction of nitroxides may be limited by their cell membrane penetration rate. After administration of 0.1 mmol/kg to the dog, the metabolic clearance was 2 to 4 mL/min-kg for TAP (Chapter 5) compared to 7 to 8 mL/min-kg for PCA (Chapter 4). This indicates that the bioreduction was slower for TAP than for PCA *in vivo*, yet the reducibility of TAP *in vitro* was higher than for PCA (Chapter 6). These apparently contradictory results prompted examination of cell membrane permeability to nitroxides.

The erythrocyte, an easily accessible cell that presents an excellent model for membrane transport studies (Ellory and Lew, 1977), was selected as a model biomembrane. In the first part of the study, the membrane permeability of the human erythrocyte to TAP and PCA was determined using an extracellular quenching agent to selectively measure the intracellular ESR signal. The penetration rate was then studied for several nitroxide derivatives. The octanol : buffer distribution coefficients of the derivatives were determined and used as indices of their polarities. Nitroxide reduction by erythrocytes was examined to determine if this bioreduction was influenced by the membrane penetration rate of the nitroxides.

Materials and Methods

Nitroxides

The nitroxides used in this study were: TAP, PCA, CAT, TPH, 6-OH, 6-NH_2, 6-COOH, 5-OH, 5-NH_2. See Chapter 2 for structures and sources. The nitroxides were stored as 25-85 mM aqueous solutions at 4 °C. Under these conditions, the nitroxide moiety was found to be stable (no detectable change) for at least a month as measured by ESR spectroscopy.

Determination of Membrane Penetration

Nitroxide concentration in intracellular fluid was selectively measured and monitored over time in erythrocyte suspension by the addition of potassium ferricyanide, an extracellular quenching agent , as described in Chapter 2. The nitroxides were added to make an inital concentration of 0.1 mM.

The erythrocyte suspension was prepared in buffer as described in Chapter 2. In the initial experiment with TAP, PCA and CAT, the buffer was phosphate buffered (7.4 mM and pH 7.4) in isotonic saline. In subsequent experiments, 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 ("buffer A").

Reduction in Human Erythrocyte Suspension and Lysate

Reduction rates of the nitroxide derivatives were determined in erythrocyte suspension and lysate at room temperature and at an initial

nitroxide concentration of 0.01 mM with ESR spectroscopy as described in Chapter 2. The erythrocyte suspension and lysate were prepared in buffer A as described in Chapter 2. The erythrocyte suspension and lysate were prepared by the addition of one volume of buffer and distilled water, respectively, to an equal volume of packed cells. Protein concentration in erythrocyte lysate and supernatant fluid separated from erythrocyte suspension was determined (Lowry et al., 1951). 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 at the time of preparation and after completion of experiments. At the end of the experiments, the oxidant potassium ferricyanide was added to the erythrocyte suspensions and lysates at a concentration of 2 mM. The ESR signal was measured and compared to freshly-prepared standards.

Determination of Octanol : Buffer Distribution Coefficients

Two milliliters of nitroxide solution (0.2-1.0 mM) in 67 mM 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 nitroxide concentrations in the octanol and buffer phases were measured by ESR spectroscopy using solutions of the nitroxides in octanol and buffer, respectively, as references. The distribution coefficient was calculated by dividing the concentration in the octanol phase by that in the buffer phase.

Results

Determination of Membrane Penetration

The results of the erythrocyte-membrane permeability experiments with TAP, PCA and CAT are shown in Figure 8.1. An intracellular ESR signal was observed for PCA (spectrum 2B in Figure 8.2), in erythrocyte suspension with the extracellular quencher, potassium ferricyanide, added. The intracellular signal was about 30 % of the total ESR signal (intracellular and extracellular) observed for PCA in erythrocyte suspension without the quencher (spectrum 1B). No intracellular ESR signal was observed within 60 minutes for the membrane impermeable nitroxide, CAT, or for TAP (spectra 2A and 2C, respectively, in Figure 8.1). In the erythrocyte lysates the ESR spectra showed essentially complete quenching for all three nitroxides (spectra 4A, 4B and 4C).

The time-course of membrane penetration is illustrated in Figure 8.2 for the carboxylic acid derivatives, PCA and 6-COOH. The half-times of equilibration were 0.6 min for 6-COOH and 1.6 min for PCA. The penetration rates for the amine and alcohol derivatives were too rapid to be determined. Half-times of equilibration must be less than 10 seconds because full equilibration was obtained at the time of the first measurement (about 30 seconds following addition of the nitroxide). For the derivatives CAT and TPH, no intracellular ESR signal was detected within 60 min, indicating that the erythrocyte membrane is impermeable to these nitroxides.

For the permeable nitroxides, PCA and 6-COOH, the intracellular ESR signal was followed until it reached an apparently constant value. Reduction of the nitroxides by erythrocytes in this time period was negligible. This value was therefore assumed to represent the final equilibrium. The intracellular ESR

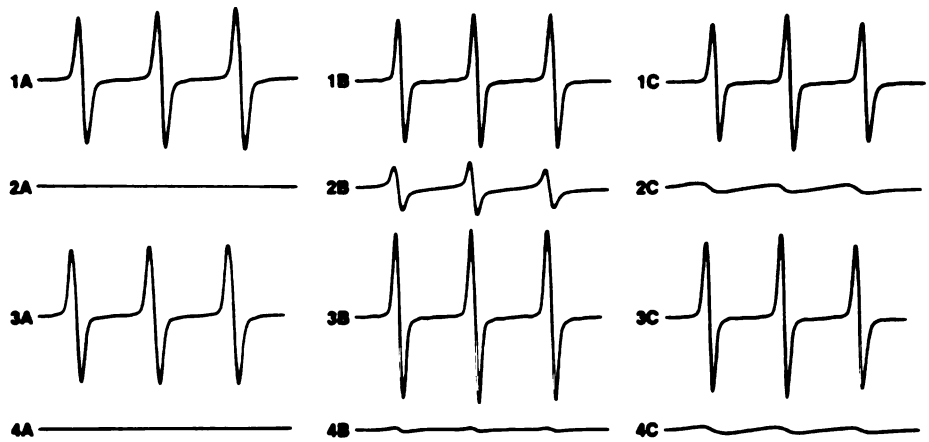


Figure 8.1 Erythrocyte membrane permeability to the nitroxides. The ESR spectra are shown for the derivatives, CAT (left panel), PCA (middle panel) and TAP (right panel). The nitroxides were added to make 0.1 mM concentrations in erythrocyte suspensions (spectra 1 and 2) and erythrocyte lysates (spectra 3 and 4) in the absence (spectra 1 and 3) and presence (spectra 2 and 4) of potassium ferricyanide. The bottom spectra (3 and 4) are controls in erythrocyte lysates to show how completely the extracellular ESR signal is broadened in the cell suspension by the addition of potassium ferricyanide. The ESR spectrum of CAT in erythrocyte suspension (spectrum 1A), the impermeable nitroxide derivative, was completely quenched (spectrum 2A), because of broadening of the peaks by potassium ferricyanide, when the extracellular quenching agent was added. For the permeable nitroxide, PCA, the intracellular ESR spectrum, protected from the extracellular quencher, was observed to increase within 5 minutes to a maximum (spectrum 2B) that was approximately 30 % of the ESR signal observed in the erythrocyte suspension (hematocrit 50 %) without the quencher (spectrum 1B), indicating that membrane penetration of PCA is rapid. For TAP, the broad lines observed in the erythrocyte suspension (spectrum 2C) are similar to those observed in the erythrocyte lysate (spectrum 4C). Therefore, no intracellular signal was observed for TAP even after a 60-minute incubation.

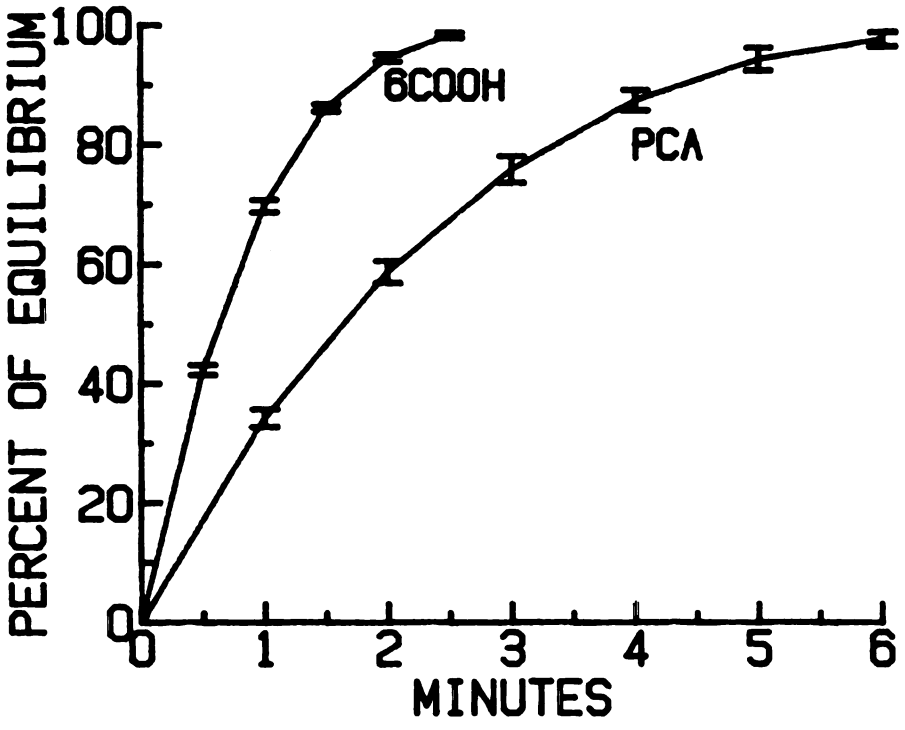


Figure 8.2 The carboxylic acid derivatives enter the erythrocyte at measurable rates. Half-times of equilibration were 0.6 min for 6-COOH and 1.6 min for PCA. The intracellular ESR signal is expressed as a percent of the equilibrium value reached in less than 8 min. The bars represent standard deviations of data from three experiments.

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

The carboxylic acid derivatives gave a stronger intracellular signal (33 and 29 percent for 6-COOH and PCA, respectively) than the hydroxyl derivatives (16 and 13 percent for 6-OH and 5-OH, respectively). The amine derivatives gave an even weaker intracellular signal (4 and 2 percent for 6-NH_2 and 5-NH_2, respectively) than the hydroxyl derivatives. From these results, the cell volume and intracellular pH of the erythrocyte can be estimated (Quintanilha and Mehlhorn, 1978).

Assuming that the hydroxyl derivatives equilibrate to the same intracellular and extracellular concentrations, the cell volume accessible to these nitroxides is approximately 15 percent of the total volume of the erythrocyte suspension or 30 percent of the packed cell volume. This may be expected because the packed cell volume includes spaces between cells. Furthermore, only part of the cell volume is accessible because the erythrocyte contains a high concentration of hemoglobin.

The ratio of intracellular and extracellular concentration for the acids and bases, calculated by dividing intracellular signal for the carboxylic acid and amine derivatives with the intracellular signal for the hydroxyl derivatives, can be used to estimate the pH gradient across the cell membrane (Quintanihla and Mehlhorn, 1978; Mehlhorn and Probst, 1982; Mehlhorn and Packer, 1983). The pKa values 4.4 and 9.4 were used for the carboxylic acid and amine derivatives, respectively. These pKa values were previously determined for the pyrroline analog of PCA and 6-NH_2 (Mehlhorn and Probst, 1982). The extracellular pH was measured (7.1 ± 0.06) and the intracellular pH was estimated to be 7.6 ± 0.2. This estimate is in agreement with a previous study in which the

intracellular pH of erythrocytes was found to be 7.8 at low temperatures (4 °C) and in the presence of impermeable anions (Minakami et al., 1975).

Reduction in Human Erythrocyte Suspension and Lysate

The loss of ESR signal in erythrocyte suspension and lysate is assumed to be caused by a one-electron reduction of the nitroxide moiety resulting in formation of the corresponding hydroxylamine. This conclusion is based on the observations that the peak width, of the low field line of the nitroxide ESR spectrum, remained constant during the experiment and that the original ESR signal was restored upon addition of 2 mM potassium ferricyanide, an oxidizing agent (see Chapter 2). Reductions of the piperidine derivatives, 6-COOH, 6-OH and 6-NH_2, in erythrocyte suspension and lysate are shown in Figure 8.3 (top panel). 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 the pyrrolidine derivatives showed the same rank order. The carboxylic acid derivatives were reduced more slowly than the hydroxyl derivatives which in turn were reduced more slowly than the amine derivatives. These differences in reduction rates do not appear to be related to differences in penetration rates because reduction is not rate limited by penetration. Quantitation of penetration was not conducted for the pyrrolidine derivatives because 90 percent or more remained after 30 min, i.e., they were all more stable than the piperidine derivatives.

Figure 8.3 (bottom panel) shows that the reduction rates of CAT and TPH are more rapid in erythrocyte lysate than in erythrocyte suspension. This indicates that the rate of reduction in erythrocyte suspension is limited by membrane penetration.

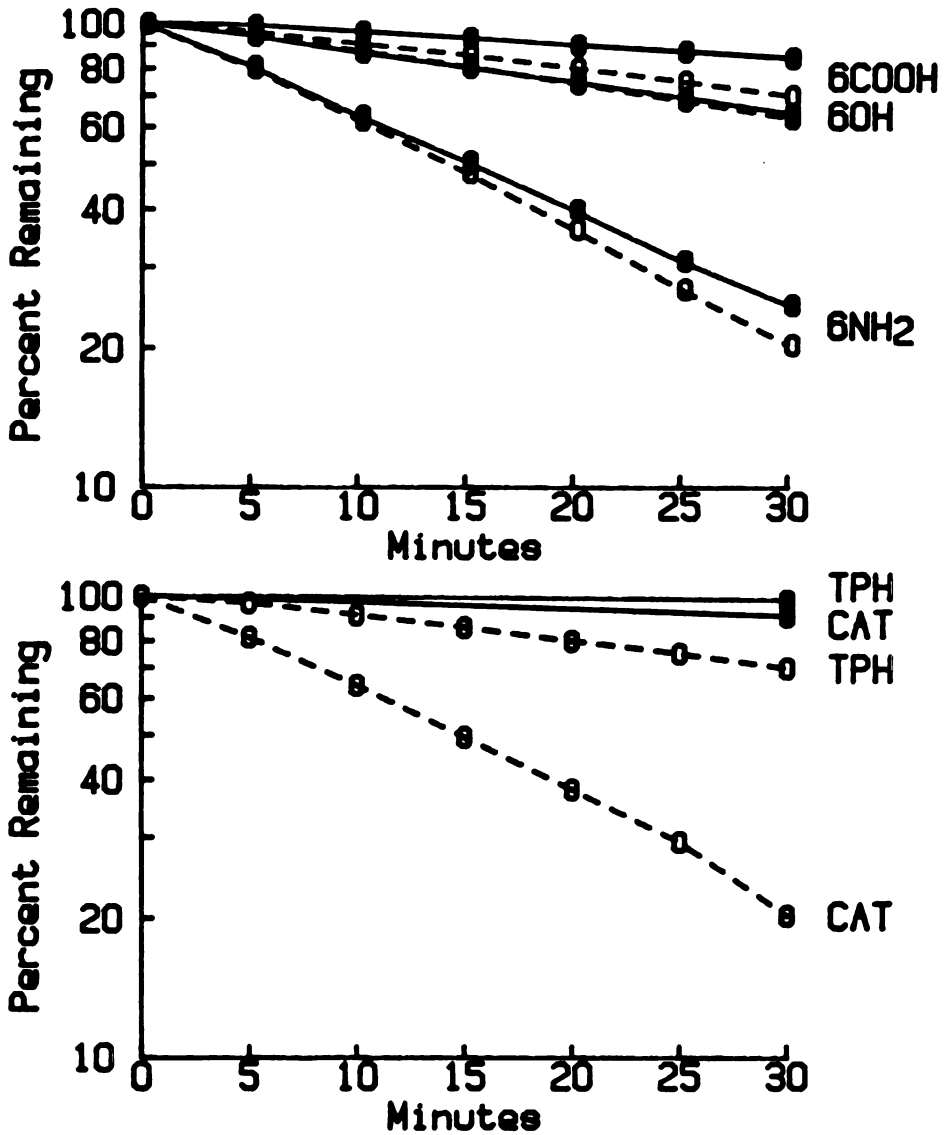


Figure 8.3 Differences in reduction rates between erythrocyte suspension (solid lines) and erythrocyte lysates (dashed lines) indicate penetration limitations. Top panel: nitroxide derivatives with little or no observed differences. Bottom panel: nitroxide derivatives with penetration limitations. Each data point is an average of at least three experiments; lines connect successive points. Initial nitroxide concentration was 0.01 mM. The coefficients of variation of the data (not shown) increased from 1-2 % at the first time point to a maximum of 15 % at the last. Experiments were run at room temperature (22.5 ± 1 °C).

The slow reduction of TPH and CAT in erythrocyte suspension may be explained by leakage of a reducing agent from the cell. All derivatives were stable (no detectable decrease in the ESR signal in 30 min) in buffer solution as well as in supernatant fluid separated from freshly-prepared erythrocyte suspension. However, when supernatant fluid was separated from an erythrocyte suspension prepared 8 hours earlier, reducing activity was observed in the supernatant fluid (2.5 and 11.1 percent reduced in 30 min for TPH and CAT, respectively). This reducing activity was comparable to that in erythrocyte suspension (2.0 and 9.6 percent reduced in 30 min for TPH and CAT , respectively). No significant lysis of the erythrocytes in the suspensions appeared to occur because the protein concentrations in supernatant fluids were about 0.6-0.8 percent of that in erythrocyte lysates.

Determination of Octanol : Buffer Distribution Coefficients

The distribution coefficients between 1-octanol and buffer are shown in Table 8.1 for buffer values of 2.5 and 7.0.

Table 8.1 *Octanol : Buffer Distribution Coefficients of Nitroxides.* [a]

Nitroxide Derivative	pH 2.5 (b)	pH 7.0
6-OH	3.9	4.2
6-NH2	0.0084	0.043
6-COOH	18.6	0.019
CAT	<0.0004	0.0004
TPH	0.046	0.01
5-OH	2.1	1.7
5-NH2	0.064	0.25
PCA	10.3	0.008

[a] Average of at least two determinations that differed by less than 20 %.

[b] The pH of the phosphate buffer phase.

Discussion

Determination of Membrane Penetration

The extracellular quenching agent potassium ferricyanide provides a means of selectively measuring the intracellular ESR signal. This technique, described in a review by Mehlhorn and Packer (1983), has been utilized for determination of cell volume, electrochemical and pH gradients across cell membranes, intracellular viscosity and membrane permeability (Lin et al., 1983; Lomax and Mehlhorn, 1985; Mehlhorn and Probst, 1982; Mehlhorn et al., 1982; Mehlhorn et al., 1985; Melandri et al., 1984; Morse II, 1971; Quintanilha and Mehlhorn, 1978). No damage to the cell membrane by the quenching agent was reported. The 200 mM ferricyanide concentration creates a hypertonic environment, but the cell volume estimated in the present study 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.

Low cell membrane permeability to TAP compared to PCA could be a reason for the slow bioreduction of TAP *in vivo* in the dog and the rat (Chapter 5). Several mechanisms of bioreduction have been suggested (Rauckman et al., 1984), including intracellular enzymatic reduction by electron transport systems such as the cytochrome P450 in microsomes (Rosen and Rauckman, 1977; Rosen et al., 1977) and the respiratory chain in mitochondria (Quintanilha and Packer, 1977), that may be limited by the ability of the nitroxide to penetrate cell membranes.

The slow penetration of TAP across the human erythrocyte membrane (Figure 8.1) is in contrast to that obseved for PCA. No intracellular ESR signal

was observed for TAP within 60 min. The reduction of TAP in erythrocyte suspension is less than 5 percent in 30 min. The equilibrium half-life for penetration of PCA across the erythrocyte membrane was 1.6 minutes (Figure 8.2). Thus, the slower bioreduction of TAP compared to PCA may be related to its lower cell membrane permeability. The impermeable nitroxide, CAT, was previously found to have relatively low clearance in the rat compared to other piperidine derivatives (Griffeth et al., 1984). However, *in vitro* studies have shown cationic nitroxides to be reduced more rapidly than neutral and anionic derivatives (Keana and VanNice, 1984; Couet et al., 1985a; 1985b). These previously reported results for CAT also support the theory that bioreduction occurs mainly intracellularly and that membrane permeability can be a rate-limiting step.

Reduction in Human Erythrocyte Suspension and Lysate

The erythrocyte suspension is an *in vitro* system in which the bioreduction and membrane permeability of nitroxides can be simultaneously studied. Erythrocytes have been demonstrated to reduce nitroxides (Blackett at al., 1974; Giotta and Wang, 1972). Reduction of the nitroxides in erythrocyte suspension is assumed to occur inside the cell and can, therefore, be limited by the membrane permeability. When the rate constant for reduction inside the cell is greater than that for transport into the cell, then the rate of reduction observed in the erythrocyte suspension represents the penetration rate. Because the susceptibility of nitroxides to reduction varies with structure, it is important to determine the reduction rate in both erythrocyte suspension and lysate. The reduction rate in erythrocyte lysate is assumed to be the same as that inside the intact erythrocyte. This approach to study membrane permeability of the

erythrocyte membrane based on the reducing capability of the cell was first reported by Ross and McConnell (1975).

In this study, the assumptions of the model were validated. The impermeable nitroxide derivatives, TPH and CAT, were not reduced siignificantly in the erythrocyte suspension indicating that the reduction occurs intracellularly. For the permeable derivatives, reduction was found to be slow in comparison with penetration in that reduction rates were essentially the same in erythrocyte suspension and lysate. These observations agree with the results of the experiments in which the peneration rates were determined. However, using the quenching agent, the penetration rate could be determined even when it was faster than the reduction process. The limiting factor in these experiments is how soon the intracellular signal can be monitored after addition of the nitroxide. In our studies this was about 30 seconds. Consequently, half-times of equilibration shorter than 10 seconds cannot be determined. The penetration rates of the amine and hydroxyl derivatives were estimated to be less than 10 seconds because the maximum intracellular ESR signal was observed instantaneously. The carboxylic acid derivatives had intermediate penetration rates while CAT and TPH appeared to be impermeable.

The model described by Ross and McConnell (1975) has been used in recent studies of erythrocyte membrane permeability (Bartosz, 1981; Bartosz and Gwozdzinski, 1983; Gwozdzinski et al., 1981; 1983). In these studies, the penetration rates of the nitroxide were assumed to be slow in comparison with reduction. Our findings show that this assumption was incorrect for the nitroxide derivatives used and that the results of these previous studies 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 observed previously in ascorbic acid

solution (Keana and VanNice, 1984; Couet et al., 1985a) and rat tissue homogenates (Couet et al., 1985b). The structural dependencies of reduction in these systems were discussed in Chapters 6 and 7. Ascorbic acid has been suggested to be the reducing agent in bovine erythrocytes (Bartosz et al., 1981). In a study of the reduction of nitroxides in mice erythrocytes, it was concluded that sulfhydryl-containing compounds, such as cysteine, were responsible for the reduction (Giotta and Wang, 1972). These small molecular weight-reducing agents could conceivably leak out of the cell. The observations that supernatant fluid separated from erythrocyte suspension 8 hours after its preparation could be an indication that reducing agents leak out of the erythrocyte. Lysis of the cell did not appear to occur. Furthermore, the buffer or supernatant fluid separated from a freshly-prepared erythrocyte suspension did not reduce the nitroxides.

Determination of Octanol : Buffer Distribution Coefficients

Distribution coefficients were determined as indices of lipophilicity of the nitroxide derivatives. Lipophilic compounds are expected to penetrate the phospholipid structure of the cell membrane faster than hydrophilic ones, unless specific carriers facilitate the transport (Stein, 1967).

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

The relatively rapid penetration rate for the carboxylic acid and amine derivatives may be explained by the lipophilicity of the uncharged form. For these derivatives, the distribution coefficients are low (Table 8.1) which is expected because at the pH of 7.4 these derivatives are essentially completely

in their respective charged form. For the carboxylic acids, the distribution coefficients are about 100 fold higher at pH 2.5 than at pH 7.4. These distribution coefficients at pH 2.5 are presumably those of the uncharged protonated forms of the carboxylic acids. Previously determined pKa values for 6-NH2 and the pyrroline analogue of PCA were 9.37 and 4.4, respectively (Mehlhorn and Probst, 1982).

In conclusion, different types of experiments were performed to evaluate the cell membrane permeability of nitroxide derivatives. Membrane penetration rates were assessed using two approaches: by appearance in intracellular fluids in the presence of an extracellular quencher, potassium ferricyanide; and by rates of reduction in lysate and cell suspension. The two techniques gave observations that correlated well with each other: the reduction of the hydrophilic nitroxide derivatives, CAT and TPH, for which no intracellular ESR signal was observed within 60 min, was rate limited by membrane permeability; for the more lipophilic nitroxide derivatives, penetration across the cell membrane appeared to be more rapid than their reduction rates. Reduction of nitroxides in erythrocyte suspension was shown to occur intracellularly. The observed erythrocyte membrane permeability to TAP and PCA support the hypothesis that reducing activity *in vivo* is predominantly present intracellularly and that reduction may be limited by membrane permeability.

Conclusions

The pharmacokinetics was examined in the dog after intravenous doses of 0.1 and 2.5 mmol/kg of TES, PCA or TAP. In this dose range, the clearance of TES decreased at the higher dose while the pharmacokinetic parameters of PCA and TAP were independent of dose. The pharmacokinetics of TAP was also examined in the rat. The clearance of TAP was about two-fold higher per kg in the rat than in the dog the steady-state volume of distribution per kg was comparable to that in the dog. The rates of elimination (half-lifes of 10 to 50 min) were compatible with the use of these nitroxides as MRI contrast agents.

For the nitroxide derivatives studied, the metabolic fates in both animal species were similar as follows: urinary recoveries of the unchanged nitroxides and their corresponding hydroxylamines were essentially complete (range of 83 to 98 percent of the dose for urine collected during 24 hours after adminstration); urinary recovery was independent of dose; the hydroxylamine was the only metabolite identified in urine; and anticipated reduction of the nitroxide moiety to the corresponding amine or conjugation of the carboxylic acid or hydroxyl functional groups of the nitroxide derivatives were not observed. As expected from the relatively rapid appearance in urine, contrast enhancement of the bladder and kidneys was demonstrated for these nitroxide derivatives in concurrent studies in animals. The renal clearances of PCA and TAP, estimated in the dog, were approximately equal to the glomerular filtration rate.

The reduction of the nitroxide moiety in these compounds has implications for their use as MRI contrast agents because the hydroxylamine, the product of this bioreduction, lacks contrast enhancing activity. Bioreduction appeared to be influenced by the chemical structure of the nitroxides. Reducibility, examined in ascorbic acid, rat tissue homogenates and human erythrocytes, was greater for piperidine derivatives than for pyrrolidine derivatives.

Membrane permeability may also influence bioreduction of nitroxides which, at least in part, appears to occur intracellularly. This was supported by the relatively low clearance in the dog observed for TAP, for which the penetration rate across the erythrocyte membrane was slow, compared to PCA which rapidly penetrated the erythrocyte membrane.

Reducing activity in rat tissue homogenates was highest in liver and kidney. The reduction in these tissues was partly caused by ascorbic acid. The remaining reducing activity was related to sulfhydryl-containing macromolecules. This activity may be enzymatic in origin because the reduction appeared to be more rapid at lower nitroxide concentrations and was dependent on NADPH, a electron donor to microsomal and mitochondrial enzyme systems.

Of the three nitroxide derivatives studied, TAP appeared to be the best candidate for use as an MRI contrast agent because of its low metabolic clearance, presumably due to low reducibility and slow cell membrane penatration, and the high fraction of the dose excreted unchanged. Furthermore, the high urinary recovery and absence of dose dependencies of the pharmacokinetic parameters favors the use of TAP.

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Appendix

Table A3.1 *TES Experiment in a Mongrel Dog*

Date: 10/12 - 83

Body Weight: 27.2 kg

Dose: 0.5375 mmol/kg of TES

Sampling Times (min)	Plasma Concentrations (mM)		
	TES	Hydroxylamine	Total
5.0	2.550	0.059	2.609
10.0	1.700	0.189	1.889
15.0	1.310	0.064	1.374
30.0	0.674	0.241	0.915
45.0	0.304	0.378	0.682
60.0	0.190	0.367	0.557
90.0	0.059	0.291	0.350
120.0	0.029	0.195	0.224
146.0	---	0.170	0.170
180.0	---	0.113	0.113
210.0	---	0.074	0.074
240.0	---	0.047	0.047
270.0	---	0.016	0.016

Table A3.2 *PCA Experiment in a Mongrel Dog.*

Date: 10/25 - 83

Body Weight: 28.2 kg

Dose: 0.5375 mmol/kg of PCA

Sampling Times (min)	Plasma Concentrations (mM)		
	PCA	Hydroxylamine	Total
5.0	2.150	0.248	2.398
10.0	1.378	0.339	1.716
15.0	1.064	0.296	1.359
30.0	0.699	0.338	1.037
45.0	0.522	0.409	0.931
60.0	0.376	0.160	0.536
120.0	0.120	0.402	0.522
145.0	0.072	0.338	0.409
180.0	0.037	0.231	0.268
210.0	0.023	0.210	0.233
242.0	0.016	0.198	0.214
270.0	0.011	0.138	0.149
304.0	0.009	0.119	0.127
330.0	0.008	0.104	0.112
360.0	0.006	0.057	0.063

Table A3.3 *TES Concentrations in Dog Plasma Measured with the ESR and HPLC Assays.*

Date: 3/9 - 83

Mongrel Dog

Body Weight: 29.2 kg

Dose: 0.5535 mmol/kg of TES

Sampling Times (min)	Plasma Concentrations (mM) ESR	HPLC
5.5	1.772	1.922
10.3	1.373	1.459
15.3	1.148	1.185
30.0	0.466	0.611
45.5	0.405	0.398
59.8	0.214	0.208
90.7	0.226	0.224
119.6	0.121	0.121
150.0	0.105	0.100

Table A3.4 *PCAConcentrations in Dog Plasma Measured with the ESR and HPLC Assays.*

Date: 3/8 - 83

Mongrel Dog

Body Weight: 30.5 kg

Dose: 0.5375 mmol/kg of PCA

Sampling Times (min)	Plasma Concentrations (mM) ESR	HPLC
5.0	1.322	1.335
10.0	1.019	0.991
15.0	0.934	0.907
30.0	0.789	0.759
45.0	0.692	0.647
60.0	0.619	0.591
90.0	0.535	0.469
120.0	0.426	0.396
150.5	0.329	0.290
180.0	0.292	0.254
210.0	0.238	0.203
240.0	0.196	0.181
270.0	0.170	0.159
300.0	0.148	0.134
330.0	0.134	0.131
360.0	0.109	0.092

Table A4.1 *Low-Dose TES Experiment in Dog #1.*

Date: 4/17 - 84

Body Weight: 11.8 kg

Dose: 0.1 mmol/kg of TES

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	TES	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
4.9	0.203	0.048	0.251	0-1	44.2
10.0	0.119	0.083	0.201		
15.0	0.075	0.079	0.154	1-2	14.0
20.0	0.047	0.068	0.115		
25.0	0.031	0.061	0.092	2-6	10.4
30.0	0.021	0.067	0.088		
40.0	0.010	0.055	0.065	6-24	1.5
50.0	0.005	0.051	0.055		
60.0	0.002	0.048	0.050	Total	73.8
90.0	---	0.038	0.038		
120.0	---	0.029	0.029		
150.0	---	0.022	0.022		
184.5	---	0.018	0.018		
210.0	---	0.017	0.017		

Table A4.2 *High-Dose TES Experiment in Dog #1.*

Date: 5/29 - 84

Body Weight: 11.8 kg

Dose: 2.5 mmol/kg of TES

Sampling Times (min)	Blood Concentration TES (mM)	Urinary Recovery Collection Period (hours)	Urinary Recovery Percent of Dose
4.9	6.024	0-1	69.6
10.0	4.413		
15.0	3.565	1-2	18.7
20.0	3.106		
30.0	2.189	2-6	10.5
45.0	1.322		
60.0	0.824	6-24	2.6
75.0	0.476		
90.0	0.270	Total	101.3
105.0	0.133		
120.0	0.063		
135.0	0.033		
150.0	0.021		
165.0	0.012		
180.0	0.008		

Table A4.3 *Low-Dose TES Experiment in Dog #2.*

Date: 4/10 - 84

Body Weight: 14.6 kg

Dose: 0.1 mmol/kg of TES

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	TES	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.0	0.199	0.073	0.272	0-1	56.1
10.0	0.111	0.098	0.209		
15.0	0.079	0.082	0.161	1-2	15.2
20.0	0.054	0.088	0.142		
25.0	0.038	0.096	0.133	2-6	18.3
30.0	0.025	0.090	0.115		
40.0	0.013	0.092	0.105	6-24	6.7
50.0	0.007	0.083	0.089		
60.0	0.004	0.072	0.076	Total	96.5
90.0	---	0.050	0.050		
120.0	---	0.035	0.035		
150.0	---	0.032	0.032		
180.0	---	0.023	0.023		
210.0	---	0.019	0.019		
240.0	---	0.016	0.016		

Table A4.4 *High-Dose TES Experiment in Dog #2.*

Date: 5/17 - 84

Body Weight: 14.5 kg

Dose: 2.5 mmol/kg of TES

Sampling Times (min)	Blood Concentration TES (mM)	Urinary Recovery Collection Period (hours)	Urinary Recovery Percent of Dose
5.3	6.658	0-1	56.9
9.9	4.711		
14.9	4.009	1-2	20.3
20.1	3.284		
25.3	2.811	2-6	14.7
30.0	2.534		
40.0	1.735	6-24	1.4
50.4	1.166		
60.1	0.849	Total	93.2
75.1	0.457		
85.0	0.317		
95.5	0.197		
105.0	0.120		
120.0	0.055		

Table A4.5 *Low-Dose TES Experiment in Dog #3.*

Date: 4/19 - 84

Body Weight: 14.8 kg

Dose: 0.1 mmol/kg of TES

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	TES	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.0	0.208	0.105	0.313	0-1	66.3
10.2	0.109	0.085	0.193		
15.1	0.061	0.103	0.163	1-2	15.1
19.9	0.031	0.083	0.115		
25.0	0.023	0.088	0.111	2-6	8.0
30.0	0.012	0.064	0.076		
40.5	0.003	0.064	0.067	6-24	0.9
50.2	0.001	0.064	0.065		
59.6	---	0.053	0.053	Total	90.3
90.0	---	0.033	0.033		
120.0	---	0.020	0.020		
150.0	---	0.015	0.015		
181.0	---	0.010	0.010		

Table A4.6 *High-Dose TES Experiment in Dog #3.*

Date: 5/24 - 84

Body Weight: 15.0 kg

Dose: 2.5 mmol/kg of TES

Sampling Times (min)	Blood Concentration TES (mM)	Urinary Recovery Collection Period (hours)	Urinary Recovery Percent of Dose
5.0	5.121	0-1	43.0
10.1	3.461		
20.0	2.615	1-2	9.8
30.0	2.183		
45.0	1.215	2-6	12.4
60.0	0.725		
75.0	0.328	6-24	4.5
90.0	0.173		
105.0	0.855	Total	69.7
120.0	0.041		
135.0	0.026		
151.0	0.013		
165.0	0.008		

Table A4.7 *Low-Dose PCA Experiment in Dog #1.*

Date: 5/15 - 84

Body Weight: 12.0 kg

Dose: 0.1 mmol/kg of PCA

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	PCA	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.0	0.240	0.041	0.281	0-1	37.2
10.0	0.184	0.023	0.207		
15.0	0.147	0.035	0.181	1-2	20.0
20.0	0.124	0.041	0.165		
25.0	0.105	0.057	0.163	2-6	16.3
30.0	0.088	0.060	0.149		
50.0	0.063	0.056	0.119	6-24	0.1
60.0	0.045	0.067	0.113		
75.0	0.035	0.059	0.095	Total	73.6
91.0	0.022	0.059	0.081		
105.0	0.015	0.060	0.075		
120.0	0.011	0.061	0.072		
150.0	---	0.047	0.047		
180.0	---	0.041	0.041		
210.0	---	0.040	0.040		
240.0	---	0.027	0.027		
270.0	---	0.021	0.021		
300.0	---	0.017	0.017		
330.0	---	0.012	0.012		
360.0	---	0.012	0.012		

Table A4.8 *High-Dose PCA Experiment in Dog #1.*

Date: 2/8 - 84

Body Weight: 10.5 kg

Dose: 2.5 mmol/kg of PCA

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	PCA	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.0	6.093	---	5.370	0-6	81.4
10.0	4.486	---	3.884		
20.0	3.189	0.017	3.205	6-24	2.1
30.0	2.499	0.282	2.780		
60.0	1.222	0.892	2.114	Total	83.5
90.0	0.622	0.818	1.440		
120.0	0.314	0.791	1.105		
150.0	0.175	0.634	0.809		
180.0	0.101	0.515	0.617		
210.0	0.605	---	0.490		
251.0	---	0.303	0.303		
270.0	---	0.252	0.252		
300.0	---	0.190	0.190		

Table A4.9 *Low-Dose PCA Experiment in Dog #2.*

Date: 2/2 - 84

Body Weight: 11.2 kg

Dose: 0.1 mmol/kg of PCA

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	PCA	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.0	0.236	0.017	0.254	0-6	78.4
10.0	0.177	0.025	0.203		
15.0	0.138	0.036	0.174	6-24	---
20.0	0.116	0.050	0.167		
25.0	0.096	0.057	0.153	Total	78.4
30.0	0.086	0.069	0.155		
40.0	0.058	0.087	0.146		
50.0	0.045	0.086	0.131		
60.0	0.031	0.085	0.116		
75.0	0.019	0.085	0.104		
90.0	0.011	0.080	0.091		
120.0	---	0.071	0.071		
180.0	---	0.061	0.061		
240.0	---	0.037	0.037		
300.0	---	0.030	0.030		
360.0	---	0.019	0.019		

Table A4.10 *High-Dose PCA Experiment in Dog #2.*

Date: 2/23 - 84

Body Weight: 12.0 kg

Dose: 2.5 mmol/kg of PCA

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	PCA	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.0	5.770	0.340	6.110	0-6	89.8
10.0	4.245	0.425	4.670		
20.0	3.110	0.820	3.930	6-24	3.9
30.0	2.314	0.957	3.270		
45.0	1.736	1.059	2.795	Total	93.7
60.0	1.202	1.418	2.620		
75.0	0.781	1.270	2.050		
90.0	0.592	1.399	1.990		
105.5	0.403	1.098	1.500		
120.5	---	1.270	1.270		
150.0	---	1.015	1.015		
180.0	---	0.740	0.740		
210.0	---	0.650	0.650		
240.0	---	0.370	0.370		
270.0	---	0.385	0.385		
300.0	---	0.305	0.305		
330.0	---	0.245	0.245		
360.0	---	0.215	0.215		

Table A4.11 *Low-Dose PCA Experiment in Dog #3.*

Date: 4/5 - 84

Body Weight: 15.2 kg

Dose: 0.1 mmol/kg of PCA

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	PCA	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.0	0.243	---	0.195	0-1	20.1
10.0	0.154	0.007	0.161		
15.0	0.136	---	0.128	1-2	44.4
20.0	0.110	0.004	0.114		
25.0	0.094	0.005	0.099	2-6	20.6
40.0	0.081	0.021	0.102		
50.0	0.057	0.043	0.100	6-24	1.6
60.0	0.042	0.053	0.095		
75.0	0.035	0.039	0.074	Total	86.7
90.0	0.021	0.047	0.068		
105.0	0.015	0.043	0.058		
150.0	---	0.040	0.040		
180.0	---	0.035	0.035		
210.0	---	0.024	0.024		
240.0	---	0.025	0.025		
370.0	---	0.019	0.019		
300.0	---	0.016	0.016		

Table A4.12 *High-Dose PCA Experiment in Dog #3.*

Date: 2/29 - 84

Body Weight: 13.3 kg

Dose: 2.5 mmol/kg of PCA

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	PCA	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.0	5.050	0.450	5.500	0-6	91.7
10.0	4.250	0.635	4.885		
20.0	2.935	1.097	4.032	6-24	0.4
30.0	2.390	0.741	3.131		
45.0	1.673	0.928	2.600	Total	92.1
60.0	1.045	1.032	2.077		
75.0	0.629	1.038	1.667		
90.0	0.557	1.086	1.642		
105.0	0.400	0.964	1.364		
120.0	---	1.383	1.383		
180.0	---	0.845	0.845		
210.0	---	0.557	0.557		
240.0	---	0.417	0.417		
276.0	---	0.335	0.335		
316.0	---	0.200	0.200		
336.0	---	0.163	0.163		

Table A5.1 *Low-Dose TAP Experiment in Dog #1.*

Date: 6/13 - 84

Body Weight: 11.6 kg

Dose: 0.1 mmol/kg of TAP

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	TAP	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.0	0.272	0.107	0.379	0-2	60.4
10.0	0.224	0.069	0.293		
15.0	0.186	0.022	0.208	2-6	18.4
20.0	0.169	0.043	0.212		
30.0	0.134	0.064	0.198	6-24	5.6
40.0	0.099	0.022	0.121		
50.0	0.088	0.039	0.127	Total	84.4
60.0	0.073	0.042	0.115		
75.0	0.056	0.055	0.111		
90.0	0.045	0.018	0.063		
105.0	0.034	0.024	0.058		
120.0	0.026	0.031	0.057		
150.0	0.017	0.021	0.038		
180.0	0.012	0.018	0.030		
210.0	---	0.011	0.011		
240.0	---	0.012	0.012		

Table A5.2 *High-Dose TAP Experiment in Dog #1.*

Date: 5/6 - 85

Body Weight: 11.4 kg

Dose: 2.5 mmol/kg of TAP

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	TAP	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.4	5.963	---	5.945	0-2	73.1
10.3	4.807	0.392	5.198		
15.0	3.916	0.657	4.573	2-6	17.2
19.9	3.724	0.493	4.216		
30.1	3.126	0.339	3.466	6-24	4.4
45.0	2.533	0.442	2.975		
60.0	1.794	0.484	2.278	Total	94.7
75.0	1.610	0.393	2.003		
90.0	1.229	0.482	1.711		
105.5	0.942	0.571	1.513		
120.1	0.800	0.515	1.314		
136.0	0.639	0.444	1.083		
150.4	0.566	0.408	0.974		
180.2	0.382	0.317	0.699		
211.0	0.299	0.269	0.568		
240.8	0.164	0.256	0.419		
270.0	0.130	0.209	0.339		
300.0	0.074	0.192	0.266		
331.4	0.061	0.154	0.215		
358.0	0.049	0.126	0.175		

Table A5.3 *Low-Dose TAP Experiment in Dog #2.*

Date: 6/4 - 84

Body Weight: 14.5 kg

Dose: 0.1 mmol/kg of TAP

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	TAP	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
4.9	0.287	0.030	0.317	0-2	63.7
10.0	0.246	0.042	0.288		
15.0	0.229	---	---	2-6	18.5
20.0	0.194	0.071	0.265		
30.0	0.161	0.057	0.218	6-24	0.3
40.0	0.136	0.029	0.165		
50.0	0.111	0.084	0.195	Total	82.5
60.5	0.098	0.041	0.139		
75.0	0.069	0.063	0.132		
90.0	0.056	0.074	0.129		
105.0	0.042	0.042	0.084		
120.0	0.031	0.029	0.060		
150.0	0.017	0.053	0.070		
180.0	0.015	0.031	0.046		
210.0	0.008	0.027	0.035		
240.5	0.004	0.029	0.033		
270.0	0.002	0.023	0.025		
300.0	---	0.017	0.017		
330.0	---	0.022	0.022		
360.0	---	0.013	0.013		

Table A5.4 *High-Dose TAP Experiment in Dog #2.*

Date: 6/20 - 84

Body Weight: 14.2 kg

Dose: 2.5 mmol/kg of TAP

Sampling Times (min)	Blood Concentrations (mM)			Urinary Recovery	
	TAP	Hydroxylamine	Total	Collection Period (hours)	Percent of Dose
5.0	7.367	0.608	7.974	0-2	67.6
10.0	5.921	0.499	6.421		
15.0	4.998	0.654	5.653	2-6	19.6
20.0	4.413	0.426	4.839		
30.0	3.375	0.752	4.127	6-24	6.1
45.0	2.911	0.544	3.455		
60.0	2.339	0.514	2.854	Total	93.3
75.0	1.872	0.802	2.674		
90.0	1.346	0.773	2.119		
105.0	1.081	0.770	1.851		
120.0	0.988	0.667	1.655		
135.0	0.861	0.491	1.352		
150.0	0.557	0.531	1.088		
165.0	0.440	0.631	1.071		
180.0	0.375	0.499	0.873		
210.0	0.251	0.471	0.722		
240.0	0.186	0.408	0.594		
270.0	0.149	0.392	0.541		
300.0	0.077	0.380	0.458		
330.0	0.076	0.300	0.377		
360.0	0.057	0.241	0.299		

Table A5.5 *Low-Dose TAP Experiment in Rat #1.*

Body Weight 0.267 kg

Dose: 0.1 mmol/kg

Sampling Times (min)	Blood Concentrations (mM)		
	TAP	Hydroxylamine	Total
5.0	0.215	0.018	0.233
10.0	0.157	0.021	0.178
20.0	0.094	0.018	0.112
30.0	0.058	0.018	0.076
40.0	0.034	0.016	0.050
60.0	0.022	0.013	0.035
90.0	0.008	0.008	0.016
120.0	0.004	0.006	0.010

Table A5.6 *Low-Dose TAP Experiment in Rat #2.*

Body Weight: 0.295 kg

Dose: 0.1 mmol/kg of TAP

Sampling Times (min)	Blood Concentrations (mM)		
	TAP	Hydroxylamine	Total
5.0	0.216	0.002	0.218
10.0	0.160	0.025	0.185
20.0	0.093	0.024	0.117
30.0	0.057	---	---
40.0	0.040	0.021	0.061
60.0	0.022	0.010	0.032
90.0	0.010	0.012	0.022
120.0	0.007	0.006	0.013

Table A5.7 *Low-Dose TAP Experiment in Rat #3.*

Body Weight 0.261 kg

Dose: 0.1 mmol/kg

Sampling Times (min)	Blood Concentrations (mM)		
	TAP	Hydroxylamine	Total
5.0	0.216	0.003	0.219
10.0	0.163	0.013	0.176
20.0	0.104	0.011	0.115
30.0	0.076	0.020	0.096
40.0	0.059	0.018	0.077
60.0	0.038	0.019	0.057
90.0	0.017	0.012	0.029
120.0	0.010	0.008	0.018

Table A5.8 *High-Dose TAP Experiment in Rat #4.*

Body Weight 0.262 kg

Dose: 2.5 mmol/kg of TAP

Sampling Times (min)	Blood Concentrations (mM)		
	TAP	Hydroxylamine	Total
10.0	4.590	2.172	6.762
20.0	2.580	1.167	3.747
40.0	1.230	0.657	1.887
60.0	0.807	0.771	1.578
90.0	0.284	0.344	0.628
120.0	0.209	0.150	0.359
180.0	0.067	0.081	0.148
240.0	0.029	0.027	0.056
300.0	0.011	0.017	0.028
360.0	0.006	0.015	0.021

Table A5.9 *High-Dose TAP Experiment in Rat #5.*

Body Weight 0.257 kg

Dose: 2.5 mmol/kg of TAP

Sampling Times (min)	Blood Concentrations (mM)		
	TAP	Hydroxylamine	Total
10.0	4.380	0.553	4.933
20.0	2.870	0.622	3.492
40.0	1.030	1.214	2.244
60.0	0.662	0.430	1.092
90.0	0.202	0.318	0.520
120.0	0.117	0.177	0.294
180.0	0.042	0.082	0.124
240.0	0.021	0.028	0.049
300.0	0.013	0.030	0.043
360.0	0.007	0.014	0.021

Table A5.10 *High-Dose TAP Experiment in Rat #6.*

Body Weight 0.250 kg

Dose: 2.5 mmol/kg of TAP

Sampling Times (min)	Blood Concentrations (mM)		
	TAP	Hydroxylamine	Total
10.0	3.174	0.823	3.997
20.0	2.344	0.659	3.003
40.0	1.241	0.611	1.852
60.0	0.733	0.425	1.158
90.0	0.328	0.269	0.597
120.0	0.240	0.281	0.521
180.0	0.056	0.104	0.160
240.0	0.023	0.085	0.108
300.0	0.013	0.045	0.058
360.0	0.007	0.018	0.025

Table A5.11 *Urinary Recovery of TAP and its Hydroxylamine in the Rat.*

Collection Period (hours)	0.1 mmol/kg Rat #7	Rat #8	Rat #9
0-6	87.2	84.8	93.2
6-24	3.5	3.1	3.2
Total	90.7	87.9	96.4
	2.5 mmol/kg		
	Rat #10	Rat #11	Rat #12
0-6	92.9	90.1	90.0
6-24	5.0	9.2	5.6
Total	97.9	99.3	95.6

Table A5.12 *TAP Concentration in Dog and Rat Urine Measured with the ESR and HPLC Assays.*

Urine Concentration (mM)			
Dog		Rat	
HPLC	ESR	HPLC	ESR
173.5	184.6	41.8	41.8
79.0	84.3	28.0	26.5
45.4	49.7	15.6	15.6
33.0	35.0	4.0	4.1
17.1	18.1	2.1	1.9
16.0	14.7	1.5	1.6
7.5	8.6	1.5	1.6
3.2	3.4	1.1	0.9
2.5	2.7	0.9	1.1
1.2	1.4		