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INTERACTION OF SPIN LABELS WITH TRANSITION METALS *

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CONTENTS

* Metal—Nitroxyl Interactions, Part 5.

ABBREVIATIONS *

acac	acetylacetonate
ADP	adenosine diphosphate
aq	aqueous
ATP	adenosine triphosphate
CoA	coenzyme A
CPK	Corey—Pauling—Koltun
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTBN	di-(tert-butyl)nitroxyl
en	ethylenediamine
EPR	electron paramagnetic resonance
hfac	hexafluoroacetylacetonate
hipip	high potential iron protein
MISP	model iron sulfur protein
porph	porphyrin
tfac	trifluoroacetylacetonate
tRNA	transfer ribonucleic acid
VB_{12r}	vitamin B-12 reduced form

A. INTRODUCTION

The electron paramagnetic resonance (EPR) spectrum of a nitroxyl radical (spin label) attached to biological macromolecules serves as a probe of the structure and dynamics of the biological molecule. For example, such probes are being used to study allosteric interactions in proteins, and molecular dynamics and organization in membranes [1—20]. In most such applications, the spin label is the only paramagnetic species present, and interpretation of the lineshape can be made in terms of the orientation and motion of the spin label [1—20]. In application of the nitroxyl spin label technique to biological systems which contain a paramagnetic metal, interaction of the nitroxyl unpaired electron with the metal unpaired electron(s) can contribute to the lineshape of the nitroxyl EPR spectrum [5,6,20—23].

Transition metal—nitroxyl radical species also provide a means to study interactions between non-equivalent unpaired electrons, i.e., electrons with different g values, coupling constants and/or relaxation times. Thus the compounds surveyed in this review are potentially of interest to specialists in biochemistry, inorganic chemistry and magnetic resonance. An attempt has been made in this review to pull together observations from a wide variety of sources published through July 1977 (with a few later papers added after the

* Nitroxyl, nitroxide, spin label, or spin probe are used in various contexts to denote a free radical species containing the $\geq N\text{—}O$ moiety.

basic review was written) which relate to the problem of interpreting the EPR spectrum of a nitroxyl radical (or other organic free radical) in the presence of a paramagnetic transition metal.

The review includes some studies involving systems containing both a nitroxyl radical and a paramagnetic metal even though the original paper did not discuss metal—nitroxyl interactions. Thus an attempt has been made to cover the literature of metal—nitroxyl interactions thoroughly. However, in spite of systematic searches using Chemical Abstracts and Science Citation Index, many important papers were located serendipitously, so thoroughness surely has eluded us.

There is a large literature on EPR lineshapes and relaxation phenomena which is certainly relevant to the topic of metal—nitroxyl interactions, but coverage of that literature is beyond the scope of this review.

The theoretical analysis of lineshapes and relaxation times in systems composed of interacting nonequivalent paramagnetic centers is in its infancy. There are few theoretical predictions with which the experimental data can be compared. The dipolar interaction model of Leigh [21,22] has been used extensively in the biochemical literature and will be mentioned frequently in this review. Smith and Pilbrow [24] recently reviewed the status of the theory of EPR spectra of interacting dissimilar transition metal ions, and Buettner and Coffman [25] have treated in some detail the interaction between Co(II) and a free radical in a coenzyme B-12—enzyme reaction. However, treatments of this type have not been applied to metal—nitroxyl interactions. Because of the lack of essential quantitative EPR spectra, the experimental results presented in many papers are in as rudimentary a stage of development as the theoretical interpretation.

The first spin label experiments, reported by Ohnishi and McConnell in 1965, used the cation radical of chloropromazine to probe the intercalation of aromatic molecules in DNA [26]. Subsequent reports used nitroxyl radicals, which were more stable than chloropromazine and had simpler, more easily interpretable EPR spectra [1]. Rozantsev and co-workers had shown that standard organic functional group reactions can be effected in molecules containing nitroxyl radicals without destroying the nitroxyl radical [27]. This observation has led to the development of a wide range of nitroxyl radicals bearing functional groups that facilitate selective covalent labeling of specific locations in biomolecules [2—5,7,9,10,16,19,20,28—33]. A large number of nitroxyl-containing analogs of steroids, fatty acids and phospholipids have been prepared and used to study molecular association and motion in membranes and model membrane systems [13—19]. The information on molecular motion and orientation which can be obtained from the lineshape of the nitroxyl EPR spectrum has been discussed in detail [1,2,6,16,19,20,34—36].

In the following survey of the literature, spin-labeled heme proteins will be discussed first, followed by other classes of biological macromolecules. The numerous examples of the interpretation of Mn(II)—nitroxyl interactions in spin-labeled enzymes using Leigh's formula [21,22] are treated as a unit,

followed by similar studies involving other metals. The more "inorganic" studies involving discrete transition metal complexes containing nitroxyl radicals and studies of collisions between paramagnetic metals and nitroxyl radicals are given separate treatment. Finally, a few papers are surveyed in which paramagnetic transition metals interact with radicals other than nitroxyl radicals.

B. SPIN-LABELED HEME PROTEINS

(i) Hemoglobin

Most spin label studies of hemoglobin have involved attachment of a nitroxyl radical to the β-93 sulfhydryl group or to the reactive sites on the periphery of the porphyrin ring of the heme group. The first published suggestion that there could be observable interaction between a nitroxyl spin label and a paramagnetic transition metal in a spin-labeled molecule was made by Symons in the discussion of a paper by McConnell and Boeyens [37]. At that time no interaction had been detected [37], but a search was instituted [38]. Subsequently a large number of papers reported changes in the nitroxyl EPR spectrum upon changing the oxidation state and/or spin state of the iron in spin-labeled hemoglobin, and attributed the changes entirely to allosteric effects. Many of the papers did not mention the possibility of metal—nitroxyl interaction. Ogawa and McConnell [39] concluded that the spin label spectral changes did not result from spin—spin interactions between the iron and the nitroxyl because (a) there was no evidence of significant line broadening in deoxyhemoglobin, and (b) the nitroxyl EPR spectrum was unchanged when the oxyhemoglobin (iron spin $S = 0$) was converted to methemoglobin azide or cyanide ($S = 1/2$). It was argued that the relaxation times of the iron atoms in these molecules "must be so different" from those in deoxyhemoglobin as to make a spin—spin interaction model inconsistent with the observed spectra [39]. They concluded that the spin label was more than 15—20 Å away from the iron, but did not indicate how this estimate was obtained. Later workers used the low spin cyano heme complex as a "diamagnetic control" [40].

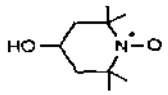
Likhtenshtein and coworkers described lineshape changes in spin-labeled hemoglobin upon changes in the oxidation state and spin state of the iron [41—43]. They concluded that the lineshape changes were not due to iron—nitroxyl spin—spin interactions "since the effects do not correlate with the number of unpaired iron electrons" [41,43]. When they labeled hemoglobin with both a nitroxyl radical and a copper complex the nitroxyl EPR spectrum was decreased in amplitude and broadened [42]. Comparison of the EPR spectra of doubly labeled hemoglobin in solution and in a solid matrix with EPR spectra of frozen solutions of mixtures of the nitroxyl radicals and the copper complexes led to the conclusion that the lineshape changes were due to exchange broadening upon collision of the nitroxyl with a copper complex bound sufficiently close. Static dipole—dipole interaction was excluded [42].

The solid water—glycerine matrix studies were interpreted as indicating that dipole—dipole and exchange interactions between the Cu(II) and the nitroxyl are not observed at distances greater than 12—15 Å [42]. Broadening of the nitroxyl spectra was observed when various paramagnetic ions were added to solutions containing spin-labeled hemoglobin, serum albumin or lysozyme. The results were interpreted as indicating that the spin labels were located on the protein surface [44].

In a series of papers beginning in 1969, Asakura and co-workers have demonstrated interaction between heme iron and nitroxyl spin labels [40,45—52]. Chronologically, it is important at this point to note that Leigh's dipolar interpretation of manganese—nitroxyl interactions (discussed below) was first published in 1969 [21] and influenced the post-1969 papers cited in this survey. Leigh's analysis of the effect of metal—nitroxyl interaction on the nitroxyl EPR spectrum predicts that in a rigid lattice there will be orientation-dependent line broadening proportional to $\mu_2^2 \tau r^{-6}$ where μ_2 is the metal magnetic moment, τ is the metal electron relaxation time and r is the distance between the metal and nitroxyl electron spins. Leigh points out that in certain cases the effect is manifested as a decrease in nitroxyl EPR spectrum amplitude without apparent change in lineshape [21,22]. Pre-1969 papers did not discuss explicitly the absolute amplitude of the spin label EPR signal. Asakura and coworkers observed that when spin labels were attached to the propionic acid side chains of the heme group in hemoproteins, changes in the spin state of the iron (in addition to conformational changes) caused changes in the amplitude of the nitroxyl EPR spectrum [40,46,52]. Interpretation as dipolar interaction effects in accordance with Leigh's treatment [21,22] resulted in estimates of the distance between the iron and the nitroxyl of 12.5 Å for hemoglobin, 12.0 Å for myoglobin, 9.0 Å for horseradish peroxidase and ~14 Å for cytochrome *c* peroxidase. The details of the analysis involve an estimate of 20° as the angle between the nitroxyl p_z orbital and the nitroxyl—iron vector, based on the shape of the nitroxyl EPR spectrum. Asakura et al. did not present a physical model consistent with both the distance and angle estimates, and none is obvious. Based on inspection of CPK molecular models the estimate of ~12.5 Å is reasonable for the iron—nitroxyl distance in the spin-labeled heme which Asakura et al. used. The iron—nitroxyl distances in hemoglobin were subsequently corrected to ~11.5—11.8 Å with the difference attributed to an impurity in the earlier study [50]. It is not clear, however, how with the same spin-labeled heme the iron—nitroxyl distance in cytochrome *c* peroxidase can be too large to show "significant interaction" — estimated as 14 Å. Asakura observed that the change in amplitude of the spin label EPR signal was greater for the change from oxyhemoglobin (magnetic moment $\mu = 0$) to deoxyhemoglobin ($\mu = 4.9$ BM) than for the change from oxyhemoglobin to acid methemoglobin ($\mu = 5.92$ BM). Since the opposite would be expected based on the magnetic moments according to Leigh's treatment [22] the effect was attributed to either a change in distance or in metal relaxation time [49]. Subsequently an estimate that the iron electron spin relaxa-

tion time in non-spin-labeled deoxyhemoglobin was longer than that in methemoglobin provided consistent rationalization, using Leigh's formula, for these differences. Although Asakura used the iron EPR signals to estimate the electron relaxation time for the iron in some hemoglobins [40,49], no mention was made of the iron EPR signal in the spin-labeled hemoglobin. Similar spin state dependent nitroxyl EPR spectral changes were observed when the spin-labeled heme was studied free in solution instead of bound in hemoglobin [50]. The tumbling rate estimated from the shape of the EPR spectrum was $\sim 10^9\ s^{-1}$, which is too fast to be consistent with the rigid-lattice assumptions of Leigh's analysis [22,50]. Nevertheless, based on a personal communication from Leigh it was assumed that the calculation would give the average distance between the metal and the nitroxyl for systems moving in solution [50]. The temperature dependence of the nitroxyl EPR spectra observed in this study [50] possibly results in part from changes in intermolecular collision rates and changes in modulation of spectral densities due to intramolecular (conformational) motions. Unfortunately integrated intensities of the EPR peaks were not reported.

Although, as described above, clear evidence for iron—nitroxyl interaction was observed for side-chain spin-labeled hemoglobin, no quantitative evidence for interaction of iron with a spin label attached to the β-93 sulfhydryl group was reported until 1972 [53]. Kokorin et al. observed that the ratio of the sum of the heights of high- and low-field lines to the height of the center line of the 3 line nitroxyl pattern at 77 K (d_1/d) was greater for spin-labeled methemoglobin than for spin-labeled oxyhemoglobin. Based on a correlation between d_1/d and the concentration of I in glycerine—water at 77 K, the values of d_1/d for the spin-labeled hemoglobins were attributed to dipolar interaction between the nitroxyl label and the iron [53].



I

Another study using this lineshape correlation multiplied the values of the distances obtained by 0.7 to convert from the random distribution of radicals inherent in the experimental correlation to isolated pairs of radicals [54].

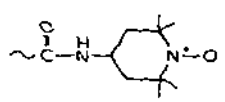
Kulikov described a method for obtaining the heme iron—nitroxyl distance from the power saturation curve of the nitroxyl radical. "Good agreement" was found for iron—nitroxyl distances in spin-labeled hemoglobin calculated by various methods, but significant uncertainty exists in measurement of relaxation times and in methods of accounting for angular dependence of the interactions [55].

Subsequently Kulikov and Likhtenshtein [56] provided additional details on these results. Using four different spin labels good agreement on nitroxyl—iron distances in the range ~14 to ~20 Å was obtained using five methods of estimating the distance. In addition to the lineshape parameter d_1/d used in

TABLE 1

Metal—nitroxyl distances estimated from changes in nitroxyl relaxation times [56]

Metal-containing species		Metal—nitroxyl distance (Å)	Method *
Hemoglobin, with various spin labels	II	⩾19.6	a
		13.7 ± 0.4	b
		⩾15.2	c
	III	17.0 ± 1.1	a
		15.1 ± 1.2	b
		15.9 ± 0.8	c
	IV	15.2 ± 1.1	a
		15.2 ± 1.2	b
		15.2 ± 0.8	c
	V	16.5 ± 2.0	a
		15.1 ± 1.2	b
		15.8 ± 1.1	c
Myosin/spin label II/Mn^{2+}		47.0 ± 2	a
Myosin/spin-labeled ATP/Mn^{2+}		42.0	a



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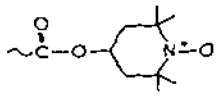

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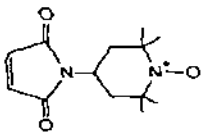

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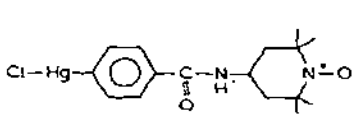


V

* a, $\Delta(1/T_1)$; b, $\Delta(1/T_2)$; c, $\Delta(1/T_1) \times \Delta(1/T_2)$

[53], and the Leigh formula [22], three methods based on measuring changes in T_1 and T_2 for the nitroxyl were used (see Table 1).

Kulikov and Likhtenshtein have emphasized the importance of relaxation studies for investigation of metal—nitroxyl interactions [55—57]. For a nitroxyl, experimentally

$$\frac{1}{T_1^0} < \frac{1}{T_2^0} \tag{1}$$

where T_1^0, T_2^0 are the relaxation times in the absence of metal. The authors then assert that dipole—dipole interaction gives approximately identical increments Δ_1, Δ_2

$$\frac{1}{T_1} = \frac{1}{T_1^0} + \Delta_1 \tag{2}$$

$$\frac{1}{T_2} = \frac{1}{T_2^0} + \Delta_2 \tag{3}$$

from which it is evident that T_1 is more sensitive than T_2. Consequently saturation curves are more sensitive indicators of metal—nitroxyl interaction than are nitroxyl line shapes [56,57]. It was found that the order of effectiveness in changing T_1 of nitroxyl radicals was $Ni^{2+} < Fe(CN)_6^{3-} \approx Co^{2+} < Mn^{2+}$. The microwave intensity, H_1^*, at which the amplitude of the EPR spectrum is equal to one-half of the maximum amplitude, was used to characterize the

effect of the metal on the nitroxyl. The H_1^* value was noticeably changed at mean Mn^{2+}—nitroxyl distances of 100 Å, whereas the shape of the nitroxyl EPR spectrum is not changed even at a mean Mn^{2+}—nitroxyl distance which results in an increase in H_1^* by a factor of 10 [56].

Expressions for T_1 and T_2 considering the total Hamiltonian of the dipole—dipole interaction in solid matrices were presented

$$\frac{1}{T_1} = \frac{1}{T_1^0} + \frac{\mu^2\gamma^2}{6r^6}\left[(1 - 3\cos^2\theta)^2 \frac{\tau_2}{1 + (\omega - \omega_i)^2\tau_2^2} + \frac{9}{2}\sin^2 2\theta \frac{\tau_1}{1 + \omega^2\tau_1^2} + 9\sin^4\theta \frac{\tau_2}{1 + (\omega + \omega_i)^2\tau_2^2}\right] \equiv \frac{1}{T_1^0} + \frac{A(\theta)}{r^6} \tag{4}$$

$$\frac{1}{T_2} = \frac{1}{T_2^0} + \frac{\mu^2\gamma^2}{6r^6}\left[2(1 - 3\cos^2\theta)^2\tau_1 + \frac{9}{2}\sin^2 2\theta \frac{\tau_2}{1 + \omega_i^2\tau_2^2} + \frac{1}{2}(1 - 3\cos^2\theta)^2 \frac{\tau_2}{1 + (\omega - \omega_i)^2\tau_2^2} + \frac{9}{4}\sin^2 2\theta \frac{\tau_1}{1 + \omega^2\tau_1^2} + \frac{9}{2}\sin^4\theta \frac{\tau_2}{1 + (\omega + \omega_i)^2\tau_2^2}\right] \tag{5}$$

where ω is the nitroxyl resonance frequency, ω_i is the metal resonance frequency, r is the distance between the spins, θ is the angle between the external field and the vector between the spins, μ is the magnetic moment of the metal, τ_1 is the metal longitudinal relaxation time and τ_2 is the metal transverse relaxation time. The superscript "0" denotes nitroxyl relaxation times in the absence of metal. These formulas were stated to be valid if $\mu^2\gamma^2\tau^2/r^6 << 1$, i.e., when the metal relaxation time is short (for short enough relaxation, $\tau_1 \approx \tau_2 = \tau$). If the further condition that $(\omega - \omega_i)^2\tau^2 >> 1$ is met, integration over all angles yields

$$\Delta\frac{1}{T_1} = \frac{\mu^2\gamma^2}{6r^6\tau}\left[\frac{4}{5(\omega - \omega_i)^2} + \frac{24}{5(\omega + \omega_i)^2} + \frac{12}{5\omega^2}\right] \tag{6}$$

$$\Delta\frac{1}{T_2} = \frac{4}{15}\frac{\mu^2\gamma^2\tau}{r^6} \tag{7}$$

from which it can also be seen that r can be calculated from the product $\Delta(1/T_1) \times \Delta(1/T_2)$ which does not depend on τ [56]. It was pointed out that the Leigh formula [22] differs from the first term of the expression for $1/T_2$ only by a numerical factor [56]. (In the above expressions some typographical errors in the original have been corrected.)

It was noted that when dipolar relaxation is dominant H_1^* is more sensitive than lineshape to the presence of the metal, but that in the case of spin-labeled nitrogenase (Section C) the lineshape changed markedly while H_1^* increased relatively little. These observations contradict the assumption of dominant dipolar interaction and require that exchange interactions be taken into account [56]. However, Dr. J.H. Hyde has pointed out in a personal com-

munication that it is important to recognize that this conclusion is valid only for certain values of τ_1. Hyde further recognized that exchange with a rapidly relaxing metal (short τ_1) is an effective "apparent" T_1 mechanism for the nitroxide. Thus if τ_1 is short enough an observation that H_1^* changes relatively little is proof that exchange is insignificant.

The addition of Cu(II) to hemoglobin, spin-labeled at the β-93 sulfhydryl group, caused a decrease in the amplitude of the nitroxyl signal, which was interpreted, using Leigh's formula, as indicating a copper—nitroxide distance of 13 Å [58]. The observed broadening of the nitroxyl spectrum was attributed to conformational changes, not copper—nitroxyl interaction. Microwave power saturation curves showed that T_1 for the nitroxyl decreased upon addition of Cu(II) [58]. Unfortunately, no information about the EPR spectrum of the Cu(II) was given in this paper. Prior work demonstrated that Cu(II) bound to hemoglobin exhibits well-resolved hyperfine structure interpreted as interaction with four nitrogens [59]. It would appear that the Cu(II) EPR spectrum is potentially important to the interpretation of the copper—nitroxyl interaction in this system.

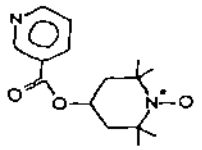
A recent paper by Artyukh et al. reported broadening of the high-field ($N_1 = -1$) nitroxyl EPR line in spin-labeled hemoglobin [60]. One "possible" explanation they suggested was spin—spin interaction with the iron of the heme [60]. Preferential broadening of one line is not consistent with spin—spin interaction. Unfortunately, this paper makes no reference to prior work in the area and does not provide sufficiently clear data to permit its use to amplify prior results at this time.

Finally it should be noted that many of the papers concerning spin-labeled hemoglobin emphasized in the spectra selected for the figures the "isosbestic points" in the nitroxyl EPR spectra. As Griffith has pointed out [49,61] these "isosbestic" points are actually only points of equal slope in the absorption curve, and cannot be used in the same way as isosbestic points in the absorption curve. Consequently many of the earlier papers on spin-labeled hemoglobin need reinterpretation.

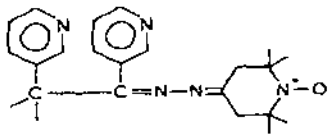
(ii) Cytochrome

The studies of spin-labeled porphyrins by Asakura, Drott and co-workers discussed in Section B(i), also included cytochrome *c* reconstituted with the spin-labeled porphyrin [45]. It was concluded that the distance between the iron and the nitroxyl was too large to permit observable dipole—dipole interaction between them. In this case both the iron and the nitroxyl resonances were observed [45].

Two analogs of metyrapone, **VI** and **VII**, have been used as spin labels which bind to the heme of cytochrome P-450 [62—64]. Griffin and co-workers [62,63] ascertained that **VI** coordinates through its pyridine nitrogen to the heme iron of cytochrome P-450. Addition of cytochrome P-450 to a solution of label **VI** substantially reduced the concentration of unbound label



VI

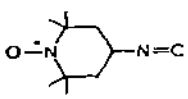


VII

($K_a \approx 4 \times 10^5$ M^{-1}) and yielded a new, broad signal. Analysis using Leigh's formula yielded an estimate of 6.7 Å as the distance between the iron and the nitroxide. Some line broadening of the bound nitroxide relative to a reference sample of nitroxide **VI** in glycerol was observed. Taking this broadening into account increases the distance estimate to ≈ 8.8 Å [62,63]. The iron EPR signal was "quite complicated" but certain features were identified as a resolved doublet due to splitting of the iron by the nitroxyl [62,63].

Nitroxyl **VII** was also judged to bind to the cytochrome P-450 via a pyridine nitrogen [64]. Use of the Leigh formula yielded an estimate of 11 ± 1 Å for the iron to nitroxyl distance. It was argued that exchange interactions could be ignored at distances >6 Å. The same type of analysis was used to estimate a distance of >8 Å between nitroxide **VII** bound to cytochrome P-450 and $Fe(CN)_6^{3-}$ added to the solution (measurements were made on frozen solutions). In this case it was recognized that the condition, in Leigh's treatment, that $\tau\omega_0^{-1} >> 1$ was not met. It was concluded that in this case Leigh's approach underestimates the dipolar coupling [64]. Ruf and Nastainczyk also demonstrated that the spin labels bound to cytochrome P-450 did not saturate as readily as free spin label and attributed the difference to enhanced relaxation of the nitroxyl by interaction with iron. It is noteworthy that the double integral of the nitroxyl **VII** bound to cytochrome P-450 was found to be smaller than the double integral of the free nitroxyl [64] and the ratio of the amplitudes of the bound and free signals was 63 ± 8%. This result is potentially significant, since the area under the spectrum predicted by Leigh's analysis should remain constant.

Ruf and Nastainczyk [64] also note, without details, that they found strong iron—nitroxyl interaction when **VIII** was bound to the heme of cytochrome



VIII

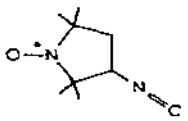
P-450, although Reichman * et al. [65] did not report any magnetic interaction between this spin label and the ferric heme. Raikhman et al. applied the broadening effect of $Fe(CN)_6^{3-}$ on spin labels to a study of cytochrome P-450 [66,67]. During this study visible spectral changes indicated a spin state change for the iron in cytochrome P-450, but there was no comment on any effect of the iron spin state on the spin label EPR spectrum [66,67].

A series of spin-labeled n-alkyl amines were bound to cytochrome P-450 in a recent study by Pirwitz et al. [211]. The observed broadening of the nitroxyl EPR spectrum for the shorter alkyl side chain spin labels was attributed to dipole—dipole interactions between the nitroxyl and nearby heme iron [211]. Estimation of the distance between the iron and the nitroxyl was deferred to a later paper.

C. SPIN-LABELED NON-HEME IRON PROTEINS

Sulfhydryl groups of a model iron—sulfur protein (MISP) were labeled with nitroxyl radicals in order to determine whether the iron atoms were clustered or dispersed [68]. The results were interpreted as indicating no interaction between the nitroxyl radical and the high-spin Fe(III) atoms in the MISP, even though the spin labels reacted with cysteine residues at the iron clusters. Most of the spin labels on each molecule were close enough together to exhibit exchange interactions yielding a single broad line, but 1 to 2 labels per molecule did not interact with other nitroxyls. These "non-interacting" nitroxyls yielded a triplet signal which was broadened beyond detection (note that it was superimposed on the broad signal due to the other nitroxyls) when $Fe(CN)_6^{3-}$ was added to the solution [68].

A closely related study was conducted on nitrogenase with a variety of spin labels to probe the ATPase center and the iron-containing center [69]. The EPR spectrum of V changed upon labeling nitrogenase. Analysis of changes in lineshape and relaxation times did not fit an assumption of dipole—dipole interaction between the nitroxyl and the iron in nitrogenase. However, based on comparison with model experiments, it was concluded that iron—nitroxyl spin exchange interaction makes an appreciable contribution to the parameters of the nitroxyl EPR spectrum [69]. A distance of 12—14 Å between the iron and the SH group of the ATPase center to which the spin label was bound was estimated based on the assumption that exchange is manifested only when the iron and the nitroxyl are within several Å [69]. The label IX apparently bonded



IX

* This author's name is variously transliterated Reichman or Raikhman.

to a cluster of iron atoms at the ATPase center yielding a single 18G-wide line consistent with exchange among a cluster of seven nitroxyls [69].

Spin-labeled derivatives of malonate have been used as the anion in ternary Fe—transferrin—anion complexes. The EPR signal of the spin label in the complex is "broadened beyond detectability". Addition of bicarbonate, which binds more tightly, displaces the spin-labeled malonate. Calculation, using Leigh's equation, (8), indicated that the iron—nitroxyl distance in the complex was about 11 Å [195,196].

D. SPIN-LABELED BIOMOLECULES CONTAINING Mn(II)

Taylor, Leigh and Cohn [21] observed that when Mn^{2+} and ADP were added to creatine kinase which had been spin-labeled with a nitroxyl radical, the amplitude of the nitroxyl radical EPR spectrum decreased without apparent change in lineshape [21,22,70]. A detailed treatment of the effect of Mn^{2+} on the nitroxyl EPR spectrum was developed by Leigh [22] using Redfield theory for the case of the nitroxyl and metal fixed in a rigid lattice with relaxation times such that $T_2 > \tau > \omega_0^{-1}$ where T_2 is the spin—spin relaxation time, τ is assumed to be equal to the spin—lattice relaxation time T_1 of the metal interacting with the nitroxyl and ω_0 is the microwave frequency. The effect was assumed to be due to the secular part of the dipolar interaction, with the nitroxyl relaxation caused by the fluctuating magnetic field due to the dipole of the unpaired electrons on the metal. The essential features of the result are summarized in the expression for the linewidth of the nitroxyl radical δH

$$\delta H = C(1 - 3\cos^2\theta'_R)^2 + \delta H_0 \tag{8}$$

$$C = (g\beta)_1 \mu_2^2 \tau r^{-6} \tag{9}$$

where θ'_R is the angle between the applied magnetic field and the line joining the two electron spins, g is the electron g-factor, β is the Bohr magneton, μ is the magnetic moment, τ is the metal relaxation time, r is the distance between the electron spins, δH_0 is the nitroxyl linewidth in the absence of paramagnetic metals, 1 denotes nitroxyl and 2 denotes metal. Sample computed nitroxyl spectra for various values of the above parameters were published [21,22]. By comparing observed spectra with computed spectra an estimate of distance between the nitroxyl and the metal was obtained [21,22]. In creatine kinase the distance between the Mn^{2+} and the nitroxyl was estimated initially to be 7—10 Å [21,71], and later separate distances for the ternary complexes with ADP were estimated as 8 Å [22] and 7.5 ± 1.5 Å [72] and with ATP as 13 Å [22] and 11.5 ± 0.6 Å [72]. Apparently no significant impact of the nitroxyl spin on the Mn^{2+} EPR spectrum was observed since no mention of the Mn^{2+} spectrum was made except to note that the superimposed spectrum of the Mn^{2+} was subtracted digitally to obtain the nitroxyl spectrum [21,72]. It should be noted that in the cases for which concentrations were published, the Mn^{2+} is present in much higher (~10-fold greater) concentration than the nitroxyl [21,72]. At a "saturating

TABLE 2

Manganese(II)—nitroxyl distances estimated using Leigh's formula

System [a]	Distance (Å)	Reference
Creatine kinase/Mn^{2+}/ATP	11.5 ± 0.6	21, 22, 71, 72 [b]
Creatine kinase/Mn^{2+}/ADP	7.5 ± 1.5	21, 22, 71, 72 [b]
Myosin/Mn^{2+}/ATP	≥13—15	73
Phosphorylase b/Mn^{2+}	16	74, 75 [b], 76
Phosphofructokinase/Mn^{2+}	12	77, 78
Mitochondrial membrane/Mn^{2+}/ATP	18—25	79
Mitochondrial membrane/Mn^{2+}/ATP	~20, >35	80
Myosin/Ni^{2+} or Mn^{2+}/with or without ADP	>15	81
Myosin subfragment 1/Mn^{2+}/ADP	>20	82
Actin/Mn^{2+}	—	83
tRNA/Mn^{2+}	<10—~25	84 [c]
ATP/Mn^{2+}	13—16	85

[a] The biological macromolecule is spin-labeled. [b] Value quoted is from this reference. [c] Did not use the Leigh formula.

concentration of Mn ADP" the amplitude of the nitroxyl EPR spectrum decreased about 95% [21]. The measured dissociation constants for the ternary complexes are consistent with the conclusion that the major effect on the nitroxyl EPR spectrum was due to Mn^{2+} bound in the ternary Mn—ADP—spin-labeled enzyme complex [72]. In another study double integration of the nitroxyl spectrum showed an apparent loss of 90% of the spin in the presence of a 77-fold excess of MnADP [72]. Spectra illustrating reduction in amplitude of the nitroxyl EPR spectra of spin-labeled creatine kinase in ternary complexes with CoADP and NiADP similar to but smaller than that caused by Mn ADP were published [21,70] but not interpreted beyond a statement that the value of C in eqn. (8) "falls in the order Mn > Co > Ni" [21]. It seems unlikely that the relaxation time for Ni^{2+} would be long enough to satisfy the assumption that $\tau > \omega_0^{-1}$. How critical this assumption is cannot be ascertained without further work, which is in progress.

Since the observed and calculated spectra for the Mn^{2+} interaction with spin-labeled creatine kinase were "gratifyingly similar" [21], the Leigh theory was used to interpret other systems containing both a metal and a nitroxyl radical. Indeed it has stimulated, and provided a framework for interpreting a large number of investigations into metal—nitroxyl distances in spin-labeled biological molecules, and has also been applied to other metal—radical and metal—metal interactions. The Mn(II)—nitroxyl distances that have been calculated using the Leigh formula are summarized in Table 2. Each paper is briefly mentioned in the following paragraphs. References to Tables and graphs useful for application of the Leigh formula are compiled in Table 3.

The nitroxyl EPR spectrum of spin-labeled myosin changes upon adding ATP and the bivalent ions Mg^{2+}, Ca^{2+}, or Mn^{2+} to form a ternary complex

TABLE 3

Tabulated data regarding the Leigh formula

Item	Reference
1. Calculated spectra for various values of C (denoted D in ref. 21)	21, 22
2. Calculated spectra for various values of θ_R, ϕ_R	22
3. Plot of relative amplitude vs. $C/\delta H_0$	22
4. Plot of relative linewidth vs. $C/\delta H_0$	22
5. Plot of Mn^{2+}—nitroxyl distance, r, vs. relative amplitude of center peak for various values of the correlation time, τ	72
6. Plot of relative amplitude vs. C	75
7. Plot of distance vs. relative amplitude of the center peak for various values of τ	79, 197

[73]. Mg^{2+}, Ca^{2+} and Mn^{2+} all have about the same effect on the nitroxyl radical EPR spectrum [73]. Using the Leigh theory the magnitude of the spectral changes indicated a lower limit of 13—15 Å between the Mn^{2+} and the nitroxyl [73]. It is not obvious how to interpret these spectral changes, since the spectra illustrated in the article and the tabulated parameters indicate differences between the spectra of the spin-labeled myosin samples prior to adding the metal ions in each case. From the text of the paper it would seem that these values should all be the same. Apparently the uncertainties in the results of this paper are as large as some of the results. In a separate series of experiments reported in this paper $Fe(CN)_6^{3-}$ and $(C_6H_6)_2Cr^+$ were added to solutions of the spin-labeled myosin and to solutions of various small nitroxyl radicals [73]. The broadening observed was attributed to exchange relaxation [73]. The charged metal ion complexes apparently were attracted to charged sites near the spin label on the myosin, resulting in more effective broadening of the bound spin label than of a neutral small nitroxyl radical in solution [73]. Other papers dealing with collision broadening are surveyed in Section H. It should be noted that the possibility of charge-accentuated effects of excess cations (e.g. Mn^{2+}) in solution should be investigated in studies of the type considered in this survey.

Cooke and Duke prepared a spin-labeled analog of ATP and observed that the EPR spectrum of a 10^{-4} M solution decreased in amplitude and broadened when the solution was also made 10^{-4} M in Mn^{2+} [85]. Interpretation using the Leigh theory provided an estimate of 13—16 Å between the Mn^{2+} and the nitroxyl, as expected for Mn^{2+} bound to the triphosphate moiety of ATP [85]. This agreement may be misleading. Two major assumptions of Leigh's theory are not met by Cooke and Duke's system: (a) Leigh assumes a fixed angular orientation of the metal and the nitroxyl, but the spectra presented are characteristic of rapid isotropic tumbling, and (b) Leigh assumes a fixed distance between the metal and the nitroxyl, but Cooke and Duke state that the distance between the two spins is not a constant in the case of their nitroxyl—Mn^{2+}

complex. It is possible that at least part of the lineshape changes observed in this case are due to exchange effects, as in the collision complex studies discussed in Section H, rather than purely dipolar effects as assumed by Leigh's theory.

Dwek and co-workers studied spin-labeled phosphorylase *b* in the presence of Mn^{2+}, which had been shown to bind specifically to phosphorylase *b* [74—76,86]. Addition of a 10-fold molar ratio of Mn^{2+} to the spin-labeled enzyme led to an 18% reduction in the amplitude of the nitroxyl EPR signal [74,75]. Using Leigh's theory a distance of 16 Å [75] between the Mn^{2+} and the nitroxyl was estimated. These authors recognized that the Leigh theory assumed a rigid lattice but that the nitroxyl in their system was reorienting rather rapidly ($\tau \approx 3 \times 10^{-9}$ s). They concluded that although "the validity of such a calculation is difficult to assess", the value calculated for the metal—nitroxyl distance "will be an upper limit" [75,76]. They also reported that the EPR spectrum has a broad background due to Mn^{2+} superimposed on the nitroxyl spectrum [75], but did not interpret the Mn^{2+} EPR spectrum.

Dwek and co-workers also observed interactions between Mn^{2+} and the nitroxyl in spin-labeled phosphofructokinase [77,78]. From the decrease in amplitude of the nitroxyl EPR spectrum a metal—nitroxyl distance of 12 Å was estimated using Leigh's theory [77,78]. The rapid motion of the nitroxyl radical ($\tau \approx 5 \times 10^{-9}$ s) was stated to decrease the dipolar interactions leading to an overestimate of the metal—nitroxyl distance [78]. However, since there are two MnATP binding sites and interaction with only one Mn(II) was assumed in the calculation, the mean distance to the two Mn(II) sites may be greater than 12 Å [78]. Since the measurements were made in the presence of excess Mn^{2+}, the effect of MnATP in solution on the amplitude of the EPR signal of free and bound nitroxyl was investigated and concluded to be small [78]. However, the details of the data presented indicate that the effect described as "small" may be greater than 10%.

MnATP also interacts with nitroxyl spin label bound to mitochondrial membrane fragments, causing a decrease of 30% in the amplitude of the nitroxyl EPR spectrum, which corresponds to a Mn^{2+}—nitroxyl distance of 18—25 Å, according to the Leigh theory [79]. The major uncertainty cited was a need to guess the electron spin relaxation time for Mn^{2+} in the membrane [79]. A broad signal due to the large excess of Mn^{2+} present was subtracted.

Ether extraction of buffered aqueous solutions of mitochondrial membrane fragments to which MnATP was bound increased the distance between the nitroxyl and the MnATP from about 20 Å to more than 35 Å, as estimated from the Leigh theory [80].

Sleigh and Burley spin-labeled actin with succinyl[*N*-imidazole]-[*N*-(4-amino-1-oxy-2,2,6,6-tetramethylpiperidine)] and with *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)maleimide [87,88]. When F-actin was spin-labeled with the succinyl reagent and converted to G-actin, addition of Mn^{2+} caused a 20% reduction in the amplitude of the nitroxyl EPR signal. Use of the Leigh equation suggested that the Mn^{2+} and nitroxyl "are a considerable distance apart"

[87]. They expressed doubt about the applicability of the Leigh equation to a system involving a relatively mobile label [87]. Sleigh and Burley observed a decrease of about 50% in the amplitude of the nitroxyl EPR spectrum of actin spin-labeled using the maleimide reagent with no observable change in line-shape when coordinated Mg^{2+} or Ca^{2+} was replaced by Mn^{2+}. Depending on the electron relaxation time assumed for Mn^{2+}, the Leigh theory yields Mn^{2+}—nitroxyl distances of 16—23 Å [88].

The Leigh theory was used to place an upper limit of 18 Å on the distance between Mn^{2+} and the nitroxyl radical of spin-labeled adenylate kinase in the presence of MnATP [89]. It was reported that Mn^{2+} had no effect on the nitroxyl EPR spectrum in the absence of ATP [89].

As part of the study of spin-labeled nitrogenase discussed in Section C, Mn^{2+} was added [69]. The line shape parameter d_1/d was unchanged, but the nitroxyl relaxation time was greatly influenced [69]. Based on a calibration curve obtained from model experiments it was estimated that the Mn^{2+} binds to nitrogenase ~50 Å away from the spin label [69].

Addition of Ni^{2+} or Mn^{2+} to spin-labeled myosin did not change the nitroxyl EPR spectra, so it was concluded that the metal binding site was >15 Å from the nitroxyl radical [81].

Myosin-dependent changes in the interaction between Mn^{2+} and a nitroxyl spin label, as evidenced by nitroxyl EPR amplitude changes, were used to monitor conformational changes in actin [83]. In the same system, when the nitroxyl and Mn^{2+} were bound to the myosin rather than the actin, no interaction between the Mn^{2+} and the nitroxyl was observed, leading to the conclusion that in myosin subfragment 1 the Mn^{2+}—nitroxyl distance is at least 20 Å [82].

Additional results for myosin are given in Table 1 [56].

Conformational transitions were observed in spin-labeled tRNA as increasing numbers of Mn^{2+} ions coordinated to the tRNA [84]. The initial Mn^{2+} added caused conformational changes which resulted in increased mobility (narrower EPR lines) of the spin label. Also, the conformational changes caused by addition of Mn^{2+} appeared to produce new coordination sites for Mn^{2+}. The Mn^{2+} binding sites were estimated to be at distances from <10 Å to about 25 Å from the spin label [84]. The distance estimates were made by considering the broadening of the nitroxyl EPR spectrum due to dipolar interaction with the Mn^{2+}, according to the formula [84]

$$\Delta H = \frac{\gamma \mu^2 \tau_c}{3r^6} \tag{10}$$

where ΔH is the broadening of a hyperfine component of the nitroxyl EPR, γ is the electron magnetogyric ratio, μ is the magnetic moment of the Mn^{2+}, r is the Mn^{2+}—nitroxyl distance and τ_c is the correlation time of the dipole—dipole coupling. The formula is stated to be valid if

$$(\omega_I - \omega_S)^2 \tau_c^2 << 1; \qquad \omega_I^2 \tau_c^2 >> 1$$

where ω_I is the Larmor frequency of the nitroxyl and ω_S is the Larmor frequency of the Mn^{2+}. Thus, in this paper, which did not cite Leigh's paper [22] or related work, the decrease in amplitude of the nitroxyl EPR spectrum is considered to be related to the square of the line width. Angular dependence of the interaction is not invoked. The observed spectra are thus interpreted to be the net result of (a) changes in mobility resulting from conformational changes in the tRNA, and (b) broadening of some of the nitroxyl spectra as a function of the distance between the nitroxyl and various coordinated Mn^{2+} ions [84].

E. SPIN-LABELED BIOMOLECULES CONTAINING METALS OTHER THAN IRON OR MANGANESE

Calculated distances are summarized in Table 4.

(i) Vitamin-B_{12}

Quite a different use of the interaction between paramagnetic metals and nitroxyl radicals was made by Wood and co-workers [94,95]. A nitroxyl radical was coordinated to the Co(III) in an alkylcobinamide. Ethanolamine—ammonia—lyase apoenzyme was reconstitued with this spin-labeled cobinamide and treated with ethanolamine. The nitroxyl EPR spectrum disappeared during the reaction, and reappeared when alcohol dehydrogenase was added to remove the spin-labeled cobinamide from the lyase. This sequence of observations was interpreted as meaning that homolysis of the Co—C bond occurs yielding low-spin Co(II) [95]. The disappearance of the nitroxyl EPR signal was postulated to be due to "weak exchange interaction between the spins which decreases the relaxation time such that the nitroxyl signal is too broad to detect" [95]. The Co(II) EPR spectrum, however, is "very similar" to that previously observed for Co(II) in VB_{12r} [95]. Unfortunately, irreversible side reactions prevented the type of quantitative EPR data needed to interpret this

TABLE 4

Metal [a]—nitroxide distances estimated using the Leigh formula

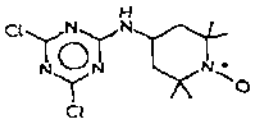
System [b]	Distance (Å)	Reference
Glyceraldehyde-3-phosphate dehydrogenase/Gd^{3+}	11—14, 18—23	96
Liver alcohol dehydrogenase/Co^{2+}	5.8 ± 0.3, 7.4 ± 0.4	90
Myeloma protein MOPC 315 fragment F_v/Gd^{3+}	12 [d]	91, 92 [c]
Transcarboxylase/Co^{2+}	⩾9	93

[a] This table includes metals other than manganese. Table 3 gives manganese—nitroxide distances. [b] The biological macromolecule is spin-labeled or interacting with a spin-labeled substrate. [c] Value quoted is from this reference. [d] Tentative, see text.

system fully being obtained — *e.g.*, whether the area under the EPR curve is equivalent to one or two unpaired electrons.

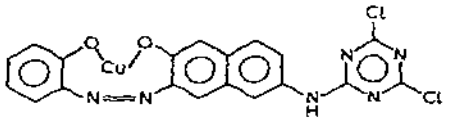
(ii) Cu(II)—nitroxyl: lysozyme and aspartate aminotransferase

Lysozyme was spin-labeled at histidine-15 with X and at a nearby (5—8 Å



X

away) lysine ϵ-NH_2 with XI. Upon adding the Cu(II) label, the intensity of



XI*

the central peak of the nitroxyl EPR spectrum decreased to only a few percent of the intensity prior to adding the Cu(II). The decrease in intensity was attributed to strong broadening "for example, by the mechanism of exchange relaxation" [143].

Copper—nitroxyl interactions were also observed when Cu^{2+} was added to spin-labeled aspartate aminotransferase (AAT). At a Cu^{2+}/AAT ratio of 8 : 1 there was a 25% decrease in intensity of the nitroxyl EPR signal, which was interpreted using the Leigh theory as indicating a lower limit of 19 Å for the Cu^{2+}—nitroxyl distance [169].

(iii) Co(II)—nitroxyl: carbonic anhydrase and alcohol dehydrogenase

When a spin-labeled sulfonamide was bound to Co^{2+} or Zn^{2+} derivatives of carbonic anhydrase, the nitroxyl EPR spectra were "almost identical" [97,98]. It is known from X-ray diffraction data that the sulfonamide group lies within the coordination sphere of the Zn^{2+} ion and that Co^{2+} occupies the same site as the Zn^{2+} ion [98]. Based on molecular models direct coordination of the spin-labeled sulfonamide to the Co^{2+} would provide a Co^{2+}—nitroxyl distance of ~10—12 Å. Taylor et al., envisage a binding mode (not delineated) which would provide a distance of 15—20 Å and thus rationalize the lack of interaction between the Co^{2+} and the nitroxyl [97]. Another paper dealing with a spin-labeled enzyme containing Co^{2+} did observe Co^{2+} interaction with the nitroxyl [90]. Drott et al., used the Leigh theory to estimate

* The formula of XI is given as in ref. 143 since not enough characterization data were presented to ascertain the copper coordination environment.

that the Co^{2+} in cobalt-substituted spin-labeled liver alcohol dehydrogenase is 5.8 ± 0.3 Å and 7.4 ± 0.4 Å from the nitroxyl in two preparations with different degrees of substitution of Co for Zn [90]. The spectra reproduced in the paper indicate a roughly 60% decrease in the amplitude of the nitroxyl EPR spectrum in the presence of Co^{2+} relative to Zn^{2+} [90].

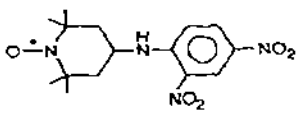
If the sulfonamide were coordinated to the Co^{2+} in the study by Coleman and coworkers [97,98], and all other parameters were the same as for the Drott et al. calculation [90], the Leigh formula would predict at least a 10% reduction in the amplitude of the nitroxyl EPR spectrum upon changing from Zn^{2+} to Co^{2+}.

(iv) Gd(III)—nitroxyl interactions

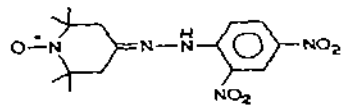
Dwek and co-workers have explored the interaction of Gd(III) with nitroxyl spin labels as a means of estimating distances in proteins [91,92,96,197].

Spin-labeled glyceraldehyde-3-phosphate dehydrogenase exhibits nitroxyl EPR spectra typical of two different degrees of immobilization of the label [96]. Addition of Gd^{3+}, which binds in the active site, reduces the amplitude of the EPR spectrum of the more mobile label to a greater extent than that of the less mobile label. Using the Leigh formula it was calculated that the mobile label is 11—14 Å from the Gd^{3+} and the immobilized label is 18—23 Å from the Gd^{3+} [96].

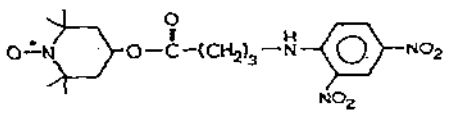
The interaction of myeloma protein MOPC 315 fragment Fv with the spin-labeled haptens XII—XV was studied with and without addition of Gd(III),



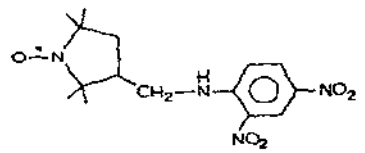
XII



XIII



XIV



XV

La(III) and Eu(III), which bind to the Fv fragment. As Gd(III) was added to a solution of the Fv fragment in the presence of spin-labeled hapten, changes in the intensity of the EPR signal of both bound and free hapten occurred. After accounting for displacement equilibrium effects, the limiting relative amplitude of the bound nitroxyl EPR signals in the presence of various lanthanides was La(III) (diamagnetic), 0.65; Gd(III), 0.4; Eu(III), 0.5. Thus it was concluded that a significant amount of the decrease in the nitroxyl EPR spectrum ampli-

tude was due to a mechanism other than interaction with the paramagnetism of the lanthanide ions [92]. Furthermore, it was noted that the Leigh theory suggests that Eu(III) should have a much smaller relative effect than was observed. It was suggested that there may be some mechanism of quenching nitroxyl EPR spectra by paramagnetic ions with very short relaxation times which is not accounted for by the Leigh theory [92]. Thus the estimate based on the Leigh theory that Gd(III) was 12 Å from the nitroxyl of the spin-labeled hapten was considered tentative [92].

The free nitroxyl EPR spectrum initially increased in amplitude due to displacement as Gd(III) was added, and then decreased again. The decrease was attributed to dipolar interactions between the two paramagnetic species free in solution, and was also observed in a control solution containing no protein [92].

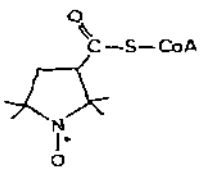
In a recent paper Dwek and co-workers have examined in more detail the effect of Gd(III) and La(III) on spin-labeled dinitrophenyl haptens bound to immunoglobulin A (IgA) myeloma protein MOPC 315 [197]. The intensity of the bound spin label nitroxyl EPR signal is decreased by both Gd(III) and La(III). It was judged that the main effect of La(III) was displacement of bound spin label. The percent decrease of the bound spin label EPR signal of, e.g. **XII**, was 70 ± 2% in the presence of Gd(III) and 45 ± 2% in the presence of La(III). For **XIII** the comparable values are Gd(III), 95—100%, La(III), 70—80%. The greater effect of Gd(III) compared with La(III) was attributed to the phenomena described by Leigh's theory, and distances between nitroxyl and Gd(III) calculated accordingly. Depending on the correlation times assumed for the calculation, Gd(III)—nitroxyl distances of <8 to <12, and 12.0 ± 0.05 to 17.5 ± 0.05 Å were estimated for various spin labels. A caution was added that for spin labels undergoing motion the calculated distances can only be considered an upper limit [197].

(v) Lipoamine dehydrogenase

Treatment of spin-labeled lipoamine dehydrogenase with Cu^{2+} "did not give any unique results" [99]. Apparently the distance between the Cu^{2+} binding site and the spin-labeled site is not known. Very little information was reported beyond a statement that "both EPR species were present" [99]. When the distance between the sites can be estimated the study of this system could be important for understanding metal—nitroxyl interactions because Cu(II) usually has an easily observed EPR spectrum.

(vi) Transcarboxylase

Spin-labeled CoA (**XVI**) bound to transcarboxylase exhibited no depend-

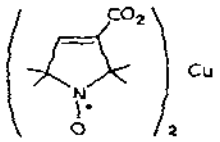


XVI

ence of nitroxyl EPR amplitude or width (≤7%) on the relative amounts of Zn(II), Co(II) and Cu(II) contained in the transcarboxylase [93]. The Leigh formula estimates that the nitroxide is ≥9.0 Å from the Co(II) [93]. NMR relaxation measurements estimate the distance from the Co(II) at the active site to the nitroxide label as ~10 Å [93].

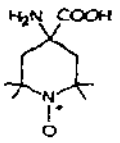
F. DISCRETE COORDINATION COMPLEXES OF PARAMAGNETIC METALS WITH LIGANDS CONTAINING NITROXYL RADICALS

Credit for the first coordination complex formed by a paramagnetic metal with a ligand containing a nitroxyl radical goes to Krinitskaya and Dobryakov [100], who in 1966 reported in a half-page communication that compound **XVII** does not give an EPR signal in the solid state. This observation has not yet been explained.

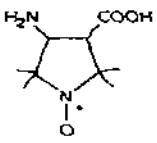


XVII

The second report was by Rassat and Rey who prepared copper and zinc complexes of the amino acid derivatives **XVIII** and **XIX** [101]. The solution EPR spectra were described as "comparable" to other nitroxyls and the spectra of the solid complexes were described as *characteristic powder spectra* [101].

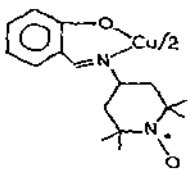


XVIII



XIX

Three groups independently prepared salicylaldimine-type Schiff base ligands containing nitroxyl radicals and obtained EPR spectra of their complexes with paramagnetic metals [102–105]. Medshidov et al. suggested that the EPR spectrum of **XX** could be interpreted in terms of a "weak exchange



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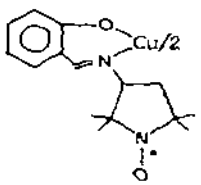


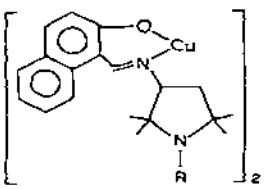
XXI

interaction between the Cu^{2+} and the nitroxyl so long as the sharp three line

pattern observed was attributed to nitroxyl radicals "which do not participate in the exchange" [103]. They also prepared Fe(III), Co(II) and Ni(II) complexes, but EPR data were given only for the Cu(II) complexes [102]. Schwartzhans and coworkers also obtained spectra of **XX** and the related Co(II) and Ni(II) complexes and judged that there was no interaction between the metal and the nitroxyl [104]. Independently, we studied the EPR of the same compound and the related Co(II), Co(III), Ni(II) and Zn(II) complexes and concluded that measurement of line intensities as well as lineshapes would have to be obtained to elucidate the interactions in these spin systems [105]. Substantial metal—nitroxyl interactions are evident in the complexes with paramagnetic metals. For example, the Cu(II) complex, **XX**, is an example of strong electron—electron exchange with resolved nuclear hyperfine structure.

No EPR spectrum was observed at 20°C for a crystalline sample of the Co(II) analog of **XX**, but non-Curie magnetic susceptibility data were interpreted as indicating weak electron exchange [213]. The X-ray diffraction crystal structure of the amino analog of **XX** has been reported [106].

Compound **XXI** is diamagnetic in the solid state, as is the analogous compound, **XXII**, prepared from 2-hydroxy-naphthaldehyde [102,103,107]. It



R = O XXI
R = OH XXII

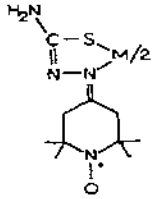
was suggested that the diamagnetism results from the formation of dimeric species in which bonds are formed between the copper and the nitroxyl such that all spins are paired [102,103]. It is not obvious how this can be achieved in view of the steric requirements of the molecules. The elemental analysis of **XXII** leads to a proposal sketched in ref. 107, for a species with two copper atoms and two nitroxyl-containing ligands in a molecular species such that the nitroxyl groups are bonded to the copper atoms. It would appear that a polymeric chain-type structure with nitroxyl bridges between dimeric units would offend fewer steric arguments. Another report of a diamagnetic complex of copper with a related ligand has appeared. In this case the aldehyde used to form the Schiff base was 3-formyl-5-methyl-salicylaldehyde [212]. These results are unique among metal—nitroxyl complexes and need to be reinvestigated. It is possible that some of these compounds are examples of internal redox yielding diamagnetic copper and diamagnetic ligand.

Another interesting feature of **XXI** is that it yields an EPR spectrum in CH_2Cl_2 solution which at 295 K is an apparent superposition of a "normal" four-line Cu(II) spectrum and a three-line nitroxyl spectrum, but at 77 K the hyperfine structure disappears [103]. These observations were attributed to dissociation of dimeric or polymeric compounds [103]. The related complex

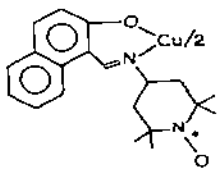
XXII is transformed in $CHCl_3$ solution into a complex identified as **XXIII** [107].

Schwarzhans and coworkers reported Ni(II) and Cu(II) complexes of **XXIV** and concluded in this case also that there was no metal—nitroxyl interaction [104].

The complex **XXV** yields an EPR spectrum in $CHCl_3$ solution very similar



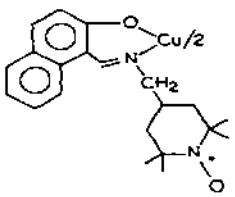
XXIV



XXV

to that given by **XX** with g and $\langle a \rangle$ values a weighted average of those for copper and nitroxyl, indicating exchange interaction between the copper and the two nitroxyls [107]. The exchange was envisioned to be made possible by a structure in which the oxygen atoms of the nitroxyl approach the copper closely enough to permit slight overlap of orbitals [107]. Subsequently, a single crystal X-ray diffraction structure determination demonstrated that in the solid phase the nitroxyl oxygens are 6.53 and 6.80 Å from the copper [108]. The closest nitroxyl—copper contacts in the crystal are intermolecular. The temperature dependence of the magnetic susceptibility and EPR of the solid in the liquid helium temperature range were interpreted in terms of an intermolecular three spin system with exchange energy ≈ 10 cm^{-1} [108].

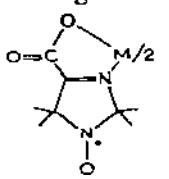
The related compound **XXVa** exhibited an EPR spectrum described as a



XXV a

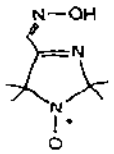
superposition of the exchange-free spectra of Cu(II) and the nitroxyl radical [213]. However the published spectrum and values of g and $\langle a \rangle$ are fully consistent with an interpretation as strong exchange between Cu(II) and two nitroxyls. Note that this is also the interpretation given to the spectrum of **XXV** by a group including one of the same authors five years earlier [107].

Sagdeev et al. prepared the complexes **XXVI—XXVIII** in which the nitroxyl



M = Cu^{2+} XXVI
M = Ni^{2+} XXVII
M = $CrCl^{2+}$ XXVIII

is very close to the metal [109,110]. The EPR spectrum of the Cu^{2+} complex, XXVI, was interpreted as a case of strong exchange between the metal and the



XXIX

nitroxyl, but it was only possible to estimate the magnitude of the coupling parameter J as $0.057\ cm^{-1} < J < 67\ cm^{-1}$ [109,110]. Limits on the range of values for J consistent with the data were estimated as follows: (a) g and $\langle a \rangle$ values were weighted averages of normal copper and nitroxyl values, as expected for the strong exchange limit, which requires $J >> \nu_0\ \Delta g/g$. Since $\Delta g \simeq 0.1$ and $\nu_0 = 3.5 \times 10^{10}$ Hz (Q-band), $J >> 1.7 \times 10^9$ Hz ($0.057\ cm^{-1}$); (b) the EPR signal intensity corresponded to three uncoupled spins at 77 K, which indicates that

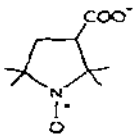
$$J < \frac{kT}{h}$$

hence, $J < 2 \times 10^{12}$ Hz ($66.7\ cm^{-1}$).

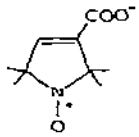
An energy level diagram for a strong exchange three spin system and predicted X- and Q-band frozen solution spectra are given in [110]. The authors presumed that they did not detect the predicted outer lines because they were too broad [110]. The relative intensities of the EPR signal expected for various coupling schemes (three independent $S = 1/2$, or $S = 1/2$, 3/2 from coupling of 3 spins) as given in ref. 110 are in error. The published results could have been obtained by ignoring terms in M_s in the matrix elements involving the ladder operators. The correct ratios in the order of the coupling schemes given above are: 1 : 1/3 : 10/3. This paper bases a comparison of the Cu^{2+}, Ni^{2+} and $CrCl^{2+}$ complexes (XXVI—XXVIII) on the erroneous statement that planar Ni^{2+} has "unpaired $d_{x^2-y^2}$ electrons". If there are unpaired d electrons on Ni^{2+} the complex must be non-planar. The paper does not contain the magnetic susceptibility information necessary to judge the spin state of the nickel, but it is not unreasonable that the molecule could be pseudo-tetrahedral and thus contain paramagnetic nickel. No EPR spectrum was observable for the Ni^{2+} complex. The EPR spectrum of the $CrCl^{2+}$ complex was observable at Q-band but not at X-band [109,110]. These papers make the important contribution of pointing out that intramolecular spin exchange between an organic free radical and a paramagnetic metal with short spin lattice relaxation time can average the electron spin states of the free radical rapidly enough to facilitate observation of the NMR spectrum of the radical. NMR spectra of these complexes are presented. The NMR linewidths of the Ni^{2+} and $CrCl^{2+}$ complexes are independent of concentration but the linewidths of the Cu^{2+} complex decrease with increasing concentration because of the long electron spin relaxation of the Cu^{2+} [109,110].

Larionov et al. claim that **XXIX** forms complexes with Ni^{2+}, Cu^{2+}, Pd^{2+}, "and ions of certain other metals", but the paper presents information on only the Pd^{2+} complex [111]. The structural similarity of **XXIX** to the ligand used in the complexes **XXVI—XXVIII** makes the paramagnetic metal complexes of **XXIX** important for comparison with the properties of **XXVI—XXVIII**.

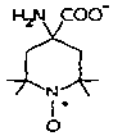
Schwarzhans and coworkers recently published [112,113] an extension of their work cited above [103]. Some of the intriguing results reported in this paper are given in tabular form below. The values given under each ligand are the 296 K EPR linewidths for a polycrystalline sample of the complex with the metal listed on the left. There does not seem to be any pattern to this data, and no explanation is offered in the paper [113]. The copper complex of **XXXI** is reported to have the formula $Cu(\mathbf{XXXI})_2H_2O$ [113]. This may be the same compound as **XVII**, which was reported as the anhydrous analog. The discrepancy between the relatively narrow EPR lines reported by Weissgerber and Schwarzhans [113] and the lack of an EPR signal in the sample prepared by Krinitskaya and Dobryakov [100] remains unresolved.



XXX



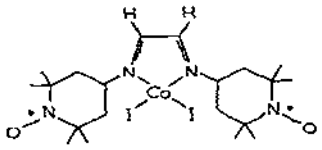
XXXI



XVIII

Cu^{2+}	340 G	120 G	50 G
Co^{2+}	390	710	13
Ni^{2+}	410	95	no signal

Indeed, Weissgerber and Schwarzhans did not reference the work by Krinitskaya and Dobryakov. The complex **XXXII** is particularly interesting



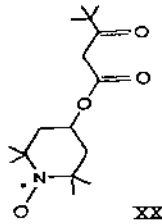
XXXII

because the EPR signal is 1000 G wide at room temperature, while a zinc complex of the same ligand exhibits a 10.5 G wide line [113]. Apparently interaction between the cobalt and the nitroxyl has substantially altered relaxation times in this complex. Unfortunately, EPR spectral intensities were not reported so it is not possible to ascertain whether the Co^{2+} contributes to the observed EPR spectrum. Schwarzhans and co-workers conclude that metal nitroxyl interaction is not observed in solution for any of their complexes but that in certain cases EPR linewidth changes reveal interactions in polycrystalline samples [112,113].

Studies of the amino acid nitroxyl II continue [101,112,114], most recently with emphasis on understanding the solution equilibria and complexa-

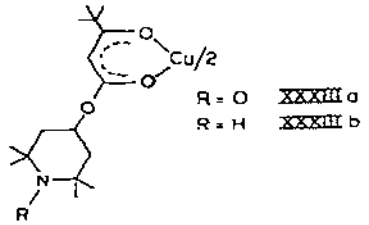
tion with Co^{2+} and Mn^{2+} preparatory to analyzing magnetic interactions [114].

Veyret and Blaise tested several models of magnetic interaction against the solid state magnetic susceptibility data for nitroxyl radicals and biradicals [115]. The magnetic susceptibility of the Cu^{2+} complex of **XXXIII** was fitted



XXXIII

to an expression involving copper—nitroxyl and nitroxyl—nitroxyl exchange interactions of 9.99 cm^{-1} and 0.0139 cm^{-1}, respectively [115]. This copper—nitroxyl exchange energy falls within the range estimated by Sagdeev et al. for **XXVI** [109,110]. No information on the EPR spectrum of the copper complex of **XXXIII** was reported by Veyret and Blaise [116].

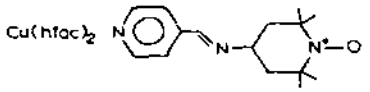
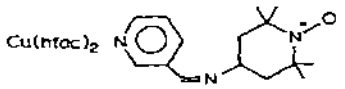
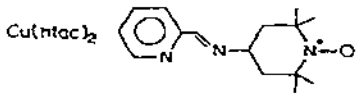
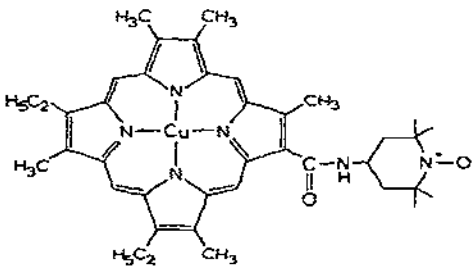
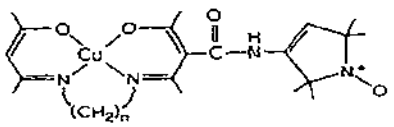
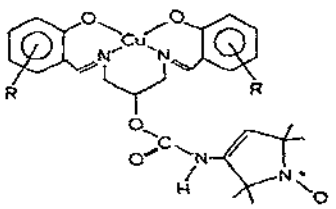


Recently Brier, Rassat, and Rey reported the EPR spectrum of **XXXIII a** and b [216]. The observed g and $\langle a \rangle$ values of **XXXIIIa** are as expected for strong exchange [216]. As in the case of, e.g., **XX** and **XXVa**, the copper hyperfine was approximately one-third the value observed for the analogous copper complex with diamagnetic ligand (**XXXIIIb**, in this case). The authors of ref. 216 (as well as the authors of refs. 198 and 215) claimed the first example of such hyperfine, apparently having overlooked the earlier work cited above. Many spectra of copper complexes of nitroxyl-containing ligands, including the spectra of **XXXIIIa**, have contained the three line pattern with $\langle a \rangle \approx 15$ G that is characteristic of nitroxyl radicals. The uncertainty expressed as to whether the "non-interacting" or "isolated" nitroxyl should be attributed to an impurity in the sample, decomposition in solution, or reversible dissociation is answerable by the proper sequence of measurements checking reproducibility and the reversibility of concentration dependence. It should be noted that due to linewidth differences, these very prominent signals may be due to only 1% or so of the sample and may reveal aspects of the chemistry not easily studied by other methods.

Recent results from our laboratory include several examples of metal—nitroxyl interactions which may be described as displaying high resolution electron—electron spin—spin splitting in EPR spectra analogous to the well known nuclear spin—spin splitting in NMR. Some of the compounds we have

TABLE 5

Cu complexes exhibiting metal—nitroxyl exchange interactions in solution

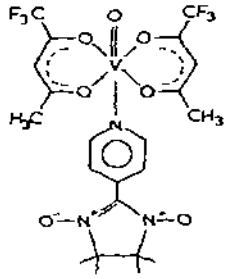
Complex		J^a (cm^{-1})	Reference
		0.0044 [b]	117
		0.0092 [b]	118
		large	118
		0.0074 [c]	119
	$n = 2$ $n = 3$	0.045 [d] 0.035 [d]	120 120
	R = H R = 5 t-Bu	0.00058 [d] 0.0011 [d]	217 217

[a] The values given are solvent dependent and are subject to future refinement. [b] CCl_4 soln. [c] $CHCl_3$ soln. [d] THF soln.

studied are listed in Table 5 together with an estimate of the magnitude of the metal—nitroxyl exchange interaction (J). The cases listed all involve a single nitroxyl radical in a d^9 (spin 1/2) Cu(II) complex. Thus we are dealing with the interaction of two spin 1/2 species, and a treatment parallel to that commonly used in high resolution NMR [121] is appropriate, yielding "AB" type four-line patterns. This approach has also been recognized by others [122—124]. Since the $^{65,63}Cu$ and ^{14}N (both for the nitroxyl nitrogen and nitrogens in the copper coordination sphere) nuclear hyperfine interactions must also be included in the analysis of the spin system, it is of the $ABMXY_n$ type. Furthermore, since the EPR experiment is done at constant frequency, the

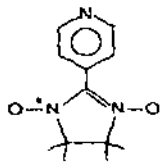
formulae available in the NMR literature which assume constant magnetic field can give only a rough approximation to J. The values of J given in Table 5 are interim results obtained from computer simulation of the spectra using a program which is still evolving. Thus they should only be considered indicative of the range of values exhibited by these complexes. Our work is continuing on a wide range of compounds with studies designed to ascertain the relative contributions of dipolar and exchange interactions to the EPR spectra.

The EPR spectrum of **XXXIIIc** exhibits an 8-line pattern with g- and $\langle a \rangle$-values the average of the nitroxyl and vanadyl starting materials, as expected for strong exchange. Due to partial dissociation, the EPR spectra of the reactants superimpose on those of the complex. The temperature dependence of the area of the EPR spectrum is consistent with a singlet ground state with a singlet—triplet splitting ($2J$) of -386 cm^{-1}. Line broadening was attributed to

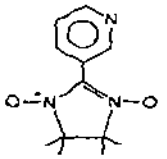


XXXIII c

incompletely averaged electron—electron dipolar interaction [198]. The complexes of Cu(hfac)$_2$ with **XXXIIId** and **XXXIIIe** similarly yielded spectra



XXXIII d



XXXIII e

indicative of strong exchange ($-2J = 310 \pm 50$ cm^{-1} and 299 ± 10 cm^{-1}, respectively), and the temperature dependence of the EPR signal area showed that the ground state is a singlet [215]. Partial dissociation was observed in solution. The related complex of Cu(hfac)$_2$ with **XLIV**, the *ortho* pyridine isomer, was diamagnetic and exhibited no EPR signal. This was accounted for by suggesting charge transfer to form Cu(III) and anionic ligand [215].

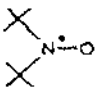
Except for the work described in this Section, exchange has been invoked to explain metal nitroxyl EPR spectra in fluid solution only in the case of collision interactions since it is generally assumed that exchange interactions are important only when the distance between spins is less than a few Å [56]. Note, however, that Zamaraev et al. found that spin exchange between Cu^{2+}(aq) and Mn^{2+}(aq) or VO^{2+}(aq) was effective even when the distance between individual cations is greater than 12 Å [125].

Wagner and coworkers have studied the dynamic nuclear polarization of solvent molecules in the presence of **XX** and the Co(II) and Ni(II) analogs but the results have not yet been published [126].

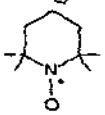
G. COMPLEXES OF TRANSITION METALS IN WHICH THE NITROXYL GROUP FUNCTIONS AS A LEWIS BASE

This section covers discrete complexes which have been isolated as solids. Studies which involve interactions between nitroxyls and metal ions or complexes dissolved in the same solution are considered in Section H.

Shortly after the characterization of di-tert-butylnitroxide (DTBN), **XXXIV**, was published, Beck et al. prepared cobalt complexes in which **XXXIV** functions as a Lewis base, $Co(DTBN)_2X_2$ (X = Cl, Br, I) [127]. The polycrystalline samples exhibited EPR spectra described by *g*-values between 2.3 and 2.7. In solution a three-line EPR spectrum characteristic of a nitroxyl radical was observed. The relative intensity of the three line spectrum increased with dilution, indicating extensive dissociation in solution [127].



XXXIV



XXXV

Other reports of isolated crystalline complexes in which nitroxyls act as Lewis bases toward paramagnetic metal ions include cobalt and copper complexes of **XXXV** [112] and the DTBN (**XXXIV**) complex of copper bis(hexafluoroacetylacetonate), $Cu(hfac)_2(DTBN)$ [128,129]. Magnetic susceptibility measurements indicate that $Cu(hfac)_2(DTBN)$ has a singlet ground state and a triplet excited state 645 cm^{-1} higher in energy [128]. Labes and coworkers formed complexes between **XXXV** and Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} and interpreted their, and prior, results as indicating that in nitroxide "complexes of d^n metal ions the metal in the complex is effectively d^{n+1}" [130]. This is a curious interpretation and does not appear to be consistent with the properties of any of the compounds whose descriptions have been published in enough detail to permit judgement. The Cu(II) complex of **XXXV** is unstable [130].

Drago and co-workers [131] re-examined the magnetic properties of $Co(DTBN)_2Br_2$, and concluded that the magnetic moment was 4.23 BM, not 2.7 BM as reported by Beck et al. [127]. The magnetic data did not distinguish between 3 and 5 unpaired spins in the molecule, but the overall judgement was that the molecule consists of tetrahedral Co^{2+} with independent radical ligands [131].

The copper bis(hexafluoroacetylacetonate) complex of **XXXV** was found to be antiferromagnetically coupled (J = 736 cm^{-1} at 298 K) and to dissociate slightly in CCl_4 solution, based on magnetic susceptibility and EPR measurements [132].

Transition metal perchlorate complexes of **XXXV** have magnetic susceptibilities in the solid state somewhat lower than expected based on independent

spins for the nitroxyl and the metal [133]. Co(II) and Ni(II) perchlorates yielded 1 : 1 complexes with **XXXV**, while Fe(II) and Zn(II) perchlorates yielded 1 : 2 complexes [133]. Polycrystalline samples exhibited *g*-value anisotropy too large for simple nitroxyls, and dissolved samples gave a three-line EPR spectrum "very similar" to uncomplexed nitroxyl radicals [133]. This paper claims that interaction with transition metals broadens and *enhances* the intensity of the nitroxyl EPR spectrum. Nothing in the literature supports this claim. Presumably it is a proof-reading error.

H. COLLISION INTERACTION BETWEEN NITROXYLS AND PARAMAGNETIC METAL IONS IN SOLUTION

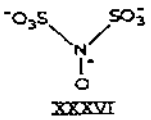
Many examples have been reported of interactions via collisions between nitroxyl radicals and paramagnetic transition metal ions in solution.

It is well known that EPR lines are broadened when the concentration of the observed (or some other) paramagnetic species is increased. In some of the papers cited below the investigators assumed that the broadening was due to exchange interactions. In other papers it was assumed that the broadening arises from dipolar interactions. A few of the studies were designed to determine the relative contributions of exchange and dipolar interactions to the broadening. There appears to be no chronological correlation between the papers of the latter type and the assumptions made by the remainder of the papers.

Much of the work in this area has been published in the Russian literature. The results in these papers are typically stated to be consistent with a simple formula which is presented without derivation. Sometimes the formulae are empirical and sometimes they are stated to be theoretical predictions. Discussion of assumptions which led to the simple formula, or of the range of parameters to which the data analysis could be applicable, is conspicuously absent in this literature.

Not all of the interaction pairs reported in each paper are discussed in the text below, but a comprehensive list of metals used is included in Table 6.

The first study of collision interactions between nitroxyl radicals and paramagnetic transition metal ions was by Pearson and Buch in 1962 [139]. The following is a brief outline of the theory presented in their paper. Consider a paramagnetic ion A, in this case the nitroxide XXXVI known as Fremy's radi-



XXXVI

cal, in a collision with another paramagnetic ion B, in this case various first transition series ions or lanthanides. As a consequence of the collision B will provide a relaxation mechanism for A through dipole and/or exchange interactions. The lifetime of A will thereby be reduced, resulting in a broadening of the EPR spectrum of A. The nature of the broadening of A will depend on two characteristic times: T_{2B} — the spin relaxation time of A in the complex

TABLE 6[a]

Collisions of nitroxyls with paramagnetic metals

Metal ion	Observation	Interpretation	Reference
$VO(acac)_2$	B	D, E	134
$VO(acac)_2$	B	E	135
$VOSO_4(aq)$	B	E	136
VO(porph)[b]	B	E	137
$VO(ClO_4)_2$	B	E	125
$Cr(H_2O)_6^{3+}$	B	D, E	138
$Cr(H_2O)_6^{3+}$	B	D	139
$Cr(acac)_3$	B	D, E	134
$Cr(acac)_3$	B	E	135
$Cr(NO_3)_3(aq)$	B	D, E	140
$CrCl_3(aq)$	B	E	136
$CrCl_3$(pyridine)	B	E	136
$[Cr(NH_3)_5Cl]^{2+}$	B	D	139
$[Cr\ en\ (C_2O_4)_2]^-$	B	D	139
$[Cr(en)_2(NCS)_2]^+$	B	D	139
$[Cr(en)_3]^{3+}$	B	D	139
$Cr(CN)_6^{3-}$	B	D	139
$Cr(C_6H_6)_2^+$	B	E	44, 54, 73, 141—143
Mn^{2+}	R	—	57
Mn^{2+}	R	D	69
Mn^{2+}	Q	D	78
Mn^{2+}	B	E	144
$[Mn(H_2O)_6]^{2+}$	B	D, E	138
$Mn(NO_3)_2(aq)$	B	D, E	125, 140
$Mn(acac)_3$	B	E	135
$Mn(acac)_3$	B	D, E	134
MnATP complex	Q	D	78
$MnCl_2(aq)$	B	E	136
$MnCl_2$(pyridine)	B	E	136
$Mn(CNS)_2$(picoline)$_4$	B	E	136
$FeCl_2(aq)$	B	E	136
$Fe(NO_3)_3(aq)$	B	E	136
$FeSO_4(aq)$	B	E	140
$Fe(acac)_3$	B	D, E	134
$Fe(acac)_3$	B	E	44, 141
$Fe(acac)_3$	B	E	135
Fe(III)(porph)[b]	B	E	137
$Fe(CN)_6^{4-}$	R	—	57
$Fe(CN)_6^{3-}$	R	D	69
$Fe(CN)_6^{3-}$	B	E	44, 54, 64—68, 73, 141—143, 148a, 165, 200, 201, 208—210
Co^{2+}	R	D	69
$Co(H_2O)_6^{2+}$	B	D	139
$Co(H_2O)_6^{2+}$	B	D, E	138

TABLE 6 (continued)

Metal ion	Observation	Interpretation	Reference
$Co(H_2O)_6(ClO_4)_2$	B	E	145
$CoCl_2$(aq)	B	E	136, 202
$Co(NO_3)_2$(aq)	B	E	140
$Co(CH_3OH)_6(ClO_4)_2$	B	E	145
$Co(C_3H_7OH)_6(ClO_4)_2$	B	E	145
$Co(C_3H_7OH)_6Cl_2$	B	E	145, 202
$Co(DMSO)_6(ClO_4)_2$	B	E	145
$Co(acetone)_6(ClO_4)_2$	B	E	145
$[Co(acac)_2]_4$	B	D,E	134
$Co(acac)_2$(pyridine)	B	E	135
$CoCl_2$(pyridine)	B	E	136
$Co(CNS)_2$(picoline)$_4$	B	E	136
Ni^{2+}	R	—	57, 203
Ni^{2+}	R	E	69
$Ni(H_2O)_6^{2+}$	B	D	139
$NiCl_2$(aq)	B	D	146—148, 149
$NiCl_2$(aq)	B	E	136
$NiCl_2$(aq)	B	—	148a
$Ni(NO_3)_2$(aq)	B	E	140
$NiCl_2$(pyridine)	B	E	136
Ni tris[c] (aq)	B	D	150
$Ni(acac)_2$	B	E	135
$Ni(acac)_2$(aniline)$_2$	B	E	145
$Ni(acac)_2$(pyridine)$_2$	B	E	145
$Ni(CNS)_2$(picoline)$_4$	B	E	136
$[Ni(en)_2(H_2O)_2]^{2+}$	B	D, E	138
Cu^{2+}(aq)	Q	—	100
$[Cu(H_2O)_4]^{2+}$	B	D	139
$Cu(NO_3)_2$(aq)	B	E	125, 151, 152
$CuSO_4$(aq)	B	E	140
$Cu(acac)_2$	B	E	135
$Cu(acac)_2$	B	D, E	134
$Cu(tfac)_2$	^{19}F NMR	E	128, 129
$Cu(hfac)_2$	^{19}F NMR	E	128, 129
$[Cu(en)_2(H_2O)_2]^{2+}$	B	D,E	138
Cu(porph)[b]	B	E	137
Cu complex XI	B	E	143
Cu complex XXXVIII	B	E	42
Ce^{3+}(aq)	B	D	139
Pr^{3+}(aq)	B	D	139
Nd^{3+}(aq)	B	D	139
Gd^{3+}(aq)	Q	D	92
$[Gd(H_2O)_6]^{3+}$	B	D	139

[a] This table contains references to articles which reported interaction between nitroxyls and transition metals under conditions such that it is believed that the interaction was via collision encounters. Notation: B, broadening; R, relaxation time changed; Q, "quenching" (peak height decreased); E, exchange; D, dipole—dipole interaction. [b] A variety of substituted porphyrins was used. [c] Tris = tris(hydroxymethyl)aminomethane.

formed between A and B; τ_B — the lifetime of the state in which A and B are close enough for the spin interactions to be effective in relaxing A. It is assumed that in ion pairs exchange is minor and magnetic dipole forces dominate. The relaxation of A due to dipole—dipole interaction between the spins on A and B is given as

$$\frac{1}{T_{2B}} = \left(\frac{4}{3}\frac{\mu_A^2}{\hbar^2}\frac{\mu_B^2}{r^6}\right)\tau_c \tag{11}$$

where μ_i are the magnetic moments, r is the distance between the unpaired electrons and

$$\tau_c^{-1} = \tau_r^{-1} + \tau_s^{-1} + \tau_B^{-1} + \ldots \tag{12}$$

where

$$\tau_r = \frac{4\pi\eta r^3}{3kT} \tag{13}$$

in which r is the hydrodynamic radius, η is the viscosity and τ_s is the shorter of the spin state lifetime for A or B. The kinetic expressions for ion pair formation are combined with the form of the Bloch equations appropriate to chemical exchange effects to yield expressions for T_2 of the nitroxyl for several limiting cases. If relaxation of the spins in the complex is rate determining for nitroxyl relaxation, the increase of the linewidth of the EPR spectrum of A over that of free A is proportional to the concentration of B for low concentrations of B and levels off at high concentrations of B. The limiting value should be proportional to μ_B^2. If the lifetime of the complex is rate determining for nitroxyl relaxation the effect of metal ions should be independent of μ_B but should depend on the ionic charge. The effects of ion charge are considered in detail [139].

It was found that for various cations of Cr, Co, Ni, Cu and Gd (see Table 6) interacting with **XXXVI** the kinetics of complexation was rate-determining for relaxation of A. At low concentrations the broadening of A was linearly dependent on concentration of B with the effect being dependent on the charge (+3, +2, +1) of B. For the lanthanides (except Gd^{3+}) broadening was much less than for the ions listed above, and it was concluded that relaxation in the complex was rate determining for nitroxyl relaxation.

It is of interest to note that the formula for T_{2B}, upon evaluating the numerical constants for the case of μ_A = 1.73 BM (one unpaired electron as in the nitroxyl radicals) and using the linewidth expression

$$(\Delta H)^2 = \frac{4}{3\gamma^2 T_2^2} \tag{14}$$

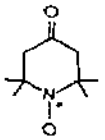
$$\text{yields } \Delta H = 1.74 \times 10^{15}\frac{\mu_B^2\tau_c}{r^6} \tag{15}$$

with μ_B in BM, τ_c in s and r in cm. Recall that Leigh's formula (Section D) upon similar numerical substitution becomes

$$\Delta H = 1.51 \times 10^{15}(1 - 3\cos^2\theta_R')\frac{2\mu^2\tau}{r^6} \tag{16}$$

Pearson's formula was derived for rapid isotropic tumbling in aqueous solution and Leigh's formula was derived for the case of a rigid lattice.

Molin and coworkers [135,138,140,153] studied the effect on the EPR spectrum of XXXVII of collisions with various paramagnetic transition metal



XXXVII

complexes. Some of the equations used in these studies to analyze exchange interactions were introduced in a paper concerning collisions between metal complexes and between DPPH and metal complexes [154]. A molecule with a readily observable EPR spectrum, such as DPPH, is used as an "indicator" of the collisions. The frequency, ν_e, of exchange interaction of the "indicator" with the metal complex was obtained from the formula

$$\nu_e = 1.52 \times 10^7 \, \Delta H_m \tag{17}$$

where ΔH_m is the peak-to-peak broadening of the EPR spectrum of the "indicator" in gauss. The bimolecular rate constant for exchange interaction, K_{exch}, is

$$K_{exch} = \frac{\nu_e}{N} = \frac{1.52 \times 10^7 \, \Delta H_m}{N} \tag{18}$$

where N is the concentration in mol l^{-1}. To measure the effectiveness of an individual collision, F, correction is made for the viscosity, η, of the solvent with the formula

$$K_{exch} = \frac{K \, T \, F}{\eta} \tag{19}$$

When each collision is effective, K should be close to 1 [154]. Derivation of these expressions was not given, but they were stated to be based on the work of Anderson [155] and Pake and Tuttle [156].

In another paper [90], Molin presents formulae for the estimation of dipole—dipole broadening, based on the development in Abragam's book [157]. For ions with long electron relaxation times (Cr(III), Mn(II) and Cu(II)), the correlation time for dipole—dipole interaction is determined by diffusion times, and the dipolar broadening is given by

$$\Delta H = \frac{128\pi^2}{15\sqrt{3}} N\mu^2 \, \frac{\gamma\eta}{kT} \, \frac{a_1 a_2}{(a_1 + a_2)^2} \tag{20}$$

where a_1 and a_2 are the effective ("Stokes") radii of the metal ion and the indicator molecule. For ions with short electron spin relaxation times (Fe(II), Co(II), Ni(II)) the correlation time for dipole—dipole interaction is determined by the electron spin relaxation times of the ion, and the broadening is given by

$$\Delta H = \frac{32\pi}{9\sqrt{3}} N\mu^2\gamma\theta \, \frac{1}{(a_1 + a_2)^3} \tag{21}$$

where θ represents the metal electron spin relaxation time (usually denoted T_1). The calculated dipole–dipole broadening for collision of **XXXVII** with various metal ions in aqueous solutions is the following percentage of the observed broadening: Cr^{3+}, 57%; Mn^{2+}, 77%; Fe^{2+}, <22%; Co^{2+}, <8%; Ni^{2+}, ≤6%; Cu^{2+}, 11% [140]. Thus the dipolar contribution to the broadening of **XXXVII** was deemed to be inconsequential except when interacting with Cr(II) and Mn(II) [140]. Hyde and Sarna criticized this paper for using the formula originally derived for nuclear relaxation because the assumption that $\omega\tau << 1$ is not satisfied for the EPR situation to which it was applied [158].

To relate the exchange rate constant K (called K_{exch} in ref. 154) to the exchange integral J, a version of a formula derived by Currin [159, formula 3.5] was introduced

$$K = A\frac{kT}{\eta}\frac{(J\tau_e)^2}{1+(J\tau_e)^2} \tag{22}$$

where $A \sim 1$ is a dimensionless constant and τ_e is the time of exchange, assumed to be the collision lifetime. Thus the exchange constant K is predicted to be independent of the value of the exchange integral if $J\tau_e > 1$ [140].

A subsequent paper examined in more detail the effect of metal electron spin relaxation time on the effectiveness of a collision for broadening the nitroxyl EPR spectrum [135]. For the cases chosen, metal acetylacetonates interacting with I, DPPH and bis-diphenylchromium cation, it was estimated that dipole–dipole interactions contributed only ~20% of the line broadening. Consequently the analysis was conducted entirely in terms of exchange interactions. The notation in this paper differs from that in the previous papers by the same authors, as follows (a) ref. 135 uses I instead of J to denote the exchange integral; we convert it to J in the formulas we quote from the paper, (b) ref. 135 uses τ_c rather than τ_e for the collision time, (c) a parameter p, the effectiveness of an individual collision is introduced in ref. 135, apparently the same as Pake and Tuttle's parameter p [156]. The previously [140,154] defined factor F is stated to be "close to" p. For the cases of interest several different sets of inequalities were considered:

(1) if $T_1 > \tau_c$, $J^2 > \delta^2$ and $J^2T_1^2 > 1$ where δ is the difference between the resonance frequencies of the two spins, τ_c is the collision time within which the interaction occurs and τ_e is "some average correlation time for the exchange interaction during a collision" [159], it is found that

$$p = \frac{1}{2}\frac{J^2\tau_e^2}{1+J^2\tau_c^2} \tag{23}$$

(2) if $\tau_c > T_1$ and $J^2T_1\tau_c < 1$

$$\text{then } \frac{1}{T_2} = \frac{J^2S(S+1)}{6}\left(T_1 + \frac{T_1}{1+\delta^2T_1^2}\right) \tag{24}$$

$$p = \frac{J^2S(S+1)T_1\tau_c}{6}\left(1+\frac{1}{1+\delta^2T_1^2}\right) \tag{25}$$

(3) if $J^2 < \delta^2$ and $\sigma^2 T_1^2 > 1$

case (a) if $J^2 T_1^2 < 1$ and $T_1 < \tau_c$

then $p = \frac{J^2 T_1 \tau_c}{8}$ (26)

case (b) if $J^2 T_1^2 > 1$ and $\tau_c < T_1$

then $p = 2 \sin^2 \frac{J\tau_c}{4}$ (27)

case (c) if $J^2 T_1^2 > 1$ and $\tau_c > T_1$

then $p \simeq 1$ (28)

The major conclusion is that if T_1 is sufficiently small, the exchange broadening should decrease with a decrease in T_1 [135]. It was found that the effectiveness of the collisions was low in the cases in which the metal T_1 is $<10^{-11}$ s, which is also estimated to be the value of τ_c. This was concluded to be confirmation of the theoretical treatment. The prediction cited above, that for weak exchange with $T_1 < \tau_c$ the effectiveness of a collision is proportional to $J^2 T_1 \tau_c$ was contrasted with the prediction of Currin's treatment that $p = J^2\tau_c^2/1 + J^2\tau_c^2$ [135].

Anisimov et al. [138] noted that (a) it was known from nuclear magnetism [157] that dipole–dipole contributions to line broadening increase as the factor η/T increases and (b) Currin [159] had shown that the contribution of exchange interactions in collisions decreases as η/T increases. They defined a broadening coefficient, A,

$$\Delta H = \Delta H_0 + A \cdot n \tag{29}$$

$$A = A_{ex} + A_{d-d} \tag{30}$$

where ΔH is the nitroxyl EPR linewidth, ΔH_0 is the concentration independent contribution to the nitroxyl linewidth, n is the concentration of the broadening agent, A_{ex} is the broadening due to exchange interactions and A_{d-d} is the broadening due to dipolar interactions. For slow or fast T_1, formulae were presented for the dipolar contribution to the linewidth, denoted as ΔH in [138] but for internal consistency in notation it is apparent that $A_{d-d} \cdot n$ is intended:

(a) if $T_1 > 10^{-7}$ s, $\Delta H(= A_{d-d} \cdot n) = \frac{3.8}{\sqrt{3}} \mu \cdot n$ (31)

where μ is the magnetic moment of the metal ion,

(b) if $T_1 < 10^{-10}$ s, $\Delta H(= A_{d-d} \cdot n) = \frac{32}{9\sqrt{3}} \gamma\mu^2 T_1 \frac{n}{r_{min}^3}$ (32)

where r_{min} is the distance of closest approach between the metal and the "indicator" (nitroxyl). Experimental results for Cr(III), Mn(II), Co(II), Ni(II) and Cu(II) complexes were presented. The line broadening caused by the Ni(II)

and Co(II) complexes, which have $T_1 < 10^{-10}$ s, is in good agreement with the theoretical prediction. This is expressed as a "strong neutralization of the dipole interactions by spin lattice relaxation". The self-broadening of XXXVII provides an example of $T_1 > 10^{-7}$ s, in which relaxation does not lead to neutralization of the dipole–dipole interactions. Mn(II) and Cr(III) have $T_1 \approx 10^{-9}$ s, which constitutes an intermediate case for which neither case (a) nor (b) is expected to predict the results, in agreement with experiment [138]. However, the authors cite $T_1 = 2.7 \times 10^{-9}$ s for the Cu(II) complex, but make no comment about the fact that the experimental linewidth agrees well with that calculated for case (a). At viscosities <1 cP the broadening of an EPR line is concluded to be determined primarily by exchange interactions. The relative contribution of dipole–dipole broadening is particularly small for ions with short relaxation times. At viscosities >50 cP the dipole–dipole interactions are not being neutralized by translational motion, and dipole broadening may be comparable in magnitude with exchange broadening for species with long T_1 [138].

A more detailed study of exchange broadening using the kinetic equation for density matrices was conducted by Salikhov et al. [144,153]. For a spin 1/2 species (S_1) (e.g., a nitroxyl) interacting with a second spin 1/2 species (S_2) in the limiting case of $|J| >> |\delta|$, $|\delta|\tau_c << 1$, the exchange broadening, $\Delta\omega$, of the nitroxyl is given by

$$\Delta\omega(S_1) = \frac{1}{2\tau_0(S_1)} \frac{J^2\tau_c^2}{1 + J^2\tau_c^2} \tag{33}$$

where $\tau_0(S_1)$ is the average time between two successive collisions of S_1 with S_2 particles, δ is the difference in resonance frequencies and τ_c is the duration of an individual collision. The theory was also applied to the case of a nitroxyl ($S_1 = 1/2$) colliding with Mn(II) ($S_2 = 5/2$) yielding

$$\Delta\omega(S_1) = \frac{2}{3} \frac{S_2(S_2+1)}{\tau_0(S_1)} \frac{J^2\tau_c^2}{1 + 9J^2\tau_c^2} \approx \frac{35}{6} \frac{1}{\tau_0(S_1)} \frac{J^2\tau_c^2}{1 + J^2\tau_c^2} \tag{34}$$

$$\Delta\omega(S_2) = \frac{2}{3} \frac{S_1(S_1+1)}{\tau_0(S_2)} \frac{J^2\tau_c^2}{1 + 9J^2\tau_c^2} \approx \frac{1}{2} \frac{1}{\tau_0(S_2)} \frac{J^2\tau_c^2}{1 + J^2\tau_c^2} \tag{35}$$

The spin exchange rate constants obtained from the linewidths of the nitroxyl and the Mn(II) differed, as predicted; the experimental ratio being 9 ± 3 compared with the predicted ratio of 11.7. The results were generalized to arbitrary S_2. An important insight stimulated by these relationships is that when the interacting spins are different the concept of exchange frequency loses the physical picture of the "flip–flop" model [144].

For the case of very short metal electron spin relaxation time, $T_1 << \tau_c$, and with $JT_1 << 1$, the exchange broadening of a nitroxyl EPR spectrum line was given as

$$\Delta\omega_{ex}(1) = Z_1 \frac{2}{3} \frac{J^2 S_2(S_2+1) T_1 \tau_c}{1 + \frac{2}{3} J^2 S_2(S_2+1) T_1 \tau_c} \tag{36}$$

where Z_1 is the frequency of collisions of a nitroxyl with metal complexes. Thus T_1 for the metal strongly influences the nitroxyl linewidth under these conditions [153].

An extended outline of the density matrix treatment [153] examines a number of limiting cases and identifies conditions that must be met for the simplified expressions to be valid. Among the interesting conclusions not covered above is that for complexes with short relaxation times $T_2(2)$ (1 = nitroxyl, 2 = complex) the EPR spectra do not reveal exchange narrowing effects when $\max(Z_1p_1, Z_2p_2) << T_2(2)^{-1}$ even though the condition $\delta << \max(Z_1p_1, Z_2p_2)$ is satisfied. Here, Z_i is the mean frequency of collisions of a spin S_i with all the spins S_2; δ is the difference between the resonance frequencies of 1 and 2; p_1,p_2 characterize the efficiency of phase variation processes during collisions. This result is explained by the fact that when T_2 is short enough complete relaxation can occur between collisions so that phases of spins in two subsequent collisions are not correlated. This paper summarizes experimental data from several prior papers [135,138,144,154].

In summary, by measuring the effect of viscosity on the interaction and comparing the results with the contributions calculated for exchange and dipolar interactions, the following conclusions were reached in this series of papers by Molin et al.:

(1) The calculated dipole—dipole broadening for collision of **XXXVII** with various metal ions was important for Cr(III) and Mn(II), but not for Fe(II), Co(II), Ni(II) and Cu(II) [140].
(2) Dipole—dipole interaction accounts for about 20% of the broadening of I by metal acetylacetonates in $CHCl_3$ solution [135].
(3) If the electron relaxation time, T_1, for the metal is less than the collision lifetime, exchange broadening of the EPR line should decrease with T_1. This is claimed to be the case for Mn^{3+}, Co^{2+} and Ni^{2+} [135]. However, the T_1 values used were for the aquo complexes, and no explicit recognition was made of the variety of solution structures for the acetylacetonates, except that $Co(acac)_2$ was used in pyridine to dissociate polymers.
(4) At low viscosities the broadening of an EPR line is determined primarily by spin exchange interactions [138].
(5) The relative contribution of dipole—dipole broadening is particularly small for ions with short relaxation times [138].
(6) For species with long relaxation times the contributions to broadening from exchange and dipole—dipole interactions could be comparable under certain circumstances [138].
(7) Broadening decreases with increased viscosity due to a decrease in the frequency of collisions [153].
(8) At high viscosity the linewidth reaches a limit equal to the dipole—dipole contribution [153].
(9) For collisions of nitroxyls with metal complexes both strong exchange and weak exchange situations can occur [153].

In a series of papers Likhtenshtein and coworkers have explored the broaden-

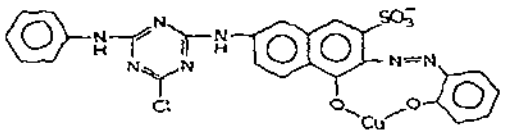
ing of free nitroxyl radicals and nitroxyl labels on proteins in the presence of paramagnetic metal ions in solution [44,136,143]. The broadening of the EPR spectrum of **XXXVII** upon collision in solution with metal complexes was found to be linearly related to the the number of unpaired electrons on the metal [136]. The broadening upon collision also depended on the geometry of the metal complex and on the unpaired spin density on the periphery of the molecule accessible to the nitroxyl in collisions [136].

Collision of dibenzene chromium cation, $(C_6H_6)_2Cr^+$, with nitroxyl radicals in spin-labeled lysozyme caused broadening of the nitroxyl EPR lines "without a change of the integral intensity of the signal and shape of the line" [143]. The broadening was interpreted as an exchange interaction, and the rate constant for exchange relaxation, K, was calculated using the formula

$$K = \frac{2.6 \times 10^7\ \Delta H}{C}\ \text{l mol}^{-1}\ \text{sec}^{-1} \qquad (37)$$

where ΔH is the broadening (G) and C is the concentration (mol l^{-1}). This formula differs in the numerical coefficient from others used by Likhtenshtein.

Interaction of the Cu(II) complex **XXXVIII** with nitroxyl radicals **IV** and



XXXVIII *

V in aqueous solution resulted in broadening of the nitroxyl EPR spectra in accordance with the formula [42]

$$K \cdot p = \frac{2.8 \times 10^5\ \delta\Delta H}{m} \qquad (38)$$

where K is the collision rate constant, p is the effectiveness of exchange relaxation during collision, $\delta\Delta H$ is the broadening due to exchange interaction and m is the concentration. A very similar study used the formula [44]

$$K = \frac{1.52 \times 10^7\ \Delta H_0}{C} \qquad (39)$$

where K = rate constant of exchange relaxation, ΔH_0 = broadening of the central component of the EPR spectrum and C = molar concentration of the paramagnetic complex. The broadening was estimated both from the change in the peak-to-peak width and from the decrease in intensity (h) assuming the proportionality $h(\Delta H)^2$. The similar dependence of width on concentration using both methods indicated that the EPR signal changes were entirely exchange broadening due to collisions [44]. Actually there are some nonlinearities in some of the plots of data, but since there is no indication of

* The formula of **XXXVIII** is given as in ref. 42 since not enough characterization data were presented to ascertain the copper coordination environment.

experimental uncertainty the significance, if any, of the non-linearities cannot be judged. It was also pointed out that spin labels on the surface of proteins can move such that the nitroxyl describes a figure of fairly large volume — in one case estimated to be a sphere of 16—18 Å diameter — and collide with paramagnetic ions in solution almost as freely as unbound nitroxyl [44].

By using charged and neutral metal complexes, $Fe(CN)_6^{3-}$, $Fe(acac)_3$ and $(C_6H_6)_2Cr^+$, the existence of charged groups near (<10—12 Å) a spin-labeled site on a protein can be shown [141]. Collision broadening was demonstrated to be more effective, at the same concentrations, between species of opposite charge in solution and thus, by inference, on the protein [141]. Addition of $Fe(CN)_6^{3-}$ to spin-labeled myosin broadened the initially sharp EPR spectrum due to a weakly immobilized spin label while not significantly affecting a strongly immobilized spin label EPR spectrum [160]. This was interpreted as indicating that collisions occurred more readily between the $Fe(CN)_6^{3-}$ and the weakly immobilized label than the strongly immobilized label [160].

The rate constants for spin exchange between **XXXVII** and a series of substituted porphyrin complexes of Fe(III), Cu(II), Ni(II) and VO(II) were measured [137]. The Fe, Cu and V complexes all had one unpaired electron, but there was no obvious overall pattern to the rate constant data. Comparison of the rate constants for these porphyrin complexes with similar experiments involving hemoglobin indicated that the heme group in hemoglobin was not readily accessible to the nitroxyl [137].

In contrast to the exchange interpretation of Likhtenshtein, Rozantsev and coworkers, Oakes assumed that Mn^{2+} broadened nitroxyl EPR spectra in micellar solutions by a dipole—dipole interaction mechanism [161]. The spin exchange term was stated to be negligible in this system [161].

A density matrix treatment of the effects of exchange among several species on the EPR spectra was performed by Parmon et al. [151]. Data for broadening of **XXXVII** by Cu^{2+} in aqueous solution fit the calculated predictions only for a set of equations which do not assume that the broadening effects are additive [151]. The contributions to the linewidth due to exchange are additive when calculated only to terms first order in concentration, but are not additive when calculated to second order in concentration. The second order corrections can be substantial if the collision frequency is comparable to the hyperfine splitting constant. Furthermore, the change in nitroxyl linewidth for a given change in metal concentration depends on the concentration of the nitroxyl. This effect is appreciable when nitroxyl—nitroxyl exchange leads to overlap of the hyperfine components of the nitroxyl. The calculations assuming additivity of exchange broadening contributions agreed with experiment up to 0.03 M nitroxyl, but deviated substantially at 0.05 M nitroxyl. However, at 0.05 and 0.06 M nitroxyl the formula assuming non-additivity did agree with experiment [151].

The problem of ascertaining metal—nitroxyl collision rates in the high concentration region where non-additivity of exchange broadening contributions must be considered was examined in a subsequent paper [152]. In the absence

of paramagnetic complexes, the effect of self-exchange of the nitroxyl on T_2 is given by the expression

$$\frac{1}{T_2} = \frac{2}{3} k_{11} C_1 \left[1 - \frac{1}{1 + 3\left(\frac{a^{(1)}}{k_{11} C_1}\right)^2} \right] \tag{40}$$

rather than

$$\frac{1}{T_2'} = \frac{2I}{2I+1} \nu_e^{(11)} = \frac{2}{3} k_{11} C_1 \tag{41}$$

which ignores the effects discussed in ref. 151. In these expressions $1/T_2 = \sqrt{3} g\beta \Delta H_{pp}/2h$ where ΔH_{pp} is the peak-to-peak linewidth, C_1 is the nitroxyl concentration, $a^{(1)}$ is the nitroxyl hyperfine splitting constant and k_{11} is the spin exchange constant related to the exchange frequency $\nu_e^{(11)}$ by the expression $\nu_e^{(11)} = k_{11} C_1$. (In subsequent expressions 2 denotes a metal complex in obvious extensions of these symbols.) It is seen that the correction in brackets in the expression for T_2 becomes significant when $a^{(1)} \cong k_{11} C_1$. This is also the condition under which the coefficient f in the expression for the metal–nitroxyl spin exchange rate constant, K_{12}, differs from $\sqrt{3}/2$ (as it would be for a Lorentzian line).

$$K_{12} = f \frac{g\beta}{h} \frac{\delta(\Delta H_{pp})}{\delta C_2} \tag{42}$$

Here $\delta(\Delta H_{pp})$ is the increase in nitroxyl linewidth due to an increase δC_2 in the metal concentration. In this case f depends on $K_{11} C_1/a^{(1)}$, $(1/a^{(1)})(1/T_2)_0$ and C_2. $(1/T_2)_0$ is the linewidth of the nitroxyl at infinite dilution. Thus the estimation of K_{12} depends on calculation of f. Graphical presentation of certain results is given in ref. [152].

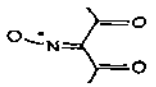
Skubnevskaya and Molin investigated the possibility of using the temperature dependence of the line broadening to distinguish between weak and strong spin exchange in collisions [145]. They present formulas showing that (a) strong exchange is characterized by a diffusional activation energy for the spin exchange constant K_e and by the absence of any temperature dependence for F, $F = K_e/K_D$, where K_D is the bimolecular diffusion constant for collisions of the radical with the complex

$$K_D = \frac{8}{3} \frac{kT}{\eta} \tag{43}$$

(b) weak exchange is characterized by a negative activation energy for K_e and F. Dipole–dipole broadening was considered insignificant in the systems examined since T_1 was short. They conclude that $Co(C_3H_7OH)_2Cl_2$ interacting with **XXXVII** in C_3H_7OH solution corresponds to the strong exchange case. Similarly $CoCl_2$ (aq) exhibits strong exchange with **XXXVII**. However, $Co(ClO_4)_2$ collisions with **XXXVII** in propanol, methanol, dimethylsulfoxide and acetone are all weak exchange cases. $Ni(acac)_2L_2$ collisions with **XXXVII** in $CHCl_3$ solution are an interesting case, being strong when L is aniline and weak when L is pyridine [145].

Hyde and Sarna have measured T_1 and T_2 for nitroxyls colliding with Cu(II), Gd(III) and other lanthanides [158]. They concluded that the interaction between Cu(II) and nitroxyl is predominantly Heisenberg exchange, but that dipole—dipole spin relaxation is the major contribution to broadening of nitroxyl in collisions with Gd(III) [158]. Exchange between a fast relaxing metal and a slow relaxing radical (e.g., nitroxyl) contributes equally to T_2^{-1} and T_1^{-1} of the radical [158]. In all of the lanthanide—nitroxyl experiments dipole—dipole contributions were judged to dominate T_1^{-1} [158]. Hyde and Sarna [158] and Kulikov and Likhtenshtein [56] emphasize that when T_1 for the metal is short enough, interaction of a nitroxyl with the metal provides an efficient equilibration of the nitroxyl spin with the lattice.

The radical **XXXIX** in the presence of $Cu(hfac)_2$ exhibited EPR spectral



XXXIX

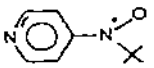
changes which were interpreted in terms of π-complex formation between the radical and the $Cu(hfac)_2$ with some orbital mixing between the radical and the copper [162]. This proposal is surprising in view of the claim that the copper EPR spectrum was not changed significantly by interaction with the radical [162]. However, no indication is given of what "significantly" means.

In a paper which became the subject of debate because of its conclusion that cellular protoplasm has a viscosity several times that of water, Keith and Snipes used the broadening effect of Ni^{2+} on nitroxyl radicals in solution to eliminate the interfering signals from nitroxyl radicals outside the cell [146, 163, 164]. (The technique of broadening nitroxyl EPR signals via collisions with paramagnetic metal species has also been used by Likhtenshtein [44,141], Oakes [161] and Rozantsev [66,67].) Keith and Snipes assumed that broadening was due to dipolar interactions, but presented no substantiation for the assumption that exchange effects did not contribute [146]. Broadening of the spectra of nitroxyls outside cells or vesicles by $NiCl_2$ has found utility in several studies [147—149]. The Ni(II) complex of tris(hydroxymethyl)aminomethane has been used for the same purpose [150]. $K_3Fe(CN)_6$ also has been proposed as a broadening agent for extracellular nitroxyl radicals. It is stated to be effective at 8×10^{-2} M and to have advantages relative to Ni(II) for biological systems [165]. $K_3Fe(CN)_6$ has also been added to these systems to prevent bioreduction of the nitroxyl [147,149].

Zelonka and Baird obtained NMR spectra of acetylacetonate-type complexes of VO^{2+}, Cr^{3+}, Fe^{3+}, Ru^{3+} and Cu^{2+} in the presence of DTBN (**XXXIV**) [128,129]. They found little or no narrowing of lines in the NMR spectra except in certain Cu complexes, and concluded that spin exchange, if any, was not sufficient to shorten the metal electron spin relaxation time significantly for any of the complexes except copper bis(hexafluoroacetylacetonate), Cu-

$(hfac)_2$, and copper bis(trifluoroacetylacetonate), $Cu(tfac)_2$ [128,129].

Wilbur and Kreilick also examined the broadening of the EPR spectra of I and **XXXIV** upon collision with metal acetylacetonates [134]. They concluded that the variations they observed in the broadenings "may be due to differences in either the dipolar or exchange terms" [134]. They predicted that exchange interaction would be enhanced by a longer metal electron relaxation time [134].



XL

Cazianis and Eaton [166] noted changes in the EPR spectrum of the spin-labeled ligand **XL** when coordinated to the metal complexes $Ni(hfac)_2$ and $Co(hfac)_2$. A change in coupling constant and linewidth was observed, with the lines broader in the Co complex than in the Ni complex [166].

I. RELATED STUDIES ON INTERACTIONS BETWEEN PARAMAGNETIC METALS AND RADICALS OTHER THAN NITROXYL RADICALS

In this section brief mention will be made of studies which involve free radicals other than nitroxyls in proximity to paramagnetic metals. These examples were selected because the interpretations given in the papers are relevant to the analysis of metal—nitroxyl interactions.

(i) Coenzyme B-12

EPR studies of coenzyme B-12-enzyme reactions reveal the presence of two lines of unequal intensity separated by about 70 to 150 G in the $g = 2$ region of frozen solution spectra [25,122,167,168]. Two papers [167,168] suggested that the Leigh theory might be applicable to certain details of the EPR spectra. It is important to note that the Leigh formula does not imply a reduction in the integrated area of the EPR peaks as implied by these papers [167,168]. Schepler et al. [122] proposed that the "doublet" EPR spectrum results from a weak exchange interaction between an organic free radical and low spin Co(II). The analysis was made in terms of an "AB" splitting pattern for two spin 1/2 species by analogy with standard high-resolution NMR spectral interpretation [122]. Buettner and Coffman showed that both exchange interaction and magnetic dipole—dipole interaction are present by performing an exact computation of the spectrum directly from the spin Hamiltonian rather than using a perturbation method [25]. They also demonstrated the importance of examining the spectra at more than one microwave frequency [25].

(ii) Horseradish peroxidase

A weak EPR signal, whose intensity was proportional to the concentration of Compound I, was observed in the reactions producing Compound I of horse-

radish peroxidase [170]. The *g*-value and width of the signal were consistent with an organic free radical, but the short relaxation time was interpreted to indicate interaction with iron [170]. It was also suggested that the Leigh theory could account for the reduced intensity of the signal [170].

(iii) Succinate dehydrogenase

The treatment of Leigh was applied to the EPR spectrum of succinate dehydrogenase in beef heart mitochondria [171]. A distance of approximately 6 Å was estimated between the Hipip-type iron sulfur center, center S-3, and a radical of ubiquinone [171].

(iv) Photosystem II

The relaxation time of signal II_{vf} in photosystem II of spinach chloroplasts was decreased by the presence of $NiCl_2$ in solution and increased by removal of Mn^{2+} [172]. These observations were stated to support the postulate that the species giving rise to signal II_{vf} is in close proximity to a transition metal which relaxes it via a dipolar interaction mechanism [172].

(v) Irradiated DNA

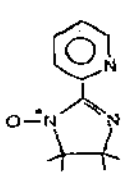
The effect of metal ions on the EPR spectra of irradiated DNA was examined by Riesz and coworkers [173,174]. They concluded that spin—spin interaction as described by Leigh did not account for the relative signal intensities observed since the intensities did not correlate with the metal—radical interaction as measured by the effect of the metal on the relaxation time of the radical [173,174]. These papers contain references to related studies that are beyond the scope of this review.

(vi) Melanin

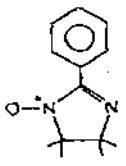
Sarna, Hyde and Swartz observed that the EPR signal of the natural free radical in melanin is reduced in intensity by paramagnetic transition metal and lanthanide ions [175]. The results were interpreted as confirming that the dipolar interaction theory of Leigh adequately describes the EPR signal intensity changes [175]. A large molar excess of the metal was required to saturate all of the metal binding sites and, in one experiment, Cu^{2+} in bulk frozen solution was observed to decrease the free radical signal intensity [175]. They also examined the dependence of the melanin free radical relaxation behavior on the relaxation time of the added metal and found agreement with Bloembergen's calculation of the effect of paramagnetic metals in solids on the relaxation of nearby nuclei [175,176].

(vii) SO_2^-

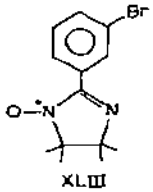
The SO_2^- radical produced by dissociation of $S_2O_4^{2-}$ in solution interacts with Mn^{2+} causing broadening of the EPR spectra. The equilibria in this



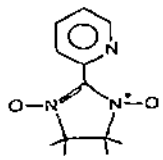
XLI



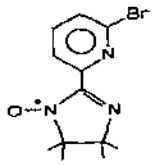
XLII



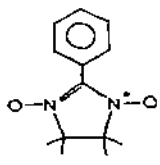
XLIII



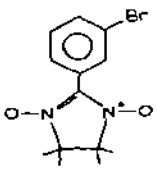
XLIV



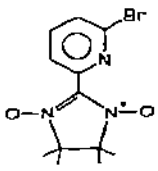
XLV



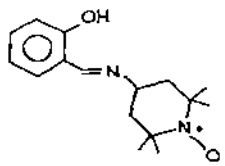
XLVI



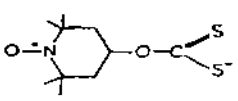
XLVII



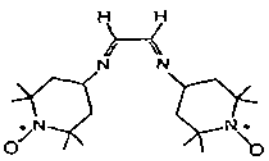
XLVIII



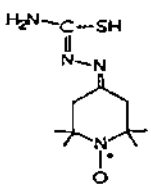
XLIX



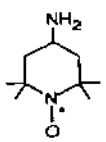
L



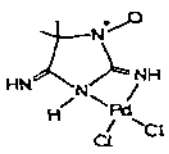
LI



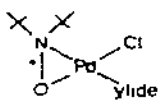
LII



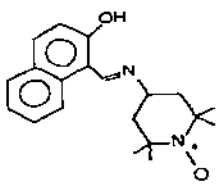
LIII



LIV



LV



LVI

system are fairly complicated, but the authors judge that the major effect on the Mn^{2+} EPR is the formation of $[Mn(H_2O)_4S_2O_4]$ with distortion of the symmetry at Mn, and that the major effect on the SO_2^- EPR is dipolar interaction between the unpaired spins in an ion pair, $Mn^{2+}(H_2O)_6 \cdot (SO_2^-)_m$ [199]. The dipolar interaction was treated with the approach used by Pearson and Buch (see eqn. (11), Section H).

J. INTERACTION OF NITROXYL RADICALS WITH DIAMAGNETIC METALS

Several papers have reported complexes of nitroxyl radicals with diamagnetic metals. These papers are not discussed exhaustively here because they are of importance to the present work primarily when they provide comparison data for evaluation of results concerning a complex of the same ligand with a paramagnetic metal. References to many of these papers are listed in Table 7.

TABLE 7

Interaction of nitroxyl radicals with diamagnetic metals

Metal	Nitroxyl	Reference
Li(I)	XLI	177
Li(I)	XLII	177
Li(I)	XLIII	177
Li(I)	XLIV	177
Li(I)	XLV	177
Li(I)	XLVI	177
Li(I)	XLVII	177
Li(I)	XLVIII	177
BF_3	XXXIV	178, 179
BF_3	XXXV	179
BCl_3	XXXIV	179
BCl_3	XXXV	179
BBr_3	XXXIV	179
BBr_3	XXXV	179
$AlCl_3$	XXXIV	178—182
$AlCl_3$	XXXV	178, 179, 205
$AlCl_3$	XXXVII	183, 184
$AlBr_3$	XXXVII	183, 184
AlI_3	XXXVII	183, 184
$Al(i\text{-}Bu)_3$	XXXV	185
Al(silica alumina surface)	XXXIV	186, 187
Al_2O_3	XXXV	204—207
SiF_4	XXXV	185
SiF_4	XXXIV	185
$SiCl_4$	XXXV	185
$SiCl_4$	XXXIV	181, 182, 185

TABLE 7 (continued)

Metal	Nitroxyl	Reference
$TiCl_4$	XXXIV	181,182, 185
$Ti(OPh)_4$	XXXIV	181, 182, 185
Co(III)	XLIX	105
Co(III)	L	188
Co(III)	LVI	213
Ni(II)	L	188
Ni(II)	XLIX	213
Cu(I)	LI	112
Zn(II)	XVIII	101, 112
Zn(II)	XIX	101
Zn(II)	XLIX	104, 105
Zn(II)	LII	104
Zn(II)	XXXV	133
Zn(II)	LI	113
Zn(II)	XLI	189, 190
Zn(II)	XLV	190
Zn(II)	L	188
$ZnCl_2$	XL	166
$ZnBr_2$	XL	166
ZnI_2	XL	166
$Zn(hfac)_2(H_2O)_2$	XL	166
$Zn(acac)_2H_2O$	XL	166
$Zn(thiourea)_2Cl_2$	XL	166
$Zn(\phi NH_2)_2Cl_2$	XL	166
$Zn(\phi NH_2)_2Br_2$	XL	166
$Zn(\phi NH_2)_2I_2$	XL	166
$Zn(py)_2Br_2$	XL	166
$Zn(py)_2(SCN)_2$	XL	166
$Zn(py)_2(CNO)_2$	XL	166
$GaCl_3$	XXXVII	183
$GaBr_3$	XXXVII	183
GaI_3	XXXVII	183
$GaBr_3$	XXXV	205
Ga_2O_3	XXXV	205
$GeCl_4$	XXXIV	181, 182, 185
$GeCl_4$	XXXV	185
$[Rb(tfa)]_2$	XXXV	218
Pd(II)	XVIII	112
Pd(II)	XXIX	111
Pd(II)	XXX	113
Pd(II)	XXXI	113
Pd(II)	XXXV	112

TABLE 7 (continued)

Metal	Nitroxyl	Reference
Pd(II)	LII	112
Pd(II)	LI	112
Pd(II)	XLIX	113
Pd(II)	LIV	191
$Pd(NO_3)_2$	XL	166
$Pd(tfac)_2$	XL	166
$Pd(hfac)_2$	XL	166
$[PdCl(XXXIV)]_2$	XXXIV	192
$[PdBr(XXXIV)]_2$	XXXIV	192
PdCl(XXXIV)-(ylide), LV	XXXIV	193
Ag(I)	XLI	190
Ag(I)	XLII	190
Ag(I)	XLIII	190
Ag(I)	XLV	190
Cd(II)	XLI	189, 190
Cd(II)	XLV	190
CdI_2	XL	166
$InBr_3$	XXXVII	194
InI_3	XXXVII	194
$SnCl_4$	XXXIV	181, 182, 185
$SnCl_4$	XXXV	181, 182, 185
$SnBr_4$	XXXIV	185
$(CO)_5$-Cr-Sn(t-Bu)$_2$	XXXIV	182, 185
Pt(II)	XVIII	112
Pt(II)	LII	113
Pt(II)	LIII	113, 214
Pt(II)	LI	113
Hg(II)	XLI	189, 190
Hg(II)	XLII	190
Hg(II)	XLIII	190
Hg(II)	XLV	190
Hg(II)	L	188
$HgCl_2$	XL	166
HgI_2	XL	166
Pb(II)	XLI	190
Pb(II)	XVL	190

K. SUMMARY

The experimental results summarized in this review indicate considerable promise of significant application of metal—nitroxyl interactions to understanding a wide variety of phenomena, including:

(a) distances in biological systems — the effect of a paramagnetic metal on

the EPR spectrum of a nitroxyl spin label, both in specific locations in a biological macromolecule, has been used to estimate the distance between the metal and the nitroxyl spin label.

(b) location of charged groups in biological systems — the effect of charged metal complexes in solution on the EPR spectrum of a nitroxyl spin label can be used to find out about the proximity of the spin label to charged groups on the biological macromolecule or membrane.

(c) heterogeneous environments — broadening of the EPR spectrum of a nitroxyl upon collision with a paramagnetic metal complex in solution can be used to differentiate between nitroxyls according to their accessibility to collisions with the metal complex. Thus spin labels on the surface of a macromolecule can be distinguished from those "buried" in a location less accessible to the metal. Nitroxyls inside cells or membranes can be distinguished from nitroxyls in the surrounding fluid.

(d) microviscosity — the viscosity dependence of metal—nitroxyl interactions can be used to probe the details of microviscosity as experienced by molecules of various sizes, shapes, charges, etc.

(e) solution dynamics — fundamental information relating to the kinetics of reactions is accessible via metal—nitroxyl interactions. This approach has an advantage over more traditional studies of chemical kinetics for obtaining certain information in that the reaction is studied at equilibrium (in a macroscopic sense) and the "reagents" are not chemically changed by the reaction.

(f) spin—spin interactions — fundamental understanding of the interactions between pairs of nonequivalent unpaired electrons is accessible via study of metal—nitroxyl interactions. These studies may yield improved quantitative results to be used in interpreting studies in areas (a)—(e). It is also important to recognize that the spin exchange interaction is the most elementary chemical reaction and thus is of inherent interest in its own right.

At the present time it appears that lack of electron spin relaxation time data is the most serious experimental impediment to interpretation of metal—nitroxyl interaction results. Hopefully there will be increased emphasis on T_1 measurements in EPR.

Theoretical advances are also urgently needed. There does not exist a general theory appropriate to interaction of nonequivalent spins (e.g., metal and nitroxyl) in fluid solution. Some limiting cases have been studied, but even here more attention must be given to careful definition of the conditions under which the simplified expressions are valid before they can be routinely applied by the experimentalist.

ACKNOWLEDGEMENTS

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REFERENCES

1 T.J. Stone, T. Buckman, P.L. Nordio and H.M. McConnell, Proc. Natl. Acad. Sci. U.S., 54 (1965) 1010.
2 C.L. Hamilton and H.M. McConnell, in A. Rich and N. Davidson (Eds.), Structural Chemistry and Molecular Biology, W.H. Freeman and Co., San Francisco, 1968, p. 115.
3 O.H. Griffith and A.S. Waggoner, Acc. Chem. Res., 2 (1969) 17.
4 H.M. McConnell and B.G. McFarland, Quart. Rev. Biophys., 3 (1970) 91.
5 M. Cohn, Quart. Rev. Biophys., 3 (1970) 61.
6 M. Cohn and J. Reuben, Acc. Chem. Res., 4 (1971) 214.
7 P. Jost and O.H. Griffith, in C.F. Chignell (Ed.), Methods in Pharmacology, Vol. 2, Appleton—Century—Crofts, N.Y. 1972, p. 223.
8 P.F. Knowles, Essays in Biochemistry, 8 (1972) 79.
9 I.C.P. Smith, in H.M. Swartz, J.R. Bolton and D.C. Borg (Eds.), Biological Applications of Electron Spin Resonance, 1972, p. 483.
10 C.F. Chignell, Life Sciences, 13 (1973) 1299.
11 H. Dugas, Can. J. Spectrosc., 18 (1973) 110.
12 A.E. Kalmanson and G.L. Grigoryan, in C. Nicolau (Ed.), Experimental Methods in Biophysical Chemistry, Wiley, N.Y., 1973, p. 589.
13 A.L. Buchachenko, A.L. Kovarskii and A.M. Vasserman, in Z.A. Rogovin (Ed.), Advances in Polymer Science, Wiley, N.Y., 1974, p. 26.
14 J.C. Metcalfe, in L. Bolis et al. (Eds.), Permeability and Function of Biological Membranes, North—Holland, Amsterdam, 1970, p. 222.
15 J.C. Metcalfe, in D. F. Hölzl-Wallach and H. Fischer (Eds.), The Dynamic Structure of Cell Membranes, Springer—Verlag, N.Y., 1971.
16 P. Jost, A.S. Waggoner and O.H. Griffith, in L. Rothfield (Ed.), Structure and Function of Biological Membranes, Academic Press, N.Y., 1971, p. 83.
17 L. Packer, in L. Packer (Ed.), Biomembranes, Academic Press, N.Y., 1973, p. 147.
18 B.J. Gaffney, Methods Enzymol., 32 (1974) 161.
19 L.J. Berliner (Ed.), Spin Labeling: Theory and Applications, Academic Press, N.Y., 1976.
20 G.I. Likhtenshtein, Spin Labeling Methods in Molecular Biology, Wiley, N.Y., 1976.
21 J.S. Taylor, J.S. Leigh Jr. and M. Cohn, Proc. Nat. Acad. Sci. U.S., 64 (1969) 219.
22 J.S. Leigh Jr., J. Chem. Phys., 52 (1970) 2608.
23 L.J. Berliner (Ed.), Spin Labeling: Theory and Applications, Academic Press, N.Y., 1976, p. 304.
24 T.D. Smith and J.R. Pilbrow, Coord. Chem. Rev., 13 (1974) 173.
25 G.R. Buettner and R.E. Coffman, Biochim. Biophys. Acta, 480 (1977) 495.
26 S. Ohnishi and H.M. McConnell, J. Am. Chem. Soc., 87 (1965) 2293.
27 M.B. Neiman, E.G. Rozantsev and Yu. G. Mamedova, Nature, 196 (1962) 472.
28 E.G. Rozantsev, Free Nitroxyl Radicals, Plenum, N.Y., 1970.
29 E.G. Rozantsev and V.D. Sholle, Russ. Chem. Rev., 40 (1971) 233.
30 E.G. Rosantsev and V.D. Sholle, Synthesis, (1971) 190.
31 E.G. Rozantsev and V.D. Sholle, Synthesis, (1971) 401.
32 A.R. Forrester, MTP Int. Rev. Sci. Organic Chem., Series 1, 10 (1973) 139.
33 H.G. Aurich and W. Weiss, Topics Current Chem., 59 (1975) 65.
34 P. Jost, L.J. Libertini, V.C. Herbert and O.H. Griffith, J. Mol. Biol., 59 (1971) 77.
35 G. Poggi and C.S. Johnson Jr., J. Magn. Reson., 3 (1970) 436.
36 I.V. Alexandrov, A.N. Ivanova, N.N. Korst, A.V. Lazarev, A.I. Priklozhenko and V.B. Stryukov, Mol. Phys., 18 (1970) 681.

37 H.M. McConnell and J.C.A. Boeyens, J. Phys. Chem., 71 (1967) 12.
38 J. Chien and H.M. McConnell, work in progress (see ref. 2).
39 S. Ogawa and H.M. McConnell, Proc. Nat. Acad. Sci. U.S., 58 (1967) 19.
40 T. Asakura, J.S. Leigh Jr., H.R. Drott, T. Yonetani and B. Chance, Proc. Nat. Acad. Sci. U.S., 68 (1971) 861.
41 G.I. Likhtenshtein, A.P. Pivovarov, P.Kh. Bobodzhanov, E.G. Rozantsev and N.B. Smolina, Biophysics, 13 (1968) 474.
42 G.I. Likhtenshtein and P.Kh. Bobodzhanov, Biophysics, 13 (1968) 891.
43 G.I. Likhtenshtein, P.Kh. Bobodzhanov, E.G. Rozantsev and V.I. Suskina, Molec. Biol., 2 (1968) 280.
44 G.I. Likhtenshtein, Yu. B. Grebenshchikov, P.Kh. Bobozhanov and Yu. V. Kokhanov, Molek. Biol., 4 (1970) 682 (p. 550 in translation).
45 T. Asakura, H.R. Drott and T. Yonetani, J. Biol. Chem., 244 (1969) 6626.
46 T. Asakura and H.R. Drott, Biochem. Biophys. Res. Commun., 44 (1971) 1199.
47 H.M. McConnell, Ann. Rev. Biochem., 40 (1971) 227.
48 T. Asakura, M. Tamura and M. Shin, J. Biol. Chem., 247 (1972) 3693.
49 T. Asakura, Ann. N.Y. Acad. Sci., 222 (1973) 68.
50 T. Asakura, J. Biol. Chem., 249 (1974) 4495.
51 T. Asakura and M. Tamura, J. Biol. Chem., 249 (1974) 4504.
52 T. Asakura, Biochem. Biophys. Res. Commun., 48 (1972) 517.
53 A.I. Kokorin, K.I. Zamarayev, G.L. Grigoryan, V.P. Ivanov and E.G. Rozantsev, Biofizika, 17 (1972) 34 (p. 31 in transl.).
54 P.V. Sergeev, T.I. Ul'yankina, R.D. Seifulla, Yu. B. Grebenshchikov and G.I. Likhtenshtein, Molek. Biol., 8 (1974) 206 (p. 162 in transl.).
55 A.V. Kulikov, Molek. Biol., 10 (1976) 132 (p. 109 in transl.).
56 A.V. Kulikov and G.I. Likhtenshtein, Adv. Mol. Relaxation Interact. Processes, 10 (1977) 47.
57 A.V. Kulikov and G.I. Likhtenshtein, Biophys., 19 (1974) 424.
58 A.I. Kyaivyaryainen, V.P. Timofeev and M.V. Vol'kenshtein, Molec. Biol., 6 (1972) 709.
59 G. Benski, T. Arends and G. Blanc, Biochem. Biophys. Res. Commun., 35 (1969) 599.
60 R.I. Artyukh, B.P. Atanasov, M.V. Volikenshtein and K. Gerzonde, Molek. Biol., 9 (1975) 452 (p. 369 in transl.).
61 T.B. Marriott and O.H. Griffith, J. Magn. Reson., 13 (1974) 45.
62 B.W. Griffin, S.M. Smith and J.A. Peterson, Arch. Biochem. Biophys., 160 (1974) 323.
63 B.W. Griffin, J.A. Peterson, J. Werringloer and R.W. Estabrook, Ann. N.Y. Acad. Sci., 244 (1975) 107.
64 H.H. Ruf and W. Nastainczyk, Eur. J. Biochem., 66 (1976) 139.
65 L.M. Reichman, B. Annaev and E.G. Rozantsev, Biochim. Biophys. Acta, 263 (1972) 41.
66 L.M. Raikhman, B. Annaev, A.B. Shapiro and E.G. Rozantsev, Biokhimiya, 37 (1972) 548 (p. 449 in transl.).
67 L.M. Reichman, B. Annaev, E.G. Rozantsev and A.B. Shapiro, Chem.—Biol. Interactions, 5 (1972) 243.
68 G.I. Likhtenshtein, E.N. Frolov, N.F. Neznaikov, L.A. Levchenko and Yu. S. Sklyar, Mol. Biol., 6 (1972) 153.
69 A.V. Kulikov, L.A. Syrtsova, G.I. Likhtenshtein and T.N. Pisarskaya, Mol. Biol., 9 (1975) 162.
70 M. Cohn, in B. Chance et al. (Eds.), Probes of Structure and Function of Macromolecules and Membranes, Vol. I, 1971, p. 97.
71 M. Cohn, J.S. Leigh Jr. and G.H. Reed, Symposia on Quantitative Biology, 36 (1971) 533.

72 M. Cohn, H. Diefenbach and J.S. Taylor, J. Biol. Chem., 246 (1971) 6037.
73 Yu. B. Grebenshchikov, G.G. Charkviani, N.A. Gachechiladze, Yu. V. Kokhanov and G.I. Likhtenshtein, Biofizika, 16 (1971) 794 (p. 826 in transl.).
74 A. Bennick, I.D. Campbell, R.A. Dwek, N.C. Price, G.K. Radda and A.G. Salmon, Nature New Biol., 234 (1971) 140.
75 I.D. Campbell, R.A. Dwek, N.C. Price and G.K. Radda, Eur. J. Biochem., 30 (1972) 339.
76 A. Bennick, R.A. Dwek, J.R. Griffiths and G.K. Radda, Annals N.Y. Acad. Sci., 222 (1973) 175.
77 R. Jones, R.A. Dwek and I.O. Walker, FEBS Lett., 26 (1972) 92.
78 R. Jones, R.A. Dwek and I.O. Walker, Eur. J. Biochem., 34 (1973) 28.
79 A. Azzi, M.A. Bragadin, A.M. Tamburro and M. Santato, J. Biol. Chem., 248 (1973) 5520.
80 C. Montecucco and A. Azzi, J. Biol. Chem., 250 (1975) 5020.
81 L.A. Blumenfeld and L.G. Ignateva, Eur. J. Biochem., 47 (1974) 75.
82 C.R. Bagshaw and G.H. Reed, J. Biol. Chem., 251 (1976) 1975.
83 J. Loscalzo, G.H. Reed and A. Weber, Proc. Nat. Acad. Sci. U.S., 72 (1975) 3412.
84 S.V. Vocel, I.A. Slepneca and J.M. Backer, Biopolymers, 14 (1975) 2445.
85 R. Cooke and J. Duke, J. Biol. Chem., 246 (1971) 6360.
86 S.J.W. Busby and G.K. Radda, Current Topics in Cellular Regulation, 10 (1976) 80.
87 R.W. Burley and R.W. Sleigh, in Basic Research in Mycology, Excerpta Medica, Amsterdam, 1972, p. 260. (International Congress Series no. 294; Part I of the Proceedings of the Second International Congress on Muscle Diseases, Perth, November 1971).
88 R.W. Burley, J.C. Seidel and J. Gergely, Arch. Biochem. Biophys., 150 (1972) 792; R.W. Sleigh and R.W. Burley, Arch. Biochem. Biophys., 159 (1973) 792.
89 N.C. Price, M. Cohn and R.H. Schirmer, J. Biol. Chem., 250 (1975) 644.
90 H.R. Drott, D. Santiago and J.D. Shore, FEBS Lett., 39 (1974) 21.
91 R.A. Dwek, R. Jones, D. Marsh, A.C. McLaughlin, E.M. Press, N.C. Price and A.I. White, Phil. Trans. R. Soc. London, Ser. B, 272 (1975) 53.
92 R.A. Dwek, D. Givol, R. Jones, A.C. McLaughlin, S. Wain-Hobson, A.I. White and C. Wright, Biochem. J., 155 (1976) 37.
93 C.H. Fung, R.K. Gupta and A.S. Mildvan, Biochem., 15 (1976) 85.
94 T. Buckman, F.S. Kennedy and J.M. Wood, Biochem., 8 (1969) 4437.
95 P.Y. Law, D.G. Brown, E.L. Lien, B.M. Babior and J.M. Wood, Biochem., 10 (1971) 3428.
96 R.A. Dwek, H.R. Levy, G.K. Radda and P.J. Seeley, Biochim. Biophys. Acta, 377 (1975) 26.
97 J.S. Taylor, P. Mushak and J.E. Coleman, Proc. Nat. Acad. Sci. U.S., 67 (1970) 1410.
98 P. Mushak and J.E. Coleman, J. Biol. Chem., 247 (1972) 373.
99 H.J. Grande, A.J.W.G. Visser, J.L. de Wit, F. Müller and C. Veeger, Z. Naturforsch. Teil B, 27 (1972) 1058.
100 L.A. Krinitskaya and S.N. Dobryakov, Izv. Akad. Nauk SSSR, Ser. Khim., (1966) 582 (p. 558 in transl.).
101 A. Rassat and P. Rey, Bull. Soc. Chim. Fr., (1967) 815.
102 A.A. Medzhidov, L.N. Kirichenko and G.I. Likhtenshtein, Izv. Akad. Nauk SSSR, Ser. Khim, (1969) 698 (p. 629 in transl.).
103 A.A. Medshidov, Yu. G. Mamedova, R.B. Lyubovskii and L.N. Kirichenko, Teor. Eksp. Khim., 6 (1970) 133 (p. 124 in transl.).
104 D. Jahr, K.E. Schwarzhans, D. Nothe and P.K. Burkert, Z. Naturforsch. Teil B, 26 (1971) 1210.
105 G.R. Eaton, Inorg. Nucl. Chem. Lett., 8 (1972) 647.
106 N.I. Golovina, G.A. Klitskaya, A.A. Medzhidov and L.O. Atovmyan, Zh. Strukt. Khim., 16 (1975) 145 (p. 132 in transl.).

107 Yu. G. Mamedova, A.A. Medzhidov and L.N. Kolomina, Russ. J. Inorg. Chem., 17 (1972) 1548.
108 L.O. Atovmyan, N.I. Golovina, G.A. Klitskaya, A.A. Medzhidov, A.V. Zvarykina, V.B. Stryukov and D.N. Fedutin, Zh. Strukt. Khim., 16 (1975) 624 (p. 579 in transl.).
109 R.Z. Sagdeev, Yu. N. Molin, G.A. Kutikova and L.B. Volodarsky, Proc. XVIth Colloque Ampere, Bucharest, 1970, p. 1159.
110 R.Z. Sagdeev, Yu. N. Molin, R.A. Sadikov, L.B. Bolodarsky and G.A. Kutikova, J. Magn. Reson., 9 (1973) 13.
111 S.V. Larionov, V.I. Ovcharenko, R.A. Sadykov and L.B. Volodarskii, Izv. Akad. Nauk SSSR, Ser. Khim., (1975) 1922 (p. 1804 in transl.).
112 D. Jahr, H. Rebhan, K.E. Schwarzhans and J. Wiedemann, Z. Naturforsch. Teil B, 28 (1973) 55.
113 R. Weissgerber and K.E. Schwarzhans, Z. Naturforsch. Teil B, 31 (1976) 208.
114 G. Terzian, A. Commons, M. Asso and D. Benlian, J. Chim. Phys., 73 (1976) 146.
115 C. Veyret and A. Blaise, Mol. Phys., 25 (1973) 873.
116 A search of Chemical Abstracts and Science Citation Index reveals no mention of XXXIII or its copper complex. No information on the preparation or properties of these complexes other than the graph of the magnetic susceptibility data in ref. 115 has been published, until very recently, see ref. 216.
117 P.M. Boymel, J.R. Chang, D.L. DuBois, D.J. Greenslade, G.R. Eaton and S.S. Eaton, J. Am. Chem. Soc., 99 (1977) 5500.
118 P.M. Boymel, S.S. Eaton and G.R. Eaton, unpublished results.
119 G.A. Braden, K.T. Trevor, J.M. Neri, D.J. Greenslade, G.R. Eaton and S.S. Eaton, J. Am. Chem. Soc., 99 (1977) 4854.
120 D.L. DuBois, S.S. Eaton and G.R. Eaton, unpublished results.
121 R.J. Abraham, The Analysis of High Resolution NMR Spectra, Elsevier, New York, 1971, Chpt. 2.
122 K.L. Schepler, W.R. Dunham, R.H. Sands, J.A. Fee and R.H. Abeles, Biochem. Biophys. Acta, 397 (1975) 510.
123 D.J. Lowe, R.M. Lynden-Bell and R.C. Bray, Biochem. J., 130 (1972) 239.
124 A. Abragam and B. Bleaney, Electron Paramagnetic Resonance of Transition Metals, Oxford, 1970, pp. 509–514.
125 K.I. Zamaraev, A.T. Nikitaev and G.A. Senyukova, Kinet. Catal., 13 (1972) 54 (p. 45 in transl.).
126 B.E. Wagner, personal communication, 1974.
127 W. Beck, K. Schmidtner and H.J. Keller, Chem. Ber., 100 (1967) 503.
128 R.A. Zelonka and M.C. Baird, J. Am. Chem. Soc., 93 (1971) 6066.
129 R.A. Zelonka and M.C. Baird, Chem. Commun., (1970) 1448.
130 C.M. Paleos, N.M. Karayannis and M.M. Labes, Chem. Commun., (1970) 195.
131 D.G. Brown, T. Maier and R.S. Drago, Inorg. Chem., 10 (1971) 2804.
132 Y.Y. Lim and R.S. Drago, Inorg. Chem., 11 (1972) 1334.
133 N.M. Karayannis, C.M. Paleos, C.M. Mikulski, L.L. Pytlewski, H. Blum and M.M. Labes, Inorg. Chim. Acta, 7 (1973) 74.
134 D. Wilbur and R. Kreilick, J. Chem. Phys., 52 (1970) 1643.
135 G.I. Skubnevskaya, K.M. Salikhov, L.M. Smirnova and Yu. N. Molin, Kinet. Katal., 11 (1970) 888 (p. 733 in transl.).
136 G.I. Likhtenshtein, Yu. B. Grebenshchikov and A.A. Medzhidov, Russ. J. Phys. Chem., 44 (1970) 453.
137 Yu. B. Grebenshchikov, G.V. Ponomarev, R.P. Yevstigneyeva and G.I. Likhtenshtein, Biofizika, 17 (1972) 910 (p. 956 in transl.).
138 O.A. Anisimov, A.T. Nikitaev, K.I. Zamaraev and Yu. N. Molin, Teor. Eksp. Khim., 7 (1971) 682 (p. 556 in transl.).
139 R.G. Pearson and T. Buch, J. Chem. Phys., 36 (1962) 1277.

140 G.I. Skubnevskaya and Yu. N. Molin, Kinet. Katal., 8 (1967) 1192 (p. 1012 in transl.).
141 Yu. B. Grebenshchikov, G.I. Likhtenshtein, V.P. Ivanov and E.G. Rozantsev, Molek. Biol., 6 (1972) 498 (p. 400 in transl.).
142 G.I. Likhtenshtein, Yu. B. Grebenshchikov and T.V. Avilova, Molek. Biol., 6 (1972) 67 (p. 52 in transl.).
143 G.I. Likhtenshtein and Yu. D. Akhmedov, Molek. Biol., 4 (1970) 551 (p. 445 in transl.).
144 K.M. Salikhov, A.T. Nikitaev, G.A. Senyukova and K.I. Zamaraev, Teor. Eksp. Khim, 7 (1971) 619 (p. 503 in transl.).
145 G.I. Skubnevskaya and Yu. N. Molin, Kinetika i Kataliz, 13 (1972) 1383 (p. 1236 in transl.).
146 A.D. Keith and W. Snipes, Science, 183 (1974) 666.
147 P.D. Morse, II, M. Ruhlig, W. Snipes and A.D. Keith, Arch. Biochem. Biophys., 168 (1975) 40.
148 R.H. Hammerstedt, R.P. Amann, T. Rucinsky, P.D. Morse, II, J. Lepock, W. Snipes and A.D. Keith, Biol. Reprod., 14 (1976) 381.
148a J.R. Lepock, P.D. Morse, II, R.J. Mehlhorn, R.H. Hammerstedt, W. Snipes and A.D. Keith, FEBS Lett., 60 (1975) 185.
149 S.A. Henry, A.D. Keith and W. Snipes, Biophys. J., 16 (1976) 641.
150 A.D. Keith, W. Snipes and D. Chapman, Biochemistry, 16 (1977) 634.
151 V.N. Parmon, A.T. Nikitaev, G.M. Zhidomirov and K.I. Zamaraev, Zh. Strukt. Khim, 13 (1972) 400 (p. 378 in transl.).
152 V.N. Parmon, A.T. Nikitaev, G.M. Zhidomirov and K.I. Zamaraev, Zh. Strukt. Khim., 13 (1972) 607 (p. 566 in transl.).
153 K.M. Salikhov, A.B. Doctorov, Yu. N. Molin and K.I. Zamaraev, J. Magn. Reson., 5 (1971) 189.
154 G.I. Skubnevskaya, E.E. Zaev, R.I. Zusman and Yu. N. Molin, Dokl. Acad. Nauk SSSR, 170 (1966) 386 (p. 603 in transl.).
155 P.W. Anderson, J. Phys. Soc. Jpn., 9 (1954) 316.
156 G.E. Pake and T.R. Tuttle Jr., Phys. Rev. Lett., 3 (1959) 423.
157 A. Abragam, The Principles of Nuclear Magnetism, Oxford, 1961.
158 J.S. Hyde and T. Sarna, personal communication, 1977.
159 J.D. Currin, Phys. Rev., 126 (1962) 1995.
160 L.G. Ignat'eva, T.M. Seregina, L.A. Blyumenfel'd, E.K. Ruuge, R.I. Aryukh and G.B. Postnikiva, Biofizika, 17 (1972) 533 (p. 556 in transl.).
161 J. Oakes, Nature, 231 (1971) 38.
162 W.H. Wolodarsky, J. Faniran and J.K.S. Wan, Can. J. Chem., 51 (1973) 4072.
163 E.D. Finch and J.F. Harmon, Science, 186 (1974) 157.
164 W. Snipes and A.D. Keith, Science, 186 (1974) 158.
165 P.D. Morse, II, Biochem. Biophys. Res. Commun., 77 (1977) 1486.
166 C.T. Cazianis and D.R. Eaton, Can. J. Chem., 52 (1974) 2454.
167 S.A. Cockle, H.A.O. Hill, R.J.P. Williams, S.P. Davies and M.A. Foster, J. Am. Chem. Soc., 94 (1972).
168 T.H. Finlay, J. Valinsky, A.S. Mildvan and R.H. Abeles, J. Biol. Chem., 248 (1973) 1285.
169 V.P. Timofeev, O.L. Polyanovskii, M.V. Vol'kenshtein, O.V. Preobrazhenskaya, G.I. Likhtenshtein and Yu. V. Kokhanov, Molek. Biol., 6 (1972) 377 (p. 302 in transl.).
170 R. Aasa, T. Vanngard and H.B. Dunford, Biochim. Biophys. Acta, 391 (1975) 259.
171 W.J. Ingledew and T. Ohnishi, FEBS Lett., 54 (1975) 167.
172 J.T. Warden, R.E. Blankenship and K. Sauer, Biochim. Biophys. Acta, 423 (1976) 462.

173 E. Rotlevi, H.M. Moss, S. Kominami and P. Riesz, Annals N.Y. Acad. Sci., 272 (1973) 387.
174 S. Kominami, V.T. Wee and P. Riesz, Rad. Res., 62 (1975) 422.
175 T. Sarna, J.S. Hyde and H.M. Swartz, Science, 192 (1976) 1132.
176 N. Bloembergen, Physica, 15 (1949) 386.
177 B.E. Wagner, J.W. Linowski, J.A. Potenza, R.D. Bates Jr., J.N. Helbert and E.H. Poindexter, J. Am. Chem. Soc., 98 (1976) 4405.
178 B.M. Hoffman and T.B. Eames, J. Am. Chem. Soc., 91 (1969) 5168.
179 B.M. Hoffman and T.B. Eames, J. Am. Chem. Soc., 93 (1971) 3141.
180 B.M. Hoffman and T.B. Eames, J. Am. Chem. Soc., 91 (1969) 2169.
181 A.H. Cohen and B.M. Hoffman, J. Am. Chem. Soc., 95 (1973) 2061.
182 A.H. Cohen and B.M. Hoffman, J. Phys. Chem., 78 (1974) 1313.
183 G.A. Abakumov, V.D. Tikhonov and G.A. Razuvaev, Dokl. Akad. Nauk. SSSR, 187 (1969) 571 (p. 561 in transl.).
184 G.A. Razuvaev, V.D. Tikhonov and G.A. Abakumov, Izv. Akad. Nauk SSSR, Ser. Khim, No. 8 (1970) 1732 (p. 1634 in transl.).
185 A.H. Cohen and B.M. Hoffman, Inorg. Chem., 13 (1974) 1484.
186 G.P. Lozos and B.M. Hoffman, J. Phys. Chem., 78 (1974) 200.
187 G.P. Lozos and B.M. Hoffman, J. Phys. Chem., 78 (1974) 2110.
188 L.N. Kirichenko and A.A. Medzhidov, Izv. Akad. Nauk SSSR, Ser. Khim., No 12 (1969) 2849 (p. 2685 in transl.).
189 B.E. Wagner, J.N. Helbert, R.D. Bates Jr. and E.H. Poindexter, J. Chem. Soc. Chem. Commun., (1973) 748.
190 J.N. Helbert, P.W. Kopf, E.H. Poindexter and B.E. Wagner, J. Chem. Soc., Dalton Trans., (1975) 998.
191 W. Beck, K. Schorpp and K.H. Stetter, Z. Naturforsch. Teil B, 26 (1971) 684.
192 W. Beck and K. Schmidtner, Chem. Ber., 100 (1967) 3363.
193 M. Okunada, G. Matsubayashi and T. Tanaka, Inorg. Nucl. Chem. Lett., 12 (1976) 813.
194 G.A. Abakumov and V.D. Tikhonov, Zh. Strukt. Khim., 13 (1972) 150 (p. 136 in transl.).
195 P. Aisen and A. Leibman, Adv. Chem. Ser., 162 (1977) 104.
196 D.C. Harris and P. Aisen, in R.R. Crichton (Ed.), Proteins of Iron Storage and Transport, North—Holland, Amsterdam, pp. 59—66, 1975.
197 B.J. Sutton, P. Gettins, D. Givol, D. March, S. Wain-Hobson, K.J. Willan and R.A. Dwek, Biochem. J., 165 (1977) 177.
198 P.F. Richardson and R.W. Kreilick, Chem. Phys. Lett., 50 (1977) 333.
199 L. Burlamacci and E. Tiezzi, J. Mol. Struct., 2 (1968) 261.
200 A.N. Kuznetsov and V.A. Livshits, Russ. J. Phys. Chem., 48 (1974) 1754 (p. 2995 in Russian).
201 E.N. Frolov, N.V. Kharakhonycheva and G.I. Likhtenshtein, Molek. Biol., 8 (1974) 886 (p. 707 in transl.).
202 V.V. Voevodskii, Yu. N. Molin and K.I. Zamaraev, Zh. Strukt. Khim., 8 (1967) 864 (p. 778 in transl.).
203 A.I. Kotelnik, G.I. Likhtenshtein and R.I. Gvozdev, Studia Biophysica, 49 (1975) 215.
204 V.I. Evreinov, V.B. Golubev and E.V. Lunina, Russ. J. Phys. Chem., 47 (1973) 118 (p. 215 in Russian).
205 V.I. Evreinov, E.V. Lunina and V.B. Golubev, Russ. J. Phys. Chem., 47 (1973) 578 (p. 1018 in Russian).
206 V.I. Evreinov, V.B. Golubev, E.V. Lunina, Le-Viet-Fu and A.K. Selivanovskii, Russ. J. Phys. Chem., 50 (1976) 400 (p. 684 in Russian).
207 V.I. Evreinov, A.K. Selivanovskii, E.V. Lunina and V.B. Golubev, Russ. J. Phys. Chem., 50 (1976) 734 (p. 1217 in Russian).

208 I.G. Kharitonenkov, M.L. Khristova and E.K. Ruuge, Molek. Biol., 11 (1977) 432 (p. 330 in transl.).
209 A.N. Kuznetsov, V.A. Livshits, G.G. Malenkov, L.A. Mel'nik, V.I. Suskina and B.G. Tenchov, Russ. J. Phys. Chem., 48 (1974) 1757 (p. 3000 in Russian).
210 A.N. Kuznetsov, V.A. Livshits, G.G. Malenkov, L.A. Mel'nik and B.G. Tenchov, Russ. J. Phys. Chem., 48 (1974) 1760 (p. 3005 in Russian).
211 J. Pirrwitz, G. Lassman, H. Rein, O. Ristan, G.R. Janig and K. Ruckpaul, FEBS Lett., 83 (1977) 15.
212 V.S. Aliev, A.A. Medzhidov, P.Sh. Mamedova and Yu. G. Mamedova, Dokl. Akad. Nauk Azerbaidzhanskoi, 29 (1973) 24 (Chem. Abs., 80 70631g), (not sighted).
213 A.A. Medzhidov, A.B. Shapiro, P.Sh. Mamedova, A.M. Musaev and E.G. Rozantsev, Izv. Akad. Nauk SSSR, Ser. Khim., 538 (1977) (p. 483 in transl.).
214 Y.H. Chao, A. Holtzer and S.H. Mastin, J.Am. Chem. Soc., 99 (1977) 8024.
215 P.F. Richardson and R.W. Kreilick, J. Am. Chem. Soc., 99 (1977) 8183.
216 R. Briere, A. Rassat and P. Rey, J. Am. Chem. Soc., 100 (1978) 343.
217 D.L. DuBois, S.S. Eaton and G.R. Eaton, J. Am. Chem. Soc., 100 (1978) 2686.
218 R.M. Richman, T.C. Kuechler, S.P. Tanner and R.S. Drago, J. Am. Chem. Soc., 99 (1977) 1055.