FACTORS RESTRICTING DIFFUSION OF WATER-SOLUBLE SPIN LABELS

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ABSTRACT Line broadening of spin label signals is treated in terms of concentration, viscosity, charge, and temperature dependencies. Line broadening of spin label signals may be caused either by spin label interactions or by the interaction between a spin label and a second paramagnetic species. Line broadening has been related to collision frequency in the literature and is treated in that way here. Collision frequency is related to diffusion processes in a way that allows information to be obtained about the diffusion environment. Several potential spin label line-broadening agents are compared as to their effectiveness. Small polymer beads with graduated pore sizes are used to show that collisional broadening has a marked dependence on the long-range structure of the diffusion environment. Application of these results to biological diffusion processes is considered.

INTRODUCTION

The potential usefulness of paramagnetic interactions that cause line broadening of spin label signals is considerable. Such interactions make possible the selective removal of spin label signals originating from specific zones of heterogeneous systems, while leaving the signal unimpaired from other selected zones (1). Spin label-spin label or spin label-paramagnetic ion interactions can be used to obtain information about diffusion processes. Previous experiments have used spin label interactions to estimate lateral diffusion rates in model (2–6) and biological membranes (7, 8). The present work indicates that it is possible to obtain additional information about the nature of diffusion environments by relating rotational diffusion measurements to spin label collision frequencies.

It is important to characterize the interactions between paramagnetic species. Such characterizations allow these measurements to be useful in a variety of biophysical experiments. The fundamental concepts of relating concentration effects (9–13), viscosity (11, 14, 15), and charge interaction (13, 15) to paramagnetic interactions have already been presented in the literature. The present report is an attempt to carry out the appropriate experiments to illustrate the usefulness of paramagnetic interactions to biophysical and biological experiments in general, and in particular to show the rela-

tionship between rotational motion and translational diffusion in a heterogeneous diffusion environment.

The spin exchange interaction between paramagnetic species depends on the collision frequency and the exchange integral describing the wave function overlap of the colliding particles. These parameters in turn depend on ligands surrounding the paramagnetic species, on constraints imposed on translational diffusion by physical barriers, and by electrostatic forces limiting the nearest approach of the ionic species.

MATERIALS AND METHODS

Spectra were taken on a Japan Electron Optics electron paramagnetic resonance spectrometer (JES-ME-1X, JEOL USA, Electron Optics Div., Medford, Mass.) fitted with a laboratory-constructed variable temperature device calibrated and continuously monitored to an accuracy of approximately ±0.5°C. Samples were routinely placed in 50-μl precalibrated capillaries with an inside diameter of 0.9 mm.

Part of a large, five-times-recrystallized sample of 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPONE) was further purified by preparative thin-layer chromatography using silica gel g as stationary phase and diethylether:chloroform (1:1, vol/vol) as the moving phase. The eluted TEMPONE was dried under vacuum over phosphorus pentoxide to a constant weight. 1.8300 g of this product was dissolved in water to a final concentration of 10^{-2} M. This served as a quantitative standard for other experiments by the expression $A = CW^2h$, where A is the area under the absorption line (obtained as a first derivative), W is the first-derivative line width (taken from expanded second-derivative spectra), h is the first-derivative line height, and C is a line shape constant equal to unity for present purposes. Agreement between this quantitative standard and the Varian weak pitch standard is within 30% (Varian Associates, Palo Alto, Calif.). TEMPONE used in line-broadening experiments was synthesized from deuterated acetone and ammonia by the procedure of Rosantsev (16).

Small quantities of the spin label 2,2,6,6-tetramethylpiperidinol-*N*-oxyl (TEMPOL) was purified by thin-layer chromatography, as previously described (17). The phosphate ester of TEMPOL, TEMPOPHOSPHATE, was purchased from Syva Chemical Co., Palo Alto, Calif. The spin label 2,2,6,6-tetramethylpiperidineamine-*N*-oxyl (TEMPAMINE) was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. The trimethyl derivative of TEMPAMINE, trimethyltempammoniumiodide, was synthesized from TEMPAMINE by exhaustive treatment of TEMPAMINE with methyl iodide. The product was purified by extraction of the product into a large excess of ether from 1 M sodium hydroxide. This product gave a single spot when assayed on cellulose thin-layer chromatographic plates.

TEMPOSULFATE was synthesized by reacting TEMPOL with sulfonylchloride in ether. The product was purified by solvent-solvent extraction.

Samples were prepared by dissolving spin labels and salts of the various ions in distilled water. The pH of these solutions was maintained between 4 and 6.

Bio-Gel P filtration beads of different pore sizes were obtained from Bio-Rad Laboratories, Richmond, Calif., and were used as described in the legend to Fig. 7. All beads were 100 mesh.

Rotational correlation time (τ_c) of TEMPONE was calculated essentially as described in the method of Kivelson (18) and as presented in equation form elsewhere (19).

$$\tau_c = K W_0[(h_0/h_{-1})^{1/2} - 1]$$

In this expression W_0 is the first-derivative mid-field line width; h_0 and h_{-1} are the mid- and high-field first-derivative line amplitudes. K is a constant that depends on the spin label's spectral parameters and the microwave frequency. We used the value 6.5×10^{-10} for K

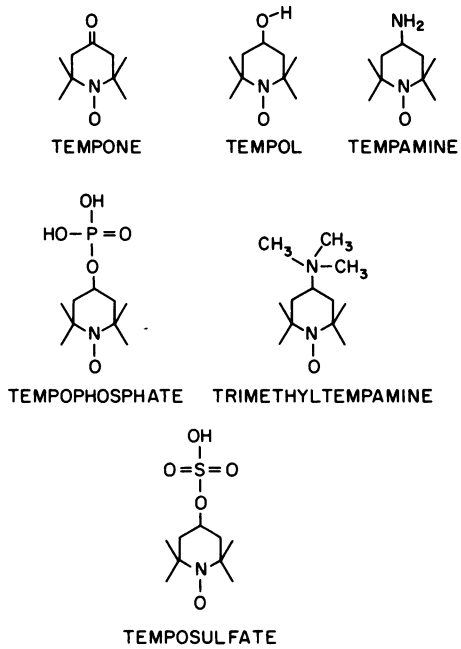


FIGURE 1

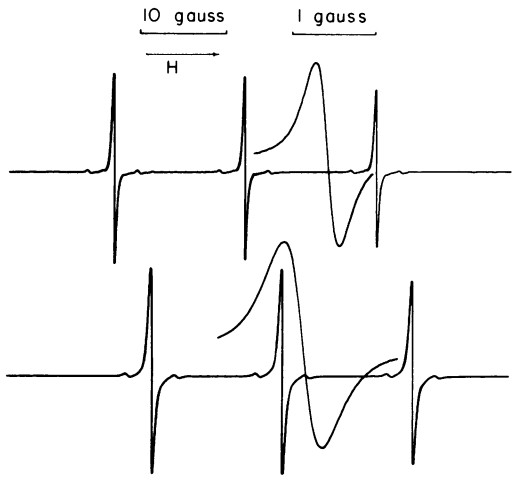


FIGURE 2

FIGURE 1 Spin label structures. The five spin labels used are all derivatives of 2,2,6,6-tetramethylpiperidine-*N*-oxyl. The five functional groups are denoted in the abbreviated names shown in the figure.

FIGURE 2 Spectra of TEMPONE and deuterated TEMPONE. The spectrum of fully deuterated TEMPONE at 10^{-4} M in water is shown in the upper spectrum and of TEMPONE at 10^{-4} M in water in the lower spectrum. An amplification of the low-field line of each is also shown. Gauss markers are presented for both scales used.

here. Ref. 19 presents the proper form of the equation for generation of K values for any spin label at any microwave frequency.

Data concerning measurement accuracy of first- or second-derivative line widths are included in the legend to Fig. 3.

RESULTS

Fig. 1 shows the structures of all spin labels used in the present investigation. The structure of TEMPONE shown represents both the protonated and fully deuterated (^{2}H) forms. [^{2}H]TEMPONE was used throughout. Fig. 2 shows spectra of TEMPONE and [^{2}H]TEMPONE taken in water at 10^{-4} M. The low-field line width of [^{2}H]TEMPONE at 10^{-3} M in water at room temperature is approximately 0.28 G, and 0.16 G at 10^{-5} M. Comparative values for TEMPONE are approximately 0.55 G and 0.42 G, respectively.

Concentration Dependence for Line Broadening

Eight broadening agents are compared in Fig. 3 as to their concentration dependence with respect to broadening the TEMPONE hyperfine lines in water. A deuterated

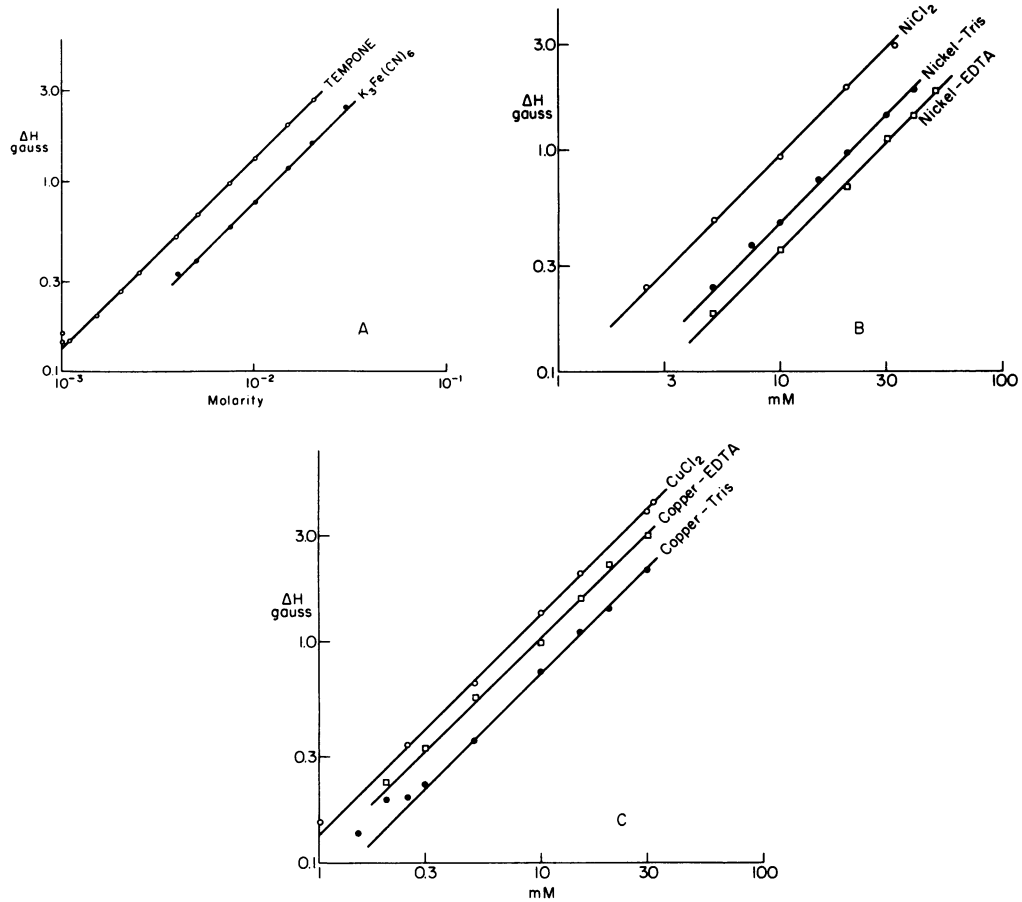


FIGURE 3 Concentration dependence of the line broadening of TEMPONE. All spectra were taken at near 25°C. Line width measurements were carried out by using expanded second-derivative spectra to an estimated accuracy of more than ±0.02 G.

TEMPONE molecule was used for this purpose so that the minimum line width could be reduced as much as possible. The TEMPONE concentration was maintained at 10^{-3} M in all the samples shown except for the TEMPONE-TEMPONE sample, in which the TEMPONE concentration was varied. The line-broadening efficiency of TEMPONE, Ni^{++}, and Cu^{++} are reasonably similar. The order of broadening efficiency among these three is Cu^{++} > TEMPONE > Ni^{++}. They all obey the same general concentration-dependent function and all three have the same slope, as shown in Fig. 3. The chelates formed by complexing an organic moiety with a metallic salt broaden less efficiently, in general, with copper-EDTA being the best.

Due to the combined considerations of effectiveness, charge, and toxicity, $NiCl_2$, TEMPONE, and $K_3Fe(CN)_6$ are the broadening agents that receive most attention in the present report.

Viscosity Dependence for Line Broadening

It has been previously shown that the small nitroxide radical di-tert-butyl nitroxide (DTBN) has a viscosity dependence that strongly affects the magnitude of the exchange interaction (11). Plachy and Kivelson (11) showed that for a T/η range of a factor of 10, the line broadening of DTBN dissolved in a hydrocarbon solvent increased smoothly as viscosity increased. These measurements were carried out at low spin label concentrations, 3 and 7 $\times$ 10^{-5} M, in a range where the solvent viscosity was also low. This basic relationship has also been shown for some paramagnetic transition element chelates interacting with TEMPONE (14, 15). These latter experiments were carried out with organic solvent-soluble chelates and were primarily done in chloroform. The relationship between concentration-dependent line broadening and solvent viscosity is shown in Fig. 4 for TEMPONE-TEMPONE line broadening. These experiments were carried out in water-glycerol mixtures at room temperature. This plot illustrates that there is a strong dependence of collision-dependent line broadening on solvent viscosity.

The τ_c measurements were carried out on samples of sufficiently dilute spin label concentration that ΔH was less than 0.1 G. τ_c values have only a slight line width dependence unless the line broadening is extreme.

Temperature Dependence of Line Broadening in Water

The expected increase in ω_{ex} as the temperature of water increases, based on classic equations, is just the ratio of the η/T values at the two temperature extremes. Over the

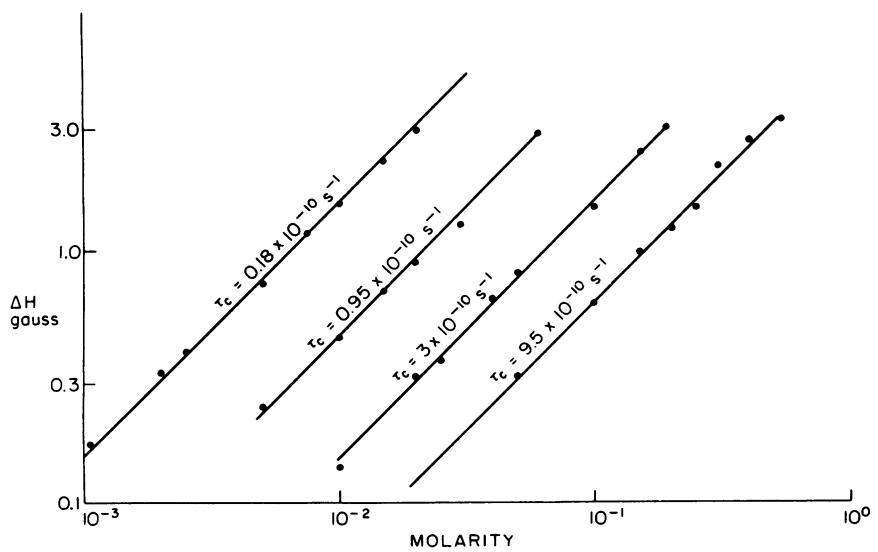
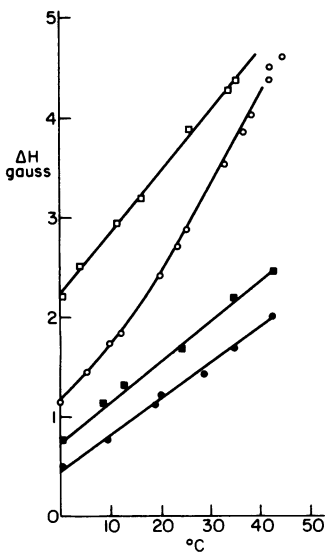


FIGURE 4 Concentration dependence of line broadening at four different viscosities. The four lines reading from left to right are: water, 65% aqueous glycerol, 75% aqueous glycerol, and 85% aqueous glycerol. τ_c values for each line are shown. The ΔH values for spectra used for τ_c measurements were less than 0.1 G.

temperature range from 0°C to 40°C this value is 3.3. The increases in ΔH for nickel-Tris and copper-Tris are 2.8 and 2.7, respectively, for the line broadening of TEMPONE (Fig. 5). The chelates formed by the complexing of copper and nickel with Tris have been discussed (20). This approximate value indicates that no interfering hydration shell or pronounced dissociation from the chelate complexes occurs over this temperature range. The chloride salts of nickel and copper, however, change more (Fig. 5). The value for $CuCl_2$ is just the expected value of 3.3. $NiCl_2$ is somewhat higher. This increase may be accounted for by progressive loss of hydration water, allowing closer approach and consequently more effective electron exchange between



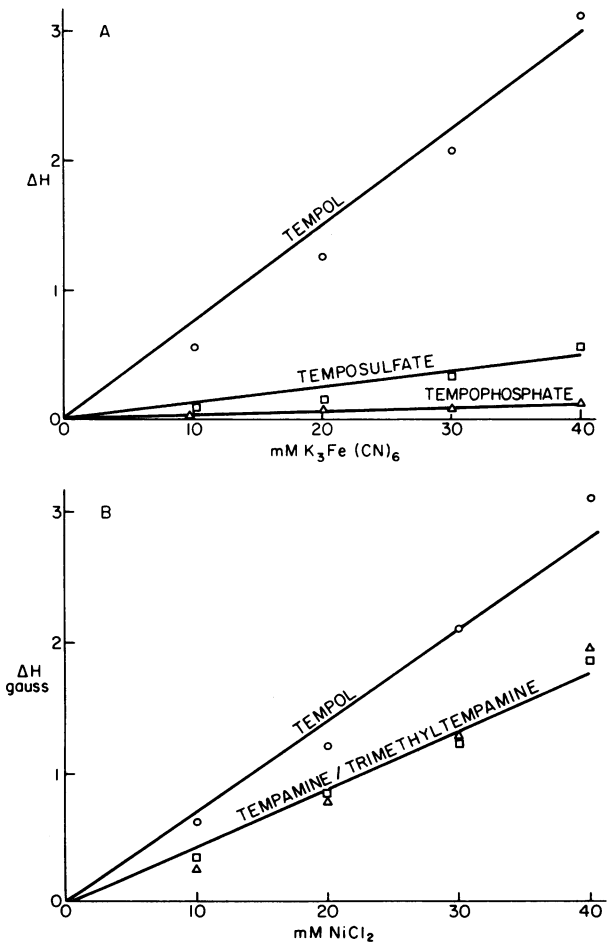


FIGURE 5

FIGURE 6

FIGURE 5 Temperature dependence of line broadening of TEMPONE. Open circles, $NiCl_2$; closed circles, nickel-Tris; open squares, $CuCl_2$; closed squares, copper-Tris. Spin labels were maintained at 10^{-3} M.

FIGURE 6 Charge dependence for line broadening. Spin labels were maintained at 10^{-3} M. (A) $K_3Fe(CN)_6$ is used as the broadening agent; (B) $NiCl_2$ is used as the broadening agent.

paramagnetic ion and spin label. $NiCl_2$ has a noticeably greater slope for this temperature dependence.

Charge Interactions Affecting Line Broadening

Fig. 6 illustrates the concentration dependence of line broadening of the TEMPOL molecule by $K_3Fe(CN)_6$. The uncharged TEMPOL molecule interacts with $K_3Fe(CN)_6$ so that as the concentration of the broadening agent is doubled, the line broadening due to collision frequency between broadening agent and TEMPOL also doubles. A negatively charged derivative of TEMPOL, TEMPOSULFATE, shows much less line broadening than TEMPOL at an equivalent concentration of broadening agent, as can be seen in Fig. 6 A. TEMPOL made into a still more negatively charged derivative, TEMPOPHOSPHATE, has even less line broadening at equivalent concentrations. This plot illustrates that charge repulsion is an extremely important parameter and that it is highly effective between charged paramagnetic broadening agents and charged spin labels.

Fig. 6 B illustrates another aspect of charge-charge interaction by using the positively charged nickel ion as the broadening agent and positively charged spin labels. The interaction between the nickel ion and TEMPOL has the expected concentration dependence, illustrating that as the concentration of Ni^{++} is doubled, the exchange frequency also approximately doubles. With a charged amine at neutral pH, TEMPAMINE, or a trimethyl-substituted amine, the concentration dependence for line broadening is increased. The magnitude of the charge-charge interactions for the positively charged species is less than for the negatively charged species. The negatively charged broadening agent, ferricyanide, has a charge of minus three, whereas

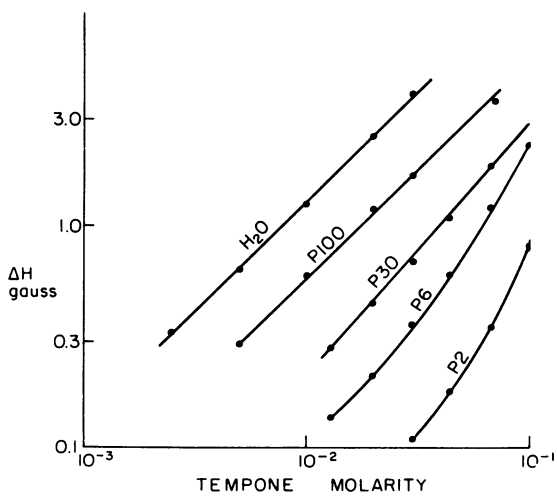


FIGURE 7 Concentration dependence for line broadening in confining volume. The P-notation refers to the minimum molecular weight in thousands excluded from that bead size. All beads were 100 mesh.

the nickel ion has a charge of plus two. A consideration of Coulomb's law leads to the expectation that ferricyanide would be more effective in the process of charge repulsion than the nickel ion.

Diffusion in a Heterogeneous Environment

We have so far treated concentration, viscosity, temperature, and charge dependencies of spin label line broadening. All of these data assume an isotropic solvent with diffusion occurring over different dimensions with equal rates. We also measured a heterogeneous environment by using a series of beads with pore sizes of different dimensions to illustrate the effects of diffusion barriers. Fig. 7 shows the effect of this extra variable. Beads containing different pore sizes were used as the matrix to measure the concentration dependence of line broadening in the same way as in any other series. All beads were used in aqueous medium and therefore the solvent for rotational diffusion of the spin label was water in all cases. For a uniform and isotropic solvent, one expects that translational diffusion will be linearly proportionate to $1/\tau_c$. The data plotted in Fig. 8 (open and closed circles) show a smooth relationship between collision-dependent line broadening and $1/\tau_c$. The present case shows a very different relationship, in that $1/\tau_c$ increases only slightly, while the collision-dependent line broadening increases drastically. The concentration dependence of TEMPONE line broadening in the beads with small pore diameters reveals a nonlinear relationship compared to that of the spin label in water; therefore, it is necessary to present the data in Fig. 8 even though it largely duplicates that in Fig. 7.

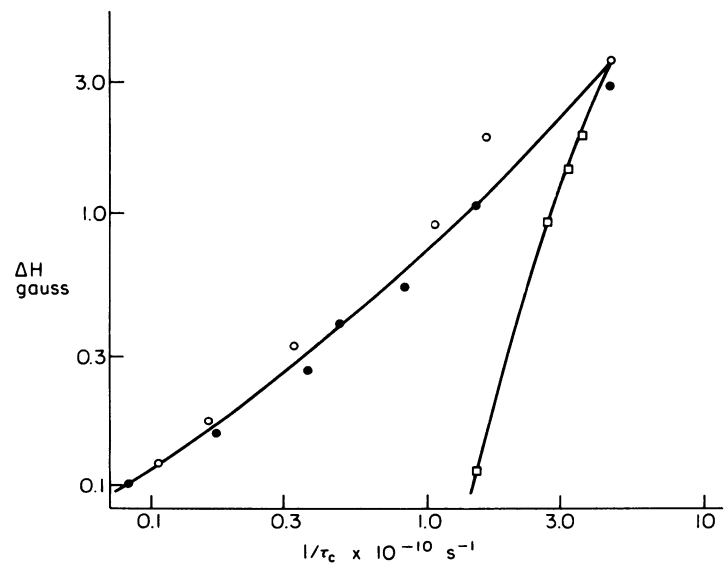


FIGURE 8 The relationship between line broadening (ΔH) and rotational correlation time (τ_c). Open and closed circles, 30 mM TEMPONE in mixtures of glycerol and water; open squares, 30 mM TEMPONE in the different sized beads shown in Fig. 7. The top square is for P100; next to the top, P30; second from the top, P6; and the bottom square is for P2.

Fig. 8 shows concentration-dependent line broadening (ΔH) plotted against $1/\tau_c$ and illustrates for such relatively isotropic media as aqueous mixtures of glycerol that there is a uniform relationship between $1/\tau_c$ and ΔH. For present purposes the line broadening agent is kept constant while the viscosity is varied. The case of the beads shows that there is only a small change in $1/\tau_c$, while the change in ΔH is drastic. This illustrates that in systems containing diffusion barriers the straightforward relationship between rotational and translational diffusion no longer exists. These data may also indicate that it may be possible to measure both the τ_c and the ΔH and then infer the average distance between diffusion barriers. Fig. 7 illustrates that for each τ_c there is a unique degree of concentration-dependent line broadening.

DISCUSSION

The general ideas and a considerable amount of data relating to the information presented in Figs. 3, 4, and 6 have been dealt with in a number of papers, many of which are cited here (2–16, 21). Excellent general treatments of spin exchange have also been presented by several authors (for examples, 22–26). Our main purposes are to present comparative data, to present data in a manner more relevant to experiments applicable to biological systems, and to place into perspective data relevant to diffusion in heterogeneous systems.

Electron Dipole Compared with Spin Exchange Contributions to Line Broadening

The total amount of line broadening due to concentration effects of paramagnetic species is the sum of electron-electron dipole and spin exchange interactions. The spin exchange interaction depends on the near encounter frequency. The electron-electron dipole interaction is more complex, having relaxation, lifetime, translational diffusion, rotational diffusion and concentration dependencies. Several authors have pointed out that low solvent viscosity resulting in rapid diffusion tends to average away the net dipole field experienced by a paramagnetic species. Increased concentration has the same effect, so that at high concentrations the net dipole field as experienced by a single molecule or ion is inverse to a further increase in concentration. The dipole field experienced by a point in space is equal to the sum of β/r^3 (27) irradiations from all paramagnetic species in the neighborhood, where β is the Bohr magneton. As the number of paramagnetic neighbors increases, this vector sum changes until at high concentrations it approaches zero. In fact, at very dilute concentrations the dipole field experienced by any point is also near zero. As the concentration increases from very dilute, the net dipole field reaches a maximum at some intermediate concentration and then again falls off as the concentration continues to increase. Concentration effects are dependent upon the relaxation time of the paramagnetic species responsible for the dipole field. In an interacting system between two paramagnetic species where the paramagnetic line-broadening agent has a much shorter relaxation time than the paramagnetic species whose signal is being detected, the average dipole field experienced by the species being detected will usually tend to be small or not detectable. If the line-

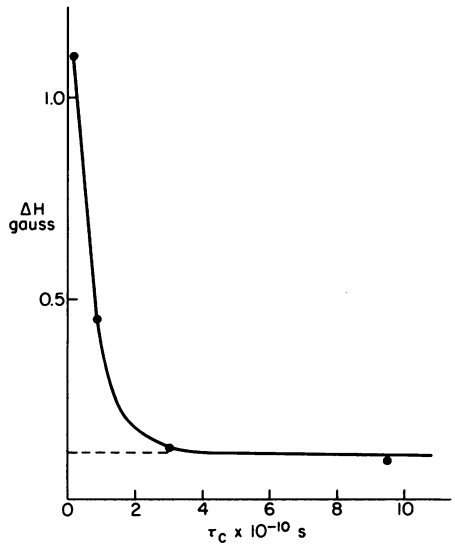


FIGURE 9 Electron dipole line broadening. TEMPONE dissolved in different mixtures of glycerol and water at 10 mM was used to obtain τ_c and ΔH values. The dotted line shown should be the electron-electron dipole contribution to line broadening under these conditions.

broadening species is so short-lived that no diffusion can occur during its own lifetime, then essentially none of the dipole field emanating from the species being used as the broadening agent should be experienced by nitroxides. Under these conditions concentration-dependent line broadening experienced by nitroxides should be due to spin exchange from both species and possibly some dipole broadening from members of the species being detected. This condition is the case when nickel is used as a broadening agent and the TEMPONE signal is measured. The relaxation time for nickel is $<10^{-11}$ s (15) and the T_1 for TEMPONE is $>10^{-7}$ s (28). Salikhov et al. (15) reported that copper chelate complexes in chloroform can cause electron-electron dipole line broadening of the TEMPONE signal but that nickel under the same conditions cannot. Fig. 9 shows data for the TEMPONE-TEMPONE interaction plotted in the same way used by the above authors, except τ_c of rotational motion is used for the abscissa, rather than bulk viscosity. This plot, constructed from data for TEMPONE dissolved at 0.01 M in glycerol-water mixtures, illustrates that electron-electron dipole broadening is dominant only when the τ_c reaches about 2×10^{-10} and greater. The exact contribution to line broadening from dipole effects depends on many variables and is difficult to determine accurately. Except for this section, dipole effects are discounted; however, for specific analyses it may be possible to make assignments as to the relative contributions of dipole and spin exchange effects.

Concentration Effects

Fig. 3 illustrates that some species are more effective than others as spin label broadening agents. $CuCl_2$ at room temperature broadens the TEMPONE line 1.5 G at 10 mM

in aqueous medium, whereas copper-Tris broadens the TEMPONE line by only 0.57 G. At this temperature almost three times as much copper-Tris as $CuCl_2$ would be required to achieve the same degree of line broadening.

In a two-compartment aqueous system where both compartments are of equal volume and are equally available to TEMPONE but only one of the compartments is available to the broadening agent, the spin label minimum line width is also very important. The amplitude of a first-derivative absorption line is inversely proportional to the square of the width; therefore, [^{2}H]TEMPONE will have its amplitude decreased by a factor of 100 at room temperature in the region available to 10 mM $CuCl_2$, while the amplitude is not lowered at all in the region available only to TEMPONE. A different spin label whose minimum line width is 1 G would differentiate between the two zones by only a factor of 6.

There are also a number of other features to take into account with respect to the concentration of a broadening agent. Many of these factors are determined by the system being analyzed. For microorganisms or cellular systems in general, toxicity to the system is important. Reducing the concentration of a broadening agent as much as possible may reduce artifacts to the extent that the experiment would otherwise be invalid. What type of interaction the broadening agent may have with the surface of any system to be analyzed must be considered. Electron microscopy is a partial aid in determining if the broadening agent causes any gross deformation to the system being analyzed. Light scattering may also be used in analyzing a particular system having characteristic light-scattering properties.

Viscosity Effects

The measurable parameters relating to translational diffusion using spin labels are line broadening (ΔH) and spin label concentration. ΔH, expressed in units of frequency, is determined by measuring the line width at a given concentration and subtracting the line width at a sufficiently dilute concentration so that the line width is judged to be at its minimum value for that motional state. This line width is proportional to the electron spin exchange frequency (ω_{ex}) and has been related to the collision frequency (K) between spin labels as $K = 2\omega_{ex}$. It has been assumed that two colliding paramagnetic molecules must be in opposite spin states to make the effects of electron spin exchange visible. This condition being true, only one-half the collisions would result in observable electron spin exchange. For the above condition to be true, the spin lattice relaxation time of the unpaired electrons involved must be longer than the encounter time. Because of the complications involved in determining such terms as the minimum distance for an effective encounter, we use the relationship where $\omega_{ex} = K$ in estimating spin label collision frequency. This estimation has quantitative characteristics, since doubling the concentration of a given species, for example, doubles the ΔH values.

Consideration of the data presented in Fig. 3, showing that a given concentration of different paramagnetic species results in a different magnitude of line broadening, suggests additional limiting factors. Hindrance from certain groups on chelates and spin

labels must also affect the efficiency of spin exchange. The relaxation time of a paramagnetic broadening agent is also important. Nickel has a relaxation time of $<10^{-11}$ s (15), at least 10^4 times faster than nitroxide spin labels (28). This very rapid relaxation time would almost certainly insure that Ni^{++} would be in several spin states during every nickel-spin label collision.

After consideration of such problems of constructing a diffusion equation based on ω_{ex}, we decided to use an empirical form. From the data presented it can be seen that the ω_{ex} varies with viscosity (Fig. 4) and concentration (Fig. 3). Therefore the diffusion constant (D) would vary directly with ω_{ex}. The ω_{ex} varies directly with concentration in a constant medium; therefore, the equation $D = (\omega_{ex}/M)k$ is adopted, where M is molarity of the broadening agent and k is an empirical constant. k is determined by measuring ω_{ex} under conditions where M and D are known. For example, [^{2}H]TEMPONE has a mol wt of 192. Glucose is approximately the same mol wt and has a diffusion constant in water at 20°C of 6.67×10^{-6} cm^2/s (29). With the D of glucose, k becomes 2×10^{-14} cm^{-1} and subsequent calculations of this spin label in aqueous medium use the same k. Changes in molarity result in different values of ω_{ex} than are usually expected for uncharged spin labels. Therefore, from plots of ΔH vs M the slope (m) of the relationship for the necessary concentration interval, $\Delta H_1 - \Delta H_0/\Delta M_1 - \Delta M_0 = m$, times the molarity being used, corrects for charge-charge interactions. The modified equation, $D = \omega_{ex}k/mM$, corrects for charge interactions. Eastman, et al. (13) treat charge effects quantitatively and present an expression using ionic and charge terms.

Diffusion in a Heterogeneous Environment

Figs. 7 and 8 show the relationship between line broadening and spin label concentrations after the introduction of diffusion barriers. The system of choice for our purposes was Bio-Gel P beads with different pore sizes. The largest pore size used, P100, restricts the passage of a molecule with a mol wt of 100,000 and larger. All smaller molecules are trapped in the diffusion channels inside the beads. The quantitative dispersion of pore sizes in this bead and others we used is unknown. The largest channel in the P100 beads allows the passage of a molecule somewhat less than 100,000 mol wt (diameter of about 100 Å), but that may not comprise a large percentage of the internal volume. Considerable space may be occupied by channels of smaller dimensions. Nevertheless, the graded pore sizes should have a smooth change with P2 having a smaller average pore size diameter than any of the other beads used.

The most interesting feature of these data is that the rotational correlation time increases only slightly while the collision-dependent line broadening decreases drastically with decreasing pore size. It is expected that the internal zones of living cells may have such a heterogeneous composition that effective diffusion channels may exist. Therefore, depending upon the molecular size of the rotating molecule, the barriers to rotational diffusion may not vary colinearly with the barriers to translational diffusion.

All the data presented here indicate that the measurement of diffusion processes in heterogeneous medium is difficult by the usual procedures and that the effective diffu-

sion constant may be different over different dimensions. Since the spin label technique lends itself to use of a range of spin label concentrations, it is potentially a good way to characterize the structure of heterogeneous aqueous environments.

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