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DIPOLAR BROADENING OF EPR SPECTRA DUE TO SOLUTE SEGREGATION IN FROZEN AQUEOUS SOLUTIONS*

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Abstract

The possibility that concentration effects due to segregation may occur in the spectroscopy of frozen solutions is often neglected. In EPR spectroscopy, segregation of paramagnetic solute can cause dipolar broadening of the spectra. EPR spectra of Mn^{2+} and Gd^{3+} in a number of frozen aqueous solutions have shown that this occurs frequently, and published spectra of frozen aqueous Mn^{2+} have been reinterpreted accordingly. The causes and consequences of inhomogeneity, and selection of frozen solution matrices to avoid it, are briefly discussed.

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INTRODUCTION

Solutions frozen to low temperatures are frequent subjects for a variety of spectroscopic investigations. The most familiar of these are the electronic spectra of molecules frozen into an organic solvent glass. Freezing has also been used to prevent the rapid recombination of reactive elements in radiation chemistry[1] and photochemistry.

In the past three years frozen solutions have also been subjects for investigation by electron paramagnetic resonance (EPR). Most of these studies have used an organic matrix,[2-4] but recently some use has been made of frozen aqueous solutions.[5-7]

The possibility that freezing may cause high local concentrations of the molecule under study is often not considered. While concentration effects may not significantly affect some parameters, others may be drastically altered. Electronic spectra of metal ions are relatively insensitive to high concentration,[8] but the intensity of spin forbidden transitions in aromatic compounds may be greatly affected by the proximity of another solute molecule.[9] Local concentrations of reactive species in radiation experiments may greatly increase reaction yields of certain products.[10,11]

In EPR spectroscopy high local concentrations of paramagnetic species will result in electron spin dipole-induced variations in the local magnetic fields of the ions studied, causing broadening of the EPR spectrum.

This paper considers the requirements for a fluid sample remaining homogeneous on solidification, and presents evidence that segregation

of solute molecules does occur in frozen aqueous solutions which had been previously assumed to be essentially homogeneous. Some published EPR observations have been reinterpreted in the light of our experiments.

FROZEN SOLUTIONS

The concentration of solute on cooling may be considered as being due to three mechanisms: solute aggregation, solute crystallization, and solvent crystallization. The molecules of interest, $\underset{\sim}{A}$, may aggregate as the equilibrium $n\underset{\sim}{A} = \underset{\sim}{A}_n$ is shifted to the right by lower temperatures. Related to aggregate formation is the appearance of crystals of $\underset{\sim}{A}$ due to a decreased solubility on cooling.

Solvent may also separate as a crystalline phase, restricting solute to the remaining volume. Particularly in aqueous solutions, where strong hydrogen bonding makes the ice structure very reluctant to include a foreign ion, crystallization of solvent as a virtually pure phase will greatly concentrate solute species in the interstices of solvent crystals. Subsequent to this concentration, the solute $\underset{\sim}{A}$ may aggregate or crystallize, or rapid cooling may trap individual molecules in a disordered phase.

If solute - solute interactions are to be avoided, aggregation and crystallization of $\underset{\sim}{A}$ must be hindered, and the volume of the disordered phase(s) containing $\underset{\sim}{A}$ must be maximized. Aggregation may be minimized by a judicious choise of medium, and solute crystallization can be discouraged by staying well below saturation concentrations. A disordered phase containing $\underset{\sim}{A}$ may be crystals of solvent or other solute with $\underset{\sim}{A}$ occurring as defects, or the phase may be non-crystalline. In experiments in frozen solution, one usually hopes that the molecules of interest are in a non-crystalline matrix.

Non-crystalline ("glass") regions will result on cooling a liquid only if diffusion is halted by increasing viscosity before chemical equilibrium can be attained. Glass formation will be favored if the diffusion rate is slow at the freezing point, if crystal nucleation is difficult, or if the rate of crystal growth is slow.[12,13]

Glass formation on cooling a pure liquid is favored by high viscosity, low freezing point, low molecular symmetry, complex crystal structure, the possibility of inter-molecular bonds which do not correspond to portions of the crystalline structure, and difficult nucleation. Individual components of a mixture to be used in glass formation should be selected with these features in mind also.

The use of a mixture also introduces a number of other possibilities for favoring glass formation: Freezing points can be lowered. Eutectic mixtures may be stable crystalline phases, causing crystal growth to be slower because of the more complex crystal structure. All rates of crystal growth will be reduced by the presence of foreign molecules, which must diffuse away from a growing crystal plane before a new layer can be added. The amount of inter-molecular bonding not corresponding to a stable structure might be increased.

Aqueous solutions are of particular interest because of the ubiquitousness of this solvent, particularly in biological systems. The reasons for doing experiments at low temperatures are as compelling for water as for any other solvent, but the problems may be greater.

The kinetics of ice crystal growth have been extensively investigated.[14,15] An ice crystal in water supercooled by 5° will grow at 1 cm/sec; at -10° it will grow at 7 cm/sec. Frozen aqueous solutions

are frequently obtained by plunging sample tubes with a bore of several millimeters into liquid nitrogen; this convenient but relatively slow means of cooling requires several seconds to freeze a sample,[16] and we can anticipate significant ice crystal formation.

In their recent paper on the EPR spectrum of manganous ion in frozen solutions, Allen and Nebert[5] suggest that resolution of hyperfine structure on the addition of chloride before freezing aqueous solutions is due to a change in crystal field parameters. We suggest, rather, that the broad structureless line observed by Wakim and Nolle,[6] and by Allen and Nebert, for frozen solutions of a manganous salt alone in water is caused by manganese - manganese dipolar broadening. Addition to the solution of other solute, whether or not a chloride, decreases the local concentrations of the paramagnetic ions on freezing, and hence diminishes dipolar interactions.

With some knowledge of glass chemistry, and some physical data on aqueous systems, one may be able to surmise which additives will be more effective in decreasing the volume occupied by ice crystals. Doping agents which will interact significantly with the molecules studied must naturally be avoided.

Lusena[17] studied the effect of different solutes on the rate of growth of ice crystals from aqueous solutions supercooled by 10°. He found alcohols and sugars to have the greatest growth retarding effect. Ethanol 25% by weight reduced the rate of linear growth by 1000-fold.

Extensive information is available on the freezing points of binary mixtures with water.[18] Again, the alcohols, with eutectics below -100°, should be relatively effective in preventing ice crystallization. The

common mineral salts and dioxane have eutectics in the -3° to -25° range, and might not work as well.

Glycerol, which is extraordinarily difficult to crystallize, might be expected to be quite effective. This compound is routinely used to protect biological cells on freezing, and it has been proposed that its primary mode of action is prevention of cell damage caused by greatly increased ionic concentrations on freezing.[19]

EXPERIMENTAL METHOD

EPR spectra were obtained with an X-band spectrometer operating at 9.1 Gc. A derivative presentation of the spectrum resulted from phase-sensitive detection of the absorption with 100 Kc field modulation of 3 oe amplitude. A rectangular TE_{102} cavity (Varian V-4531) was used with a quartz jacketed gas flow cooling system (Varian V-4547 slightly modified). Liquid samples at room temperature were placed in 3 mm bore quartz sample tubes, and cooled by immersion in liquid nitrogen before placement in the cavity. Sample temperature in the cavity was about 90° K, and was not a critical parameter.

All solutions used were saturated with air. Attempts to observe a line broadening effect in frozen aqueous solution due to the paramagnetism of O_2 were unsuccessful.

Gadolinium perchlorate was made by the addition of 3 M perchloric acid to Gd_2O_3, and diluted to the desired strength. The stock solution used was 0.0040 M in $Gd(ClO_4)_3$, and was 0.040 M in $HClO_4$ to hinder hydrolysis.

EXPERIMENTAL RESULTS

Spin dipole-dipole interactions are not the most sensitive effect dependent on sample homogeneity, but they have the advantage that the effect is understood theoretically, and is easily related to experimental parameters. The dipolar interaction between two paramagnetic species is given by the classical relation $E_d = \mu r^{-3} (1 - 3 \cos^2\phi)$. Van Vleck[20] has considered the effect of this interaction on the shape of an EPR line. Its contribution to the width of the line between points of maximum slope is given by the relationship

$$\Delta H = 2g\beta[3S(S+1)/5]^{\frac{1}{2}} \left\langle \left(\sum_{i \neq j} r_{ij}^{-6}\right)^{\frac{1}{2}} \right\rangle_{\text{average over } j}$$

for a sample with randomly oriented ions.

The value of the r^{-3} summation over all ions depends markedly on the randomness of ion - ion distances. For a completely random distribution, this average is approximately equal to 5C, where C is the volume concentration of paramagnetic ions. If the sample is not homogeneous, the EPR line width due to this interaction is a relatively good measure of the mean local concentration.

For a spin N/2 system, the line width contribution is approximately N oe for a 0.02 M concentration. Exchange narrowing has not been considered, so this model will break down for very high concentrations. The effect of paramagnetic species other than the one being observed is reduced by a factor of 2/3.[20]

Figure 1 displays the effect of adding varying concentrations of sodium perchlorate to 0.005 M manganous nitrate frozen. We find that as line widths are reduced by a decrease in dipolar interactions, peak

intensities increase. The dependence of spectrum on concentration of additive was also investigated in detail for methanol, perchloric acid, and nitric acid. The results were similar to those displayed for sodium perchlorate, but with varying degrees of effectiveness in producing line narrowing.

The effect of a variety of different additives on the spectrum of 0.005 M manganese were investigated. Spectra for this concentration in 25 and 75% volume percent aqueous methanol were equivalent, and were as distinct as any spectra obtained, indicating that no noticeable dipolar broadening occurred in these frozen solutions. If the intensity of the derivative EPR spectrum (absolute maximum minus absolute minimum slope of the absorption) of the 25% methanol is assigned the value 100, various frozen aqueous solutions yield the intensities listed in Table I.

Regardless of the additive used, the same characteristic spectrum is dominant. As the only species common to all the systems tried are Mn^{++} and H_2O, hydrated manganous ion is probably responsible for the spectrum. Manganese complexes involving non-water ligands presumably contribute at most a broad background signal because of the much greater crystal field splittings caused by a first coordination sphere which is not composed of all identical ligands.

Two different phenomena are responsible for the effect of a given additive on signal height; reduction of dipolar broadening will narrow lines, increasing signal height; complex formation will decrease the amount of hydrated manganese present. Dipolar broadening causes a decrease in clarity of detail in a spectrum. A convenient measure of

this clarity in the Mn^{++} spectrum is the ratio of difference between the first minimum and second maximum to the difference between the first maximum and last minimum. The 25% methanol spectrum has a clarity index of 1.06. Values for other frozen solutions (in percent) are given in Table I.

As anticipated, methanol and glycerol are quite effective in preventing solute segregation, and dioxane and potassium nitrate are very ineffective. Solutions of potassium acetate are relatively viscous, and extrapolation of the available freezing point data[18] places the eutectic in the vicinity of -70° C, so the clarity produced by this additive is not unexpected. Sulfuric acid has five eutectics with water, so one is not too surprised that the water molecules are successfully confused.

By comparing the intensity and clarity figures for the different additives, we estimate that 75 to 85% of the Mn^{++} in the 2 M HCl solution is a chloride complex. In all of the other solutions, at least 40% of the manganese seems to remain as the hydrate producing the characteristic spectrum. This is true even for 2 M pyridine and sulfate, which are reported to complex Mn^{++} moderately strongly in aqueous solution.[21,22]

To check the assumption that dipolar broadening is responsible for the phenomena observed, the effect of added Ni^{++} on the Mn^{++} spectrum was compared with the effect of increased Mn^{++} concentration. The effect of added Ni^{++} on the clarity and specific intensity is about the same as only 5% as much added Mn^{++}. Considering the difference in magnetic moments and the statistical factor of 2/3, Ni^{++} should be 36% as effective in broadening Mn^{++} as manganese itself. The remaining factor of 7 may be due to a tendency for the two kinds of ions to go into different phases

on freezing; an alternate possibility is that rapid relaxation of the Ni^{++} electrons may reduce the effective magnitude of the nickel magnetic moment.

Frozen aqueous solutions of gadolinium^{3+} were also found to have an EPR signal which progressively narrows and increases in intensity on the addition of diamagnetic solutes before freezing. Our hopes of using Gd^{3+} to obtain a clear cut dipolar broadening effect on a single EPR line were muddled by a broad background absorption, probably due to the presence of more than one chemical species. The unpaired electrons of an ^8S rare earth ion are sufficiently insulated from the ligands that a complex of the type $Gd(H_2O)_{n-1}Cl^{2+}$ has only a little greater crystal field splitting than $Gd(H_2O)_n^{3+}$. The EPR absorption of frozen aqueous Gd^{3+}, illustrated in Figure 2, may also be complicated by the presence of ions with different numbers of ligands coordinated.

Unlike gadolinium, the manganous ion has consistently been found to have a coordination number of six. Single crystal EPR studies of the $Mn(H_2O)_6^{++}$ ion have been made in several diamagnetic hosts.[23] Values found for the axial splitting parameter, D, are in the range 0.014 to 0.027 cm^{-1}. Comparison of the powder spectrum of $Mn(H_2O)_6^{++}$ in a Tutton salt, $(NH_4)_2Zn(SO_4)_2 \cdot 6H_2O$, at room temperature (D = +0.024 cm^{-1}), with that of Mn^{++} in a frozen solution shows that the frozen solute has a mean crystal field assymetry equal to or slightly smaller than that for $Mn(H_2O)_6^{++}$ in the Tutton salt. We conclude from this and the chemical evidence which has been presented that the spectrum in the frozen aqueous solution is due to $Mn(H_2O)_6^{++}$.

DISCUSSION AND CONCLUSIONS

When glasses were designed for use in visible and ultra-violet spectroscopy, the primary consideration was optical clarity. Besides being a necessity for optical investigations, this clarity is evidence of the absence of large crystals. The absence of easily noticeable light scattering is not, however, a guarantee of the absence of either solute aggregation or crystallization, or solvent ordering in micro-crystals. An instrumented investigation of radiation scattering by the "glassy" frozen solutions commonly used would reveal information about their homogeneity, but the relation between scattering and the parameter of interest in a given spectroscopic experiment would be rather indirect.

Solvent crystallization-induced aggregation is usually not serious in the commonly used organic glass systems.[24,25] Fluorescence spectra of aromatic systems in glasses generally do not contain peaks known to be characteristic of the dimer or of aggregates.[9]

In a recent study on aromatic triplet-triplet energy transfer, Siegel and Judeikis[26] found that transfer was independent of solvent for most glasses. The rate of transfer was significantly higher only when methyl-cyclohexane, a relatively poor solvent for aromatics, was used. They concluded that solute distribution was essentially uniform except in this case. This work, and the successful resolution of ligand hyperfine splittings in the EPR spectra of Cu^{++} [2,3] and VO^{++} [4] complexes in some organic matrixes indicates that a properly chosen organic glassing mixture can be quite satisfactory.

However, the choice of organic glass has been shown to affect the sharpness of both EPR[2] and fluorescence spectra,[9] which should caution

experimenters to check homogeneity even in these systems. Solute segregation due to a shifted dimerization equilibrium, or decrease in solubility, is, of course, quite possible in the best of glasses.

We have found that moderately rapid cooling of dilute aqueous solutions does not prevent extensive segregation of solute caused by ice crystal formation. Addition of large quantities of experimentally inert solute can greatly help to prevent this, but it seems unlikely that ice crystallization can be completely halted even in the best experimental conditions.

Wang[10] has pointed out that the enhanced dimerization of thymine on irradiation of a frozen aqueous solution is due to solvent crystallization-induced segregation of the monomer.[27] Despite this observation, however, some recent research in radiation and photochemistry does not seem to consider adequately the possibility of inhomogeneity in frozen solution.

Very rapid freezing of aqueous solutions has become popular in EPR studies of enzyme kinetics.[28,7] The freezing is accomplished by squirting a 0.2 mm jet of solution at 30 meters per second into isopentane at -145° C. Palmer, Bray, and Beinart[7] have found that chemical reactions can be quenched in about 10 milliseconds, roughly 100 times faster than plunging a sample tube into liquid nitrogen.

The possibility for dipolar interactions in frozen enzyme systems is smaller than in the manganese situation for two reasons besides the greater cooling rate. The sites of paramagnetism are somewhat buried within a protein, preventing close approach of two spins. Also, the relatively slow diffusion rate of a protein may prevent it from being

segregated as effectively. Nevertheless, decreasing the cooling rate by increasing the jet diameter has altered relative EPR peak heights by up to 30% for a doubled "quenching time".[7] This has been tentatively attributed to different reaction activation energies causing different effective quenching times for the various reactions within a single system,[7] but the possibility that some dipolar interactions are entering should be considered.

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Table I. Intensity and clarity of detail of the derivative EPR spectrum of 0.005 M Mn^{++} in different frozen aqueous solutions. Scales are arbitrary, and are defined in the text.

Solution	Intensity	Clarity
25% methanol	100	106
2M methanol	67	83
2M K acetate	45	85
2M H_2SO_4	45	82
25% glycerol	44	84
25% pyridine	42	74
2M HNO_3	39	73
2M $NaClO_4$	32	75
2M $HClO_4$	17	36
25% dioxane	16	5
2M HCl	9	69
2M KNO_3	8	5

Figure 1. Effect of added sodium perchlorate on the derivative EPR spectrum of 0.005 M Mn^{++} in frozen aqueous solution. "Other ions" refers to total concentration of anions and cations not Mn^{++}.

Figure 2. EPR derivative spectrum of Gd^{3+} in frozen aqueous solution. (a) 0.002 M $Gd(ClO_4)_3$, 0.02 M $HClO_4$; (b) a + 1.0 M $NaClO_4$. Spectrum is centered on g = 2.

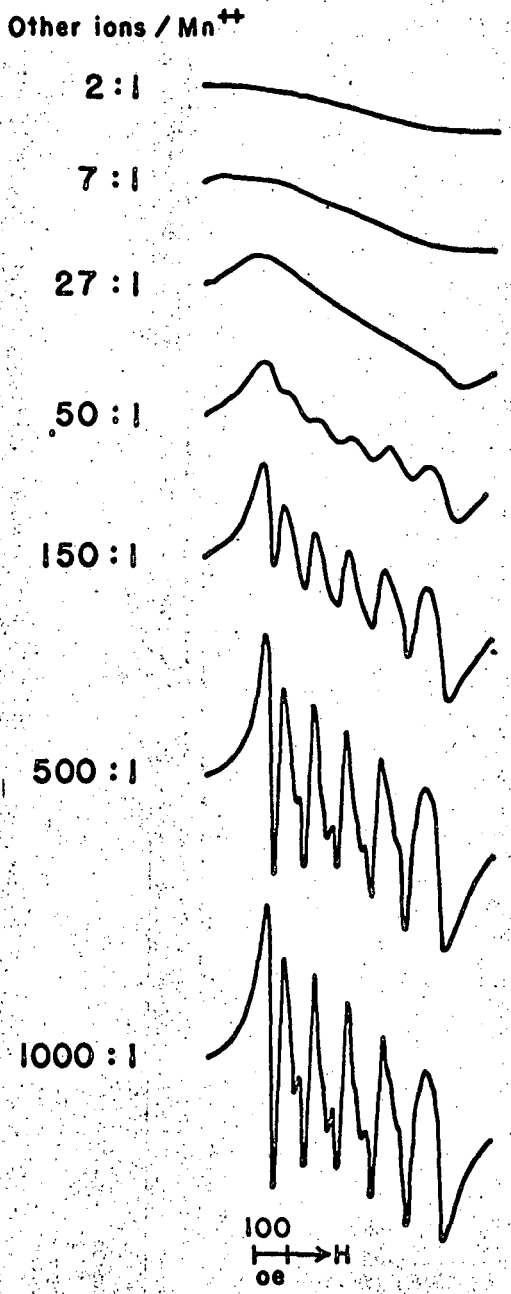

Fig. 1

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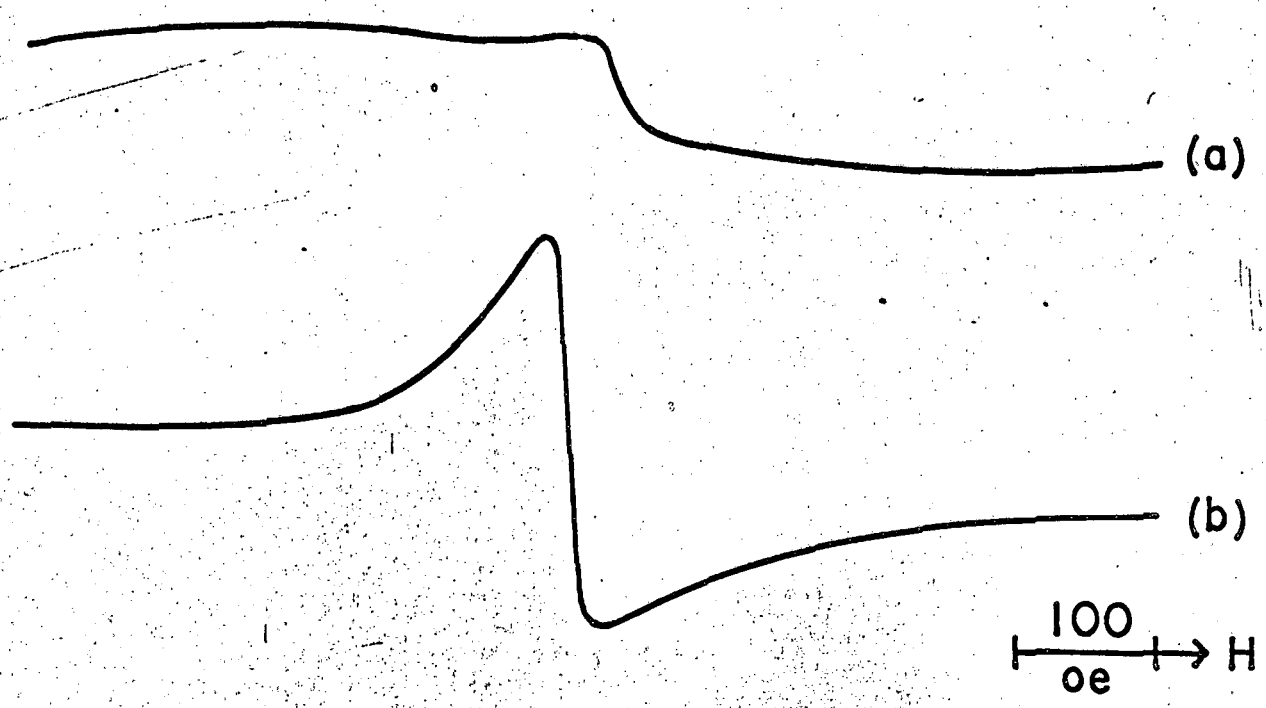

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Fig. 2