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THE LOCATION OF SPIN PROBES IN TWO PHASE MIXED LIPID SYSTEMS*

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SUMMARY

We observed electron spin resonance (ESR) spectra of either a cholestane or a stearic acid spin probe in liposomes formed of egg, yeast or dipalmitoyllecithins individually or in mixtures and found:

(1) The "liquid lines" often observed with the stearic acid probe and liposomes originate with the probe free in solution.

(2) The lipid–water partition coefficient of the stearic acid probe increases with increasing fluidity of the lipid. In mixed systems this causes the probe concentration to be higher in the more fluid component and leads to overestimation of the fluidity of the system.

(3) The lipid–water equilibrium of the stearic acid probe is rapid. This prevents removal of the free probe from the system by dialysis or washing and ensures that movement of the probe to the more fluid environment upon phase separation in the lipid will be rapid.

(4) Even with fluid lipids "liquid lines" can appear in the ESR spectra at high dilutions or high spin probe: lipid ratios.

(5) The cholestane spin probe produces spectra with no "liquid lines", which in most cases represent the fluid and crystalline phases of mixed systems in proportion to their relative concentrations.

INTRODUCTION

Spin probes, stable free radicals and free radical derivatives of steroids, fatty acids, and phospholipids, are used to measure molecular architecture and fluidity in model and biological membranes. A perpetual question is whether spin probes reflect an accurate picture of overall membrane properties or whether they are confined to limited, more fluid patches of membrane [1].

Oldfield et al. [2] have shown that the spectrum of the *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 12-ketostearic acid ("stearic acid spin label") in liposomes

Abbreviations: Stearic acid spin label, the *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 12-ketostearic acid; cholestane spin label, the analogous derivative of cholestane-3-one; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

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formed from a mixture of dioleoyllecithin and dibehenoyllecithins (22 : 0) resembles the spectrum of stearic acid spin label in pure dioleoyllecithin liposomes. In addition, if stearic acid spin label was incorporated in dibehenoyllecithin liposomes which were then mixed with unlabeled dioleoyllecithin liposomes, the spin probe could be seen to move from the less to the more fluid liposome.

The solubility of the spin probe 2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPO) in lipids increases as the fluidity of the lipid increases [3]. The lipid–water partition has been used to measure the extent of fluid regions in biological membranes [4] and in liposomes formed of lipid mixtures [5–7].

We have investigated the ESR spectra of stearic acid spin label and the *N*-oxyl-4',4'-dimethyloxazolidine derivative of cholestane-3-one ("cholestane spin label") to see if both these probes partitioned into the more fluid phases. We have done so in dipalmitoyllecithin, which is below its phase transition at room temperature, egg lecithin, which is above its transition temperature and yeast lecithin, which has been shown to contain 85–90 % monounsaturated fatty acids [8] and is very fluid at room temperature. Calorimetric [9] and surface pressure [10] studies have shown that lecithins with highly different transition temperatures form two phase systems at temperatures between the transition temperatures of the two lipids.

MATERIALS AND METHODS

The dipalmitoyllecithin was purchased from General Biochemicals, Chagrin Falls, Ohio. A single spot was shown on thin layer chromatography when developed in two different solvent systems and C 16 : 0 was the only fatty acid observed by gas chromatography. Stearic acid spin label was purchased from Syva Associates, Palo Alto, Calif. and cholestane spin label was prepared by the method of Keana et al. [11]. Baker's yeast purchased from Morrison Lamothe, Ottawa, Canada, was blended in a Gifford Wood homogenizer with glass beads in methanol. The dried filtrate was washed by the Folch procedure [12]. The total lipids were taken to dryness and redissolved in chloroform : methanol (1 : 1, v/v). The lecithin was isolated by silica gel thin layer chromatography and the fatty acid composition was assayed by gas chromatography (Table I).

Liposomes were formed by evaporating chloroform solutions of lipids plus spin probes with a stream of nitrogen. The lipid was placed under a vacuum for two hours to remove residual solvent, a buffer consisting of 0.14 M NaCl and 0.01 M Tris, pH 7.4, was added, and the tubes were gassed with nitrogen and sealed. The

TABLE I

FATTY ACID COMPOSITION OF YEAST LECITHIN

	%
16 : 0	6.2
16 : 1	47.8
18 : 0	5.7
18 : 1	40.3
Satd./unsatd.	10 : 74

tubes were then shaken and placed in a water bath sonicator (Heat Systems 5 cm × 5 cm, New York) for about one hour with several changes of water. The temperature was kept below 40 °C for egg and yeast lecithin preparations and below 50 °C for preparations containing dipalmitoyllecithin. After the liposomes had been allowed to equilibrate for at least one hour, ESR spectra were obtained with a Varian E9 spectrometer (9.5 GHz, 100 KHz field modulation) equipped with a Varian 620 i computer for signal averaging and spectral addition. All experimental procedures were subsequently performed at room temperature (23 °C).

RESULTS

Mixed liposomes

Liposomes were formed from yeast and dipalmitoyllecithin containing 0, 25, 75 or 100 mole% dipalmitoyllecithin plus 1 % spin probe. With stearic acid spin label, the separation between the outermost resonances and the widths of the peaks increased with increasing content of dipalmitoyllecithin (Fig. 1). At 100 % dipalmitoyllecithin and 1 % stearic acid spin label, the "liquid line" [13, 14) was very prominent. The spectra of stearic acid spin label in pure yeast lecithin and in pure dipalmitoyllecithin were added together in the ratios of 1 : 3 and 3 : 1. These computer generated spectra differed from the corresponding experimental spectra in that the peaks were broader and the narrow components ("liquid lines") were much more prominent.

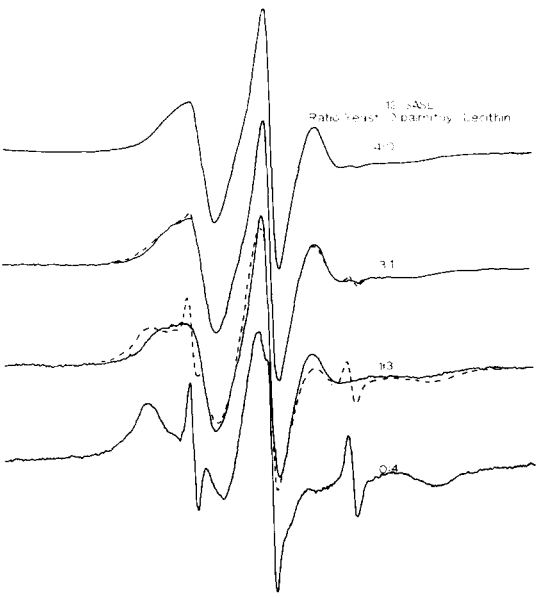

Fig. 1. Spectra of stearic acid spin label in lecithin liposomes in 0.14 M NaCl, 0.01 M Tris–HCl, pH 7.4, at 23 °C. The ratio of probe to lipid was 1 : 100. Spectra are plotted at a constant total amplitude. ———, spectra of liposomes of the indicated composition. – – –, are the results of adding spectra of stearic acid spin label in yeast lecithin liposomes and in dipalmitoyllecithin liposomes in the ratios 3 : 1 and 1 : 3, respectively. 12 SASL, stearic acid spin label.

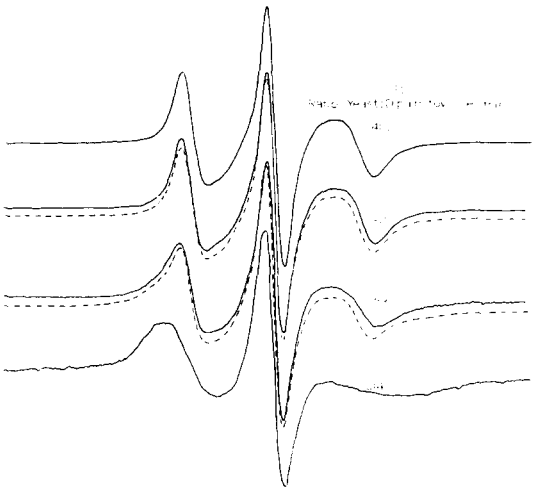

Fig. 2. Electron spin resonance spectra of cholestane spin label in lecithin liposomes. The experimental conditions and plots are as in Fig. 1. The computer generated spectra are displaced downward so as to be visible. CSL, cholestane spin label.

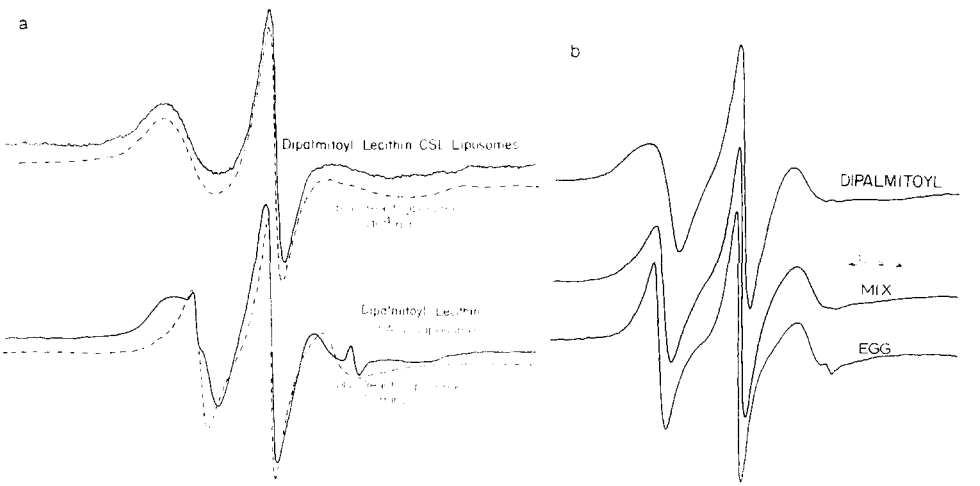

Fig. 3. (a) Electron spin resonance spectra of cholestane spin label and stearic acid spin label in dipalmitoyllecithin liposomes before (solid spectra) and after (dashed spectra) mixing with an equimolar quantity of unlabelled yeast lecithin liposomes. The time after mixing is indicated below the dashed spectra. The mole ratio of lipid to spin probe was 100 : 1. (b) Spectra of stearic acid spin label in liposomes of dipalmitoyllecithin (probe: lipid is 1 : 1000) alone and mixed with an equal quantity of unlabelled egg lecithin liposomes. The bottom spectrum is of stearic acid spin label in egg lecithin for comparison. All spectra were run at 23 °C in 0.14 M NaCl, 0.01 M Tris, pH 7.4. CSL, cholestane spin label; 12 SASL, stearic acid spin label.

This experiment was repeated using the cholestane derivative as the spin probe. The ESR line widths and maximum hyperfine separation increased with increasing concentrations of dipalmitoyllecithin. However, no "liquid lines" appeared. When the spectra of cholestane spin label in yeast or in dipalmitoyllecithin were added together in the ratios 1 : 3 or 3 : 1, the computer generated spectra closely resembled the experimental spectra (Fig. 2). The computer spectra were displaced vertically with respect to the experimental spectra in the figure to make them more visible.

Liposomes of dipalmitoyllecithin plus spin probe were mixed with liposomes of yeast lecithin. With cholestane spin label there was virtually no change in the spectra with time. Even after 10^4 min the spectrum of the mixed system closely resembled that of the probe in dipalmitoyllecithin (Fig. 3a). With stearic acid spin label the spectrum changed rapidly, the peaks becoming narrower and the liquid line disappearing. The change was virtually complete in two minutes, the shortest time in which the experiment could be performed. The experiment with stearic acid spin label was repeated using a lipid–spin probe ratio of 1000 : 1 instead of 100 : 1 in case the capacity of dipalmitoyllecithin for stearic acid spin label was limited (Fig. 3b). At the 1000 : 1 ratio, the liquid line was of very low relative intensity and could not be seen on a single scan. The top spectrum plotted in Fig. 3b is the result of the addition of 265 scans. Even at this high ratio of lipid to probe, a rapid migration of the probe to the more fluid phase was noted.

It has been noted that some lipid systems are too tightly packed to include cholestane spin label [15]. Liposomes of dipalmitoyllecithin formed in 1 M $CaCl_2$ gave an exchange broadened ESR spectrum for cholestane spin label, whereas those of yeast lecithin did not (Fig. 4). This is probably due to formation of patches

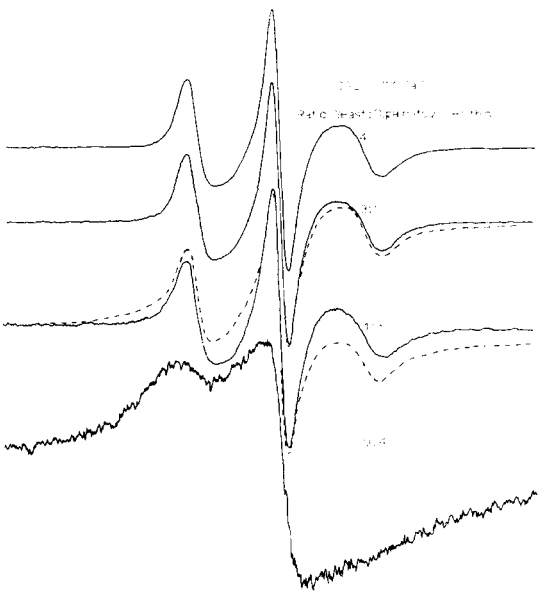

Fig. 4. Spectra of cholestane spin label in lecithin liposomes run in 1 M $CaCl_2$ at 23 °C. The ratio of probe to lipid was 1 : 100. The computer-generated summation spectra are dashed. CSL, cholestane spin label.

of probe within the rigid lipid, although the possibility of vesicle formation by excluded probe cannot be discounted. Similar behaviour has been observed in oriented films where any vesicles formed would have been removed [15]. The ESR spectra of cholestane spin label in liposomes formed of 3 : 1 and 1 : 3 mixtures of yeast and dipalmitoyllecithin did not match spectra generated by the 3 : 1 and 1 : 3 computer addition of the spectra of cholestane spin label in liposomes of yeast and dipalmitoyllecithins. As can be seen by the displacement of the base line on the high field side of the spectra, the computer generated spectra contain an excess of the exchange broadened component. Thus in this extreme case even cholestane spin label does not produce an ESR spectrum truly representative of the relative proportions of the two phases, but complete segregation of cholestane spin label into only the more fluid phase does not occur.

Stearic acid spin label "liquid line"

In Fig. 1 the "liquid line" was visible when stearic acid spin label was incorporated in liposomes of dipalmitoyllecithin, but not in yeast lecithin. However, when liposomes of egg or yeast lecithin containing 1 mole% stearic acid spin label were diluted with buffer, a "liquid line" became apparent (Fig. 5)a. This effect is due to dilution and not to sonication because the liposomes were sonicated, allowed to

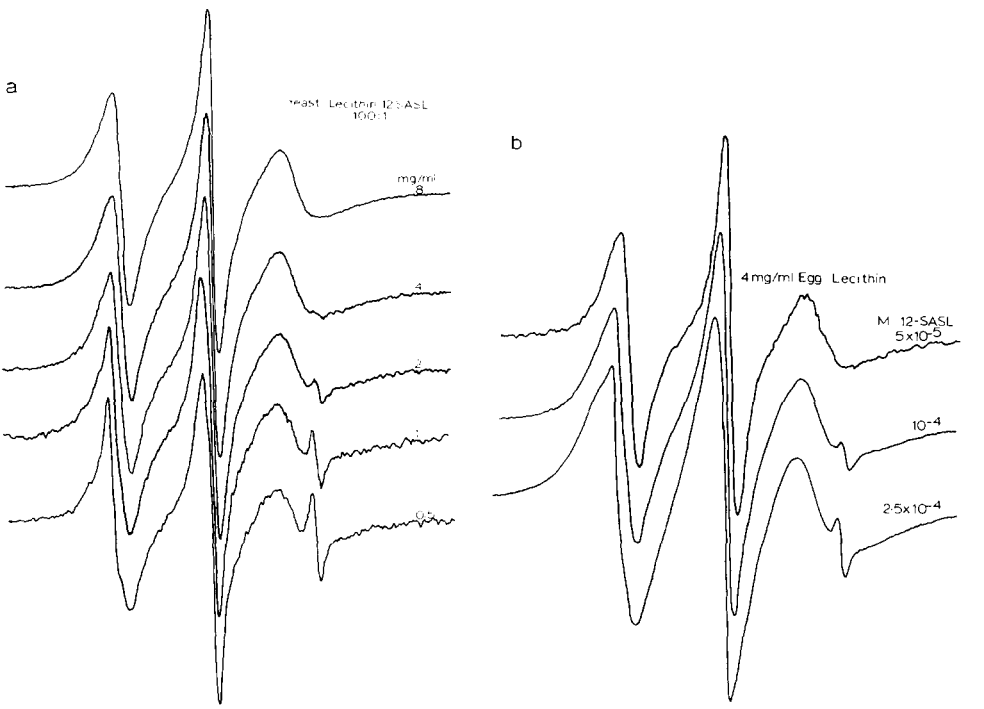

Fig. 5. Spectra of stearic acid spin label in lecithin liposomes in 0.14 M NaCl, 0.01 M Tris–HCl at pH 7.4 and 23 °C. (a) Effect of diluting the yeast lecithin liposome suspension with buffer. (b) Effect of varying the ratio of probe to egg lecithin at a constant lecithin concentration. 12-SASL, stearic acid spin label.

equilibrate, diluted, allowed to re-equilibrate, and then the ESR spectra were taken. Increasing the ratio of stearic acid spin label to egg or yeast lecithin at a constant lipid–buffer ratio also brought about the appearance of the liquid line (Fig. 5b).

These results indicate that stearic acid spin label partitions between the lipid and water and that the liquid line is due to probe in aqueous solution. In confirmation of this it was found that for stearic acid spin label in hexane the isotropic hyperfine splitting was 14.4 G and the width of the high field line (w_{-1}) was 3.2 G. For stearic acid spin label in water and the "liquid lines" of stearic acid spin label in dipalmitoyl-lecithin the corresponding values were 15.9 G and 1.2 G, respectively.

The partition coefficient could not be measured accurately because the spectrum of the probe in the lipid overlaps the "liquid lines". An approximation was made by mixing various concentrations of unlabeled liposomes with stearic acid spin label in buffer. The amount of the free probe was assayed by comparing the amplitude of the high field "liquid line" in the mixture and the corresponding resonance of the probe dissolved in pure buffer. The amount of the probe in the lipid was measured as the difference between the total amount of probe added and the amount in the aqueous phase. The distribution coefficient, K, was measured as

$$K = \frac{n_l \cdot [H_2O]}{n_w \cdot [\text{lipid}]}$$

where n_l is the number of moles of probe in the lipid, n_w is the number of moles of probe in the water, and $K \simeq 10^6$ for egg or yeast lecithin and 10^5 for dipalmitoyl-lecithin at 23 °C.

DISCUSSION

The "liquid lines" have been observed for stearic acid spin label in influenza virus and erythrocytes [13, 14] in egg lecithin liposomes (Schreier-Muccillo, S. and Smith, I. C. P., unpublished results), and in rabbit sarcoplasmic reticulum [4]. The observations that the splittings and peak widths of the "liquid lines" match those of an aqueous solution of stearic acid spin label, but not those of a non-polar solution of the probe, and that the relative amplitudes of these resonances are dependent on the lipid–water ratio strongly indicate that these resonances are due to free probe in solution. Monolayer studies have revealed that stearic acid spin label forms stable films with either the carboxyl group alone, or with both the carboxyl and the nitroxide groups at the water interface [16]. Nitroxide groups at the aqueous interface might exhibit spectral properties similar to those of free spin probe in solution, but since water is present to large excess, there is no reason to suppose that the proportion of spin probe so located would increase as the liposomes concentration was decreased from 8 to 0.5 mg lecithin/ml.

The partition coefficient of stearic acid spin label is difficult to measure accurately because of spectral overlap and its large magnitude, two to three orders greater than the lipid : water coefficient of TEMPO [7]. Like the partition coefficient of TEMPO, that of stearic acid spin label is higher with a lipid above than with a lipid below its chain melting transition temperature. If a lipid system consists of two phases, one below and one above its transition temperature, stearic acid spin label does not partition entirely into the more fluid phase. However, the probe concen-

tration in the more fluid phase will be higher than in the less fluid phase and the spectra will not accurately reflect the fluidity of the entire system. This partition may take place via lateral diffusion within a bilayer as well as by diffusion through the aqueous phase.

The cholestane spin probe shows no sign of water solubility and little or no tendency to transfer from one liposome to another. In liposomes formed of a mixture of two lecithins, one above and one below the transition temperature, this spin probe accurately reflects the fluidity of its environment. However, when one phase is so rigid as to exclude the spin probe, the cholestane spin label mainly reports the fluidity of the other phase.

Cholestane spin label has been shown to produce angular-dependent spectra when dissolved in planar films of phosphatidylserine [15], phosphatidylethanolamine [15], lecithins with a range of fatty acid compositions [17, 18], phosphatidylinositol [19], ceramide [19], sphingomyelin [19], and total lipids isolated from brain [20], morning glory [20], erythrocytes [20], and *Escherichia coli* [21]. Cholestane spin label dissolves and produces angular-independent spectra in films of monoolein, phosphatidic acid, mixed diglycerides at room temperature [15] and cerebrosides [19]. Films of monolaurin, monopalmitin and monostearin exclude cholestane spin label at room temperature, but exhibit anisotropic spectra at higher temperatures [15]. Cholesterol generally increases spectral anisotropy of cholestane spin label in films of lipids above their transition temperature [15, 22–24] and induces the solubility of cholestane spin label in monolaurin [11] and sphingosine at room temperature [19], systems that normally exclude cholestane spin label.

In general then, there are few lipids that exclude cholestane spin label, and fewer still that exclude cholestane spin label in the presence of cholesterol. This is not surprising, considering the similarity in structure and behavior in films of this probe to cholesterol [25], which mixes well with a variety of lipids.

The transfer of stearic acid spin label from liposomes of relatively rigid lipids to liposomes of fluid lipid undoubtedly takes place due to the finite concentration of free probe in aqueous solution. In the case of cholestane spin label, the solubility of the probe in solution is negligible, and interliposome transfer could only be effected by vesicle fusion. The absence of transfer of cholestane spin label indicates that such fusion does not take place to any appreciable extent under the conditions of these experiments.

It has been observed that the electrical conductivity of dipalmitoyllecithin on a sintered glass support [26] and the self permeability to $^{22}Na^{+}$ of dipalmitoyllecithin and phosphatidylglycerol vesicles [27] exhibit local maxima at the transition temperatures of the lipid involved. Linden et al. [28] have observed that the sugar uptake of *E. coli* enriched with elaidic acid also exhibits a maximum at the transition temperature of the lipid. They suggest that it may be important for the transport processes of some organisms to have fluid and solid phases in equilibrium.

Quite another form of heterogeneity has been postulated in which the membrane consists of a mosaic of phospholipid and protein [29, 30]. Fatty acid spin probes show the presence of both unmodified phospholipid bilayer and protein bound lipid in mixtures of cytochrome oxidase and membrane lipid [31, 32].

Thus, when spin probes are used to study intact membranes, it must be kept in mind that only the more fluid regions may be monitored by fatty acid probes such as

stearic acid spin label whereas cholestane spin label may probe several regions of differing fluidity to yield a composite spectrum.

CONCLUSIONS

In agreement with earlier findings [2] the probe stearic acid spin label tends to migrate to the more fluid lipid phase in multiphase systems. This is because the lipid–water partition coefficient of the probe is higher when the lipid is fluid. Measurements of membrane fluidity obtained with this probe in heterogeneous systems containing no cholesterol or other similar sterol are therefore not necessarily representative of the entire membrane. The stearic acid spin probe has the additional disadvantage that it is sufficiently water soluble that the aqueous component contributes appreciably to the ESR spectrum. The high field "liquid line" is sufficiently resolved as to be readily apparent. The low field and central resonances serve to complicate the spectra. The equilibrium of the probe between the lipid and the aqueous phases is sufficiently rapid that the "liquid line" cannot be washed or dialyzed away. The cholestane spin probe has no "liquid line" in any spectrum so far observed and is compatible with a wide variety of lipids. This probe generally represents an accurate picture of the average state of the membrane. However, there are a few systems that will exclude even this probe. This can be detected by examining spectra of the probe in the component parts of the system for broadening due to electron–electron exchange interaction.

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