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LOCATION AND ORIENTATION RELATIVE TO THE MICELLE SURFACE FOR GLUCAGON IN MIXED MICELLES WITH DODECYLPHOSPHOCHOLINE

EPR AND NMR STUDIES

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Summary

The spin labels, 5-doxylstearate, 12-doxylstearate, 16-doxylstearate and 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine, have been incorporated into dodecylphosphocholine micelles and mixed dodecylphosphocholine/glucagon micelles. The EPR spectral parameters for the different spin labels and the ^{1}H- and ^{13}C-NMR relaxation rates for nuclei of the detergent molecules indicated that inclusion of up to one spin label molecule per micelle had little influence on the spatial organization of the micelles. Furthermore, the location and environment of the spin labels in the dodecylphosphocholine micelles were not noticeably affected by the addition of glucagon and the ^{1}H-NMR spectra observed for glucagon in mixed spin label/deuterated dodecylphosphocholine/glucagon micelles showed that the different spin labels had essentially no effect on the conformation of glucagon. Approximate spatial locations within the micelle for the nitroxide moieties of the different spin labels were determined from the NMR relaxation rates observed for different nuclei of dodecylphosphocholine. On this basis, the line broadening of individually assigned glucagon ^{1}H-NMR lines by the different spin labels was used to determine the approximate orientation of the polypeptide chain with respect to the micelle surface. Overall, the data indicate that the glucagon backbone runs roughly parallel to the micelle surface, with the depth of immersion adjusted so that polar and apolar side chains can be oriented towards the surface or interior of the micelle, respectively.

Introduction

In previous studies it was shown that mixed micelles of glucagon and dodecylphosphocholine contain one polypeptide and approx. 40 detergent molecules, and that in the ^{1}H-NMR spectrum of glucagon bound to fully deuterated dodecylphosphocholine micelles, numerous resonance lines can be resolved and assigned to specific locations in the polypeptide covalent structure [1,2]. Detailed proton-proton nuclear Overhauser enhancement studies for the assigned resonances showed that micelle-bound glucagon adopts an extended form rather than a compact, globular overall structure [2,3]. For the segment 19—27 of micelle-bound glucagon, a preliminary molecular geometry was proposed by combining the Overhauser effect data with a distance-geometry algorithm [4]. Thus, considerable information is now available on the conformation of micelle-bound glucagon.

In addition to the conformation, a further important aspect of structure-function relationships for membrane-bound proteins is the orientation of the protein relative to the lipid membranes. In the present study, the location and orientation relative to the micelle surface of glucagon bound to deuterated dodecylphosphocholine micelles are investigated by measurements of the paramagnetic effects caused by micelle-bound nitroxide spin labels on the ^{13}C- and ^{1}H-NMR spectra of dodecylphosphocholine and glucagon in the mixed micelles.

Materials and Methods

Glucagon was purchased from Calbiochem and used without further purification. The fatty acid spin labels, 5-doxylstearate, 12-doxylstearate and 16-doxylstearate were purchased from Syva. The synthesis of dodecylphosphocholine [5] and [$^2H_{38}$]dodecylphosphocholine [1] have been described previously. The synthesis of 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine is described below. Samples containing spin labels were prepared by vacuum drying a known quantity of spin label in organic solvent and adding the desired amount of dodecylphosphocholine solution or dodecylphosphocholine/glucagon solution. Dissolution of the spin labels was sometimes aided by warming to approx. 50°C and brief sonication on a water bath sonifier.

EPR measurements were performed on a Varian E4 X-band instrument. The temperature was 37°C for all experiments. Rotational correlation times, τ_r, for the spin labels were calculated from the EPR spectra using the equation [6,7]:

$$\tau_r = 6.5 \cdot 10^{-10} \cdot \Delta H \cdot \left\{ \left(\frac{I(0)}{I(+1)} \right)^{1/2} + \left(\frac{I(0)}{I(-1)} \right)^{1/2} - 2 \right\}$$

where $I(0)$, $I(+1)$ and $I(-1)$ are the peak-to-peak heights of the 0, +1 and −1 transitions in the first-derivative EPR spectrum and ΔH is the linewidth (in G) of the 0 transition.

^{13}C-NMR spectra at 25.16 MHz were obtained on a Varian XL-100 instrument and ^{1}H-NMR spectra at 360 MHz were obtained on a Bruker HX-360

instrument. Both the ^{13}C spectra and the ^{1}H spectra were obtained in $^{2}H_2O$, which was also used for the lock system. Chemical shifts for ^{13}C- and ^{1}H-NMR spectra are given relative to sodium 3-trimethylsilyl[2,2,3,3-$^{2}H_4$]propionate at p^{2}H 7.0 [8]. Values of p^{2}H are pH meter readings uncorrected for isotope effects. In some cases the resolution of the ^{1}H-NMR spectra was improved by multiplying the free induction decay with a phase-shifted sine bell [9,10].

Synthesis of 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine

Dodecylphosphoric acid (Labimex) and 1-oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine (Aldrich) were coupled with 2,4,6-triisopropylbenzenesulfonyl chloride (Fluka) according to the method of Aneja and Chadha [11]. Dodecylphosphoric acid (0.13 g, 0.5 mmol) and 1-oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine (0.17 g, 1 mmol) were mixed and dried under vacuum over P_2O_5. Anhydrous pyridine (10 ml) and 2,4,6-triisopropylbenzenesulfonyl chloride (0.45 g, 1.5 mmol) were added and the mixture, protected from moist atmosphere, was stirred at room temperature for 12 h. Water (0.5 ml) was added, stirring continued for 5 min and then the reaction mixture was dried by rotary evaporation. Further drying on a vacuum line aided in removal of pyridine. The dry material was extracted with diethyl ether and the ether extract taken to dryness by rotary evaporation. The oily material obtained was purified by chromatography on silica gel 60 (Merck) using chloroform/methanol/triethylamine (80 : 20 : 2, v/v) for elution. The column fractions which contained product were pooled and rechromatographed on silica gel using chloroform/methanol/water (65 : 25 : 4, v/v) for elution. After pooling and drying the product-containing fractions, a thick, oily, orange-red material was obtained. This material showed no detectable impurities in thin-layer chromatography on silica gel with various solvent systems. ^{1}H-NMR spectra before and after reduction with dithionite indicated that the product had the proper chemical structure and was fully paramagnetic (yield 0.06 g = 27%).

Results

In most studies of the influence of paramagnetic species on NMR spectra the binding sites for paramagnetic probes are part of the molecule studied [12,13]. In the present investigation of mixed dodecylphosphocholine-glucagon complexes by use of paramagnetic nitroxide spin labels, a more complex situation is encountered. The detergent molecules may exist in either monomeric or micellar forms and both the spin label and the polypeptide may be either free or micelle-bound. Exchange of detergent, polypeptide and spin label between the various environments as well as diffusion within the micelle of the detergent, spin label and polypeptide molecules relative to one another may influence the paramagnetic effects observed in the NMR spectra. In the first two sections of Results, we therefore present the results of EPR and NMR experiments designed to demonstrate which solution species and relaxation mechanisms dominate the paramagnetic effects observed in mixed spin label/dodecylphosphocholine micelles or in mixed spin label/dodecylphosphocholine/glucagon micelles. Evidence that spatial information can be obtained

from such mixtures is also presented. The last section presents the results of ^{1}H-NMR experiments with spin label/deuterated dodecylphosphocholine/glucagon mixtures which have been used to orient glucagon relative to the dodecylphosphocholine micelle.

EPR measurements for spin labels bound to dodecylphosphocholine micelles and mixed dodecylphosphocholine/glucagon micelles

Fig. 1 shows EPR spectra measured for $1 \cdot 10^{-4}$ M 5-doxylstearate as a function of the concentration of dodecylphosphocholine. Below the critical micelle concentration of the detergent (approx. $1.2 \cdot 10^{-3}$ M [5]), the EPR spectrum is identical to that observed for the spin label in the absence of detergent (Fig. 1A and B). At dodecylphosphocholine concentrations just above the critical micelle concentration, the EPR spectrum consists of the superposition of a sharp and a broad spectrum (Fig. 1C). For $2.5 \cdot 10^{-3}$ M detergent essentially only the broad component is present (Fig. 1D). As the detergent concentration is further increased, the broadening of the EPR spectrum becomes less pronounced (Fig. 1E) and at sufficiently high detergent concentration, the EPR spectrum is insensitive to further increases in the detergent concentration. We interpret the spectra in Fig. 1 as follows. Detergent concentrations below the critical micelle concentration represent one limiting situation where there is little or no interaction between the detergent and the spin label. The opposite limit is attained when the detergent concentration is sufficiently high so that, on the average, one or less spin label molecule is bound per detergent micelle and an EPR spectrum characteristic of an isolated spin label in a milieu provided by the detergent micelle is obtained.

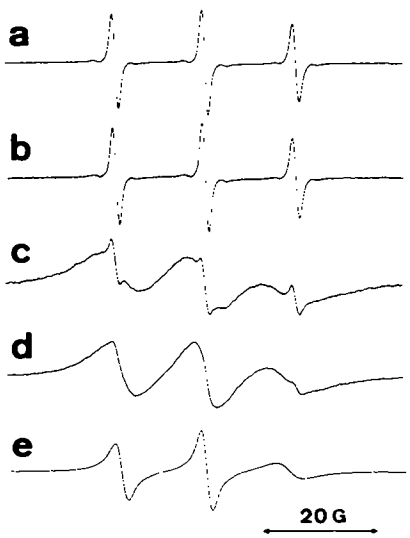

Fig. 1. X-band EPR spectra for $1 \cdot 10^{-4}$ M 5-doxylstearic acid as a function of the concentration of dodecylphosphocholine. The spectra were measured in 0.05 M phosphate buffer at pH 7.0 and 37°C for dodecylphosphocholine concentrations of: (a) 0, (b) $3 \cdot 10^{-4}$ M, (c) $2 \cdot 10^{-3}$ M, (d) $2.5 \cdot 10^{-3}$ M and (e) $2.5 \cdot 10^{-2}$ M. The critical micelle concentration for dodecylphosphocholine is approx. $1.2 \cdot 10^{-3}$ M [5].

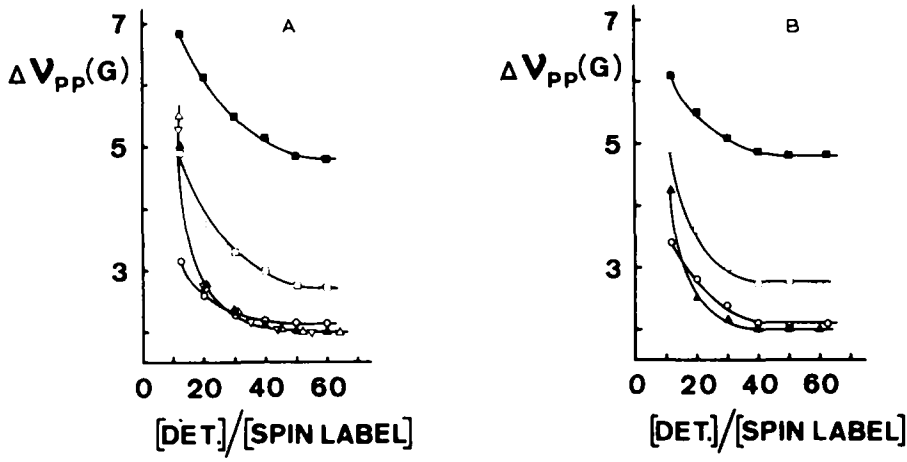

Fig. 2. Plots of the peak-to-peak linewidth, $\Delta\nu_{pp}$, of the $I = -1$ transition in the X-band EPR spectra of four different spin labels as a function of the ratio of the molar concentrations of dodecylphosphocholine and spin label. (A) Titration with pure dodecylphosphocholine for: (■) $4 \cdot 10^{-3}$ M 5-doxylstearate, (□) $4 \cdot 10^{-3}$ M 12-doxylstearate, (○) $4 \cdot 10^{-3}$ M 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine; (△, ▲, ▽) $1 \cdot 10^{-3}$, $4 \cdot 10^{-3}$ and $1 \cdot 10^{-2}$ M 16-doxylstearate, respectively; DET., detergent. (B) Titration with a 50 : 1 mixture of dodecylphosphocholine and glucagon for: (■) $1 \cdot 10^{-3}$ M 5-doxylstearate, (□) $1 \cdot 10^{-3}$ M 12-doxylstearate, (○) $1 \cdot 10^{-3}$ M 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine, (▲) $4 \cdot 10^{-3}$ M 16-doxylstearate. All measurements were performed in 0.05 M phosphate buffer at pH 7.0 and 37°C.

At intermediate detergent concentrations, micelles are formed which contain more than one spin label molecule. This leads to broadening of the EPR spectrum through interaction between spin labels so that the degree of broadening depends on the average number of spin labels per micelle [14]. At detergent concentrations just above the critical micelle concentration, appreciable proportions of the spin label occur in both free and micelle-bound states. When exchange of the spin label between these states is slow on the EPR time scale, a superposition of EPR spectra from free spin label and from spin label in micelles containing more than one spin label molecule is then observed.

The experiments in Fig. 1 show that for $1 \cdot 10^{-4}$ M 5-doxylstearate, dodecylphosphocholine concentrations of approx. $3 \cdot 10^{-3}$ M are sufficient to bind virtually all of the spin label to the detergent micelles. This ensures that at the considerably higher detergent concentrations used for NMR experiments (see below), it may safely be assumed that very little of the spin label is free in solution. For the other spin labels used in this study, i.e., 12-doxylstearate, 16-doxylstearate and 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine, similar behavior was observed. As demonstrated in the following, observation of the line broadening for conditions where there is more than one spin label molecule per micelle provides a means for determining the number of detergent molecules per micelle.

Fig. 2A shows plots of the peak-to-peak linewidth of the $I = -1$ transition in the EPR spectra as a function of the detergent-to-spin label ratio. High concentrations of spin label ($4 \cdot 10^{-3}$ M) and detergent (0.05—0.25 M) were used in these experiments. This ensured that the spin label was essentially fully bound to the micelles at all detergent concentrations and furthermore

that these measurements would provide a direct control of the micelle size at the high detergent concentrations used in NMR experiments (see below). For each of the spin labels, the linewidth of the $I = -1$ transition decreased sharply as the detergent-to-spin label ratio was increased from approx. 10 : 1 to 40 : 1 (Fig. 2A). The linewidth became independent of the detergent-to-spin label ratio at approx. 50—60 detergent molecules per spin label, indicating that at this ratio approx. 1 spin label molecule was bound per micelle [14]. Hence, micelles consisting of 50—60 detergent molecules were formed for dodecylphosphocholine concentrations of approx. 0.25 M. For 16-doxylstearate, very similar curves were obtained by using $1 \cdot 10^{-3}$, $4 \cdot 10^{-3}$ or $1 \cdot 10^{-2}$ M spin label (Fig. 2A), suggesting that the size of the dodecylphosphocholine micelles is virtually the same for detergent concentrations between 0.06 and 0.6 M. This supports and extends previous ultracentrifugation and quasi-elastic light-scattering measurements [2], which showed that for 0.02 M dodecylphosphocholine there are 50—60 detergent molecules per micelle and that the size of the dodecylphosphocholine micelles was essentially unchanged for detergent concentrations up to 0.1 M.

The size of mixed spin label/dodecylphosphocholine/glucagon micelles has been investigated by titration of $1 \cdot 10^{-3}$ M 5-doxylstearate, $1 \cdot 10^{-3}$ M 12-doxylstearate, $1 \cdot 10^{-3}$ M 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine and $4 \cdot 10^{-3}$ M 16-doxylstearate with a dodecylphosphocholine/glucagon mixture containing 50 detergent molecules per glucagon molecule. Fig. 2B shows that for all four spin labels, the peak-to-peak linewidth of the $I = -1$ transition of the EPR spectrum became independent of the concentration of the dodecylphosphocholine/glucagon mixture at a detergent-to-spin label ratio of approx. 40 : 1. This coincides with earlier results on mixed dodecylphosphocholine/glucagon micelles obtained with different methods [2], indicating that the stoichiometry of the glucagon/dodecylphosphocholine complex is not appreciably altered by inclusion of up to one spin label molecule per micelle.

Further information on the organization of the dodecylphosphocholine micelles and the mixed dodecylphosphocholine/glucagon micelles has been obtained from studies of the ^{14}N hyperfine splittings, a, and the rotational correlation times, τ_r (Table I). Comparison of the values for free spin label and for dodecylphosphocholine micelles containing one or less spin label molecule per micelle shows that there is a decrease of 0.7—0.9 G in the hyperfine splitting and a 4—15-fold increase in the rotational correlation time when the spin label is incorporated into the micelles. The decrease in the hyperfine coupling constant is indicative of a less polar environment for the nitroxide moiety of the micelle-bound spin label [15] and the increase in the rotational correlation time indicates that the rotational mobility of the nitroxide moiety is reduced compared to the free spin labels. Similar features have previously been observed for fatty acid spin labels bound to other types of detergent micelles [14,16—18]. Table I further shows that the hyperfine splittings and the rotational correlation times measured for dodecylphosphocholine micelles with or without bound glucagon are very similar and therefore indicates that within the micelle the local environment of the nitroxide moiety of the spin label is largely uninfluenced by the presence of glucagon.

TABLE I

ISOTROPIC HYPERFINE SPLITTING CONSTANTS (a) AND ROTATIONAL CORRELATION TIMES (τ_r) MEASURED FOR FREE SPIN LABELS AND FOR SPIN LABELS INCORPORATED INTO DODECYLPHOSPHOCHOLINE MICELLES OR MIXED DODECYLPHOSPHOCHOLINE/GLUCAGON MICELLES

Measurements in 0.05 M phosphate buffer at pH 7.0 and 37°C. See Materials and Methods for the procedure used to calculate the rotational correlation time.

Spin label	$1 \cdot 10^{-4}$ M spin label		$1 \cdot 10^{-4}$ M spin label $5 \cdot 10^{-2}$ M dodecylphosphocholine *		$1 \cdot 10^{-3}$ M spin label $5 \cdot 10^{-2}$ M dodecylphosphocholine $1 \cdot 10^{-3}$ M glucagon	
	a_{14N} (G)	τ_r (s) ($\times 10^{10}$)	a_{14N} (G)	τ_r (s) ($\times 10^{10}$)	a_{14N} (G)	τ_r (s) ($\times 10^{10}$)
16-Doxylstearic acid	15.7	0.82	14.9	3.5	14.8	4.4
12-Doxylstearic acid	15.7	1.8	14.8	9.2	14.8	9.2
5-Doxylstearic acid	15.7	1.3	14.8	18	14.8	16
1-Oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine	17.0	0.81	16.3	3.9	16.3	4.4

* For spin label bound to dodecylphosphocholine micelles, the hyperfine splitting and the rotational correlation time were constant, within experimental error, for dodecylphosphocholine concentrations between 0.02 and 0.6 M.

^1H- and ^{13}C-NMR relaxation for dodecylphosphocholine in mixed spin label/dodecylphosphocholine micelles and mixed spin label/dodecylphosphocholine/glucagon micelles

The locations within the micelle of the nitroxide moieties of the different spin labels have been determined by measuring the paramagnetic contributions to the relaxation rates of individually assigned nuclei in the detergent molecules. Fig. 3 shows ^{13}C-NMR spectra obtained for 0.2 M dodecylphosphocholine (Fig. 3A) and for 0.2 M dodecylphosphocholine plus $8 \cdot 10^{-3}$ M 16-doxylstearate (Fig. 3B) or $8 \cdot 10^{-3}$ M 5-doxylstearate (Fig. 3C). Comparing Fig. 3A and B, it is seen that addition of 16-doxylstearate results in increased linewidths for the detergent ^{13}C resonances, with some resonances showing considerably more line broadening than others. Similar effects are observed when 5-doxylstearate is bound to the dodecylphosphocholine micelles (Fig. 3C), except that the most strongly broadened resonances are different for 5-doxylstearate and 16-doxylstearate. This is most clearly seen when resonances 1 and 15 are compared in the three spectra of Fig. 3. These results show that the paramagnetic relaxation caused by the different spin labels is selective for different ^{13}C nuclei of the detergent molecules.

When fast exchange conditions prevail and when the number of spin label molecules per micelle is small enough that interactions between spin label molecules can be neglected, the paramagnetic contributions to the observed relaxation rates, $1/T_1^p$ and $1/T_2^p$, should increase linearly with the average number of spin label molecules per micelle. We have measured the dependence of the ^{13}C spin-lattice relaxation rates for 0.2 M dodecylphosphocholine on the 16-doxylstearate concentration in the range from $1 \cdot 10^{-4}$ to $4 \cdot 10^{-3}$ M. For 56 dodecylphosphocholine molecules per micelle [5], this corresponds to

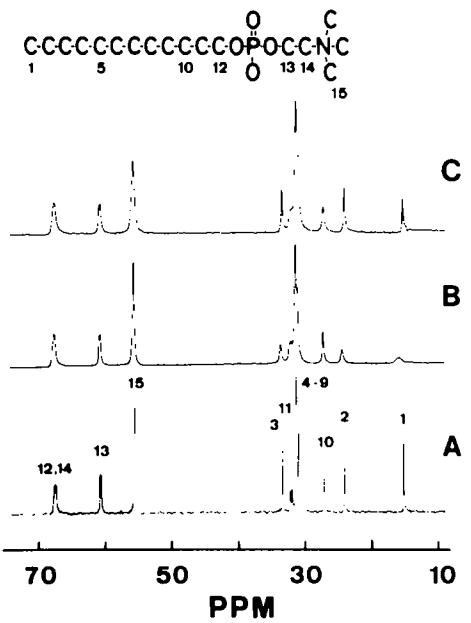

Fig. 3. 25.16 MHz ^{13}C-NMR spectra of: (A) 0.2 M dodecylphosphocholine, (B) 0.2 M dodecylphosphocholine plus $8 \cdot 10^{-3}$ M 16-doxylstearate, (C) 0.2 M dodecylphosphocholine plus $8 \cdot 10^{-3}$ M 5-doxylstearate. The assignments of the resonances [19,20] are given by the numbers shown in A and in the molecular structure at the top of the figure. The spectra were obtained in $^{2}H_2O$ solutions containing 0.05 M phosphate buffer, p^{2}H 7.0, at 37°C.

an average number of spin label molecules per micelle of approx. 0.03—1.1. Over this range, within experimental error, $1/T_1^p$ for the different ^{13}C resonances of dodecylphosphocholine was found to vary linearly with the spin label concentration. For resonances 1 and 2, which show pronounced line broadening in the presence of 16-doxylstearate (Fig. 3), linewidth measurements showed that $1/T_2^p$ also varied linearly with the spin label concentration. Hence, the rate of exchange of spin label between the free and micelle-bound states was greater than 10^2 s^{-1}. Thus, rapid exchange conditions prevailed in these NMR experiments.

In order to compare the effects caused by the different spin labels, we have measured the paramagnetic contributions to the ^{13}C-NMR linewidths, $\Delta\nu_{1/2}^p$, for 0.2 M dodecylphosphocholine in the presence of $8 \cdot 10^{-3}$ M concentrations of four spin labels. Plots of $\Delta\nu_{1/2}^p = \Delta\nu_{1/2} - \Delta\nu_{1/2}^d$, where $\Delta\nu_{1/2}$ and $\Delta\nu_{1/2}^d$ are the linewidths observed in the presence and absence of the spin label, respectively, versus the position of the observed ^{13}C nuclei in the covalent structure of the detergent molecules are shown in Fig. 4A. For 12-doxylstearate and 16-doxylstearate, the paramagnetic contributions to the ^{13}C linewidths as a function of the covalent positions are rather similar (Fig. 4A). The resonance corresponding to carbon 1 (resonance 1, Fig. 3) is strongly broadened, there is a progressive decrease in $\Delta\nu_{1/2}^p$ for carbons 2—10 and nearly equal values of $\Delta\nu_{1/2}^p$ are seen for carbons 12—15 (Fig. 4A). In contrast, for 5-doxylstearate and 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine, carbons 1—3 show appreciably smaller values of $\Delta\nu_{1/2}^p$ than carbons 10—15. In the Appendix

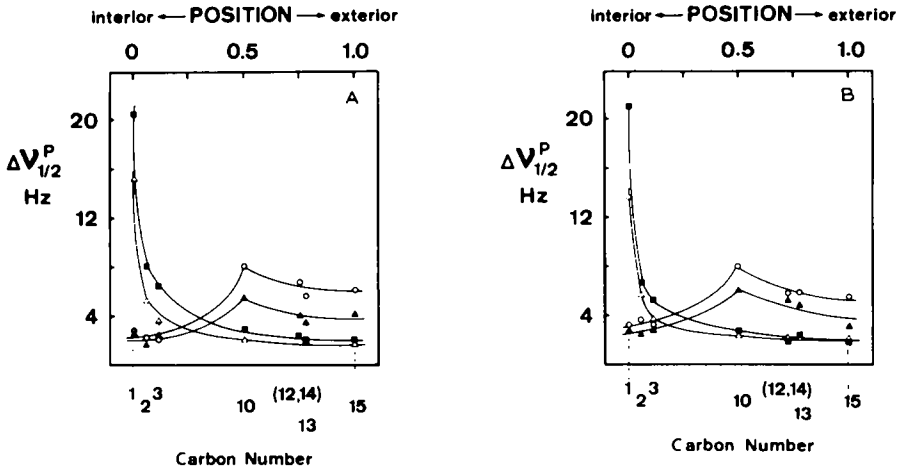

Fig. 4. Plots for four different spin labels of the paramagnetic contributions, $\Delta\nu^P_{1/2}$, to the ^{13}C-NMR linewidths of the resonance of dodecylphosphocholine as a function of the positions of the different ^{13}C nuclei in the covalent structure of the detergent molecules. (A) 0.2 M dodecylphosphocholine, $8 \cdot 10^{-3}$ M spin label. (B) 0.1 M dodecylphosphocholine, $4 \cdot 10^{-3}$ M spin label, $2 \cdot 10^{-3}$ M glucagon. The different spin labels are: (■) 16-doxylstearate, (△) 12-doxylstearate, (○) 5-doxylstearate and (▲) 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine. All measurements were performed at 25.16 MHz in 0.05 M phosphate buffer at p^{2}H 7.0 and 37°C. The numbers given at the bottom of the figure refer to the resonance assignments in Fig. 3. The solid lines have been drawn to visualize the connectivities between the datum points obtained for the individual spin labels. At the top of the figure, approximate positions of the different ^{13}C nuclei with respect to the micelle surface are indicated. These were estimated by assuming that in micellar dodecylphosphocholine, the terminal methyl of the dodecyl chain is located at the center of the micelle and the N-methyl groups of the choline moiety are located at the exterior surface of the micelle. Other carbon nuclei were located relative to the two types of methyl group by counting the number of intervening bonds (see Discussion and the Appendix).

it is shown that the data in Fig. 4A are qualitatively consistent with localization of the nitroxide moieties of 12-doxylstearate and 16-doxylstearate near the center of the micelle, and the nitroxide moieties of 5-doxylstearate and 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine near the phosphate groups of the detergent molecules in the micelle. Fig. 4B shows the paramagnetic contributions to the linewidths of the detergent ^{13}C resonances for mixtures containing 0.1 M dodecylphosphocholine, $2 \cdot 10^{-3}$ M glucagon and $4 \cdot 10^{-3}$ M spin label. The close similarity of the plots in Fig. 4A and B indicates that glucagon does not appreciably alter the influence of the spin labels on the detergent ^{13}C resonances, suggesting that the spatial orientation of the detergent and the different spin labels within the micelle is largely unchanged when glucagon is also bound to the micelle.

In the ^{13}C-NMR spectra, the resonances of carbons 11, 12 and 14 were overlapped with other resonances and carbons 11—14 showed scalar couplings of several Hz to ^{31}P. Since the linewidth measurements were less accurate for these resonances, the selectivity of the line broadening effects caused by 5-doxylstearate and 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine could not be determined as reliably as for the other spin labels. Therefore, for both these spin labels, the selectivity of the paramagnetic effects was also studied by measurements of the ^{1}H spin-lattice relaxation times, T_1. In the

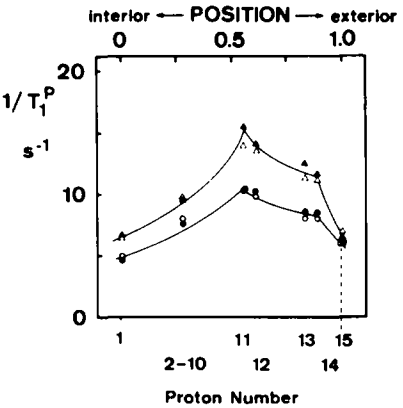

Fig. 5. Plots for two different spin labels of the paramagnetic contribution to the ^1H spin-lattice relaxation rates, $1/T_1^P$, for the ^1H-NMR lines of dodecylphosphocholine as a function of the positions of the different ^1H nuclei in the covalent structure of the detergent molecules. The measurements were performed at 360 MHz with $5 \cdot 10^{-2}$ M dodecylphosphocholine and $1 \cdot 10^{-3}$ M spin label in 0.05 M deuterated phosphate buffer at p^2H 7.0 and 37°C. Filled symbols: no glucagon present. Open symbols: $1 \cdot 10^{-3}$ M glucagon added. The spin labels are: (▲, △) 5-doxylstearate and (●, ○) 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine. The numbers at the bottom of the figure correspond to hydrogen atoms attached to the carbon nuclei shown in the molecular structure of dodecylphosphocholine in Fig. 3. Because these resonances were overlapped in the ^1H-NMR spectrum, methylene groups 2—10 have been plotted at an average position. The solid lines have been drawn to visualize the connectivities between the datum points obtained for a given spin label. The approximate positions of the hydrogen atoms relative to the micelle surface indicated at the top of the figure have been taken to be the same as those of the carbon atoms to which they are attached (see legend to Fig. 4 and the Discussion and Appendix).

absence of spin label, all of the ^1H resonances had T_1 values between 0.6 and 0.8 s, with the exception of methyl group 1, where T_1 was 1.3 s. Some selectivity for different protons could be observed for the paramagnetic contributions to the spin-lattice relaxation times, $1/T_1^P$, (Fig. 5). For both 5-doxylstearate and 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine, the largest values of $1/T_1^P$ corresponded to the methylene groups 11 and 12 (Fig. 5). This would be consistent with location of the nitroxide moieties of these spin labels near the phosphate group of the detergent (see the Appendix). Fig. 5 also shows that addition of glucagon caused little change in the plots of $1/T_1^P$ versus position, thereby further confirming that the presence of bound glucagon causes no major change in the location within the micelle of the spin label nitroxide moiety.

^1H-NMR studies of glucagon in mixed deuterated dodecylphosphocholine/glucagon micelles and mixed spin label/deuterated dodecylphosphocholine/glucagon micelles

Fig. 6A shows the 6.8—8.0 ppm region of the ^1H-NMR spectrum obtained in ^2H_2O for glucagon bound to deuterated dodecylphosphocholine micelles. Since all labile protons were pre-exchanged with deuterium, this spectrum contains only resonances from the non-labile protons of the aromatic rings present in glucagon. Many of the resonances in the spectrum shown in Fig. 6A have previously been assigned to specific protons in the polypeptide

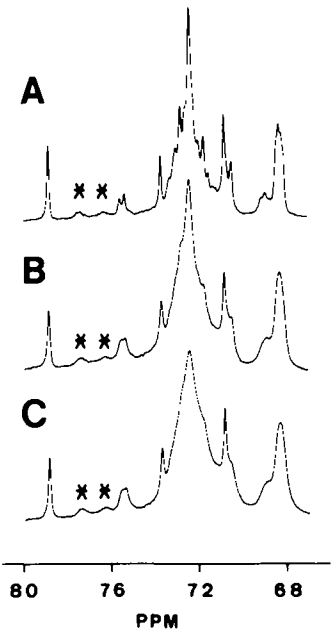

Fig. 6. Spectral region from 6.8 to 8.0 ppm of the 360 MHz ^{1}H-NMR spectrum obtained for $1 \cdot 10^{-3}$ M glucagon and $5 \cdot 10^{-2}$ M [$^2H_{38}$]dodecylphosphocholine in 0.05 M phosphate buffer at p^{2}H 7.0 and 37°C in the presence and absence of spin label. (A) No spin label. (B) $3.2 \cdot 10^{-4}$ M 5-doxylstearate. (C) $3.2 \cdot 10^{-4}$ M 16-doxylstearate. Since all labile polypeptide protons were pre-exchanged with deuterium, all the resonances in the figure correspond to protons of the aromatic rings present in glucagon. The two weak lines marked by asteriks correspond to impurities.

covalent structure [2]. Fig. 6B and C shows the spectra obtained for micelle-bound glucagon in the presence of 5-doxylstearate or 16-doxylstearate, respectively, where on the average 0.3 spin label molecules were bound per micelle. Two important features are apparent in the spectra of Fig. 6. Firstly, although the presence of the spin labels caused broadening of the glucagon resonances, no change in the chemical shifts was observed. Since this was also the case for a large number of assigned resonances in the spectral regions of the aliphatic side chains and the polypeptide backbone, the ^{1}H-NMR spectra imply that the presence of the spin label has little or no influence on the conformation of micelle-bound glucagon. Secondly, the spin labels broaden the glucagon resonances to various extents. This is clearly seen, e.g., for the singlet resonance of the C2H proton of His-1 at 7.88 ppm and the C7H doublet of the indole ring of Trp-25 at 7.56 ppm (Fig. 6). For those assigned resonances which are well resolved in the ^{1}H-NMR spectrum of micelle-bound glucagon, Table II summarizes the line broadening effects observed in the presence of the different spin labels. In view of the evidence presented in the previous section for different locations within the micelle of the nitroxide moieties of the different spin labels, the results in Table II suggest rather different positions within the micelle for different hydrogen atoms of micelle-bound glucagon (see Discussion).

TABLE II

QUALITATIVE SURVEY OF PARAMAGNETIC LINE BROADENINGS FOR THE ^{1}H-NMR RESONANCES OF GLUCAGON IN MIXED SPIN LABEL/DEUTERATED DODECYLPHOSPHOCHOLINE/GLUCAGON MICELLES

Measurements were made with $1 \cdot 10^{-3}$ M glucagon, $5 \cdot 10^{-2}$ M [$^2H_{38}$]dodecylphosphocholine and $3.2 \cdot 10^{-4}$ M spin label in $5 \cdot 10^{-2}$ M phosphate buffer at p^{2}H 7.0 and 37°C.

Residue *	Relative line broadening for different spin labels			
	Category I: little broadening for all spin labels	Category II: greatest broadening for 5-doxylstearate or 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphospho-piperidine	Category III: roughly equal broadening for all spin labels	Category IV: greatest broadening for 12-doxylstearate or 16-doxylstearate
His-1	ring C2H, C4H			
Thr-I (5 or 7)		γCH_3		
Thr-II (5 or 7)		γCH_3		
Tyr-I, II (10 and 13)			ring C3H, C5H	
Tyr-II (10 or 13)			ring C2H, C6H	
Lys-12	ϵCH_2			
Leu-14				$\delta_{1,2}$ CH_3
Arg-17	δCH_2			
Arg-18	δCH_2			
Ala-19			βCH_3	
Val-23			αCH, $\gamma_2 CH_3$	
Trp-25		ring C2H	ring C6H, C7H	
Leu-26				$\delta_{1,2}$ CH_3
Met-27				ϵCH_3
Thr-29	γCH_3			

* Resonance assignments taken from Ref. 2.

Discussion

The present EPR studies of spin labels and the measurements of paramagnetic effects on the ^{1}H and ^{13}C relaxation rates for dodecylphosphocholine served to characterize further the multimolecular species formed in aqueous solutions containing dodecylphosphocholine and glucagon [3]. Previous investigations with various physico-chemical methods had shown that at detergent concentrations above the critical micelle concentration, mixed micelles of approx. 40 dodecylphosphocholine molecules and one glucagon molecule with a molecular weight of approx. 17 000 were formed [3]. The present spectroscopic data provided direct evidence that the glucagon-dodecylphosphocholine complex can be regarded as a polypeptide bound to a micelle with a typical micellar organization. While the EPR spectra showed that the different spin labels used in these studies are incorporated into the micelles, both the NMR relaxation measurements (Figs. 4 and 5) and the EPR parameters (Table I) indicated on the one hand that the spin labels caused no appreciable perturbation of the organization of the glucagon/dodecylphosphocholine micelles and on the other hand that the presence of glucagon had very little influence on the environment or location within the micelle of the nitroxide moieties of the different micelle-bound spin labels. A further

important result of these experiments (Fig. 2) was that they demonstrated that micellar species with the above stoichiometry also prevailed at the relatively high concentrations used for high-resolution ^{1}H-NMR studies of the micelle-bound glucagon [1—4] (Fig. 6).

Since the lipid spin labels were incorporated into the mixed dodecylphosphocholine/glucagon micelles without noticeable perturbations of the micellar organization, studies of paramagnetic relaxation effects in the ^{1}H-NMR spectrum of micelle-bound glucagon seemed a viable way to characterize the location of the polypeptide chain relative to the micelle surface. In Table II, individually assigned ^{1}H-NMR lines of micelle-bound glucagon are divided into four categories on the basis of the paramagnetic line broadening observed with the different spin labels. Several resonances showed only weak line broadening from all four different spin labels (category I, Table II), indicating that none of the spin labels could closely approach the corresponding polypeptide protons. Since the spin labels were incorporated into the micelles, this saturation could arise either for protons which are external to the micelle or for protons which are in the interior of a compact, globular polypeptide structure formed within the micelle. Since earlier ^{1}H-NMR studies had shown that the overall structure of micelle-bound glucagon is extended rather than compact and globular [2,3], the latter explanation can be excluded and it therefore appears that the resonances which show weak line broadening for all four different spin labels must be ascribed to protons which are mainly exterior to the micelle. For His-1, Lys-12 and Thr-29 a position mainly exterior to the micelle, and therefore exposed to the aqueous solution, was also indicated by the observation that the pK_a values for these residues in micelle-bound glucagon correspond to those for an extended polypeptide chain in aqueous solution [2]. On the basis of the experimental results (Figs. 4 and 5) and theoretical considerations (Appendix), the protons grouped into categories II—IV (Table II) must be located increasingly deeper in the micelle interior. The peripheral methyl groups of Leu-14, Leu-26 and Met-27 would thus be among the polypeptide fragments which are most deeply immersed in the lipid micelle. Overall, however, since the present experiments showed that the peripheral atoms of the side chains of His-1, Lys-12, Arg-17, Arg-18 and Thr-29 are all exterior to the micelle, it appears that the polypeptide chains runs roughly parallel to the micelle surface with different segments immersed at somewhat different depths, but that glucagon does not really penetrate through the interior of the dodecylphosphocholine micelle.

Fig. 7 shows a schematic drawing for the orientation of the glucagon fragment 18—29 relative to the micelle surface. The segment 19—27 has the conformation previously obtained from ^{1}H-NMR studies [4] and was oriented to obtain the best possible consistency with the data of Table II. Qualitative agreement with the data of Table II was obtained for all amino acid residues expect Val-23 which appears in category IV in the figure rather than in category III (Table II). While no further data on the conformations of Arg-18 and Thr-29 are available, location of the peripheral groups of these side chains on the micelle surface provided additional constraints on the depth of immersion of the segment 19—27 into the micelle. Fig. 7 illustrates for the C-terminal region the general qualitative picture of micelle-bound glucagon

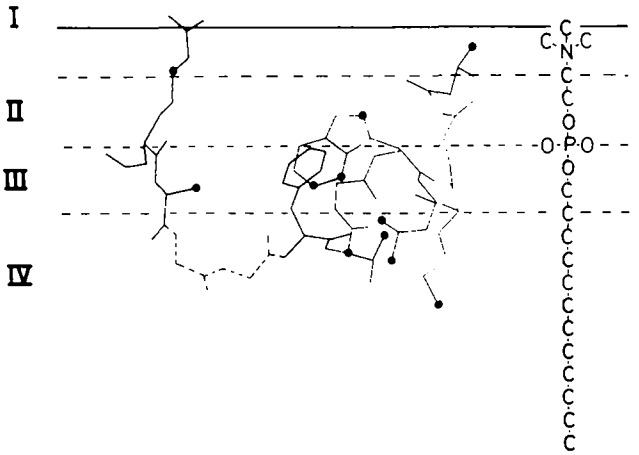

Fig. 7. Schematic presentation of the orientation with respect to the micelle surface of residues 18—29 of glucagon bound to dodecylphosphocholine micelles. The conformation for residues 19—27 obtained from previous ^{1}H-NMR studies [4] was oriented to provide the best possible consistency with the data in Table II. Residues 18, 28 and 29 have been given conformations which allow the peripheral protons of residues 18 and 29 to be near the micelle surface. To give a scale of approximate locations relative to the micelle surface, a fully extended dodecylphosphocholine molecule, with a reduced length of 18 Å rather than 25 Å, is also shown. The reduced length corresponds to the approximate radius of the dodecylphosphocholine/glucagon micelle determined previously [2]. The micelle surface is indicated by the solid line. For the sequence : Arg-18—Ala-19—Gln-20—Asp-21—Phe-22—Val-23—Gln-24—Trp-25—Leu-26—Met-27—Asn-28—Thr-29—OH, all heavy atoms are shown except for the side chains of Gln-20, Asp-21, Gln-24 and Asn-28, for which ^{1}H-NMR resonance assignments have not yet been obtained. For residues 20 and 21, where no direct conformational information was obtained either from previous nuclear Overhauser enhancement measurements [2—4] or from the present experiments, the backbone is drawn with broken lines. For those ^{1}H-NMR resonances where the measured line-broadening effects (Table II) were used to orient the polypeptide chain, the corresponding carbon atoms are indicated by the filled circles. The locations of nuclei in categories I—IV (Table II) are indicated by the dashed lines and the Roman numerals at the left.

which emerged from the present study, i.e., that the polypeptide backbone is mainly parallel to the micelle surface, with the polar side chains oriented preferentially towards the lipid/water interface and the apolar side chains mainly into the interior of the micelle.

Appendix

For a rigid molecule containing a paramagnetic center, the dipole-dipole contribution from the paramagnetic species to the nuclear spin-lattice or spin-spin relaxation rates, i.e., $1/T_1^p$ and $1/T_2^p$, respectively, is given by [21]:

$$\frac{1}{T_{1,2}^p} = f_{1,2}(\omega_S \omega_N \tau) \cdot \frac{1}{r^6} \qquad \text{(A1)}$$

where f_1 and f_2 are functions of the Larmor frequencies for the electron and the nucleus, ω_S and ω_N, and the correlation time τ for the electron-nucleus interactions, and r is the electron-nuclear separation. In the present experiments, diffusion of the polypeptide, detergent and spin label within the micelle will lead to averaging of the distance r. Assuming that the frequency for modulation of the distance r is slow compared to the nuclear Larmor frequency,

but rapid compared to the nuclear relaxation rate, then leads to the equations:

$$\frac{1}{T^{P}_{1,2}} = f_{1,2}(\omega_S \omega_N \tau) \cdot \left\langle \frac{1}{r^6} \right\rangle \tag{A2}$$

where $\langle 1/r^6 \rangle$ denotes the time average over all possible electron-nuclear separations within the micelle. Provided that in the presence of a given spin label all nuclei within the micellar complex have the same value of τ, Eqn. A2 can be used to obtain relative values of the expectation value $\langle 1/r^6 \rangle$ for different nuclei from measurements of $1/T^{P}_{1}$ or $1/T^{P}_{2}$.

To show that the present measurements are qualitatively consistent with the form of Eqn. A2 and the assumption that different nuclei have very similar τ values, we have calculated the values of $\langle 1/r^6 \rangle$ for a model in which it is assumed that the micelle is spherical and that the unpaired electron and the nucleus diffuse on the surface of concentric spheres with radii less than or equal to the micelle radius. It is then straightforward to show that:

$$\left\langle \frac{1}{r^6} \right\rangle = \frac{r_S^2 + r_N^2}{(r_S^2 - r_N^2)^4} \quad \text{if } |r_S - r_N| \geqslant d \tag{A3}$$

$$\left\langle \frac{1}{r^6} \right\rangle = \frac{1}{8 r_S r_N} \left[\frac{1}{d^4} - \frac{1}{(r_S + r_N)^4} \right] \quad \text{if } |r_S - r_N| < d,\ r_S \text{ and } r_N \neq 0$$

where r_S and r_N are, respectively, the radii of the spheres on which the electron and the nucleus diffuse and d is the minimum possible separation between the unpaired electron and the nucleus. It should be noted that in this simple model the center of micelle cannot simultaneously be occupied by the unpaired electron and a nucleus.

With the above assumptions, plots of the relative values of the paramagnetic relaxation rates, $1/T^{P}_{1}$ or $1/T^{P}_{2}$, for various possible positions of the unpaired electron and the nuclei are shown in Fig. 8. The experimental points shown are the line-broadenings measured for the dodecylphosphocholine ^{13}C nuclei in the presence of 5-doxylstearate normalized to a maximum line broadening of 12 Hz. Comparison of the experimental points and the calculated curve B (Fig. 8) indicates that the above model for the paramagnetic effects in the mixed spin label/dodecylphosphocholine micelles reproduces qualitatively the characteristics of the experimental observations. This interpretation then suggests further that within the micelle the unpaired electron of 5-doxylstearate is located near $r_N = 0.7$, which is the location of the phosphorus atom in the fully extended dodecylphosphocholine molecule. Although the spherical model is not entirely adequate for describing the paramagnetic effects when the spin label is near the center of the micelle, the curves in Fig. 8 provide justification for locating the unpaired electrons of 16-doxylstearate and 12-doxylstearate near the micelle center (Figs. 4, 5 and 8). It may be noted that the curves in Fig. 8 also suggest that the major reason that the paramagnetic effects observed for 5-doxylstearate or 1-oxyl-2,2,6,6-tetramethyl-4-dodecylphosphopiperidine (Figs. 4 and 5) do not show greater selectivity is that the minimum possible separation, d, is appreciable compared to the size of the micelle.

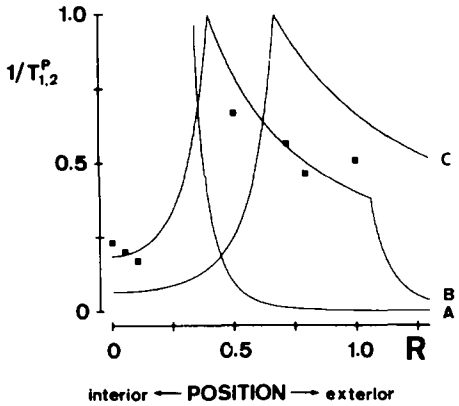

Fig. 8. For three different positions of the unpaired electron within a model spherical micelle (see text), plots are shown of the relative paramagnetic contributions to nuclear spin-lattice or spin-spin relaxation rates, $1/T_1^p$ or $1/T_2^p$, as a function of the location of different nuclei. The ordinate shows the radial nuclear positions, R, for a spherical micelle in which detergent molecules are oriented along radii of the sphere and in which $R = 1$ corresponds to the length of the detergent molecules. The curves have been extended to values of $R > 1$ to accomodate the possibility that nuclei in a micelle-bound polypeptide may be external to the detergent micelle. For each curve the abscissa has been normalized to unity for the radial location which shows the largest paramagnetic contribution to nuclear relaxation rates. The radial location of the unpaired electron, r_s, for the individual curves is: (A) $r_s = 0.01$, (B) $r_s = 0.73$, (C) $r_s = 1.0$. The curves were calculated according to Eqn. A2 and A3 using a normalized value of 0.33 for the minimum possible electron-nucleus distance, d, i.e., for a micelle radius of 18 Å, d would correspond to 6 Å. For ^{13}C nuclei of dodecylphosphocholine, this value gave the best fit to the experimental data (see below) and is consistent with measurements on space-filling models. The filled squares represent experimental points for the paramagnetic line broadening of the ^{13}C resonances of dodecylphosphocholine in the presence of 5-doxylstearate normalized to a maximum line broadening of 12 Hz. The approximate radial positions of the different ^{13}C nuclei were determined by assuming that the detergent molecules are fully extended along the radial direction. Note that for the spherical model (see text) the center of the micelle cannot simultaneously be occupied by the unpaired electron and a nucleus.

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References

1 Brown, L.R. (1979) Biochim. Biophys. Acta 557, 135—148
2 Bösch, C., Brown, L.R. and Wüthrich, K. (1980) Biochim. Biophys. Acta 603, 298—312
3 Wüthrich, K., Bösch, C. and Brown, L.R. (1980) Biochem. Biophys. Res. Commun. 95, 1504—1509
4 Braun, W., Bösch, C., Brown, L.R., Gō, N. and Wüthrich, K. (1981) Biochim. Biophys. Acta 667, 377—396
5 Lauterwein, J., Bösch, C., Brown, L.R. and Wüthrich, K. (1979) Biochim. Biophys. Acta 556, 244—264
6 Stone, R.J., Buckman, T., Nordio, P.L. and McConnell, H.M. (1965) Proc. Natl. Acad. Sci. U.S.A 54, 1010—1017
7 Cannon, B., Polnaszek, C.F., Butler, K.W., Eriksson, L.E.G. and Smith, I.C.P. (1975) Arch. Biochem Biophys. 167, 505—518
8 De Marco, A. (1977) J. Magn. Reson. 26, 527—528
9 De Marco, A. and Wüthrich, K. (1976) J. Magn. Resonance 24, 201—204
10 Wagner, G., Wüthrich, K. and Tschesche, H. (1978) Eur. J. Biochem. 86, 67—76

11 Aneja, R. and Chadha, J.S. (1971) Biochim. Biophys. Acta 248, 455—457
12 LaMar, G.N., Horrocks, W.DeW., Jr and Holm, R.H. (1973) NMR of Paramagnetic Molecules, Academic Press, New York
13 Wüthrich, K. (1976) NMR in Biological Research: Peptides and Proteins, North-Holland, Amsterdam
14 Kwan, C.L., Atik, S. and Singer, L.A. (1978) J. Am. Chem. Soc. 100, 4783—4786
15 Griffith, O.H. and Jost, P.C. (1976) in Spin Labeling — Theory and Applications (Berliner, L.J., ed.), chapter 12, Academic Press, New York
16 Schreier, S., Ernandes, J.R., Cuccovia, I. and Chaimovitch, H. (1978) J. Magn. Resonance 30, 283—298
17 Waggoner, A.S., Keith, A.D. and Griffith, O.H. (1968) J. Phys. Chem. 72, 4129—4132
18 Yoshioka, H. (1979) J. Am. Chem. Soc. 101, 28—32
19 Birdsall, N.J.M., Feeney, J., Lee, A.G., Levine, Y.K. and Metcalfe, J.C. (1972) J. Chem. Soc. Perkin II, 1441—1445
20 Roberts, J.D., Weigart, F.J., Kroschwitz, J.I. and Reich, H.J. (1970) J. Am. Chem. Soc. 92, 1338—1347
21 Solomon, I. (1955) Phys. Rev. 99, 559—565