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SPIN LABEL PARTITIONING IN LIPID VESICLES

A MODEL STUDY FOR DRUG ENCAPSULATION

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Summary

In this paper we attempt to outline some features which determine the encapsulation of small molecules into lipid vesicles. Spin labels derived from five carboxylic acids of different lengths were synthesized and incorporated in varying amounts into multilamellar and unilamellar vesicles made up of four different phosphatidylcholines. The influence on the release process of the bilayer rigidity and of the hydrophobicity of the entrapped molecule was systematically studied. The hydrophobicity is of critical importance and was estimated by measuring the partition coefficient (P) between octanol and buffer. In multilamellar vesicles, molecules characterized by extreme P values ($\log P < -0.3$ and $\log P > 5$) can be efficiently entrapped. The rate of leakage is related to the P value according to a bell-shaped curve. Moreover, gel state of the bilayer and long acyl chains of the lipids are properties which favor a good entrapment.

Small unilamellar vesicles may be formed in the presence of high concentra-

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Abbreviations: DMPC, dimyristoyl-DL-α-phosphatidylcholine; DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DSPC, distearoyl-DL-α-phosphatidylcholine; Tempocholine, *N,N*-dimethyl-*N*-(2′,2′,6′,6′-tetramethyl-4′piperidinyl-1′-oxyl)-2-hydroxyethylammonium; C_2, 4-acetamido-2,2,6,6-tetramethylpiperidine-1-oxyl; C_4, 4′-butyramido-2,2,6,6-tetramethylpiperidine-1-oxyl; C_8, 4-capramido-2,2,6,6-tetramethylpiperidine-1-oxyl; C_{12}, 4-lauramido-2,2,6,6-tetramethylpiperidine-1-oxyl; C_{16}, 4-palmitamido-2,2,6,6-tetramethylpiperidine-1-oxyl; ESR, electron spin resonance; T_c, transition temperature of the bilayer crystalline-liquid crystalline phase transition; P, partition coefficient in the system octanol/buffer.

tions of hydrophilic and lipophilic spin labels. However, the formation of unilamellar vesicles produces a significant reduction of the internal volume and of the entrapped water-soluble spin labels. High fractions of lipid-soluble spin labels can be incorporated in unilamellar vesicles but the vesicle stability is diminished.

Introduction

The use of lipid vesicles as carriers has led to some interesting results in recent years [1—6]. In some cases, the vesicles were merely used as probe carriers but the main emphasis concerned the entrapment of therapeutic agents since this new technique should provide the possibility of delivering suitable drugs to specific targets without harmful side effects [3—6].

This implies that the following should be controlled experimentally: (1) the physico-chemical conditions required for a stable entrapment and (2) the parameters which determine a specific interaction between the loaded vesicles and a living tissue.

Many studies have been devoted to an approach of these two problems. With respect to the entrapment problem, various drugs have been tested but it seems rather difficult to correlate the data available at present not only because an analysis of the drug chemical properties determining the entrapment process is usually missing, but also because the lipid composition as well as the technique of preparation of the vesicles vary greatly from paper to paper.

It was felt that a systematic approach should be undertaken in order to determine some general encapsulation conditions and this paper reports on such a study. The study only deals with small molecules (M_r = 213—410), since the entrapment of large molecules such as proteins has been successfully achieved [7—10,34]. We have used nonelectrolyte molecules which differ essentially by their hydrophilic-hydrophobic balance. In order to follow the dynamics of these molecules in the vesicles, the molecules were spin-labeled since the corresponding ESR spectra yield valuable information regarding the probe environment.

The vesicles were made from four different phosphatidylcholines and the influences of the vesicle size, fluidity and temperature were investigated in some detail.

Materials and Methods

Materials. L-α-Phosphatidylcholine from egg yolk (type VII-E), dimyristoyl-DL-α-phosphatidylcholine (DMPC), dipalmitoyl-DL-α-phosphatidylcholine (DPPC), distearoyl-DL-α-phosphatidylcholine (DSPC), caprylic, lauric and palmitic acids were purchased from Sigmal Chemical Co. Butyric anhydride and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl were obtained from Aldrich. Acetic anhydride was a product of Merck. 5-Doxyl-stearic acid was purchased from Syva, Palo Alto, CA, U.S.A. These chemicals were used without further purification. All reagents and solvents were proanalysi products.

Synthesis of spin labels. Tempocholine chloride was prepared and purified

according to the method of Kornberg and McConnell [11]. The other spin labels studied were derived from 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl, with this saturated carboxylic acids of different lengths were reacted. The spin label 4-palmitamido-2,2,6,6-tetramethylpiperidine-1-oxyl (C_{16} derivative) was prepared and purified according to Rozantsev [12]. The other spin labels, i.e., 4-lauramido-2,2,6,6-tetramethylpiperidine-1-oxyl (C_{12} derivative), 4-capramido-2,2,6,6-tetramethylpiperidine-1-oxyl (C_8 derivative), 4-butyramido-2,2,6,6-tetramethylpiperidine-1-oxyl (C_4 derivative) and 4-acetamido-2,2,6,6-tetramethylpiperidine-1-oxyl (C_2 derivative) were obtained by reaction of the corresponding acid anhydrides with 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl. Lauric and caprylic anhydrides were synthesized and purified according to Wallace and Copenhaver [13]. Spin labels were purified by column chromatography on Al_2O_3 using $CHCl_3$ as eluent and recrystallized from diethyl ether (C_2 and C_4 derivative), hexane (C_8 derivative) or a mixture of both (C_{12} derivative).

Preparation of multilamellar vesicles and small unilamellar vesicles. Lipids were dissolved in $CHCl_3$ in a spherical flask. The solutions were evaporated to dryness with a rotary evaporator at 30°C (water bath) and further dried under vacuum overnight. Lipophilic spin labels (C_8, C_{12}, and C_{16} derivatives) were dissolved in $CHCl_3$ and mixed with the lipid prior to evaporation. Hydrophilic spin labels (tempocholine, C_2 and C_4 derivatives) were incorporated into the liposomes by dissolving them in buffer (100 mM Tris-HCl (pH 7.4), 0.9% NaCl). Multilamellar vesicles were obtained by mechanical stirring (Vortex mixer) of the lipid film in buffer at temperatures above the corresponding lipid phase transition (T_c). Small unilamellar vesicles were obtained by sonication of the multilamellar vesicles (Branson sonifier B12). During sonication the temperature was kept above T_c.

Chromatography. The multilamellar vesicles containing the encapsulated spin labels were separated from free labels by rapid filtration on a short PD 10 column (Pharmacia, 1.5 × 5 cm) loaded with Sephadex G-25 M and equilibrated with buffer.

ESR measurements. They were made with a Varian E3 spectrometer. The temperature was regulated with a Varian variable temperature controller. The samples were introduced in 50-μl Drummond microcapillaries. Spectra were usually recorded with a modulation amplitude of 1 G and a microwave power of 4 mW.

Permeability measurements. After chromatography on a PD 10 column (3—4 min), the multilamellar vesicles suspension was homogenized and incubated at the required temperature. After various time intervals 100-μl aliquots were cooled in melting ice and all subsequent operations were performed with samples and reagents at 0°C. At this temperature, the vesicle membrane is impermeable to ascorbate [11]. 20-μl aliquots of 0.35 M ascorbate [11,14] were added to the sample and 20 μl buffer added to the controls. The samples and controls were then transferred to the microcapillaries and ESR spectra were recorded at 0°C. Ascorbate reduces the nitroxide spin label to the non-paramagnetic hydroxylamine and the rate of decay of the height of the low field line of the spin probe spectrum was used to monitor the kinetics of the release of the spin labels [14].

Effects of sonication on the amount of tempocholine trapped within the

vesicles. 8 mg/ml DPPC were dispersed in 10^{-2} M Tempocholine in buffer and sonicated. After various sonication times, duplicate 50-μl aliquots were cooled in an ice/water bath and further treated as for permeability measurements. Reduction of the h_{+1} amplitude as a function of the sonication duration gives a measurement of the changes of the internal volume.

Results

The partition coefficient (P) of the spin labels between octanol and buffer was used as a measurement of their hydrophilic-lipophilic balance. The values corresponding to C_2 and C_4 derivatives were directly measured. From these data, the contribution of the N—O˙ group to the molecular hydrophobicity could be estimated in the Rekker formalism [15]. This latter result was utilised subsequently in order to evaluate the partition coefficient corresponding to Tempocholine, C_8, C_{12} and C_{16} derivatives. The P values of the molecules studied are given in Table I. In this report, the labels will be arbitrarily classified into two groups: the so-called water-soluble (C_2 and C_4 derivatives) and the lipid-soluble (C_8, C_{12} and C_{16} derivatives). Tempocholine, which is known to be essentially water-soluble ($\log P < -2.66$) will serve as a reference.

a) Partitioning of spin labels in multilamellar vesicles

Some typical spectra corresponding to the six spin labels incorporated in DPPC multilamellar vesicles appeared in Fig. 1. The spectra A and B were recorded at 0°C in presence of sodium ascorbate.

Ascorbate treatment ensures that the radicals solubilized in the aqueous external phase of the multilamellar vesicles are chemically reduced. Consequently, the remaining components arise from label molecules sequestered in the vesicle interior, i.e. in the aqueous and the lipid phase.

Going from tempocholine to C_2 derivative and from C_2 to C_4 derivative (Fig. 1A), the line width increase can be attributed to a partial incorporation of the label in the lipid phase. This results from: (1) an anisotropic motion of the label; (2) a modification of the polarity of the label environment affecting the hyperfine splitting constant [16].

These effects increase with P as shown for the C_8 derivative ascorbate-treated sample (spectrum B). The spectrum results from the superposition of a narrow triplet due to C_8 derivative located in the vesicles inner aqueous phases on a broad underlying singlet (- - - - -) attributed to high local concentrations of C_8 derivative in the lipid phase.

It should be underlined here that the effect produced by ascorbate on labels incorporated in the lipid phase is less than 10%. Indeed, it has been proved that in multilamellar vesicles, the lipid fraction which is in contact with the extra vesicular buffer varies between 5 and 8% [17]. Moreover, we measured the ascorbate reduction of the 5-doxyl-stearic acid incorporated in multilamellar vesicles of DPPC. We observed a reduction less than 5% during the time course of our measurements (6 min) indicating a negligible penetration of ascorbate in the vesicles external bilayer under our experimental conditions.

Lipid-soluble molecules such as C_{12} derivative and C_{16} derivative ($\log P > 5$) are essentially entrapped in the lipid bilayers; indeed, it can be seen in Fig. 1

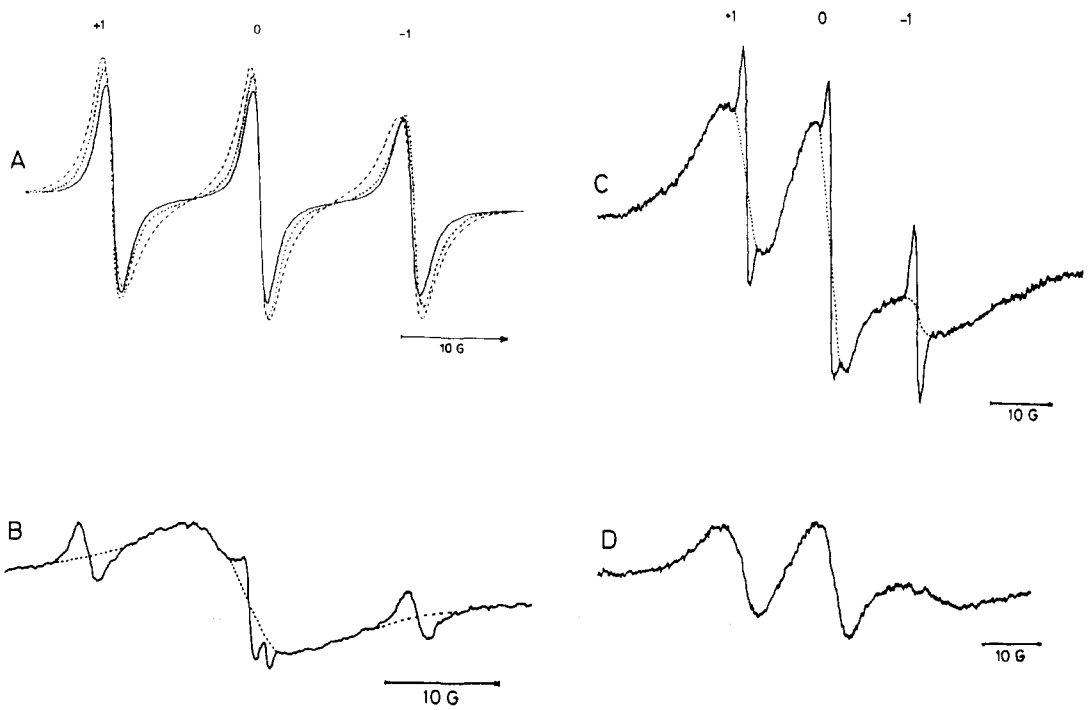


Fig. 1. ESR spectra of the spin labels incorporated in multilamellar vesicles of DPPC. (A) Tempocholine, (———); C_2 derivative, (· · · · · ·); and C_4 derivative, (- - - - - -). 50 μl of vesicles suspensions (20 mg DPPC/ml, $2.5 \cdot 10^{-2}$ spin label) +20 μl 0.35 M sodium ascorbate at 0°C, gain = $8 \cdot 10^4$. (B) 20 mol% C_8 derivative after chromatography on a PD-10 column and ascorbate treated at 0°C, gain = $6.2 \cdot 10^5$. Lipid concentration, 20 mg DPPC/ml. (C) 5 mol% C_{12} derivative. The sharp triple †1, 0, —1 corresponds to C_{12} derivative in the aqueous phase. Lipid concentration, 10 mg DPPC/ml. Temperature, 25°C, gain = $5 \cdot 10^5$. (D) 5 mol% C_{16} derivative. Lipid concentration, 10 mg DPPC/ml. Temperature, 25°C, gain = $3.2 \cdot 10^5$.

that the spectra corresponding to unchromatographed suspensions of DPPC vesicles exhibit little (C_{12} derivative spectrum C) or no (C_{16} derivative spectrum D) aqueous component. Moreover, high speed centrifugation confirmed that most (C_{12} derivative) or all (C_{16} derivative) labels are incorporated in vesicles as judged from ESR spectra of both vesicles and supernatants. This conclusion is valuable for all concentrations tested in the range of 1—40%. For C_{12} derivative and C_{16} derivative, an increase in label concentration produces the coalescence of the sharp triplet into a broad singlet due to radical-radical interaction. A quantitative analysis of the molecular organization of the C_{16} derivative molecules in the DPPC matrix membrane will appear elsewhere [18].

A study of the release of the entrapped spin labels indicate that the partition coefficient determines the rate of leakage out of the vesicles. This is shown in Fig. 2 which compares the reduction time course of the h_{+1} lines of multilamellar vesicles of DPPC loaded respectively with Tempocholine, C_2 derivative and C_4 derivative. The vesicles are incubated at 23°C for various periods of time and then treated with sodium ascorbate at 0°C. The line height reduction measures the release of the spin labels from the vesicle interior. Both C_2 and C_4 derivatives are in equilibrium between the membrane and the inner aqueous

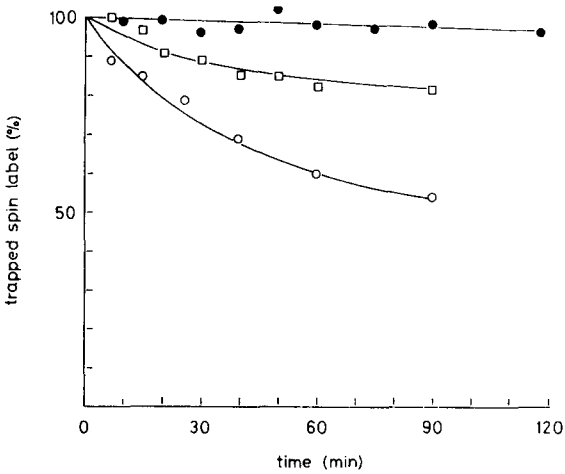
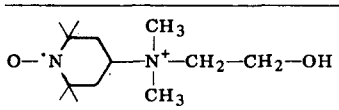
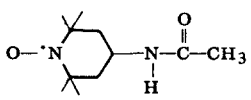
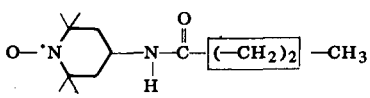
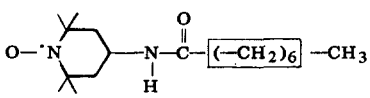
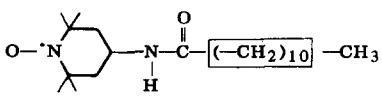
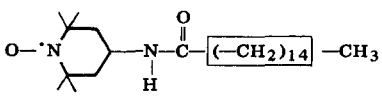


Fig. 2. Release time course from DPPC-multilamellar vesicles. Effect of the spin label hydrophobicity. Temperature, 23°C. Tempocholine (●); C_2 derivative, (□), and C_4 derivative, (○).

phase [16] but C_4 derivative which is characterized by a larger P value than C_2 derivative (Table I) solubilizes more freely in the lipid phase. The results indicate that a small P value favors a good maintenance of the trapped molecules within the vesicles [19], but even after 90 min 50% of the C_4 derivative

TABLE I

STRUCTURES AND PARTITION COEFFICIENTS OF THE SPIN LABELS

Spin label	Abbreviation	log P(octanol/buffer)
	Tempocholine	<—2.66 **
	C_2 derivative	0.52 *
	C_4 derivative	1.35 *
	C_8 derivative	3.36 **
	C_{12} derivative	5.47 **
	C_{16} derivative	7.57 **

* Measured values.
** Calculated values.

molecules remain encapsulated. The influence of the lipid chain length on the leakage process is shown in Fig. 3. At 23°C, DPPC and DSPC are in the gel state. It can be seen that the DSPC vesicles which are much further below their transition temperature than the DPPC vesicles retain more efficiently the C_4 derivatives molecules.

At the same temperature, the release of C_4 derivative from DMPC multilamellar vesicles (not shown) occurs so fast that after an incubation time of 6 min an ascorbate treatment abolishes completely the spectrum. This temperature is very close to the corresponding lipid phase transition [14]. Our observation confirms previous findings that the membrane permeability is drastically increased at this critical temperature [14,20]. Experiments were performed with egg phosphatidylcholine at 23° C and with DMPC at 37° C, i.e., at temperatures above the corresponding T_c. Results indicate that in both cases the C_4 derivative release is very fast. It should be stressed, however, that even below T_c the temperature has a profound effect on the membrane permeability. Fig. 3 compares the release of C_4 derivative from DSPC multilamellar vesicles at 23 and 37°C. The two systems are in the gel state since the DSPC phase transition occurs at 58°C [21]. It can be seen that a 14°C increase accelerates 3-fold the C_4 derivative release after 100 min. Similar results were obtained with DPPC at 4 and 23°C. It can be concluded that the maintenance of a given molecule in multilamellar vesicles is favored by lipids of long saturated acyl chains, by low temperature and by a lipid matrix in the gel state.

The C_8 derivative molecules, although essentially soluble in the lipids and only slightly soluble in water, tend to diffuse from the liposomal membrane into the buffer until reaching an equilibrium which depends both upon P and on V_L/V_W [22]. This is due to the volume of the water phase (V_W) largely exceeding the corresponding volume of the lipid phase (V_L) (50—500-fold). With respect to molecules characterized by intermediate P values, i.e., when the molecules are soluble both in buffer and lipid, the entrapment seems thus difficult to achieve.

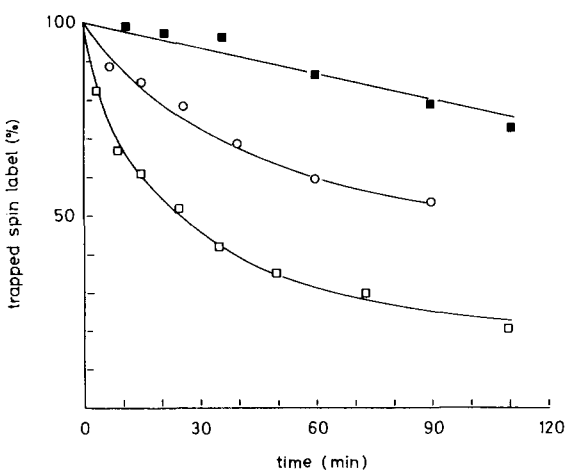


Fig. 3. Release time course of C_4 from multilamellar vesicles. Effect of temperature and of fatty acid chain length of liposomes. DSPC, 23° C, (■); DPPC, 23° C, (○); DSPC, 37° C, (□).

Fig. 4 gives a general description of the influence of *P* since it describes the release of the entrapped molecules from DPPC multilamellar vesicles as a function of the partition coefficient. Measurements were performed after a 60 min incubation time at 23°C.

b) Partitioning of spin labels in small unilamellar vesicles

These vesicles have only been sporadically tested as possible drug carriers [23], but by contrast they have been used extensively in the study of liposome-cell interactions [2,23].

A straightforward situation is encountered when the drug and the lipid molecules are in weak interaction. Such a situation characterizes molecules with very small *P* values such as Tempocholine which does not diffuse through lipid bilayers [1,11,14]. Consequently, such molecules can be entrapped without difficulty in unilamellar vesicles [1,11,14]. However, the use of small vesicles is somewhat disadvantageous as compared to multilamellar vesicles, relating to the volume to the internal aqueous phase [23,24].

Under our experimental conditions, the internal phase of the DPPC multilamellar vesicles measured from a comparison of the amplitudes of the Tempocholine ESR spectra, before and after ascorbate treatment corresponds to 0.92 l/mol DPPC and this value drops to 0.23 after sonication. These values are in agreement with data previously reported [25,26] and are worth considering in relation to the use of these vesicles as drug carriers. It has been demonstrated in the previous section that these molecules characterized by a log *P* value between −0.3 and 5 tend to diffuse rapidly from the interior of the vesicles to the external medium, although in multilamellar vesicles, the molecules have to cross several lipid layers. Obviously, unilamellar vesicles would be even more disadvantageous for entrapping these molecules.

We then examined the entrapment of lipid-soluble molecules in small unilamellar vesicles. Fig. 5 depicts the chromatogram after passage through a Sepharose 4B column of a sonicated DPPC suspension containing 40 mol% of C_{12}

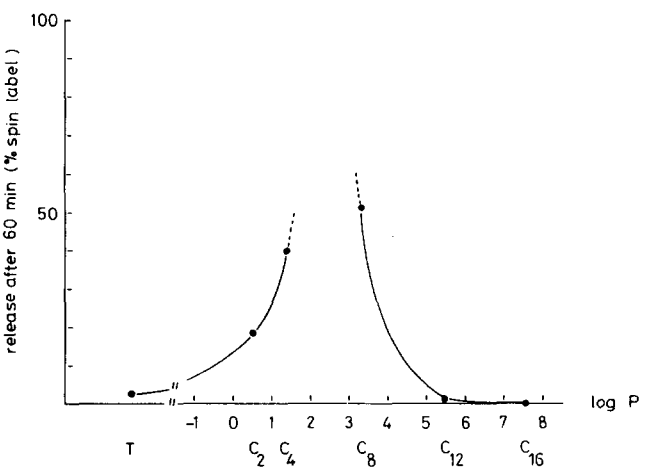


Fig. 4. Percent release of the spin labels from DPPC multilamellar vesicles after 1 h incubation time at 23° C. Effect of the partition coefficient *P* of the labels.

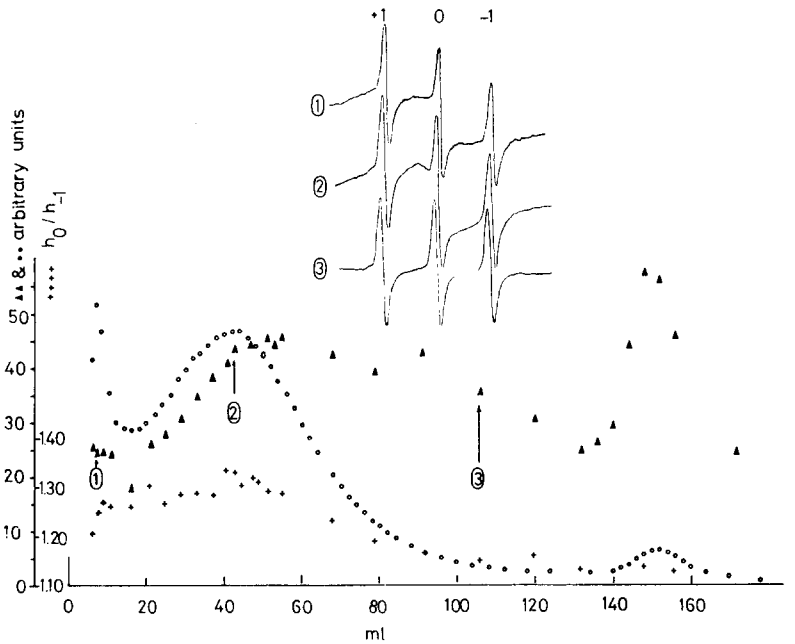


Fig. 5. Sepharose 4B chromatogram of DPPC unilamellar vesicles (10 mg/ml) sonicated for 1 h in presence of 40 mol% of C_{12} derivative. ○, absorbance at 254 nm given in arbitrary units. ▲, amplitude profile of the high field line of the ESR spectra; arbitrary unit. +, profile corresponding to h_0/h_{-1} ratio. The inset shows typical spectra associated with multilamellar vesicles fractions (①, gain = $4 \cdot 10^5$); unilamellar vesicles fractions (②, gain = $2.5 \cdot 10^5$); and fractions without vesicles (③, gain = $2.5 \cdot 10^5$).

derivative. This graph demonstrates that it is possible to form small unilamellar vesicles under these experimental conditions. Indeed, the absorbance measurements at 254 nm reveal the presence of three peaks in the elution profile: the first one (6—12 ml) corresponds to multilamellar vesicles, the second one (25—70 ml) to unilaminellar vesicles, and the third one (140—160 ml) to free lipid and free label molecules. This profile is in agreement with classical published data [27].

Besides the absorbance measurements, the ESR spectra corresponding to each fraction collected were also recorded. Some typical spectra are shown in Fig. 5 (inset). Spectra 1 and 2 are composed of a superposition of two components: one corresponds to C_{12} derivative in buffer and the other to C_{12} derivative in the lipid phase. The third spectrum of the inset corresponds to C_{12} derivative in the aqueous phase only (no vesicles in these fractions). In this last case the h_0/h_{-1} ratio is constant and equals 1.12 under our experimental conditions. In the presence of lipids, however, the h_0/h_{-1} amplitude varies and can be used to detect the presence of C_{12} derivative in the lipid phase of the vesicles. Indeed, the partitioning of C_{12} derivative in the bilayer produces a broad singlet in the center of the spectra and this influences the amplitude of the middle field line. It can be seen in Fig. 5 that the profile of h_0/h_{-1} is similar to the one corresponding to the absorbance measurements. In contrast, the profile which describes the amplitude variations of the h_{-1} lines reveals a significant discrepancy with respect to the liposome elution profile. This parameter

measures mainly the presence of label in buffer. After elution of the unilamellar vesicles, the concentration of C_{12} derivative remains high in the subsequent fractions. The tail of the profile indicates that during chromatograpphy the label is released from the membrane and is subsequently retarded on the gel.

The stability of unilamellar vesicles formed in the presence of lipophilic spin labels is drastically diminished. It was found that, at a given label concentration, the bilayer instability is linearly related to the logarithm of P (Defrise-Quertain, F., Chatelain, P., Ruysschaert, J.M. and Delmelle, M., unpublished results).

Discussion

The most favorable conditions for the maintenance of a substance in the carrier correspond to extremely low or extremely high P values (Fig. 4). Indeed, the strong hydrophilic tempcholine derivative ($\log P < -2.66$) leaks at a very low rate from multilamellar vesicles maintained at a temperature below T_c, while soluble molecules characterized by $\log P$ values ranging between -0.3 and 1.70 (C_2 derivative, C_4 derivative) tend to flow out rapidly. The release process can, however, be reduced by using multilamellar vesicles composed of lipids of long saturated acyl chains, maintained in the gel state.

The use of Tempocholine also allowed ut to demonstrate that very short periods of sonication are sufficient for forming small unilamellar vesicles and consequently reducing up to 4-fold the internal water volume of the DPPC vesicles. It seems reasonable to claim that the use of small unilamellar vesicles would be suitable only for water soluble molecules with $\log P$ less than -0.3. This finding explains the successful entrapment of metothrexate ($\log P = -1.85$) [28] and cytosine arabinoside ($\log P = -0.79$) [28] in small unilamellar vesicles [29,30]. It has to be kept in mind, however, that the use of small unilamellar vesicles supposes that the drug can be dissolved at a high concentration in buffer during vesicle formation.

Incorporation of molecules characterized by intermediate P values ($1.70 < \log P < 4$) corresponds to a very unfavorable situation (Fig. 4). Indeed, the substance is poorly soluble in water and must therefore be mixed with the lipids during the vesicle preparation procedure. By adding the buffer, however, a redistribution of the molecules occurs between the two phases as a result of the solubility in lipids and in water.

To the best of our knowledge, drugs with intermediate P values have been successfully entrapped in liposomes only when they form a molecular complex with at least one constituant of the lipid matrix. This explains why actinomycin D ($\log P = 1.96$) and vinblastine ($\log P = 1.97$) could be trapped and maintained in vesicles [24,30]. When specific interactions are absent, molecules with intermediate P value diffuse continuously out of the carrier.

With lipid soluble molecules such as C_{12} derivative and C_{16} derivative ($\log P > 5$), the degree of incorporation in liposomes is virtually complete and time independent. We feel that the most promising results concern the entrapment of those molecules but surprisingly very few compounds of this type have actually been tested. One exception concerns cholesterol which has been used to modulate the cell membrane fluidity through its transfer from vesicles [23].

High P values which ensure a successful entrapment might be obtained by modifying the chemical structure of the drugs, provided that the therapeutic potential remains unaltered. Some promising results have recently been reported with hydrocortisone and two modified derivatives; hydrocortisone itself has an intermediate P value ($0.59 < \log P < 1.93$) [28] and hence cannot be efficiently entrapped [31]. A higher drug incorporation and retention were obtained with esters of hydrocortisone such as octanoate ($\log P = 4.75$) [28] and palmitate ($\log P \simeq 9$). The hydrocarbon chains anchor the corticosteroid in the liposome bilayer [31,32]. It should be mentioned, however, that high P values do not necessarily guarantee a successful incorporation into a bilayer. Indeed, the use of the partition coefficient between octanol and buffer does not take into account the anisotropic properties of the bilayer structure. Squalene, for example, is characterized by a high P value although it cannot be solubilized in the acyl chains because of steric hindrance [33].

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