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EXCITATION ENERGY TRANSFER FROM TRYPTOPHAN RESIDUES OF PEPTIDES AND INTRINSIC PROTEINS TO DIPHENYLHEXATRIENE IN PHOSPHOLIPID VESICLES AND BIOLOGICAL MEMBRANES

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An efficient excitation energy transfer from tryptophan residues of intrinsic membrane proteins to an extrinsic fluorescent probe (diphenylhexatriene) has been demonstrated in rat erythrocyte ghosts. To correlate this transfer with the localization of the probe, a model system has been investigated. It consists of peptides containing lysine and tryptophan residues bound to negatively charged phosphatidylserine vesicles. Absorption and fluorescence spectroscopies were used to follow peptide binding and diphenylhexatriene incorporation. Peptide binding is accompanied by a blue shift of the tryptophan fluorescence together with an increase of the quantum yield and of the fluorescence decay time. An experimental Föster critical distance value of 4.0 nm was found for energy transfer from tryptophan residues of peptides to diphenylhexatriene which approaches the range of calculated values (3.1–3.7 nm) using a two-dimensional model. These results demonstrate that efficient energy transfer can occur from tryptophan residues of intrinsic proteins to diphenylhexatriene without any interaction between diphenylhexatriene and proteins in biological membranes.

Introduction

In a recent study, we have investigated membrane alterations linked to hypertension in spontaneously hypertensive rats [1–3]. Diphenylhexatriene fluorescence polarization was used to monitor these alterations. In the course of these studies we observed an efficient energy transfer from the tryptophan residues of intrinsic proteins to 1,6-diphenyl-1,3,5-hexatriene. This raised the question of whether diphenylhexatriene was bound to the hydrophobic core of membrane proteins or located along the phospholipid chains in the middle of the bilayer.

Theoretical and experimental studies of energy transfer between randomly and non-randomly distributed donors and acceptors in bidimensional structures have been carried out [4–10]. Protein-phospholipid interactions in membranes have been investigated using energy transfer to fluorescent probes such as *n*-anthroyloxy stearic acids [4,11], parinaric acid isomers [12] and trinitrophenyl or dansyl groups attached to phospholipids [13].

In the present paper we have investigated (i) energy transfer from tryptophan residues of intrinsic membrane proteins to diphenylhexatriene in erythrocyte ghosts, (ii) the binding of oligopeptides containing lysine and tryptophan residues to negatively charged phosphatidylserine vesicles and (iii) energy transfer processes between tryptophan

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residues of peptides and diphenylhexatriene. The Föster critical distance derived from experimental measurements (4.0 nm) was found to be close to the calculated value (3.4 nm). These results are discussed in relation to the localization of diphenylhexatriene with respect to membrane proteins.

Materials and Methods

Materials. Lysyl-tryptophanyl-methyl ester (Lys-Trp-OMe) was a gift of Dr. Mayer (Centre de Biophysique Moléculaire, Orléans); lysyl-tryptophanyl-lysine acetate (Lys-Trp-Lys), lysyl-tryptophanyl-glycyl-lysyl-tertiobutyl ester (Lys-Trp-Gly-Lys-OtBu), lysyl-glycyl-tryptophanyl-lysyl-tertiobutyl ester (Lys-Gly-Trp-Lys-OtBu) were purchased from Bachem, 1,6-diphenyl-1,3,5-hexatriene was obtained from Aldrich and phosphatidylserine (PS) from Lipid Products, Nutfield (U.K.). All solutions were prepared in a degased standard buffer: 1 mM NaCl/1 mM sodium cacodylate/0.2 mM EDTA, pH 7.0. Stock solutions of peptides were made with the above buffer; diphenylhexatriene was dissolved in tetrahydrofuran (Merck spectroscopic grade).

Preparation of membranes and vesicles. PS vesicles were prepared under nitrogen flow by sonication of suspensions of phospholipids in the above buffer. The sonication was performed in a glass tube surrounded by a water bath maintained at 20°C for 20 min [10 times (1 min sonication + 1 min stand-by)] to avoid local overheating. After sonication, the vesicle solution was centrifuged at $10\,000 \times g$ for 30 min at 20°C. Using a ^{3}H-radiolabelled phosphatidylcholine, the total phospholipid loss in this vesicle preparation was found to be less than 10%. Erythrocyte ghosts from Wistar rats were prepared as previously reported in a 5 mM phosphate buffer, pH 8, following the method of Steck and Kant [14]. Diphenylhexatriene was dissolved in tetrahydrofuran ($2 \cdot 10^{-3}$ M) and then mixed with buffer to obtain a final concentration of $2 \cdot 10^{-6}$ M. Diphenylhexatriene labelling was obtained by mixing an equal volume of the $2 \cdot 10^{-6}$ M dispersion in buffer with a suspension of plasma membranes ($7 \cdot 10^{-8}$ mg protein/ml).

Absorption and fluorescence measurements. Absorption spectra were recorded with an Uvikon 820 spectrophotometer (Kontron). Fluorescence spectra were recorded on a FICA 55 differential spectrofluorimeter or an Aminco SPF 500 spectrofluorimeter. Sample bleaching was avoided by using narrow excitation slits (1 nm). Solution absorbance was maintained below 0.05 for $\lambda_{exc} = 280$ nm to minimise the inner filter effect. All spectroscopic measurements were carried out at 20°C. Fluorescence polarization was obtained with an Elscint fluoropolarimeter model MV-1a.

Fluorescence decays were measured at 20°C using a single-photon counting nanosecond spectrofluorometer, model 199 M (Edinburgh Instruments). The excitation flash lamp was filled with hydrogen (0.5 B) and run at 8 kV and 50 kHz. Full width at half maximum was 1.2 ns. Excitation and emission wavelengths were 280 and 340 nm (band width: 11 nm).

Energy transfer measurements. Typical energy transfer experiments were carried out in a 1×1 cm fluorescence cuvette containing 2 ml buffer solution and a glass coated magnet stirring bar. The solution contained appropriate concentrations of vesicles and peptide (see Fig. 9 for details). The lipid to peptide ratio R_i (R_i = moles of PS (on the basis of an average molecular weight of 811 per phospholipid)/mole of peptide) was kept constant and equal to 20. Diphenylhexatriene in tetrahydrofuran solution was added with a 2-μl glass micropipet under stirring to favour maximum contact of diphenylhexatriene molecules and vesicles. Spectroscopic measurements were then performed after 5 min equilibration. Final concentrations of tetrahydrofuran were kept below 0.5% (v/v). Fluorescence decays of the peptide bound to vesicles ($R_i = 50$) were recorded in the absence and in the presence of increasing amounts of diphenylhexatriene. In each case, counting was stopped when 10 000 counts were accumulated at the maximum channel. After each experiment, the emission decay from vesicles labelled with the same amount of diphenylhexatriene but without peptide was recorded under identical conditions for the same time duration as in the presence of peptide. The decays were then subtracted to correct for the contribution of diphenylhexatriene-labelled vesicles to the peptide decay.

Experimental data were exploited following the formalism proposed by Fung and Stryer [5] for the two-dimensional Förster's theory of fluorescence

energy transfer. The distance R_0 (in m) at which the rate of excitation energy transfer between the donor and acceptor is equal to the sum of the rates of all other modes of deactivation is given by Eqn. 1:

$$R_0(\mathrm{m}) = 9.79 \cdot 10^{-6} \left(J K^2 Q_0 n^{-4} \right)^{1/6} \quad (1)$$

where J is the spectral overlap integral in $\mathrm{M}^{-1}\ \mathrm{m}^3$, K^2 the dipole-dipole orientation factor, Q_0 the quantum yield of the donor in the absence of the acceptor and n the refractive index of the intervening medium. Energy transfer efficiency (ET) was calculated from the reduction of the donor emission according to Eqn. 2

$$\mathrm{ET} = 1 - (Q_A / Q_0) = 1 - (\tau_A / \tau_0) \quad (2)$$

where Q_A and τ_A are the quantum yield and the fluorescence lifetime of the donor in the presence of the acceptor, respectively, and τ_0 the fluorescence lifetime of the donor in the absence of the acceptor.

Plotting ET versus the acceptor surface density σ expressed as moles of acceptor per mole of phospholipid, makes it possible to determine an experimental value of R_0 using theoretical curves calculated by Fung and Stryer [5].

Results

1. Energy transfer from tryptophan residues of intrinsic membrane proteins to diphenylhexatriene in rat erythrocyte ghosts

Adding diphenylhexatriene to erythrocyte ghosts leads to a partial quenching of the tryptophan fluorescence of intrinsic proteins excited at 280 nm. The fluorescence from incorporated diphenylhexatriene is observed at wavelengths longer than tryptophan fluorescence. Only diphenylhexatriene incorporated in the membrane emits fluorescence; diphenylhexatriene remaining in the buffer does not fluoresce appreciably. At 20°C the fluorescence lifetimes for diphenylhexatriene in tetrahydrofuran solution and in the erythrocyte ghosts are 8.8 ns and 10.4 ns, respectively (data not shown). Assuming that the ratio of fluorescence quantum yields and lifetimes are identical in these two environments, the effective concentration of diphenylhexatriene incorporated in the membrane can be evaluated from a comparison of fluorescence intensities of diphenylhexatriene-labelled membranes excited at 365 nm with tetrahydrofuran solutions of known diphenylhexatriene concentration. Thus in the experiment reported in Fig. 1 we determined that a concentration of $4 \cdot 10^{-9}$ M diphenylhexatriene was incorporated in the membrane.

To determine whether energy transfer was taking place from tryptophan residues to diphenylhexatriene the fluorescence excitation spectrum of diphenylhexatriene-labelled erythrocyte ghosts was compared with that of a $4 \cdot 10^{-9}$ M diphenylhexatriene solution in tetrahydrofuran for an emission wavelength of 430 nm. At this wavelength, the more emissive species is diphenylhexatriene but tryptophan residues still contribute to the fluorescence emission (Fig. 1a). As demonstrated on excitation spectra (Fig. 1c') a marked contribution of tryptophan excitation to diphenylhexatriene emission measured at 430 nm was observed even when the tryptophan contribution of intrinsic proteins was subtracted. It is then possible to estimate an equivalent diphenylhexatriene concentration in tetrahydrofuran which would be required to give the same fluorescence intensity measured at 430 nm under excitation at 280 nm: a $5 \cdot 10^{-8}$ M value was found. The excitation coefficients of diphenylhexatriene and tryptophan at 280 nm are 3000 and 5500, respectively. Therefore the ghost system behaves as if an equivalent tryptophan concentration (C_{Trp}) of about $2.5 \cdot 10^{-8}$ M had completely transferred its excitation energy to diphenylhexatriene ($\varepsilon^{280}_{\mathrm{Trp}} \cdot C_{\mathrm{Trp}} = \varepsilon^{280}_{\mathrm{DPH}} \cdot C_{\mathrm{DPH}}$). This leads to a rough estimate ($2.5 \cdot 10^{-8} / 4 \cdot 10^{-9}$) of 6 to 7 tryptophans totally transferring their energy to one diphenylhexatriene molecule.

2. Energy transfer in model systems

Binding of peptides to phosphatidyl vesicles. Binding of several peptides containing lysine and tryptophan residues to PS vesicles was studied by absorption and emission spectroscopy. At pH 7.0 adding negatively charged PS vesicles to a solution of peptides resulted in a shift of the absorption spectrum to longer wavelengths together with a shift to shorter wavelengths of the emission spectrum. The tryptophan fluorescence quantum yield

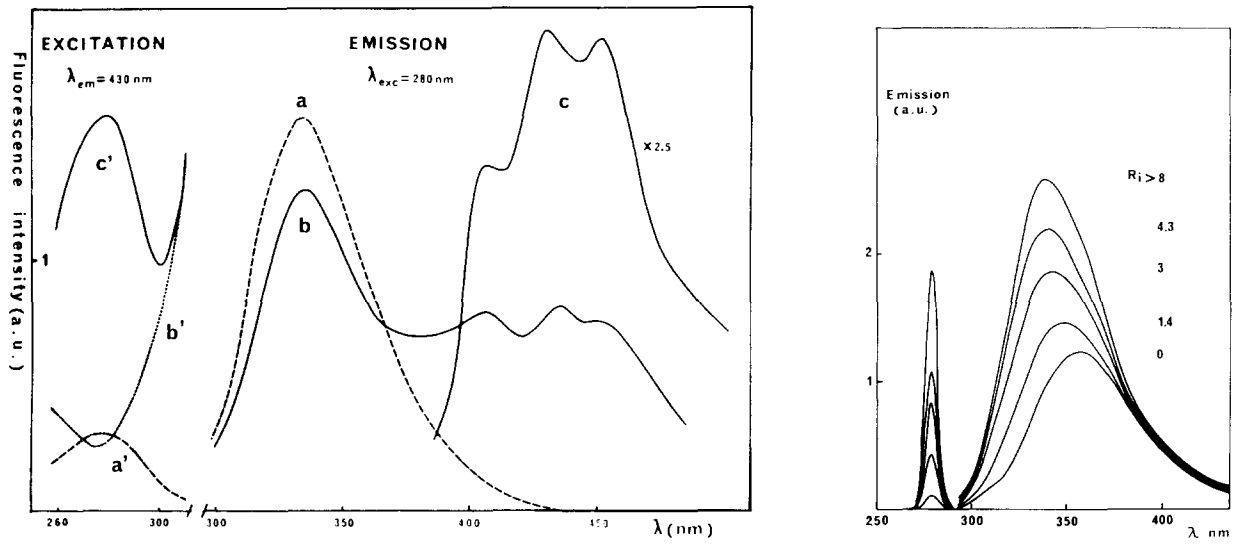

Fig. 1. Right side: Emission spectra recorded with an excitation wavelength of 280 nm at 20°C. (a) Spectrum from rat erythrocyte ghosts ($2 \cdot 10^{-8}$ mg protein/ml). (b) Spectrum from rat erythrocyte ghosts ($7 \cdot 10^{-8}$ mg protein/ml) labelled with diphenylhexatriene (10^{-6} M in buffer). (c) Difference emission spectrum recorded under the same conditions as (b) with erythrocyte ghosts in the reference beam. Left side: Excitation spectra recorded at an emission wavelength of 430 nm. (a') Spectrum from tryptophan residues of intrinsic proteins in rat erythrocyte ghosts. (b') Spectrum from a $4 \cdot 10^{-9}$ M diphenylhexatriene solution in tetrahydrofuran. (c') Spectrum from diphenylhexatriene-labelled erythrocyte ghosts with unlabelled erythrocyte ghosts in the reference beam at the same concentration. The tryptophan contribution at 430 nm was thus directly subtracted.

Fig. 2. Uncorrected emission spectra of Lys-Gly-Trp-Lys-OtBu in the absence ($R_i = 0$) and in the presence ($R_i > 0$) of increasing concentrations of PS vesicles in standard buffer pH 7. The peptide concentration was kept constant ($7.6 \cdot 10^{-6}$ M). Vesicles were added to the peptide solution and the mixture incubated at 20°C for 1 h before recording the spectra. Excitation conditions: $\lambda = 280$ nm: slit width = 1 nm. Scattering of the solutions at 280 nm was also recorded using a 40-times lower sensitivity of the recorder.

was found to be enhanced upon binding of the peptides to vesicles as shown on Fig. 2. Light scattering at the excitation wavelength increased linearly with vesicle concentration. In Fig. 3 are presented the binding curves for several peptides as a function of increasing vesicle concentration expressed as R_i, the ratio of phospholipid to peptide concentrations. The fluorescence intensity measured at a fixed wavelength (340 nm) increased linearly up to an R_i of approx. 5 and then reached a plateau. Extrapolation of the linear part of the binding curve (low R_i) allowed us to determine that the minimum number of lipids required for each bound peptide molecules was between 5 and 6. The fluorescence quantum yield enhancement was determined by comparison of the area of the fluorescence spectrum of the free peptides (S_f) with that of the bound peptide (S_b) at saturation i.e. in the plateau region ($R_i > 6$) taking into account the absorbance differences observed in absorption spectra (see below and Fig. 4). Different S_b/S_f values were found depending on the nature of the peptides. The highest quantum yield enhancement was found for Lys-Trp-OMe (2.36) whereas comparable values were found from Lys-Gly-Trp-Lys-OtBu (1.41), Lys-Trp-Gly-Lys-OtBu (1.50) and Lys-Trp-Lys (1.24). In the case of Lys-Gly-Trp-Lys-OtBu fluorescence lifetime measurements confirmed the quantum yield enhancement calculated above; the ratio of the lifetimes of the free and bound peptides was found to be 1.6.

Difference absorption spectra have been recorded for free and bound peptides (Fig. 4) showing a shift of 2 nm toward longer wavelengths for Lys-Gly-Trp-Lys-OtBu, Lys-Trp-Gly-Lys-OtBu and Lys-Trp-Lys and of 4 nm in the particular case of Lys-Trp-OMe. Consequently, a hyperchromism was observed in the 270–305 nm region for all bound peptides.

Adding KCl to the complexes formed at low

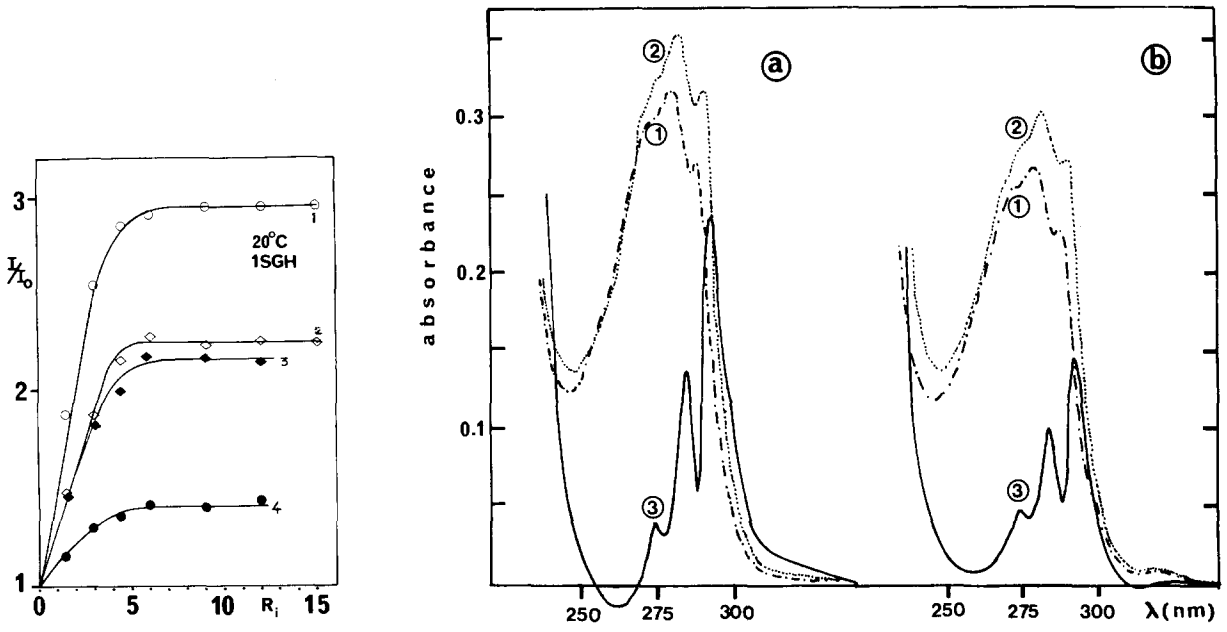

Fig. 3. Relative fluorescence intensity (I/I_0) at 20°C of several peptides measured at fixed wavelength ($\lambda = 337$ nm) versus R_i; I_0 is the fluorescence intensity of the peptide in the absence of vesicles and I that of the peptide in the presence of vesicles. Experimental points represent results of three independent measurements. 1, Lys-Trp-OMe; 2, Lys-Gly-Trp-Lys-OtBu; 3, Lys-Trp-Gly-Lys-OtBu; 4, Lys-Trp-Lys.

Fig. 4. Absorption spectra of (a) $5.7 \cdot 10^{-5}$ M Lys-Gly-Trp-Lys-OtBu and (b) $4.8 \cdot 10^{-5}$ M Lys-Trp-Lys in the presence (· · · · · ·) and in the absence (· – · – ·) of PS vesicles ($R_i = 20$). Difference spectra (———) are also shown in each case (scale multiplied by 2.5).

salt concentration shifted the fluorescence maximum to higher wavelengths, and decreased the fluorescence intensities showing that electrostatic interactions were involved in the binding process (results not shown). For Lys-Gly-Trp-Lys-OtBu we observed that half-dissociation occurred at 0.15 M KCl (pH 7.5) while Dufourcq et al. [15] have observed half dissociation at 0.3 M KCl in the same conditions for Lys-Trp-OMe. It should be noted that vesicles tend to aggregate upon adding monovalent ions. This process has been kinetically studied by Day et al. [16]. We followed vesicle aggregation by measuring the scattering peak intensity as a function of KCl concentration. The fluorescence decrease was measured in parallel and it was found that the fluorescence intensity reached a plateau which was higher than that expected for the free peptide (corresponding to a complete dissociation). These results seem to indicate that part of the peptide remained embedded in vesicle aggregates and was not accessible to potassium ions.

Incorporation of diphenylhexatriene in phosphatidylserine vesicles. An accurate determination of energy transfer parameters requires a precise knowledge of the surface density of diphenylhexatriene in the phospholipid vesicles. The concentration of diphenylhexatriene effectively incorporated into vesicles was determined from absorption and fluorescence spectra recorded at different diphenylhexatriene concentrations in solutions containing peptide-bound vesicles as shown in Figs. 5 and 6.

Absorption spectra for increasing concentrations of diphenylhexatriene added to vesicle solutions are shown in Fig. 5. Structured spectra were characterized by peak maxima at 340, 358 and 377 nm. Shifts of the peak maxima observed in different media are presented in Table I.

Molar extinction coefficients (ε_M) of diphenylhexatriene were found to be identical in tetrahydrofuran and in hexane. Except for the slight shifts observed for the peak positions, absorption spectra of diphenylhexatriene in tetrahydrofuran

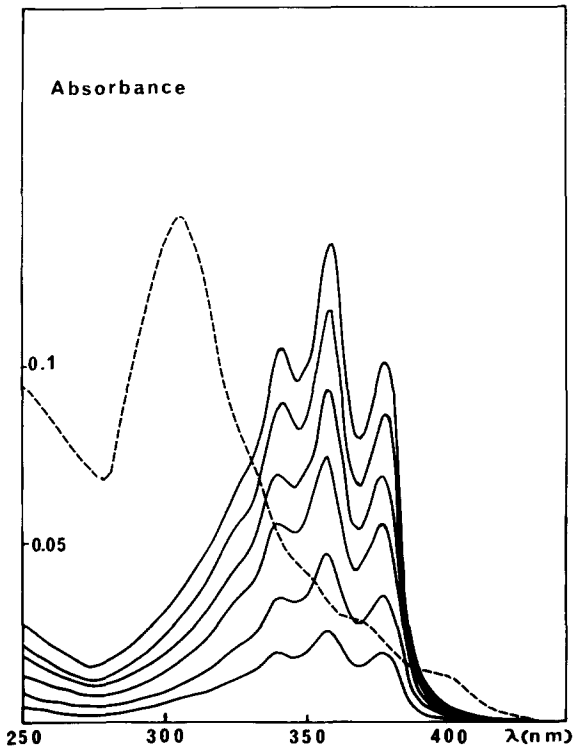

Fig. 5. Absorption spectra of dephenylhexatriene in the presence of Lys-Gly-Trp-Lys-OtBu-bound vesicles ([Lys-Gly-Trp-Lys-OtBu] = $7.6 \cdot 10^{-6}$ M, [PS] = $1.52 \cdot 10^{-4}$ M). A cuvette containing the same amounts of peptide bound to vesicles is used as reference to cancel out the peptide and vesicle absorptions. Volumes of 2-μl aliquots of diphenylhexatriene in tetrahydrofuran solution ($4.6 \cdot 10^{-4}$ M) were added to the peptide-vesicles solution (2 ml) under stirring. The dotted line represents the diphenylhexatriene absorption spectrum in the buffer, for an added concentration of $2.3 \cdot 10^{-6}$ M.

and in hexane were very similar to those observed in PS vesicles (see Table I). Electronic transitions occurring during the absorption process were then

TABLE I

ABSORPTION SPECTRAL DATA FOR DIPHENYLHEXATRIENE IN ORGANIC SOLVENTS AND IN PS VESICLES

ε_1, ε_2 and ε_3 are the extinction coefficients at the wavelengths of the three peaks. THF, tetrahydrofuran.

Medium	Absorption maxima (nm)			Relative absorbance	
	λ_1	λ_2	λ_3	$\varepsilon_1/\varepsilon_2$	$\varepsilon_3/\varepsilon_2$
Hexane	369.5	350.5	334.0	0.73	0.76
THF	374.5	355.0	338.5	0.75	0.74
PS vesicles	377	358	340	0.75	0.76

assumed to be similar in organic solvents and in the PS bilayer, and a value of 80000 $M^{-1} \cdot cm^{-1}$ was taken for ε_M at the absorption maximum (358 nm) of diphenylhexatriene spectrum in the presence of vesicles. The absorption spectrum of diphenylhexatriene in the buffer is also presented on Fig. 5 (dotted line). A well-defined maximum was observed at 305 nm, with less defined peaks in the 350–410 nm region. A long wavelength maximum is observed around 400 nm which is not present in the spectra obtained in organic solvents. Increasing the diphenylhexatriene concentration increased the absorption peak at 305 nm as well as the solution scattering. The scattered intensity of diphenylhexatriene in water was found to be 30-times greater than in tetrahydrofuran for the same diphenylhexatriene concentration (about $2 \cdot 10^{-6}$ M). Adding tetrahydrofuran to the aqueous solution converted instantaneously the 'aqueous' diphenylhexatriene spectrum to the 'organic' diphenylhexatriene spectrum (that found in pure tetrahydrofuran solution) and decreased simultaneously the scattering intensity. These results are likely due to the formation of aggregates by the hydrophobic diphenylhexatriene molecules in water. The same phenomenon was observed by Bolard et al. [17] with the polyene antibiotic amphotericin B in phospholipid vesicles.

However, the diphenylhexatriene concentrations measured from absorption spectra (Fig. 6a) were found to be less than those expected from the known concentration of the diphenylhexatriene solution added to the vesicle suspension. This is very likely due to a third form of diphenylhexatriene, that we called 'adsorbed' diphenylhexatriene, which was detected by emptying the cuvette and then washing the cell with the same volume (2 ml) of tetrahydrofuran. The amount of diphenylhexatriene adsorbed on the quartz walls at saturation was estimated to be around 10^{-7} mol/cm^2 of wall when the aqueous concentration of diphenylhexatriene varied from 1.5 to 5.5 μM. This corresponds approximately to the quantity of diphenylhexatriene outside the vesicles for added diphenylhexatriene concentration higher than $2 \cdot 10^{-6}$ M and a vesicle concentration of $1.52 \cdot 10^{-4}$ M.

Fluorescence spectra were recorded for each diphenylhexatriene addition using an excitation

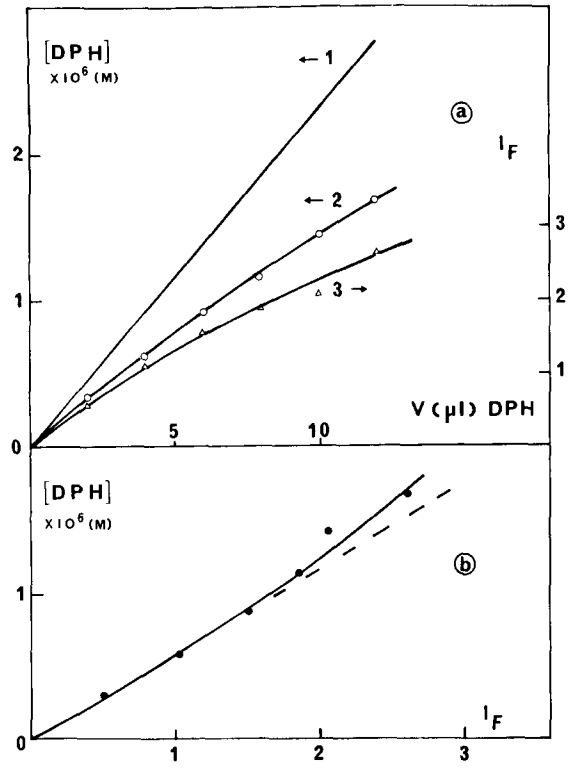

Fig. 6. Incorporation of diphenylhexatriene (DPH) in vesicles containing Lys-Gly-Trp-Lys-OtBu. Same conditions as in Fig. 5. (a) Curve 1: total diphenylhexatriene concentration in the vesicles solution; curve 2: incorporated diphenylhexatriene in vesicles as derived from absorption spectra (Fig. 5, using ε_M^{358} = 80000 $M^{-1} \cdot cm^{-1}$); curve 3: corresponding fluorescence intensities measured at 430 nm (λ_{exc} = 390 nm) versus added volumes of diphenylhexatriene solution in tetrahydrofuran. (b) Concentration of diphenylhexatriene incorporated into vesicles (from absorption spectra) versus diphenylhexatriene fluorescence intensity. The slope of the linear part of the calibration curve was used to calculate the true incorporated diphenylhexatriene concentration. For more details see text.

wavelength of 390 nm in order to selectively excite diphenylhexatriene and to avoid inner filter effects (the absorbance at this wavelength was less than 0.01). No detectable shift was observed in the fluorescence spectrum of diphenylhexatriene in the vesicles compared with that observed in tetrahydrofuran or hexane solutions. The relative intensities of the vibronic bands in the fluorescence spectrum slightly changed with the solvent. The fluorescence of diphenylhexatriene in water (excitation wavelength 305 nm) is rather weak, about 10^{+3} times lower than that of diphenylhexatriene in tetrahydrofuran. Therefore the fluorescence observed in mixtures of diphenylhexatriene and vesicles should correspond only to the diphenylhexatriene species incorporated into the vesicle bilayer. Fluorescence intensities were then plotted versus the added diphenylhexatriene concentrations (Fig. 6a, curve 3). As expected from what we observed in absorption measurements, the diphenylhexatriene fluorescence did not increase linearly with the added diphenylhexatriene concentration. Plotting fluorescence intensities versus diphenylhexatriene concentrations determined from absorption spectra showed that the fluorescence intensity of diphenylhexatriene varied linearly with 'inside' diphenylhexatriene concentration up to approx. 10^{-6} M (Fig. 6b). The curve deviates from linearity for higher concentrations. This result confirms that, for high diphenylhexatriene concentrations, the solution contains increasing amounts of non-incorporated diphenylhexatriene which contribute to the absorbance measured at 358 nm, but not to the fluorescence. The linear part of this calibration curve can be used to determine the concentration of incorporated diphenylhexatriene through fluorescence and absorption measurements.

Energy transfer measurements. Increasing diphenylhexatriene amounts were added to a cuvette containing a fixed concentrations of the peptide Lys-Gly-Trp-Lys-OtBu and of PS vesicles (R_i = 20). Incorporated diphenylhexatriene was determined as described above. Energy transfer from tryptophan to diphenylhexatriene was demonstrated by the quenching of tryptophan fluorescence and the sensitization of diphenylhexatriene fluorescence (about 5 times) upon excitation at 280 nm (Fig. 7). Further evidence for energy transfer was provided by the appearance of the donor (tryptophan) contribution in the acceptor (diphenylhexatriene) excitation spectrum recorded at the acceptor emission maximum (430 nm) as shown on Fig. 8. Emission spectra (λ_{exc} = 280 nm) for different amounts of added diphenylhexatriene are presented in Fig. 9. An isoemissive point was observed at 385 nm. Diphenylhexatriene fluorescence spectra as displayed on Fig. 9 were found to be very close to those observed in tetrahydrofuran solution (see above). The emission spectra also show that the presence of diphenylhexatriene dis-

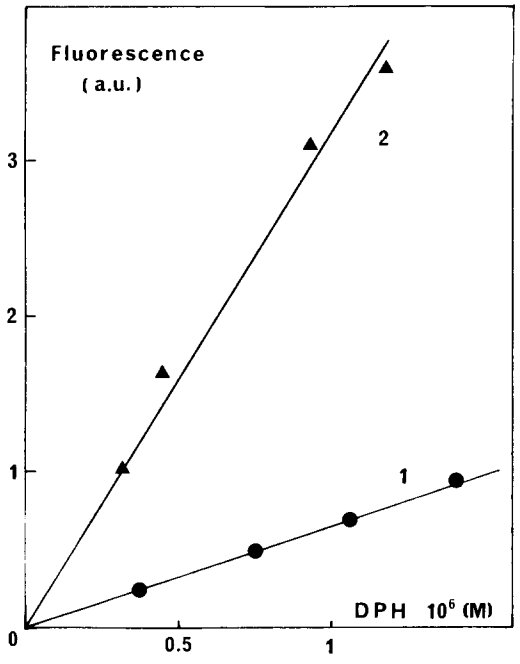

Fig. 7. Sensitized fluorescence at 430 nm of diphenylhexatriene (DPH) versus diphenylhexatriene concentrations in PS vesicles. Curve (1) represents the fluorescence of diphenylhexatriene excited at 280 nm when added to vesicles which did not contain the peptide. Curve (2) corresponds to the fluorescence of diphenylhexatriene upon excitation at 280 nm of both diphenylhexatriene and the peptide ([Lys-Gly-Trp-Lys-OtBu] = $7.6 \cdot 10^{-6}$ M; $R_i = 20$). The ratio of the slopes of the two straight lines is about 5 indicating an efficient energy transfer from tryptophan to diphenylhexatriene.

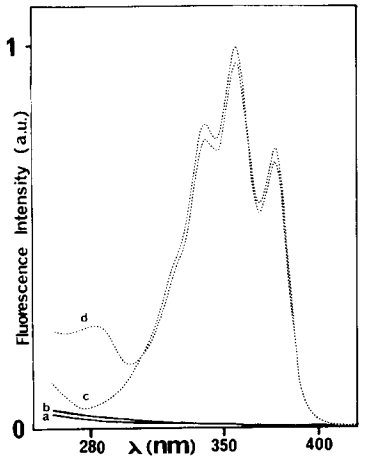

Fig. 8. Excitation spectra ($\lambda_{em} = 430$ nm) of solutions at 20°C in standard buffer of: (a) PS vesicles (PS stands for a concentration of 10^{-4} M in phospholipid); (b) PS + Lys-Gly-Trp-Lys-OtBu ($5 \cdot 10^{-6}$ M); (c) PS + diphenylhexatriene ($5 \cdot 10^{-7}$ M); (d) PS + Lys-Gly-Trp-Lys-OtBu ($5 \cdot 10^{-6}$ M) + diphenylhexatriene ($5 \cdot 10^{-7}$ M).

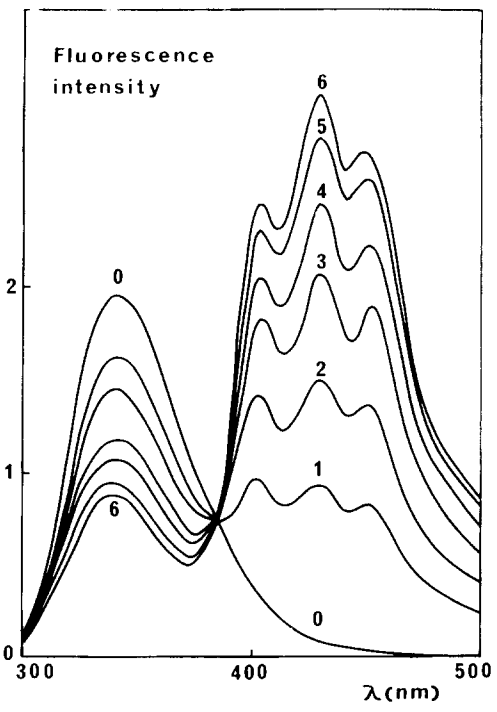

Fig. 9. Emission spectra showing energy transfer from Lys-Gly-Trp-Lys-OtBu to diphenylhexatriene (DPH) in PS vesicles as a function of incorporated diphenylhexatriene concentration into vesicles. Experimental conditions were: [PS] = $1.52 \cdot 10^{-4}$ M, $R_i = 20$, DPH/PS as follows 0 = no DPH; 1 = 0.0021; 2 = 0.0041; 3 = 0.0059; 4 = 0.007; 5 = 0.0078; 6 = 0.010. Samples were excited at λ = 280 nm, slit width = 1 nm at 20°C. Isoemissive point = 385 nm.

solved in the bilayer did not alter peptide binding as the fluorescence maximum (340 nm) was not shifted upon increasing diphenylhexatriene concentration (in the buffer the fluorescence maximum of the free peptide was observed at 355 nm).

Fluorescence decays were measured on solutions containing a ratio of phospholipid to peptide (Lys-Gly-Trp-Lys-OtBu) equal to 50 as a function of diphenylhexatriene concentration. As shown on Table II, all fluorescence decays can be accounted for the the superposition of two exponential functions. Binding of the peptide to phosphatidylserine vesicles increases the average lifetime by a factor 1.6 in agreement with the fluorescence quantum yield enhancement. Adding diphenylhexatriene to the peptide-vesicle complex leads to a shortening of both components of the tryptophan fluorescence decay. Within experimental errors, the re-

TABLE II

FLUORESCENCE LIFETIMES OF Lys-Gly-Trp-Lys-OtBu ($1.5 \cdot 10^{-6}$ M) FREE OR BOUND TO PS VESICLES ($R_i = 50$) IN THE ABSENCE AND IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF DIPHENYLHEXATRIENE (σ = [DPH]/[PS]) AT 20°C IN STANDARD BUFFER pH 7

The fluorescence decays were analyzed as $I(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$. The average lifetime $\langle\tau\rangle$ is defined as $\langle\tau\rangle = (a_1\tau_1 + a_2\tau_2)/(a_1 + a_2)$, χ is the sum of the squares of the weighted residuals. Energy transfer efficiencies (ET) were calculated as described in Materials and Methods.

Sample	σ ($\times 10^3$)	τ_1 (ns)	a_1	τ_2 (ns)	a_2	$\langle\tau\rangle$ (ns)	χ^2	ET
Free peptide	–	0.94	0.55	2.51	0.45	1.65	1.00	
Peptide bound to PS vesucles	0	1.88	0.68	4.52	0.32	2.72	1.25	0
	2.3	1.59	0.64	4.19	0.36	2.53	1.65	7.0
	4.6	1.21	0.71	3.57	0.29	1.89	1.96	30.5
	6.9	1.06	0.70	3.15	0.30	1.69	1.64	37.9
	8.2	0.98	0.70	2.92	0.30	1.56	1.94	42.6
	9.8	1.04	0.75	2.96	0.25	1.52	1.95	44.1

spective contributions of the two components remain constant. The decrease of the average lifetime is nearly identical to the decrease of the tryptophan fluorescence quantum yield measured under steady-state conditions (see above). Transfer efficiency (ET) was calculated as indicated under Materials and Methods from the quenching of the peptide fluorescence and plotted versus the surface density of acceptor σ = [DPH]/[PS]. The theoretical curves published by Fung and Stryer [5] were replotted as ET versus R_0 for different σ values. This made it possible to determine R_0 values from our experimental results as indicated on Fig. 10 and Table III.

A theoretical value of R_0 may be computed through Eqn. 1. From the corrected and normalised emission spectrum of the peptide bound to vesicles and the absorption spectrum of incorporated diphenylhexatriene the overlap integral was calculated to be $J = 7.4 \cdot 10^{-20}\ M^{-1} \cdot m^3$. The fluorescence quantum yield Q_f of the free peptide (Lys-Gly-Trp-Lys-OtBu) at 20°C was calculated by comparison with tryptophan as follows: $Q_f = \langle\tau_f\rangle Q_T / \langle\tau_T\rangle$; $\langle\tau_f\rangle$ and $\langle\tau_T\rangle$ are the peptide and tryptophan average lifetimes in water, respectively, and Q_T is the tryptophan fluorescence quantum yield in water. $\langle\tau_T\rangle = 3$ ns at pH 7, 20°C as determined by Gudgin et al. [19], $Q_T = 0.13$, value found by Chen [20] and $\langle\tau_f\rangle = 1.65$ ns, value measured by us for the free peptide in the absence

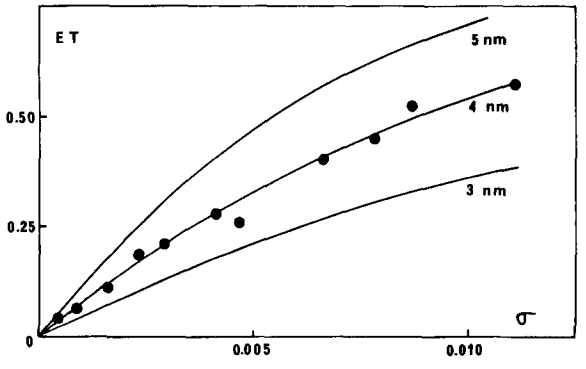

Fig. 10. Energy transfer efficiency $ET = 1 - (Q_A/Q_0)$ as a function of σ the surface density of energy acceptor. Lines correspond to calculated curves for $R_0 = 3$, 4, and 5 nm according to Fung and Stryer (1978). These calculations assumed that the closest distance of donor-acceptor approach was 0.84 nm. In our system the distance of closest approach between the tryptophan residue of the peptide and the diphenylhexatriene molecule is not likely to be shorter than the value used in the theoretical calculations.

TABLE III

R_0 VALUES DETERMINED FROM PLOTS OF ET VERSUS R_0 FOR DIFFERENT σ VALUES (see text)

$\sigma = \frac{[DPH](M)}{[PS](M)}$ ($\times 10^3$)	R_0 (nm)
2	3.6–3.8
4	3.8–4.0
6	3.9–4.1
8	3.9–4.1

of vesicles at 20°C in standard buffer, pH 7 (see Table II). Q_f was then calculated to be 0.072. The refractive index n was determined according to Sklar et al. [21] and found to be equal to 1.45. The fluorescence quantum yield of the bound peptide Q_b was then derived considering that $Q_b = Q_f \cdot (\tau_b/\tau_f) \cdot (\tau_f^r/\tau_b^r)$ where τ^r are the radiative lifetimes and subscripts f and b refer to the free and bound states of the peptide. As the peptide absorbance increases (approx. 10%) upon binding to vesicles as does the refractive index (1.45 in the lipidic phase instead of 1.33 in water) the ratio τ_f^r/τ_b^r was calculated to be equal to 1.32. As presented in Table II, the value of τ_b is 2.72 ns. This allowed us to compute a value of 0.16 for Q_b. Through Eqn. 1 a value of 3.4 nm was calculated for R_0. In this calculation the value of $\langle K^2 \rangle$ was assumed to be equal to 2/3. The variation of $\langle K^2 \rangle$ due to the restricted motion of the donor and acceptor during the donor lifetime has already been studied [18]. These calculations define the limits of variation of $\langle K^2 \rangle$ compatible with polarization data of the donor and acceptor. Measured values of peptide and diphenylhexatriene polarizations in vesicles were equal to 0.20 (λ_{exc} = 280 nm; λ_{em} = 342 nm) and 0.125 (λ_{exc} = 356 nm; λ_{em} = 430 nm) at 20°C, respectively. The maximum error limit in the evaluated distance between the two chromophores was thus estimated to be around 10% when $\langle K^2 \rangle$ was chosen as 2/3. This defines the actual range of variation of calculated R_0 values as 3.1–3.7 nm.

Fluorescence quenching of the donor in the presence of acceptor is presented on Fig. 10. The best fit of experimental data was obtained for a value of R_0 equal to 4.0 nm. The latter value is reasonably close to the above calculated R_0 values.

Discussion

The peptides used in this work were purposedly chosen for studies of electrostatic and hydrophobic interactions with the phospholipid bilayer. The carboxylic end of the peptide was blocked thus conferring a maximum number of positive charges. In the case of Lys-Gly-Trp-Lys-OtBu for example the presence of a tryptophan residue together with the glycine residue and the terminal tertiobutyl group confer hydrophobic properties to the peptide. Hydrophobic interactions involving tryptophan were evidenced by a shift to shorter wavelengths of the fluorescence maximum and an increase of the fluorescence quantum yield upon binding. In the case of phosphatidylserine vesicles, it was found that the quantum yield of the bound peptide was 1.6-times higher than that of the free peptide. The fluorescence shift was about 15 nm and the binding stoichiometry of six lipids per bound peptide. The observed fluorescence changes are consistent with a partially buried location of the tryptophan residue within the hydrophobic part of the phospholipid bilayer, Dufourcq et al. [15] have found that for the peptide Lys-Trp-Lys at pH 3.0 when the terminal carboxylic group is protonated (net charge 3 +) or the peptide Lys-Trp-OMe at pH 7.5 (net charge 2 +), the fluorescence shift was about 15 nm and the stoichiometry of four lipids per bound peptide using either phosphatidylserine or phosphatidylinositol vesicles. The association constant was estimated in either case to be at least of the order of 10^7 M^{-1}. The peptide Lys-Gly-Trp-Lys-OtBu used in this work bears three positive charges and exhibits very similar binding characteristics as compared with the peptides used by Dufourcq et al. [15]. The peptide-phospholipid complexes were dissociated by increasing the ionic strength. In view of the present results it can be concluded that positively charged peptides bind strongly to negatively charged vesicles and that these electrostatic interactions allow the aromatic ring of tryptophan to insert in a more hydrophobic environment.

Adding an extrinsic fluorescent molecule such as diphenylhexatriene does not alter the complexes as judged from the stability of the scattered light intensity (no vesicle alteration) and that of the fluorescence maximum of the peptide. Incorporation of diphenylhexatriene into the phospholipid bilayer was demonstrated by absorption and fluorescence measurements. Absorption spectra showed that diphenylhexatriene was randomly dispersed in the hydrophobic part of PS vesicles as its spectrum was very similar to that observed in organic solvents. The slight absorption shift observed in the presence of a phospholipid bilayer may be related to the polarizability of the medium as observed by Sklar et al. [21] with diphenylpolyenes in various solvents.

The fluorescence of diphenylhexatriene was found to be less dependent on the medium; we did not observe any fluorescence shift in the emission spectra of diphenylhexatriene in hexane, tetrahydrofuran or in PS vesicles. Sklar et al. [21] also observed that the emission characteristics of *cis*- and *trans*-parinaric acids were not dependent on the medium. Both parinaric acids and diphenylhexatriene fluorescence were found to be negligible in water medium. This result allowed accurate determinations of the diphenylhexatriene concentrations inside the vesicles as already discussed in Results.

In addition, fluorescence polarization measurements of diphenylhexatriene in peptide-bound vesicles have shown that the diphenylhexatriene polarization upon excitation at 356 nm does not vary with the peptide/phospholipid ratio. This result shows that under our experimental conditions peptide binding to, and diphenylhexatriene incorporation in the phosphatidylserine bilayer do not significantly perturb its structure.

Since there exists a good overlap of the tryptophan emission spectrum and the diphenylhexatriene absorption spectrum, one should expect energy transfer to occur between the two fluorophors. Results presented on Fig. 9 show that upon adding diphenylhexatriene to peptide-bound vesicles, the diphenylhexatriene fluorescence increased in parallel to the fluorescence decrease of the peptide. The presence of an isoemissive point at 385 nm indicates that the presence of either compound in the vesicle bilayer does not perturb the shape of the emission spectrum of the other.

The model of Föster transfer in a three-dimensional space can be applied to fluorescent donors and acceptors randomly distributed in a plane provided the following conditions are met [5,8]. (i) There is no transfer between energy donors: at the rather high lipid/peptide ratio value used in our transfer experiments ($R_i = 20$) the mean distance between tryptophan residues is of the order of 6 nm which is much larger than the Föster distance for Trp-Trp energy transfer (0.6 nm) [22]. (ii) The number of acceptors in the excited state is small compared with the number in the ground state: a low excitation energy has always been used in all our experiments ($\lambda_{exc} = 280$ nm, slit width = 1 nm); therefore the above condition is certainly fulfilled. (iii) The distance between donors and acceptors does not change during the excited state lifetime of the donor: during the average donor lifetime ($\langle\tau\rangle = 2.72$ ns), one donor molecule diffuses over a distance of $(4D\ \langle\tau\rangle)^{1/2} = 0.10$ nm (assuming the donor diffusion coefficient D to be of the order of 10^{-12} m^2/s) which is small compared with the average distance between donor and acceptor molecules (approx. 4 nm under our experimental conditions). (iv) The critical distance R_0 is the same for all donor-acceptor pairs: this condition requires a single population of donors and acceptors. As a first approximation it seems reasonable to assume that all peptide molecules have the same environment for their tryptophan residues as demonstrated by their fluorescence spectrum. The observation that fluorescence quenching of the peptide by transfer to diphenylhexatriene is not accompanied by any shift of the fluorescence spectrum points to the involvement of only one donor species. As shown on Table II, the fluorescence decay of the peptide (Lys-Gly-Trp-Lys-OtBu) in the presence of the energy acceptor (diphenylhexatriene) can always be represented by a superposition of two exponentials the respective contributions of which do not change with acceptor concentration eventhough the average lifetime may be reduced by as much as 50%. The origin of the two components in the fluorescence decay has not been elucidated yet. They exist for the free peptide in aqueous solutions and might represent two conformers of the peptide [23]. The fact that both lifetimes are increased upon binding to phospholipid vesicles seems to indicate that the tryptophan residue has a similar environment in the hydrophobic area of the phospholipids for both conformers of the bound peptide. The equilibrium between the two conformers appears to be slightly shifted as revealed by the change in the contributions of the two decay components (Table II). A similar shortening of the two lifetimes is observed upon energy transfer to diphenylhexatriene. The two conformers thus appear to transfer with similar efficiency to diphenylhexatriene.

An average value of 4.0 nm for the critical Förster distance R_0 was determined by the quenching of the donor fluorescence to be compared with the calculated value of 3.4 nm. Kimelman et al. [12] also found R_0 values in this range

(3.6–4.3 nm) for energy transfer from tryptophan residues of the M 13 coat protein to isomers of parinaric acids in synthetic vesicles. The overlap integral for this system is smaller than in our case and corresponds to a theoretical R_0 value of 3.1 nm. The agreement of experimental and theoretical values of R_0 found in our system testifies to the validity of the method we worked out for accurate determination of the surface density of the acceptor.

The results obtained with erythrocyte ghosts demonstrate that tryptophan residues of intrinsic membrane proteins efficiently transfer their excitation energy to diphenylhexatriene incorporated in the biological membrane. This observation could have been taken as evidence for direct binding of diphenylhexatriene to proteins. However, the results obtained with the model system peptide/vesicle/diphenylhexatriene demonstrates that an efficient energy transfer takes place over rather long distances without any close interaction between donor and acceptor. The experimental critical distance determined in the model system is 4.0 nm. The average distance between protein centers assumed to be randomly distributed in the membrane bilayer can be estimated to be about 7 nm for rat erythrocyte ghosts. Therefore diphenylhexatriene randomly distributed in the membrane phospholipids is certainly close enough to tryptophan residues of proteins to act as an efficient energy acceptor. The energy transfer process obviously does not require binding of diphenylhexatriene to proteins.

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